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Outer Membrane Proteins from *Legionella pneumophila* Serogroups and Other *Legionella* Species

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Outer membranes were isolated from eight serogroups of *L. pneumophila* and five other *Legionella* species. The protein composition of the membranes was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A single, disulfide stabilized protein with a molecular size of 29,000 to 30,000 daltons was found to be the major outer membrane protein (MOMP) of all the serogroups. The equivalent of the *L. pneumophila* MOMP was not observed in any of the other *Legionella* species examined. Silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels revealed distinctive patterns for each serogroup and other *Legionella* species that were not observed by staining with Coomassie blue and may result from the presence of lipopolysaccharide in the membrane preparations. The MOMP from serogroup 1 was isolated by exposing crude peptidoglycan to detergent in the presence of heat and reducing agent and was found to be tightly associated with lipopolysaccharide. Antibodies to this complex were used to probe the outer membranes of the remaining, *L. pneumophila* serogroups and other *Legionella* species by Western blotting. Serogroup 1 anti-MOMP antibodies were found to react with the MOMP from the remaining seven serogroups examined, whereas antibodies directed against the lipopolysaccharide of serogroup 1 only reacted with lipopolysaccharide from two of the remaining seven serogroups.

The gram-negative bacterium *Legionella pneumophila* is a facultative intracellular parasite that has been shown to multiply within human peripheral blood monocytes (18) and block phagosome-lysosome fusion (17). When in an intracellular environment that contains inhibitors of bacterial protein synthesis, the organism ceases to multiply, yet remains viable (19). This observation suggests that the cell surface may play a vital role in maintaining the fusion-resistant phagosome.

The cell envelope of *L. pneumophila* has several interesting properties that may be related to the ability of the organism to parasitize phagocytic cells. The peptidoglycan (PG) of *L. pneumophila* serogroup 1 (Philadelphia 2) is highly cross-linked, and protease-resistant proteins have been shown to be tightly associated with the glycan backbone of this structure (1). A similar structural relationship in the PG of the intracellular parasite *Coxiella burnetii* has recently been demonstrated (4). The outer membrane of *L. pneumophila* serogroup 1 Knoxville 1 (LPK-1) contains a single major outer membrane protein (MOMP) that exists as a large aggregate stabilized by disulfide linkage. The apparent molecular size of the monomeric form of this protein is 28,000 to 29,000 daltons (8, 12, 16). Disulfide-stabilized outer membrane proteins have been observed in the chlamydiae (24); interestingly, *Chlamydia psittici* belongs to a select group of organisms, including *L. pneumophila*, that inhibit lysosome-phagosome fusion (17).

Given the similarities of the *L. pneumophila* serogroup 1 cell envelope with those of the intracellular parasites mentioned above and the consideration that the cell surface may be an essential factor in intracellular survival, we chose to compare the outer membrane proteins of various *Legionella* species in detail. The outer membranes from eight *L. pneumophila* serogroups and five other *Legionella* species were isolated, and outer membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with both Coomassie blue and silver as staining reagents. With these two methods, clear differences between the outer membrane profiles of all organisms examined were detected. The MOMP from *L. pneumophila* serogroup 1 (LPK-1) was then isolated from cell envelopes based on the susceptibility of the aggregate form of this protein to heat and reducing agents (16). The isolated MOMP was used to generate antibodies to characterize the antigenic similarities between this protein and outer membrane proteins from serogroups and species within the genus *Legionella*.

MATERIALS AND METHODS

Organism and culture conditions. The following *Legionella* serogroups and species were maintained in charcoal-yeast extract broth (25) (modified as previously described [16]) containing 20% glycerol at −70°C. *L. pneumophila* strains included Knoxville 1 (serogroup 1), Togus 1 (serogroup 2), Bloomington 2 (serogroup 3), Los Angeles 1 (serogroup 4), Dallas 1E (serogroup 5), Chicago 2 (serogroup 6), Chicago 8 (serogroup 7), and Concord 3 (serogroup 8). *Legionella* species other than *L. pneumophila* used in this study included *L. bozemanii*, *L. dumoffi*, *L. gormanii*, *L. longbeachae*, and *L. micdadei*. A human pneumonia clinical isolate identified as *L. pneumophila* serogroup 1 was kindly provided by Washington Winn Jr., University of Vermont Medical College. Plates containing charcoal-yeast extract agar were streaked with stock cultures and incubated in a 5% CO_2 incubator at 37°C for 48 h. Batch cultures were prepared as described previously (16).

Isolation of outer membranes. Outer membranes were isolated from all *L. pneumophila* serogroups and other *Legionella* species as previously described (16), except that recovered cell envelopes were layered onto sucrose gradients. Briefly, 100-ml overnight cultures were harvested by centrifugation at 4°C and washed twice with cold 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4). Cell pellets were suspended in 2 ml of

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10 mM HEPES buffer, and DNase I (Sigma Chemical Co., St. Louis, Mo.) and RNase A (Sigma) were added to 50 μg/ml each. The suspension was passed through a French pressure cell twice at 10,000 lb/in^2 and was centrifuged at 1,000 × *g* for 15 min to remove whole cells. The supernatant was centrifuged at 50,000 rpm in a Beckman 60Ti rotor for 1 h. The cell envelope-containing pellet was washed two times in cold HEPES buffer by centrifuging at 50,000 rpm in the Beckman 60Ti rotor. The pellet was suspended in 1.5 ml of HEPES buffer, and 0.8 ml was loaded onto sucrose gradients consisting of 2 ml of 70% sucrose, 3 ml of 64% sucrose, 3 ml of 58% sucrose, 2 ml of 52% sucrose, and 1 ml of 46% sucrose. The gradients were centrifuged at 25,000 rpm in a Beckman SW41 rotor for 18 h at 4°C. The lower two bands from each gradient were pooled, diluted to 25 ml in HEPES buffer, and centrifuged at 50,000 rpm in a Beckman 60Ti rotor for 1 h. The recovered outer membranes were washed twice in HEPES buffer by centrifugation at 50,000 rpm in a 60Ti rotor for 1 h and suspended in 1.5 ml of HEPES buffer. Protein concentrations were determined by the method of Lowry et al. (22). The amount of 2-keto-3-deoxyoctonic acid (KDO) in outer membranes was assessed by the method of Waravdekar and Saslaw (30) with KDO (Sigma) as a standard.

Isolation of the LPK-1 MOMP. LPK-1 whole cells were broken as previously described (16). After whole cells were removed by centrifugation at 1,000 × *g* for 15 min, cell envelopes were collected by centrifuging the suspension at 50,000 rpm in a Beckman 60Ti rotor for 1 h. The pellet was suspended in 50 ml of 50 mM Tris (pH 8.0) containing 2% SDS–10 mM EDTA–50 mM NaCl and incubated at 60°C for 30 min. The suspension was centrifuged at 150,000 × *g* for 30 min at 20°C. The supernatant was decanted, and the pellet was suspended in 50 ml of the same buffer, incubated at 60°C for 30 min, and centrifuged at 150,000 × *g* for 30 min. The pellet was suspended in 5 ml of 10 mM Tris (pH 8.0), diluted with 5 ml of 50 mM Tris (pH 8.0)–2% SDS–1% 2-mercaptoethanol–2 mM EDTA, and heated to 100°C for 5 min. After cooling, the suspension was centrifuged at 150,000 × *g* for 30 min. The supernatant containing 2-mercaptoethanol solubilized material was decanted, and 1 ml of the supernatant was applied to a 1.5- by 45-cm Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) column that had been previously equilibrated at 32°C in 50 mM Tris–2% SDS–10 mM EDTA–50 mM NaCl. Fractions were collected at 50 drops per fraction and analyzed for the presence of the MOMP by SDS-PAGE.

Electroblotting of outer membrane proteins. The cross-reactivity of antibodies directed against the LPK-1 MOMP with outer membrane proteins from other *L. pneumophila* serogroups and other *Legionella* species was examined by using the Western blot method of Towbin et al. (28). Briefly, purified outer membranes or the purified MOMP from LPK-1 were applied to an SDS-polyacrylamide gel containing 12.5% acrylamide, and 30 mA was applied until the bromophenol blue dye front reached the bottom of the gel. After the gel was overlaid with nitrocellulose paper (Schleicher & Schuell Co., Keene, N.H.), the proteins were transferred to the paper by placing the sandwich in a Trans-Blot chamber (Bio-Rad Laboratories, Richmond, Calif.) containing a buffer consisting of 0.025 M Tris base–0.192 M glycine–20% methanol. A voltage of 55 V was applied to the chamber for 2 h. After electrotransfer, the nitrocellulose was incubated in 0.01 M Tris–0.9% NaCl (pH 7.4)–3% bovine serum albumin at 37°C for 30 min. The nitrocellulose was rinsed in 200 ml of TN (0.01 M Tris, 0.9% NaCl [pH 7.4]) and was then incubated in 100 ml of TN containing 1% bovine serum albumin and 100 μl of antiserum for 2 h at 37°C. After rinsing in TN, the nitrocellulose was incubated in 100 ml of TN containing 1% bovine serum albumin and 50 μl of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Cappel Laboratories, Cochranville, Pa.) for 2 h at 37°C. After a brief rinse in water, the nitrocellulose was incubated in a solution containing 0.05 M Tris–0.15 M NaCl–0.005 M EDTA–0.05% Triton X-100 (pH 7.4) for 15 min at room temperature followed by incubation in TN for 10 min at room temperature. The nitrocellulose was then placed in 60 ml of TN, and 20 ml of methanol containing 2 mg of 4-chloro-1-napthol (Bio-Rad) per ml was added. A 40-μl sample of 30% hydrogen peroxide was added, and the reaction was allowed to proceed for 5 min at room temperature. The reaction was stopped by rinsing in water.

Isolation of LPS. Lipopolysaccharide (LPS) was isolated from LPK-1 by a modification of the method of Darveau and Hancock (7). One liter of late-exponential-phase cells grown in charcoal-yeast extract broth was harvested and washed twice with 10 mM HEPES buffer by centrifugation. The washed pellet was suspended in 15 ml of 10 mM Tris (pH 8.0) and DNase and RNase were added to 200 and 50 μg/ml, respectively. The suspension was passed through a French pressure cell twice at 10,000 lb/in^2, followed by centrifugation at 1,000 × *g* to remove whole cells. The supernatant was decanted, and additional DNase and RNase were added to 200 and 50 μg, respectively, followed by incubation at 37°C for 1 h. The suspension was then sonicated for 1 min (two 30-s bursts) with a Biosonik IV sonicator (Bronwill Scientific Inc., Rochester, N.Y.). A 5-ml sample of 0.5 M EDTA in 0.01 M Tris (pH 8), 2.5 ml of 20% SDS in 0.01 M Tris, and 2.5 ml of 0.01 M Tris were added. After vortexing, the suspension was centrifuged at 50,000 × *g* for 30 min at 15°C. The supernatant was decanted, protease (from *Streptomyces griseus*; Sigma) was added to 200 μg/ml, and the suspension was incubated at 37°C for 12 h. Two volumes of 0.375 M $MgCl_2$ in ethanol was added, and the suspension was cooled to 0°C and centrifuged at 12,000 × *g* for 15 min at 0°C. The pellet was suspended in 0.01 M Tris (pH 8)–2% SDS–0.1 M EDTA, sonicated for 1 min, and incubated in an 85°C water bath for 30 min. After cooling, 2 volumes of 0.375 M $MgCl_2$ in ethanol was added, and the suspension was cooled to 0°C. The suspension was centrifuged at 12,000 × *g* for 15 min at 0°C. The pellet was suspended in 10 ml of 0.01 M Tris (pH 8), sonicated briefly, and centrifuged for 10 min in a tabletop clinical centrifuge. The supernatant was diluted to 25 ml with 0.01 M Tris (pH 8) and centrifuged for 2 h at 42,000 rpm in a Beckman 60Ti rotor. The pellets were washed twice with 0.01 M Tris (pH 8) by centrifugation at 42,000 rpm. The pellet was suspended in 2 ml of 0.01 M Tris (pH 8), protease was added to 100 μg/ml, and the suspension was incubated for 18 h at 37°C. The suspension was centrifuged at 42,000 rpm in a Beckman 60Ti rotor for 1 h. The pellet was washed twice with 0.01 M Tris (pH 8) by centrifugation. The final pellet was suspended in 1 ml of distilled water, and the suspension was stored at −20°C.

SDS-PAGE. SDS-PAGE was performed as described previously (16). Gels were stained with either Coomassie blue in a solution containing 25% methanol and 10% acetic acid or by the silver stain method of Tsai and Frasch (29).

Preparation of antiserum. After prebleeding, white New Zealand rabbits were injected subcutaneously with 100 μg of the isolated LPK-1 MOMP in Freund complete adjuvant. Two weeks after the initial injection, 100 μg of the protein

suspended in Freund incomplete adjuvant was injected subcutaneously. After an additional 2 weeks, rabbits were boosted with 10 μg of the antigen administered intravenously. Serum was collected 5 days after the final challenge. The presence of antibody to the MOMP in the antiserum was assessed with Western blots (28) of isolated *L. pneumophila* outer membranes. This antiserum contained antibody that reacted with a protein that was considerably smaller than the MOMP. Antibody to this protein was removed by absorbing the antiserum with whole cells of an *Escherichia coli* clone which expressed this *L. pneumophila* antigen on its cell surface (Hindahl and Iglewski, manuscript in preparation).

RESULTS

Purification and protein composition of outer membranes. Application of isolated cell envelopes from the *L. pneumophila* serogroups, the other *Legionella* species, and a clinical isolate to sucrose gradients resulted in the separation of this material into four distinct bands. The gradient patterns were identical to that reported earlier for LPK-1, except the uppermost CM-1 band was absent owing to the loading of isolated cell envelopes instead of whole cell lysates onto the gradients (16). The lower two bands representig the OM-1 and OM-2 (16) were pooled and used to characterize the outer membrane proteins. KDO analysis of these two bands confirmed their identity as outer membrane. The amount of KDO in the *L. pneumophila* serogroup outer membrane ranged from 0.019 to 0.029 μmol/mg of protein. These data are in agreement with earlier reports for the KDO content of isolated LPK-1 outer membrane (16). The outer membranes of the other *Legionella* species contained lesser amounts of KDO (0.007 to 0.016 μmol/mg of protein) than the *L. pneumophila* serogroups. The *L. pneumophila* serogroup 1 clinical isolate outer membrane contained 0.022 μmol of KDO per mg protein.

Analysis of outer membranes by SDS-PAGE revealed that a single protein with an apparent molecular size of 29,000 to 30,000 daltons was the MOMP in all *L. pneumophila* serogroups and the clinical isolate (Fig. 1). The major outer membrane protein from serogroups 1 through 8 and the clinical isolate all required heating in the presence of a reducing agent (2-mercaptoethanol) to be resolved as monomers. The MOMPs did not appear to enter the gel as a recognizable band when outer membranes were solubilized at 100°C for 5 min in the absence of a reducing agent (data not shown). A protein of this size was not observed in any of the outer membrane preparations from species other than *L. pneumophila*, with the exception of the *L. micdadei*. The outer membrane from this organism contained three protein species with molecular sizes of approximately 30K, 38K, and 39K (Fig. 1, lane p). The proportion of any one of these proteins relative to that represented by the MOMP of the various *L. pneumophila* serogroups was noticeably less.

Owing to the relative abundance of the 29K to 30K protein in the outer membrane profiles of the *L. pneumophila* serogroups and the lack of a similar protein in the other *Legionella* species, the silver stain method of Tsai and Frasch (29) was employed. The silver staining was performed with and without prior treatment of the gel with periodic acid. Staining the *L. pneumophila* serogroups in this fashion allowed for the detection of differences in the outer membrane profiles not observed with the less sensitive Coomassie blue staining (Fig. 2). Although the higher molecular size range (i.e., greater than 30K) of the gel showed

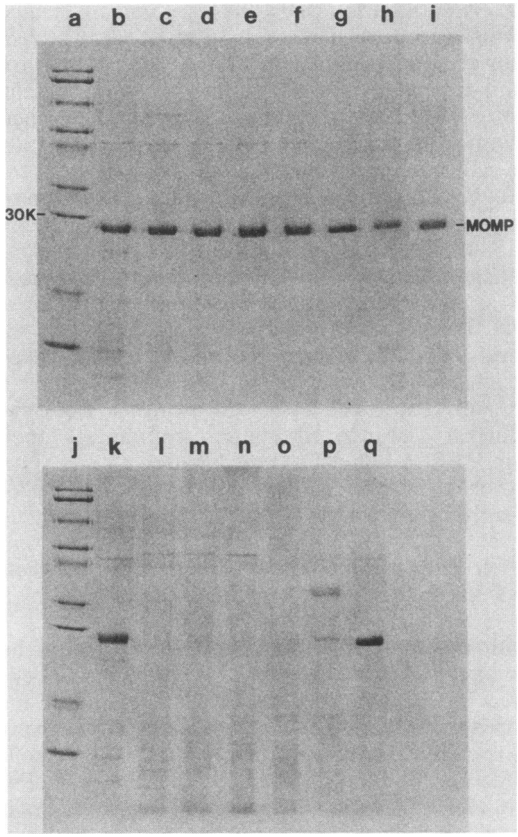

FIG. 1. SDS-PAGE profiles of outer membranes from *L. pneumophila* serogroups and other *Legionella* species stained with Coomassie blue. Samples were solubilized in buffer containing 2-mercaptoethanol at 100°C for 5 min, and 10 μg of membrane protein was loaded per lane. Lanes: b through i, *L. pneumophila* serogroups 1 through 8, respectively; k, *L. pneumophila* serogroup 1; l, *L. bozemanii*; m, *L. dumoffii*; n, *L. gormanii*; o, *L. longbeachae*; p, *L. micdadei*; q, *L. pneumophila* clinical isolate. Lane a contained the following molecular size standards: beta-galactosidase (130K), phosphorylase B (93K), bovine serum albumin (68K), glutamate dehydrogenase (50K), ovalbumin (43K), lactate dehydrogenase (36K), carbonic anhydrase (30K), soybean trypsin inhibitor (21K), and lysozyme (14K).

a great deal of similarity between the *L. pneumophila* serogroups, striking differences were observed in the lower-molecular-weight range of the gel. Heavily staining areas in the 20K to 30K region of the gel clearly distinguished the serogroup outer membranes from one another. These distinctive patterns were greatly enhanced after pretreatment of gels with periodic acid (Fig. 2, lanes b through i), indicating that a carbohydrate moiety (i.e., LPS) was responsible for these areas of heavy stain deposition. In addition, the MOMP in all serogroups did not stain with this method when the gel had been exposed to periodic acid before staining. Similarly, oxidation of gels containing outer membranes from species other than *L. pneumophila* before staining revealed distinctive heavily staining patterns for each organism. Several of these patterns had ladder like appearances suggestive of LPS (Fig. 2A, lanes l through p). Each of these outer membranes revealed a characteristic pattern that allowed for differentiation between the strains tested; collectively, these patterns were much different than those of the *L. pneumophila* serogroups.

When the outer membranes from a clinical isolate were

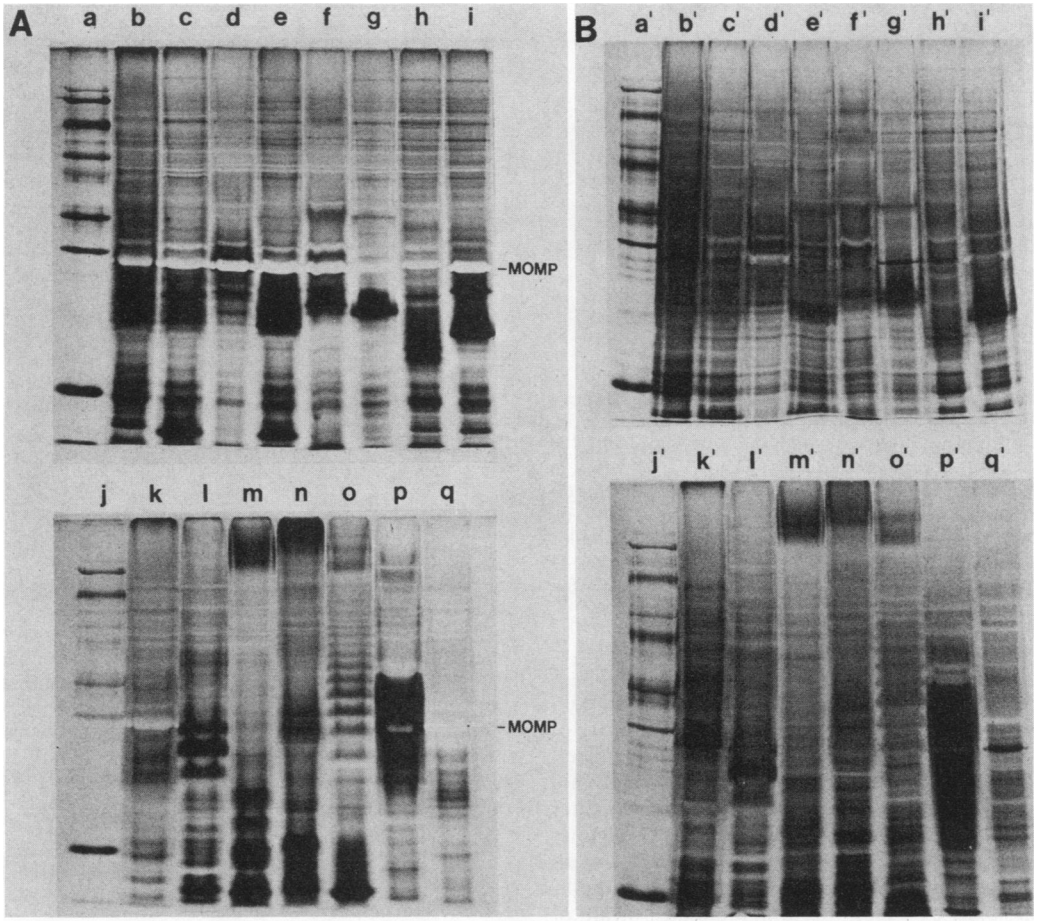

FIG. 2. Silver staining of SDS-PAGE outer membrane profiles. The solubilization conditions, amount of membrane protein applied, and order of loading the gels were as described in the legend to Fig. 1. Gels were fixed overnight in a solution of 40% ethanol–5% acetic acid and were oxidized for 5 min in a solution containing 0.7% periodic acid before silver staining. Lanes: a through q, gels treated with periodic acid before staining; a′ through q′, gels, with no pretreatment before staining.

examined by either Coomassie blue or silver staining, the pattern of staining was very similar to *L. pneumophila* serogroup 1, including the lack of silver staining of the MOMP after periodate oxidation (Fig. 1 and 2A, lanes q).

Although equivalent amounts of protein were loaded onto the SDS-PAGE gels, seemingly less protein was observed for the outer membranes from other *Legionella* species relative to *L. pneumophila* outer membranes (Fig. 1). This result could be a reflection of a greater number of protein species in the total membrane protein. Furthermore, when compared with *L. pneumophila* outer membranes in Fig. 2B (lanes b′ through i′), a larger proportion of the total membrane protein from species other than *L. pneumophila* appeared to be localized in the high- and low-molecular-weight regions of the gel (lanes l′ through p′).

Isolation of the LPK-1 MOMP. Solubilization of cell envelopes in buffer containing SDS resulted in the recovery of insoluble material (i.e., crude PG) with which the MOMP was associated. Subjecting the SDS-insoluble material to elevated temperatures in the presence of reducing agent followed by centrifugation resulted in the release of the MOMP in a soluble form. The vast majority of the proteinacious material released from the SDS-insoluble material when analyzed by SDS-PAGE was that of the MOMP. Fractions collected after application of the solubilized MOMP to gel filtration chromatography in the presence of SDS were analyzed by SDS-PAGE (Fig. 3). Using this criterion to assess the degree of homogeneity of the fractions, those with a retention coefficient of 0.69 were consistently found to be highly enriched in the MOMP. Scanning densitometry of gels stained with Coomassie blue revealed that the amount of contaminating material in this fraction was less than 0.1%. To further examine the purity of these preparations, fractions with a retention coefficient of 0.69 were subjected to SDS-PAGE; after periodic acid oxidation, gels were examined by the silver stain method of Tsai and Frasch (29). A fraction with a retention coefficient of 0.69 stained by this method is shown in Fig. 4 (lane b). The results of this procedure revealed the presence of ladder like bands suggestive of LPS in association with the isolated MOMP.

Isolation of LPS. Because LPS appeared to be associated with the MOMP, the LPS was isolated from LPK-1 to use as a basis for comparing silver-stained SDS-PAGE gels containing outer membranes and the isolated MOMP. The LPS isolation method of Darveau and Hancock (7) was employed because we were unable to recover LPS from hot phenol extractions of whole cells. In spite of rigorous proteolytic treatments (see Materials and Methods), the LPS remained

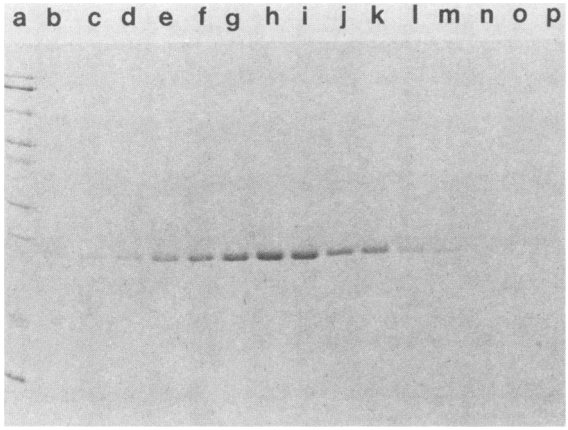

FIG. 3. Gel filtration fractions of material released from the crude peptidoglycan of *L. pneumophila* serogroup 1 by heating in the presence of SDS and reducing agent (see Materials and Methods). A highly purified form of the MOMP was identified by SDS-PAGE in fractions with a retention coefficient of 0.69 (lane k). Lanes: a, molecular weight standards; b through e, fractions 44 through 50, respectively; f through j, fractions 52 through 60, respectively; k through p, fractions 62 through 72, respectively.

contaminated with some protein. The isolated LPS contained 0.072 μmol of KDO per mg of protein. The majority of associated protein was the MOMP (Fig. 4, lanes d and e). Extraction of the LPS recovered by this method with phenol failed to remove the MOMP from the LPS, thereby further emphasizing the tight association between these two molecules (data not presented). These findings are in agreement with those of Gabay and Horwitz, who reported similar results for the isolated LPS from the Philadelphia 1 strain of *L. pneumophila* serogroup 1 (12) and confirmed the fact that LPS was associated with the isolated LPK-1 MOMP.

Western blot analysis. We utilized the isolated MOMP to generate antibodies to probe the antigenic similaries or lack thereof between the serogroup 1 MOMP-LPS complex and the outer membranes of the various *L. pneumophila*

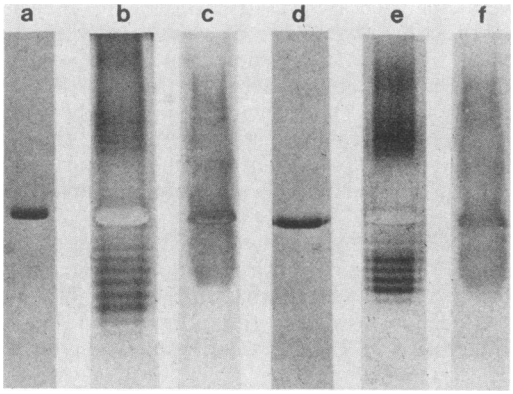

FIG. 4. Isolated MOMP of *L. pneumophila* serogroup 1. The isolated MOMP (retention coefficient, 0.69) and LPS were examined with SDS-PAGE by staining gels with Coomassie blue or with silver after periodate oxidation. Western blots of these fractions were reacted with anti-MOMP antiserum. Lanes: a, MOMP stained with Coomassie blue; b, MOMP stained with silver; c, Western blot of MOMP; d, LPS stained with Coomassie blue; e, LPS stained with silver; f, Western blot of LPS.

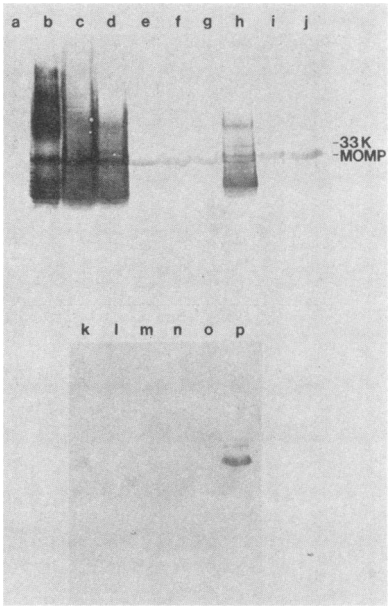

FIG. 5. Western blots of outer membranes from *L. pneumophila* serogroups and other *Legionella* species. Outer membranes were subjected to SDS-PAGE, electrotransferred to nitrocellulose paper, and incubated with antiserum directed against the MOMP from *L. pneumophila* serogroup 1. Lanes: a, molecular weight standards; b, purified *L. pneumophila* serogroup 1 MOMP; c through j, outer membranes from *L. pneumophila* serogroups 1 through 8, respectively; k through o, outer membranes from *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, and *L. micdadei*, respectively; p, clinical isolate of *L. pneumophila* serogroup 1. Samples were loaded onto gels at 10 μg of membrane protein per lane.

serogroups and other *Legionella* species. Absorbed serum from rabbits challenged with the LPK-1 MOMP was examined for antibody to the antigen by the Western blot technique (28). Antibodies directed against both the isolated LPK-1 MOMP and the LPS (Fig. 4, lanes c and f) associated with this protein were detected by using absorbed antiserum at a working dilution of 1:1,000. The absorbed antiserum to the LPK-1 MOMP was utilized to examine the outer membranes from the remaining *L. pneumophila* serogroups and other *Legionella* species. The absorbed antiserum reacted with a broad area of the outer membranes from *L. pneumophila* serogroups 1 and 2 (Fig. 5, lanes c and d) that corresponded to the LPS associated with the MOMP (Fig. 4, lanes c and f; Fig. 5, lane b). The only other serogroup that exhibited this type of smearing was serogroup 6 (Fig. 5, lane h), although the intensity of the reaction relative to serogroups 1 and 2 was slightly less. The anti-MOMP antiserum reacted with the MOMP from all of the serogroups, including that from the clinical isolate (Fig. 5, lane p). However, the LPS of the clinical isolate (serogroup 1) did not react with this antibody. A membrane component with a molecular size similar to that of the MOMP that reacted with the absorbed antiserum was not detected in the outer membranes from the other *Legionella* species. An outer membrane protein with a molecular size of 33K was found to cross-react with the anti-MOMP antibody. This result could be due to contamination of the antigen preparation with this component. This seems unlikely, since scanning densitometry and silver staining of gels containing fractions used as antigen indicated that, although the MOMP

was clearly associated with LPS, additional proteins were not present. However, trace quantities of this component may have been present in the preparation that were highly immunogenic, or the LPS may have masked its presence. Prebleed serum did not react with outer membranes or the isolated MOMP.

DISCUSSION

The protein profiles of the outer membranes from *L. pneumophila* serogroups and the other *Legionella* species as determined by SDS-PAGE were similar to those reported in previous study of sodium lauryl sarcosinate-insoluble proteins from various *L. pneumophila* serogroups and other *Legionella* species (8). Treatment of cell envelopes with sodium lauryl sarcosinate has been shown to selectively solubilize the cytoplasmic membrane from *E. coli* (10). However, owing to the distant taxonomic relationship of *L. pneumophila* with the *Enterobacteriaceae* (5), we felt that mechanical disruption of cells followed by density gradient centrifugation would yield preparations containing the most accurate representation of the outer membrane proteins. Comparison of our results and those of Ehret et al. (8) indicates that solubilization of cell envelopes with detergent results in preparations yielding SDS-PAGE protein patterns similar to those seen for isolated outer membranes when visualized by Coomassie blue staining. The effect of this detergent on the silver staining patterns is unknown, because this staining method was not utilized in the previous study. The outer membranes from all of the *L. pneumophila* serogroups could be easily distinguished from those of the other *Legionella* species by the presence of the abundant disulfide-stabilized MOMP in the former and its absence in the latter. The major differences observed in the SDS-PAGE profiles of outer membranes from all serogroups and species stained with silver after periodate oxidation may be a reflection of LPS present in the membranes. These results allowed for further differentiation among outer membrane profiles of these organisms.

Absorbed antiserum to the LPK-1 MOMP contained antibody that reacted with the MOMP from all serogroups, suggesting a similarity in composition among these proteins. A cross-reactive component with a similar molecular size was not observed in the species other than *L. pneumophila*, thereby further distinguishing the *L. pneumophila* serogroups from these organisms. Gosting et al. (13) have identified a species-specific 29K antigen in *L. pneumophila* by using a monoclonal antibody to probe Western blots of whole cell lysates from various serogroups and species within the genus *Legionella*. These investigators speculated that this antigen was the MOMP from these organisms, and our results appear to confirm this suggestion. Owing to its similarity in size and recognition by antibody, the MOMP of *L. pneumophila* would appear to be an unlikely candidate for the serogroup specific antigen, which is in agreement with suggestions that serogroup specificity may be determined by a carbohydrate moiety (11, 20). The LPS associated with the LPK-1 MOMP isolated in this study proved to be highly immunogenic. We found that absorbed antiserum that reacted with the LPS from serogroup 1 reacted similarily with serogroup 2 only and to a lesser extent with serogroup 6, lending further support to the consideration that a carbohydrate moiety (i.e., LPS) may be the serogroup-specific antigen. Owing to the requirement of heat and reducing agent to resolve the MOMP as a 29K monomer, the aggregate form of this protein with associated LPS may be a major constituent of the high-molecular-weight antigen described by Johnson et al. (20).

The clinical isolate used in this study was originally identified outside of our laboratory as belonging to serogroup 1, yet the LPS of the clinical isolate failed to react with the antibody generated against the LPK-1 MOMP-LPS complex (Fig. 5, lane p). This is not necessarily surprising in light of reports that have identified several subtypes within *L. pneumophila* serogroup 1 (21, 31, 33). Based on the results we report here, subtleties in LPS composition may account for subtype differentiation between these two organisms.

Several of our observations conflict with those of a recent report by Butler et al. (6), who describe a MOMP common to 9 of 10 *Legionella* species examined (including five of the other *Legionella* species in this study) that has an apparent molecular size of 24K. With antibody generated against the purified 24K MOMP, cross-reactivity to the 24K MOMP was demonstrated in 9 of the 10 species examined. LPS associated with the 24K MOMP was not described in that report. The reason for the discrepencies in the molecular size of the MOMP, its occurence in various species, and its association with LPS are unclear at this time, but may result from differences in isolation procedures. Butler et al. reported that small amounts of MOMP could be detected in outer membranes from species other than *L. pneumophila* only after treatment of the membranes with lysozyme (6). This method was not employed in the preparation of outer membranes and the isolated form of the MOMP utilized in this study.

The LPK-1 MOMP remains tightly associated with crude PG after solubilization of cell envelopes in 2% SDS at 100°C for 15 min and does not dissociate from crude PG in high salt (16), a treatment that has been shown to release PG-associated proteins (i.e., porins) in several gram-negative bacteria (23, 27, 32). Yet when we isolated LPS from LPK-1, the MOMP was released in association with the LPS in a soluble form after cell envelopes were extracted with SDS. Therefore, it appears that the MOMP-LPS complex may exist in both a free and a bound form. Heating the crude PG in the presence of reducing agent was the only effective method to release the MOMP from the PG, indicating that at least some of the MOMP may be linked to the PG via disulfide linkage. Amano and Williams reported that trypsin-insensitive proteins are associated with the PG of *L. pneumophila* (1) and that solubilization of isolated PG in 2% SDS and 1% 2-mercaptoethanol released 30% of the PG-associated proteins (2). A similar relationship between protease-resistant proteins and the PG of the intracellular parasite *Coxiella burnetii* has been reported (3, 4). Amino acid analysis of the *L. pneumophila* PG failed to detect the presence of cysteine (1), thereby decreasing the likelihood of a disulfide linkage between the MOMP and the PG. However, PG-associated proteins have been proposed to be associated with outer membrane components (2) which may include the MOMP.

The strain of *L. pneumophila* used by Amano and Williams (Philadelphia 2) was maintained by passage through fertile hen eggs (1). The strains used in this study had all been grown on laboratory medium in the absence of eucaryotic cells (i.e., in an extracellular state). Structural differences in the cell envelope, including the PG composition, may be associated with the difference in maintenance of these organisms. Previous studies have shown that the cell envelope proteins of elementary bodies from the intracellular parasite *Chlamydia psittici* are solubilized in SDS only after exposure to a reducing agent (15). In addition, unique

differences in the protein composition of outer membranes from the elementary bodies and reticulate bodies of this organism have been described. Cysteine-rich outer membrane proteins and the MOMP require solubilization in the presence of reducing agent for resolution via SDS-PAGE in the extracellular, infective elementary body form. The comparative amount of these cysteine-rich proteins is greatly reduced in the metabolically active, intracellular reticulate body outer membranes, and the majority of the MOMP does not require a reducing agent for solubilization (14). These observations indicate that regulation of disulfide linkage in the outer membrane proteins of *C. psittici* may play a crucial role during the transition from the extracellular to the intracellular environment (24). Also, cysteine deprivation has been shown to greatly reduce the efficiency of conversion from the reticulate body form to the elementary body form (26). In this regard it is curious that L-cysteine is an essential growth requirement of *L. pneumophila* (9). Future comparisons of the outer membrane proteins from intracellular and extracellular forms of *L. pneumophila* may reveal differences similar to those seen in *C. psittici*.

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