CHARACTERIZATION OF THE OUTER MEMBRANES FROM *LEGIONELLA PNEUMOPHILA* AND FIVE NON-PNEUMOPHILA SPECIES

by

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ABBREVIATIONS

Ap	Ampicillin
BSA	Bovine Serum Albumin
CM-1	Cytoplasmic Membrane-1
CM-2	Cytoplasmic Membrane-2
CL	Cardiolipin
CTB	Hexadecyltrimethylammonium bromide
CYE	Charcoal Yeast Extract
CYEA	Charcoal Yeast Extract Agar
DAP	Diaminopimelic Acid
DFA	Direct Fluorescent Antibody
EB	Elementary Body
EDTA	Ethylene diaminetetraacetic acid
g/cc	Grams per cubic centimeter
ELISA	Enzyme-linked Immunosorbent Assay
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High Pressure Liquid Chromatography
IM	Intermediate Band
IFA	Indirect Fluorescent Antibody
K	Kilodaltons
Kb	Kilobase
KDO	2-keto-3-deoxyoctonic acid
Λ	Conductance
LDB	Legionnaires' Disease Bacterium
LPK-1	Legionella pneumophila Knoxville-1
LPS	Lipopolysaccharide
µCi	Microcurie
µmole	Micromole
mS	Millisiemens
mV	Millivolt
MOMP	Major Outer Membrane Protein
NADH	Nicotinamide Adenine Dinucleotide
OM-1	Outer Membrane-1
OM-2	Outer Membrane-2
nm	Nanometer
nS	Nanosiemen
pg	Picogram
PBS	Phospate Buffered Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol (manuscript 1 only)
PG	Peptidoglycan
PHB	Polyhydroxybutyrate
RB	Reticulate Body
REL	Readily Extractible Lipid
σ	Bulk Conductance
SDH	Succinate Dehydrogenase
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TLC	Thin Layer Chromatography
TN	Tris-NaCl buffer

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I. Introduction and Statement of the Problem

Legionella pneumophila is a gram-negative bacterium that is one of a growing number of species within the genus *Legionella* that exists as a facultative intracellular parasite. Various species within this genus have been associated with severe, often fatal human pneumonias. *L. pneumophila* has also been shown to survive and multiply within the normally inhospitable environment of human peripheral mononuclear cells and macrophages. In addition, the organism prevents phagosome-lysosome fusion within the phagocyte and is refractory to a wide variety of antibiotics both *in vitro* and *in vivo*. These properties and the preference of the intracellular environment by the organism lead to pulmonary infections that are difficult to diagnose and manage therapeutically. The cell envelope [i.e. cytoplasmic membrane (CM), peptidoglycan (PG), and outer membrane (OM)] and in particular the outer membrane may be related directly to the antibiotic resistance of the organism and/or the ability of the organism to survive intracellularly.

The primary objective of this research project was to characterize the cell envelope of *L. pneumophila* with emphasis on the outer membrane of the organism. *L. pneumophila* serogroup-1 (Knoxville-1) was studied as a prototypical strain to examine the composition of the outer membrane relative to other gram-negative bacteria. The specific aims of this research were as follows:

i. Isolate the outer membrane from L. pneumophila and characterize this moiety by chemical, enzymatic, physical, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses.

ii. Isolate and characterize the major outer membrane protein(s) from L. pneumophila.

iii. Compare outer membranes from L. pneumophila serogroups and five non-pneumophila species by SDS-PAGE analysis.

iv. Establish a genomic library of L. pneumophila in Escherichia coli strain HB101 and identify E. coli clones expressing L. pneumophila antigen(s).

II. Literature Review

A. Legionellosis

Legionella pneumophila or Legionnaire's disease bacterium (LDB) is a gram-negative bacillus that causes a severe, often fatal pneumonia in humans. Prior to 1976, the organism had escaped detection as a recognized bacterium. However, in July of 1976 a localized outbreak of pneumonia in individuals attending the American Legion convention at the Bellevue-Stratford hotel in Philadelphia, Pennsylvania led to the eventual isolation of this pathogen. As it became apparent that the etiological agent involved in this outbreak could not be readily identified by local investigators, a team from the Center for Disease Control (CDC) in Atlanta, Georgia was assigned to the case. Exhaustive epidemiological studies revealed that the mode of transmission of the agent appeared to be airborne, and the source of the agent was the cooling system of the hotel air conditioning system (52). While the source and mode of transmission were determined, the identification of the etiological agent remained a mystery. McDade and co-workers at the CDC finally isolated a bacterium from autopsy lung tissue. The bacterial agent was isolated by injecting autopsy lung material into guinea pigs, removing and pulverizing the spleen from inoculated animals, and introducing this suspension to embryonated chicken eggs. This procedure resulted in the successful growth and microscopic identification of a bacillus that was termed the Legionnaire's disease bacterium. Individuals infected during the Philadelphia outbreak exhibited seroconversion to antigens of the isolated bacterium, thus confirming the identity of the LDB as the causative agent of the disease (100).

Subsequent to its identification and the developement of reliable diagnostic procedures, LDB has been identified as the agent involved in numerous isolated outbreaks of pneumonia and many individual cases in the United States and throughout the world (13). Within the United States the incidence of Legionella related pneumonia appears to be highest in areas east of the Mississippi river (45). While the general population appears to be at risk of contracting Legionellosis, the infection tends to occur most frequently in males, older individuals, immunosuppressed patients, smokers, and heavy consumers of alcohol (21, 52). L. pneumophila has also been shown to colonize individuals with cystic fibrosis (40). Although the above factors appear to predispose individuals to a greater risk of acquiring Legionella pneumonia, accumulating epidemiological data makes it apparent that Legionellosis is a serious pulmonary threat to the population at large.

An individual with Legionellosis typically presents with a sudden onset of chills, high fever, dry cough, and headache. This condition is followed by increasing pulmonary distress to the point where the individual may have difficulty talking (6). The site of infection was at first thought to be localized within the lower respiratory tract. However, 36% of patients infected with the bacterium exhibit gastrointestinal distress in the form of diarrhea and 26% exhibit neurological disorders (88), indicating that a greater diversity of tissues may be affected by the organism itself or products elaborated by the organism. Further evidence for systemic involvement of the organism during infection has been presented by the identification of L. pneumophila in peripheral blood (35). In addition, the organism has been recovered from a surgical wound (16) and direct fluorescent antibody

(DFA) testing has demonstrated the presence of the organism in heart, kidney, spleen (143) and brain tissue (57). L. pneumophila has also been identified in a case of bacterial endocarditis (99) in which pneumonia was not observed.

In contrast to the severe, usually rapid pulmonary infiltration associated with Legionella pneumonia; a second, distinctly different clinical presentation of Legionella infection referred to as Pontiac fever has been described (59,86). This infection is characterized by a sudden onset of chills, fever, and headache, and is self-limiting within 3-5 days. Pneumonia is absent from this type of infection and the attack rate, unlike Legionella pneumonia, is typically independent of pre-disposing factors and is extremely high (>95%) (59). Pontiac fever has been shown to be caused by L. pneumophila serogroups 1 (86) and 6 (133) and by a non-pneumophila species L. feeleii (69). The mechanism(s) by which the same organism can generate such diverse clinical manifestations remains to be elucidated.

A major problem involved with Legionellosis has been the inability to make a rapid and reliable diagnosis of the infection. Originally, the only laboratory method available for diagnosis was the demonstration of at least a four-fold increase in titre between acute and convalescent serum (13). This information meets standards of confirmed diagnosis of Legionellosis as outlined by the Centers for Disease Control, yet is of little use to the physician attempting to manage a rapidly progressing case of fulminant pneumonia, in that often times, three to four weeks are required before the 4-fold titre increase is realized (13). Direct fluorescent antibody (DFA) testing of samples from individuals suspected of Legionellosis has greatly reduced the time required to recognize this

infection. DFA testing has been shown to distinguish between 35 strains of 4 serogroups of L. pneumophila and is able to recognize both a common and a specific antigen displayed by the individual strains (103). The effectiveness of the DFA assay is limited however, to the efficiency with which the organism can be recovered from the patient. The use of sputum samples and transtracheal aspirates from patients infected with LDB that have been tested by DFA has only been moderately successful in recovering identifiable organisms (20, 34). An alternative rapid screening method for the diagnosis of Legionellosis has been developed based on the observation that Legionella specific antigen is detectable in the urine of infected patients (8). Antigen in urine has been detected by enzyme-linked immunosorbent assay (ELISA) (8, 11, 91) and reversed passive hemagglutination (96, 134) assays. The antigen has been detected in urine for as long as 326 days after the initiation of therapy for Legionellosis (91) and as little as 0.2 ng can be detected by ELISA (96) Antigen has also been detected in the serum of patients (91). Although these assays are exquisitely sensitive, their practical application in the typical clinical laboratory is limited. The most widely utilized and best standardized method to date for diagnosis of Legionellosis remains the indirect fluorescent antibody (IFA) test (105).

The members of the Legionellaceae are clearly resistant to a wide variety of antibiotics in vitro (33, 36, 114, 120, 121, 149) and in vivo. Treatment of Legionellosis is restricted to management of the infection with the antibiotic erythromycin (105) and occasionally rifampin or doxycycline (13, 38). Part of the antibiotic resistant nature of these organisms stems from the production of β-lactamase which has been demonstrated in most of the Legionella species examined (19, 97). In

addition, the intracellular environment preferred by the organism may prevent many of the antimicrobial agents from reaching their target sites.

As mentioned, the success of this organism in escaping eradication by the host defense system and/or by antibiotics may in part be related to the fact that the organism is a facultative intracellular parasite. In vitro experiments have shown that Legionella species successfully replicate within HeLa cells and other human cell lines (29), chick embryo cells, Vero cells (123), primate alveolar macrophages (80, 89), and human peripheral blood monocytes (75). The presence of L. pneumophila in the cytoplasm of alveolar macrophages recovered from infected lung tissue has also been demonstrated (24).

The apparent preference of the organism for the intracellular environment of eukaryotic cells raises questions regarding the role of humoral immunity in response to Legionella infections. It is known that patients infected with the bacterium show a rise in IgG and IgM class antibodies that specifically recognize Legionella antigens (114) and the use of IFA testing of serum for detection of Legionellosis is well documented (38, 105). However the importance of humoral immunity in the actual clearance of the organism has been disputed. Using a highly sensitive guinea pig animal model, Eisenstein et al (41) have shown that high titres of anti Legionella antibodies do not protect animals from contracting pneumonia after exposure to aerosolized L. pneumophila. Similar implications have resulted from studies using an entirely different experimental system. The in vitro studies of Horwitz and Silverstein have shown that L. pneumophila specific antibody in the presence of complement is not bacteriocidal for the organism (76). These

studies led to the observation that specific antibody enhanced binding of the organism by monocytes, yet opsonized organisms were no more readily killed by the phagocytes than the untreated controls (77). Finally, monocytes exposed to soluble factors released from activated monocytes in immune serum were much more efficient at preventing the replication of L. pneumophila than unactivated monocytes (78). The mechanism by which the bacterium escapes the antimicrobial actions of the phagocyte are poorly understood. However, it is known that the organism exists in a membrane bound vacuole that is resistant to fusion with lysosomes (74) and that intracellular survival appears to be independent of protein synthesis (79).

These findings (41, 74, 76, 77, 78) indicate that cellular immunity is largely responsible for host defense against Legionellosis. This consideration was addressed in an elegant study of the pulmonary inflammatory response in guinea pigs challenged with aerosols of viable L. pneumophila (30). Briefly, this study revealed a dose-related influx of polymorphonuclear leukocytes (PMNs) immediately following aerosol challenge. Concommitant with this influx of PMNs, the number of recoverable alveolar macrophages was reduced. Three days following the initial challenge, PMN numbers began to drop and macrophage numbers increased until reaching a maximum density 11 days following challenge. The majority of viable bacteria (i.e. 86%, 16 hr post-infection) recoverable from lung lavage were associated with the macrophage population. The bacteria were found to be located within the macrophages and this association was consistent throughout the time course of the experiment. The antibody response relative to the inflammatory response was also monitored and demonstrable antibody was not observed until 7

days following challenge. These observations greatly strengthen the contention that humoral immunity may play a secondary role relative to the celluar defense system in the clearance of Legionella infections.

B. The Genus Legionella

1. Taxonomy

The original criterion utilized to identify L. pneumophila as a new species was an extremely thorough comparison of the extent of DNA homology that this organism shared with a wide variety of other bacteria (19). As mentioned periodically throughout the body of this text, there are different serogroups within the species L. pneumophila. To date, nine distinct serogroups have been identified. (10, 22, 39, 44, 103, 104). Also, numerous serotypes and strains within these serogroups have been identified (84,144, 150)

In addition to L. pneumophila (17), "Legionella-like organisms" (27) (non-pneumophila species) have been isolated bringing the total number of species withing the genus Legionella to 11. These species include L. bozemanii (18), L. dumoffii (18), L. feeleii (69), L. gormanii (107), L. jordanis (26), L. longbeachae (102), L. micdadei (65), L. oakridgensis (116), L. sainthelensi (23), and L. wadsworthii (37).

The continual addition of new serogroups to the species L. pneumophila and species within the genus Legionella has made interpretation of earlier literature relative to all currently known members within the genus Legionella cumbersome. Some investigations have utilized only one serogroup and numerous serotypes within the serogroup, others three serogroups, while others have compared some serogroups with

some non-pneumophila species. Although L. pneumophila can be distinguished from non-pneumophila species by its ability to hydrolyze hippurate (19, 64) the general phenotypic characteristics of all Legionella species are quite similar making typical laboratory differentiation between the various species difficult. All of the above species have been recovered from patients with pneumonia with the exception of L. gormanii, L. jordanis, and L. oakridgensis, and L. sainthelensi. Of these four, only L. oakridgensis and L. sainthelensi have not been serologically associated with human pneumonia (19). The basis of species differentiation has been the extent of DNA relatedness exhibited between these organisms. DNA relatedness among these species ranges from 0 to 46% (19). In addition to these described species, an additional 13 species have been tentatively identified based on DNA-DNA hybridization studies. Eleven of these organisms were environmental isolates and the other two were recovered from cases of pneumonia. As of yet, these species remain unnamed, yet it is clear that the taxonomic status of the genus Legionella is far from complete (19).

2. Growth Conditions

Originally, the major difficulty in isolating L. pneumophila from the Philadelphia outbreak was the fact that the organism did not grow on common laboratory media. Consequently, new growth media were developed to isolate and support the growth of the organism. The first successful medium was a Mueller-Hinton agar supplemented with Isovitalex and hemoglobin (46). The requirement of these additions suggested that exogenous cysteine and iron were necessary supplements. The requirement of cysteine has been confirmed by numerous laboratories and emphasized by

Hoffman (72) who found two key enyzmes in cysteine biosynthesis lacking in L. pneumophila and Reeves who verified the requirement for added iron (122). A GC-FC agar (140), charcoal yeast extract agar (47), and an enriched blood agar (60) were also developed. Broth media were also developed that adequately supported the growth of L. pneumophila. These include a complex medium containing proteose peptone #3 (140), a chemically defined medium (125), and a charcoal yeast extract broth medium (124). An improvement of the charcoal yeast extract medium has been developed that employs the buffer N-2-Acetamido-2-aminoethanesulfonic acid (ACES) (119) and this medium has been routinely employed in most current investigations. The requirement for charcoal in autoclaved yeast extract preparations is not fully understood although it has been suggested that the charcoal acts as a free radical scavenger for hydrodgen peroxide and superoxide generated by the autoclaving process (71). While most of the media described for the growth of Legionella require a pH of less than 7.0, the chemically defined medium of Ristroph (125) indicates that L. pneumophila will grow well at pH = 6.3.

The means by which L. pneumophila is maintained in the laboratory has been found to be critical relative to the state of the virulence of the organism. It has been demonstrated that a culture of L. pneumophila that has been passed repeatedly on Mueller-Hinton agar loses a significant degree of virulence (101). When virulent L. pneumophila is passed repeatedly on Mueller-Hinton agar, plaque formation on chick embryo monolayers and fever and/or death in guinea pigs is greatly reduced (115). Conversely, avirulent organisms can be converted to virulent by infecting human lung fibroblast cultures with avirulent L. pneumophila.

This "attained" virulence has been maintained by the organism throughout twelve passages on charcoal yeast extract agar (147).

3. Ecology

Contrary to its fastidious laboratory medium growth requirments, L. pneumophila has been shown to remain viable in nothing but distilled water for up to 139 days and in tap water for periods up to one year (130). The organism has been isolated from various fresh water lakes and salt water sources in the environment with temperatures as low as 17.0°C. From one of these source, the organism was found in a concentration of 9.6×10^3 cells/ml (51). Also, a number of different Legionella species have been recovered from water sources in the recently devasted area surrounding mount St. Helens (23) indicating that the organism is capable of inhabiting a harsh aqueous environment.

Given the location in which the organism is found (51) and its ability to survive and multiply within eukaryotic cells, efforts have been put forth to determine if a parasitic relationship exists between L. pneumophila and a natural host within the aqueous environment. L. pneumophila appears to be able to utilize extracellular slime produced by the blue-green algae Fischerella sp. as both a carbon and energy source (14, 139). The organism has also been shown to multiply within the freshwater amoebae Acanthamoeba castellanii (73, 129), A. palestinensis (2), and the ciliated protozoan Tetrahymena pyriformis (48). It would appear therefore that the ecological niche of L. pneumophila is water and that the organism may rely on the presence of other organisms for survival within this environment.

As mentioned, localized outbreaks and individual cases appear to be distributed world-wide (13) and the organism has been isolated from a variety of environmental sources (51). This information suggests that the organism may be ubiquitous in nature. This consideration was addressed by examining a small population in Iowa for serum antibody to L. pneumophila serogroups 1 - 4 (68). Prior to this study, local physicians attested to the fact that no abnormal respiratory illness had been observed within the population up to six weeks prior to the study. The results of this study indicated that 23 percent of the 517 subjects tested had serum titres of 1/16 against more than one serogroup of L. pneumophila. This observations supports the suspected ubiquitous nature of the organism. However, the authors of this study point out that the possibility of cross-reacting antigens between L. pneumophila and other bacterial species cannot be discounted. A similar study was performed on various non-human primates. Sixteen different old and new world species of primates were tested for antibody to L. pneumophila. Of the sixteen species tested, all exhibited antibody titres to L. pneumophila (67). These studies in conjunction with observations that the organism is readily recovered from numerous environmental sources lend futher credence to the suggested ubiquity of the organism.

4. Ultrastructure

The ultrastructure of L. pneumophila has been well characterized. The organism has been shown to replicate by nonseptate pinching (13, 66) and the cell envelope is typical of that seen in most gram-negative bacteria. The fine structure of the cell envelope appears to be similar for both in vivo and in vitro cultures (87). Various investigators have

reported the presence of cytoplasmic and outer membrane (24, 61, 66, 87, 126). A peptidoglycan layer has only recently been demonstrated (66) by transmission electron microscopy. These observations agree with the biochemical demonstration of the typical peptidoglycan components diaminopimelic acid, muramic acid, and glucosamine in L. pneumophila (1, 50, 87). A single, polar flagellum and the presences of pili have been observed on the L. pneumophila cell surface (127, 137). Flagella have been isolated from L. pneumophila and appear to be antigenically similar among L. pneumophila serogroups and various Legionella species (43, 128). Furthermore the "tumbling" motion associated with flagellated organisms has been observed microscopically in L. pneumophila by use of a direct fluorescent antibody assay (137). These observations are in agreement with the general property of motility which has been ascribed to the genus Legionella (19). A recent report by Hebert et al (66) has described extracellular material associated with the cell surface of L. pneumophila, L. bozemanii, L. dufmoffii, and L. micdadei that absorbs the stain ruthenium red suggesting an acidic polysaccharide capsule may be present in these organisms. These results are in agreement with an earlier report that identified capsular-like antigens in L. pneumophila using immunological methodology (132). Failure to observe this material in previous investigations may be a reflection of the maintenance conditions of the organisms in the various studies. As discussed previously, the virulence of the organism is greatly dependent on its maintenance history (101, 115, 147). In addition, the state of virulence of the organism has been correlated with the physical length of the organism. L. pneumophila has been shown to exist in varying degrees of pleiomorphism depending on the growth medium. The length of the organism

has been shown to range from 0.3 μm to greater than 10 μm, often forming long filaments, depending on the growth medium. A recent report has established that the shorter forms (x = 1.94 μm) of the organism are approximately 3 logs more virulent than longer forms (x = 9.30 μm) (15). These observations re-emphasize the need for adherence to a convention of culture mainetenance when making comparative studies.

5. Physiology

DNA - DNA homology studies were utilized extensively in comparing the original legionnaires' disease bacterium isolate with other known bacteria before the conclusion was reached that L. pneumophila represented a new species (17). From these studies, the genome of L. pneumophila has been estimated to have a size of 2.5×10^9 daltons and a Guanosine + Cytosine (G + C) content of 39%. As mentioned, DNA homology studies have also been used to determine the identification of many non-pneumophila species which as a group have a G + C content ranging from 39 - 45% (19). Plasmids have been identified from a large number of L. pneumophila species (2, 22, 81, 90, 95, 106, 113) and several non-pneumophila species (3). The size of these plasmids varys greatly (1.9 - 85 megadaltons [Md]) from report to report and as of yet, the function of these plasmids remains cryptic. Similarly, very little is known regarding the genetics of the Legionella. A recent report has demonstrated conjugal transfer of plasmids from compatibility groups C, P, and W by Escherichia coli into L. pneumophila (32). Also, the plasmid RP4 has been transferred by E. coli into L. pneumophila and non-pneumophila species (25). These studies provide the first substantial basis for initiating genetic studies of these organisms.

Clearly much further work is required to clarify the function of the cryptic plasmids in the Legionella and to begin to make inroads toward establishing an understanding of Legionella genetics.

Several laboratories have investigated the metabolism of L. pneumophila. Although several reports have indicated that L. pneumophila utilizes small amounts of glucose (136, 141), carbohydrates are not fermented by the organism (72). Glutamate has been shown to serve as a carbon source and its is generally agreed that amino acids serve as the primary carbon source of the organism (7). Arginine, cysteine, isoleucine, methionine, serine, threonine, tyrosine, and valine are essential for growth (58, 135). A continuation of these investigations revealed that glutamate, serine, threonine and tyrosine contribute greatly to the energy production of the cell (136). Oxidative metabolism of amino acids by L. pneumophila occurs by the tricarboxylic acid cycle (70) and the respiratory apparatus contains cytochromes of the c, b, a, o, and d, types (70, 92). The finding that the metabolism of L. pneumophila is based on amino acid utilization corresponds with the detection of a protease produced by the organism (109, 138). Several non-pneumophila species have also been shown to produce proteases which are immunologically similar (9). The extracellular hemolytic enzyme phospholipase C has also been detected in a number of Legionella species (4, 5). Although the release of both a protease and a phospholipase by the organism clearly would lead to destruction of surrounding tissues in the human host, the contribution, if any, of these factors to the virulence of Legionella is unknown at this time.

An additional extracellular product associated with toxicity for chinese hamster ovary cells has been identified. The toxin is methanol soluble and is stable to both heating and a pH range of 5 - 8 (53). To date, the toxin has been associated with the species L. pneumophila only. It is believed that the toxin contributes to the ability of the organism to survive within phagocytes (54). Lochner et al have demonstrated that L. pneumophila toxin disrupts events leading to the generation of oxygen free radicals in polymorphonuclear leukocytes (PMNs) that are normally anti-bacterial (94).

C. The Bacterial Outer Membrane

The outer membrane is the outer most structure of the gram-negative bacterial cell envelope, which includes the inner or cytoplasmic membrane and the peptidoglycan layer. The outer membrane serves as the initial permeability barrier between the organism and the environment and also serves to enclose the area between the outer and cytoplasmic membrane which is referred to as the periplasmic space. The periplasmic space is the area in which the peptidoglycan layer is located, and is rich in degradative enzymes which process metabolites prior to entry into the cytoplasm and it also contains enzymes associated with drug resistance (i.e. beta-lactamase) (28). Therefore, the outer membrane not only functions as a barrier bewtween the organism and its environment, but serves as a means of containing the compartment between the cytoplasm and the exterior of the cell.

The structural and functional properties of the outer membrane are determined by its chemical composition. Our current understanding of

gram-negative outer membrane organization and function is based primarily on extensive studies of the outer membranes from Escherichia coli and Salmonella typhimurium (148). The outer membrane from these organims has been shown to consist of three major components. These are phospholipid, protein, and lipopolysaccharide (LPS). The first two constituents are typical biomembrane entities, but LPS is essentially found only in gram-negative bacterial outer membranes.

Based on statistical calculations (131), degradation studies (85), and freeze fracture ultrastructural studies (131), a model of the gram-negative outer membrane has been established. The model predicts that the outer half of the bilayer membrane is composed solely of protein and LPS. The inner half of the bilayer is composed of phospholipid and protein only. According to this model, variations in the relative ratios of these three components determines the degree of exclusion of hydrophobic compounds from penetrating the outer membrane (11, 112).

1. Phospholipids

The phospholipid composition of E. coli and S. typhimurium consists primarily of phosphatidylethanolamine, with smaller amounts of phosphatidylglycerol and cardiolipin (117, 142). As described earlier, the amount of phospholipid in the outer membrane is sufficient to only represent the inner face of the outer membrane (131). Hence, protein to phospholipid ratios are useful when comparing cytoplasmic and outer membranes to those of non-enteric outer membranes to determine if these properties fit within the described model of outer membrane architecture.

2. Outer Membrane Proteins

Outer membrane proteins include periperal proteins (non-transmembrane) and integral proteins (transmembrane). In addition to assuming a structural role, these proteins serve a variety of functions. The most abundant proteins found in the E. coli and S. typhimurium outer membranes have molecular weights ranging from 35,000 to 45,000 (7). These proteins, either as a single species, or up to three species form trimers (118) which results in the formation of transmembrane diffusion pores and hence are termed "porins". Porins serve to act as a permeability barrier to hydrophilic molecules and disallow the penetration of molecules exceeding a given molecular weight (31, 112). This exclusion limit varies greatly between enteric bacteria (31) and non-enteric bacteria (63). Porins are readily detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and are useful markers in assessing the success of outer membrane isolation procedures. Numerous additional outer membrane proteins from a large number of gram-negative bacteria have been isolated and their associated functions have been well characterized. However, discussion of the entirety of this group of proteins is beyond the scope of this review. Comparisons between L. pneumophila proteins with those from well characterized bacteria will be discussed in following chapters.

3. Lipopolysaccharide

Lipopolysaccharide (LPS), the third major constituent of the enteric outer membrane, is thought to be ubiquitous in gram-negative bacteria and is unique to gram-negative outer membranes. The molecule consists of three major subunits. These are the distal o - side chain (O antigen)

which typically consists of repeating oligosaccharide subunits, the middle core region which typically contains the distinctive molecule 2-keto-3-deoxyoctonic acid (KDO) and often a heptose sugar; and the lipid A moiety proximal to the outer membrane. The lipid A region typically consists of a glucosamine dissacharide backbone with fatty acids in association by both amide and ester linkages. An alkali stable, acid labile (amide linked) hydroxylated fatty acid is a constituent of lipid A and can usually be utilized as a marker during the isolation of LPS from a variety of bacteria. It should be noted however, that while the chemical composition of enteric LPS is often used for comparative purposes, many non-enteric organisms contain LPS with chemical compositions that vary greatly from the well described molecules from members of the Enterobacteriaceae (145). As discussed, LPS and proteins appear to constitute the entirety of the outer half of the bilayer and therefore represent the exposed surface of the outer membrane. The distal, carbohydrate containing O-side chain presents a hydrophilic surface to the environment and thereby discourages the penetration of the outer membrane by hydrophobic molecules. It has been shown that certain mutant strains of S. typhimurium that lack varying degrees of carbohydrate in the o-side chains and/or core region become sensitive to antibiotics that are "hydrophobic" (110). The hypothesis has been put forth that when the carbohydrate of the LPS molecule is reduced to a certain limit, an inefficiency of protein insertion into the outer membrane results. This disruption in protein insertion in turn results in phospholipid molecules filling the gap created by the missing protein thereby effecting a phopholipid bilayer in the outer membrane. The resultant bilayer allows for the passive diffusion of hydrophobic

molecules into the cell (111). It appears therefore that LPS and proteins determine to a large extent both the hydrophilic and hydrophobic permeability properties of the outer membrane in addition to contributing to the physical integrity of this structure.

D. The L. pneumophila Cell Envelope

As mentioned in the Introduction, the specific aims of this investigation included the characterization of the cell envelope and in particular the outer membrane from L. pneumophila by chemical, physical, enzymatic, and SDS-PAGE analysis. For the purposes of this review, only information regarding the cell envelope that was available at the initiation of this study will be discussed. Relevant reports published during the course of this investigation will of course be discussed within the body of the ensuing manuscripts and in particular within the discussion and summary section.

Information regarding the lipid composition of the various serogroups and non-pneumophila species is lacking. Only the cellular lipid composition of various strains of serogroup 1 from L. pneumophila have been described in detail. The phospholipids from these organisms include phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin in order of decreasing proportion (49). The occurence of phosphatidyl choline in bacterial membranes is very unusual while it is typically a component of eukaryotic membrane systems (93). The fatty acid profile of members of the genus Legionella is distinctive and is routinely used as a means of comparison in taxonomic evaluations of suspected new Legionella species. In L.

pneumophila, greater than 68% of the cellular fatty acids are branched (108) with iso C16:0 being the predominant fatty acid component (49). Information regarding the fatty acids of various strains of L. pneumophila indicate that there is little difference between strains (98, 108). Although earlier studies failed to detect hydroxylated fatty acid(s) (108, 146), Mayberry (98) has presented evidence describing the existence of hydroxy fatty acids in L. pneumophila. The major hydroxy fatty acids of L. pneumophila (3-hydroxy-12-methyltridecanoate, 3-hydroxy-n-eicosonoate, and 2,3-dihydroxy-12-methyltridecanoate) are very unusual when compared to those found in other bacteria (145).

A peptidoglycan layer has been demonstrated by electron microscopy (50, 87) and biochemical analysis of crude peptidoglycan isolated from L. pneumophila serogroup 1 has identified the typical peptidoglycan components muramic acid and glucosamine (87). Diaminopimelic acid, another common constituent of gram-negative peptidoglycan, has also been identified in L. pneumophila (62).

A high molecular weight antigen with chemical characteristics of lipopolysaccharide (LPS) has been isolated from L. pneumophila (82) and shown to be surface localized (42). The high molecular weight antigen described by Johnson et al. (82) was isolated from L. pneumophila by washing cells with phosphate buffered saline. This simple agitation of cells yielded a high molecular weight antigen composed of 35 percent carbohydrate, 2.6 percent protein, 1.8 percent phospholipid, and 1.0 percent KDO. These data suggest that the antigen is largely composed of LPS. Similar studies have demonstrated that the antigen is isolable by washing cells of L. pneumophila with physiological saline and passing the saline extract over a Sepharose 6B column (83). This antigen has been

shown to determine serogroup specificity between L. pneumophila serogroups 1 and 2 (83). Further suggestions as to the existence of LPS or "endotoxin" have been reported. A technique commonly utilized for the isolation of LPS (hot aqueous phenol method) was applied to L. pneumophila (146). Material was recovered that contained KDO, but lacked hydroxy fatty acids. The unusual hydroxy fatty acids described in whole cells of L. pneumophila by Mayberry (98) may have been present but escaped detection by these investigators (146). These findings in conjunction with the detection of KDO in this antigen firmly suggests the presence of LPS in L. pneumophila. Biological properties of LPS include the gelling of limulus lysate, the production of a biphasic fever curve when injected into rabbits, and a necrotic reaction or Schwartzman reaction when introduced to the skin sub-dermally. All of these reactions have been demonstrated with L. pneumophila (55, 56, 82) which is in agreement with the biochemical evidence to suggest the presence of LPS in this organism.

A preliminary report on the L. pneumophila outer membrane has been presented by Flesher et al (50). The outer membrane was isolated by sonication of the organism followed by sucrose density gradient centrifugation (50). The cytoplasmic membrane was found to have a buoyant density of 1.20 g/cc. The outer membrane was shown to have a buoyant density of 1.23 g/cc and contained KDO, a further indication that LPS is a structural component of the outer membrane.

At the beginning of this study, the above information was all that was known regarding the cell envelope from members of the genus Legionella. In particular, information regarding the protein and lipopolysaccharide composition of the outer membranes from these

organisms was noticeably lacking. In view of the important structural and functional roles the outer membrane assumes in the survival of gram-negative bacteria, it was felt that the isolation and thorough chemical analysis of the outer membrane from L. pneumophila serogroups and non-pneumophila species was warranted. Such information would be useful in making intra and inter-species comparisons of these properties within and outside of the genus Legionella. While a study of this nature may not be directly applicable in elucidating the virulence of these organisms relative to the human host, information of this type may lead to further our understanding of the relationship of these organisms and their environment.

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III. Manuscripts

Manuscript 1

Isolation and Characterization of the Outer Membrane from *Legionella pneumophila*

ABSTRACT

A whole cell lysate of Legionella pneumophila was fractionated into five membrane fractions by sucrose gradient centrifugation. Membranes were characterized by enzymatic, chemical, and SDS-PAGE analysis. Two forms of cytoplasmic membrane (CM-1, CM-2), a band of intermediate density (IM), and two forms of outer membrane (OM-1, OM-2) were detected. The CM-1 fraction was the purest form of cytoplasmic membrane and fraction CM-2 was primarily cytoplasmic membrane associated with small amounts of peptidoglycan. The IM, CM-1, and CM-2 fractions were enriched in PG and the amount of carbohydrate and 2-keto-3-deoxyoctonic acid (KDO) was not appreciably greater in outer membrane relative to cytoplasmic membrane. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were found to be the major phospholipids in the membrane fractions. The major outer membrane proteins had molecular weights of 29,000 and 33,000 daltons and were both modified by heating. The 29,000 dalton protein was tightly associated with the peptidoglycan and was equally distributed in the IM, OM-1, and OM-2.

INTRODUCTION

Legionella pneumophila is a gram-negative bacillus that is sensitive to the hydrophobic antibiotic erythromycin (27). This general observation suggests that the outer membrane of L. pneumophila may present a hydrophobic surface to the environment. Recent studies on the crystal violet uptake and hexadecane affinity of L. pneumophila have shown that the L. pneumophila cell surface is distinctly more hydrophobic than wild type Salmonella cholerasuis (4). The functional properties of the outer membrane, including the degree of susceptibility to hydrophobic antibiotics, are determined by the chemical composition and relative amounts of protein, phospholipid and lipopolysaccharide (LPS).

Ultrastructure studies have revealed that the cell envelope of L. pneumophila is typical of gram-negative bacteria in that an inner or cytoplasmic membrane, a peptidoglycan layer, and an outer membrane are present. (10,29). However, with the exception of the recent report of Amano and Williams (1) on the peptidoglycan layer, information regarding the chemistry of the L. pneumophila cell envelope is limited. The peptidogylcan of L. pneumophila contains muramic acid, glucosamine, and meso-diaminopimelic acid (DAP) with 80-90% of the DAP residues cross-linked. Sodium hydroxide hydrolysis is required to release protease resistant proteins associated with the peptidoglycan, and although lysozyme effectively hydrolyzes the glycan backbone, the integrity of the peptidoglycan sacculus remains intact (1).

Preliminary reports have indicated that L. pneumophila outer membrane material can be isolated by treatment of the organism with ethylenediaminetetraacetic acid (EDTA) - lysozyme or extraction with EDTA

(10, 11). Lipopolysaccharide (LPS) has yet to be isolated from L. pneumophila although Wong et al. (38) characterized material from the organism that had properties characteristic of endotoxin. Similarly, Johnson et al. (15) have isolated a high molecular weight antigen (F-1 antigen) with a chemical composition indicative of the presence of LPS.

Considering the lack of detailed information available, we undertook a study of the L. pneumophila outer membrane. Information from such a study would be useful in assessing the molecular architecture of the outer membrane and aid in understanding the susceptibility of the organism to various antibiotics. Here we report the isolation and chemical characterization of the L. pneumophila outer membrane.

MATERIALS AND METHODS

Organism and Culture Conditions

L. pneumophila Knoxville 1 strain was obtained from Jay Hammel, Hahneman Medical College, Philadelphia, PA. The organism was passaged greater than 20 times on Charcoal Yeast Extract Agar (CYEA) (28) and maintained in Charcoal Yeast Extract broth (CYE) (28) containing 20% glycerol at -70°C. Stock cultures were streaked onto CYEA and incubated at 35°C in a 5% CO_2 incubator for 48 h. Purity of the culture was routinely assessed microscopically and by the use of fluorescent antibody provided by the Centers for Disease Control, Atlanta, GA. Large scale cultures were prepared by inoculating 50 ml CYE broth with growth from 48 h CYEA plates followed by incubation at 35°C in a shaking water bath for 24 h. The 50 ml starter inoculum was then added directly to 1 liter of CYE broth contained in a Fernbach flask. The flask was incubated at 35°C

with shaking (200 r.p.m.) in a New Brunswich incubator-shaker (New Brunswich Scientific Co., New Brunswick, N.J.) for approximately 12 h until late exponential growth was detected. Late exponential growth was equivalent to 375-425 Klett units as measured with a Klett Summerson Colorimeter (Klett Mfg. Co. Inc., New York, U.S.A.) containing a green filter. The composition of all CYE media used in this study was identical to that previously described (28) except that 1.0 g KH_2PO_4 and 5.4 g K_2HPO_4 were added per liter and ferric pyrophosphate was omitted from our preparations.

Outer Membrane Preparation

Outer membranes were prepared by a modification of the method of Hancock and Nikaido (13). All of the following manipulations were performed at 4°C. One liter of late exponential phase cells was harvested by centrifugation at 10,000 x g for 15 min. Cells were washed twice by centrifugation in a total of one liter of cold 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma Chemical Company, St. Louis, MO) buffer, pH 7.4. The cell pellet was resuspended in 15 ml of 20% sucrose in HEPES buffer and 1 mg each of deoxyribonuclease I and ribonuclease A (Sigma) were added. The suspension was passed through a French pressure cell (American Instruments Co., Silver Springs, MD) twice at 15,000 p.s.i. Remaining whole cells were removed from the suspension by centrifuging at 1,000 x g for 15 min. The cell lysate containing supernatant was decanted and diluted with an equal volume of 10 mM HEPES buffer. Two ml of the suspension was layered onto sucrose gradients consisting of 5 ml 70% sucrose, 8 ml 64% sucrose, 7 ml 58% sucrose, 4 ml 52% sucrose, 3 ml 48%

sucrose, and 3 ml 44% sucrose. All sucrose concentrations were in 10 mM HEPES buffer. The gradients were centrifuged at 25,000 r.p.m. in a Beckman SW 27 rotor for 20 h. Gradient bands were collected from above with a 12 gauge cannula attached to a 10 ml syringe, diluted in 10 mM HEPES, and centrifuged at 50,000 r.p.m. in a Beckman Ti60 rotor for 1 h. Pellets were washed 3 times by resuspension in cold, deionized water and centrifugation at 50,000 r.p.m. Membrane pellets were resuspended in 1-3 ml cold, deionized water and the suspensions were either utilized immediately for enzyme analysis or stored at -20°C for further chemical characterization.

The absorbance profile of the gradient at 280 nm (A_{280}) was determined using gradients prepared in Beckman SW 41 cellulose nitrate tubes. Volumes of sucrose concentrations and cell lysate were added proportionately to those described for SW 27 gradients. Identical centrifugal force and time of centrifugation was applied to the SW 41 gradients. Gradient tubes were punctured from below and 10 drop fractions were collected. Each fraction was diluted with 2.5 ml of deionized water and the absorbance at 280 nm was determined using a Beckman series 35 spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). Buoyant densities were determined by collecting fractions from a gradient loaded with 10% sucrose in HEPES buffer only. The refractive indices of blank fractions corresponding to A_{280} peaks were measured with a refractometer (Bausch & Lomb, Rochester, N.Y.) and compared to refractive indices of known sucrose concentrations in 10 mM HEPES.

Enzyme Assays

Membranes were assayed for reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase, succinate dehydrogenase and malate dehydrogenase activity by the method of Ellar et al. (7). Conditions for succinate and malate dehydrogenase activity determinations were identical to those in which NADH was the substrate except that 0.25 ml of phenazine methosulfate (Sigma) solution (3 mg/ml) was added to the reaction mixture.

Chemical Analysis

Protein content was determined by the method of Lowry et al. (20) using bovine serum albumin (Sigma) as the standard. Total hexose was estimated by the anthrone method of Shields and Burnett (31) using glucose (Fischer) as the standard. Uronic acid content was estimated by the method of Dische (6) using glucuronic acid (Sigma) as the standard. Hexosamine content was estimated by the method of Levvy and McAllen (19) following hydrolysis of the samples in 4N HCl at 105°C for 4 h. Glucosamine-HCl (Sigma) served as the standard. 2-keto-3-deoxyoctonic acid (KDO) content was estimated by the method of Waravdekar and Saslaw (37) after samples had been hydrolyzed in 0.1N H_2SO_4 at 100°C for 10 min. Pure KDO (Sigma) was the standard. Heptose content was estimated by the method of Osborn (25) using glucoheptose (Sigma) as the standard.

Lipid Analysis

Readily extractable lipid (REL) was extracted from membranes by the method of Bligh and Dyer (3). All glassware utilized in lipid studies was acid washed and rinsed extensively in deionized water. Membrane

(5-15 mg protein) was added to deionized water to a final volume of 15 ml in a 250 ml separatory funnel. Methanol (37.5 ml) and chloroform (18.75 ml) were added, the suspension was shaken and allowed to stand for 18 h. The lower REL containing $CHCl_3$ phase was collected in a round bottom flask and the $CHCl_3$ was removed under reduced pressure. The remaining residue was resuspended in a total of 5 ml $CHCL_3$ and passed over sodium sulfate into a tared tube. The $CHCl_3$ was evaporated under nitrogen and the weight of the REL was recorded. Analysis of individual phospholipid species within the total REL from membranes was performed by the one dimensional thin layer chromatography (TLC) method of Fine and Sprecher (9). Whatman LK5D plates (Whatman Inc., Clifton, N.J.) were pre-developed in $CHCl_3$:MeOH (1:1) and allowed to dry overnight under dessication. Plates were dipped in a solution of 1.2% boric acid in 50% ethanol, allowed to dry for 15 min., and activated at 100°C for 1 hr. The REL was resuspended to 10 mg/ml in $CHCl_3$ and 5 µl was applied to the preadsorbant area. Plates were developed in $CHCl_3$:MeOH:H_2O:NH_4OH (120:75:6:2). Lipids were visualized with iodine vapor and individual phospholipids were identified by comparison with commercial standards (Sigma). Phosphatidylcholine (PC) was further identified using Dragendorf reagent (Supelco Inc., Bellefonte, PA) and phosphatidylethanolamine (PE) was identified by eluting phospholipid from the adsorbant with $CHCl_3$:MeOH (2:1), spotting fresh TLC plates and spraying with ninhydrin. Distribution of individual phospholipids within the REL from membranes was determined by scraping iodine positive spots corresponding to phospholipids into glass tubes and assaying for phosphorus by the method of Ames and Dubin (2). Total lipid phosphorus (REL) applied to TLC plates was also determined by this method.

SDS Polyacrylamide Gel Electrophoresis

The polypeptide profiles of membranes was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (18). The stacking gel contained 5% acrylamide (acrylamide:bis, 30:8) and the separating gel consisted of 12.5% acrylamide. Gels were routinely run at 30 mA for 2.5 hr. at 4°C. Membranes were solubilized in sample buffer at either room temperature (20°C) or 100°C for 5 min and in the presence or absence of 1% 2-mercaptoethanol (2-ME). Heat modifiable proteins were identified by 2-dimensional SDS-PAGE using the gel system described above. Membranes were solubilized in sample buffer at 20°C and run on a 12.5% separating gel. One cm wide strips of the gel were cut out, placed in a sealed, glass tube containing sample buffer and heated at 100°C for 5 min. The acrylamide strip was placed in a horizontal position on top of a second 12.5% separating gel and current was applied to 30 mA for 2.5 hr. at 4°C. Proteins from both one dimensional and two dimensional gels were stained with Coomassie blue in a solution of 25% MeOH and 10% acetic acid and destained in 25% MeOH and 10% acetic acid.

Peptidoglycan associated proteins were identified based on a modification of the method of Lugtenberg et al. (21). Total membrane was recovered from cell lysate by centrifugation at 50,000 r.p.m. in a Beckman 60Ti rotor for 1 hr. The pellet was resuspended in 10mM HEPES containing 2% sodium dodecyl sulfate (SDS) and individual samples were heated at 30°C, 45°C, 60°C, 80°C or 100°C for 15 min. Crude peptidoglycan was pelleted by centrifugation at 50,000 r.p.m., resuspended in HEPES buffer, and centrifuged at 50,000 r.p.m. for 1 hr. Pellets were resuspended in 0.5 ml HEPES, solubilized in SDS-PAGE sample

buffer at 100°C for 5 min, and applied to a 12.5% acrylamide gel as described above.

RESULTS

Membrane Fractionation

Lysis of L. pneumophila with a French pressure cell followed by sucrose gradient centrifugation resulted in five distinct bands. The location of each band in the gradient corresponded with the interface of sucrose steps similar to the observation of Hancock and Nikaido (13) for Pseudomonas aeruginosa membranes. Bands were designated in order of increasing density as follows. Cytoplasmic membrane-1 (CM-1), cytoplasmic membrane-2 (CM-2), intermediate band (IM), outer membrane-1 (OM-1), and outer membrane-2 (OM-2). The CM-1 was clear orange while CM-2 had decreased levels of orange coloration and was more opaque than CM-1. The buoyant density of these fractions was less than that observed for the cytoplasmic membrane reported in an earlier study (10). The IM, OM-1, and OM-2 were diffuse, white bands. It was necessary to load whole cell lysate onto gradients in order to recover sufficient quantitites of the CM-1 and CM-2 fractions for analysis. Lysis of gram-negative bacteria with a French pressure cell is known to disrupt cytoplasmic membrane into small fragments that are difficult to recover (16). The protocol we describe (Materials and Methods) was determined to be the method of choice after we attempted to isolate outer membrane by a number of published methodology. Efforts to retrieve cell envelopes (total membrane) by preparative sucrose gradients (13) or ultracentrifugation

(17) for loading onto analytical gradients resulted in very low yields of CM-1 and CM-2. Also, in our hands, the EDTA-lysozyme method (26) was ineffective at yielding osmotically fragile spheroplasts.

The absorbance at 280 nm of membrane fractions is shown in Figure 1 and depicts the location and relative amounts of each band in the gradients. The large absorbance values seen for fractions at the top of the gradient are believed to represent soluble cytoplasmic components as a result of loading cell lysate onto the gradient. Separation of CM-1 and CM-2 was difficult regardless of manipulations of concentration or volumes of sucrose in this area of the gradient. As a result, slight cross-contamination during recovery of these fractions was unavoidable. As indicated in Figure 1, OM-2 contained the least amount of material relative to other membrane fractions.

Assessment of NADH, succinate, and malate dehydrogenase activity (Table 1) was utilized to determine the efficiency of separating cytoplasmic membrane from outer membrane. Although NADH and succinate dehydrogenase activity was highest in CM-1 and CM-2, these enzymes were also detected in the IM, OM-1, and OM-2 fractions. Specific activity of these enzymes in the upper bands was approximately five fold greater than that in OM-1. Malate dehydrogenase activity was not detected in any of the membrane fractions. In view of the concern that extensive washings of membranes had partially inactivated these enzymes, fresh membranes were prepared and washed only once in 10 mM HEPES buffer and assayed for the above enzymes. Results of these studies were equivalent to those described in Table 1.

Chemical Characterization of Membrane Fractions

The results of the chemical analysis of membrane fractions are shown in Table 1. The CM-1 contained the least amount of protein and the IM the greatest amount on a dry weight basis. Conversely, REL content was greatest in CM-1 and lowest in the IM fraction. Assays for total hexose and KDO were utilized as an indication of LPS in the membrane fractions. We observed little difference in the amount of carbohydrate and KDO between the upper two bands (CM-1,CM-2) and the lower three bands (IM, OM-1, and OM-2) (Table 1). In view of the concern that DNA was interfering with the KDO assay, absorbance scans of thiobarbituric acid positive chromophores were performed. The absorbance maximum for both pure KDO and membrane fractions was 548 nm and the lack of an increase in absorbance in the 530 nm - 540 nm range for the membrane fractions suggested that DNA was not interfering with this assay. Heptose, another common component of LPS was not detected in the membrane fractions. The hexosamine content was greatest in OM-1 and was monitored as a means of determining peptidoglycan distribution in the gradient based on the finding that glucosamine is a major constituent of the L. pneumophila peptidoglycan (1). Membranes were also examined for the presence of uronic acids to address the possibility that anionic polysaccharides were associated with the cell surface. Results of the assay were negative indicating that these compounds were not present in membrane fractions. In addition, these data indicated that uronic acids were not causing interference in the KDO assay (37).

SDS PAGE

Membranes were characterized by SDS-PAGE under several conditions of sample preparation. When membranes were solubilized at 100°C in sample buffer containing 2-ME, results seen in Figure 2 (lanes 2-6) were obtained. The profiles for CM-1 and CM-2 were virtually identical. Fractions IM, OM-1 and OM-2 also had profiles very similar to one another but distinct differences were observed between the cytoplasmic membranes (CM-1, CM-2) and the IM, OM-1, and OM-2. The major protein in the IM, OM-1, and OM-2 had an apparent molecular weight of 29,000 daltons (29K). To determine if heat modifiable proteins were present, membranes were solubilized in sample buffer at 20°C and examined under identical conditions to those described above. In the absence of heating, the 29K band did not appear to enter the gel as a recognizable band (Fig. 2, lane 8) and what appeared to be large aggregates of material were observed at the top of the separating gel for IM, OM-1, and OM-2. A second heat modifiable protein was observed in which the apparent molecular weight decreased from 45K to 33K in the absence of heating. This protein was also present in the IM, OM-1, and OM-2 fractions only. The result of heating membranes at 100°C in the absence of 2-ME is seen in Figure 2 (lane 7). The 29K protein again was not present and smearing at the top of the lanes loaded with IM, OM-1, and OM-2 was observed suggesting that both heat and reducing agent are required to release the 29K protein in monomeric form. The 33K protein increased in apparent molecular weight to 45K indicating that heat only is required for the modification of this protein. To further characterize the heat modifiable proteins, 2-dimensional SDS-PAGE analysis was performed using OM-1 as being representative of the proteins in fractions IM, OM-1, and OM-2. Heating

the sample in the presence of 2-ME prior to SDS-PAGE in the first dimension yielded a typical diagonal line with all proteins falling on the diagonal after the second dimension was run (Fig. 3a). Figure 3b shows the result of solubilizing OM-1 at 20°C with 2-ME before running the first dimension followed by heating prior to running the second dimension. The 29K protein departed markedly from the diagonal confirming that it runs at a greatly increased molecular weight in the absence of heating. The 33K protein also behaved differently under these conditions and its position above the diagonal shows that heating caused an increase in apparent molecular weight.

The 29K protein was found to be associated with SDS insoluble material (i.e. crude peptidoglycan) (Fig. 4). Heating total membrane in 2% SDS at 100°C for 15min. failed to release this protein from the crude peptidoglycan. The 33K protein however, was partially released at 60°C and was completely released from the crude PG after incubation at 100°C.

Phospholipid Analysis

Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), and cardiolipin (CL) were the major phospholipids of the REL from membranes (Table 2). These findings are in agreement with those of Finnerty et al (9) who found PC, PE, PG, and CL to be the major phospholipids of the REL from whole cells of L. pneumophila. Phospholipid distribution in membrane fractions was determined by assaying for lipid phosphorus (2). Percent recovery of total phosphorus applied to TLC plates was greater than 90% for all fractions. PG was not detected in CM-1 but was present in the remaining four fractions. PE was the most abundant phospholipid in all of the membranes with PC being the next most abundant species. CL and PG were

present in noticeably lower amounts (6-14% and 8-13% respectively). Iodine positive material running near the solvent front was detected and was believed to represent neutral lipid and free fatty acids as described by Fine and Sprecher (8). This material was not further characterized. Residue remaining at the origin of application on the TLC plates and a noticeable viscosity when the REL from IM, OM-1, and OM-2 were resuspended to 10 mg/ml in $CHCl_3$ suggested that polyhydroxybutyrate (PHB) was contaminating the REL of these fractions. Membrane associated PHB has been observed in L pneumophila in a previous study (29), and we found that ether precipitable material, a characteristic of PHB (30), was recoverable from IM, OM-1, and OM-2 REL. (data not presented).

DISCUSSION

We have described the isolation of cytoplasmic and outer membranes from L. pneumophila The protein profiles generated by SDS-PAGE demonstrate a distinct difference between CM-1, CM-2 and IM, OM-1, and OM-2, while the profiles of IM, OM-1, and OM-2 were virtually identical. Proteins forming transmembrane pores (porins) are normally within the molecular weight range of 30-45K and are abundant in purified outer membranes. A 29K protein was the most abundant protein in the IM, OM-1, and OM-2 fractions with lesser amounts of a 33K protein. The 29K protein entered the gel only after solubilization in buffer containing reducing agent at elevated temperature suggesting that the protein exists as a large aggregate stabilized by disulfide linkage. Similar conditions are required to resolve the major outer membrane protein (MOMP) from Chlamydiae. However, the MOMP from these organisms was not recoverable from SDS insoluble material (22). The 29K protein from L. pneumophila

was recoverable from SDS insoluble material (i.e. crude peptidoglycan) and was not liberated by treatment at temperatures exceeding 60°C (fig.4), a property characteristic of porins from E. coli (21). In addition, extraction of crude peptidoglycan from L. pneumophila with high salt, which normally releases porins from the peptidoglycan of Salmonella typhimirium (34) and P. aeruginosa (40), failed to release the 29K protein (unpublished observations). The 33K protein increased in apparent molecular weight to 45K upon heating, but in contrast to the 29K protein, this behavior was independent of reducing agent. The outer membrane of Haemophilus influenzae contains two heat modifiable proteins, one of which (protein a) has an apparent molecular weight of 34K in the absence of heating and has an apparent molecular weight of 47K after heating (35). Outer membrane proteins with molecular weights of 43K and 30K, have been identified in Brucella abortus (36) and a 29K protein has been reported to be a major outer membrane protein in Rickettsia prowazeki (32). It is of interest to note the similarities in the outer membrane proteins of L. pneumophila and those from the outer membranes of the intracellular parasites mentioned above (22, 32, 36). Unfortunately, the biological function of these proteins is poorly understood making further comparison difficult.

We did not observe striking differences with respect to typical chemical membrane markers (enzymatic activity, KDO, etc.) between cytoplasmic membrane (CM-1, CM-2) and outer membrane (OM-1, OM-2). The IM, OM-1, and OM-2 were expected to be enriched in total hexose relative to CM-1 and CM-2 as a result of increasing amounts of LPS associated with these fractions. However, carbohydrate content was not appreciably greater in these fractions. Hexosamines are constituents of the lipid-A

moiety of the LPS from Salmonella typhimirium and the ratio of hexosamine to KDO is 2:3 (24). However, the molar ratio of hexosamine to KDO in membrane fractions in our study ranged from 40:1 (CM-1) to 72:1 (OM-1). This suggests that the overwhelming majority of hexosamine detected was a reflection of the presence of peptidoglycan and not LPS. Heptose sugar, a common constituent of LPS was not detected in any of the fractions which is in agreement with the study of Flesher et al (11). The lack of uronic acids in membranes indicated that anionic saccharides were not associated with the membrane fractions.

Johnson et al (15) have shown that a high molecular weight antigen (F-1) is readily released from L. pneumophila by washing cells with buffer. The antigen is composed of 35% carbohydrate, 2.6% protein, 1.8% phospholipid, and 1% KDO suggesting that it contains LPS. Given this observation, a significant amount of outer membrane components similar to the F-1 antigen may have been removed during the washing of cells prior to lysis in the French pressure cell.

Alternatively, several observations have been made that suggest that L. pneumophila maintains a hydrophobic cell surface (4, 27). We found that the percent (wt/wt) total hexose in OM-2 is less than 3.5% indicating that the cell surface may be quite hydrophobic. Bohach and Snyder (4) found that the hydrophobicity of the L. pneumophila cell surface was intermediate between wild type Salmonella cholerasuis (hydrophilic) and a deep-rough LPS mutant of Salmonella typhimurium (hydrophobic). Deep rough mutants of S. typhimurium exhibit increased susceptibility to hydrophobic antibiotics including erythromycin (23) and L. pneumophila has been shown to be sensitive to erythromycin in vitro (27). Although material with a high carbohydrate content is easily

removed from whole cells (15), the antibiotic susceptibility characteristics of L. pneumophila and the findings of Bohach and Snyder (4) suggest that our observations may be an accurate reflection of the true carbohydrate content of the outer membrane.

We have confirmed the unusual phospholipid composition of L. pneumophila whole cells reported by Finnerty et al (9). PE and PC were the most abundant phospholipids in the membranes and the PE:PC ratio increased with membranes of increasing density. PE enrichment in outer membranes is well documented in E. coli (5) and as indicated by our data, appears to occur in the L. pneumophila outer membrane. PC is seldom observed in bacterial phospholipids and is a common component of eukaryotic membranes (12). However, PC has been observed in the phospholipids of Nocardia coelicia (30%) (39), Treponema pallidum (33%) (14), and Brucella abortus (34%) (33) among others. The amount of PC in L. pneumophila Knoxville strain has been reported to be 35% of the total phospholipid (9). We found that the amount of PC in membranes ranged from 27-43% (Table 2). The function of PC in the cell envelope of L. pneumophila is unclear at this time, but its presence clearly distinguishes the lipid composition of this organism from most bacteria.

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Figure 1. Absorbance profile of sucrose gradient at 280 nm. 10 drop fractions were collected from the bottom of the gradient, diluted with 2.5 ml H_2O and measured at 280 nm (A_{280}). Fraction 1; bottom of the gradient.

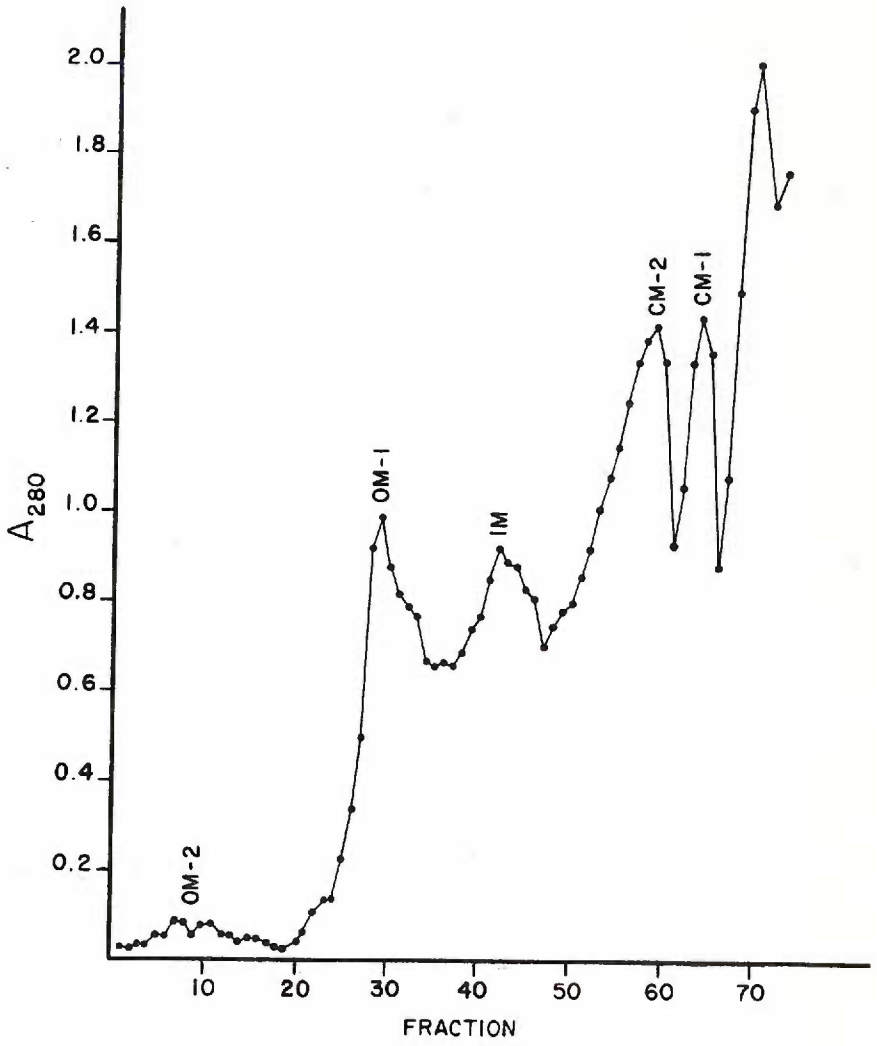
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A_{280}
OM-2
OM-1
IM
CM-2
CM-1
10
20
30
40
50
60
70
FRACTION

Figure 2. SDS-PAGE of membrane fractions. Lanes 2-6 correspond to CM-1, CM-2, IM, OM-1, and OM-2 respectively. Membranes were solubilized in sample buffer containing 2-mercapoethanol (2-ME) at 100°C for 5 min. Lane 7: OM-2 solubilized in buffer lacking 2-ME at 100°C. Lane 8: OM-2 solubilized in buffer containing 2-ME at room temperature. 10 µg protein was applied to each lane. Lane 1: molecular weight standards; beta-galactosidase (130,000), phosphorylase B (93,000), bovine serum albuimin (68,000), glutamate dehydrogenase (50,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), lysozyme (14,000).

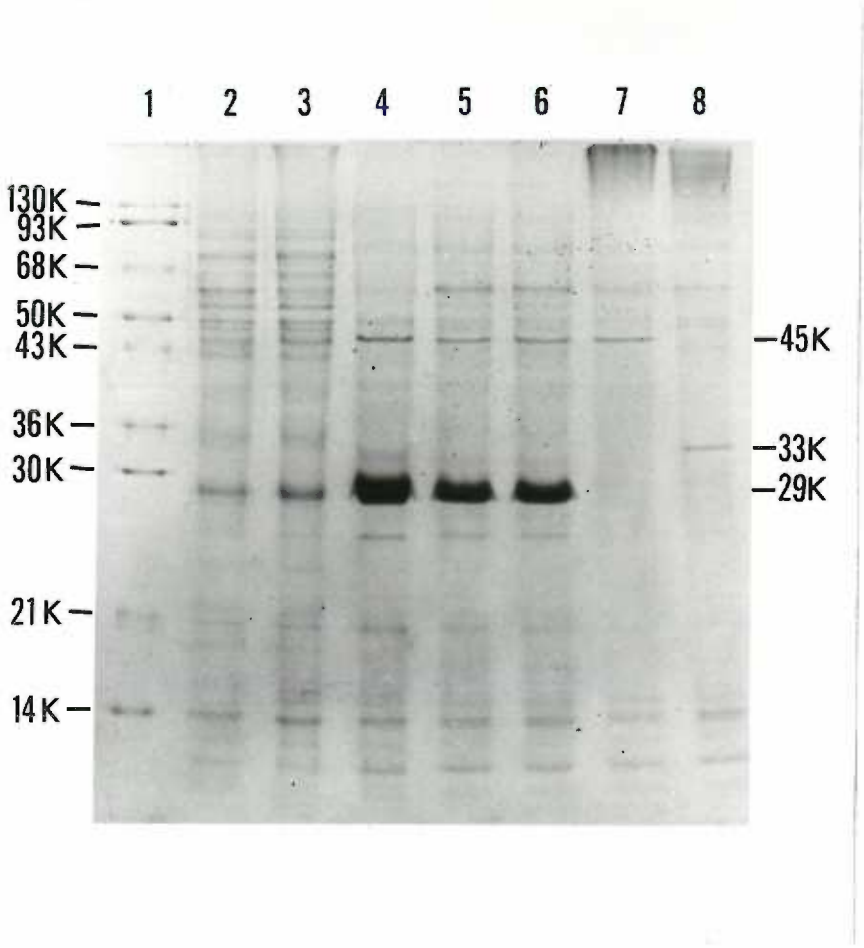
1
2
3
4
5
6
7
8
130K
93K
68K
50K
43K
36K
30K
21K
14K
45K
33K
29K

Figure 3. 2-dimensional SDS-PAGE of heat modifiable proteins. Fraction OM-1 was solubilized in sample buffer containing 2-ME at room temperature or at 100°C for 5 min. before running in the first dimension. Gel strips cut from the first dimension were placed in sample buffer containing 2-ME and heated at 100°C for 5 min. before running in the second dimension. All proteins were located on the diagonal when heated before running the first dimension (a). The results of no heating prior to running the first dimension are seen in (b). The 29K protein behaves as a large aggregate and the 33K protein increased in molecular weight to 45K upon heating. 10 μg of protein was loaded before running the first dimension.

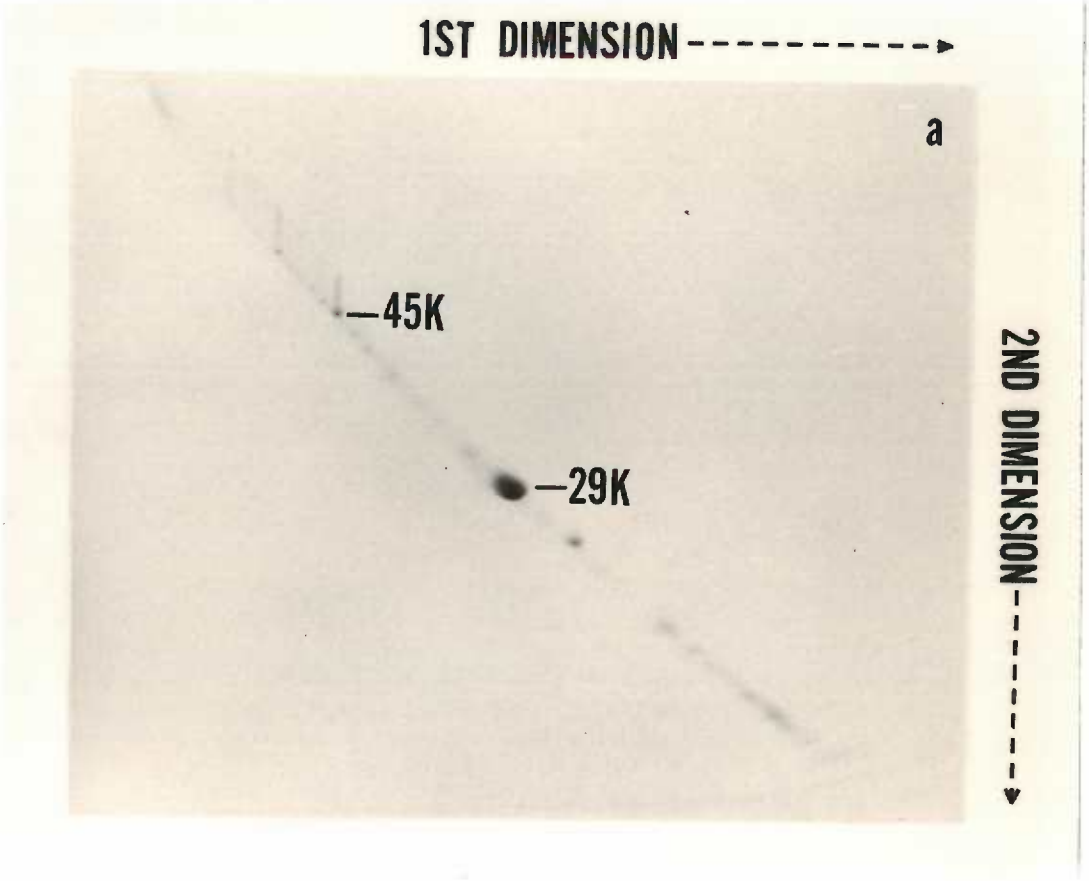
1ST DIMENSION
a
—45K
—29K
2ND DIMENSION

Figure 3. (cont.)

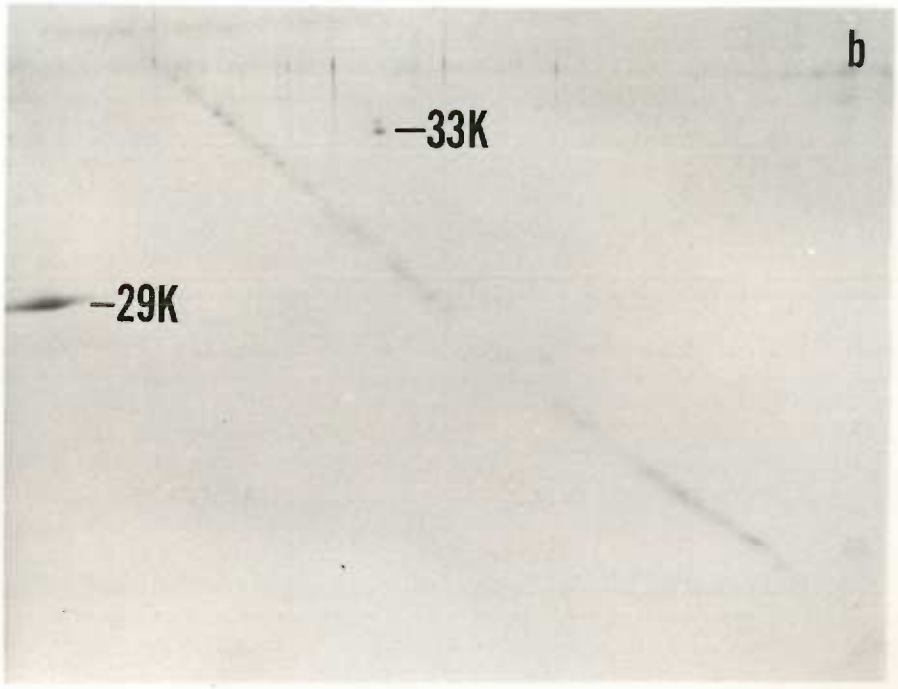
1ST DIMENSION
b
—33K
—29K
2ND DIMENSION

Figure 4. SDS-PAGE of peptidoglycan associated proteins. Cell envelopes were solubilized in SDS as described in Methods. Temperatures of SDS solubilization were as follows. Lane 1, 30°C; 2, 45°C; 3, 60°C; 4, 80°C; 5, 100°C. SDS insoluble material was solubilized in SDS-PAGE sample buffer containing 2-ME at 100°C before loading onto the gel. Protein was loaded at 10 μg per lane.

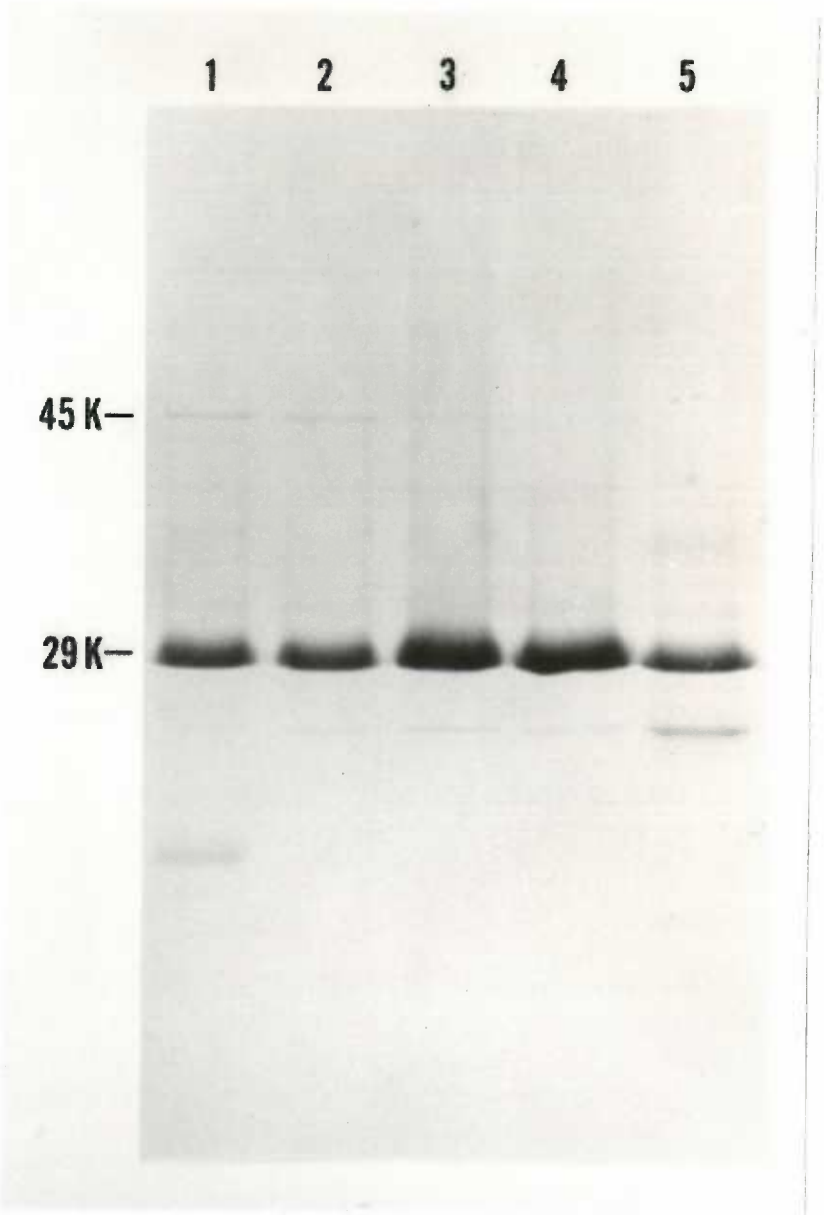
1
2
3
4
5
45 K—
29 K—

Table 1. Physical, enzymatic and chemical characteristics of membrane fractions.

Membrane Fraction	Bouyant Density[a]	NADH[b]	SDH[b]	% Protein[c]	% Readily Extractible Lipid[c]	Hexosamine[d]	KDO[d]	Hexose[d]
CM-1	1.14	158	108	45	59	0.36	0.009	0.10
CM-2	1.17	251	74	67	24	0.48	0.010	0.21
IM	1.25	69	30	77	15	1.60	0.024	0.18
OM-1	1.28	49	20	76	18	2.17	0.030	0.17
OM-2	1.32	60	26	72	23	1.45	0.023	0.27

a) expressed as g/cc

b) expressed as nanomoles Dichloroindolephenol reduced / min / mg protein. NADH, NADH dehydrogenase; SDH, succinate dehydrogenase.

c) expressed as wt/dry wt membrane

d) expressed as μmole / mg protein

Table 2. Phospholipid composition of membrane fractions

Membrane Fraction	Phosphatidylglycerol[a]	Phosphatidylcholine[a]	Phosphatidylethanolamine[a]	Cardiolipin[a,b]
CM-1	ND[c]	43	45	12
CM-2	8	35	42	14
IM	9	39	45	8
OM-1	12	33	49	6
OM-2	13	27	50	10

a) expressed as percent of total lipid phosphate recovered

b) percent lipid phosphate values for cardiolipin were adjusted by dividing μmole cardiolipin phosphate by 2

c) ND: not detected

Manuscript 2

Outer Membrane Proteins from *Legionella pneumophila* Serogroups and Non-pneumophila Species

ABSTRACT

Outer membranes were isolated from eight serogroups of L. pneumophila and five non-pneumophila species. The protein composition of the membranes was characterized by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A single, disulfide stabilized protein with a molecular size of 29,000-30,000 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 non-pneumophila species examined. Silver staining of SDS-PAGE gels revealed distinctive patterns for each serogroup and non-pneumophila species that were not observed by staining with Coomassie blue and may result from the presence of lipopolysaccharide (LPS) 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 LPS. Antibodies to this complex were used to probe the outer membranes of the remaining serogroups and non-pneumophila species by western blotting. The MOMP was found to be a cross-reactive antigen in all of the serogroups while antibodies directed against the LPS of serogroup 1 only cross-reacted with two of the remaining seven serogroups.

INTRODUCTION

The gram-negative bacterium Legionella pneumophila is a facultative intracellular parasite that has been shown to multiply within human peripheral blood monocytes (16) and block phagosome-lysosome fusion (18). When in an intracellular environment that contains inhibitors of bacterial protein synthesis, the organism ceases to multiply, yet remains viable (17). 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 (3). 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 - 29,000 daltons (8,12,15). Disulfide stabilized outer membrane proteins have been observed in the Chlamydiae (23) and interestingly, Chlamydia psittici belongs to a select group of organisms including L. pneumophila, that inhibits lysosome-phagosome fusion (18).

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 non-pneumophila species were isolated and outer membrane proteins were analyzed by SDS-PAGE using both Coomassie blue and silver as staining reagents. Using 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 agent (15). 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 (CYE) broth (24) [(modified as previously described (15)] containing 20% glycerol at -70°C. Legionella 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 Dr. Washington Winn Jr., University of Vermont Medical College. Plates containing CYE agar were streaked with stock cultures and incubated in a 5% CO_2 incubator at 37°C for 48 hr. Batch cultures were prepared as described previously (15).

Isolation of Outer Membranes. Outer membranes were isolated from all serogroups and non-pneumophila species as previously described (15) 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 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.4. Cell pellets were resuspended in 2 ml of 10 mM HEPES buffer and deoxyribonuclease I (Sigma Chemical Co., St. Louis, Mo.) and ribonuclease 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 x g for 15 min to remove whole cells. The supernatant was centrifuged at 50,000 rpm in a Beckman 60Ti rotor for 1 hr. The cell envelope containing pellet was washed 2 times in cold HEPES buffer by centrifuging at 50,000 rpm in the Beckman 60Ti rotor. The pellet was resuspended in 1.5 ml of HEPES buffer and 0.8 ml was loaded onto sucrose gradients consisting of 2 ml 70% sucrose, 3 ml 64% sucrose, 3 ml 58% sucrose, 2 ml 52% sucrose, and 1 ml 46% sucrose. The gradients were centrifuged at 25,000 rpm in a Beckman SW41 rotor for 18 hr 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 hr. The recovered outer membranes were washed twice in HEPES buffer by centrifugation at 50,000 rpm in a 60Ti rotor for 1 hr and resuspended in 1.5 ml of HEPES buffer. Protein concentrations were determined by the method of Lowry et al. (21). The amount of 2-keto-3-deoxyoctonic acid (KDO) in outer membranes was assessed by the method of Waravedekar and Saslaw (29) using KDO (Sigma) as a standard.

Isolation of the LPK-1 MOMP. LPK-1 whole cells were broken as previously described (15). After whole cells were removed by centrifugation at 1,000 x g for 15 min, cell envelopes were collected by centrifuging the suspension at 50,000 rpm in a Beckman 60Ti rotor for 1 hr. The pellet was resuspended in 50 ml of 50 mM Tris (pH 8.0) containing 2% sodium dodecyl sulfate (SDS), 10mM EDTA, and 50mM NaCl and incubated at 60°C for 30 min. The suspension was centrifuged at 50,000 x g for 30 min at 20°C. The supernatant was decanted and the pellet was resuspended in 50 ml of the same buffer, incubated at 60°C for 30 min and centrifuged at 150,000 x g for 30 min. The pellet was resuspended in 5 ml of 10mM Tris pH 8.0, diluted with 5 ml of 50 mM Tris (pH 8.0), containing 2% SDS, 1% 2-mercaptoethanol (2-ME), 2 mM EDTA, and heated to 100°C for 5 min. After cooling, the suspension was centrifuged at 150,000 x g for 30 min. The supernatant containing 2-ME solubilized material was decanted and 1 ml of the supernatant was applied to a 1.5cm x 45cm Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column that had been previously equilibrated at 32°C in 50mM Tris, 2% SDS, 10mM EDTA, and 50mM 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 serogroups and non-pneumophila species was examined using the Western blot method of Towbin (26). 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 overlayed with nitrocellulose paper (Schleicher & Schell Inc., Keene, N.H.) , the proteins were transferred to the paper by placing the sandwich in a Trans-Blot chamber (Bio-Rad, Richmond, Ca) containing a buffer consisting of 0.025M Tris base, 0.192M glycine, and 20% methanol. A voltage of 55V was applied to the chamber for 2 hr. Following electrotransfer, the nitrocellulose was incubated in 0.01M Tris, 0.9% NaCl pH 7.4 containing 3% bovine serum albumin (BSA) at 37°C for 30 min. The nitrocellulose was rinsed in 200 ml of 0.01M Tris, 0.9% NaCl (TN) pH 7.4 and was then incubated in 100 ml of TN containing 1% BSA and 100 µl of antiserum for 2 hr at 37°C. After rinsing in TN, the nitrocellulose was incubated in 100 ml of TN containing 1% BSA and 50 µl of horse-radish peroxidase conjugated goat anti-rabbit IgG antibody (Cappel Laboratories, West Chester, Pa) for 2 hr at 37°C. After a brief rinse in water, the nitrocellulose was incubated in a solution containing 0.05 M Tris, 0.15M NaCl, 0.005M EDTA, and 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/ml of 4-chloro-1-napthol (Bio-Rad) was added. Forty µl 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 Lipopolysaccharide. Lipopolysaccharide was isolated from LPK-1 using a modification of the method of Darveau and Hancock (7). One liter of late exponential phase cells grown in CYE broth was harvested and washed twice with 10 mM HEPES buffer by centrifugation. The washed pellet was resuspended in 15 ml of 10 mM Tris pH 8.0 and DNase (Sigma) and RNase were added to 200 and 50 μg/ml respectively. The suspension was passed through a French pressure cell twice at 10,000 psi followed by centrifugation at 1,000 x g to remove whole cells. The supernatant was decanted and additional DNase and RNase were added to 200 μg and 50 μg respectively followed by incubation at 37°C for 1 hr. The suspension was then sonicated for 1 min (2-30 sec bursts) with a Biosonik IV sonicator (Bronwill, Rochester, N.Y.). Five ml of 0.5M EDTA (in 0.01 M Tris, pH 8), 2.5 ml 20% SDS in 0.01 M Tris, and 2.5 ml of 0.01 M Tris were added and after vortexing the suspension was centrifuged at 50,000 x 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 hr. Two volumes of 0.375 $MgCl_2$ in ethanol was added, the suspension was cooled to 0°C and centrifuged at 12,000 x g for 15 min at 0°C. The pellet was resuspended 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, two volumes of 0.375 M $MgCl_2$ in ethanol were added and the suspension was cooled to 0°C. The suspension was centrifuged at 12,000 x g for 15 min at 0°C. The pellet was resuspended in 10 ml of 0.01 M Tris (pH 8), sonicated briefly, and centrifuged for 10 min. in a

table top clinical centrifuge. The supernatant was diluted to 25 ml with 0.01 M Tris (pH 8) and centrifuged for 2 hr 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 resuspended in 2 ml of 0.01 M Tris (pH 8), protease was added to 100 μg/ml, and the suspension was incubated for 18 hr. at 37°C. The suspension was centrifuged at 42,000 rpm in a Beckman 60Ti rotor for one hr. The pellet was washed twice with 0.01 M Tris (pH 8) by centrifugation. The final pellet was resuspended in 1 ml of distilled water and the suspension was stored at -20°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. This was performed as described previously (15). Gels were stained with either Coomassie blue in a solution containing 25% MeOH and 10% acetic acid or by the silver stain method of Tsai and Frasch (27).

Preparation of Antiserum. Following pre-bleeding, white New Zealand rabbits were injected subcutaneously with 100 μg of the isolated LPK-1 MOMP in Freund's complete adjuvant. Two weeks after the initial injection, 100 μg of the protein suspended in Freund's incomplete adjuvant was injected subcutaneously. After an additional two weeks, rabbits were boosted with 10 μg of the antigen administered intravenously. Serum was collected 5 days following the final challenge. The presence of antibody to the MOMP in the antiserum was assessed with western blots (26) 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 (M. S. Hindahl and B. H. Iglewski, manuscript in preparation).

RESULTS

Purification and Protein Composition of Outer Membranes. Application of isolated cell envelopes from the L. pneumophila serogroups, the non-pneumophila 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 (15). The lower two bands representing the OM-1 and OM-2 (15) were pooled and used to characterize the outer membrane proteins. KDO analysis of these 2 bands confirmed their identity as outer membrane. The amount of KDO in the L. pneumophila serogroup outer membrane ranged from 0.019 - 0.029 μmole KDO/mg protein. These data are in agreement with earlier reports for the KDO content of isolated LPK-1 outer membrane (15). The non-pneumophila outer membranes contained lesser amounts of KDO (0.007-0.016 μmole/mg protein) than the L. pneumophila serogroups. The L. pneumophila serogroup 1 clinical isolate outer membrane contained 0.022 μmole KDO/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 major outer membrane protein in all L. pneumophila serogroups and the clinical isolate (Figure 1). The major outer membrane protein from serogroups 1-8 and the clinical isolate all required heating in the presence of reducing agent (2-ME) 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 five minutes in the absence of reducing agent (data not shown). A protein of this size was not observed in any of the outer membrane preparations from non-pneumophila species 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 noticebly less.

Owing to the relative abundance of the 29K-30K protein in the outer membrane profiles of the L. pneumophila serogroups and the lack of a similar protein in the non-pneumophila species, the silver stain method of Tsai and Frasch (27) was employed. The silver stain 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 by the less sensitive Coomassie blue staining (Fig 2). Although the higher molecular size range (i.e. greater than 30K) of the gel showed 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 20-30K region of the gel clearly distinguished the serogroup outer membranes from one another. These distinctive patterns were greatly enhanced after pre-treatment of gels with periodic acid (Fig 2, lanes b-i) indicating that a carbohydrate moiety (i.e. LPS) was responsible for these areas of heavy stain deposition. In addition, the major outer membrane protein in all serogroups did not stain using this method when the gel had been exposed to periodic acid prior to staining.

Similarly, oxidation of gels containing non-pneumophila outer membranes prior to staining revealed distinctive heavily staining patterns for each organism. Several of these patterns had "ladder" like appearances suggestive of LPS (Fig.2, lanes l-p). Each non-pneumophila outer membrane revealed a characteristic pattern that allowed for differentiation between the strains tested and collectively these patterns were much different than those of the L. pneumophila serogroups.

When the outer membranes from a clinical isolate were 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 following periodate oxidation (Figs. 1 & 2, lane q).

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 protein 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 following application of the solubilized MOMP to gel filtration chromatography in the presence of SDS were analyzed by SDS-PAGE as seen in Figure 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 and following periodic acid oxidation, gels were examined by the silver stain method of Tsai and Frasch (27). A fraction with a retention coefficient of 0.69 stained by this method is shown in figure 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. Owing to the indication that LPS was 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. In spite of rigorous proteolytic treatments (see Methods), the LPS remained contaminated with some protein. The isolated LPS contained 0.072 μmole KDO/mg protein. The majority of associated protein was the MOMP (Fig. 4, lanes d and e). These findings are in agreement with those of Gabay & 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 in order to probe the antigenic similaries or lack therof between the serogroup 1 MOMP / LPS complex and the outer membranes of the various L. pneumophila serogroups and non-pneumophila species. Absorbed serum from rabbits challenged with the LPK-1 MOMP was examined for antibody to the antigen using the western blot technique (26). Antibodies directed against both the isolated LPK-1 MOMP and the LPS

(Fig. 4, lanes c and f) associated with this protein were detected using absorbed antiserum at a working dilution of 1:1000. The absorbed antiserum to the LPK-1 MOMP was utilized to examine the outer membranes from the remaining L. pneumophila serogroups and non-pneumophila species. The absorbed antiserum reacted with a broad area of the outer membranes from L. pneumophila serogroups 1 and 2 (Fig. 5; lanes c, 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 major outer membrane protein 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 protein that reacted with the absorbed antiserum was not detected in the outer membranes from the non-pneumophila 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 althought 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. Pre-bleed 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 non-pneumophila species as determined by SDS-PAGE were similar to those reported in a previous study of sodium lauryl sarcosinate-insoluble proteins from various L. pneumophila serogroups and non-pneumophila species (8). Treatment of cell envelopes with sodium lauryl sarcosinate has been shown to selectively solubilize the cytoplasmic membrane from Escherichia 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 non-pneumophila 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 following periodate oxidation may be a reflection of LPS present in the membranes. These results allowed for further differentiation between 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 non-pnemophila species thereby further distinguishing the L. pneumophila serogroups from these organisms. 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 (11,19). The LPS associated with the LPK-1 MOMP isolated in this study proved to be highly immunogenic. We found 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. (19).

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. pneumophlia serogroup 1 (20,30,32). 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 non-pneumophila species in this study) that has an apparent molecular size of 24,000 daltons. Using 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 employed to generate outer membranes and isolated forms of the MOMP.

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 (15), a treatment that has been shown to release PG associated proteins (i.e. porins) in several gram-negative bacteria (22,28,31). 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-ME 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 peptidoglycan failed to detect the presence of cysteine (1) thereby decreasing the liklihood 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 the current study had all been grown on laboratory medium in the absence of eukaryotic 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 (EB) from the intracellular parasite Chlamydia psittici are solubilized in SDS only after exposure to reducing agent (13). In addition, unique differences in the protein composition of outer membranes from the EB and reticulate bodies (RB) of this organism have been described. Cysteine-rich outer membrane proteins and the major outer membrane protein require solubilization in the presence of reducing agent for resolution via SDS-PAGE in the extracellular, infective EB form. The comparative amount of these cysteine rich proteins is greatly reduced in the metabolically active, intracellular RB outer membranes and the majority of the major outer membrane protein does not require 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 (23). Also, cysteine deprivation has been shown to greatly reduce the efficiency of conversion from the RB form to the EB form (25). 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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Figure 1. SDS-PAGE profiles of outer membranes from L. pneumophila serogroups and non-pneumophila species stained with Coomassie blue. Samples were solubilized in buffer containining 2-mercaptoethanol at 100°C for 5 min. and 10 μg of membrane protein was loaded per lane. Lanes b-i, L. pneumophila serogroups 1-8; lane k, L. pneumophila serogroup 1; lane l, L. bozemanii; lane m, L. dumoffii; lane n, L. gormanii; lane o, L. longbeachae; lane p, L. micdadei; lane q, L. pneumophila clinical isolate. Lane a, 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).

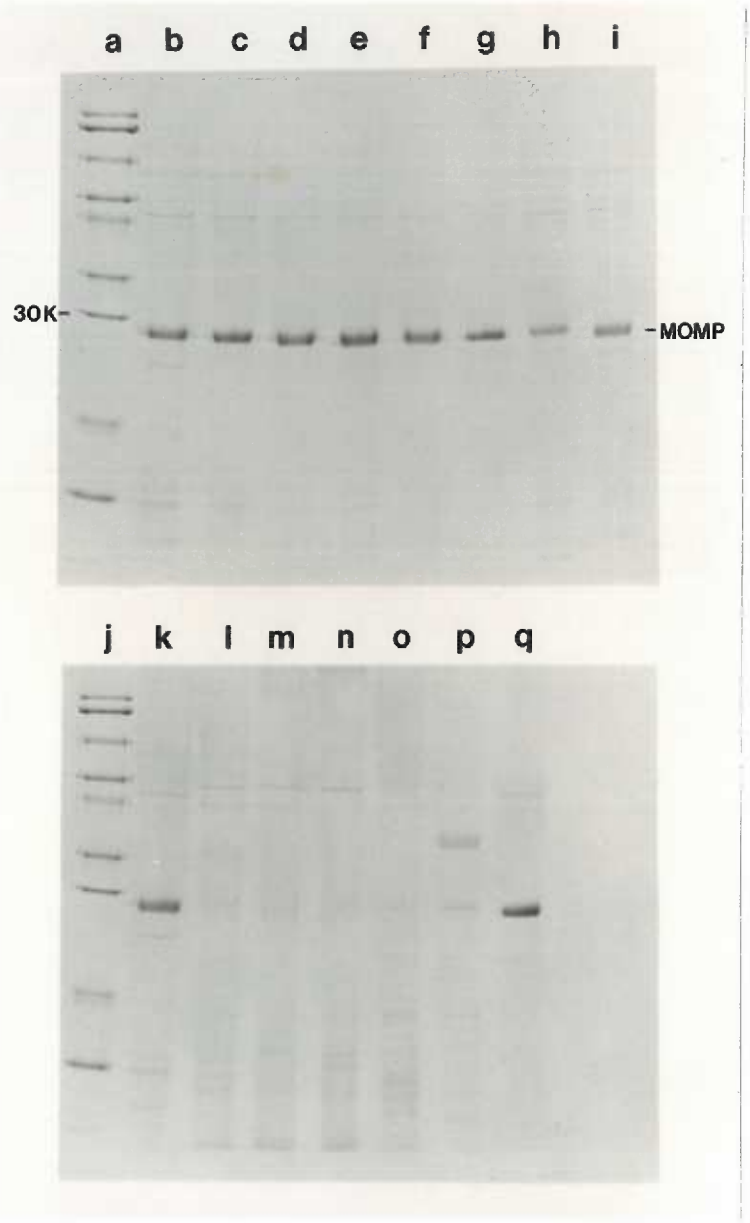
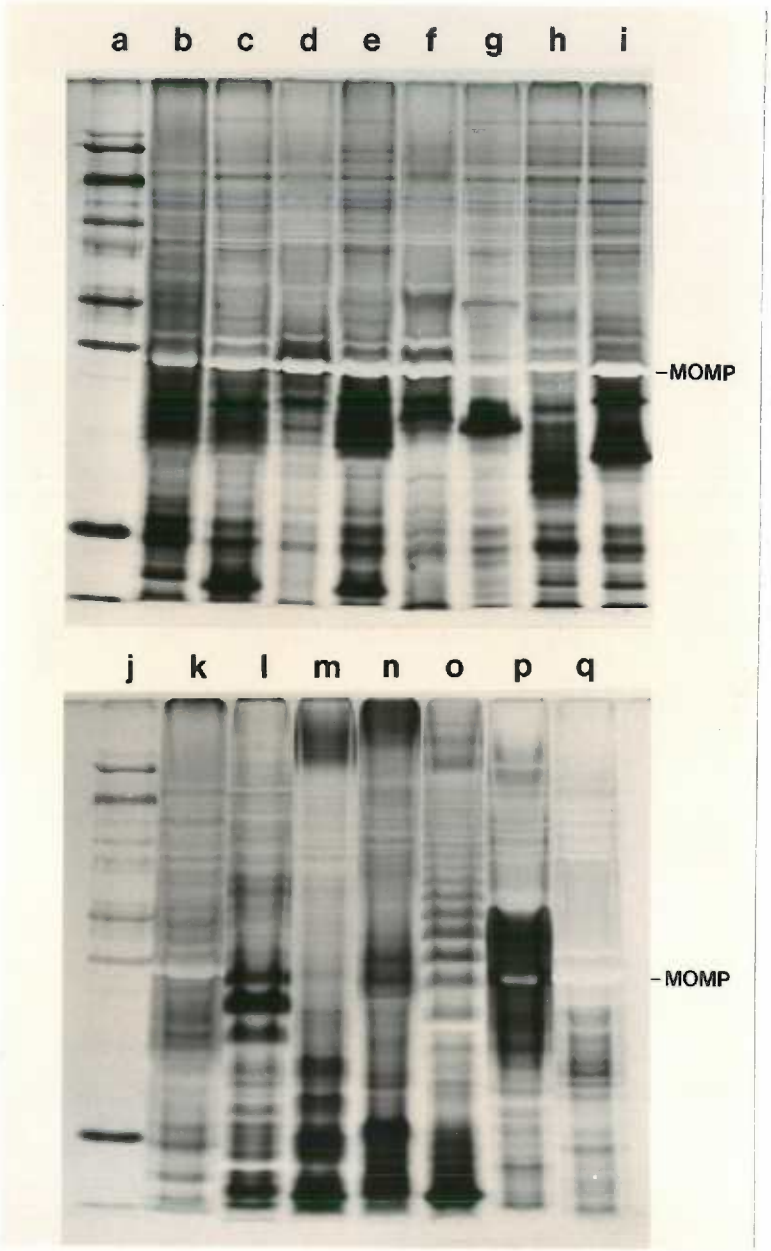

a b c d e f g h i
30K-
-MOMP
j k l m n o p q

Figure 2. Silver staining of SDS-PAGE outer membrane profiles. Solubilization conditions, amount of membrane protein applied, and the order of loading the gels were identical to that described in 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 prior to silver staining. Lanes a-q; gel treated with periodic acid prior to staining. Lanes a'-q' gel with no pretreatment prior to staining.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MOMP

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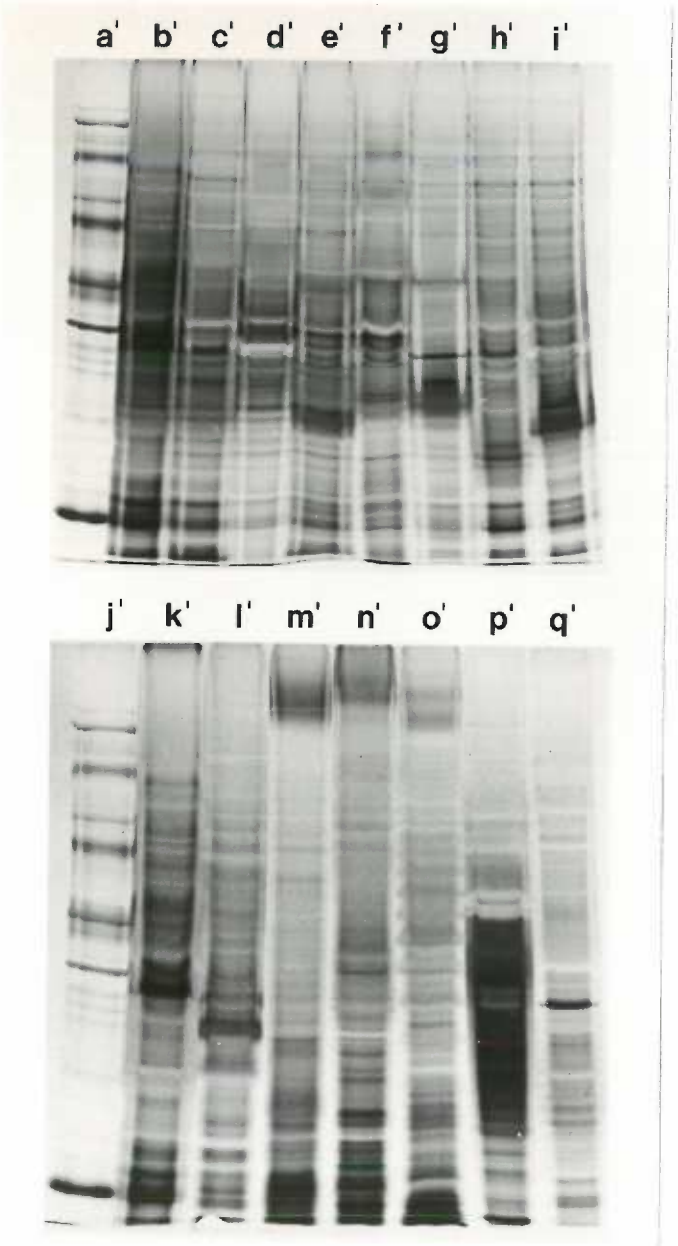

a' b' c' d' e' f' g' h' i'
j' k' l' m' n' o' p' q'

Figure 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 methods). A highly purified form of the MOMP was identified by SDS-PAGE in fractions with a retention coefficient of 0.69 (lane k). Lane a, molecular weight standards; lanes b-e, fractions 44-50; lanes f-j, fractions 52-60; lanes k-p, fractions 62-72.

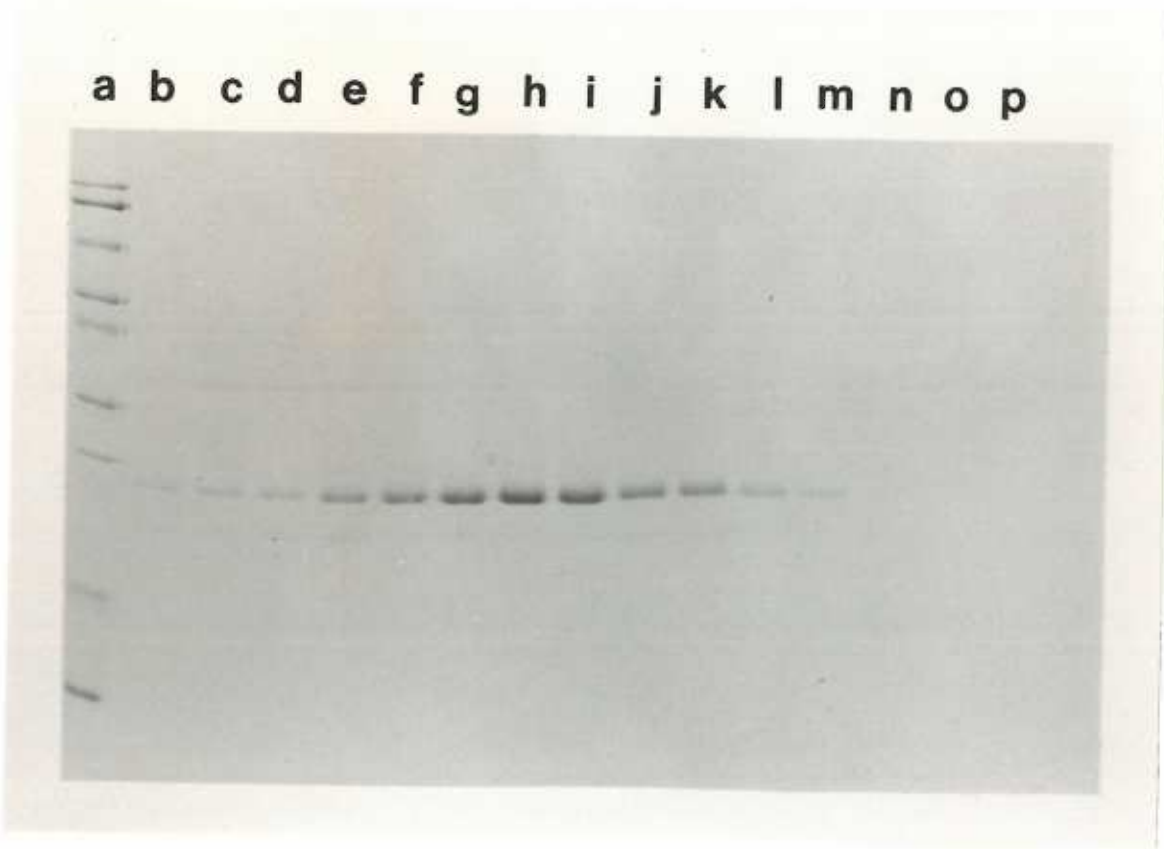

a b c d e f g h i j k l m n o p

Figure 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 following periodate oxidation. Western blots of these fractions were reacted with anti-MOMP antiserum. Lane a, MOMP stained with coomassie blue; lane b, MOMP stained with silver; lane c, Western blot of MOMP; lane d, LPS stained with coomassie blue, lane e, LPS stained with silver; lane f, Western blot of LPS.

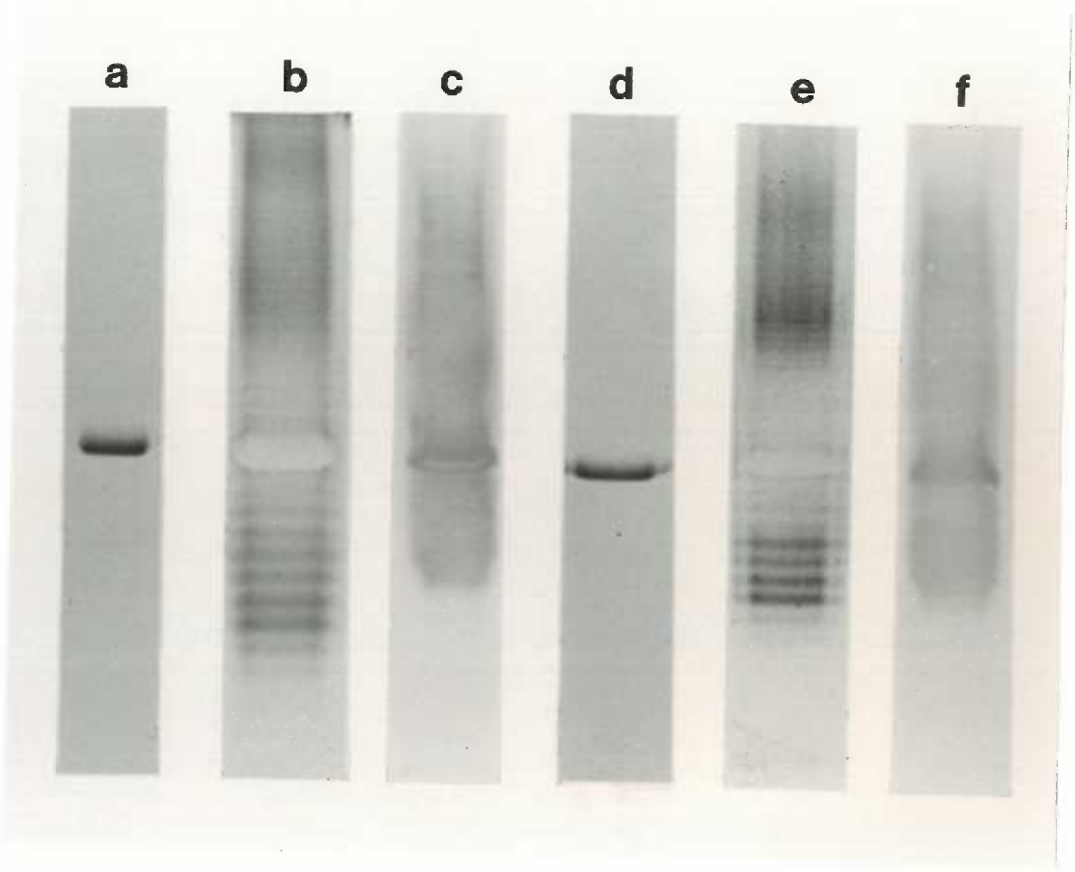
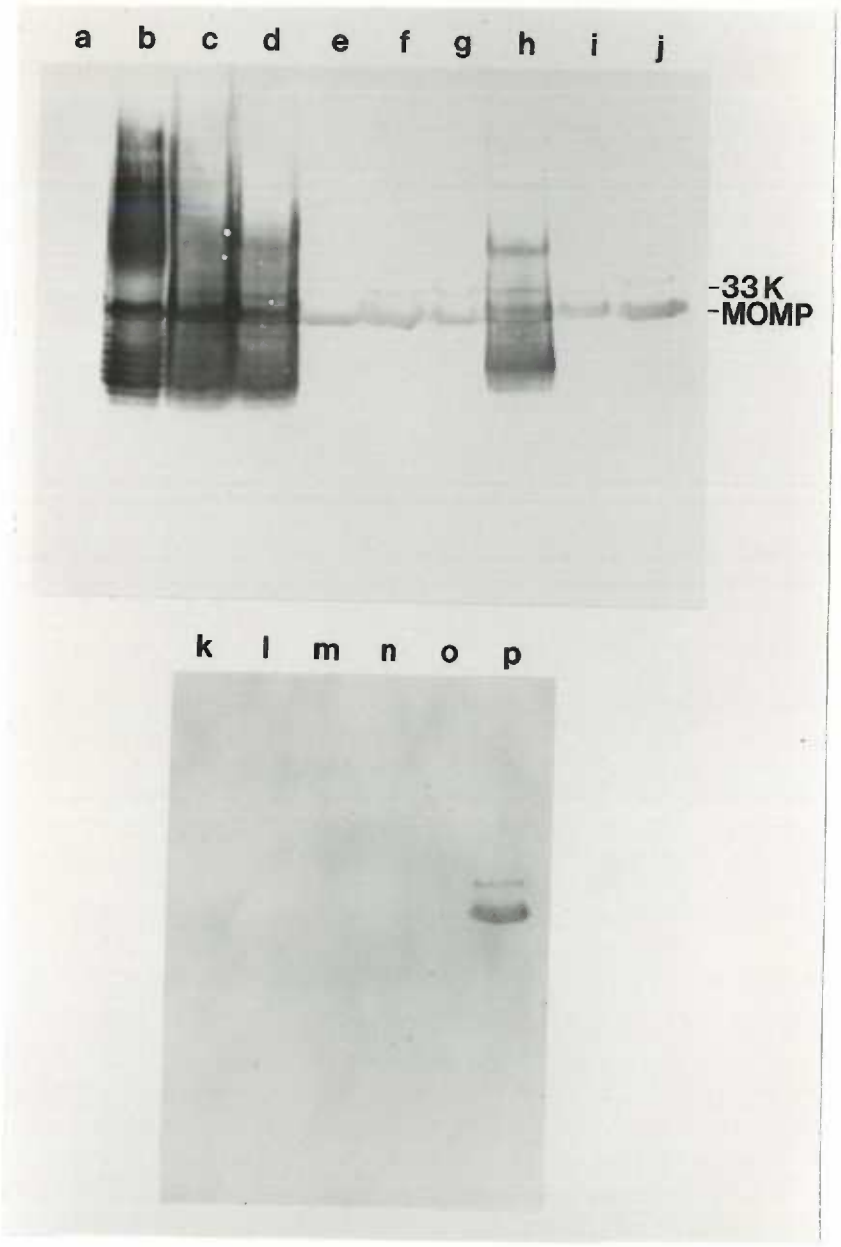

a
b
c
d
e
f

Figure 5. Western blots of outer membranes from L. pneumophila serogroups and non-pneumophila 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. Lane a, molecular weight standards, lane b, purified L. pneumophila serogroup 1 MOMP; lanes c-j, outer membranes from L. pneumophila serogroups 1-8; lanes k-o, outer membranes from L. bozemanii, L. dumoffii, L. gormanii, L. longbeachae, and L. micdadei respectively; lane p, clinical isolate of L. pneumophila serogroup 1. Samples were loaded onto gels at 10 μg of membrane protein per lane.


a b c d e f g h i j
-33K
-MOMP
k l m n o p

Manuscript 3

Cloning and Expression of a Common *Legionella* Antigen in *Escherichia coli*

ABSTRACT

A genomic library of Legionella pneumophila was constructed by inserting L. pneumophila knoxville-1 strain (LPK-1) chromosome fragments generated by digestion with restriction enzyme Eco RI into cosmid vector pHC79. Subsequent in vitro packaging by phage lambda and infection of Escherichia coli HB101 resulted in the recovery of ampicillin resistant colonies which when taken together represented the entirety of the LPK-1 genome. Screening of the library with antibodies directed against the isolated LPK-1 major outer membrane protein allowed for the identification of six clones that reacted with this antiserum. Western blot analysis of these clones resulted in the finding that a 19,000 dalton (19K) component was the reactive antigen in all of the clones. Western blot analysis of outer membranes from L. pneumophila serogroups and non-pneumophila species indicated that the cloned 19K antigen was common to all serogroups and all but one of the non-pneumophila species examined. Incubation of one of the clones (pMH5) with whole antiserum removed antibodies specific for the 19K antigen indicating that the 19K antigen was exposed on the surface of the E. coli clone. Clone pMH5 was used as an immunoabsorbent to recover antibody to the cloned 19K antigen by elution of the antibody from the cells with a low pH glycine buffer confirming the surface localization of this L. pneumophila antigen in E. coli.

INTRODUCTION

The gram-negative bacterium Legionella pneumophila causes a severe, often fatal pneumonia in humans and is resistant to a number of antimicrobial agents (30). The ability of the organism to successfully escape eradication by the immune system and most antibiotics may be directly related to the fact that L. pneumophila is an intracellular parasite of phagocytic cells. L. pneumophila has been shown to multiply within human peripheral monocytes (22) and interfere with the cascade of phagocytic events by preventing phagosome-lysosome fusion (23). This latter phenomenon occurs in the presence of erythromycin, indicating that protein synthesis may not be required to disrupt phagocytosis and that maintenance of the fusion-resistant vacuole in which the organism resides may in part be mediated by the cell surface of the organism. An ongoing interest of our laboratory concerns the cell surface and in particular, the protein composition of the L. pneumophila outer membrane.

The protein profile of the L. pneumophila Knoxville-1 (LPK-1) outer membrane as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is dominated by a disulfide stabilized major outer membrane protein (MOMP) that has an apparent molecular size of 29,000 daltons (29K) (19). The size of this protein and its disulfide stabilization is common to all eight serogroups of L. pneumophila and when isolated from LPK-1 crude peptidoglycan, is tightly associated with lipopolysaccharide (LPS). A MOMP with similar properties has not been

observed in outer membranes from L. bozemanii, L. dumoffii, L. gormanii, L. longbeachae, or L. micdadei (M. S. Hindahl and B. H. Iglewski, submitted for publication).

Several antigens from L. pneumophila have been described including a high molecular weight (F-1) antigen (24) and a serogroup-specific antigen (14,25). The composition of these antigens share the common property of being chemically complex, with all preparations containing varying amounts of lipid, protein, and carbohydrate (14,24,25). Recently L. pneumophila cellular antigens have been successfully cloned and expressed in Escherichia coli and several of these antigens have been shown to be translocated to the cell surface E. coli (12,13). However, the MOMP was not one of the antigens described in these reports. Owing to the preponderance of this protein relative to the total amount of membrane protein and its location in the outer membrane we were interested in the potential antigenicity of this protein in comparison to other outer membrane proteins. We studied the LPK-1 MOMP by constructing a cosmid genomic library of the LPK-1 chromosome in Escherichia coli strain HB101. Screening of the library with anti-MOMP antiserum failed to detect a MOMP clone. However, six clones expressing a 19K L. pneumophila antigen were identified. In this report we describe the identification and characterization of E. coli clones that successfully expressed this LPK-1 antigen.

MATERIALS AND METHODS

Organisms and Culture Conditions

Legionella pneumophila serogroups 1-8 used in this study included Knoxville-1 (serogroup 1), Togus 1 (serogroup 2), Bloomington 2 (serogroup 3), Los Angeles 1 (serogroup 4), Dallas 1E (Serogorup 5), Chicago 2 (Serogroup 6), Chicago 8 (serogroup 7), and Concord 3 (serogroup 8). Non-pneumophila species included *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, *L micdadei*. A human pneumonia clinical isolate identified as *L pneumophila* serogroup 1 was provided by Dr. Washington Winn Jr., University of Vermont Medical College. Organisms were maintained in a modified characoal yeast extract (CYE) broth (19) containing 20% glycerol at -70°C. Stock cultures were grown on CYE agar at 37°C in a 5% CO_2 atmosphere for 48 hr. prior to use. Broth cultures were grown in CYE broth after inoculation from 48 hr CYE agar at 37°C for ca. 12 hr. *Escherichia coli* strain HB101 was grown in either NY broth [0.8% nutrient broth (Difco laboratories), 0.5% yeast extract (Difco)] or T broth [1% tryptone (Difco Laboratories), 0.5% NaCl).

Isolation of Outer Membranes

Legionella exponential phase cultures (100 ml) were harvested and washed once in 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

(HEPES) (Sigma Chemical Co., St. Louis, Mo.) buffer, pH 7.4. Outer membranes were isolated from Legionella species by lysing cells with a French pressure cell followed by sucrose density gradient centrifugation as described previously (19). Sucrose was removed from outer membranes by resuspension of the membranes in HEPES buffer and centrifugation at 50,000 rpm in a Beckman Ti60 rotor for one hr. Washed membranes were resuspended in 1.0 ml of HEPES buffer and protein content was determined by the method of Lowry et al. (27). Identity of outer membranes was confirmed by 2-keto-3-deoxyoctonic (KDO) acid content of the membranes as estimated by the method of Waravdekar and Saslaw (36). Following lysis of washed whole cells with a French pressure cell, outer membranes were isolated from Escherichia coli HB101 by sucrose gradient centrifugation according to the method of Koplow and Goldfine (26).

Antibody Preparation

The MOMP from LPK-1 was isolated by exposing detergent insoluble material derived from cell envelopes to heat and reducing agent followed by gel filtration chromatography. Lipopolysaccharide (LPS) was found to be tightly associated with the isolated MOMP and when this preparation was used to generate MOMP antibodies, anti-LPS antibodies were also generated (M. S. Hindahl and B. H. Iglewski, submitted for publication). as seen in figure 4, lanes b, c, d, and h of this report. For descriptive purposes, antiserum generated by exposure to the MOMP/LPS complex will be referred to as anti-MOMP antiserum. The isolated MOMP (100 μg) in Freund's complete adjuvant was injected subcutaneously into white New Zealand rabbits. Two weeks following the initial injection,

rabbits received an additional 100 μg of MOMP in Freund's incomplete adjuvant subcutaneously. After two weeks 10 μg of the MOMP was administered intravenously and serum was collected five days following the final challenge.

Genomic Library Construction

A genomic library of LPK-1 was generated by insertion of chromosome fragments into the cosmid vector pHC79 (20) packaging with phage lambda, and subsequent infection of E. coli strain HB101 as follows. LPK-1 chromosome was isolated from 250 ml of exponential phase cells grown in CYE broth by the method of Nakamura et al (29). Chromosomal DNA (300 μg) was digested with 100 units of restriction endonuclease Eco RI (Bethesda Research Laboratories [BRL], Bethesda, Maryland) at 37°C for 10 min. The reaction was terminated by extracting the mixture twice each with phenol, chloroform:isoamyl alcohol (24:1), and ether equilibrated with 0.01 M Tris pH 8.0, 0.001 M EDTA (TE). Fragments in the digestion mixture were separated according to size by sucrose gradient centrifugation (28). Fractions of the gradient were electrophoresed in 0.7% agarose gels and those fractions containing fragments with lengths of 30-50 Kb were pooled. Pooled fragments were diluted with one volume of sterile distilled water and precipitated with ethanol. The pellet was resuspended in 20 μl of 0.02 M Tris, pH 7.4. Cosmid vector pHC79 (5 μg) was digested with 20 units of Eco RI at 37°C for 1 hr. The linearized cosmid was extracted twice with phenol, twice with chloroform:isoamyl alcohol (24:1), and four times with ether. The cosmid DNA was recovered by

ethanol precipitation and resuspended in 20 µl of 0.02 M Tris, pH 7.4. Chromosome fragments and Eco RI digested cosmid were mixed in a ratio of 20:1 respectively in a buffer consisting of 0.02 M Tris, 0.01 M $MgSO_4$, and 0.0006 M ATP pH 7.4 . Three units of T4 DNA ligase (BRL) were added and the mixture was incubated at 18°C for 24 hr. The ligation mixture was extracted once with chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. The pellet was washed once with cold 70% ethanol and following centrifugation at 12,000 x g for 10 min, the pellet was dried under vacuum. The pellet was resuspended in 10 µl of 10 mM Tris, pH 7.8 and 6 µl of the hybrid cosmid was added to a lambda packaging extract (Promega Biotech, Madison, Wisc.) and the mixture was incubated at 22°C for 2 hr. Phage dilution buffer (0.1 M NaCl, 0.01 M Tris, 0.01 M MgSO4, pH 7.9) was added (0.5 ml) followed by 25 µl of chloroform and the mixture was centrifuged at 12,000 x g for 3 sec. The aqueous phase was removed and added to 1 ml of E. coli HB101 that had been grown overnight in tryptone broth containing 0.2% maltose. Following infection at room temperature for 20 min., aliquots of 0.1 ml were spread on L-agar plates containing 100 µg/ml ampicillin (Ap^{100}) and incubated at 37°C for 36 hr. Colonies expressing resistance to Ap (Ap^r) were picked onto NYA plates (Ap^{100}) in grids of 100 colonies per plate.

Cosmid Isolation and Agarose Gel Electrophoresis

The presence of cosmids in E. coli HB101 was originally demonstrated by growing Ap^r colonies in 5 ml NY Ap^{50} broth and treating the cells by the method of Holmes and Quigley (21) followed by agarose gel electorophoresis. All agarose gels in this study contained 0.7% agarose

(Bio-Rad) in a Tris borate buffer (0.089 M Tris-Borate, 0.002 M EDTA pH 8) and were electrophoresed in a mini-sub chamber (Bio-Rad) at 75 volts constant voltage for 2 hr. Cosmids from HB101 clones that reacted with anti-MOMP antibody were recovered from cultures grown in one liter of NY broth containing 50 µg/ml Ap following alkaline lysis (5) by cesium chloride gradient centrifugation (28). Bands corresponding to the cosmids were removed from the gradients and subjected to a second round of cesium gradient centrifugation. Cosmids were removed from the gradients, extracted with isopropanol to remove remaining ethidium bromide, and dialyzed against 0.01 M Tris, 0.001 M EDTA pH 8.0 (TE) for 24 hr. Cosmids were concentrated by ethanol precipitation, resuspended in 100 µl of TE, and stored at $4^{o}C$.

Colony Blots of the LPK-1 Genomic Library

Ampicillin resistant colonies of E. coli HB101 were screened for reactivity to anti-MOMP antiserum as follows. Grids of Ap^{r} colonies on NYA plates were overlayed with nitrocellulose (NC) discs that had been pretreated by heating to $60^{o}C$ in the presence of 0.2% SDS for 15 min. followed by rinsing in phosphate buffered saline (PBS) and distilled water, and drying overnight at room temperature. Colony containing discs were placed in a chamber containing chloroform vapor for 20 min. Following drying for 5 min., the NC discs were individually placed into petri dishes containing 10 ml of a solution consisting of 0.05 M Tris, 0.15 M NaCl, 0.005 M $MgCl_2$, and 3% bovine serum albumin (BSA) (Sigma), pH 7.5 . Lysozyme (Sigma) and DNase I (Sigma) were added at 40 µg and 10 µg per plate respectively and the plates were incubated on a rocking

platform at 37°C for 1 hr. The NC discs were washed briefly in water and were then resuspended in 10 ml of 0.01 M Tris, 0.01M NaCl pH 7.4 (TN) containing 1% BSA. To each plate 10 µl of anti-MOMP antiserum was added (1:1000) and the plates were incubated on a rocking platform at 37°C for 2 hr. After a brief rinse in distilled water, the NC discs were resuspended in TN containing 1% BSA. Five µl of peroxidase conjugated goat anti-rabbit IgG (Cappel Laboratories, West Chester, Pa.) were added (1:2000) and the discs were incubated at 37°C on a rocking platform for 2 hr. The discs were rinsed briefly in water and 10 ml of a buffer containing 0.05 M Tris, 0.15 M NaCl, 0.005 M EDTA, and 0.05% Triton X-100, pH 7.4 for 15 min. was added and plates were incubated at room temperature for 15 min. The discs were rinsed with water, 10 ml of TN was added and the discs were incubated at room temperature with rocking for 10 min. The TN was removed and 6 ml of fresh TN was added to the plates followed by 2 ml of methanol containing 4-chloro-1-napthol 2 mg/ml). The reaction was initiated by the addition of 4 µl of H_2O_2 and terminated by rinsing in H_2O.

Recovery of anti-Legionella Antibodies from E. coli HB101

Antibodies reacting with a 19K L. pneumophila antigen were recovered using an E. coli clone from the genomic library as follows. A clone from the library that contained a hybrid cosmid yet did not express Legionella antigens (pED1) was used to pre-absorb the anti-MOMP antiserum to remove cross-reactive antibodies directed against E. coli. A twelve hour culture of pED1 grown in 250 ml of NY Ap^{50} broth was harvested by centrifugation. The cell pellet was resuspended in 500 ml of sterile

phosphate buffered saline (PBS) and collected by centrifugation. The washed pellet was resuspended in 3 ml of anti-MOMP antiserum in a 50 ml sterile tube and place on a tube rotator at 4°C for 10 hr. The suspension was centrifuged at 8,000 x g for 10 min., the serum containing supernatant was filter sterilized, and labled E. coli (antigen negative, cosmid containing) pre-absorbed serum.

An E. coli HB101 clone expressing a 19K L. pneumophila antigen (pMH5) was grown in 100 ml NY Ap^{50} broth for 8 hr. Cells were harvested and washed in 500 ml of sterile PBS by centrifugation. The washed pellet was resuspended in 1.5 ml of E. coli (antigen negative, cosmid containing) pre-absorbed serum and placed on a tube rotator at 4°C for 12 hr. The suspension was placed in a sterile eppendorf tube and cells were removed by centrifugation at 12,000 x g for 5 min. The supernatant was removed, filter sterilized, and labled E. coli (antigen positive, cosmid containing) absorbed serum. Antibody remaining bound to the pellet was recovered using the method of Engleberg et al (13). The cell pellet was washed three times with 1.0 ml aliquots of sterile, cold PBS by centrifugation. The washed pellet was resuspended in 1.0 ml of an elution buffer consisting of 0.2 M glycine, 0.2 M NaCl, pH 2.8, and incubated on a tube rotator at room temperature for 30 min. (13). The bacteria were removed from the suspension by centrifugation at 12,000 x g. The supernatant was filter sterilized and the pH was slowly adjusted to 7.4 with 2 M Tris base. The eluate was filter sterilized, labled pMH5 eluted antibody, and stored at 4°C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western Blotting.

Proteins from the outer membranes of Legionella species and whole cell lysates from E. coli HB101 were separated by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (19). Proteins were electrophoretically transferred from SDS-PAGE gels to NC sheets in a transblot chamber (Bio-Rad, Richmond, Ca) containing 0.025 M Tris base, 0.192 M glycine, and 20% methanol by application of 55V (constant voltage) to the chamber for 2 hr. Following electrotransfer, the NC sheets were incubated at 37°C in TN containing 3% BSA for 1 hr. After rinsing in H_2O, NC sheets exposed to rabbit anti-MOMP antiserum were treated identically to the NC discs used in colony blots as described above except that all volumes were increase by a factor of 10.

Western blots were probed with antibody eluted from clone pMH5 exactly as described for the anti-MOMP antiserum except that the working dilution of antibody was 1:100. Antibody bound to NC western blots was detected using ^{125}Iodine labled protein-A (New England Nuclear, Boston, MA). NC sheets were placed in 50 ml of TN containing 1% BSA. ^{125}I protein-A was added (1 μCi per blot) and the blots were incubated at 37°C on a rocking platform for 4 hr. The NC sheets were each rinsed with 3 liters of TN, placed on absorbant paper, and allowed to air dry. The NC sheets were placed under Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N. Y.) in an X-ray cassette and incubated at -70°C for 18 hr.

RESULTS

Genomic Library Construction

Packaging hybrid cosmids consisting of the vector pHC79 plus large fragments of the LPK-1 chromosome (i.e. c.a. 35-45 Kb) and infection of E. coli HB101 resulted in 1150 Ap^r colonies. The size of the L. pneumophila chromosome has been estimated at 2.5×10^9 daltons (6). Assuming an average size of 40 kilobases for the LPK-1 fragments packaged and using the equation of Clarke and Carbon (8), 860 colonies would be required to attain a 99.9% certainty of having the entire chromosome represented by the library. Based on these calculations the cosmid library was considered to represent the entirety of the LPK-1 chromosome. Six Ap^r colonies were picked randomly from the library and grown in L-broth containing Ap^{50} for 12 hr. Agarose gel electrophoresis of nucleic acids recovered from these cultures by the method of Holmes and Quigley (21) resulted in the observation that each Ap^r colony contained an extrachromosomal element that appeared to have a molecular weight of approximately 40 - 50 Kb (data not shown). The presence of the orginal vector pHC79 in Ap^r clones was demonstrated by digesting cesium purified high molecular weight hybrid cosmids with Eco RI followed by agarose gel electrophoresis (fig.1). Screening the library with anti L. pneumophila MOMP antibody resulted in the identification of 6 clones that bound antibody. Subsequent analysis revealed that these colonies bound antibody with or without lysis of the colonies prior to exposure to antibody suggesting that perhaps LPK-1 antigens were exposed on the E.

coli surface. Large scale cosmid preparations from four of these clones (pMH1, pMH2, pMH5, pMH6) and subsequent digestion with Eco RI resulted in the identification of the original vector, and fragments which collectively equaled a total size suitable for packaging by phage lambda (Fig. 1) for each of the clones.

SDS-PAGE and Western Blot Analysis

The SDS-PAGE protein profiles of four clones expressing antigen (pMH1, pMH2, pMH5, and pMH6) and a clone containing cosmid but not expressing antigen (pED1) were compared. No observable differences were detected between the clones expressing antigen and the clone lacking antigen when SDS-PAGE gels containing cell lysates (fig. 2A) and outer membranes (fig. 2B) were stained with coomassie blue. Silver staining of gels containing outer membranes following periodic acid oxidation also revealed no discernable differences between the clones (data not shown). When whole cell lysates from clones pMH5 and pMH6 were applied to SDS-PAGE gels followed by western blotting with anti-MOMP antiserum, a band corresponding to a molecular size of 19K was found to react with the antiserum (fig. 3, lanes a and b). Western blots of cell lysates from the cosmid containing, antigen negative clone pED1 failed to react with the antiserum (fig. 3, lane e). The 19K antigen was also identified by western blots of cell lysates from clones pMH1, pMH2, pMH3, and pMH4 (data not shown). Western blots of isolated cell envelopes and purified outer membranes from pMH5 revealed an enrichment of the 19K antigen (fig. 3, lanes c and d) indicating that the antigen was localized in the E. coli outer membrane.

Western blots of outer membranes from L. pneumophila serogroups and Legionella species reacted with anti-MOMP antiserum indicated that a 19K antigen was common to all serogroups and species (Fig. 4, lanes b-p) with the exception of L. gormanii and that the 19K antigen from pMH5 outer membranes co-migrated with this Legionella antigen (Fig. 4, lane q).

Recovery of 19K Antibodies

The finding that lysis of colonies was not required for antibody binding and the fact that the E. coli outer membrane appeared to be enriched with the 19K L. pneumophila antigen indicated that the antigen was expressed on the E. coli cell surface. To examine this possibility we attempted to absorb the 19K specific antibody from the anti-MOMP antiserum used to probe western blots of Legionella outer membranes. Antiserum that had been absorbed with a cosmid containing, antigen negative clone (pED1) followed by absorption with an antigen positive clone (pMH5) was used to probe western blots as described above. Detectable amounts of anti-19K antibody were present after absorption with the antigen negative clone pED1 (fig. 4, lanes a-q). However, following absorption of the antiserum with clone pMH5, antibody was no longer observed at this working dilution of antiserum indicating that the 19K L. pneumophila antigen was expressed on the surface of the E. coli clone (fig 4. lanes a'-q').

To further characterize the surface expression of this L. pneumophila antigen in E. coli we attempted to use pMH5 as a solid phase

immunoabsorbent and recover the antibody by eluting it from the E. coli cell surface according to the method described by Engleberg et al. (13). Using this eluate to probe western blots of the outer membranes from the Legionella serogroups and species it was found that anti-19K antibody was recovered from pMH5 using the acidic elution buffer. The antibody bound to the 19K antigen present in western blots of outer membranes of the Legionella species and pMH5 (Fig. 5). The fact that the antibody was detected using staphlycoccal protein-A suggested that the anti-19K antibody was of the IgG class. Ouchterlony and HPLC analysis of the eluted antibody using purified IgG as a standard confirmed that IgG class antibodies were present in the glycine eluate (data not shown).

(fig. 4, lane b) or eluted antibody (fig. 5, lane b), it was present in sufficient amounts to be identified in the LPK-1 outer membrane under both conditions (fig. 4, lane c; fig. 5, lane c). The 19K antigen appears to be a common antigen to all but one of the six species of Legionella we have examined and was found to be associated with the Legionella outer membranes (Fig. 4) indicating that it is a membrane protein. Furthermore, the cloned 19K antigen appeared to be localized in the E. coli outer membrane and was expressed on the cell surface. The 19K antigen can now be added to a list of common antigens in the Legionellae including a 24K major outer membrane protein common to all species (7) and a 29K species specific antigen (18) in L. pneumophila.

Several L. pneumophila serogroup 1 antigens have recently been cloned in E. coli including those with molecular sizes of 17K, 19K, 24K, 61K, 66K and 68K (12). Of these antigens, all but the 17K antigen have been shown to be surface expressed in E. coli (13). We have not been able to determine if the antigen we have characterized is identical to that described by Engleberg et al. (12, 13). If the antigens are one in the same, it is curious that this particular gene is so readily transcribed and expressed on the cell surface by E. coli, an organism that has been shown to have less than 3% DNA homology with L. pneumophila (6) and as discussed previously maintains a cell envelope considerably different from that of L. pneumophila (13). These differences include those associated with peptidoglycan structure (1,2), lipid composition (19), and outer membrane protein composition (7,10,19). Although such large differences in DNA homology and cell envelope architecture exists

between these two organisms it is clear that the expression, processing, and translocation of at least some L. pneumophila proteins may occur by similar mechanisms as those employed by E. coli. Comparisons of sequence analysis of the isolated 19K product and/or the subcloned 19K gene with those of E. coli outer membrane proteins or their genes may show similarities in composition. Such information would be a useful beginning in understanding the protein synthetic machinery of L. pneumophila, an organism that secretes a variety of exoproducts (3,4,15,34).

The function of the 19K antigen is unclear at this time. However, its apparent localization in both the L. pneumophila and E. coli outer membranes and its molecular size suggests that perhaps the 19K antigen is a pilin subunit. Ultrastructure studies have demonstrated the presence of pili and a single polar flagellum on the L. pneumophila cell surface (9, 31). Pilin subunits having molecular sizes of 15K in Pseudomonas aeruginosa (37), 17.5K - 21K in Neiserria gonorrhoeae (33), and from 13K - 29.5K for the various host specific E. coli pili (16) have been reported. The 19K antigen appears to be a common antigen in the L. pneumophila serogroups and all but one of the non-pneumophila species we examined. This property is similar to that observed for isolated flagella from a wide variety of serogroups and species within the genus Legionella (11, 32). The ability to recover antibody to the 19K antigen from the E. coli clone should allow for straightforward affinity purification of the antigen. Subsequent biochemical and immunological comparisons of the isolated antigen with purified L.

pneumophila pili will allow for further consideration of the possible relationship between these entities.

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Figure 1. Agarose gel electrophoresis of cesium gradient purified cosmids from E. coli HB101 clones expressing the L. pneumophila 19 K antigen digested with restriction enzyme Eco RI. Lane a, Hind III digested lambda DNA standards; 23.1 Kilobase (Kb), 9.4 Kb, 6.6 Kb, 4.4 Kb, 2.3 Kb, and 2.0 Kb. Lane b, original cosmid vector pHC79 digested with Eco RI; lanes c-F, Eco RI digested cosmids pMH1, pMH2, pMH5, pMH6.

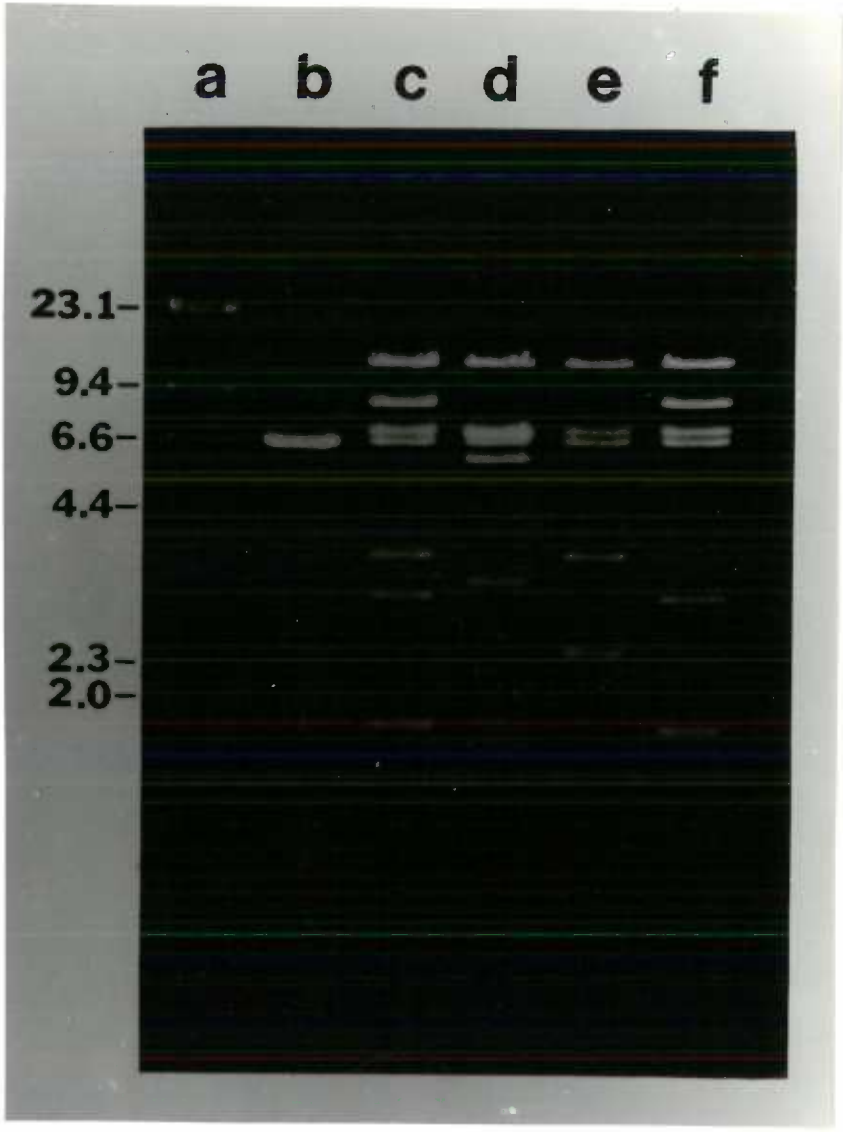

a
b
c
d
e
f
23.1–
9.4–
6.6–
4.4–
2.3–
2.0–

Figure 2A. SDS-PAGE analysis of whole cell lysates from four clones expressing the 19K antigen and one clone not expressing antigen. Lane a, molecular weight standards in kilodaltons. Lane b, antigen negative clone (pED1). Lanes c-f, 19K antigen expressing clones pMH1, pMH2, pMH5, and pMH6 respectively. All lanes were loaded at 10 μg protein per lane.

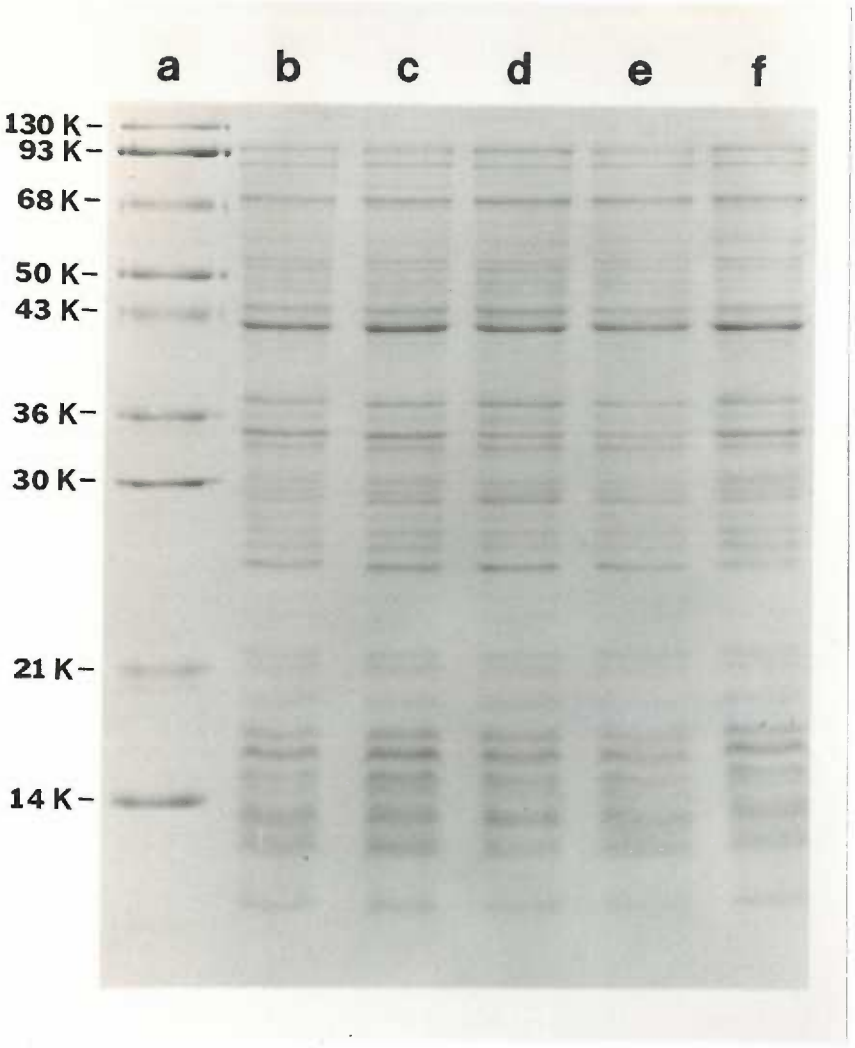

a
b
c
d
e
f
130 K–
93 K–
68 K–
50 K–
43 K–
36 K–
30 K–
21 K–
14 K–

Figure 2B. SDS-PAGE analysis of outer membranes from four clones expressing the 19K antigen and one clone not expressing antigen. Lane a, pED1; lanes b-e, clones pMH1, pMH2, pMH5, and pMH6. All preparations were loaded onto gels at 10 μg per lane.

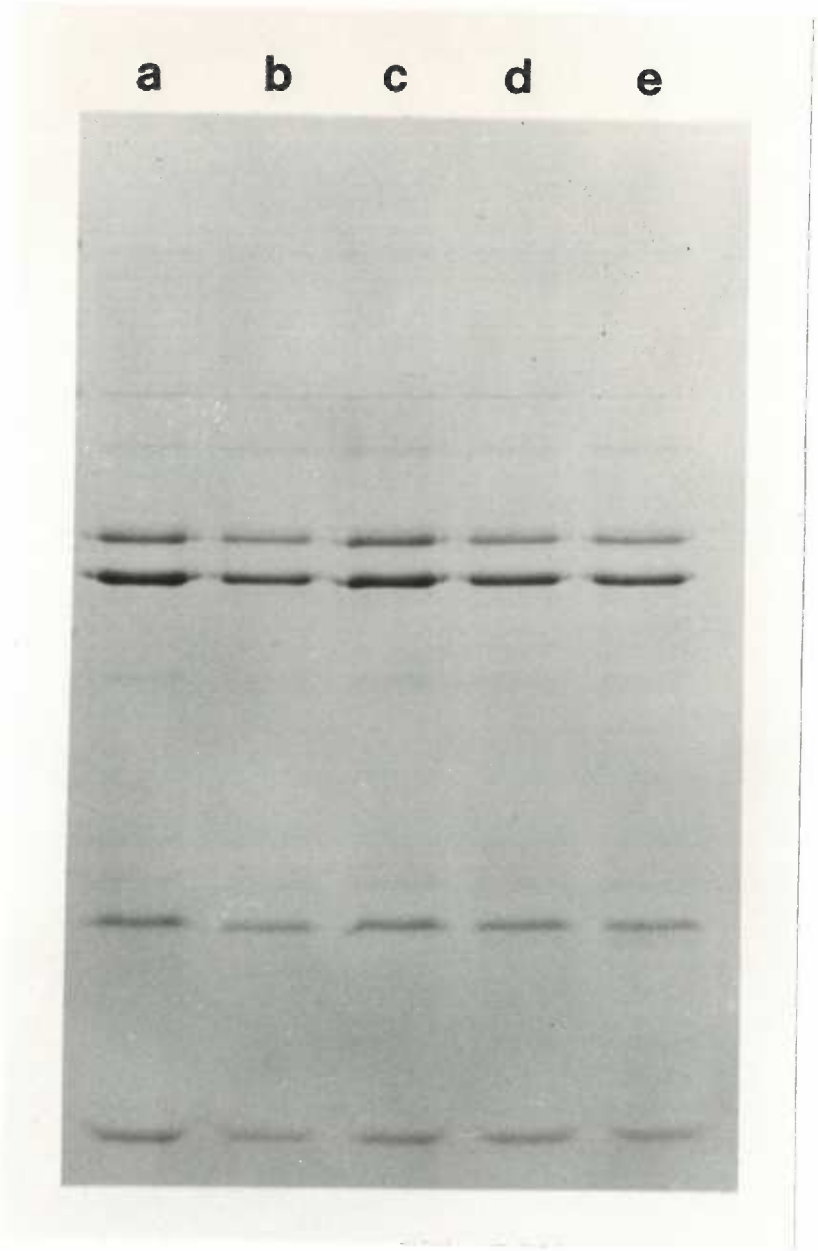
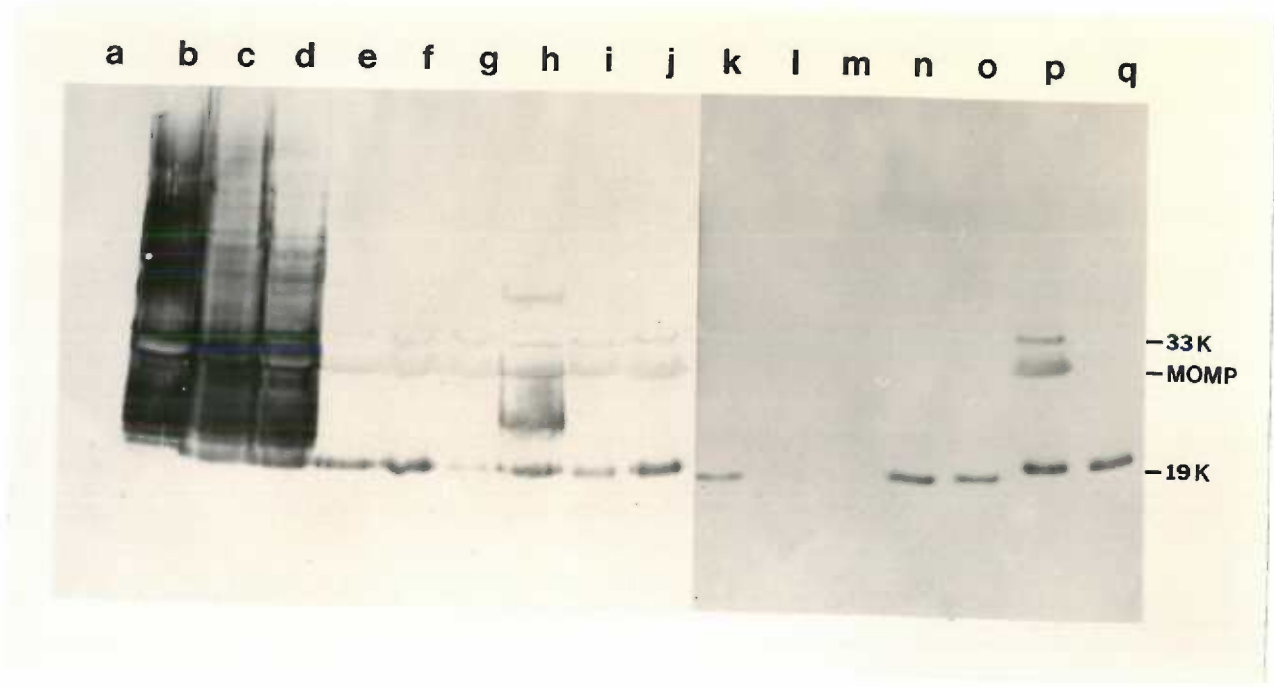

a
b
c
d
e

Figure 3. Western blots of E. coli HB101 clones reacted with antiserum directed against the LPK-1 MOMP. Lanes a and b, whole cell lysates of clones pMH5, and pMH6. Lanes c and d, isolated cell envelopes and outer membranes respectively from clone pMH5. Lane e, whole cell lysate from HB101 containing cosmid but not expressing antigen (pED1). Lane f, molecular weight standard as described in figure 2. All preparations were loaded onto SDS-PAGE gels at 10 μg per lane.


a b c d e f

Figure 4. Western blots of outer membranes from Legionella species and HB101 clone pMH5 reacted with antiserum directed against the MOMP. 10 μg of membrane protein from each sample was applied to SDS-PAGE and following electrophoresis was transferred to nitrocellulose as described in Methods. Lane a, molecular weight standards identical to those described in figure 2. Lane b, isolated MOMP complex. Lanes c - j, L. pneumophila serogroups 1 - 8 respectively. Lane k, L. bozemanii; lane l, L. dumoffii; lane m, L. gormanii; lane n, L. longbeachae; lane o, L. micdadei; lane p, L. pneumophila serogroup 1 clinical isolate; lane q, HB101 clone pMH5. Lanes a - q, blots reacted with antiserum pre-absorbed with a antigen negative, cosmid containing HB101 clone (pED1). Lanes a' - q', blots reacted with antiserum pre-absorbed with pED1 followed by absorption with HB101 clone pMH5.


a b c d e f g h i j k l m n o p q
33K
MOMP
19K

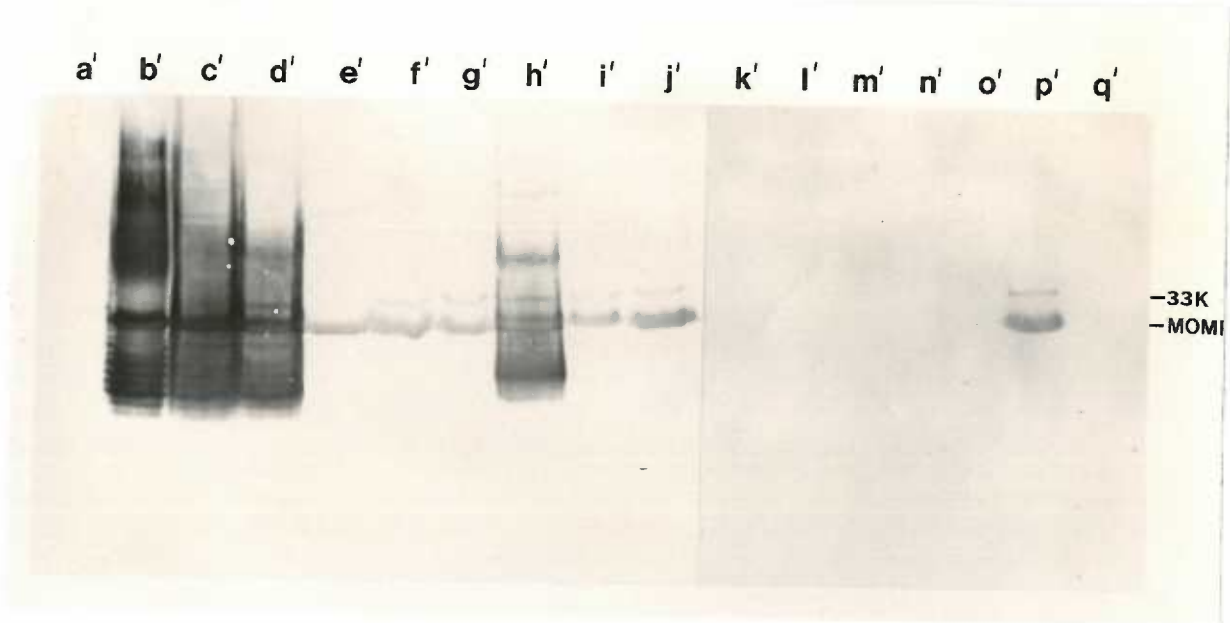
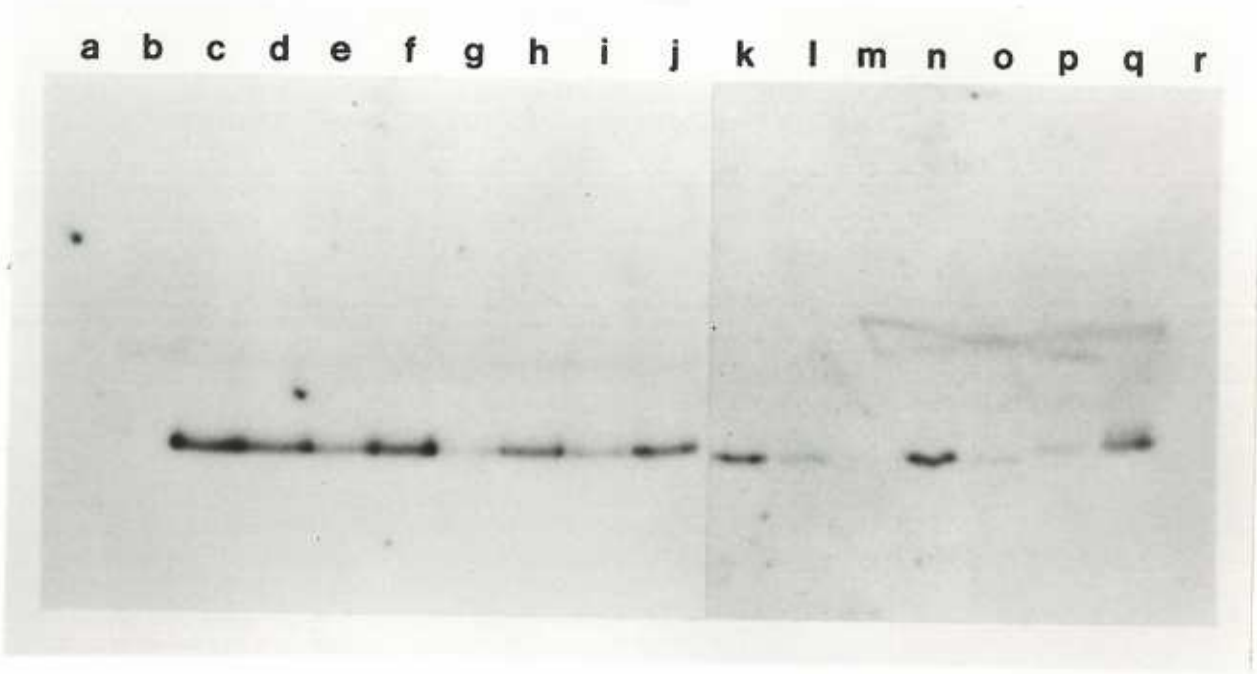

a' b' c' d' e' f' g' h' i' j' k' l' m' n' o' p' q'
33K
MOMI

Figure 5. Western blots of outer membranes from Legionella species and HB101 clones reacted with antibody eluted from the surface of clone pMH5. SDS-PAGE and western blotting of outer membranes was performed as described in Methods. Antibody eluted from the surface of HB101 clone pMH5 after absorption of anti MOMP/LPS antiserum with the clone was reacted with blots and bound antibody was detected with ^{125}I protein-A. Lane a, molecular weight standards as described in figure 3. Lane b, isolated MOMP/LPS complex. Lanes c-j, L. pneumophila serogroups 1 - 8; lane k, L. bozemanii; lane l, L.dumoffii; lane m, L. gormanii; lane n, L. longbeachae; lane o, L. micdadei; lane p, L. pneumophila serogroup 1 clinical isolate; lane q, HB101 clone pMH5; lane r, HB101 clone pED1. All samples were loaded onto SDS-PAGE gels at 10 μg per lane.


a b c d e f g h i j k l m n o p q r

IV. Summary and Discussion.

A tremendous body of information has accumulated concerning the genus Legionella subsequent to the identification of L. pneumophila as the causative agent of the 1976 pneumonia epidemic in Philadelphia. Taxonomic studies have indicated that a large number of species, and as of yet unnamed species, exist within the genus (4). These organisms appear to be ubiquitous in nature and are found in a variety of aqueous environments.

The continual addition of species and suspected species to the ever-growing list of members within the genus Legionella has indirectly deflected the focus of many investigations. Although a very systematic characterization protocol has routinely been employed following the suspicion that a new Legionella isolate has been recovered, some very basic questions regarding these organisms remain partially answered at best.

As evidenced by the various species characterized in the studies described in this thesis, at least some of the non-pneumophila species were recognized at the beginning of this study. However, in light of the difficulties arising from attempting to interpret numerous reports covering a wide range of serogroups and species relative to any given aspect of the organism, I felt that a systematic characterization of the L. pneumophila cell envelope was warranted. The cell envelope and in particular the outer membrane from L. pneumophila knoxville-1 (LPK-1) were characterized in detail to establish a biochemical baseline

for purposes of comparing this information with that from other serogroups and species.

A number of methodologies were utilized in efforts to isolate the outer membrane from LPK-1. The first approach taken was to attempt to lyse whole cells with lysozyme-EDTA followed by separation of cell envelope components by sucrose gradient centrifugation. This method, as described in manuscript 1, was found to be unsatisfactory in that lysozyme appeared to be ineffective at forming osmotically fragile spheroplasts. This apparent resistance to lysozyme mediated destruction of the peptidoglycan is in agreement with the studies of Amano and Williams who speculated that this phenomenon was due to the presence of proteins covalently bound to the peptidoglycan on the L. pneumophila (1) The use of a French pressure cell was found to be the most effective method for lysing whole cells of LPK-1 and it was found that loading of whole cell lysates onto sucrose gradients was necessary to recover workable quantities of cytoplasmic membrane (manuscript 1).

Biochemical analysis of the outer membrane resulted in the detection of the common LPS component 2-keto-3-deoxyoctonic acid (KDO) and hexosamine which is often a component of LPS and is typically a major constituent of peptidoglycan. Uronic acids, which are indicators of bacterial capsular material, were not detected in isolated outer membranes from LPK-1. Capsule-like material has, however, recently been observed in association with the cell surface of several Legionella species (15). The failure to chemically detect this material in isolated outer membranes may be the result of loss of this material during outer membrane preparation. The phospholipid content of the LPK-1 outer membrane was distinquished from that of most bacteria by the presence of

phosphatidylcholine. This finding is in agreement with an early report that described this lipid as being common to the cellular lipids from a number of L. pneumophila serogroup 1 strains. While the functional significance of this observation remains unclear, it continues to serve as a means of differentiating this organism from most other bacteria (21).

Analysis of the outer membrane proteins by SDS-PAGE revealed that the protein profile was dominated by a single major outer membrane protein (MOMP) with an apparent molecular size of 29,000 daltons. Two-dimensional SDS-PAGE analysis of outer membranes resulted in the observation that this protein required heating in the presence of reducing agent to be resolved as a monomer. The MOMP was also found to be tightly associated with the peptidoglycan layer and again was found to require heating in the presence of reducing agent to dissociate this protein from the peptidoglycan.

The major outer membrane proteins from many gram-negative bacteria are often pore-forming proteins called porins. Based on methodologies commonly used to isolate porins from other bacteria, the tight association of the L. pneumophila 29K protein with the peptidoglycan was exploited in an effort to purify this protein. A purification method based on release of this protein from SDS insoluble material (crude peptidoglycan) by increased ionic strength was ineffective. Subjecting LPK-1 cell envelopes to extraction methods that utilized either Triton X-100 or hexdecyltrimethylammmonium bromide (CTB) detergents did, however, result in the recovery of highly enriched 29K protein preparations that could be utilized to assess the possibility that this

protein contained pore-forming functions in a black lipid bilayer membrane system (appendix B).

The performance of the black lipid bilayer apparatus was monitored using a purified outer membrane protein (NmpC) from E. coli suspected of having pore-forming properties (appendix A). This protein was indeed found to be a porin and although the diameter of the channel formed by this protein was slightly less than those formed by other E. coli proteins, the properties of this protein in the black lipid bilayer apparatus were essentially identical to those for a number of other gram-negative porins. These properties are demonstrated in appendix A and include: a) an orderly, stepwise increase in conductance increments with the magnitude of the conductance increases being very similar in the majority of the recorded events; b) a linear relationship between total current passing through the membrane as a function of time after addition of the pore-forming protein; c) channel forming events independent of voltage; d) a linear relationship between conductance and salt concentration at a fixed potential across the membrane.

The channel forming events that occurred when the isolated L. pneumophila 29K protein was introduced into the black lipid bilayer apparatus were unusual when compared to that of the NmpC protein. The conductance increments were neither uniform in magnitude nor in stepwise incorporation into the membrane (appendix B, figs. 1 and 2). Both the instability of the channels formed and the fluctuations in conductance can also be seen in figure 3 of appendix B which represents the total increase in current over time after the introduction of isolated 29K protein to the black lipid bilayer. The reason for the instability of the channels formed remains unclear. However, it was felt that the

fluctuations in the magnitude of conductance increments were due to the incorporation of aggregate forms of the protein of varying sizes. The 29K protein also exhibited a linear relationship between conductance and salt concentration and channel forming events were found to be independent of applied voltage. The results of single channel event studies were inconclusive regarding determining the average diameter of the channels formed. However, this study clearly demonstrates that the 29K protein from LPK-1 forms channels in black lipid bilayers and is a porin. Subsequent to this study, Gabay et al. (11) isolated the MOMP from L. pneumophila philadelphia-1 strain (serogroup 1) using the detergent CTB and assayed this preparation in black lipid bilayers for pore-forming activity. The results of this study were similar to those discussed above for the LPK-1 MOMP confirming that this protein is a porin.

A continuation of the investigation of the outer membrane proteins from members of the genus Legionella revealed that the 29K protein (MOMP) was common to all eight serogroups examined within the species L. pneumophila. The requirement of heating in the presence of reducing agent to resolve this protein as a monomer by SDS-PAGE was also common to all serogroups within the species. A protein of this molecular size was not detected in the five non-pneumophila species however. The SDS-PAGE profiles of the serogroup outer membrane proteins were very similar when visualized by coomassie blue staining, yet could readily be differentiated from one another by staining identical gels with silver. The basis for these differences appears to be the result of differences in the LPS within the outer membranes. The differences in silver staining patterns between the various Legionella species is even more

striking than those seen for the L. pneumophila serogroups. These distinct patterns were also attributed to the presence of LPS in the outer membranes from these organisms. In addition to DNA hybridization methodology, the utilization of this technique provides a powerful identification tool for the differentiation of Legionella species.

The immunological relationships between the outer membrane proteins of L. pneumophila serogroups and Legionella species was studied by exposing the outer membrane proteins from these organisms to antibody directed against the LPK-1 29K protein (manuscript 2). In view of the concern that the methods employed to isolate the MOMP from LPK-1 for use in porin studies resulted in the recovery of aggregates of varying size, efforts were made to recover a more homogeneous preparation of this protein for the generation of anti-MOMP antibodies. Subjecting the SDS-insoluble material (crude peptidoglycan) to heat and reducing agent released the MOMP in a soluble form which was then isolated by gel filtration chromatography. Antibodies directed against this preparation were found to cross-react with the MOMP from all of the eight L. pneumophila serogroups. The antiserum also contained antibodies to LPS which were found to cross react with the LPS from two other serogroups. The fact that these were LPS antibodies was verified by isolation of LPS from LPK-1 to utilize for comparison in western blots of the 29K protein. Several interesting observations resulted from this experiment. The 29K protein co-purified with the LPS indicating that two forms of the 29K protein exist; an SDS soluble form, and a form associated with SDS insoluble material (crude peptidoglycan). In addition, the 29K protein was resistant to extremely rigorous proteolytic treatments. Both of these properties are identical to those described for the LPS from L.

pneumophila philadelphia-1 strain (10). The MOMP/LPS antibody containing antiserum did not cross-react with the outer membranes from any of the non-pneumophila species allowing for further distinction between these species.

The 29K outer membrane protein may be the same species specific antigen detected in western blots of L. pneumophila whole cell lysates with a monoclonal antibody reagent (13). In contrast to the recognition of a 29K protein as a species specific antigen, a 24K peptidoglycan associated protein has recently been reported as being common to 9 of the 10 Legionella species examined and has been suggested as being a genus specific antigen (5). The results of studies such as those described above clearly emphasize the useful taxonomic information gained from an increased definition and subsequent understanding of the compostion of the outer membranes from members of the genus Legionella.

Thorough analysis of the L. pneumophila peptidoglycan has shown that this structural entity of the cell envelope is chemically complex when compared to other gram-negative bacteria. The glycan polymer is highly cross-linked and contains covalently linked proteins. (1). In addition to this mesh-like network exterior to the cytoplasmic membrane, the outer membrane contains the disulfide stabilized 29K protein that is at least partially linked to the peptidoglycan layer. The additive structural effect of the peptidoglycan layer and the highly cross-linked MOMP in the outer membrane results in a complex cell envelope composition that presents an extremely rigid barrier between the interior of the cell and the environment. I felt that the best way to examine the relationship of the L. pneumophila MOMP with the other cell envelope components was to isolate this protein from the remainder of the L. pneumophila proteins by

cloning the gene for the MOMP and searching for the expression of the gene product in E. coli. To this end, a genomic library of the LPK-1 chromosome was constructed by ligating large fragments of the LPK-1 chromosome fragments into a cosmid vector, packaging the constructs with phage lambda, and infecting E. coli strain HB101 (manuscript 3). Screening of the resulting library with the anti-MOMP/LPS antibody described in the second manuscript failed to detect a clone expressing this protein. However, several clones were identified that did react with the antibody and were found to be expressing the same antigen, which had an apparent molecular size on SDS-PAGE of 19K. Western blot analysis of outer membranes from eight L. pneumophila serogroups and five non-pneumophila species indicated that this antigen was common in the outer membranes of all serogroups and all but one of the species examined suggesting that it was common antigen. Further analysis of one of these clones revealed that the gene encoding this antigen was expressed on the surface of E. coli indicating that not only was the mRNA specifying this antigen translated by this organism, but the protein was processed and transported across the cytoplasmic membrane and periplasmic space to be properly inserted in the outer membrane. Several other L. pneumophila antigens have also been cloned in a different vector system and shown to be surface expressed in E. coli (7,8). The implications of these findings are far-reaching in that the expression of these genes and the proper processing of their products by an organism with which L. pneumophila shares less than 3% DNA homology seems highly unlikely.

The ultimate goal of a study of this type is to be able to understand structure-function relationships of the outer membrane of L. pneumophila. A function mediated by the outer membrane that is of

obvious concern is the relationship between the actual physical surface displayed by the organism and its ability to survive within its environment.

L. pneumophila is described as a facultative intracellular parasite and is found in a wide variety of aqueous habitats. However, a number of in vitro studies have demonstrated that the organism replicates intracellularly within common aquatic protozoa and a number of human cell types (including professional phagocytes). Furthermore, the organism has been observed within phagocytes taken from in vivo settings. These data convincingly suggest that in both its natural ecological niche and in the human host, the organism prefers to exist in an intracelluar environment.

In human infections, the organism apppears to have evolved several mechanisms by which it is able to evade destruction by professional phagocytes [i.e. monocytes, macrophages, polymorphonuclear leukocytes (PMNs)], the very cells of the immune system designed to destroy the bacterium. The actual events leading to the successful residency of these organisms in such an inhospitable environment are not fully understood. It is known that L. pneumophila produces an extracellular product that interferes with the respiratory burst event in polymorphonuclear leukocytes (PMNs) that normally leads to the production of oxygen containing compounds that are bacteriocidal (22). L. pneumophila has been shown to be highly sensitive to these products in vitro (16) indicating that it is of great advantage to the organism to be able to directly combat this aspect of the host defense system.

As an alternative to confronting PMNs by the production of a toxin that neutralizes an essential antibacterial component of these cells, L. pneumophila is able to survive and replicate within monocytes and

macrophages (17, 20). Once phagocytosed, the organism is known to prevent phagosome-lysosome fusion by a yet to be elucidated mechanism and maintain itself within a membrane bound vacuole. The ability of L. pneumophila to prevent phagosome-lysosome fusion places the organism in a select group of bacteria including Mycobacterium tuberculosis and Chlamydia spp. that also have this property.

Comparison of some of the cell surface properties of these organisms with those of L. pneumophila results in several interesting observations. Prevention of phagosome-lysosome fusion in M. tuberculosis has been shown to be mediated by strongly anionic glycolipids (sulfatides) resulting in the suggestion by one investigator that the presence of a polyanionic surface may lead to subtle changes in the phagosome membrane and thereby prevent fusion with the lysosome (12). The outer membrane of L. pneumophila is dominated by the presence of the disulfide stabilized 29K MOMP which has been shown to be a porin (appendix b, 11). It is worthy of note that bacterial porins share the property of having acidic isoelectric points (3). If one were to assume that the isoelectric point of the L. pneumophila porin is also acidic, the presence of the MOMP in conjunction with the recently described anionic capsule-like material associated with the L. pneumophila cell surface (15) may result in the presentation of a highly anionic surface to the phagocyte and potentially prevent phagosome-lysosome fusion.

The Chlamydiae also prevent phagosome-lysosome fusion in professional phagocytes (9, 23). The ability of these organisms to prevent this function is independent of protein synthesis [as seen for L. pneumophila (19)], and has been shown to be mediated by the surface of the organism. Both heat inactivation of the organism (which causes a

structural change in the cell surface) and opsonization of the organisms with anti-Chlamydial antibody, eliminates the ability of the organism to prevent fusion and the organism is rapidly eliminated by the phagocyte (9). Given the apparent central role that the cell surface plays in the intracellular survival of these organisms, it should be recognized that a high degree of similarity exists between the major outer membrane protein of these organisms and that from L. pneumophila. The MOMPs from both C. psitaci and C. trachomatis have apparent molecular sizes on SDS-PAGE gels of 40 K, are highly cross-linked by disulfide bonds (14), and the MOMP from C. trachomatis has been shown to function as a porin (2). The degree of disulfide cross-linking in this protein has been shown to vary dramatically between the extracellular elementary body (EB) form and the intracellular reticulate body form (RB) (14). Finally, whole cells of C. trachomatis exposed to IgG antibody directed against the C. trachomatis MOMP were found to penetrate HeLa cells with an efficiency equal to untreated controls, yet antibody treated cells were no longer infectious (6). This observation coupled with those discussed above indicates that the MOMP containing cell surface plays an integral role in the pathogenesis and survival of the Chlamydiae.

The findings that the cell surfaces of M. tuberculosis and Chlamydia spp. are essential in preventing the destruction of these organisms by professional phagocytes emphasizes the need for further investigation of the L. pneumophila cell surface and outer membrane. The information presented in this thesis establishes a solid framework upon which to design experiments of a more exacting nature to address the mechanisms of intracellular survival of the organism. Of interest is the comparison of the disulfide cross-linked MOMP in both the Chlamydiae spp. and

L. pneumophila which as described above are very similar to one another. The inference of this discussion is that the L. pneumophila MOMP may be of importance in the intracellular survival of this organism. Given the similarity of the two MOMPs from these organisms, approaches utilized to study the MOMP from Chlamydia spp. would seemingly be well adapted to L. pneumophila. Of immediate interest would be to determine if differences exist in the degree of cross-linking of the L. pneumophila MOMP between extracellular and intracellular forms of the organisms. Methodologies have also been established to recover Chlamydia spp. within intact, isolated phagosomes. This approach allows for the comparison of membrane proteins of the phagosome relative to the entire plasma membrane protein compliment to determine if specific proteins are recognized and selected by the bacterium during early events of phagocytosis (i.e. phagosome formation) (24). Early studies have shown that L. pneumophila opsonized with whole cell antibody are phagocytosed more rapidly than non-opsonized controls, yet survived intracellularly equally as well (18). Now that methods are available to isolate the MOMP from L. pneumophila, it should be straightforward to develop more specific immunological reagents to determine if blocking the MOMP in whole cells with MOMP specific antibody will alter the infectivity of the organism for in vitro cell cultures as seen for C. trachomotis. It should also be remembered that in manuscripts two and three the outer membranes from five non-pneumophila species were thoroughly examined and a protein with properties like the MOMP from the L. pneumophila serogroups was not observed. These organisms would seemingly be the ideal negative controls in examining their rate of intracellular survival relative to L. pneumophila serogroups in professional phagocyte cultures infected in vitro.

The demonstration of the successful cloning and expression of the LPK-1 19K antigen is encouraging with respect to future efforts to study Legionella gene products in the well characterized E. coli host. Utilization of the genomic library established in this study may prove useful in investigations to study suspected virulence factors of L. pneumophila (i.e. protease, hemolysin, toxin). In addition, the library presents an excellent opportunity to develop strategies in order to detect E. coli clones expressing a determinant or determinants involved in the intracellular survival of L. pneumophila.

It is hoped that the information presented in this study will lend to not only a basic biochemical understanding of the L. pneumophila outer membrane, but as outined above, serve as a basis for future investigations designed to explore the complexities and intracellular survival of this organism.

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Appendix A

Outer Membrane Protein NmpC of *Escherichia coli*: Pore-Forming Properties in Black Lipid Bilayers

Abstract

The purified NmpC outer membrane protein from Escherichia coli, when incorporated into planar lipid bilayers, gave rise to channels with a single channnel conductance of 1.8 nS in 1M KCl. This suggests that the NmpC protein is a porin.

The Escherichia coli outer membrane has been shown to contain a variety of polypeptides which function as transmembrane diffusion channels and bear the common name porins (10). All E. coli porin proteins studied to date share the following properties (10): the functional pore-forming unit is a trimer species which maintains its oligomeric association even in the presence of sodium dodecyl sulfate (SDS) at moderate (<60°C) temperatures; it displays a tight noncovalent association with the peptidoglycan; the monomeric molecular weights are similar and range from 35,000 to 45,000 daltons; the porin monomers have acidic isoelectric points; porins are present in high copy numbers ($>10^5$ polypeptides per cell) under the appropriate circumstances; and all form hydrophilic transmembrane pores with similar channel sizes [estimated as 1.2 to 1.5 nm in diameter (1, 1a, 2, 5)]. In addition, there is considerable nucleotide and protein sequence homology amongst some of these porins (14). The porin proteins from E. coli whose function has been well documented include the constitutive porins OmpC, OmpF (1-3, 11) and protein K (15), and the inducible porins LamB (2) and PhoE (11, Benz et al., in press). In addition, Pugsley and Schnaitman provided evidence that protein 2 (also called the Lc protein) which is encoded by the prophage PA2, can reverse some of the transport defects associated with loss of the constitutive porins OmpC and OmpF (13). Although this reversal of the transport defects was somewhat strain, growth medium, and substrate specific, it suggested that protein 2 might have a porin function. It has since been demonstrated that the NmpC protein which is produced in high levels in a pseudorevertant of an E. coli K-12 porin-deficient mutant (12) is extremely similar but not identical to protein 2 on the basis of proteolytic cleavage patterns (7).

Furthermore, the NmpC protein appears closely related to the OmpD (38,000) porin protein of Salmonella typhimurium (2, 6). Although this has provided circumstantial evidence that the NmpC protein is a porin, direct evidence has been lacking to date.

The NmpC protein From E. coli K-12 strain CS483 (12) (obtained from Barbara Bachmann of the Coli Genetic Stock Center, Yale University, New Haven, Conn., as strain CGSC6066) was purified exactly as described previously for the protein K porin (15) except that the final solubilization step was performed at 60°C for 2 h rather than at 37°C for 2 h.

The basis of this purification procedure was the resistance to denaturation by SDS and strong noncovalent association with the peptidoglycan of the NmpC protein. The NmpC protein remained bound to the peptidoglycan even after treatment with 2% SDS, high salt concentration, and EDTA at 37°C for 2 h, although the same procedure when performed at 60°C resulted in nearly quantitative release of the NmpC protein from the peptidoglycan. The purified NmpC protein formed an oligomer with an apparent mass of 74,000 daltons when analyzed by SDS - polyacrylamide gel electrophoresis (5) after solubilization in SDS at temperatures less than 60°C (data not shown). However, after solubilzation at 100°C for 5 min, the protein appeared in the gel electrophoretogram as a monomer of 39,500 daltons.

The methods used for black lipid bilayer experiments have been described previously in detail (1, 3). The apparatus consisted of a Teflon chamber with two compartments connected by a small hole (0.1 to 1 mm^2). A membrane was formed across the hole by painting on a solution of 1 to 2% (wt/vol) oxidized cholesterol in n-decane. Bilayer formation was

indicated by the membrane turning optically black to incident light. Conductance through the pore was measured after application of a given voltage, using a pair of Ag-AgCl electrode inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a preamplifier, monitored by a storage oscilloscope, and recorded on a strip-chart recorder.

Addition of moderate amounts (final concentration 46.7 pg/ml) of the purified NmpC protein to the aqueous salt solution bathing a lipid bilayer membrane resulted in an increase in specific membrane conductance of 2 orders of magnitude. After the membrane turned black, conductance increased in a time-dependent fashion for more than 60 min and failed to reach a stationary level before the membrane broke. When a large number of NmpC channels (>50) were incorporated into the membrane, current measured through the channels was a linear function of applied voltage and extrapolated to zero current at zero voltage. This suggested that the NmpC channel is not voltage induced or regulated. In all, no significant differences were observed in macroscopic conductance experiments with the NmpC protein or other E. coli porins (1, 3, 4, 15).

When small amounts (3×10^{-11} M) of purified NmpC porin were added to the aqueous phase bathing a lipid bilayer membrane, membrane conductance increased in a stepwise fashion (Fig. 1). By analogy with other lipid bilayer experiments, these increases probably represented the time-dependent incorporation of single NmpC channels into the membrane. Occasionally, increments were observed which were twofold larger than the most common increments, especially in the presence of 1 M NH_4Cl, probably due to the simultaneous incorporation of two channels into the membrane. Most of the conductance increments were directed upwards although

downwards-directed conductance steps of similar magnitude were also observed, presumably due to loss or inactivation of a previously incorporated channel.

As shown previously for other porins (1-4, 15), the single-channel increments were not of uniform size but were distributed around a mean. For example, in 1 M KCl the most common conductance increment was 1.5 nS (33% of measured channels), but a substantial number of channels with conductances of 1.0 nS (18%), 2.0 nS (23%), and 2.5 nS (19%) were observed in the 213 recorded single-channel conductance steps. Analogous observations were made for all other salts studied.

The average single-channel conductances were recorded for a variety of salts (Table 1). Despite substantial variations in the bulk conductance (i.e., the conductance σ of the salt solution in the absence of a membrane) and in the average single-channel conductance Λ, the ration of Λ to σ varied only 1.7 fold. This suggested that the NmpC channel is filled with water. In agreement with this possibility, single-channel conductance was a linear function of salt concentration, a result expected for a large water-filled channel (Table 1).

The data presented here confirm previous hypotheses that the NmpC protein from E. coli is a porin and profide the first direct evidence for this. The purified NmpC porin forms large water-filled channels as judged by the linear relationship between KCl concentration in the aqueous phase and single-channel conductance, the relatively constant Λ/σ ratios for a variety of salts, and the linear relationship between macroscopic current and applied voltage. Thus, the NmpC channel has properties similar to those of the well-studied porins of E. coli and other bacteria. Many other physical properties of the NmpC channel

resemble those of other E. coli porins, including an oligomeric (presumably trimeric) association of subunits that is stable to SDS (9), a strong noncovalent association with the peptidoglycan (7, 10), a monomeric molecular weight in the 35,000 to 45,000 range, an acidic isoelectric point and a tendency to form multiple isoelectric focusing bands (10), expression dependent on the tolC locus (8), and a similar conductance (1.8 nS) in 1 M KCl (1, 1a, 2, 15).

Single channel conductance experiments suggested that the size of the NmpC channel is similar to that of the PhoE and protein K pores but somewhat smaller than the OmpC, OmpF, and LamB pores. On the basis of the data in Table 1 for 1 M KCl and assuming a pore length of 7.5 nm (2), we could estimate the effective pore diameter as 1.2 nm. This small decrease in size over the OmpC (1.3 nm) and OmpF (1.4 nm) porin channels (2) could be quite significant when considering the effectiveness of NmpC in the uptake of larger molecules such as nucleotides, β-lactams, and di- or trisaccharides, given the substantial differences in the permeability of OmpC and OmpF toward such compounds (11).

The clear demonstration here that NmpC is a porin brings the known number of porins in E. coli to six, i.e., OmpC, OmpF, NmpC, PhoE, LamB, and protein K. Even given the conditional production of the PhoE and LamB porins and the fact that NmpC is a silent gene in wild-type E.coli K-12, a major unanswered question concerns the reason for so many genes coding for very similar proteins in the same species of bacterium.

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Figure 1. Stepwise increases in the membrane conductance after addition of 3×10^{-11} M NmpC protein to the aqueous phase (1 M NaCl) bathing a lipid bilayer membrane. The membrane was formed from 1.5% oxidized cholesterol in n-decane, the applied voltage was 20 mV, and the temperature was maintained at $25^{\circ}C$. The record begins at the left.

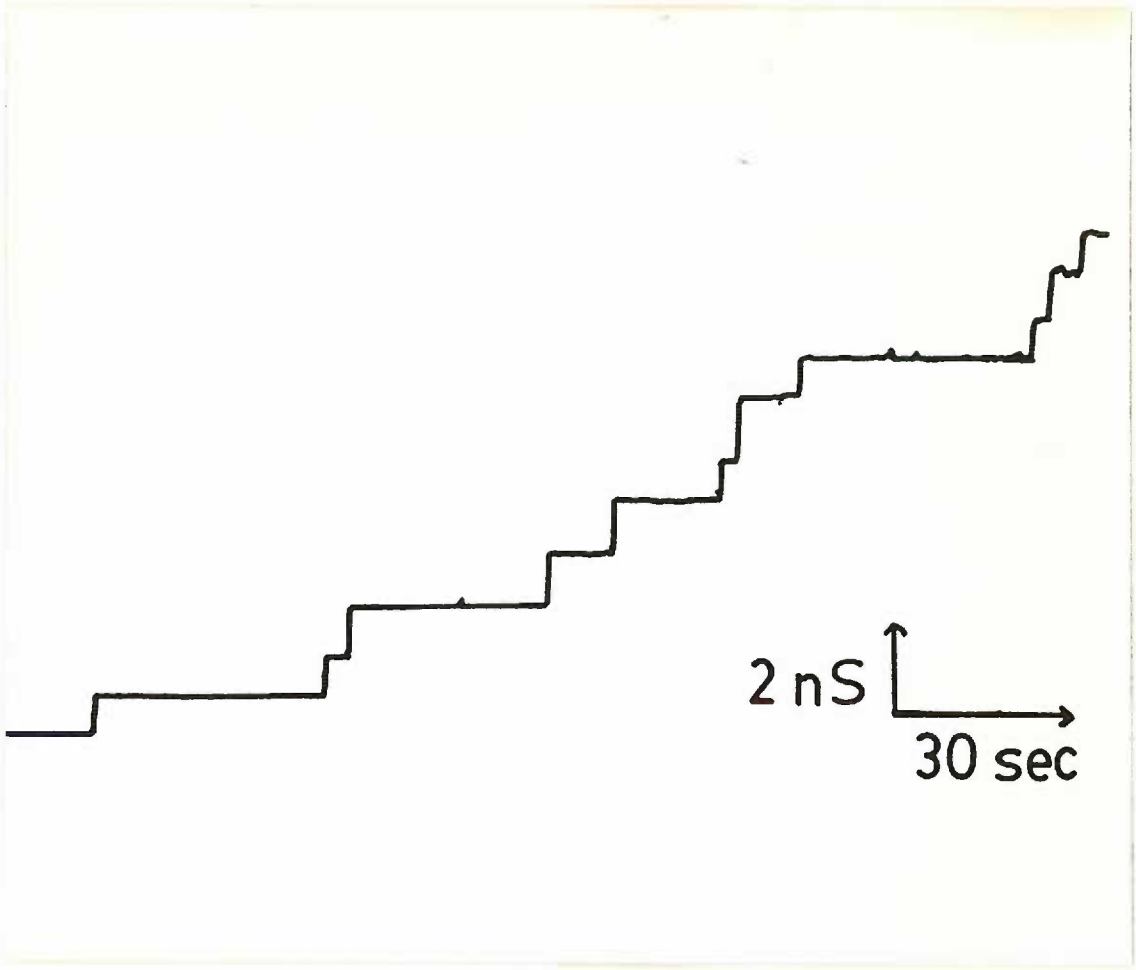
2 nS
30 sec

TABLE 1. Average single-channel conductance increments in different salt solutions. The aqueous phase contained the indicated concentration of salt and 3×10^{-11} M NmpC protein was added to initiate single-channel measurements. The applied voltage was 10 mV; membranes were made from 1 to 2% oxidized cholesterol; the temperature of the aqueous phase was 25°C. Λ was averaged for a large number of conductance increments. The bulk conductance for each salt solution was the measured conductance in the absence of a membrane. Control experiments demonstrated that the small amount of SDS added to the aquous phase together with NmpC protein did not cause membrane conductance to increase.

Salt	Conc. (M)	Avg single-channel conductance Λ (nS)	Bulk Phase conductance of salt solution (mS/cm)	Λ/σ (10^{-8} cm)	No. of increments measured
KCl	0.03	0.058	3.4	1.7	104
	0.1	0.19	11	1.7	178
	0.3	0.49	34	1.5	174
	1.0	1.80	110	1.6	213
NaCl	1.0	1.12	84	1.3	258
NH_4Cl	1.0	1.39	112	1.2	122
$MgCl^2$	0.5	0.62	64	1.0	249
Tris-chloride	0.5	0.33	30	1.1	132
K_2SO_4	0.5	1.32	76	1.7	110

Appendix B

Behavior of the Isolated *Legionella pneumophila* Major Outer Membrane Protein in Planar Black Lipid Bilayers

Methods for isolating the major outer membrane proteins (MOMPs) from a number of gram-negative bacteria have been developed based on the tight association of these proteins with the peptidoglycan layer of the cell envelope. As described in manuscript 1, the 29K outer membrane protein from L. pneumophila apparently maintains a similar relationship with the peptidoglycan of the organism. Based on this observation, efforts were made to isolate the 29K protein from L. pneumophila knoxville-1 (LPK-1) strain to determine if this protein, like many of the MOMPs from various gram-negative organisms was a pore-forming protein or porin. The basic approach to isolating porins relies upon the insoluble nature of the peptidoglycan in the presence of detergents. Typically, solubilization of cell envelopes in detergent followed by centrifugation results in the recovery of a crude peptidoglycan preparation which contains associated porins. The porins are then released from the peptidoglycan by increasing the ionic strength of the solution and following column chromatography are readily attainable in a highly purified form. Porins have been successfully isolated by solubilizing cell envelopes in the anionic detergent sodium dodecyl sulfate (SDS) (4), the non-ionic detergent Triton X-100 (3), and the cationic detergent hexadecyltrimethylamonium bromide (CTB) (2).

LPK-1 cell envelopes were subjected to all of the methods mentioned above. Extraction with the anionic detergent SDS followed by exposing the crude peptidoglycan to increasing ionic strength was found to be unsuccesful at releasing the 29K protein (manuscript 1). Treatment of LPK-1 cell envelopes with either the method utilizing Triton X-100 (3) or the cationic detergent CTB (2) resulted in highly enriched preparations of the 29K protein as judged by SDS-PAGE.

Both of these preparations were introduced into planar black lipid bilayers to assess the pore-forming capacities of these samples. The experimental conditions were identical to those described for the characterization of the NmpC protein from Escherichia coli (appendix A). Both the Triton X-100 and the CTB preparations were found to be highly active in channel formation when picogram quantities were added to the aqueous solution bathing the oxidized cholesterol membrane. However the channel forming events were extremely erratic in both the apparent size of the channels formed and the stability of the channels within the membrane. When small amounts of either the Triton X-100 (fig. 1) or the CTB (fig. 2) prepared 29K were introduced into the membrane containing Teflon Chamber a wide range of conductance increments (Λ) was observed. The majority of the channel forming events were short-lived as seen by the vertical lines which represent a channel forming event due to the incorporation of the protein(s) into the membrane followed by an immediate release of the channel from the membrane or inactivation of the incorporated channel (figs. 1 and 2). In addition, after some of the channel forming events, the lipid bilayer appeared to be destabilized to varying degrees as demonstrated by the noisy signals following some of the vertical rises (figs. 1 and 2). As noted, the size of the conductance increases resulting from channel forming events was highly inconsistent. This could be the result of the simultaneous incorporation of different channels or due to the incorporation of aggregate forms of the 29K protein into the membrane that contained different numbers of channels. Although channel forming events occured upon the introduction of either preparation to the bilayer, clearly the behavior of the 29K protein differed greatly from that of the E. coli NmpC protein described in

Appendix A. As observed for the NmpC protein, the size of conductance increments is not absolutely uniform but centers around a mean which is clearly demonstrated by histogram analysis of the number of events in a given salt solution. When greater than four hundred channel forming events resulting from the introduction of the 29K protein into a 1 M NaCl solution were analyzed in this fashion, it became apparent that channel forming events were occuring by the insertion of monmeric, dimeric, trimeric, and oligomeric channels. This indicated that the fluctuation in the size of conductance increases was not due to the simultaneous incorportation of varing numbers of channels, but that aggregates of channels were being incorporated into the membrane. This interpretation may also explain the frequent occurence of off-scale conductance increases, and the common noisy signals following channel forming events not seen with the E. coli NmpC protein (appendix A, fig. 1).

An additonal parameter usually measured during these type of studies is the increase in total current over time after the introduction of porins to the Teflon chamber. Assuming that channel incorporation is taking place in a stepwise, orderly fashion such that the integrity of the membrane remains intact, a linear relationship between total current and time after porin addition is realized. These type of studies were performed using both the Triton X-100 and the CTB preparations of the 29K protein. As can be seen in figure 3, addition of either preparation resulted in a net total current increase over time. However, the relationship between current and time showed fluctuations not seen with the NmpC protein (appendix A) and other porins (1). These fluctuations are in agreement with the single-channel studies discussed above that suggested that: a) the conductance increments covered a wide range of

sizes, and b) the channels were either released from the membrane or inactivated (denoted by the drops in total current).

The results of these studies indicate that the 29K protein from L. pneumophila is indeed a porin. This protein differs from the porins of many other gram-negative bacteria in that exposure of crude peptidoglycan to increasing ionic strength does not release the protein from this structure. However, methods employing both non-ionic and cationic detergents extractions of L. pneumophila cell envelopes did result in the recovery of a highly enriched form of this protein that formed channels in black lipid bilayers. The behavior of the 29K protein in this system differed greatly from most porins which was believed to be attributable to the tendency of the protein to exist in aggregate forms. These preliminary studies indicate that additional characterization of the 29K MOMP aggregates will be necessary to better define the channel forming properties (i.e. diameter of the channel) of this protein in black lipid bilayers.

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Figure 1. Representative channel forming events when L. pneumophila 29K protein was introduced to 1 M NaCl bathing an oxidized cholesterol black lipid bilayer membrane. The protein was isolated using a method based on the solubilization of cell envelopes in Triton-X 100 (3). The horizontal bars indicate the size of both conductance (nS) increases and conductance decreases. The record begins on the left.

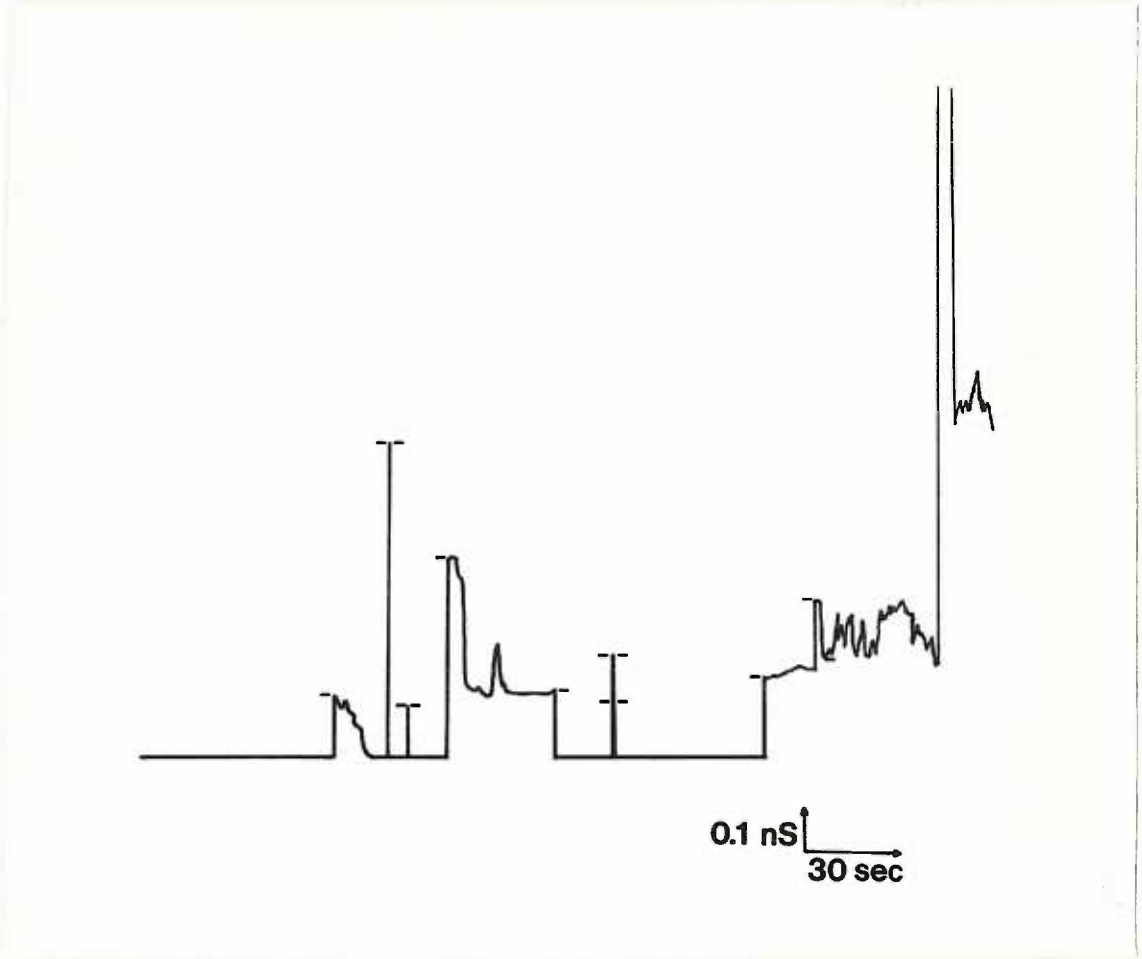
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Figure 2. Representative channel forming events when L. pneumophila 29K protein was introduced to 1 M NaCl bathing an oxidized cholesterol black lipid bilayer membrane. The protein was isolated based on the solubilization of cell envelopes in the cationic detergent hexadecyltrimethylammonium bromide (CTB) (2). The horizontal bars indicate the size of both conductance (nS) increases and conductance decreases. The record begins on the left.

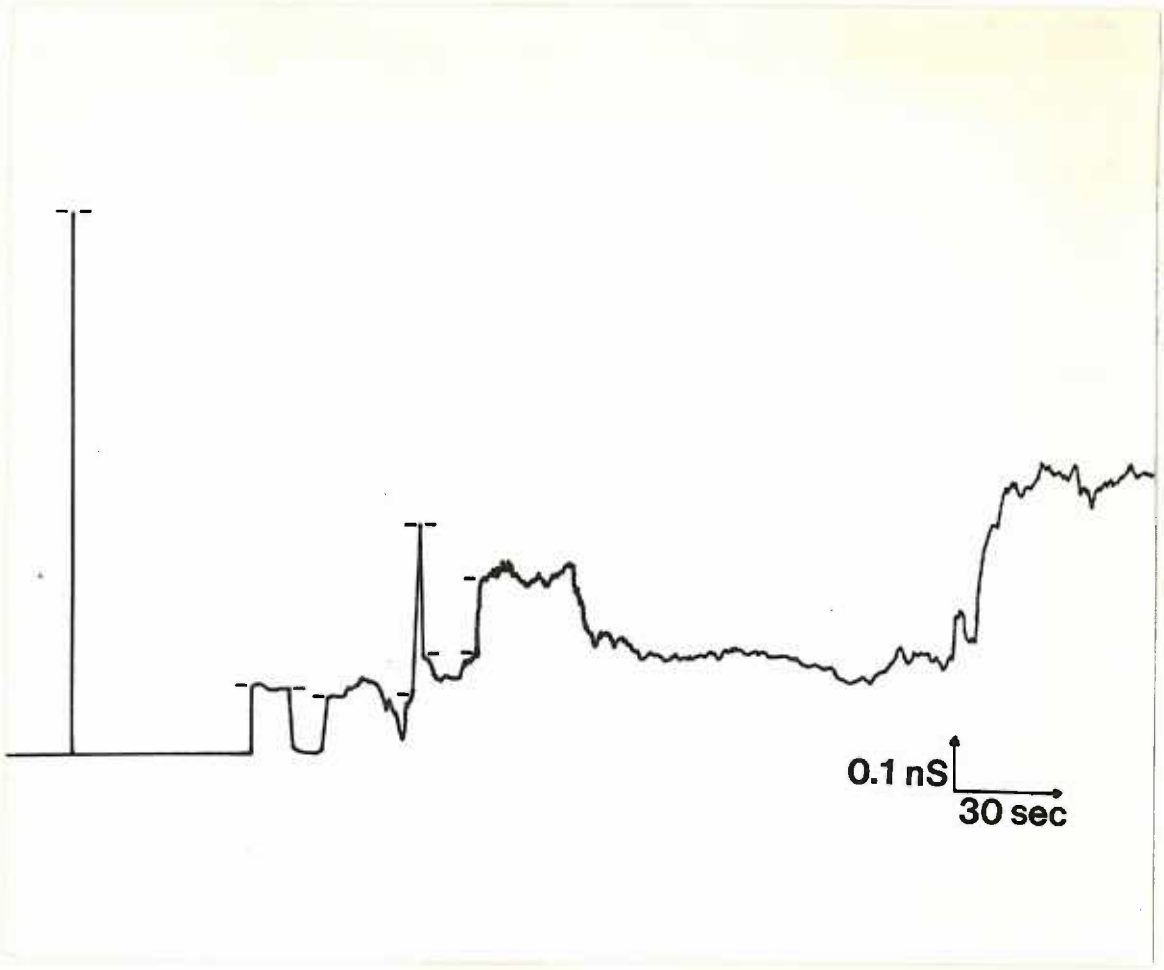
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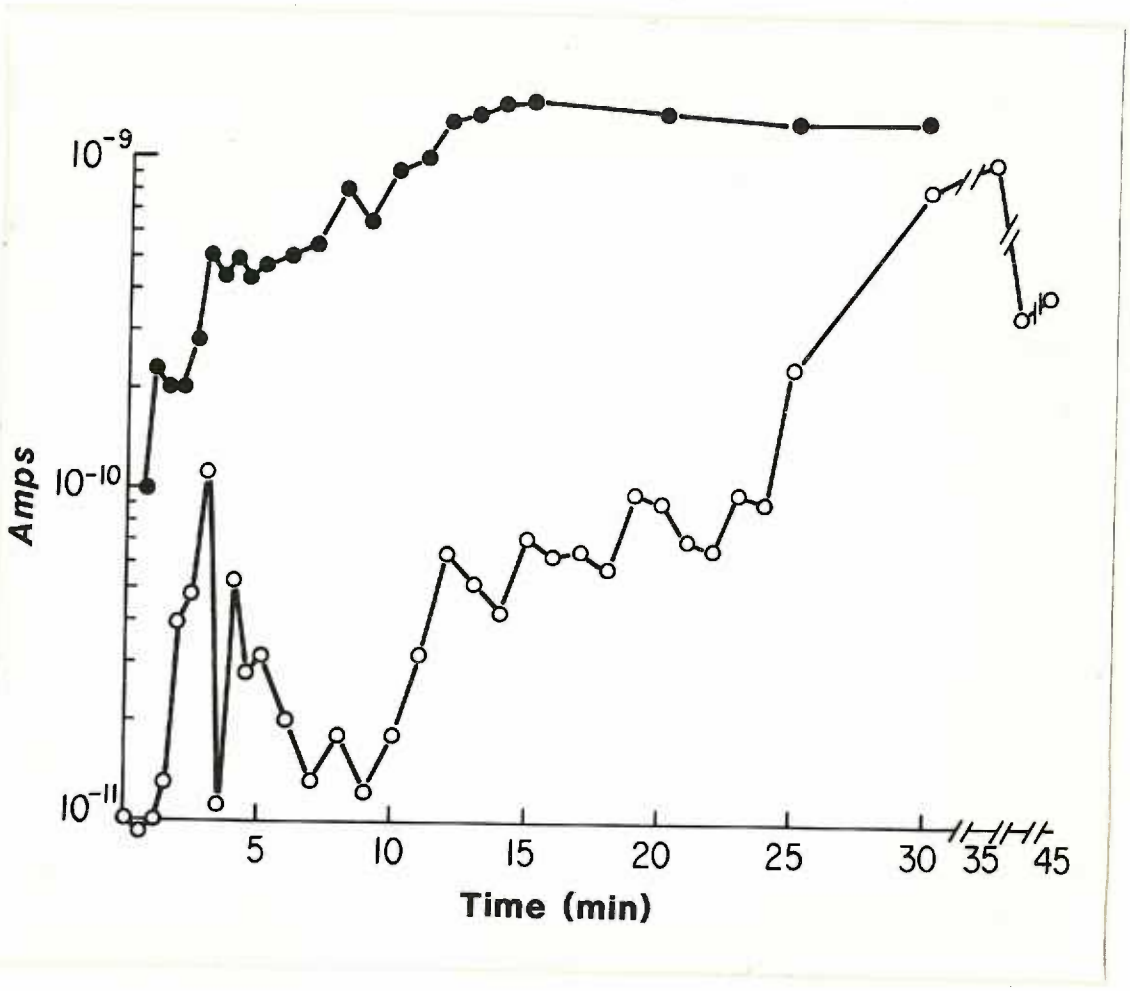
Figure 3. Current measurements in black lipid bilayers after introduction of L. pneumophila 29K protein. The 29K protein isolated by either the Triton X-100 method (3) or the CTB method (2) was introduced into 1 M NaCl bathing oxidized cholesterol membranes and current passing through the membranes was measured as a function of time. Open circles, 29K protein isolated by the CTB method. Closed circles, 29K protein isolated by the Triton X-100 method.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