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Disulfide-Bonded Outer Membrane Proteins in the Genus *Legionella*

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***Legionella pneumophila* and related species were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for outer membrane proteins. Of the 10 species examined, 9 contained a 24-kilodalton (kDa) major outer membrane protein (MOMP) that was resolvable only when outer membrane material was heated in the presence of 2-mercaptoethanol. Labeling studies with [^{35}S]cysteine indicated that the protein contained cysteine, and disulfide cross-linking of the unreduced complex was demonstrated by labeling with iodoacetamide. The unreduced outer membrane preparation contained peptidoglycan, and after treatment with lysozyme to remove peptidoglycan, a protein complex of 95 kDa was observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol. Reduction of the 95-kDa complex yielded 24-kDa monomers, suggesting that the 95-kDa complex was composed of four subunits. The 24-kDa MOMP from *L. pneumophila* was purified, and antibody produced to this protein cross-reacted with all species of *Legionella* as determined from an immunoblot of a sodium dodecyl sulfate gel. Only serogroup 1 strains of *L. bozemanii* lacked the 24-kDa MOMP and showed no cross-reactivity. These results suggest that the 24-kDa MOMP common to most species of *Legionella* contains a genus-specific epitope.**

Proteins associated with the outer membranes of pathogenic gram-negative bacteria are frequently associated with various mechanisms of pathogenesis. In the case of *Legionella pneumophila* and related species, outer membrane proteins (OMPs) are likely to be involved in a unique phagocytic process (17) as well as in resistance to intraphagocytic killing mechanisms (16). In a preliminary study of the OMPs of *L. pneumophila* by Ehret et al. (4), it was reported that all serogroups possessed a 29-kilodalton (kDa) major OMP (MOMP). This work has recently been confirmed by Hindahl and Iglewski (12), and the gene encoding for this protein has been cloned and expressed in *Escherichia coli* (5). The protein fails to enter sodium dodecyl sulfate (SDS) gels with boiling in the absence of 2-mercaptoethanol (2-ME), and the protein may be associated with the peptidoglycan (12). Peptidoglycan-associated proteins have also been reported in *L. pneumophila* by Amano and Williams (1).

Our interest in this protein is twofold. The protein may be a good antigen for use in developing more specific serological tests, and the disulfide-bonded nature of the MOMP may be associated with the oxygen sensitivity problem recently reported (15, 20) and may therefore be a growth-influencing factor. The present investigation confirms that the MOMP of *L. pneumophila* likely is bound to peptidoglycan and is present in outer membranes in a high-molecular-weight matrix held together by disulfide bonds. The results also indicate that all species surveyed contain a 24-kDa MOMP exhibiting immunologic cross-reactivity with the 24-kDa MOMP of *L. pneumophila*.

MATERIALS AND METHODS

Bacterial strains and cultivation. All *Legionella* strains used in this study were obtained from L. Pine (Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga.) and maintained on *N*-(2-acetamido)-2-aminoethanesulfonic acid (Sigma Chemical Co., St. Louis, Mo.)-buffered charcoal-yeast extract medium (BCYE) (24). All cultures were routinely tested for purity by microscopic examination and by culture on brucella agar. Batch cultures of the various *Legionella* strains were initiated by washing cells from two BCYE agar slants into 1 liter of yeast extract broth containing 0.04% cysteine and 0.025% ferric PP_i. The cultures were incubated statically in flasks for 3 to 5 h at 37°C and then shaken on a gyratory shaker (200 rpm) for an additional 15 to 20 h. Cells were collected from mid- to late-log phase (A_{680}, 0.5 to 0.9; 1-cm cuvettes) and streaked for purity on BCYE and brucella agar plates. The pellets were stored at −20°C.

Preparation of outer membranes. Cells were lysed in a French press, and crude membrane material was obtained by procedures described by Hoffman and Pine (14). The cytoplasmic and outer membrane fractions were separated by a modification of the method of Ito et al. (18). In this procedure, crude membranes were layered onto 15% (wt/vol) sucrose–3 mM EDTA (18 ml) with a cushion of 70% (wt/vol) sucrose–3 mM EDTA (3 ml) and were centrifuged in an SW28.1 rotor at 25,000 rpm for 2 h. A total membrane fraction was collected from the 15%/70% sucrose interface. The outer membrane then was separated from the inner membrane by a step gradient containing five equal-volume fractions of sucrose each containing 3 mM EDTA: 50, 55, 60, 65, and 70%. A band at the 60%/65% interface contained the outer membrane material as judged by the presence of 2-keto-3-deoxyoctonic acid (23) and the absence of NADH dehydrogenase and succinate dehydrogenase activities (13, 14). Protein was estimated by the biuret method with bovine serum albumin as the standard (10).

In a second procedure, outer membranes were obtained by extraction of whole cells with sodium *N*-lauroyl sarcosinate (Sarkosyl; Sigma). By this method, cells from a 24- to 48-h-old BYCE agar slant were suspended in 5 ml of 50 mM Tris hydrochloride (pH 7.2) to an optical density of 0.2, pelleted by centrifugation (10,000 × *g* for 15 min), and disrupted with 2% Sarkosyl in 50 mM Tris hydrochloride (pH 7.2). After 1 h of incubation at 37°C, DNA was sheared by ultrasonic vibration (15 s at a setting of 6 on a model 185 Sonifier Cell Disruptor; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Lysozyme (1 mg/ml) was added, and incubation was resumed for 1 h at 37°C. Unbroken cells were

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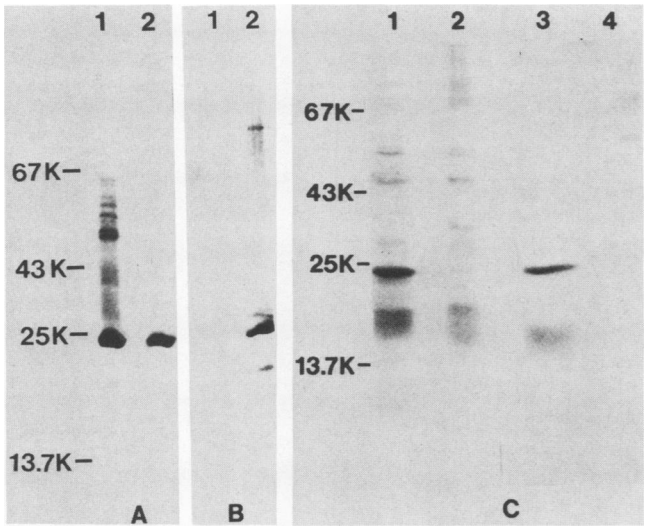

FIG. 1. Characteristics of the MOMP of *L. pneumophila*. (A) SDS-PAGE profiles of OMPs prepared by sucrose density centrifugation (lane 1) and by extraction of whole cells with 2% Sarkosyl (lane 2). Both preparations were heated in SDS containing 2-ME as described in the text. (B) Autoradiograph of the SDS-PAGE profiles of *L. pneumophila* OMPs reacted with [^{14}C]iodoacetamide. Sarkosyl-treated outer membranes were reacted with [^{14}C]iodoacetamide without (lane 1) or with (lane 2) prior treatment with dithiothreitol. (C) Effect of 2-ME on the migration of *L. pneumophila* OMPs in polyacrylamide gels. The cells were grown in the presence of [^{35}S]cysteine and subjected to electrophoresis in the presence (lane 1) or absence (lane 2) of 2-ME. Lanes 3 and 4 are the autoradiograph of the Coomassie blue-stained gel of lanes 1 and 2. Numbers on left show standard molecular weight markers described in the text (67K, 67,000).

removed by low-speed centrifugation, and outer membranes were pelleted by ultracentrifugation at 100,000 × *g* for 30 min, washed once in 2% Sarkosyl, and suspended in the solubilization buffer described below.

SDS-PAGE. Samples were heated for 3 min at 100°C in solubilization buffer containing 62.5 mM Tris hydrochloride (pH 6.8), 2% SDS, 10% glycerol, and 0.005% bromphenol blue. In some experiments, 10% (vol/vol) 2-ME or other reducing agents were added. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out in one dimension at a constant current of 30 mA in 11%, 7.5% to 12%, or 7.5% to 15% gradient vertical slab gels containing 0.1% SDS in the Tris-glycine system as described by Laemmli (19). The gels were stained with Coomassie brilliant blue R-250 by the method of Fairbanks et al. (9). The protein standards used to estimate molecular weight included: bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and RNase A (13,700) (Pharmacia Fine Chemicals, Piscataway, N.J.).

Polyclonal antibody production to MOMP and immunoblotting. The 24-kDa MOMP of *L. pneumophila* was purified from 20 g (wet weight) of packed cells. Details of the purification process and antibody production will appear elsewhere. Briefly, proteins were extracted from Sarkosyl-prepared outer membranes in SDS at 100°C and separated via gel filtration (Sephadex G-75; Pharmacia). Pooled fractions were judged for purity of MOMP by SDS-PAGE. SDS was removed by using a DE-52 (Whatman, Inc., Clifton, N.J.) anion-exchange column. Antiserum was prepared in New Zealand White rabbits by using 1 mg of purified antigen. The titer of the monospecific polyclonal antibody was determined by an indirect fluorescent-antibody assay with reagents obtained from the Centers for Disease Control and by an enzyme-linked immunosorbent assay with goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase. Wells of microtiter dishes (Becton Dickinson and Co., Oxnard, Calif.) were coated with outer membrane material or purified 24-kDa protein by methods generally described by Engvall et al. (6–8).

After SDS-PAGE, proteins were electroeluted onto nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.) by methods outlined by Towbin et al. (27). The monospecific polyclonal antibody was reacted with the blot for 1 h and then was developed with goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase.

Autoradiography. Autoradiography was used to demonstrate cysteine incorporation into proteins of the outer membrane. *L. pneumophila* (Philadelphia 1) was grown in the chemically defined medium of Reeves et al. (26) in which glutathione was omitted and the cysteine concentration was decreased to 0.01%. L-[^{35}S]cysteine (1 μCi/ml; Amersham Corp., Arlington Heights, Ill.) was added to a culture which was incubated while shaking for 24 h. At a final turbidity (optical density at 680 nm) of 0.8, the cells were collected by centrifugation, and outer membranes were prepared by the Sarkosyl procedure. After electrophoresis, the stained and dried gels were subjected to autoradiography with Kodak DEF-5 X-ray film without a screen.

Alkylation of sulfhydryl groups with [^{14}C]iodoacetamide. A modification of a method described by Hatch et al. (11) was used for alkylation of the free thiol groups. Sarkosyl-prepared outer membranes of *L. pneumophila* (Philadelphia 1) were suspended in two portions (150 μl) of solubilization buffer consisting of 0.1 mM Tris hydrochloride (pH 8.0), 2% SDS, and 10% glycerol. Dithiothreitol (final concentration, 3.8 mM) was added to one part, and both samples were heated to 100°C for 3 min. The preparations were cooled to 4°C and incubated for 1 h with [^{14}C]iodoacetamide (1.56 Ci/mol; 8 mM; Amersham). The reactions were stopped by the addition of excess 2-ME (10%, vol/vol) and by heating to 100°C for 3 min. The acetylated preparations were subjected to SDS-PAGE and autoradiography.

RESULTS

Disulfide-bonded nature of the MOMP. The SDS-PAGE profile of proteins associated with the outer membrane of *L. pneumophila* is shown in Fig. 1A. Similar protein profiles were observed for outer membrane material prepared by sucrose density centrifugation of cells lysed in a French press and by extraction of whole cells with 2% Sarkosyl. A 24-kDa MOMP was observed when outer membranes were prepared by either method. Outer membranes prepared by cell fractionation and separated on the basis of density contained several proteins in addition to the MOMP, whereas extraction with Sarkosyl yielded preparations that consisted almost exclusively of the MOMP. Extraction of whole cells with 2% SDS yielded a PAGE protein profile similar to that seen when outer membranes were prepared with Sarkosyl. The additional bands seen in the gradient-prepared outer membranes may represent contaminating cytoplasmic membrane proteins or OMPs extracted by Sarkosyl and SDS.

The PAGE profile of Sarkosyl-prepared outer membranes of *L. pneumophila* grown in the presence of [^{35}S]cysteine is shown in Fig. 1C. Radioactivity was incorporated into several minor proteins and the MOMP, indicating that these proteins contain cysteine and have the potential to form disulfide bonds. A similar result was obtained when *L.*

pneumophila was grown in the presence of [^{14}C]cysteine; however, no radioactivity was incorporated into proteins when the organisms were grown in the presence of [^{35}S]cystine (not shown). The MOMP failed to enter SDS gels unless the membrane preparations were heated to 100°C in SDS containing 10% (vol/vol) 2-ME, 10 mM dithiothreitol, or 10 mM dithionite, suggesting that the MOMP is extensively cross-linked by disulfide bonds. Incubation of the outer membrane preparations for 1 h at 37°C in SDS containing 2-ME or for 3 min at 100°C in SDS containing 10 mM glutathione or 10 mM cysteine failed to dissociate the MOMP complex to monomers.

When outer membranes were incubated with the sulfhydryl-reactive agent [^{14}C]iodoacetamide, radioactivity was associated with the MOMP only when the outer membranes were pretreated with dithiothreitol, further confirming the disulfide-cross-linked nature of the MOMP complex (Fig. 1B). Finally, the cross-linked MOMP complex appeared to be a feature of intact organisms and not the result of spontaneous formation of intramolecular disulfide bonds during cell disruption, since the high-molecular-weight complex was observed whether or not outer membranes were prepared in the presence of 25 mM iodoacetamide to block free sulfhydryl groups.

Association of MOMP with peptidoglycan. Our attempts to determine the molecular weight of the outer membrane complex were initially unsuccessful. Results from composite gel electrophoresis (22) suggested that the molecular weight was in excess of 800,000. However, when Sarkosyl-prepared outer membranes were treated with lysozyme and subjected to electrophoresis in the absence of reducing agents, an intensely staining band of an average molecular weight of 95,000 was observed. Electrophoresis of the lysozyme-treated preparations under reducing conditions resulted in the disappearance of the 95-kDa band and the appearance of the 24-kDa MOMP band (Fig. 2). A second Sarkosyl wash step was useful for removing excess lysozyme from the preparation.

OMPs of selected *Legionella* species. Since the other *Legionella* species share a variety of similarities with *L. pneumophila*, we decided to examine them for similar disulfide-bonded OMPs (Fig. 3). Although all of the outer membrane preparations shown were obtained from an equal number of cells, the preparations contained a widely variable amount of protein, and it is conceivable that Sarkosyl extracted a significant proportion of the OMP from several species. Of the 10 species, 9 possessed a 24-kDa protein which required treatment with 2-ME for optimum entry into the gel. The lack of a 24-kDa protein in *L. bozemanii* (WIGA) led us to examine other strains and serogroups of this species. Although not shown, all serogroup 1 strains examined by SDS-PAGE (3) exhibited three minor bands between 26 and 30 kDa, whereas serogroup 2 (*L. bozemanii* Toronto) possessed a 24-kDa MOMP and two additional bands similar to those seen with serogroup 1 strains. *L. oakridgensis* exhibited a 26-kDa band intensified by treatment with 2-ME and a 39-kDa band which was not modified by treatment with reducing agent. In addition to a 24-kDa protein, *L. micdadei* (HEBA) possessed an intensely staining 35-kDa protein which did not enter the gel in the absence of 2-ME. All species exhibited minor protein bands at 56, 48, and 14 kDa which were not modified by the reducing agent.

All *Legionella* species with the exception of serogroup 1 strains of *L. bozemanii* (strain WIGA shown) possessed MOMPs that cross-reacted with polyclonal antibody against the 24-kDa MOMP prepared from *L. pneumophila* (Fig. 4).

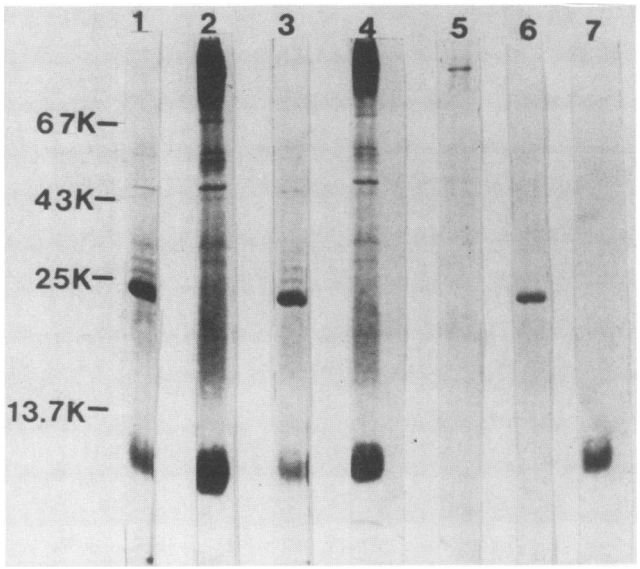

FIG. 2. SDS-PAGE profiles of lysozyme-treated outer membranes of *L. pneumophila*. Sarkosyl-prepared outer membrane material was treated with lysozyme before washing (lane 1, in the presence of 2-ME; lane 2, without 2-ME) and after washing (ultracentrifugation step two; lane 3, with 2-ME; lane 4, without 2-ME). Material in lanes 5 (without 2-ME) and 6 (with 2-ME) was not treated with lysozyme, and lane 7 contains lysozyme only. The 24-kDa MOMP is only present in lanes for which samples were reduced with 2-ME. The 95-kDa material is seen only in preparations treated with lysozyme. The low-molecular-weight material (13,000) is residual lysozyme. Numbers on the left are defined in the legend to Fig. 1.

Interestingly, the 24-kDa MOMP from the Toronto strain of *L. bozemanii* (serogroup 2) also exhibited cross-reactivity (immunoblot not shown). Minor bands, including one with a relative molecular weight of 26,000 in *L. bozemanii* (both serogroups), were seen in several species. Some cross-reactivity was also seen with a high-molecular-weight complex that may correspond to the 95-kDa protein observed when *L. pneumophila* was treated with lysozyme in the absence of 2-ME. Molecular weight markers and OMPs from *E. coli* did not cross-react.

DISCUSSION

Several researchers have recently demonstrated that the MOMP of *L. pneumophila* has a molecular mass between 24 and 29 kDA (4, 5, 12). The differences in reported molecular weight may be a function of methods of outer membrane preparation, since investigators using HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma) buffer report a 29-kDa protein (4, 12), whereas those using Tris buffer report a 24-kDa protein (5). Regardless of the apparent molecular weight, it is generally agreed that the MOMP may be bound in a macromolecular complex by disulfide bonds (12). In support of this hypothesis, we demonstrated that labeled cysteine is incorporated into MOMP and that strong reducing agents, but not glutathione or cysteine, will dissociate the complex to monomers. More rigorous evidence was provided by the observation that [^{14}C]iodoacetamide reacted with the MOMP only if *L. pneumophila* outer membranes were first reduced with dithiothreitol. Furthermore, the cross-linked complex appears to occur naturally in outer membranes, since inclusion of the sulfhydryl-alkylating agent iodoacetamide during prepara-

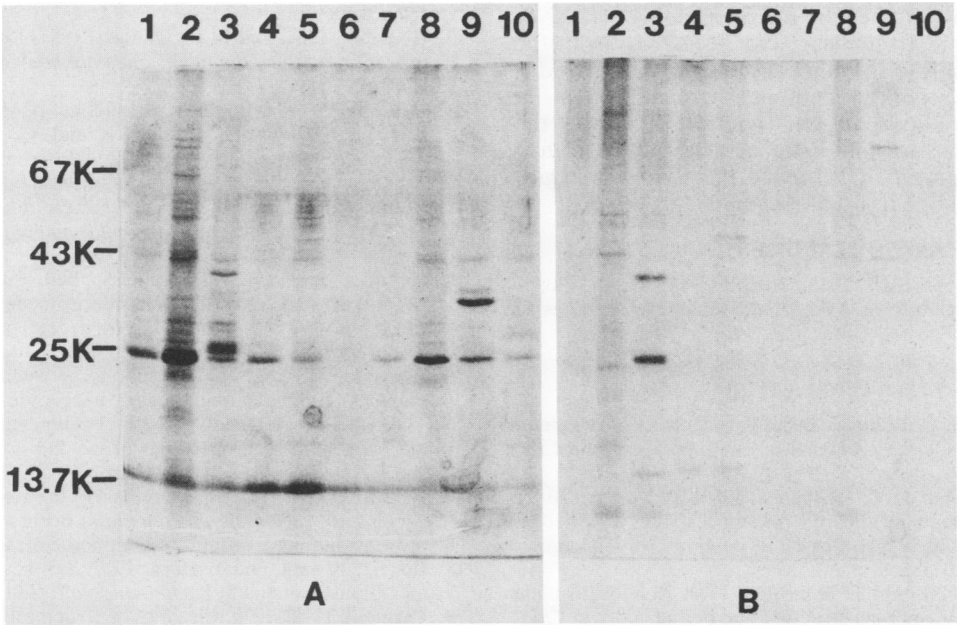

FIG. 3. MOMP of 10 *Legionella* species. Outer membrane material was prepared by Sarkosyl extraction, lysozyme treated, and digested in solubilization buffer in the presence (A) or absence (B) of 10% (vol/vol) 2-ME. The following species were run (lanes): 1, *L. longbeachae*; 2, *L. feeleyii*; 3 *L. oakridgensis*; 4, *L. wadsworthii*; 5, *L. dumoffii*; 6, *L. bozemanii* (WIGA); 7, *L. gormanii*; 8, *L. pneumophila* (Philadelphia 1); 9, *L. micdadei* (HEBA); 10, *L. jordanis*. Numbers on the left are defined in the legend to Fig. 1.

tion of outer membranes did not prevent the appearance of the complex in the preparation.

Hindahl and Iglewski (12) suggested that the 29-kDa protein from the Knoxville 1 strain of *L. pneumophila* was bound to peptidoglycan, since treatment of outer membranes with high salt and heating in SDS failed to solubilize the MOMP. Our observations support this suggestion. Although intact *L. pneumophila* cells are resistant to the action of lysozyme (2), we noted that treatment of nonreduced outer membranes of *L. pneumophila* with lysozyme resulted in the appearance on SDS-acrylamide gels of a 95-kDA complex. This complex disappeared with the concomitant appearance of the 24-kDa MOMP band when the lysozyme-treated preparations were reduced and subjected to SDS-PAGE. Although as yet unproven, the 95-kDa complex may represent a porin consisting of four disulfide-cross-linked subunits of the MOMP. Amano and Williams (1) found a 2-ME-modifiable 39-kDa protein in *L. pneumophila* which apparently is released from peptidoglycan by treatment with lysozyme. It is uncertain whether or not this protein is identical to the 24- to 29-kDa MOMP reported here and by others (4, 5, 12).

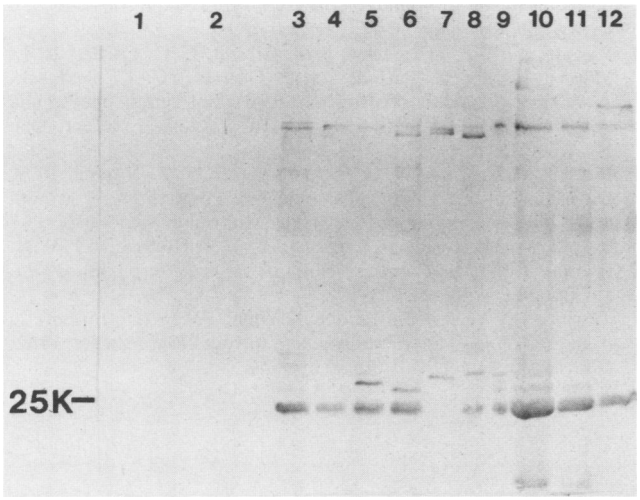

FIG. 4. Immunoblot of 2-ME-reduced MOMP of 10 *Legionella* species. Outer membrane material was prepared as described in the legend to Fig. 3A (reduced with 2-ME) and then, after SDS-PAGE, immunoblotted onto nitrocellulose and treated with monospecific polyclonal antibody prepared to the purified 24-kDa MOMP of *L. pneumophila*. The following species were run (lanes): 2, *E. coli*; 3, *L. micdadei* (HEBA); 4, *L. jordanis*; 5, *L. gormanii*; 6, *L. wadsworthii*; 7, *L. bozemanii* (WIGA); 8, *L. dumoffii*; 9, *L. longbeachae*; 10, *L. feeleyii*; 11, *L. pneumophila* (Philadelphia 1); 12, *L. oakridgensis*. Lane 1 contains the molecular weight standards. 25K, 25,000 molecular weight.

The function of the extensively disulfide-bond cross-linked MOMP complex is unknown. Relatively minor disulfide-cross-linked OMPs have been reported in *Neisseria gonorrhoeae* (22), whereas elementary bodies of *Chlamydia trachomatis* (21). and *Chlamydia psittaci* (11) possess outer membranes in which several proteins, including the MOMP, are extensively cross-linked by disulfide bonds. The MOMP complex of chlamydiae has been shown to have porin activity (3), and it has been suggested that the cross-linked nature of the chlamydial outer membrane lends structural stability to the organism (11, 21). Regardless of the physiological role of the cross-linked MOMP, it is notable that reducing agents such as 2-ME, dithiothreitol, and thioglycolate and oxidants such as hydrogen peroxide and superoxide anions may chemically alter cysteine residues in proteins, thereby inhibiting the growth of legionellae (15, 25). Hydrogen peroxide can oxidize cysteine to cystine (disulfide bond) as well as to cysteic acid and various other compounds (9) that cannot participate in disulfide bonding.

All *Legionella* species, with the possible exception of serogroup 1 strains of *L. bozemanii*, appear to possess a disulfide-cross-linked MOMP of similar molecular weight. Moreover, despite a lack of genetic relatedness between the

species, antiserum prepared to the 24-kDa MOMP of *L. pneumophila* exhibited significant cross-reactivity with the 24- to 26-kDa MOMPs of the other species. Since the 24-kDa protein is present in both virulent and avirulent *Legionella* strains, its role in pathogenesis is unclear. However, the existence of a genus-specific epitope on the MOMP may permit the development of improved diagnostic strategies with monoclonal antibodies that recognize this epitope.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Amano, K., and J. C. Williams.** 1983. Partial characterization of peptidoglycan-associated proteins of *Legionella pneumophila*. J. Biochem. **94**:601–606.
2. **Amano, K.-I., and J. C. Williams.** 1983. Peptidoglycan of *Legionella pneumophila*: apparent resistance to lysozyme hydrolysis correlates with a high degree of peptide cross-linking. J. Bacteriol. **153**:520–526.
3. **Bavoil, P., A. Ohlin, and J. Schachter.** 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. **44**:479–485.
4. **Ehret, W., G. Anding, and G. Ruckdeschel.** 1984. Characterization of membrane proteins from various strains and serogroups of *Legionella pneumophila* and other *Legionella* species, p. 265–268. *In* C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowski (ed.), *Legionella*: Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
5. **Engleberg, N. C., D. J. Drutz, and B. I. Eisenstein.** 1984. Cloning and expression of *Legionella pneumophila* antigens in *Escherichia coli*. Infect. Immun. **44**:222–227.
6. **Engvall, E., K. Jonsson, and P. Perlman.** 1971. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim. Biophys. Acta **251**:427–434.
7. **Engvall, E., and P. Perlman.** 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry **8**:871–879.
8. **Engvall, E., and P. Perlman.** 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. J. Immunol. **109**:129–135.
9. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
10. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
11. **Hatch, T. P., I. Allan, and J. H. Pearce.** 1984. Structural and polypeptide differences between envelopes and infective and reproductive life cycle forms of *Chlamydia* spp. J. Bacteriol. **157**:13–20.
12. **Hindahl, M. S., and B. H. Iglewski.** 1984. Isolation and characterization of the *Legionella pneumophila* outer membrane. J. Bacteriol. **159**:107–113.
13. **Hoffman, P.** 1984. Bacterial physiology, p. 61–67. *In* C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowski (ed.), *Legionella*: Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
14. **Hoffman, P. S., and L. Pine.** 1982. Respiratory physiology and cytochrome content of *Legionella pneumophila*. Curr. Microbiol. **7**:351–356.
15. **Hoffman, P. S., L. Pine, and S. Bell.** 1983. Production of superoxide and hydrogen peroxide in medium used to culture *Legionella pneumophila*: catalytic decomposition by charcoal. Appl. Environ. Microbiol. **45**:784–791.
16. **Horwitz, M. A.** 1984. Interactions between *Legionella pneumophila* and human mononuclear phagocytes, p. 159–166. *In* C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowski (ed.), *Legionella*: Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
17. **Horwitz, M. A.** 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell **36**:27–33.
18. **Ito, K., T. Sato, and T. Uura.** 1977. Synthesis and assembly of membrane proteins in *Escherichia coli*. Cell **11**:551–559.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **207**:680–685.
20. **Locksley, R. M., R. F. Jacobs, C. B. Wilson, W. M. Weaver, and S. J. Klebanoff.** 1982. Susceptibility of *Legionella pneumophila* to oxygen-dependent microbicidal systems. J. Immunol. **129**:2192–2197.
21. **Newhall, W. J. V., and R. B. Jones.** 1983. Disulfide-linked oligomers of the major outer membrane protein of *chlamydiae*. J. Bacteriol. **154**:998–1001.
22. **Newhall, W. J. V., C. E. Wilde III, W. D. Sawyer, and R. A. Haak.** 1980. High-molecular-weight antigenic protein complex in the outer membrane of *Neisseria gonorrhoeae*. Infect. Immun. **27**:475–482.
23. **Osborn, M. J.** 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. **50**:499–506.
24. **Pascule, A. W., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Meyerowitz, C. M. Patton, G. W. Gorman, C. L. Carmack, J. W. Ezzell, and J. N. Dowling.** 1980. Pittsburgh pneumonia agent: direct isolation from human lung tissue. J. Infect. Dis. **141**:727–732.
25. **Pine, L., J. R. George, M. W. Reeves, and W. K. Harrell.** 1979. Development of a chemically defined liquid medium for growth of *Legionella pneumophila*. J. Clin. Microbiol. **9**:615–626.
26. **Reeves, M. W., L. Pine, S. H. Hutner, J. R. George, and W. K. Harrell.** 1981. Metal requirements of *Legionella pneumophila*. J. Clin. Microbiol. **13**:688–695.
27. **Towbin, H., T. Stahelin, and J. Gordon.** 1979. Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.