# ISOLATION AND CHARACTERIZATION OF THE CYTOPLASMIC AND OUTER MEMBRANES OF THE LEGIONNAIRES' DISEASE BACTERIUM (*LEGIONELLA PNEUMOPHILA*)

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*Legionella pneumophila*, the etiologic agent of Legionnaires' disease, is an aerobic gram-negative bacillus. The bacterium multiplies intracellularly in human blood monocytes (1) and human alveolar macrophages (2). Under tissue culture conditions, multiplication is exclusively intracellular (1). Previous studies from this laboratory have described several features of *L. pneumophila*–mononuclear phagocyte interaction: (*a*) *L. pneumophila* is phagocytized by an unusual mechanism, "coiling phagocytosis", in which a phagocyte pseudopod is coiled around the bacterium as the organism is internalized (3); (*b*) *L. pneumophila* induces the formation of and multiplies within a novel phagosome that is studded with host cell ribosomes (4); (*c*) *L. pneumophila* inhibits phagosome-lysosome fusion (5); and (*d*) *L. pneumophila* inhibits phagosome acidification (6).

As a first step towards understanding these *L. pneumophila*–phagocyte interactions, we have studied the envelope of this bacterium. In this paper, we report the isolation and characterization of the *L. pneumophila* cytoplasmic and outer membranes, and of the lipopolysaccharide (LPS)[1] in the outer membrane.

## Materials and Methods

*Media.* Albumin yeast extract (AYE) broth and modified charcoal yeast extract (CYE) agar were prepared as described (7).

*Bacteria.* *L. pneumophila*, Philadelphia 1 strain, was grown in embryonated hens' eggs, harvested, tested for viability and the presence of contaminating bacteria, and stored at −70°C, as described (1). For experimental use, egg yolk–grown *L. pneumophila* was cultured one time only on CYE agar. Isolated colonies were inoculated into AYE broth and the bacteria grown at 37°C to mid-logarithmic phase. At mid-logarithmic phase, the

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[1] *Abbreviations used in this paper:* AYE, albumin yeast extract; CYE, modified charcoal yeast extract; IFA, indirect fluorescence antibody; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; MOMP, major outer membrane protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TES, 10 mM Tris-HCl buffer, pH 8, containing 0.1 M EDTA and 2% SDS.

optical density (OD) is 0.7 at 540 nm, as measured in a Coleman 44 model spectrophotometer (Perkin-Elmer Corp., Norwalk, CT).

*Escherichia coli* K12, strain MC 4100 (8), is a rough, unencapsulated bacterium without an O antigen on its LPS. *E. coli* serotype 09:K29$^-$:H$^-$ (*E. coli* K$^-$), a spontaneously occurring unencapsulated mutant of strain Bi 161-42, is a smooth, unencapsulated bacterium with an O antigen on its LPS (9). The bacteria were grown to mid-logarithmic phase (OD of 0.7 at 540 nm) in tryptic soy broth (10).

*Isolation and Separation of Cytoplasmic and Outer Membrane.* Membranes of *L. pneumophila* were obtained by preparing and lysing spheroplasts by the method of Osborn et al. (11). Briefly, bacteria were grown in AYE broth to an OD of 0.7 at 600 nm and harvested by centrifugation at 11,000 *g* for 10 min at 4°C. The bacteria were immediately suspended in 1/10 of the culture volume in 10 mM Tris-HCl buffer, pH 7.8, containing 0.75 M sucrose (4°C). Lysozyme (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.1 mg/ml, and the suspension was left on ice for 2 min. Bacteria were converted to spheroplasts by slowly diluting the suspension over a period of 8–10 min by the addition (via a peristaltic pump) of 2 vol of 1.5 mM EDTA disodium salt (Sigma Chemical Co.), pH 7.5 (4°C). In most experiments, spheroplasts were osmotically lysed by dilution in 4 vol water (4°C). In some experiments, spheroplasts were lysed by sonication until the absorbance dropped to ~10% of its original value. In isolations in which NADH oxidase activity of membrane fractions was subsequently assayed (see below), dithiothreitol was added to a final concentration of 0.2 mM before spheroplasts were lysed, and was included in all subsequent steps. Bacterial membranes were separated from intact cells by centrifuging the suspension at 1,200 *g* for 20 min at 4°C. The bacterial membranes in the supernatant fraction were pelleted by centrifugation at 360,000 *g* for 2 h at 4°C.

The cytoplasmic and outer membranes were separated by sucrose density gradient centrifugation. The bacterial membranes were carefully suspended in 1 ml 25% sucrose (wt/wt) containing 5 mM EDTA disodium salt (pH 7.5, 4°C) and subjected to sucrose density gradient centrifugation at 247,000 *g* for 16 h, as described (11). A tube was inserted to the bottom of the centrifuge tube and gradient fractions were collected with the aid of a peristaltic pump. The density of gradient fractions was calculated from the refractive index.

*Electron Microscopy. Legionella* membranes (obtained from $10^{10}$ cells) were pelleted by centrifugation at 15,600 *g* for 15–30 min in an Eppendorf centrifuge at 4°C. These membranes were then fixed and processed for electron microscopy, as described (4).

*Assays for Protein, LPS, and NADH Oxidase Activity in Sucrose Density Gradient Fractions.* Protein was determined by the method of Lowry et al. (12) using bovine serum albumin (Armour Pharmaceutical Co., Kankakee, IL) as a standard.

LPS was estimated by determining the amount of 2-keto-3-deoxyoctonate (KDO) in the membrane fractions. Membrane fractions (0.3–0.4 mg of protein) were precipitated with 10% trichloracetic acid (Fisher Scientific Co., Pittsburgh, PA), collected by centrifugation at 4°C for 10 min at 20,000 *g*, and washed twice by centrifugation with 1.5 ml $H_2O$ to remove residual sucrose. KDO was released from the membrane precipitate by hydrolysis with 0.02 N $H_2SO_4$ at 100°C for 20 min and directly assayed by the thiobarbituric acid method of Weissbach and Hurwitz (13) as modified by Osborn et al. (11).

NADH oxidase activity was measured by the method of Osborn et al. (11). 50 μl of membrane suspension containing 20–60 μg of protein was incubated with substrate (50 mM Tris HCl, pH 7.5, 0.12 mM NADH, 0.2 mM dithiothreitol; all from Sigma Chemical Co.) in a total volume of 1 ml. The rate of decrease in absorbance at 340 nm was measured at 22°C in a Zeiss PM6 recording spectrophotometer. NADH oxidase activity was expressed in units; one unit was defined as the amount of enzyme activity that converted 1 μmole substrate per minute per milliliter sample.

*Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE).* SDS-PAGE (reagents from BDH Chemicals Ltd., Poole, England) was performed according to Laemmli (14) as modified by Ames (15). For protein analysis, the separating gel contained 12.5%

acrylamide and 0.33% *N,N'* methylene bis-acrylamide. For analysis of LPS, the separating gel contained 14% acrylamide, 4 M urea, (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY), as described by Tsai and Frash (16). Samples were solubilized in an equal volume of SDS-PAGE sample buffer containing 125 mM Tris-HCl, 20% glycerol (Fisher Scientific Co., Pittsburgh, PA), 4% SDS, 4 mM EDTA disodium salt, 10% 2-mercaptoethanol (Sigma Chemical Co.), and 0.01% bromophenol blue (Sigma Chemical Co.), pH 6.8, and heated at 100°C for 5 min. Molecular weight standards were α-lactalbumin (14,200 mol wt) trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000), obtained from Sigma Chemical Co. After electrophoresis, the gels were fixed in 10% acetic acid, 25% isopropanol, and stained for proteins with 0.2% Coomassie Brilliant Blue R-250 (Sigma Chemical Co.) in fixing solution, or stained for LPS with silver nitrate by the method of Tsai and Frash (16).

*Cell Surface Iodination.* *L. pneumophila* were grown to an OD of 0.8 at 540 nm in AYE broth. Bacteria in 1 ml of the culture were pelleted by centrifugation, washed three times with phosphate-buffered saline (87.66 g NaCl, 53.61 g $Na_2HPO_4 \cdot 7H_2O$, and 27.6 g $NaH_2PO_4 \cdot H_2O$ per liter) (PBS), and resuspended in 40 μl PBS. 10 μl of $^{125}I$ (100 μCi/μl; New England Nuclear, Boston, MA) was added to the bacteria. The mixture was then transferred to a tube containing 50 μg of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril; Pierce Chemical Co., Rockford, IL) and kept on ice, with occasional shaking, for 10 min. The bacteria were then washed five times with 1 ml PBS containing $10^{-2}$ M potassium iodide. The final washed cell pellet was suspended in 200 μl of SDS-PAGE sample buffer, heated at 100°C for 5 min, and subjected to electrophoresis as described. Radiolabeled polypeptides were revealed by autoradiography of the dried gel using x-ray film (Kodak X-Omat XAR-5 film; Eastman Kodak Co., Rochester, NY).

*LPS Isolation.* LPS was isolated from *L. pneumophila* envelopes, using the method of Darveau and Hancock (17), with modifications. *L. pneumophila* membranes, prepared as described above from $10^{10}$ cells, were pelleted by centrifugation at 15,600 *g* for 30 min at 4°C in an Eppendorf centrifuge and suspended in 200 μl of 10 mM Tris-HCl buffer, pH 8, containing 0.1 M EDTA tetrasodium salt (Sigma Chemical Co.) and 2% SDS (TES buffer) to solubilize the membrane components. The suspension was sonicated in a water bath (Heat Systems-Ultrasonics Inc., Plainview, NY) to ensure complete solubilization. The preparation was centrifuged at 15,600 *g* for 5 min at room temperature in an Eppendorf centrifuge to pellet peptidoglycan. The supernate was incubated overnight at 37°C with pronase (Sigma Chemical Co.) at a final concentration of 200 μg/ml to digest membrane proteins. At the end of the incubation period, LPS was precipitated by incubating the mixture with 2 vol of 0.375 M $MgCl_2$ in 95% ethanol at −20°C for 2–3 h, and pelleted by centrifugation at 15,600 *g* for 30 min at 4°C in an Eppendorf centrifuge. The pellet was suspended in 200 μl of the TES buffer and heated at 85°C for 30 min to denature residual pronase-resistant proteins. The solution was then cooled and incubated at 37°C overnight, with pronase at a final concentration of 25 μg/ml, to digest remaining protein contaminants. After this incubation, LPS was precipitated with 2 vol of 0.375 M $MgCl_2$ in 95% ethanol and pelleted by centrifugation as described above, resuspended in 200 μl $H_2O$, and frozen at −20°C. The yield of LPS was estimated by determining the amount of KDO recovered relative to the cell dry weight. LPS from *E. coli* K12 (rough) and *E. coli* $K^-$ (smooth) were isolated by the same procedure.

*Antisera.* Antiserum against the major outer membrane protein (MOMP) of *L. pneumophila* was obtained by immunizing 2-kg New Zealand White rabbits subcutaneously with purified MOMP. The purification of this protein to homogeneity will be described elsewhere.[2] Rabbits were initially injected with 100 μg of MOMP mixed 1:1 with complete Freund's adjuvant, and 2 and 6 wk later with 100 μg of MOMP mixed 1:1 with incomplete Freund's adjuvant. After 2 wk, blood was drawn, and the serum collected and stored at −20°C.

Antiserum to the LPS was obtained by immunizing 2-kg New Zealand White rabbits

[2] Gabay, J. E., M. S. Blake, W. Niles, and M. A. Horwitz. Purification of the *Legionella pneumophila* major outer membrane protein and demonstration that it is a porin. Manuscript in preparation.

subcutaneously with LPS purified as described above. Rabbits were first injected with 50 μg LPS in complete Freund's adjuvant (Difco Laboratories Inc., Detroit, MI), and then, 2 and 4 wk later, with 50 μg LPS in incomplete Freund's adjuvant (Difco Laboratories Inc.). After 2 wk, blood was drawn, and serum collected and stored at −20°C.

*Normal and Immune Human Sera.* Human sera were obtained and stored as described (4); normal (nonimmune) human sera with indirect fluorescent antibody anti-*L. pneumophila* Philadelphia 1 titers of <1:64 were obtained from adult donors not known ever to have had Legionnaires' disease. Human immune sera were obtained from adult donors who had recently recovered from Legionnaires' disease; all had serologic and/or cultural evidence of infection by serogroup 1 *L. pneumophila*.

*Immunoblot Analysis.* For Western blotting, membrane samples were first subjected to SDS-PAGE. The separated polypeptides were then electrophoretically transferred from the gel to nitrocellulose paper (Schleicher and Schuell, Inc., Keene, NH) for 2 h at 1 A in a Bio-Rad Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, CA) using the method described by Towbin et al. (18). After the transfer, the nitrocellulose paper was incubated twice in 0.5% Tween 20 (Sigma Chemical Co.) in PBS (Tween PBS buffer) for 30 min each time, to block the remaining binding sites, and cut into strips. The strips were incubated for 3 h at room temperature with various human sera diluted 1:20 to 1:100 in Tween-PBS buffer, washed three times for 5 min, with the same buffer, and incubated for 2 h at room temperature with alkaline phosphatase–conjugated goat anti-human Ig, IgG fraction (Sigma Chemical Co.), diluted 1:1000 in Tween-PBS buffer. Afterwards, the strips were washed three times for 5 min with Tween-PBS buffer, and once with 0.15 M veronal acetate buffer, pH 9.6. The strips were then stained to reveal the antigen-antibody complexes, using 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) as the alkaline phosphatase substrate, and nitroblue tetrazolium (Sigma Chemical Co.) as the chromophore, as described by Blake et al. (19).

*Indirect Fluorescence Antibody (IFA) Assay.* Whole intact *L. pneumophila* bacteria were treated at 100°C for 20 min, washed twice in PBS, and suspended in the same buffer to a final concentration of ~$5 \times 10^8$ bacteria/ml. 50 μl of the heat-killed bacterial suspension was incubated for 1 h at 37°C with 50 μl of patient serum diluted to various concentrations in PBS. The bacteria were washed twice, resuspended in 50 μl of PBS, and incubated for 30 min at 37°C with 50 μl fluorescein-conjugated rabbit anti-human Ig, IgG fraction (Cappel Laboratories, Cochranville, PA), diluted 1:10 in PBS. The bacteria were washed once more, resuspended in 10 μl of 10 mM Tris-glycerol buffer, pH 8.0, and examined and photographed with a Nikon photomicroscope with epifluorescence optics, using a Plan Achromat 100× oil-immersion lens. The IFA titer for each patient specimen was the highest dilution of serum yielding positive fluorescence for >80% of the bacteria. In all cases, <10% of bacteria were positive for fluorescence upon a further twofold dilution of the serum.

In experiments designed to determine the proportion of patient antibodies detected in the IFA assay that are directed against LPS and the MOMP, patient sera were titered in this assay before and after incubation with excess amounts of LPS and MOMP. The amount of LPS or MOMP used corresponded to 10–50 times the amount necessary to inhibit completely the binding of rabbit anti-MOMP or anti-LPS antibodies to *L. pneumophila*, as measured by the IFA assay using fluorescein-conjugated goat anti-rabbit Ig, IgG fraction (Cappel Laboratories). For use in these studies, purified MOMP and LPS were precipitated from solution by incubation with 2 vol 100% ethanol overnight at −20°C, and pelleted by centrifugation at 15,600 *g* for 45 min at 4°C. The MOMP and LPS were resuspended directly in sera and incubated for 1 h at 37°C, and for an additional 12 h at 4°C. The treated sera were then centrifuged at 15,600 *g* for 20 min at 4°C to remove insoluble material before testing in the IFA assay. Clarifying the sera of insoluble material did not alter the IFA titers.

## Results

*Isolation of L. pneumophila Outer and Cytoplasmic Membranes.* To separate *L. pneumophila* outer and cytoplasmic membranes, we used a procedure based on

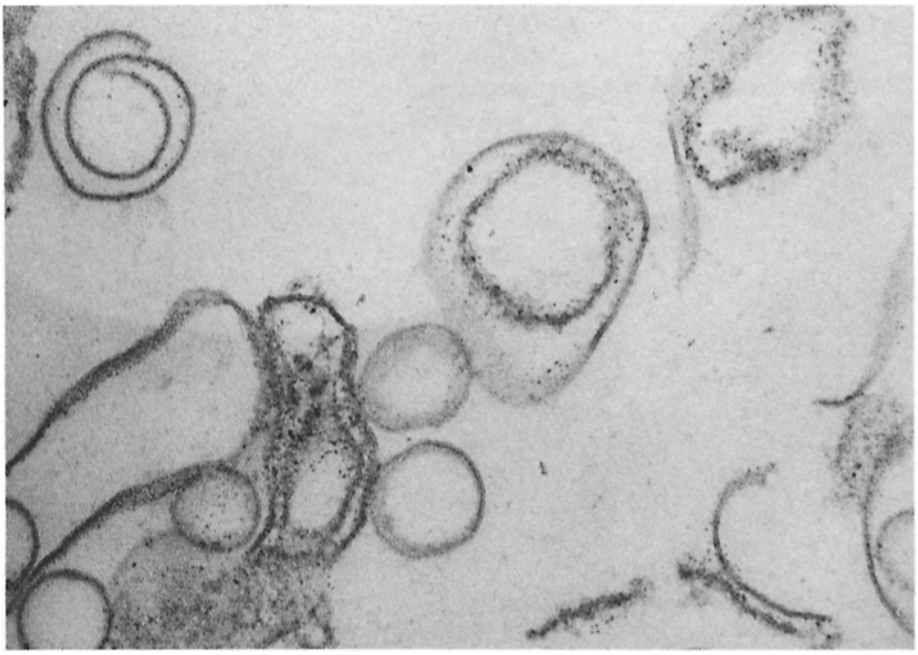

FIGURE 1. *L. pneumophila* total membrane fraction. *L. pneumophila* were converted to spheroplasts by lysozyme-EDTA treatment and the spheroplasts lysed by osmotic shock. The membranes were recovered in the pellet by ultracentrifugation and were fixed and stained for electron microscopy as described in the text. × 91,000.

equilibrium density gradient centrifugation of total membranes (11). Other procedures, based on differential membrane solubility in detergents (Sarkosyl or Triton X-100) (20), did not result in a good separation. We first converted intact *L. pneumophila* to spheroplasts by lysozyme-EDTA treatment, as described in Materials and Methods. The rate of conversion into spheroplasts, monitored by phase contrast microscopy, was 80–90%. Second, we lysed the spheroplasts, either by osmotic shock or by sonication, ultracentrifuged the lysed spheroplasts, and recovered a total membrane fraction in the pellet. By electron microscopy, these membranes appeared as empty vesicles of variable but relatively large size (Fig. 1). Third, we subjected the *L. pneumophila* total membrane fraction to isopycnic centrifugation in a sucrose density gradient.

This procedure resulted in the separation of two major protein peaks at buoyant densities 1.222 and 1.145 (Fig. 2), densities approximating those at which outer and cytoplasmic membranes, respectively, typically migrate (11). To confirm this, we tested the fractions collected from the sucrose gradient for specific membrane markers. One component of the electron transport system, NADH oxidase, which is found exclusively in cytoplasmic membrane, localized to the peak of density 1.145 (Fig. 2). Very little of this enzymatic activity was detected in the peak of density 1.222. In contrast, LPS, which is found exclusively in the outer membrane, was localized to the peak of density 1.222 (Fig. 2), and little was found in the peak of density 1.145. These results allowed us to identify the peak of buoyant density 1.222 as the outer membrane and the peak of

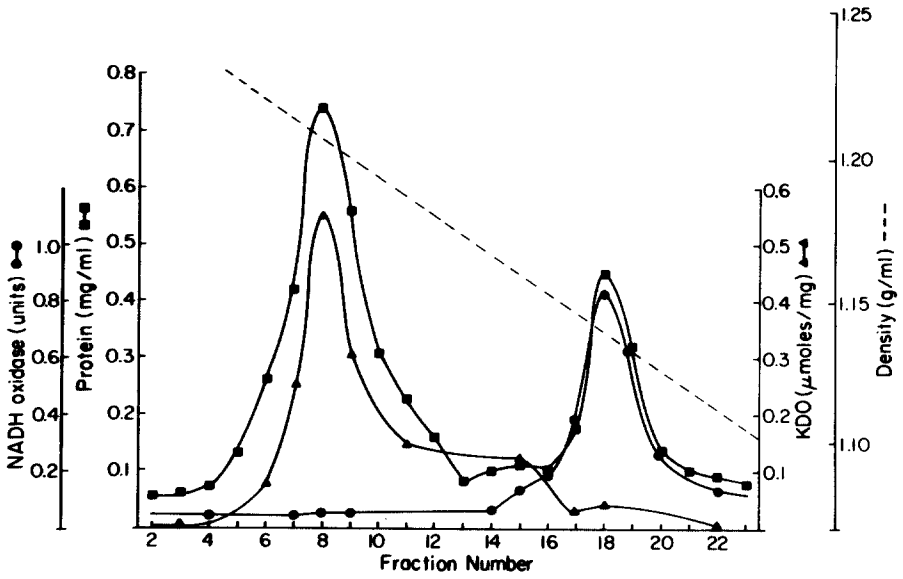


FIGURE 2. Sucrose density gradient centrifugation of total membranes of *L. pneumophila*. *L. pneumophila* membranes were subjected to isopycnic centrifugation in a sucrose density gradient. Fractions collected from the bottom of the gradient were assayed for density, protein, KDO, and NADH oxidase activity, as described in the text.

buoyant density 1.145 as cytoplasmic membrane. The highly localized distribution of the membrane markers indicated that the membranes were well separated.

*Protein Composition of L. pneumophila Outer and Cytoplasmic Membrane.* SDS-PAGE of the *L. pneumophila* total cell extract revealed that *L. pneumophila* contains three major proteins of apparent molecular weight 28,000 (28 K), 45 K, and 65 K (Fig. 3, lane *2*). The 28 K mol wt protein is the most abundant protein of the bacterium. SDS-PAGE of extract of the total membranes, obtained from lysed spheroplasts as described above, revealed that the 28 K mol wt protein is a membrane protein (Fig. 3, lane *3*). SDS-PAGE of proteins of the outer and cytoplasmic membranes, obtained by sucrose density gradient cenrifugation, revealed that the 28 K mol wt protein is located in the outer membrane (Fig. 3, *A–S*). The *L. pneumophila* outer membrane contains a single major protein, the protein migrating at mol wt 28 K, which we hereafter refer to as MOMP (the major outer membrane protein). The cytoplasmic membrane protein profile is distinct from that of the outer membrane, with one major protein of apparent molecular weight 65 K.

*Surface Iodination of Cell Envelope Components.* To see which of the outer membrane protein species are exposed at the cell surface, we treated intact live *L. pneumophila* with $^{125}I$ in Iodogen-coated tubes, lysed the $^{125}I$-labeled *L. pneumophila* by boiling them in 2% SDS, subjected the lysates to SDS-PAGE, and visualized the iodinated proteins by autoradiography. Under the conditions of labeling, proteins present at the cell surface were preferentially labeled. Fig. 4 shows that the MOMP is the major protein labeled. The inner membrane

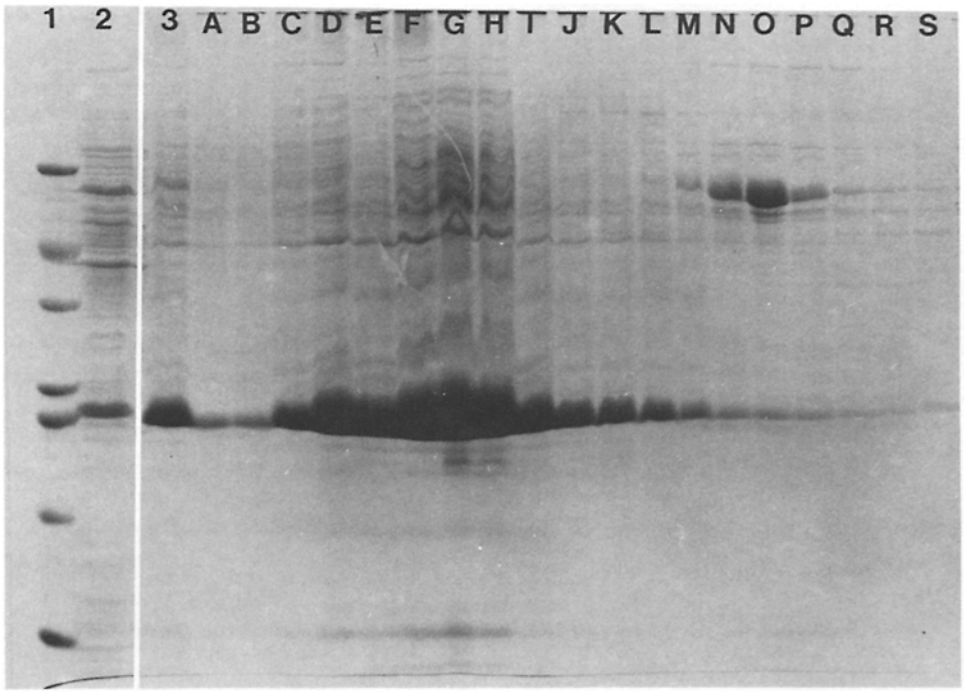


FIGURE 3. SDS-PAGE analysis of fractions collected from the sucrose density gradient shown in Fig. 2. Total cell (*2*), total membranes (*3*), and fractions from the sucrose density gradient (*A–S*) were analyzed by SDS-PAGE (12.5% acrylamide) and stained with Coomassie Blue. Molecular weight standards (*1*) were α-lactalbumin (14,200 mol wt), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine albumin (66,000).

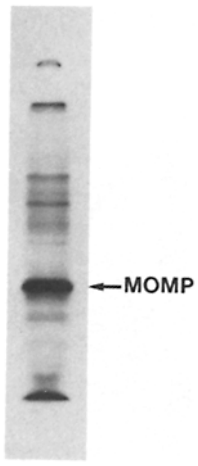


FIGURE 4. Surface iodination of *L. pneumophila*. Live intact *L. pneumophila* were treated with $^{125}I$ in Iodogen-coated tubes, washed, and boiled in 2% SDS. The labeled preparation was subjected to SDS-PAGE and the protein bands visualized by autoradiography. MOMP, major outer membrane protein.

proteins were not labeled by this procedure, suggesting that the MOMP of *L. pneumophila* is exposed at the cell surface.

To confirm that the MOMP was exposed at the cell surface, we performed two additional types of experiments using rabbit antibody against purified MOMP. First, we showed, by immunofluorescence labeling, that anti-MOMP antibody binds to the surface of intact live *L. pneumophila*. Second, we showed that anti-MOMP agglutinates intact live *L. pneumophila*. These experiments confirmed that the MOMP is exposed at the cell surface.

*LPS Isolation.* We first attempted to isolate *L. pneumophila* LPS by the hot phenol-water procedure generally used for LPS purification (21), but we were unable to detect LPS in the water phase. We therefore isolated *L. pneumophila* LPS by Darveau and Hancock's (17) method using SDS-EDTA, as described in Materials and Methods. The yield of LPS was 2.28 μg KDO/mg cell dry weight, an amount comparable to that obtained from smooth and rough *E. coli* LPS (2.1 and 1.28 μg KDO/mg cell dry weight, respectively). The protein contamination of this LPS preparation, as measured by the Lowry assay (12), was ~2% (wt/wt). SDS-PAGE followed by Coomassie Blue staining revealed only one protein contaminant, the MOMP.

We subjected the LPS to SDS-PAGE and visualized it by silver staining. As shown in Fig. 5, the *L. pneumophila* LPS displayed a typical ladder pattern. Other researchers have proposed that these orderly spaced bands represent LPS molecules, with different numbers of repeating units in their O side chains (22, 23). The size distribution of *L. pneumophila* LPS molecules was different from that of *E. coli* LPS molecules. The average size of *L. pneumophila* LPS molecules was less than that of smooth *E. coli* but greater than that of rough *E. coli*.

*Serological Responses of Patients to L. Pneumophila Cell Envelope Components.* Patients with Legionnaires' disease respond to the infection with the production of anti-*L. pneumophila* antibodies (24). To investigate patient antibody responses to cell envelope components, we studied the sera of six patients who had laboratory evidence of infection by serogroup 1 *L. pneumophila*. We first

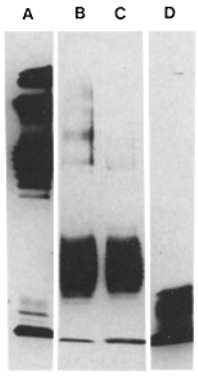


FIGURE 5. LPS of *L. pneumophila*. LPS was isolated from *L. pneumophila* Philadelphia 1 (agar-grown and broth-grown), *E. coli* K12 (rough), and *E. coli* $K^-$ (smooth), subjected to SDS-PAGE (14% acrylamide, 4 M urea), and the gel stained with silver nitrate as described in the text. 5 μg of LPS from each preparation were loaded on the gel. (*A*) *E. coli* $K^-$ (smooth); (*B*) *L. pneumophila*, agar grown; (*C*) *L. pneumophila*, both-grown; (*D*) *E. coli* K12 (rough).

TABLE I

*Majority of Patient Antibodies to L. pneumophila Philadelphia 1 Are Directed Against LPS*

| Patient | Serum IFA titer | | Reduction in titer with LPS | |
|---|---|---|---|---|
| | − LPS | + LPS | Fold | Percent |
| A | 1:1024 | 1:2 | 512 | 99.8 |
| B | 1:16384 | 1:32 | 512 | 99.8 |
| C | 1:256 | 1:4 | 64 | 98.4 |
| D | 1:1024 | 1:4 | 256 | 99.6 |
| E | 1:256 | 1:2 | 128 | 99.2 |
| F | 1:512 | 1:2 | 256 | 99.6 |

Sera from patients with serologic evidence of infection to serogroup 1 *L. pneumophila* were incubated with buffer, excess purified MOMP, or excess purified LPS. The IFA titer of each serum specimen to *L. pneumophila* Philadelphia 1 was determined as described in the text. The IFA titers of sera in the presence of MOMP were the same as with buffer alone (− LPS).

retested the sera by the IFA assay using *L. pneumophila* Philadelphia 1, a serogroup 1 organism, as antigen. All sera had significant titers against heat-killed *L. pneumophila* Philadelphia 1 (Table I).

To determine which cell envelope proteins elicit an antibody response in humans, we examined the reactivity of patient sera with outer and cytoplasmic membrane proteins of *L. pneumophila* Philadelphia 1, using Western blotting. We isolated total membrane, outer membrane, and cytoplasmic membrane of *L. pneumophila*, separated the proteins by SDS-PAGE, electrophoretically transferred the proteins onto nitrocellulose paper, and incubated the blots with nonimmune sera or sera from patients with *L. pneumophila*. The *L. pneumophila* antigen-antibody complexes were revealed histochemically (19). Normal sera contained low amounts of antibodies to *L. pneumophila* proteins (Fig. 6). Patient sera contained high amounts of antibodies to several outer and cytoplasmic membrane proteins (Fig. 6). However, patient sera contained low amounts of antibodies to *L. pneumophila* MOMP by this technique (Fig. 6).

*LPS Is the Major Antigen Recognized by Patient Sera.* To investigate what proportion of patient anti-*L. pneumophila* Philadelphia 1 antibodies is directed against the two major surface components of this bacterium, the MOMP and LPS, we assayed the capacity of purified *L. pneumophila* Philadelphia 1 MOMP and LPS to inhibit fluorescence in the IFA assay. We determined the IFA titer of patient sera before and after incubation with excess amounts of LPS and MOMP.

Preincubation of patient sera with an excess amount of MOMP had no influence on the IFA titers. In contrast, an excess amount of LPS reduced the IFA titers by >98% (Table I). Excess LPS added to rabbit anti-MOMP antiserum did not influence the IFA titer of this serum, indicating that LPS does not reduce IFA titers nonspecifically. These findings indicate that the great majority of patient antibodies that recognize *L. pneumophila* Philadelphia 1, are directed against the LPS of this strain.

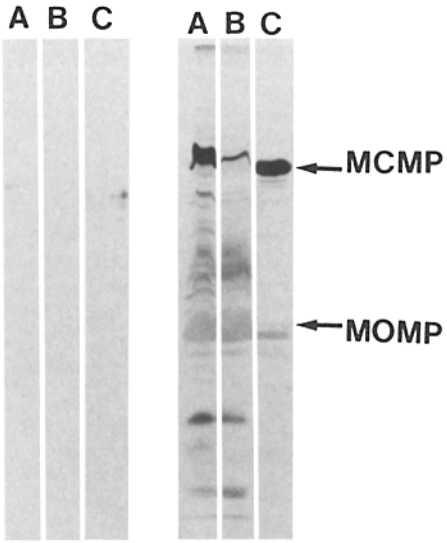


FIGURE 6. Western-blot analysis of the reactivity of patient sera with isolated outer and cytoplasmic membrane proteins of *L. pneumophila* Philadelphia 1. Total membrane proteins (*A*), outer membrane proteins (*B*), and cytoplasmic membrane proteins (*C*) of *L. pneumophila* Philadelphia 1 were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose paper. The blots were then incubated with a 1:100 dilution of either convalescent serum from a patient recovered from Legionnaires' disease (*right*) or normal serum (*left*). The *L. pneumophila* antigen-antibody complexes were revealed histochemically, using alkaline phosphatase–conjugated goat anti–human Ig antibodies MCMP, major cytoplasmic membrane protein. Sera from five other patients yielded similar results.

## Discussion

We have found that the *L. pneumophila* outer and cytoplasmic membranes can be separated by sucrose density gradient centrifugation, as with other gram-negative bacteria (20). The distribution of specific membrane markers, KDO and NADH oxidase, allowed us to distinguish the two membranes. We also found that other methods of membrane separation, based on differential membrane solubility using detergents (20), resulted in poor separation of *L. pneumophila* cytoplasmic and outer membranes.

As expected, the outer and cytoplasmic membranes had very distinct protein profiles. The *L. pneumophila* cytoplasmic membrane had one major protein species migrating at 65 K mol wt. The *L. pneumophila* outer membrane also had a single major protein species, the MOMP, of apparent molecular weight 28 K. This protein was also the most abundant protein in *L. pneumophila*. A previous study (25) reported that all *L. pneumophila* strains tested contain a major protein of approximately the same molecular weight as the MOMP. The *L. pneumophila* MOMP is exposed at the cell surface; the protein can be labeled by iodination of intact cells, and rabbit antiserum prepared against the purified MOMP bound to and agglutinated intact cells.

Our results differ from those recently reported by Hindahl and Iglewski (26). In their study, *L. pneumophila* was fractionated into five membrane fractions by sucrose gradient centrifugation; the amount of KDO was not appreciably greater in fractions designated as outer membrane than in those designated as cyto-

plasmic membrane. In contrast, in our study, *L. pneumophila* was fractionated into two distinct fractions (outer membrane and cytoplasmic membrane) by sucrose gradient centrifugation, and KDO was localized to the outer membrane fraction. It is possible that methodological differences account for the disparate results. Hindahl and Iglewski studied a different *L. pneumophila* serogroup 1 strain (Knoxville 1), cultured it in broth lacking iron pyrophosphate, and used a French pressure cell to disrupt whole bacterial cells. In contrast, we studied *L. pneumophila* Philadelphia 1, cultured it in broth containing iron pyrophosphate, and used the Osborn procedure to isolate membranes. In both studies, the outer membrane fractions contained a major protein of ~28 K mol wt.

We find that the *L. pneumophila* LPS, which could not be extracted by the phenol-water procedure, can be isolated by treating the bacteria with SDS-EDTA, as described by Darveau and Hancock (17). The LPS pattern obtained by silver staining differed markedly from the pattern observed with either *E. coli* K12 (rough) or *E. coli* $K^-$ (smooth) LPS, or other LPS described in the literature (17, 27). This finding and two other lines of evidence suggest that *L. pneumophila* LPS may be a novel type of bacterial LPS. First, lipid analysis of *L. pneumophila* has shown (28, 29) an unusual fatty acid composition; branched-chain fatty acids are the predominant type and hydroxy-fatty acids, which are generally associated with classical endotoxins as structural components of lipid A, are absent. Second, *L. pneumophila* endotoxicity seems to differ from that of classical endotoxicity of gram-negative bacteria (30) in that the endotoxin isolated from *L. pneumophila* induces a very weak pyrogenicity response in rabbits and is of relatively low toxicity for mice compared with *Salmonella* or *Neisseria gonorrhoeae* endotoxins. Further chemical and biological studies are necessary to determine if *L. pneumophila* contains a novel type of LPS.

Our study shows that convalescent sera from patients with serologic evidence of infection with serogroup 1 *L. pneumophila* (24) contain antibodies to a variety of inner and outer membrane proteins and to LPS of *L. pneumophila* Philadelphia 1, a serogroup 1 organism. The immunofluorescence studies show that few of these antibodies recognize the MOMP of *L. pneumophila* Philadelphia 1. In contrast, the great majority (>98%) of these antibodies recognized the LPS of this strain. This strongly indicates that LPS is the major serogroup antigen and the major antigen responsible for the reactivity of patient sera in the IFA assay, the principal diagnostic assay for *Legionella* infection. This result is consistent with an earlier study that found that a major *L. pneumophila* antigen recognized by patient sera was similar to endotoxin (31). Further studies are needed to define fully the role of LPS in serogroup specificity.

Our study demonstrates that patients with Legionnaires' disease generate abundant antibodies to LPS, but does not exclude the possibility that patients also generate large amounts of antibodies specific to the particular strain infecting them, i.e., antibodies that do not crossreact with other organisms within the same serogroup.

## Summary

*Legionella pneumophila*, the etiologic agent of Legionnaires' disease, is phagocytized in an unusual way and multiplies in human mononuclear phagocytes in a

novel phagosome. As a first step toward understanding these *L. pneumophila*–phagocyte interactions, we have studied the envelope of *L. pneumophila* Philadelphia 1 strain. We isolated cell envelopes by treating whole bacterial cells with lysozyme and EDTA to convert them to spheroplasts, then lysing the spheroplasts osmotically or sonically. We resolved the cell envelopes into two membrane fractions by isopycnic centrifugation. We localized NADH oxidase to the fraction of buoyant density 1.145, which we designated cytoplasmic membrane, and lipopolysaccharide (LPS) to the fraction of density 1.222, which we designated outer membrane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the *L. pneumophila* outer membrane contains a single major protein species migrating at 28,000 mol wt; this is the major protein of the bacterium. The cytoplasmic membrane also contains a single major protein species migrating at 65,000 mol wt. Surface iodination of the bacteria and agglutination and immunofluorescence studies with rabbit antibody produced against the purified major outer membrane protein (MOMP) revealed that this protein is exposed at the cell surface. We isolated LPS from *L. pneumophila* membranes by SDS-EDTA treatment. The pattern obtained by subjecting the LPS to SDS-PAGE and staining the gel with silver nitrate suggests that *L. pneumophila* LPS might be atypical.

We studied patient serologic responses to cell envelope components of *L. pneumophila* Philadelphia 1, a serogroup 1 organism. Sera from patients with evidence of infection with serogroup 1 *L. pneumophila* contained large amounts of antibody to this strain. Few of these antibodies recognized the MOMP of *L. pneumophila*. In contrast, >98% of these antibodies were directed against the LPS. This indicates that LPS is the dominant serogroup antigen and the major antigen responsible for the reactivity of patient sera in the indirect fluorescent antibody assay, currently the principal diagnostic assay for *Legionella* infection.

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