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Gene-encoded surface antigens of Legionella pneumophila and their role in pathogenicity

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GENE-ENCODED SURFACE ANTIGENS OF *LEGIONELLA PNEUMOPHILA* AND THEIR ROLE IN PATHOGENICITY

BY

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DISSERTATION

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TO MY HUSBAND AND MY PARENTS

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ABSTRACT

GENE-ENCODED SURFACE ANTIGENS OF *LEGIONELLA PNEUMOPHILA* AND THEIR ROLE IN PATHOGENICITY

BY

ANDREA S. HIGH

University of New Hampshire, December 1992

The pneumonial agent, *Legionella pneumophila*, is the predominant bacterium responsible for Legionnaires' disease. Experimentally, these organisms have demonstrated the ability to adhere to host cells without the presence of a mucopolysaccharide layer.

A recombinant plasmid, pLP 116, resulting from the ligation of a *Hae* III digest from the *L. pneumophila*, Nottingham N_7 genome and *Sma* I-digested pUC 19 vector was shown to encode for a 25 kilodalton (kDa) major outer membrane protein (MOMP) of *L. pneumophila* by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS). This protein was detected on the surface of an *E. coli* clone (LP 116) by immunoassays. Virulence testing using the fertile chicken egg lethality assay determined that the clone experienced increased virulence over that of the parent strain. The *E. coli* parent strain was found to be non-adherent to U937 cells in culture while the clone LP 116 experienced a 40-

55 % increase in adherence. The *L. pneumophila* N_7 strain demonstrated 100% binding; however, *L. pneumophila* and clone LP 116 incubated with MOMP-specific monoclonal antibody experienced a complete loss of adherence to U937 cells. Organisms coated with the monoclonal antibody did not infect fertile chicken eggs at any dilution.

The outer membrane protein (OMP) profiles of the attenuated derivative of the *L. pneumophila* isolate used in this study showed a decrease in the 25 kDa protein and the presence of a 31 kDa protein not found in the OMP profile of the virulent strain. This laboratory strain experienced an increase in lethal dose (LD_{50}) values in the chicken embryonated egg lethality assay. When the recombinant plasmid pLP 116 was electroporated (electrically transformed) into the attenuated *L. pneumophila* derivative the 25 kDa protein was produced in greater amounts and the 31 kDa band was no longer present. The LD_{50} values of the transformed attenuated *L. pneumophila* N_7 strain decreased to that of the original isolate.

This study has shown the first reported difference between what appears to be genotypically and phenotypically similar organisms. It also has demonstrated that the 25 kDa MOMP of *L. pneumophila* plays an important role in adherence of the organism and that the 25 kDa MOMP can be recognized as a virulence factor related to the ability of the organism to cause infection.

INTRODUCTION

1. General and clinical features:

1.1 The Philadelphia outbreak:

Legionella pneumophila is a facultative intracellular gram-negative bacterium that survives and multiplies within human alveolar macrophages and monocytes. This organism is the major etiologic agent responsible for Legionnaires' disease, a severe form of acute lobar pneumonia. It evades macrophage defenses by inhibiting the oxygen-dependent sequelae of phagocytosis and blocking phagosome-lysosome fusion (Horwitz, 1984). As a consequence, the organism replicates unhindered within the phagosome of infected cells utilizing host cell-derived amino acids as energy sources. Infection of these cells leads to their eventual destruction.

During the summer of 1976, 182 members of the Pennsylvania American Legion developed an acute respiratory illness after returning home from the American Legion Convention that was held at the Bellevue Stratford Hotel in Philadelphia (Winn, 1988). Although the discovery of this bacterium was the result of a subsequent intensive investigation of this mysterious outbreak that ended in 29 deaths, diseases caused by the members of the family *Legionellaceae* have been traced in retrospective studies back to the 1940s (Tatlock, 1944, Bozeman, *et al.*, 1968). Primary reasons for the organism evading detection was its inability to grow on traditional laboratory media as well as failure to stain with conventional gram-staining procedures.

In 1977 this previously unrecognized human pathogen was recovered from human lung tissue by CDC investigators using isolation procedures more typically used for

Rickettsia spp. Samples of infected human lung tissue form the Philadelphia outbreak were inoculated into the peritoneal cavity of guinea pigs. The spleens from infected animals were harvested and subsequently injected into the yolk sacs of embryonated chicken eggs. Early attempts to isolate the organism were unsuccessful until antibiotics, normally included in the assay, were eliminated. Gimenez stain containing carbol fuschin allowed the organism to be visualized (McDade, *et al.*, 1979). Since its recognition in 1977, 42 species, three subspecies and 53 serotypes have been identified (Benson, *et. al*, 1991; Rodgers and Pasculle, 1991; Thacker, *et al.*, 1991).

1.2 Clinical presentation.

Legionellosis has several markedly disparate clinical presentations. The pneumonic form, Legionnaires' disease, can occur with clinical findings including headache, fever, myalgia often associated with high mortality due to toxic shock, respiratory collapse and heart failure. The incubation period is 2-10 days prior to the symptoms appearing. Upper respiratory symptoms are usually absent although a dry cough may follow after 3-4 days. If sputum is present it is usually non-purulent. Some hemoptysis may occur and dyspnoea and respiratory distress are common (Dowling, 1985). Pneumonia caused by *L. pneumophila* is by far the most common form, but other *Legionella* species have shown to cause disease and these include *L. micdadei, L. bozemanii, L. dumoffii* and others. The illness progresses quickly and hospitalization is necessary. Legionnaires' disease is a persistent problem for the chronically and seriously ill as well as immunocompromised patients; however, sporadic cases have been reported in previously healthy people (Sharrar, 1985). Although Pontiac fever, the non-pneumonic form presents with symptoms of fever and myalgia, recovery usually occurs within 2 to 5 days (Table 1). There is no current explanation for the different clinical presentations as the causal organisms appear to be phenotypically and genotypically alike (Girod, *et al.*, 1982).

Table 1 Clinical presentation of legionellosis

Two forms: Legionnaires' disease (LD) and Pontiac Fever (PF)
Geneotypically and phenotypically identical

	LD	PF
Named for:	Philadelphia outbreak	Pontiac outbreak
Attack rate:	1-5%	95%
Incubation period:	2-10 days	1-2 days
Symtoms:	Pneumonia	Flu-like symptoms
Other organs affected	Kidney, G. I. tract, CNS	None
Case:Fatality rate	Variable 0-40%	0%

1.3 Diagnosis:

Since *Legionella* is a pneumonial agent, diagnosis may be difficult as many bacterial and viral agents produce essentially similar effects. Failure to stain and difficulties in growing the organism create further complications.

The mainstay of laboratory diagnosis in determining the presence of *Legionella* remains growth on BCYE-α agar supplemented with L-cysteine and the absence of growth on unsupplemented blood agar (Edelstein, *et al.*, 1979; Winn and Pasculle, 1982). Organisms in the blood, secretions or tissues of a patient can be detected by microscopic examination using gram-stain substituting safranin with carbol fuschin to identify long, narrow non-dividing cells. Other staining techniques such as Giemsa and Gimenez are useful for nonspecific identification of *Legionella.* Immunofluorescence can be used to determine species and serogroup identification although problems with crossreactions are reportedly common (Bartlett, *et al.*, 1986).

Biochemical assays, gas liquid chromatography of branched fatty acids, thin layer chromatography of the quinones, serological assays, agglutination tests, ELISA and indirect hemagglutination have all been employed to identify the agent. Commercial DNA probes have been developed for the detection of *Legionella* in culture (Edelstein, 1986; Wilkinson, *et al.*, 1986). These have been shown to be quite effective but because they use radioactive labeled reagents, they tend to be fairly expensive, have a short shelf life and may pose problems for smaller laboratories.

1.4 Epidemiology:

L. pneumophila and other members of the family *Legionellaceae* have been shown to be part of the "normal" ecological flora of water distribution systems, in particular, thermally polluted waters. The organism occurs widely in natural fresh water habitats including lakes and ponds and appears secondarily in air conditioning units, cooling towers, water tanks and similar human-derived aquatic habitats in hospitals, hotels and

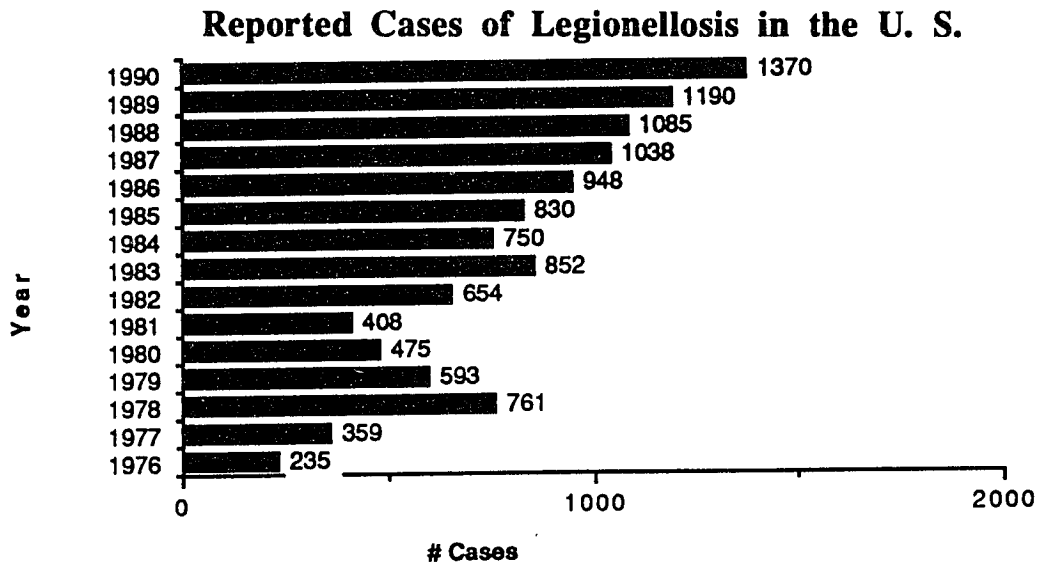
other buildings (Knudson, *et al*, 1985; Stout, *et al*, 1986). Bacteria are spread by aerosolization of contaminated water via air ducts and shower heads.

Sporadic community-acquired and nosocomial disease are common. Hospital acquired Legionnaires' disease is a persistent problem among the elderly, seriously ill or immunocompromised patients. Those most susceptible to Legionnaires' disease are men over 50 years of age. Men are affected two to three times more than women and the highest incidence is in the 40-70 year old group. Infection in children does occur although it is rare. Other risk factors for predisposition to the disease are previous lung disorders, high alcohol intake, diabetes and smoking. Persons immunocompromised due to chemotherapy or chronic illness such as AIDS (Bansorg, *et al.*, 1991) are also at great risk. Person to person transmission has not been documented.

In the United States, reported cases of legionellosis have been steadily rising as shown in Table 2. Legionellosis has been recognized in many countries throughout the world; however, some countries appear to have more cases than others but this may be due to an increased ability to recognize the disease. Although the organism has been identified since 1977 and the medical community is aware of the disease, many cases go unrecognized because *Legionella* is only detectable on special media that are not regularly used for the screening and diagnosis of pneumonial agents.

Infection by *Legionella* is more likely to be a problem in industrialized nations. Circumstances surrounding outbreaks involves colonization of water supplies with *Legionella* organisms and the presence of a nutrient source that will allow them to multiply. Aerosolization of water containing the organism coupled with human contact with the aerosol creates the environment for disease. However, the predisposition of the host appears to be the an important factor in the development and outcome of the disease state. Aerosols are the recognized vehicle for the disease but unusual cases of *Legionella* infection

Table 2



Adapted from CDC Morbidity and Mortality
Summary of Notifiable Diseases
United States, 1990

were recently documented where organisms entered an open chest wound as patients were bathed with *Legionella*-contaminated tap water (Lowry, *et al.*, 1991).

Pontiac fever, the non-pneumonic form of legionellosis has a high attack rate but no fatalities. The name of the disease derived from the incident in Pontiac, Michigan were 144 persons in the Oakland County health department were taken ill with symptoms such as fever, chills, general myalgia, malaise and headache. Persons became ill with flu-like symptoms anywhere from 5 to 66 hours after entering the building. Seventy-three percent of the cases occurred within a 3 day period. It was determined by the investigators in 1968 that the incident surrounded the event of starting the air conditioning system (Sharrar, 1985) but the agent was not discovered to be *Legionella* until after the Philadelphia outbreak. Legionnaires' disease and Pontiac fever were both caused by *L. pneumophila* serogroup 1 organisms. *L. pneumophila* is responsible for 85% of the outbreaks (Winn, 1988); however, to date, there is no explanation for the different expression of the disease.

1.5 Pathogenesis and Pathology:

Legionella species are ubiquitous in aquatic environments but in order to be pathogenic, the organism must come in contact with cells such as macrophages, specifically in the lung alveoli. This is effected by inhalation of organisms present in small sized (5-10 μm) aerosol droplets. Once organisms are engulfed they continue to multiply within the phagosome eventually causing cell death and the release of greater numbers of bacteria along with toxic products which continue the process (Horwitz and Silverstein, 1981).

Phagocytosis of the organisms is enhanced but not dependent upon opsonizing antibody. After ingestion, within phagosomes, bacteria are associated with ribosomes and mitochondria. In the presence of virulent *L. pneumophila*, phagolysomal fusion and acidification are inhibited (Horwitz, 1984). Rapid intracellular multiplication of *Legionella* is followed by cellular lysis.

Although the lung is the main organ of infection and shows the most pathological

damage, there is evidence of bacteremia and presence of soluble serogroup antigen in serum and urine. It has been shown that *Legionella* organisms do produce a variety of endotoxins from surface antigens that induce pyrogenic effects in infected individuals as well as weight loss, skin reactions and may lead to death (Bartlett, *et al.*, 1986). Low molecular weight materials from filtrates and sonicated cells have been shown to have lethal effects on mice, chicken embryos and cells in culture. These materials interfere with host cell oxidative metabolism and suppress phagocytosis (Friedman, *et al.*, 1982; Wong and Feeley, 1983). Proteases of *Legionella* may also share in the induction of lung lesions and may be responsible for the majority of the reported lung pathology in disease (Baskerville, *et al.*, 1986).

Non-pneumonic legionellosis may be due to the condition of the host or lack of virulence on behalf of the organism. It has been difficult to determine pathogenicity when comparing virulent and avirulent strains of *L. pneumophila* because both produce flagella, toxins and proteases with no appreciable differences (Winn, 1988).

There is nothing unique about *Legionella* pneumonia that is apparent in X-ray radiographs. The appearance of radiographic pathology may not occur until 3 days after the onset of symptoms and the recovery from the disease may occur long before radiographic improvement (Kirby, *et al.*, 1979; Fairbank, *et al.*, 1983). Post-mortem lungs samples have demonstrated the development of fibrino-purulent disease in one or both lungs with focal or lobar consolidation, edema, congestion and grey hepatization. In most cases, a fibrinous exudate is present the pleural cavity. Macroscopic lesions are not always present but can include hemorrhage areas and focal abscess formation (Blackmon, *et al.*, 1978).

1.6 Immunity:

Mechanisms of susceptibility to legionellosis and recovery from the disease are not known. Epidemiological evidence has suggested that *Legionella* infections are directly

related to risk factors which immunocompromise the host. Advanced age, underlying disease, cigarette smoking and immunosuppressive therapy have been shown to influence attack and fatality rates of Legionnaires' disease.

Theoretically, the introduction of *Legionella* antibodies would facilitate opsonization of bacteria with detrimental results to the host. However, animal studies have shown that passive transfer of antibody against *L. pneumophila* is effective against subsequent challenge with whole organisms (Rolstad and Berdal, 1981; Winn, 1988). Yet, antibody studies on healthy individuals who had been exposed to the organism as a result of contaminated residential tap water for 2 to 20 years, showed no difference in their antibody titers than those with no apparent exposure (Arnow, *et al.*, 1985).

Friedman, *et al.* (1984) demonstrated that lymphokine-activated macrophages did not support the growth of ingested legionellae while Horwitz *et al.* (1984) showed that non-specific activation of these cells also inhibited intracellular replication of *Legionella.* Such data suggest that cellular immunity plays an important role with *Legionella* infection.

It has been difficult to establish whether individuals who have contracted legionellosis are susceptible to re-infection. Researchers have shown that immunization of guinea pigs with killed antigen is protective in some cases, but not in others (Baskerville, *et al.*, 1983; Eisenstein, *et al.*, 1984). Winn and colleaques (1988) were able to protect guinea pigs for 1 month after infection by aerosol or intratracheal route with sub-lethal inoculum of *L. pneumophila.*

Human defenses against legionellosis most likely involve synergistic action of both the humoral and cellular immune systems. Studies that have elucidated a defense system in animals have demonstrated limited bacterial killing or bacteriostasis of *L. pneumophila.* Further work is needed to fully delineate the mechanisms of human resistance to Legionnaires' disease.

1.7 Treatment and Prevention:

The most widely used antibiotic for the treatment for *Legionella* infection is erythromycin. When the outbreak occurred at the American Legion Convention it appeared that those treated with erythromycin had greater success than those treated with penicillins, cephalosporins or aminoglycosides. Those patients on β-lactam antibiotics probably did not fair well because of β-lactamase production by *L. pneumophila*. Ampicillin was shown to be most effective in *in vitro* studies but its inability to cross the host cell membrane and attack intracellular organisms limits its usefulness (Elliott and Rodgers, 1985). Erythromycin, rifampicin and ciprofloxacin all inhibit intracellular growth of *Legionella* in cell cultures while the β-lactams do not. Rifampicin, although very effective is not widely used as problems with antibiotic resistance arise. Recent results from animals studies demonstrate that ciprofloxacin actually has greater effects on *Legionella* than the traditional erythromycin and rifampicin in experimental infection (Rodgers, *et al.*, 1990).

Early experiments using UV exposure at low dosages for short periods was shown to kill *L. pneumophila* in culture. This treatment may have been useful for water supplies; however, photoreactivation did occur when the organisms were subsequently exposed to light indicating that *L. pneumophila* possesses a very effective light-dependent DNA repair system (Knudson, 1985). It appears that chemical treatment and elevation of water temperature is necessary to inactivate the organism in water. Chlorination, the most practical solution to the eradication of *Legionella*, requires higher dosages than those necessary for the enteric bacteria. Water supplies treated with 4 ppm (4 mg of free chlorine per liter of water) of chlorine were found to be not only effective, but an inexpensive method of treatment (Baird, *et al.*, 1984). Although there are variations in heat resistance noted amongst the legionellae, Stout, *et al.*, (1986) found that simply raising the water temperature to 60°C is sufficient to kill most *Legionella* organisms. Since *Legionella* are

ubiquitous in the environment, prevention of aerosols and a reduction in exposure to aerosols are required to decrease the incidence of disease.

2. Classification and nomenclature:

The family *Legionellaecae* was established by using DNA homology and 16s ribosomal RNA studies (Table 3) as these organisms are not closely related to any other known bacteria (Brenner, *et al.*, 1979; Ludwig and Stackebrandt, 1983). Features used for the classification of *Legionella* were the estimation of nucleic acid guanine and cytosine (G +C) content which is approximately 39 mol % but has a range between 38 and 52 mol %, a genome size of 2.5 x 10^9 daltons and a DNA relatedness that must be greater than 70%. The general properties of *L. pneumophila* are listed in Table 4 and other members of the *Legionelleceae* are shown in Table 5.

Several procedures have been used for identification of *Legionella* in epidemiological studies. Differentiation of species can be accomplished by the use of dyes in media. The addition of bromocresol purple and bromothymol blue to bacteriological media can produce colonies that are white to pale green while the autofluorescing bacteria such as *L. dumoffii, L. gormanii* and *L. bozemanii* appear small round blue-grey colonies. Colonies can fluoresce when exposed to long wave (365 nm) ultraviolet. Addition of glycine to media when culturing for environmental strains is helpful as the glycine inhibits the growth of many other gram-negative bacteria but not *Legionella* (Wadowsky and Yee, 1981). Antimicrobials that can be used for the selection process are: amphotericin B, anisomycin, cefamandole, colistin, polymixin B, trimthoprim, vancomycin and ceftazidime (Bartlett, *et al.*, 1986) as well as cyclohexamide for antifungal activity (Bopp, 1981). Unfortunately, some of these treatments can also be inhibitory to some *Legionella* species and strains. Characterization of *Legionella* using typical phenotypic tests and biochemical assays has presented problems because many of the differentiating media do not support the growth of the organisms.

Table 3 Relatedness of nonlegionellae to unfractionated rRNA genes from *L. pneumophila*

Genus	Range of relatedness (%) to *L. pneumophila* rRNA genes, 75°C
Proteus, Providencia, Escherichia, Haemophilus, Serratia	58-66
Klebsiella, Pseudomonas, Aeromonas	51-57
Flavobacterium, Campylobacter,* Bacillus, Flexbacter*	30-38
Vibrio, Campylobacter, Flavobacterium**	21-29
*Cytophaga, Staphylococcus, Flavobacterium**	9-19

*Different species of these genera gave different relatedness values

Adapted from Classification of *Legionellaceae*, 1986
Israel Journal of Medical Sciences. Vol 22

Table 4 **Properties of *L. pneumophila***

Non-spore forming
Aerobic
Motile
Non-acid fast
High proportion of branched fatty acids in cell wall
Amino acids as carbon and energy sources
Oxidase (+/-)
Catalase (+)
Peroxidase (+)
Nitrate reductase (-)
Urease (-)
Hippurate hydrolysis (-, most strains)
Gelatinase (+)
β-lactamase (+)
Exoproteases (+)

Determination of antigenic properties offer more reliable methods to speciate and define strains of *Legionella*. Polyclonal antibodies (Brown, *et al.*, 1982; Garrity, *et al.*, 1982; Plouffe, *et al.*, 1984), monoclonal antibodies (Maher, *et al.*, 1983, 1987; Gosting, *et al.*, 1984), immunoelectrophoresis (Zanen-Lim *et al.*, 1984), slide agglutination/coagulation (Groothius, *et al.*, 1984; Thacker, *et al.*, 1985), immunofluorescence and immunoferritin labeling (Rodgers, 1982) have all been useful in identifying *Legionella* species and strains in nosocomial or community outbreaks. Alloenzyme analysis (Selander, *et al.*, 1985; Edelstein, *et al.*, 1986; Tompkins, *et al.*, 1987) can be used to subtype strains of *L. pneumophila*. Plasmid (Mikesell, *et al.*, 1981; Mellado, *et al.*, 1986) and peptide analysis (Brown, *et al.*, 1982, Lema and Brown, 1983; 1985; Nolte, *et al.*, 1984) has also been used for the identification of species and strains. DNA probes have been developed to identify *Legionella* strains isolated on bacteriological culture plates as well as from lung tissue and water samples (Grimont, *et al.*, 1985; Edlestein, 1986; Wilkinson, *et al.*, 1986, Starnbach, *et al.*, 1989; Bej, *et al.*, 1990). Often times many combined techniques are necessary to identify common source outbreak

The name "*Legionella*" derives from the American Legion Convention from which the bacteria were discovered and the species, "*pneumophila*" derives from the Greek "*pneumo*", lung and "*philos*", loving because of the pneumonic nature of the organism (Brenner, *et al.*, 1979). Questions as to the degree of genetic divergence acceptable to consider the formation of new genera has troubled the taxonomy of *Legionellaceae*. DNA homology is the standard by which *Legionella* are identified and by this technique, the genus *Legionella* is recognized. Earlier literature included the description of a new genus, *Fluoribacter*, which was proposed as a result of the blue-white fluorescing species found for the *L. gormanii*, *L. dumoffii* and *L. bozemanii* (Garrity, et al., 1980). The Pittsburgh pneumonia agent, *L. micdadei*, which shares less than 10% homology with other *Legionella* species, was renamed by Garrity and colleagues (1980) as the genus *Tatlockia*

Table 5 **Phenotypic characteristics**

Species	# of serogroups	Source	Catalase	β-lactamase
*L. pneumophila**	14	h/e[1]	+	+
*L. bozemanii***	2	h/e[2]	+	V
L. dumoffii	1	h/e	+	+
L. gormanii	1	e	+	+
*L. micdadei****	1	h/e	+	-
L. longbeachae	2	h	+	V
L. jordanis	1	h/e	+	+
L. oakridgensis	1	h/e	+	+[3]
L. wadsworthii	1	h	+	+
L. feeleii	2	h/e[2]	+	-
L. sainthelensi	1	e	+	+
L. hackeliae	2	h	+[3]	+
L. anisa	1	e	+	+
L. maceachernii	1	h/e	+	-
L. jamestowniensis	1	e	+	+
L. rubrilucens	1	e	+	+
L. erythra	1	e	+	+
L. spiritensis	1	e	+	+
L. parisensis	1	e	+	+
L. cherrii	1	e	+	+
L. steigerwalti	1	e	+	+
L. santicrusis	1	e	+	+
L. israelensis	1	e	+	+
L. cincinnatiensis	1	h	+	-
L. birminghamensis	1	h	+	+
L. moravica	1	e	+	+
L. brunensis	1	e	+	+
L. quinlivanii	1	e	+	-
L. tuconensis	1	h	+	+
*L. londiniensis****	1	e	+[3]	+
*L. geetiae****	1	e	+	-
*L. quarteiraensis****	1	e	+	+
*L. nautarum****	1	e	+	+
*L. worsleiensis****	1	e	+[4]	+
L. fairfieldensis	1	e	+[3]	-
L. adelaidensis	1	e	+	-

Legionella species fail to reduce nitrate and do not grow on unsupplemented blood agar. All strains will grow on L-cysteine-supplemented BCYE-α media only, with exception of *L. oakridgensis* and *L. jordanis* which will grow on L-cysteine-free BCYE-α agar after multiple passages. Sources were of human (h) or environmental (e) origin. V=variable. ND=Not determined. YG=yellow-green, R=red, BW=blue white. [1] Serogroups 8 and 11 through 14 have not been isolated from an evironmental source.

of *Legionella* species

Oxidase	Hippurate	Gelatin liquefaction	Autofluorescence	Brown pigment	Motility
V	+	+	-	+	+
V	-	+	BW	+	+
-	-	+	BW	V	+
-	-	+	BW	+	+
+	-	-	-	-	+
+	-	+	-	+	+
+	-	+	-	+	+
-	-	+	-	+	-
-	-	+	-	-	+
-	V	-	-	+[3]	+
+	-	+	-	+	+
+	-	+	-	+	+
+	-	+	BW(V)	+	+
+	-	+	-	+	+
-	-	+	-	+	+
-	-	+	R	+	+
+	-	+	R	+	+
+	+[3]	+	-	+	+
+	-	+	BW	+	+
+	-	+	BW	+	+
-	-	+	BW	+	+
+	+	+	-	+	+
-	-	+[3]	-	+	+
+	-	+	-	ND	+
V	-	+	YG	ND	+
+[3]	-	+	-	ND	+
-	-	+	-	ND	+
-	-	+	-	ND	+
-	-	+	BW	ND	+
-	-/+[3]	+	-	+	-
-	+[3]	+	-	+[3]	+
-	-	+	-	+	+
+	-	-	-	-	-
-	-	+	-	+	+
+[3]	-	-	-	-	+
-	-	+	-	-	+

[2] Serogroup 2 has not been isolated from an environmental source. [3] Strains may be weakly positive. [4] Peroxidase is positive.

**L. pneumophila* contains 3 subspecies, subspp. *pneumophila*, subspp. *pascullei* and subspp. *fraseri*. ******Fluorobacter* is the alternative genus. ******Tatlockia* is the alternative genus.

**** Proposed new species. Six additional species remain unnamed.

Adapted from Manual of Clinical Microbiology with permission, Rodgers, 1991.

originally isolated from the blood of a soldier at Fort Bragg (Tatlock, 1944). Although there still may be need for further differentiation of the family *Legionellaceae*, the use of the genus *Fluorobacter* or *Tatlockia* has not been widely accepted.

3. Molecular aspects:

3.1 Genetics:

Insight into the pathogenesis of legionellosis is rudimentary and even less is known concerning the bacterial genetic regulation of disease-inducing factors. Most genetic studies have concerned the identification of the species or strains (Brown, *et al.*, 1982, 1985; Nolte, *et al.*, 1984). Estimates of overall genetic relatedness of strains from DNA hybridization experiments have been used to define species limits within the genus *Legionella* (Selander, *et al.*, 1985, Edelstein, *et al.*, 1986). The new family *Legionellaceae* was established by DNA hybridization and 16s ribosomal RNA studies as the legionellae were not closely related to any described organism (Brenner, *et al.*, 1979; Ludwig and Stackebrandt, 1983; Saunders, *et al.*, 1988; Fry, *et al.*, 1991). Commercial DNA probes have been developed to identify *Legionella* species and strains in diagnostic and environmental settings (Grimont, *et al.*, 1985; Edelstein, 1986; Wilkinson, *et al.*, 1986; Starnbach, *et al.*, 1989; Bej, *et at.*, 1991).

Plasmids, small pieces of extranuclear DNA, were first discovered in *Legionella* by Knudson and Mikesell (1980). They have since been detected in many species and strains of *Legionella,* but they remain cryptic (Brown, *et al.*, 1982, 1985; Mellado, et. al, 1986). Although plasmid-encoded virulence factors have been reported for other gram-negative bacteria, the possession of plasmids does not appear to impart any detectable phenotype or other markers of their presence. In general, this is due to the lack of a minimal media making it difficult to cultivate the organism under minimal nutrient conditions as well as the ability of *Legionella* plasmids to resist curing. Recent findings have shown that the large, "80" megadalton (mDa) plasmid, commonly found in some isolates of legionellae does

have the ability to conjugate with a non-plasmid bearing *L. pneumophila* auxotrophic strain (Mintz and Zou, 1991) but no association with virulence has been noted. Studies of virulence and pathogenicity using plasmid-bearing strains of *Legionella* have proved unfruitful; however, the patterns of the plasmids restricted with endonucleases have been useful for epidemiological studies for identification of strains and to define their point of origin in nosocomial outbreaks.

Plasmid transfer into *Legionella* strains using well-defined, cloned plasmids in conjugative experiments with *E. coli* have shown that *Legionella*, within limits, can accept plasmids from other organisms (Chen, *et al.*, 1984; Dreyfus and Iglewski, 1985). The chromosomal mobilization plasmids from *Pseudomonas aeruginosa* (RP1, RP4, R68.45) were accepted into the cytoplasm of an *L. pneumophila* "wild type" strain but integrated into the chromosome of a laboratory-synthesized thymidine-deficient *L. pneumophila* mutant (Dreyfus and Iglewski, 1985). Although the appropriate antibiotic resistance was conferred, plaque formation by RPDI, a pilus-specific phage, did not occur in the transconjugates. The RP1 or RP4 plasmid could not transfer to any other *Legionella* but could transfer back to an engineered *E. coli* strain. Transposon mutagenesis using suicide plasmid strategies (Keen, *et al.*, 1985; Mintz and Shuman, 1987) demonstrated to some extent that *Legionella* can be mutated in a random fashion but no changes in the pathogenicity of the organism have been demonstrated.

Genes cloned into *E. coli* have proved useful for detecting proteins in *Legionella* that are highly conserved and common to other gram-negative bacteria. The Rec A protein possesses many regulatory and catalytic activities involved with homologous recombination and DNA repair mechanisms. The recA genes for *L. pneumophila* have been cloned and sequenced (Dreyfus, 1989; Xhao and Dreyfus, 1990). The genes responsible for this protein in *L. pneumophila* share sequences in common with those in *E. coli*. The groELS region responsible for the production of heat-shock proteins found in *Pseudomonas*,

Mycobacterium bovis and *E. coli* have been located in *L. pneumophila.* The genes that encode for this 58-60 kDa protein have been cloned and sequenced (Hoffman, *et al.* 1989, 1990; Sampson, *et al.*, 1990) as well as been located in another species of *Legionella, L. micdadei* (Bangsborg, *et al.* , 1989; Hindersson, *et al.*, 1990). It has been suggested that the groELS gene responsible for the heatshock protein is implicated with the virulent expression of *Legionella,* an organism found in thermally polluted waters, but thus far there has been no evidence to support this theory. The genes for a 38 kDa protein responsible for a metaloprotease of *L. pneumophila* referred to as the major secretory protein (MSP) have been shown to possess proteolytic activity in *L. pneumophila* (Quinn, *et al.*, 1989). Szeto and Shuman (1990) have since determined that this particular protein is not a virulence factor as it is not responsible for intracellular replication nor the killing of host cells. In studies where guinea pigs were injected with MSP and subsequently exposed to aerosols of *L. pneumophila,* Blander, *et al.*, (1990) found that MSP functions as an immunoprotective molecule. Other proteins discovered via cloning techniques have included the 19 kDa outer membrane peptidoglycan-associated protein originally discovered by Hindahl and Iglewski (1987) and recently sequenced by Ludwig, *et al.* (1991).

The first successful experiment with the cloning of *Legionella* proteins into *E. coli* has uncovered genes responsible for the 24 kDa *mip* (macrophage infectivity potentiator) protein (Engleberg, *et al.*, 1984a, 1984b, 1986, 1988, 1989; Cianciatto, *et al.*, 1989a, 1989b, 1990a, 1990b). These studies have shown that the absence of this protein impairs the ability of the macrophage to engulf *Legionella* organisms (Cianciotto, *et al.*, 1989b). This protein appears to have genus-wide status and interestingly, sequences from these genes have also been located in DNA encoding for the major outer membrane of *Chlamydia trachamonas* (Lundemose, *et al.*, 1991). Clearly the ability of the organism to be taken up by the macrophage is a critical feature to the survival of *Legionella.* Laboratory synthesized, thymidine-deficient auxotrophic mutants of *L. pneumophila* (Mintz, *et al.*,

1988) have been reported and these, too, were shown to have lost the ability to infect macrophages. The mechanisms by which these events take place have not been described in either study.

3.2 Outer membrane components:

Electron microscopy has shown that *Legionella* organisms attach to mammalian cells in culture prior to engulfment (Oldham & Rodgers, 1985); however, the mechanisms mediating such attachments are unknown. The presence of various structural and non-structural bacterial cell surface and membrane components (Rodgers & Davey, 1982; Rodgers, 1983) may play important roles in the process of adherence and uptake (Fig. 2).

a) Lipopolysaccharides (LPS):

The LPS is a major constituent of the outer membrane of this and other gram-negative bacteria and has been shown to be highly antigenic material. LPS has been shown to be an endotoxin responsible for fever, shock, vascular collapse and hemorrhage in other gram-negative bacteria. The LPS of *L. pneumophila* has demonstrated high antigenic activity particularly in convalescing patients (Winn, 1988). The F-1 antigen, a high-molecular weight protein located on the surface of *L. pneumophila* (Johnson, *et al.*, 1979; Elliott, *et al.*, 1981) has been found to possess endotoxic activity, inhibit serological reactions and participate in opsonization (Winn, 1988). The LPS is tightly bound to the major outer membrane (MOMP) found on the surface of *L. pneumophila.* Electrophoretic patterns of *L. pneumophila* LPS have determined that it is of the smooth type but unlike the LPS from the Enterobacteriaceae it has a tighter banding pattern (Gabay and Horwitz, 1985; Nolte, *et al.*, !986; Sonesson, *et al.*, 1989) and has been used to identify *L. pneumophila* strains in common-source outbreaks. There is no doubt that this material has influence in pathogenicity but the exact role has not been defined.

b) Proteins:

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the

outer membrane of *L. pneumophila* showed a genus-common 29 kDa protein (Ehret *et al.*, 1984) which was shown to be a component of a large aggregate protein of 95 kDa molecular mass (Butler *et al.*, 1985). This 95 kDa aggregate composed of 24-29 kDa subunits is stabilized by disulfide bonds and has been reported as structurally similar to the major outer membrane protein found in *Chlamydia* spp.; another, albeit obligate, intracellular pathogen (Newhall & Jones, 1983). Based on its folding properties and ability to form membrane channels, this *Legionella* MOMP behaves as a porin with similar properties to those found on *E. coli* (Gabay *et al.*, 1985). The *L. pneumophila* MOMP shows immunologic similarity to the other serotypes of *L. pneumophila* (Hindahl & Iglewski, 1986) as well as to other *Legionella* species (Butler *et al.*, 1985). Bellinger-Kawahara and Horwitz (1990) reported that the MOMP of *L. pneumophila* was responsible for C3 binding in opsonization studies and that this activity initiated phagocytosis. Since the intracellular existence of the organism is critical to its survival, the MOMP would appear to play a significant role in pathogenicity.

4. Morphology:

The morphological properties of *L. pneumophila* are listed in Table 6 and illustrated in Fig. 1. Organisms of the *Legionellaceae* are non-capsulated and, on the average, each cell is 0.3-0.9 μm in width, 2-20 μm in length; filaments formed in excess of 20 μm are commonly found on growth on bacteriological media (Rodgers, 1979). Such long forms have also been found in protozoan models (Fields, *et al.*, 1984) and in the embryonated chicken egg yolk sac as well as in human lung tissues (Rodgers, 1979). The bacterial ultrastructure is similar to that of other gram-negative organisms with most species and strains possessing double envelope layers each of 25 nm thickness and composed of typical lipid bilayer membranes. Peptidoglycan-like layers have been noted in the *Legionellaeceae*. Diaminopimelic acid, a component of peptidoglycan, as well as 2-keto-3 deoxyoctonate, a component of lipopolysaccharide, have been found in *L. pneumophila*

Table 6 Morphological properties of *L. pneumophila*

Short rods (from autopsied lung 0.6 x 1-2 μm, from agar 0.4 x 2-3 μm)

Tapered ends (new growth)

Vacuolated surfaces, convoluted or smooth surfaces

Long forms (*in vivo* up to 20 μm in length, *in vitro*> 50 μm)

Internal PBH granules-deformed in freeze-fracture

Division by non-septate binary fission

Flagella and fimbriae present

Blebs and granules on surface subunits, no evidence of acid polysaccharide capsule

F-1 antigen (serogroup specific) located on the bacterial surface

Limiting cell envelope, 'unit membrane', 10 nm thick

Peptidoglycan-like mucopeptide layer present after partial plasmolysis

Plasma membrane, 'unit membrane' 10 nm thick

Rich in ribosomes, 25 nm diameter

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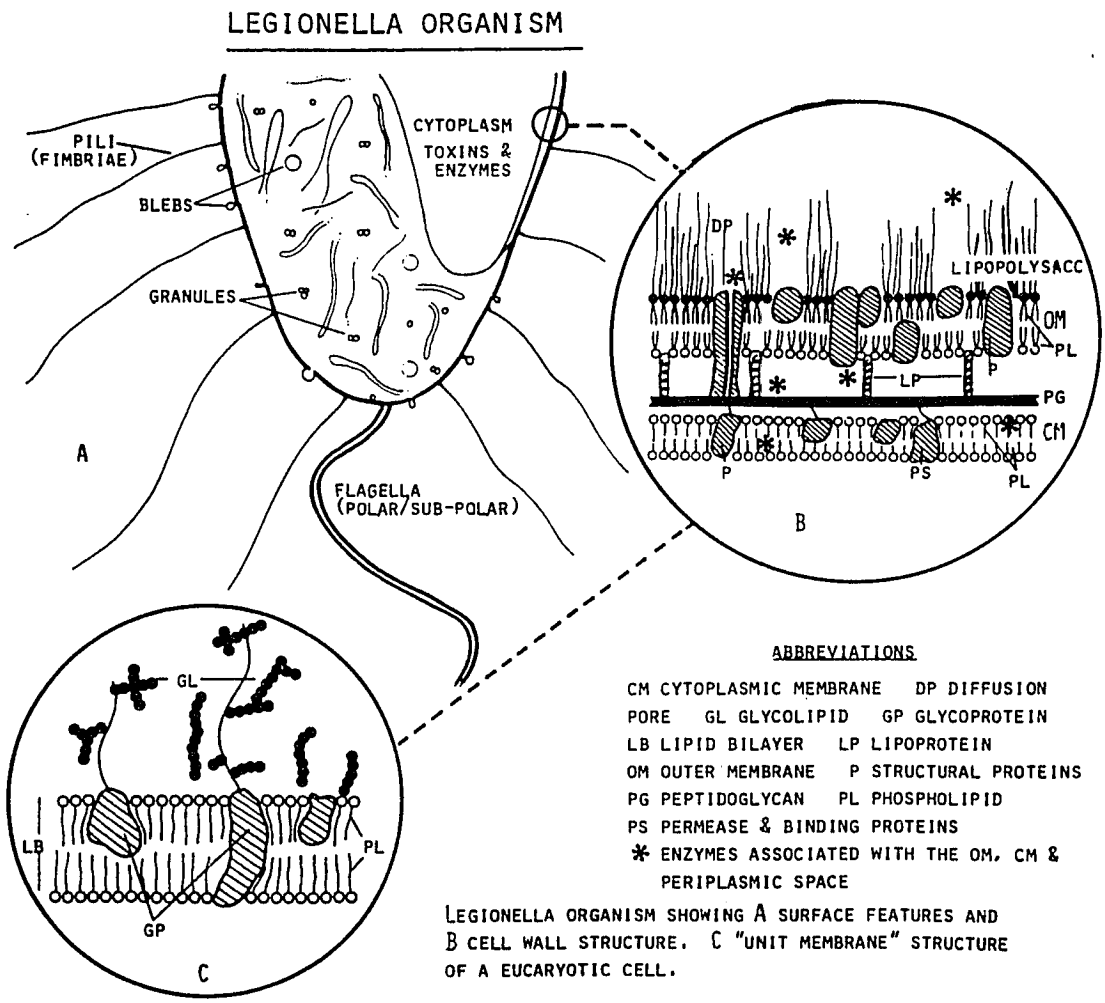

Figure 1. Schematic diagram of the *Legionella* organism showing (A) surface features and (B) cell wall structure. (C) "Unit membrane" structure of a eukaryotic cell. Abbreviations are listed under the diagrams. The diagram represents features which may be considered potential "adhesins".

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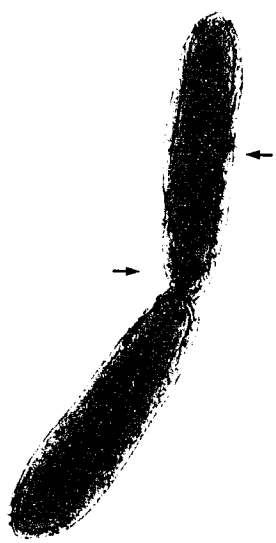


Figure 2. Dividing cells of *Legionella pneumophila*. Cells were harvested from BCYE-α agar plates after 24 h and negative stained with 0.5% uranyl acetate. Specimens were prepared on 50 micron copper grids coated with 0.2% (w/v) formvar. Loose outer membrane is evident and convoluting surface can be detected (arrows).

(Rodgers, 1979). Ribosomes measuring 25 nm in diameter, are evenly dispersed along with fine skeins of nuclear material. In freeze-fracture electron micrographs, vacuoles found in the cytoplasm measure between 30-200 nm and on occasion up to 0.5 μm. Due to the deformation patterns detected, each granule is surrounded by a non-fracturable membrane with a single ridge. These have been shown to be poly-β-hydroxybutyrate (PBH) granules (Rodgers and Davey, 1982). All species with the exception of *L. oakridgensis*, "*L. nautarum*" and "*L. londiniensis* "are motile and have at least one flagellum per cell (Rodgers, 1979; Rodgers and Pasculle, 1991). The presence of fimbriae has also been observed in *L. pneumophila* (Rodgers, 1979; Rodgers, *et al.*, 1980). The organisms have a surface that is rugose and highly convoluted and each has tapering ends; however, older cultures have a smoother surface with rounded ends. The cells divide by pinching binary fission within the lamella of the bacterial membrane (Rodgers, 1979) (Fig. 2).

5. Growth aspects:

5.1 Physico-chemical properties:

Organisms of the *Legionellaceae* grow optimally at temperatures between 35-37°C at pH 6.9 with a narrow pH range between 6.85-6.95 for clinical isolates. Such pH in media is best achieved by using potassium buffers rather than those that contain sodium as sodium can restrict or inhibit the growth of *Legionella.* Some species grow optimally in the presence of 5% CO_2 but if BCYE-α is used as the nutrient source, CO_2 may be inhibitory to growth.

5.2 Biochemical properties:

Although this is an intracellular organism, *Legionella* can be cultured on special bacteriological media. Colonies can take up to 3-5 days to appear and may vary in size but appear as grey pinpoint colonies as initial growth. The size of the colonies does not seem to be a reflection of any important features of the legionellae. Organisms can be visualized by

gram stain by either avoiding the decolorization steps or substituting carbol fuschin for safranin. *Legionella* species are non-acid-fast with the exception of *L. micdadei* which on occasion has been found to test positive by this staining method (Hilton, *et. al*, 1986). These organisms are non-sporeforming and have an unusual fatty acid and lipid composition which has been used to differentiate the family *Legionellaceae* and individual species within the family (Moss, *et al.*, 1981).

Amino acids are the major energy and carbon source for all *Legionella* species and may very well be the only characteristic the legionellae have in common with each other. Amino acids are catabolized through the TCA (Tricarboxylic Acid) cycle and sugars are synthesized by gluconeogenic enzymes of the Embden-Meyerhof-Parnas pathway (Pine, *et al.*, 1979; George, *et al.*, 1980; Hoffman, 1984). Carbohydrates are not fermented or oxidized by any of the *Legionella* species although there is evidence that these pathways exist.

Species demonstrate weak catalase and peroxidase activity. Nitrates are not reduced nor is urea hydrolyzed. *L. pneumophila* does produce gelatinase, phospholipase and β-lactamase and possess the ability to hydrolize starch. Hippurate hydrolysis can also be detected in some *Legionella* species. Studies have also shown that *L. pneumophila* produces exoproteases capable of hemolytic and cytotoxic activities. (Keen and Hoffman, 1989). Some, not all species of *Legionella* may produce a brown pigment on Feeley-Gorman media containing casein hydrolysates, beef extract and tyrosine.

There has been success in growing *Legionella* organisms on a number of existing media provided supplements were added. Meuller-Hinton media supplemented with IsoVitalex and hemoglobin supports the growth of *Legionella* as well as blood agar containing L-cysteine and ferric iron (Feeley, *et al.*, 1978). L-cysteine appears to be necessary because the organism lacks enzymes needed for serine transacetylase and o-acetylserine sulfhydralase which prevents formation of cysteine. Ferric iron appears to

enhance growth of the organism but may not be an absolute requirement as there does not appear to be a need for iron more than any other bacteria. Evidence that siderophores which enhance the solubilization and transport of iron when stringent conditions exist have been found in the legionella (Goldoni, *et al.*, 1991). The recent discovery of siderophores was the result a sensitive assay that did not depend on the production of catechol or methylhydroxamate; however other investigators have detected the presence of iron reductase and reason that this is how *L. pnuemophila* functions in an iron-deficient environment (Johnson, *et al.*, 1991).

Common growth factor for the legionellae are guanine with arginine, isoleucine, methionine, phenylanaline or tyrosine, proline, serine, threonine and valine; however, the beneficial effects of these vary throughout the different species (Pine, *et al.*, 1986). Currently, the preferred medium for growth of *Legionella* species is buffered charcoal yeast extract and α-ketoglutarate (BCYE-α) agar supplemented with L-cysteine; however, after several passages on lab media, apparently *L. jordanis* and *L. oakridgensis* can be maintained without the L-cysteine supplement (Bartlett, *et al.*, 1986). The yeast extract provides the purine and pyrimidine derivatives of which guanine is the basic requirement for growth (Pine, *et al.*, 1986). This medium also contains N-2 acetamido-1 amino-ethanesulfonic acid (ACES) which aids in the growth of *Legionella* while surpressing other organisms (Bartlett, *et al.*, 1986). Many of the components of the medium may not be essential for the growth of the organism *per se* but may improve growth conditions as the media that is suitable for *Legionella* growth is also extremely toxic to the organism. Charcoal and alpha-ketoglutarate probably function by absorbing toxic oxygen radicals present in the media formed after exposure to light or by-products of yeast extract from autoclaving (Hoffman, 1984).

6. Ecology:

Under laboratory conditions *Legionella* species are fastidious but can survive well

in their natural aquatic habitat (Arnow, et. al, 1985; Knudson, 1985; Stout, 1986). It is well established that *Legionella* can be found in many natural or human-derived fresh water habitats. It has also been isolated from mud, although it has not yet been found in dried soil.

Aerosolization of these waters containing the organisms is responsible for infectivity and disease. Investigations have revealed the presence of *Legionella* in environments containing photosynthetic bacteria, algae and protozoa. Organisms have been found in mats of cyanobacteria containing *Fisherella* species (Bohach and Snyder, 1983) in ranges of pH that exceed 6.9 to 10.6 and in thermal effluents at 45°C. Studies have shown that *Legionella* organisms can multiply in free-living protozoans commonly found in aquatic environment. Such studies suggest that these conditions only serve to amplify the number of organisms of *Legionella* associated with outbreaks (Bohach, *et al.*, 1984; Fields, *et al.*, 1984; Barbaree, *et al.*, 1986). The ability to survive aquatic environments may be enhanced by growth in various protozoans present in the same habitat (Barbaree, *et al.*, 1986). In humans, *Legionella* is usually found growing in alveolar macrophages. As a consequence, the relationship between protozoans and macrophages have been actively pursued. The ultrastructure of infected protozoans and human monocytes has been used to support the hypothesis that humans are incidental hosts for *Legionella* whereas the protozoans may be the primary hosts (Barbaree, *et al.*, 1986).

The bacteria isolated from surface waters show a variety of chemical, physical and biochemical profiles. *Legionella* spp. are more resistant to chlorine than the enteric bacteria and highly resistant strains have been isolated from plumbing fixtures as these organisms can survive on materials such as rubber and metals. Growth of *L. pneumophila* is encouraged by rubber fittings used in plumbing fixtures and filters support the growth of *Legionella* by organism build-up or metals that stimulate its growth such as zinc and iron.

Temperature is critical as *L. pneumophila* can multiply in tap water as high as 42°C.

Some species and strains have survived temperatures exceeding these temperatures in hot water heaters that maintain temperatures between 30 and 54°C. There is a difference in the tolerance of high temperatures throughout the species but protozoan existence and biofilm may help to protect *Legionella* in the environment. Location of the heating element in the water heaters has been influencial in the survival of *Legionella* organisms as gas and oil water heaters have heating elements on the bottom which severely hampers the survival of *Legionella* whereas electric heater elements are in the middle of the tank thus causing little exposure to high temperatures on the bottom of the tank (Lee and West, 1991). Unfortunately, energy saving measures such as lowering water heater temperatures and using filters that conserve water in showerheads and sinks may encourage growth and spread of *Legionella* organisms.

HYPOTHESIS

Legionella pneumophila has been recognized since 1977 as the predominant bacterium responsible for Legionnaires' disease but the mechanisms of pathogenicity have not been elucidated. It has been established that *L. pneumophila* and other members of the genus are organisms free-living in aquatic environments. Aerosolization of water containing the organisms has been implicated with infectivity and disease. Pontiac fever, also caused by *L. pneumophila*, presents many of the early symptoms of Legionnaires' disease but the symptoms disappear within a few days. Although host factors must be a consideration, to date, there is no explanation for this phenomenon.

There have been few reports linking virulence of *Legionella* to factors at the molecular level. Proteins associated with outer membranes of pathogenic gram-negative bacteria are frequently associated with various proposed mechanisms of pathogenesis. The outer aspects associated with the cell envelope are the first components of the bacterium to contact the host cell, and the nature of these thereby profoundly influence virulence at the host cell surface as well as subsequent intracellular infection. Because *L. pneumophila* possess an outer membrane that has been shown to adhere to host cells without a mucopolysaccharide layer present (Rodgers, 1979), there may be other factors involved in attachment. Investigations of the *L. pneumophila* outer surface have been made difficult because of the inability to separate these components (Ehret, *et al.*, 1984; Butler, *et al.*, 1985; Hindahl and Iglewski, 1986).

The specific aims of this study were:

1. To clone individual surface antigens of *L. pneumophila* into *E. coli* to circumvent the problems associated with the separation of outer membrane components.

2. To assess the influence of the recombinants in the infective process by using virulence assays previously established by this laboratory for the study of pathogenic mechanisms, in particular, those of adherence.

3. To seek the distinctions between what appears to be phenotypically and genotypically similar organisms.

MATERIALS and METHODS

1. Techniques used to study pathogenic aspects of *Legionella*:

1.1 Origin and maintenance of the bacterial strains:

L. pneumophila serogroup 1, strain Nottingham N_7, originally isolated from the sputum of a fatal case of Legionnaires' disease (Table 7), was passaged a total of two times on L-cysteine supplemented buffered charcoal yeast extract agar (BBL) with added α-ketoglutarate (BCYE-α) and grown at 37°C for 24 h in a humid atmosphere. The N_7 strain was passaged a further 53 times on laboratory media and determined to be attenuated by the fertile hens' egg assay (Tzianabos and Rodgers, 1989). The N_7 derivative was maintained in a similar manner as the original N_7 strain. This strain and its derivative contained no plasmids. The electroporated strains of N_7 containing the pUC 19 plasmid or the LP 116 recombinant plasmid were maintained on BCYE-α containing 50 μg/ml of ampicillin. All strains were maintained in 1% serum containing 10% sorbitol at -70°C.

E. coli JM 83, containing the β-galactosidase genes lacking the alpha-peptide was grown in nutrient broth while the strain with the pUC 19 plasmid vector (See Fig. 3) was grown in nutrient broth containing 50 μg/ml ampicillin. Both *E. coli* JM 83 preparations were grown as overnight cultures at 37°C in a shaking water bath and were stored at -70°C in nutrient broth (Difco) containing 0.8% dimethyl sulfoxide (DMSO). A description of the media used in this study is detailed in Appendix 1.

Table 7

THE SOURCE and HISTORY OF BACTERIAL STRAINS USED IN THE STUDY

L.pneumophila Nottingham N_7 (Clinical isolate)	Queens Medical Centre, University Hospital Nottingham, England
L. pneumophila Nottingham N_7 (High-passage)	Passaged at the Dept. of Microbiology, UNH, Durham, NH
E. coli JM 83 ϕ80dlacZΔM15, ara, Δ (lac-proAB), rpsL, thi, F- (With cloning vector pUC 19)	Department of Microbiology UNH, Durham, NH
***E. coli* LP 116** (Recombinant)	Produced in this study
L. pneumophila LP 116 (Transformed)	Produced in this study
L. pneumophila pUC 19 (Transformed)	Produced in this study

Figure 3. Smaller plasmids such as pUC 19 (approximately 2.7 kilobases) can accommodate larger segments of foreign DNA and replicate at higher copy number yielding a better signal for the products being produced. pUC plasmids are also useful in transformation studies as efficiency of transformation is inversely proportional to the size of the plasmid. They lack the *rop* gene located near the origin of DNA replication which is responsible for the regulation of copy number. Without these genes present, the plasmids replicate at a much higher rate. The pUC plasmids carry segments of DNA derived from the *lac* operon of *E. coli* that encodes for the amino-terminal fragment of β-galactosidase. When the plasmid is transformed into the appropriate *E. coli* strain such as JM 83 which has the β-galactosidase genes without the alpha peptide, lactose metabolism can be detected. Fragments ligated to the pUC vector will cause an interruption in this region. This system uses a histochemical detection method rather than inactivation of an antibiotic resistance marker (Maniatis, *et al.*, 1982). The diagram of pUC 19 shows restriction sites outside the lac Z' or polylinker region. The restriction sites within this area highlighted with a dark arrow, are unique.

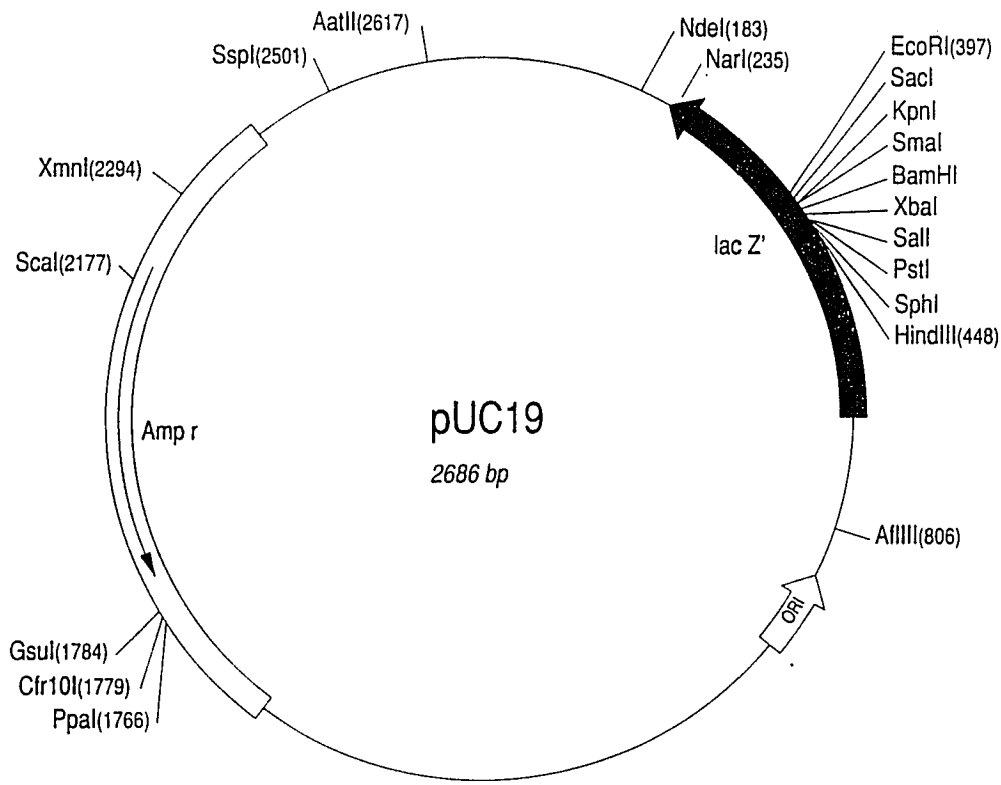


Figure 3. Schematic diagram of the pUC 19 plasmid

1.2 Preparation of DNA:

a) Genomic DNA:

Chromosomal DNA was isolated by a modification of the method described by Marmur (1961). Cells were packed by centrifugation at 5,000 x g for 10 min at 4°C and treated twice for 5 min with 0.15 M NaCl-0.1 M EDTA, pH 8.0. The lysozyme step was omitted from the original procedure as it was found that this did not lyse *L. pneumophila* effectively. Twenty-five percent sodium dodecyl sulfate (SDS) was added to the NaCl-EDTA mixture and incubated at 60°C for 15 min. On cooling, sodium perchlorate at a final concentration of 5 M was added to precipitate the proteins. The proteins were removed by treatment with equal volumes of chloroform-isoamyl alcohol (24:1) three times with wrist-action shaking at 30 min intervals. The mixture was centrifuged at 10,000 x g for 10 min at 4°C. The aqueous phase containing the nucleic acids was removed with an inverted 10 ml glass pipet to minimize DNA shearing. This nucleic acid mixture was dissolved in sodium citrate (Appendix 3.1) and treated for 30 min under static conditions with 10 µg/ml RNase (I.U.B. 2.7.7.16) at 37°C. This process was followed by three chloroform-isoamyl alcohol treatments with wrist-action shaking, each of 30 min and the resultant DNA was collected by overnight precipitation in ethanol at -20°C. The concentration of genomic DNA from this isolation procedure was determined spectrophotometrically by the diphenylamine (DPA) test (Burton, 1968) using 100 µg/ml calf thymus DNA as the standard.

b) Plasmid DNA:

The cloning vector, pUC 19, was isolated from *E. coli* JM 83 by the alkaline lysis method of Birnboim and Doly (1979). Organisms were washed in buffer containing 25 mM Tris-HCl and 25% sucrose. Lysozyme was added to the wash buffer to a final concentration of 40 µg/ml and the mixture was kept on ice for 30 min. SDS at a final concentration of 1% was added as a lysing solution for 5 min. and was followed by 150 µl

of 3 M potassium 5 M acetate (Maniatis *et al.*, 1982) for 1 h. Plasmid DNA was precipitated in 95% ethanol either overnight at -20°C or for 20 min at -70°C. Phenol/chloroform isoamyl alcohol treatments (Kado and Liu, 1981; Marmur, 1961) were used to eliminate contaminating proteins and the cloning vector was treated with 10 µg/ml RNase at 37 °C. Plasmid DNA was kept in 95% ethanol and stored at -20°C until needed. A detailed description of the solutions used for the isolation of genomic DNA is located in Appendix 3.

1.3 Transformation:

a) Chemical Transformation ($CaCl_2$):

The resultant *L. pneumophila* genomic DNA fragments and pUC 19 plasmid constructs were introduced into *E. coli* JM 83 by $CaCl_2$ transformation procedures (Maniatis *et al.*, 1982). Solutions are further described in Appendix 3. An overnight culture of *E. coli* JM 83 was diluted 1:100 in Luria-Bertani (LB) broth and grown for 3 h to a density of 10^7 organisms/ml. Cells were centrifuged at 5,000 x g for 5 min at 4°C and washed for 30 min in 50 mM $CaCl_2$ at 0°C. This procedure was repeated 3 times. Competent cells were combined with the ligation mixture containing the plasmid with inserts and 0.1 M Tris-HCl, pH 7.2. After 10 min at 0°C, the mixture was incubated at 37°C (static) for 2 min at which time prewarmed LB broth was added for 20 min. The resultant bacterial suspension was inoculated onto lactose-containing violet red bile (VRB) agar (See Appendix 1) supplemented with 50 µg/ml ampicillin. Colorless colonies indicated an inability to metabolize lactose due to the insert interruption of the ß-galactosidase region of the vector.

b) Electroporation (Electrical Transformation):

The attenuated *L. pneumophila* strain was harvested from plates and suspended in phosphate buffered saline (PBS), pH 7.0 (10^{8-9} cfu/ml). The suspension was centrifuged

at 5,000 x g for 10 min at 4°C. All electroporation conditions were performed as recommended by the manufacturer (BIO-RAD, Richmond, CA). The bacteria were washed twice in cold filter-sterilized electroporation (Ep) buffer containing 272 mM sucrose, 1 mM MgCl, 7 mM NaH_2PO_4, pH 7.4. After centrifugation at 5,000 x g for 10 min at 4°C, the cells were resuspended in Ep buffer and kept on ice for 15 min. Purified plasmids of pUC 19 with or without the *L. pneumophila* DNA inserts were suspended in Tris-EDTA (TE) buffer containing 40 mM Tris and 2 mM disodium EDTA, pH 7.5 to a final concentration of 5 μg/μl in 0.8 ml of cell suspension. This mixture was placed in a chilled 0.4 cm Gene Pulser cuvette. Cells were exposed to a single pulse (peak voltage 2.5 kV; capacitance 25 μF) which generated a field strength of 6.25 kV/cm. The time constants were 4.5-4.8 m sec in these trials. Immediately following high voltage treatment, the cuvettes were placed on ice for 30 min. Four hundred microliters of the electroporated cell suspension were spread on L-cysteine-supplemented BCYE-α and incubated overnight (18 h) at 37°C. The minimum inhibitory concentration (MIC) of ampicillin for *L. pneumophila* N_7 was established as 0.5 μl/ml (Elliott, and Rodgers, 1985). This growth was harvested the following day and inoculated onto L-cysteine-supplemented BCYE-α plates containing 50 μg /ml ampicillin to select for transformants. Electroporation buffer is described in Appendix 3.

1.4 Agarose gel electrophoresis:

a) Plasmid Analysis:

E. coli and *L. pneumophila* clones were screened for plasmid content by the method of Kado and Lui (1981). Bacteria were suspended in 125 μl 1X TE buffer and lysed with addition of 250 μl of 3% SDS in 50 mM Tris adjusted to pH 12.0 using 2 N NaOH. This mixture was incubated at 65°C for 20 min at which time phenol/chloroform/isoamyl alcohol was added and gently mixed by inverting the tube 12 times. DNA was carefully removed above the interface and precipitated overnight at -20°C in 95% ethanol. An alternative

plasmid isolation procedure was stated in **1.2.**

b) Restriction analysis:

Genomic or plasmid DNA samples were digested with restriction endonucleases according to the manufacturers instructions. Restriction enzymes used in this study are listed in Table 8.

DNA samples were loaded onto a 0.7% (w/v) agarose gels using 50% glycerol and 0.1% (w/v) bromophenol blue as a tracking dye and subjected to 5 V/cm for 3 h in a Tris-EDTA-acetate buffer system, pH 7.8 in a horizontal electrophoresis unit. The gel slabs were stained with 0.5 μg/ml ethidium bromide and viewed using an ultra-violet transilluminator. A detailed description of the materials used in this procedure is listed in Appendix 3.

1.5 DNA recovery:

Agarose gel electrophoresis was performed as stated in **1.4b.** Slices of agarose gels containing plasmids were excised by using a sterile razor blade unless otherwise stated. The recovered DNA was suspended in 1X TE buffer and treated with phenol/chloroform/isoamyl alcohol. All samples were precipitated in 95% EtOH and stored at -20°C until needed. The following methods were used to recover plasmid DNA:

a) Low temperature-melting agarose:

Low temperature-melting agarose (BIO-RAD) was employed to retrieve plasmids from agarose gels. Agarose slices were placed in sterile 1.5 ml Eppendorf tubes and heated to 65°C.

b) Electroelution by dialysis tubing:

Agarose slices containing plasmids were removed from the gel and placed in dialysis tubing with 1X TE buffer. The clamped tubing was placed at the cathode of the electrophoretic unit. The samples were subjected to 5 V/cm for 15 to 60 min in different trials. After elution, the TE buffer was removed by pipet from the dialysis tubing.

Table 8

RESTRICTION ENZYMES USED IN THE STUDY

Restriction Enzyme	Derivation
Bam HI (BRL)	*Bacillus amyloliquefaciens*
Eco RI (IBI)	*Escherichia coli* RY13
Hind III (IBI)	*Haemophilus influenzae* Rd
Pst I (IBI)	*Providencia stuartii*
Kpn I (BRL and BioLabs)	*Klebsiella pneumoniae* OK8
Xba I (BRL)	*Xanthomonas badrii*
Sal I (BRL)	*Streptomyces albus* G
Sma I (BRL)	*Serratia marcescens*
Pvu I (BRL)	*Proteus vulgaris*
Pvu II (BRL and IBI)	*Proteus vulgaris*
Acc I (BRL)	*Acinetobacter calcoaceticus*
Hae III (BRL and IBI)	*Haemophilus aegyptius*

Reaction and storage conditions for each of the restriction endonucleases were as specified by the manufacturer. All experiments used overnight digestions with approximately one unit of restriction endonuclease.

Agarose slices were viewed on an UV transilluminator to ensure that the plasmid was no longer present.

c) **Crush and soak method**:

The agarose slice containing the plasmids were placed in a sterile 1.5 ml Eppendorf tube and mashed with a pipet tip. TE buffer in 1X strength was added to the Eppendorf tube, vortexed and treated with phenol/chloroform isoamyl alcohol. The aqueous phase containing the recovered DNA was removed from the interface. This procedure was repeated one additional time. After the initial precipitation, 1 µl of tRNA and 0.25 volume of 5 M NaCl were added to the recovered DNA. The samples were stored in equal volumes of isopropyl alcohol.

d) **Unidirectional electroelution**:

The International Biotechnologies, Inc (IBI, New Haven, CT) electroelutor was employed to remove plasmid from agarose gels. The agarose slices were placed in the wells with 1X TE buffer. The traps were loaded with a high-salt buffer to prevent the DNA from escaping. The samples were subjected to 125 V for 50 min intervals as the time necessary to remove the plasmids was proportional to the size of the plasmid. The details of the high-salt buffer solution is listed in Appendix 3.

e) **Membrane collection system**:

The Schleicher and Schuell Elutrap electro-separation system was used to retrieve plasmids or DNA fragments. Gel slices were placed in the membrane trap as specified by the manufacturer and subjected to 150 V. The application time was monitored by viewing the trap with a hand-held short wave UV lamp to determine if the DNA had migrated from the gel. DNA was removed from the membrane by reversing the polarity for 20 seconds at 200 V.

f) **Trough method**:

Plasmid retrieval was performed by applying reverse polarity to agarose gels with

plasmid content. DNA was collected in wells formed by removal of an agarose slot directly above the desired plasmid (Maniatis, 1982).

1.6 Preparation of DNA for radioactive labeling:

L. pneumophila DNA partially restricted with 1 unit of *Hae* III per 1 µg DNA used in random labeling experiments was prepared for ^{32}P-α labelled dCTP using a nick translation kit (BRL). The spun column technique (Maniatis, *et al.*, 1982) removed unincorporated label from nick translated molecules through the use of equilibrated Sephadex G-50 in Saline Tris-EDTA (STE) buffer which was continually added to a 1 cc syringe packed with glass wool and spun at 5,000 x g for 4 min until 0.9 cc packed volume was reached. After two additional centrifugations with 100 µl STE the radioactively labeled sample was added to the syringe and centrifuged in the same manner and collected in a sterile 1.5 ml Eppendorf tube. This material was stored -20°C. Since this was radioactively labeled material, the tube was kept in a 50 ml glass beaker covered by another glass beaker of larger size to block the escape of radioactivity into the freezer.

1.7 Southern hybridization:

Agarose gels were transferred to nitrocellulose filters for Southern blot analysis (Southern, 1975) after being denatured and neutralized. Two layers of taped 10 ml test tubes were used to raise the glass platform from the bottom of a glass baking dish. Whatman 3 MM paper cut larger than the glass plate was placed on the platform and soaked with 10X standard sodium citrate (SSC) (Appendix 3.16). The paper was smoothed using a glass rod. The agarose gel placed face down on the Whatman filter, was smoothed with a glass rod to eliminate air bubbles. Nitrocellulose filters paper cut exactly to the dimensions of the gel, were floated on the surface of 2X SSC for several minutes at room temperature to ensure that the sheet was evenly soaked by capillary action. Filters were placed onto the gel and smoothed with a glass rod. Two pieces of Whatman 3 MM paper cut to the exact dimensions of each gel and nitrocellulose filters were placed on top of the nitrocellulose

filter and smoothed with a glass rod. Paper towels, each cut to the exact dimensions of the filters and the gel were placed on top of the Whatman 3MM filters to a height of approximately 7 cm. A glass plate was mounted on top of the towels and weighted with a corked 500 ml Erlenmeyer flask filled with water to add weight. This was secured by taping the flask in four directions onto the lab bench. After 18 h the tower structure was carefully taken down and the nitrocellulose filters were removed, soaked in 6X SSC at room temperature for 5 min and left to air dry on a fresh piece of Whatman 3MM paper. The dried filter was baked between 2 pieces of Whatman 3MM paper in a vacuum oven for 2 h at 80°C.

The baked filters were resubmerged into 6X SSC prior to the prehybridization step necessary to bind all nonspecific sites on the sheets. Each filter was placed in a heat-sealable plastic bags just large enough to accommodate the filter. Prehybridization fluid was added at 0.2 ml for each cm^2 of baked filter carefully removing any air bubbles formed inside the bag. The filters were incubated at 68°C in a water bath for 2 h. All prehybridization fluid was removed from the bag by squeezing excess material through a small slit in the bag. Hybridization fluid containing the radioactively labeled sequences was added at 50 µl per cm^2 and resealed after carefully removing all air pockets. Filters were incubated overnight at 68°C. Following these incubation periods the filters were carefully removed from the plastic bags and were washed significantly in buffer containing 2X SSC and in 0.1% SDS at room temperature for 1 h and then in buffer containing 0.2X SSC and 0.1% SDS at 68°C for 2 h when using random labeled probes. The filters were dried on Whatman 3MM paper, wrapped in sheet plastic and developed on X-OMAT-AK X-ray film (Kodak, Rochester, NY). A detailed description of the solutions used for Southern hybridization are listed in Appendix 3.

1.8 Sequencing analysis:

The *L. pneumophila* DNA insert in was sequenced directly from the plasmid using

the dideoxy chain-termination method described by Sanger, *et al.* (1977) with a Sequenase sequencing kit (U. S. Biochemical Corporation, Cleveland, OH). Samples were reacted with ^{35}S-methionine label (NEN, Boston, MA) and M13 primers (U. S. Biochemical Corporration) or primer extension sequences obtained from the novel DNA (National Biosciences, Plymouth, MN). The polymerase chain reaction (PCR) GeneAmp kit was used to amplify regions of the DNA sequence using the Perkins Elmer Cetus Thermocycler (Perkins Elmer Cetus, Norwalk, CT). The reaction conditions were as follows: 1.30 min at 94 °C, 1 min at 55 °C and 1.30 min at 72 °C for 40 cycles. The amplified products were prepared for sequencing by using Miniprep push columns (Stratagene, La Jolla, CA). The information obtained from sequencing was compared to existing sequence data registered with Gen-Bank (San Francisco, CA) and analyzed by the Genetic Computer group (GCG) Sequence Analysis Software Program.

1.9 Replica Plating:

Clones originating from the electroporation of *L. pneumophila* with pUC 19 and pLP 116 were transferred to plates containing BCYE-α agar without ampicillin and grown overnight at 37°C in a humid atmosphere. The cells were then transferred by means of replica plating (Miller, 1974) using velvet squares mounted on a wooden block sized to accommodate 100 X 15 mm plastic petri dishes containing BCYE-α with 50 μg/ml ampicillin to assay for colonies spontaneously cured of either the pUC 19 vector or the LP 116 recombinant plasmid.

1.10 Filter-binding immunoassay and dot blot analysis:

E. coli clones expressing *L. pneumophila* antigens were detected by filter binding in an enzyme immunoassay (FB/EIA) (Engleberg *et al.*, 1984) by growing the clones overnight at 37°C on nutrient agar supplemented with 50 μg/ml ampicillin. Colonies were removed by direct application onto nitrocellulose filters and these were treated by baking in a vacuum oven at 60°C. Organisms were not lysed prior to the baking process. Samples

for dot blot analysis were prepared similarly but formalin-fixed and added as drops to nitrocellulose filters which were air dried. Oven dried or formalin-fixed air dried filters were pretreated with Tris buffered saline (TBS), pH 7.5 by immersing the filters slowly into a baking dish and allowing the buffer to rise by capillary action. Premoistened sheets were treated for 1 h with a 3% gelatin blocking solution. All filters were incubated overnight at room temperature in a 1:800 dilution of *L. pneumophila* N_7 polyclonal rabbit antisera (A. O. Tzianabos, Department of Microbiology, UNH) in a 1% blocking solution. After brief rinsing in distilled water, the filters were washed four times for 10 min each in TBS and were incubated for 2 h in 1:3000 goat-anti rabbit horseradish peroxidase (HRP) conjugate (Organon Teknika Corp, Malvern, PA) in a 1% blocking solution. The solutions for this assay are located in Appendix 4.

1.11 Polyacrylamide gel electrophoresis (PAGE) and immunoblotting:

Bacterial outer membrane proteins were isolated as previously described (Wenman, 1985). Cells were lysed in phosphate buffered saline (PBS) by glass bead disruption and outer membranes were collected by differential centrifugation (Fig. 4). Following treatment with 2% (w/v) sodium lauryl sarcosinate to dissolve cytoplasmic membrane material (Filip et al., 1973), the non-solubilized outer membrane proteins were either suspended in reduced sample buffer containing 70 mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% (w/v) glycerol and 0.002% (w/v) bromophenol blue, pH 6.8 or non-reduced sample buffer which lacked 2-mercaptoethanol. Both were boiled for 5 min. prior to electrophoresis. OMP samples were also used to determine the protein content spectrophotometrically using bovine serum albumin (BSA) as the standard (Lowry et al., 1951). A constant current of 5 m amp for 18 h was used in a horizontal electrophoresis unit (Hoefer) with 12% (w/v) SDS polyacrylamide gel, 25 mM Tris-192

SCHEME FOR THE ISOLATION

OF OUTER MEMBRANE PROTEINS

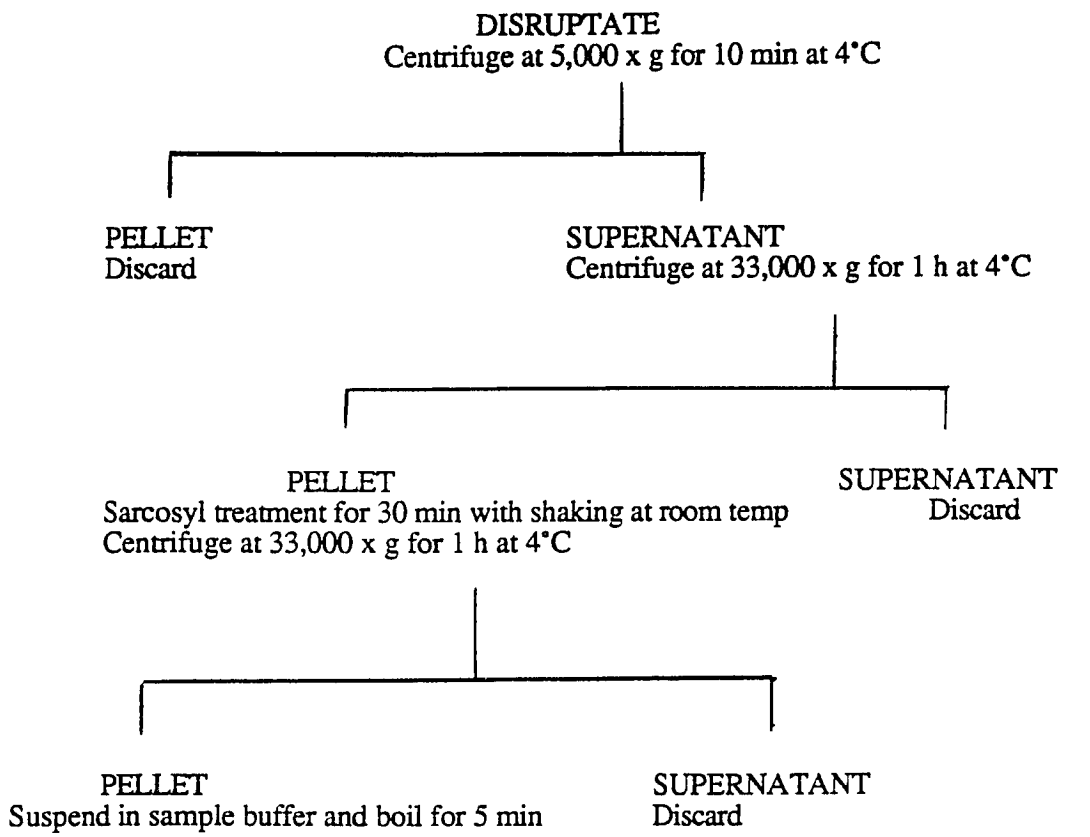


Figure 4. Differential centrifugation was used to separate the OMP from other components of the cell. Sarcosyl treatments were used to dissolve the cytoplasmic membrane. After boiling the OMP in sample buffer, they could be used immediately or stored at -20°C.

mM glycine buffer and 1% (w/v) SDS, pH 8.3 (Laemmli, 1970). Gels were stained with 19.5 % silver nitrate as described by Oakley et al. (1980).

Immunoblot analysis (Towbin et al., 1979) was accomplished by transferring polyacrylamide gels to nitrocellulose using a Transblot cell (Bio-Rad, Richmond, CA) for 18 h and the filters probed with *L. pneumophila* rabbit antisera or MOMP-specific monoclonal antibody (a gift from Dr. Larry A. Gosting, Genetic Systems, Seattle, WA) followed by HRP-conjugated antiserum as described for colony transfers in **1.9**

A description of materials necessary for polyacrylamide gel electrophoresis of proteins and immunoblotting are listed in Appendix 4.

1.12 Immunofluorescence:

a) Indirect immunofluorescence:

E. coli JM 83, *L. pneumophila* N_7 and the *E. coli* clones were tested initially in an indirect immunofluorescence assay (IFA). Bacteria were fixed *in situ* on glass slides for 15 min in 5% formalin, air-dried and treated with a 1:500 dilution of *L. pneumophila* or *E. coli* rabbit antiserum (Difco, Detroit, MI) for 30 min at 37°C in a humid atmosphere. After rinsing in PBS for 15 min, the slides were treated in similar fashion with FITC-conjugated goat anti-rabbit antiserum and air-dried in darkness. Specimens were mounted in glycerol containing diazobicyclo-2,2,2 octane (DABCO) and examined by UV epifluorescence microscopy.

b) Direct Immunofluorescence:

A direct immunofluorescence assay (DFA) was performed using a commercially prepared monoclonal antibody (Genetic Systems, Seattle, WA, USA) raised against a 29 kDa OMP common antigen found in all serogroups of *L. pneumophila* (Gosting *et al.*, 1984) and subsequently shown to have specificity for the MOMP (Nolte and Conlin, 1986). Specimens were mounted and viewed as stated above.

1.13 Chick embryo virulence assay:

Fertile white Leghorn chicken eggs (UNH Poultry Farm) were maintained at 37°C in a humid atmosphere. Bacteria that were either scraped from agar plates or grown in broth were suspended in PBS with organism densities ranging from 10^1 to 10^7 cfu/ml. These were inoculated into the yolk sac of fertile chicken eggs using 0.1 ml of the 10-fold dilutions (Fig. 5). The eggs were candled twice daily to determine embryo lethality. The lethal dose $_{50}$ (LD_{50}) for each clone and the controls was calculated by the method of Reed & Muench (1938). Experiments were performed using a minimum of 10 eggs per dilution and the results were the average of three separate experiments.

1.14 Preparation and maintenance of cell cultures:

Eagle's MEM (Irvine Scientific, Santa Ana, CA) supplemented with 7.5 % sodium bicarbonate, 300 mM glutamine (10 ml/L)(Sigma Chemicals) and 10% bovine calf serum (Hyclone Laboratories, Inc., Logan, Utah), was warmed to 37°C before use and the cells were incubated in 5% CO_2 at 37°C in plastic T-75 cm^2 flasks (Costar, Cambridge, MA). The cell culture line used in this study was a human monocyte-like cell line (U-937) derived from malignant cells obtained from a pleural effusion of a 37 year old Caucasian male with diffuse histiocytic lymphoma. This cell line was obtained from the American Type Culture Collection (ATCC), Rockville, MD. Since U937 cells are monocyte-like, they do not form monolayers so no trypsinization was necessary for passage. Cells were treated for 24 h with phorbol 12-myristate 13-acetate (PMA)(Sigma Chemicals) to allow them to adhere to the surface of plastic dishes for the subsequent studies. Once adhered to the flask surface, the cells were removed using a rubber policeman and transferred at a density of 10^5 cells/well to 6-well plates containing 1 ounce glass coverslips. Cell culture solutions and media are listed in detail in Appendix 2.

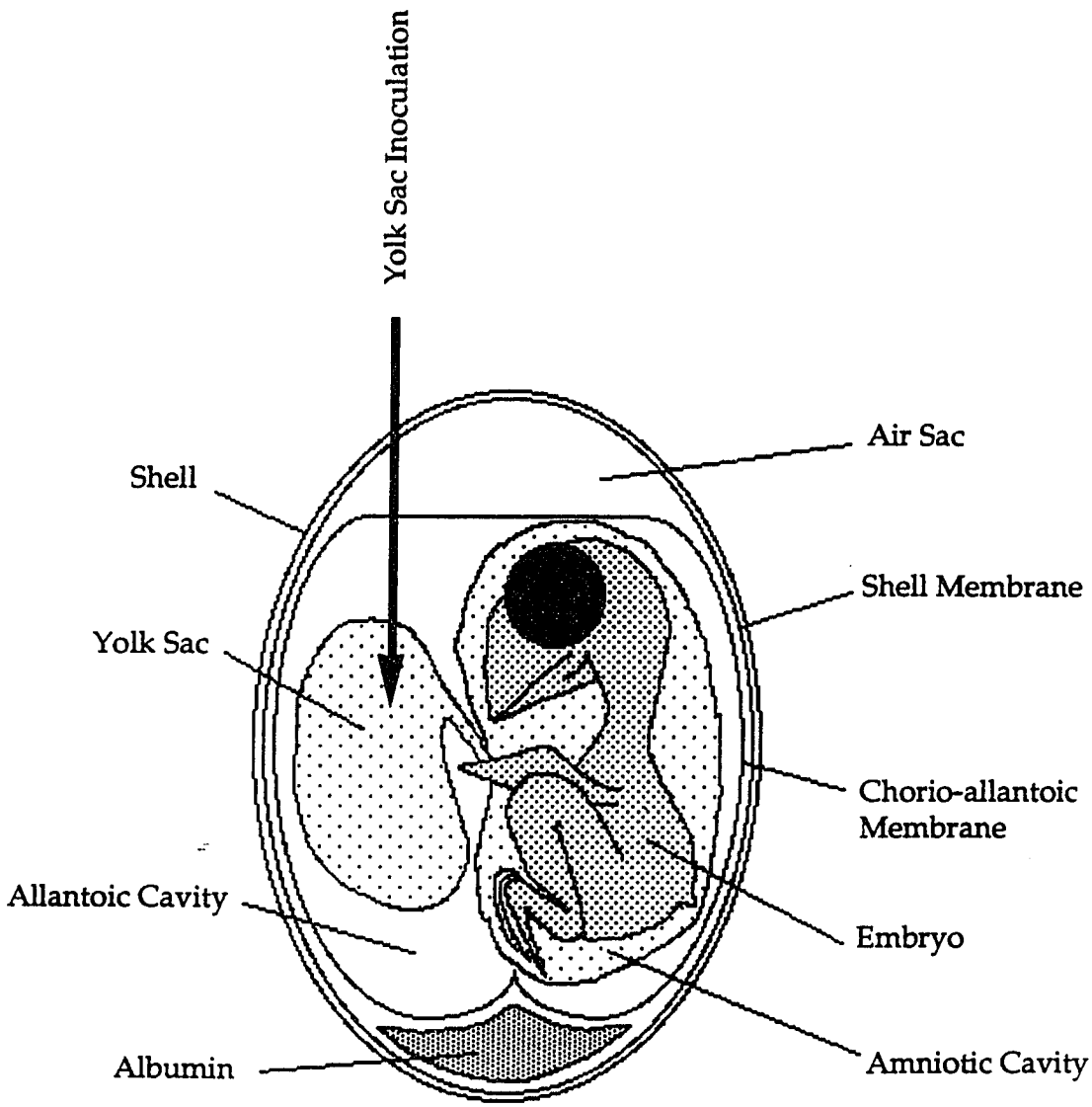


Figure 5. Chicken embryo yolk sac inoculation
The lethal dose $_{50}$ for *L. pneumophila* N_7, *E. coli* JM 83 and each of the *E. coli* clones was determined by injecting 0.1 ml of the 10-fold dilutions into the yolk sac of fertile hens' eggs. It was previously determined that the yolk sac route of inoculation was most effective for *L. pneumophila* (Tzianabos and Rodgers, 1989)

2. The plasmids of *Legionella* and virulence:

2.1 Plasmid analysis of the *Legionella* species and strains:

Legionella were grown overnight on BCYE-α agar at 37°C in a humid atmosphere. Bacteria were scraped from plates and treated as stated in **1.2b**. Recovery of plasmids from in the *Legionella* species and strains were performed as stated in **1.5.** LD_{50} studies to determine virulence of the organisms were performed on the plasmid-bearing isolates as stated in **1.13.**

3. Construction of the genomic library:

3.1 Development and screening of recombinant plasmids:

L. pneumophila DNA, isolated as stated in **1.2b,** was digested with several restriction endonucleases to determine which enzyme yielded the smallest base pair fragments. A partial digest using *Hae* III was used for *L. pneumophila* DNA while the plasmid vector was cleaved completely with *Sma* I and subsequently used in the ligation studies with the *L. pneumophila* DNA fragments using T4 ligase (BRL, Bethesda, MD) according to the procedures appropriate for blunt-end ligation. Colonies demonstrating an interruption of the α-complementary-β-galactosidase region were selected from VRB agar, containing lactose and supplemented with 50 μg/ml ampicillin. These colonies were grown overnight at 37°C in nutrient broth containing 50 μg/ml ampicillin. Surviving cultures were frozen at -70°C in 0.8% DMSO.

The clones were screened by agarose gel electrophoresis to determine if plasmids were present in the cells. Plasmid preparations of positive clones were initially digested with *Sma* I although the original cut site would be lost due to the ligation of blunt ends. Fragments that were detectable in the gel were measured and plotted on a semi-logarithmic scale to determine the size of the material released. Agarose gels containing samples of the *Sma* I-digested plasmids were transferred to nitrocellulose sheets as described in **1.7** and were probed with *L. pneumophila* DNA fragments created for the original ligation

procedure.

3.2 Recloning experiments:

Recombinant plasmids showed irregularities when digested with *Sma* I therefore *Pst* I was used on the recombinant plasmid, pLP 116 and this was examined by electrophoresis as described in **1.4**. The resultant fragments were electroeluted from the gel using an Elutrap
electroseparation chamber (Scheicher and Schuell, Keene, NH) and a horizontal gel unit (BRL) according to the manufacturers specifications. The *L. pneumophila* DNA fragments were ligated into *Pst* I digests of pUC 19 and transformed into *E. coli* JM 83 (Maniatis *et al.*, 1982). The colorless transformants were collected and maintained as described in **1.3** of this section. Characterization of the recloned samples was by FB/EIA as well as restriction analysis and Southern hybridization described in **1.10, 1.4b** and **1.7**, respectively.

3.3 Detection of surface-expressed *L. pneumophila* proteins:

Recombinant clones were assessed for their ability to express antigens of *L. pneumophila* by using the techniques described in **Section 1.10**. Clones were transferred using sterilized toothpicks and inoculated onto nutrient agar containing 50 µg/ml of ampicillin. Each plate was prepared with 5 rows of ten samples (across) and incubated overnight at 37°C. Growth was removed from the petri dishes by placing nitrocellulose sheets across the plates and allowing them stand for 5 min. Filters were baked at 60°C for 2 h in a vacuum oven and treated with *L. pneumophila* polyclonal antisera and by goat-anti rabbit conjugate, both in a 1% gelatin solution. The filters were immersed in color developer containing 0.05% (w/v) 4-chloro-1-naphthol and 0.15% (v/v) hydrogen peroxide in a 5:1 solution. Solutions used in filter transfers and dot blot assays are described in Appendix 4.

3.4 Outer Membrane Protein (OMP) analysis of the *L. pneumophila* antigen-expressing recombinant clones:

Samples of the OMP of *E. coli* JM 83, the recombinant clones containing *L. pneumophila* DNA and the *L. pneumophila* N_7 isolate were compared and measured on a semi-logarithmic scale. The techniques for outer membrane isolation and polyacrylamide gel electrophoresis are described in **1.11**. Protein samples each of approximately 5 μg/ml were loaded onto a 5% (w/v) stacking gel and subjected to SDS-PAGE (Laemmli, 1970). The molecular weight standards (BIO-RAD) were lysozyme, 14,300; β-lactoglobulin, 18,400; α-chymotrypsinogen, 25,700; ovalalbumin, 43,000; Bovine serum albumin, 68,000; phosphorylase B, 97,400; and myosin (H-chain), 200,000. Strong disulfide bonds are resistant to double boiling and, in the absence of a reducing agent, cannot migrate through the gel matrix (Ehret, *et al.*, 1984; Gabay, *et al.*, 1985, Butler, *et al.*, 1986; Hindahl and Iglewski, 1986). Duplicate OMP samples of *E. coli* JM 83, *L. pneumophila* N_7 and clone LP116 were subjected to electrophoresis after boiling for 5 min in sample buffer without 2-mercaptoethanol. Five percent (v/v) 2-mercaptoethanol was added to one set of samples followed by boiling for 5 min while the other was examined without further treatment.

The silver staining method of Tsai and Frasch (1982) using periodic acid oxidation of polyacrylamide gels and a 20% silver nitrate concentrations made it possible to better observe the lipopolysaccharides in the gels. SDS-PAGE of OMP samples of the *E. coli* clone, *L. pneumophila* N_7 and *E. coli* JM 83 parent strain were fixed overnight in 40% ethanol-5% acetic acid. Gels were soaked in 0.7% (w/v) periodic acid for 5 min and silver stained by the method of Tsai and Frasch (1982) to locate any usual changes in the banding patterns of *E. coli* LPS in LP116 in the gels. A detailed description of the LPS silver stain preparation and the solutions used in polyacrylamide gel is located in Appendix 4.

3.5 Immunofluorescence:

The procedure for IFA and DFA are stated in **1.12.** All clones were examined by both the IFA method using *L. pneumophila* polyclonal rabbit antisera and DFA using a monoclonal antibody raised to the major outer membrane protein (MOMP) of *L. pneumophila.* The *E. coli* clones and parent were compared to the *L. pneumophila* N_7 strain. The DFA kit contained a *L. pneumophila* positive control.

3.6 SDS-PAGE and immunoblot analysis:

SDS-polyacrylamide gels containing OMP samples were transferred to nitrocellulose filters as stated in **1.11.** The filters were initially treated with *L. pneumophila* polyclonal rabbit antisera to detect the protein source in the clone and determine if it was an *L. pneumophila* surface-expressed protein. To further examine if the protein was of MOMP origin, the OMP of *E. coli* LP 116 was transferred from polyacrylamide to nitrocellulose filter paper and treated with monoclonal antibody raised to the MOMP of *L. pneumophila.* The solutions and antibody are described in Appendix 4.

4. Electroporation of the attenuated *L. pneumophila* strain using pUC 19 and the pLP 116 recombinant plasmid:

4.1 Electrical transformation:

Transformation of the attenuated *L. pneumophila* strain was accomplished by the procedure stated in **1.3.** *L. pneumophila* was grown on BCYE-α agar overnight at 37°C in a humid atmosphere. The growth was scraped from the plates and suspended in PBS to approximately 10^{8-9} cfu/ml. After removing the PBS by centrifugation, cells were suspended in Ep buffer. To ensure that growth on the ampicillin-supplemented media was the result of transformation and not spontaneous mutations, controls were both the *L. pneumophila* N_7 virulent and attenuated strains that were either electroporated samples in the absence of DNA or non-electroporated samples.

Transformants were screened by plasmid analysis using isolation methods

described by Birnboim and Doly (1979) and Kado and Lui (1981). These samples were screened by plasmid analysis as stated in **1.4**. Samples with plasmids present were digested with *Pst* I and viewed by agarose gel electrophoresis after staining with ethidium bromide. Transformants containing pLP 116 or pUC 19 were cured of their plasmids by replica plating in **1.9**. No mutagenic agents were used in the curing process.
Outer membrane proteins for all *L. pneumophila* samples were isolated as stated in **1.10**, assayed for protein content and subjected to SDS-PAGE. The wells were loaded with 5 μg/ml of the OMP samples and were silver stained with 19.5% silver nitrate.

5. Virulence assay, adherence and blocking studies:

5.1 Fertile hens' egg lethality assay:

Seven day old chicken embryos were injected into the yolk sac with *E. coli* JM 83, the *E. coli* clones, *L. pneumophila* N_7, both virulent and attenuated and all electroporated samples of *L. pneumophila* as described in **1.13**. *L. pneumophila* N_7 and *E. coli* clones were coated with monoclonal antibody raised to the MOMP of *L. pneumophila* and injected as stated to assess the function of the MOMP. Control eggs were injected with PBS only and maintained up to eight days post inoculation. The original cfu/ml for each trial was determined by viable plate counts.

5.2 Adherence assay using U937 cells:

U937 cells were harvested when the culture reached late exponential phase of growth (1-2 x 10^6 cells/ml). The cells were differentiated into a non-replicative, adherent macrophage-like cell line with PMA which was added for a final concentration 10^{-8} M for 24 h. The cells were distributed into 6-well plastic plates (Costar, Cambridge, MA) containing sterilized, 1 ounce glass coverslips. The cell density per well was approximately 5 x 10^5 cells/well. Cells were treated with cytochalasin D (Sigma Chemicals) at a final concentration of 3 μg/ml to inhibit the uptake of organisms by

interfering with microfilament formation (King, *et al.*, 1991; Elliot and Winn, 1986). The cells were treated for 1 h before being exposed to *E. coli* JM 83, *E. coli* LP 116 or *L. pneumophila* organisms with or without MOMP-monoclonal antibody. U937 cells used in blocking studies were coated with immune serum to bind all available Fc receptor sites prior to exposure to any organisms. All organisms were suspended in HBSS and distributed in 10-fold dilutions to each of the wells. The ratio was approximately 100:1 for U937 cells (A. Tzianabos thesis, 1989), the multiplicity of infection (MOI) used in other studies. The organisms were in contact with the cells for 2 h after which time they were rinsed 3 times in HBSS and prepared for DFA or IFA as described in **1.12.** Unbound material was removed with 3 HBSS washings and the cells were counterstained for 5 min. at 37°C with 1% propidium iodine prior to mounting for microscopic examination in IF studies. Viable counts of the washings from the 6-well plates of U937 cells lysed with water were also used to assay adherence.

RESULTS

1. Plasmids as potential virulence factors:

Strains of *L. pneumophila*, Philadelphia 2 and Heysham 2, *L. bozemanii*, WIGA, *L. hackeliae* Lansing 2 and *L. dumoffii*, Tex-KL all contained one or more plasmids (Fig. 6). The Heysham 2 strain of *L. pneumophila* and the *L. hackeliae* species each contained a single 80 mDa plasmid. *L. dumoffii* contained both the 80 mDa plasmid as well as a 45 mDa plasmid while *L. bozemanii* had 80, 45 and 35 mDa plasmids. The Philadelphia 2 strain of *L. pneumophila* possessed only a single 21 mDa plasmid. The 80 mDa plasmid appeared to be in greater copy number in the *L. dumoffii*, Tex-KL isolate and each time the plasmids were isolated from this species, the 80 mDa plasmid appeared strongest in agarose gel electrophoresis studies. However, unlike the smaller plasmids, large plasmids are usually produced in low copy number. Attempts to retrieve the 80 mDa plasmid from any of the isolates using several recovery methods failed to yeild this plasmid. All plasmid-bearing *Legionella* isolates were compared to selected plasmidless strains by establishing LD_{50} for each of these organisms. Data are presented in Table 9.

2. The recombinant clones:

2.1 Initial examination of restriction endonuclease-digested *L. pneumophila* genomic DNA:

Several restriction enzyme digests were prepared with *L. pneumophila* N_7 genomic DNA. In all instances where the enzyme was able to cleave the DNA, only partial digestion could be achieved. *Hae* III digestions yielded the smallest base pair fragments for ligation into the pUC 19 vector while *Pst* I and *Sma* I did not appear to digest *L. pneumophila* N_7 DNA when compared to the uncut sample (Fig.7).

Figure 6. Plasmid analysis of selected *Legionella* species.
Thirty-two strains and species were subjected to agarose gel electrophoresis. Only five of these isolates were found to possess plasmids. Lanes 1 through 5 are standard molecular weight markers; 5.5, 25, 34, 62 and 96 megadaltons (mDa), respectively. Lane 6: *L. bozemanii* WIGA, ATCC #33217 (A gift from Dr. D. J. Grimes). Lane 7: *L. bozemanii* WIGA from an unknown resource. Lane 8: *L. pneumophila* Philadelphia 2. Lane 9: *L. pneumophila* Philadelphia 1. Lane 10: *L. pneumophila* Heysham 2. Lane 11: *L. hackeliae* Lansing 2. Lane 12: *L. pneumophila* Los Angeles 1. Lane 13: *L. pneumophila* Washington 1. Lane 14: Molecular weight markers 62 and 96. Lane 15: *L. feeleii* WO-44C. Lane 16: *L. micdadei* Tatlock. Lane 17: *L. pneumophila* Nottingham N_7.. Lane 18: *L. dumoffii* Tex-KL.

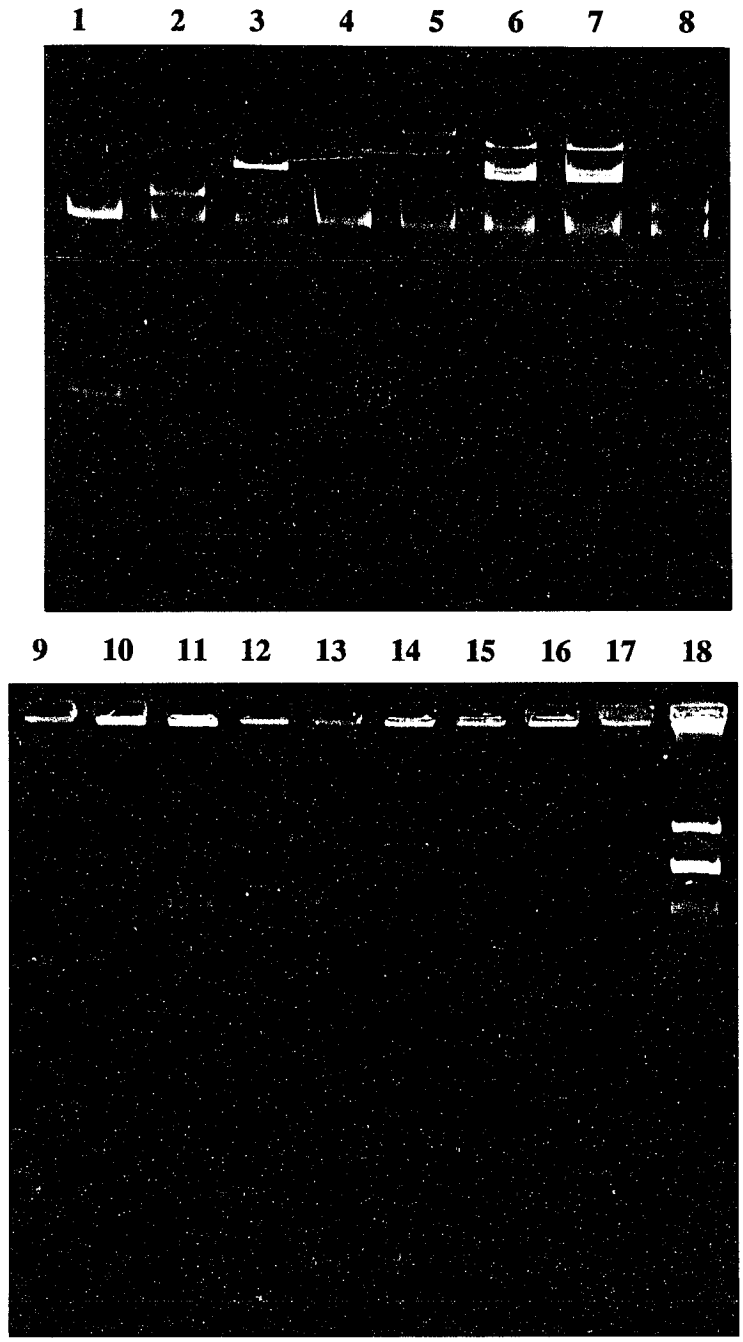


Figure 6. The plasmids of *Legionella*.

Table 9 Lethal dose $_{50}$ values for species and strains of *Legionella* inoculated into chicken embryo yolk sac

Species (strain)	Plasmids	LD_{50} Endpoints*
L. micdadei (PPA)	None	No death
L. hackeliae (Lansing 2)	80	No death
L. pneumophila (Philadelphia 2)	21	2.5×10^5
L. pneumophila (Heysham 2)	80	1.2×10^2
L. pnuemophila (Nottingham 7)	None	4.6×10^2
L. bozemanii (WIGA**)	80, 45, 35	6.7×10^2
L. bozemanii (WIGA**)	80, 45,35	5.9×10^2
L. dumoffii (Tex-Kl)	80, 45	5.2×10^2

Plasmid-bearing cells could be recovered from the yolk sac 48 h post inoculation.
*Ten eggs were inoculated per dilution with 0.1 ml samples and LD_{50} data were calculated from three separate experiments. Control eggs were injected with PBS and in this group, no death occured up to eight days post inoculation
**Two isolates on hand from different sources.

With the exception of strains of *L. micdadei*, *L. hackeliae* and *L. pneumophila*, Philadelphia 2 all isolates examined were virulent for the chick embryo assay (Tzianabos and Rodgers, 1989) and this property did not appear to correlate with the presence of identifiable plasmids. However, integration of plasmids into the *Legionella* genome cannot be excluded.

Figure 7. Restriction Analysis of *L. pneumophila* Nottingham, N_7 genomic DNA. Chromosomal DNA of *L. pneumophila* N_7 was digested with several restriction endonucleases to determine which would yield the smallest base pairs. Lane 1: *L. pneumophila* genomic DNA uncut. Lane 2: phage lambda digested with *Hind* III. Lane 3: *Pst* I. Lane 4: *Sma* I Lane 5: *Kpn* I. Lane 6: *Hae* III. Lane 7: phage lambda DNA digested with *Hind* III. Lane 8: *Bam* HI. Lane 9: uncut *L. pneumophila* DNA. Lane 10: *Eco* RI. Lane 11: *Hind* III. Note that *Pst* I (Lane 3) and *Sma* I (Lane 4) did not appear to cleave *L. pneumophila* genomic DNA.

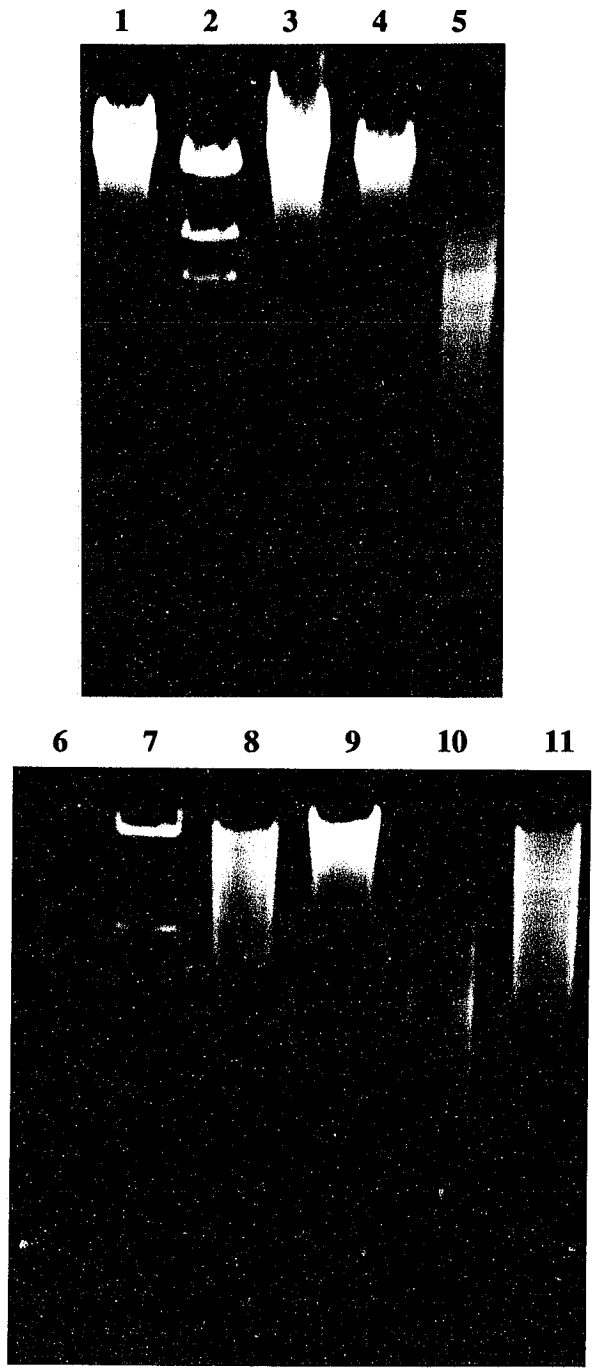


Figure 7. Digested *L. pneumophila* N_7 genomic DNA.

2.2 Preliminary examination of the *E. coli* recombinants:

A total of 1123 clones were generated from the transformation procedure; however, 129 survived transfer to nutrient broth containing 50 μg/ml of ampicillin. These recombinants were initially screened for their plasmid content. It was apparent that the plasmid profiles underwent changes in the banding pattern when compared to pUC 19 as shown in Fig.8. Eight of the surviving clones expressed *L. pneumophila* antigens in the absence of bacterial lysis using rabbit antisera in the filter binding and dot blot assay (Fig. 9). *L. pneumophila* N_7 and the clones but not *E. coli* JM 83 gave positive fluorescence when tested with *L. pneumophila* polyclonal antisera and FITC conjugate.

2.3 Restriction analysis and Southern hybridization of the *E. coli* clones demonstrating surface-expressed *L. pneumophila* proteins:

The migration patterns of the cloned plasmid DNA digested with *Sma* I were shown to differ when compared to that of the pUC 19 vector cut with the same enzyme and in some instances *Sma* I digests apparently did not appear to cut the plasmids. Restriction analysis of the eight clones using concentrated amounts of isolated plasmid revealed the presence of a band of approximately 750 base pairs when measured against the *Pst* I digested lambda DNA standard and averaged from three separate trials (Fig. 10). The fragments could only be detected by running the agarose gel from 1/2 to 3/4 to completion. It was discovered in several earlier trials that the fragments were escaping the bottom of the gel because they ran too far ahead of the tracking dye. The cut site for *Sma* I should have been lost by the blunt-end ligation but there was evidence to suggest that a *Sma* I site was still intact. It was shown by Southern hybridization using a ^{32}P-labeled *L. pneumophila* DNA probe derived from the genome of *L. pneumophila* N_7 that these fragments were *L. pneumophila* DNA (Fig. 10). Initial studies with restriction endonucleases indicated that Pst I was unable to cleave *L. pneumophila* N_7 DNA; however, this digest was able to

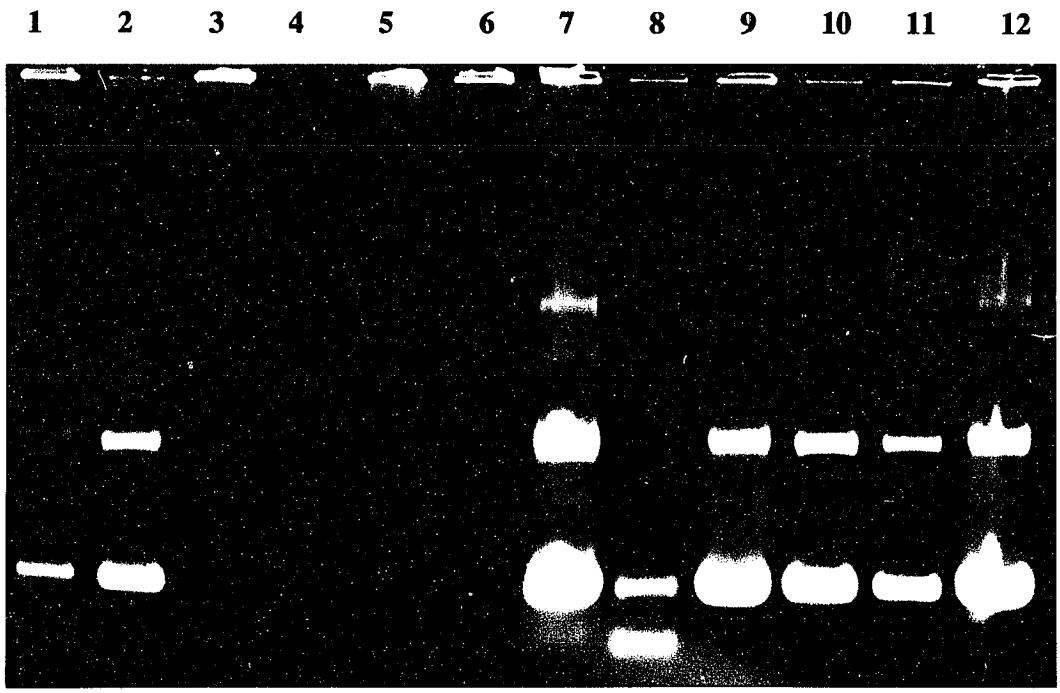


Figure 8. Initial screening of the recombinant plasmids.
The recombinant clones were analyzed for their plasmid content. Lane 1: pLP 20. Lane 2: pLP 21. Lane 3: pLP 43. Lane 4: pLP 46. Lane 5: pLP 62. Lane 6: pLP 93. Lane 7: pLP 112. Lane 8: pUC 19. Lane 9: pLP 116. Lane 10: pLP 117. Lane 11: pLP 119. Lane 12: pLP 121. Lanes 3-6 were clones that later could not be grown on nutrient agar in the presence of 50 μg/ml ampicillin.

Figure 9. Colony transfer (A) and dot-blot assay (B).
Recombinant clones were grown on nutrient agar plates containing 50 μg/ml ampicillin in rows of ten across and 5 down. After overnight growth at 37°C, nitrocellulose was placed directly onto the plate. Colonies were lifted off the nutrient agar plate by removing the nitrocellulose and the sheets were baked in a vacuum oven at 60°C for 2 h. The cells were not lysed prior to baking. To insure that no lysis occurred during the baking process, colonies were retested using formalin-killed cells in 2 μl drops directly onto nitrocellulose. Sheets were air dried prior to antibody treatments. Formalin-killed *L. pneumophila* N_7 (+) and *E. coli* JM 83 (-) cells were used as the controls.

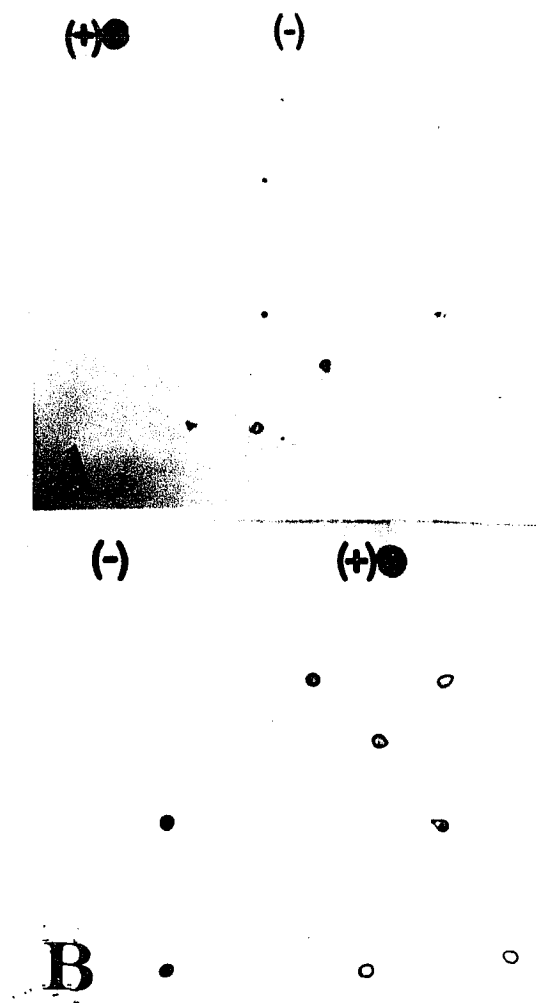


Figure 9. Detection of surface-expressed antigens of *L. pneumophila* in *E. coli*.

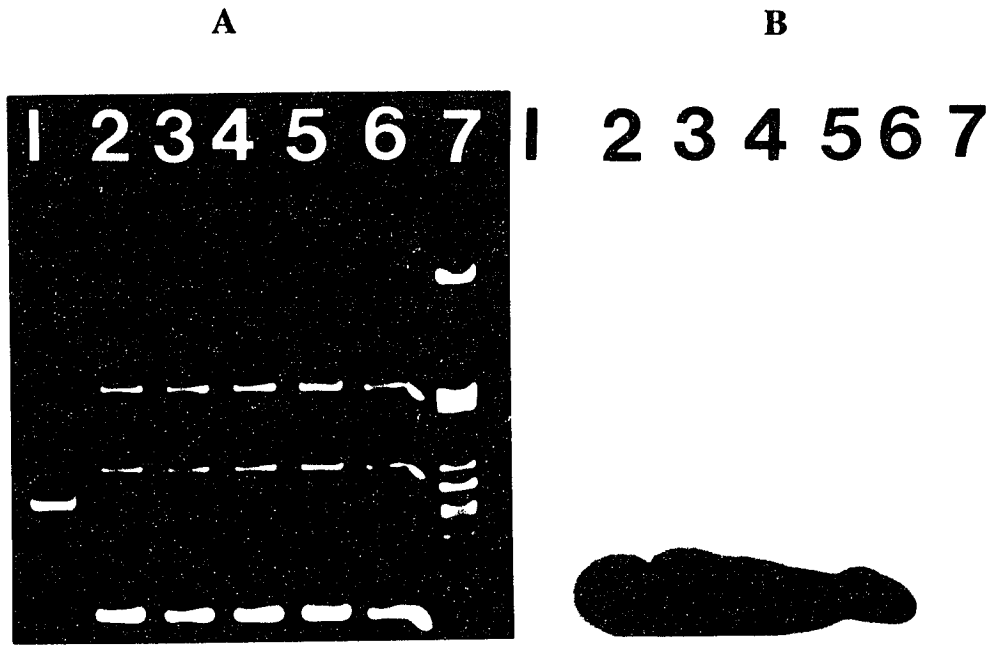


Figure 10. Restriction analysis and Southern hybridization.
Recombinant plasmids were digested with *Sma* I and in this instance, fragments were released. The agarose gel was transferred to nitrocellulose and the filter was probed with ^{32}P-labeled *L. pneumophila* N_7 genomic DNA. Picture A. Lane 1: pUC 19. Lane 2: pLP 112. Lane 3: pLP 116. Lane 4: pLP 117. Lane 5: pLP 119. Lane 6: pLP 121. Lane 7: phage lambda DNA digested with *Pst* I. Picture B. The Southern blot corresponds to the legend for Picture A.

release the fragment from the pLP 116 plasmid. A fragment approximately the same size as the original *Sma* I digests was released. This fragment, after being eluted from the agarose gel, was ligated with a *Pst* I digest of pUC 19 and transformed into *E. coli* JM 83. The recloned transformants continued to release a 750 bp fragment and Southern blotting showed that the fragment contained *L. pneumophila* N_7 DNA sequences in common (Fig. 11). These "reclones" continued to express *L. pneumophila* antigens without lysis in colony transfers and dot blot assays.

2.4 SDS-PAGE of the eight recombinants:

The outer membrane proteins of the eight *E. coli* clones were isolated and subjected to polyacrylamide gel electrophoresis to detect changes that may have occurred in the protein profile. A band of approximately 25 kDa was present in the OMP of the eight clones but not in the *E. coli* JM 83 parent strain. A band of the same molecular weight appeared in the *L. pneumophila* N_7 OMP profile (Fig. 12).

2.5 Oxidation of polyacrylamide gels:

Periodic acid treatment of polyacrylamide gels followed by silver staining showed dark lipopolysaccharide (LPS) banding patterns but did not stain the 25 kDa region for *L. pneumophila* (Fig. 13a). The clone LP 116 but not the *E. coli* JM 83 parent failed to stain in the same region. There were no apparent changes in the LPS pattern of the clone when compared to the parent *E. coli* strain.

2.6 Migration patterns of *E. coli* LP 116 under non-reduced conditions:

A composite photograph showing the electrophoresis of OMP samples of the *L. pneumophila* N_7 strain, the *E. coli* parent strain and the clone LP 116 prepared in non-reducing buffers showed that the 25 kDa protein did not enter the gel (Fig. 13b). The OMP profile of the *E. coli* parent strain under non-reduced conditions when compared to the clone LP 116 appeared to be similar in its migration pattern but was destroyed by addition

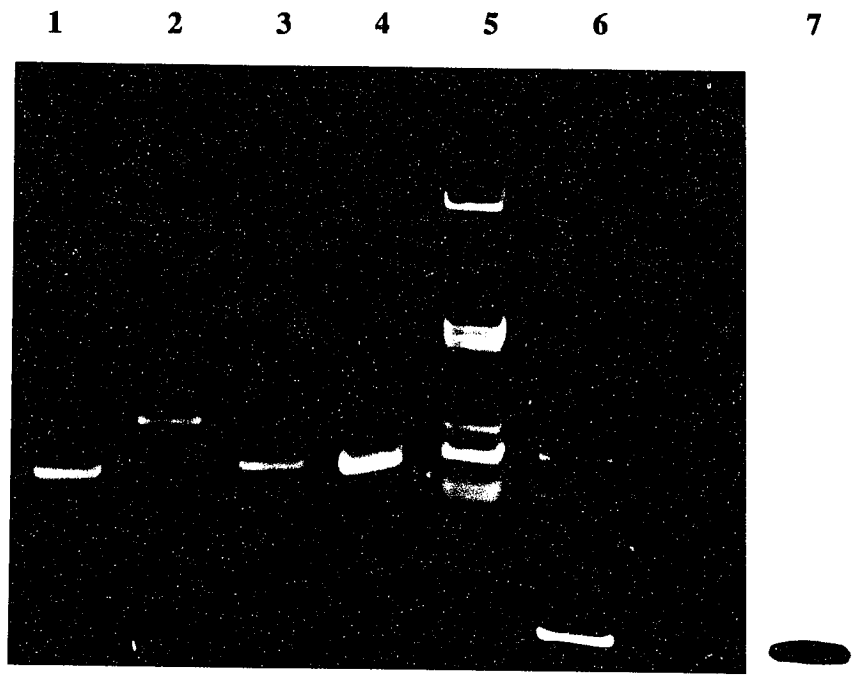


Figure 11. Recloning using *Pst* I digest.
In order to obtain fragments upon each digestion, the fragment was recloned using a *Pst* I digest. Lane 1: pUC 19. Lane 2: pLP 116. Both lanes 1 and 2 were digested with *Sma* I. Lane 3: pUC 19. Lane 4: pLP 116. Lane 5: phage lambda DNA standard. Lane 6: recloned pLP 116. Lanes 3-6 were digested with *Pst* I. Lane 7: Southern blot of the recloned pLP 116.

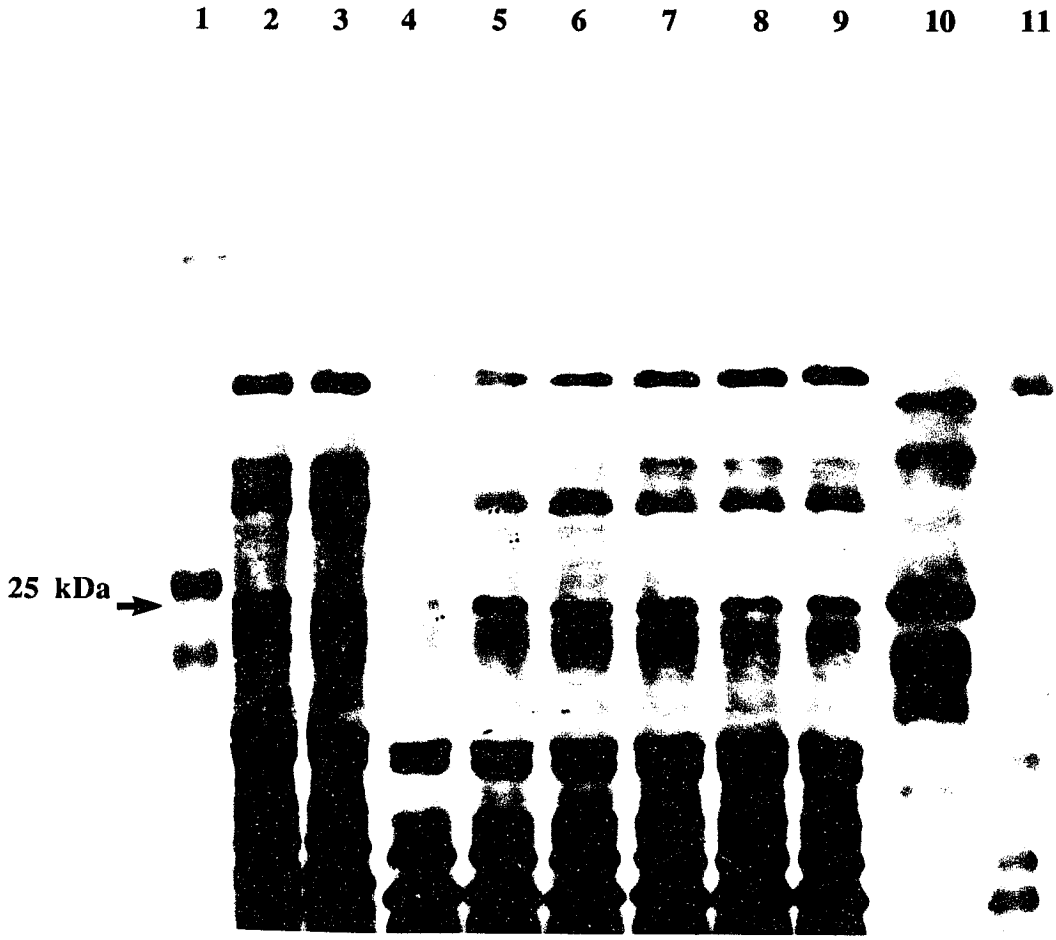
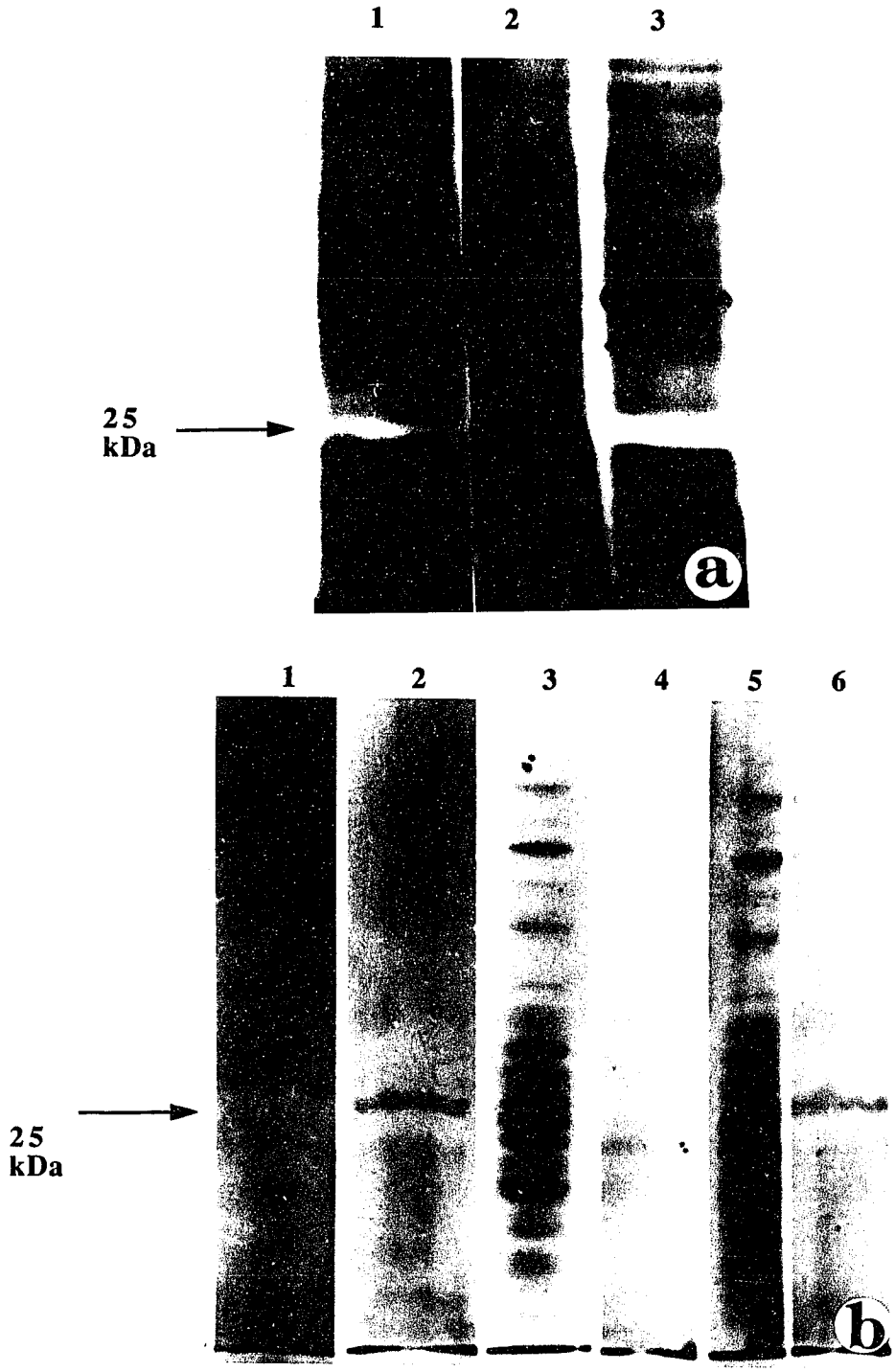


Figure 12. Outer membrane protein profiles of the recombinants. Eight recombinants expressed *L. pneumophila* antigens on their surface without lysis. To determine whether this protein was a surface protein of *L. pneumophila*, outer membrane proteins were isolated and subjected to SDS-PAGE. Lane 1: Molecular weight standard. Lane 2: *E. coli* LP 20. Lane 3: *E. coli* LP 21. Lane 4: *E. coli* LP 112. Lane 5: *E. coli* LP 116. Lane 6: *E. coli* LP 117. Lane 7: *E. coli* LP 119. Lane 8: *E. coli* LP 120. Lane 9: *E. coli* LP 121. Lane 10: *L. pneumophila* N_7. Lane 11: *E. coli* JM 83. Note that the band at 25 kDa in the clones was not present in the parent strain but was comparable to the band in *L. pneumophila* N_7 OMP profile.

Figure 13a. Periodic acid treatment of polyacrylamide gels.
Polyacrylamide gels containing OMP were oxidized to determine if the 25 kDa band could be related to changes in the LPS of any of the recombinants. Lane 1: *E. coli* LP 116. Lane 2: *E. coli* JM 83 with pUC 19. Lane 3: *L. pneumophila* N_7. There was a failure to stain in the 25 kDa region in the clones as shown by sample LP 116. This was a similar phenomenon found in *L. pneumophila* N_7 and reported in other *L. pneumophila* strains containing an MOMP.

Figure 13b. Migration patterns with non-reduced/reduced buffers.
Recombinant LP 116 migration patterns of the OMP under reduced and non-reduced conditions. Lane 1: *L. pneumophila* N_7, non-reduced. Lane 2: *L. pneumophila* N_7, reduced. Lane 3: *E. coli* JM 83 with pUC 19, non-reduced. Lane 4: *E.coli* JM 83, reduced. Lane 5: *E. coli* LP 116, non-reduced. Lane 6: *E. coli* LP 116, reduced. The 25 kDa OMP did not appear in the gel under non-reduced conditions as shown in the *L. pneumophila* sample in Lane 1 and the *E. coli* LP 116 sample in Lane 5. The same OMP preparations with addition of the reducing agent, 2-mercaptoethanol, allowed the 25 kDa OMP to enter the gel in Lane 2 and Lane 6.



Figures 13a and b. Testing for the *L. pneumophila* MOMP

of 2-mercaptoethanol and subsequent boiling (Fig. 13b, Lane 4). Under reducing conditions the 25 kDa protein, which was somewhat solubilized by these rigorous treatments, was detected in both the *L. pneumophila* and the *E. coli* clone OMP profiles.

2.7 Direct immunofluorescence with MOMP-specific monoclonal antisera:

After treatment with the commercially prepared DFA kit, the LP 116 recombinant was positive for the MOMP of *L. pneumophila*. The quality of fluorescence of the *L. pneumophila* N_7 strain and that of the clone were similar to the control supplied with the kit. The *E. coli* JM 83 parent strain did not fluorescence (Fig. 14).

2.8 Sequencing of the 25 kDa MOMP DNA:

The sequence data obtained from the dideoxy-chain termination experiments using pLP 116 are shown in Fig. 15. The actual size of the *L. pneumophila* DNA fragment was 810 base pairs inclusive of a 682 base pair open reading frame.

2.9 Comparison of the OMP profiles and immunoblots of the recombinant, *E. coli* parent, *L. pneumophila*-DNA contributing isolate and the attenuated *L. pneumophila* derivative:

The 25 kDa band showed cross reactivity in immunoblot procedures using *L. pneumophila*-specific polyclonal antisera and MOMP-specific monoclonal antibody (Fig.16). The attenuated *L. pneumophila* N_7 derivative contained a 25 kDa protein; however, it appeared to be produced in smaller amounts. There was a 31 kDa protein found in the attenuated derivative that was not present in the original *L. pneumophila* N_7 isolate (Fig.16, Lane 3). Neither the 31 kDa band nor the 25 kDa band was apparent in the polyclonal immunoblot (Fig. 16, Lane 8) but the 25 kDa region strongly crossreacted in the monoclonal immunoblot. The 25 kDa MOMP in the virulent *L. pneumophila* N_7 isolate could be observed among the LPS ladder of the polyclonal immunoblot (Fig. 16, Lane 7). The *E. coli* LP 116 clone only crossreacted in the 25 kDa region in both the polyclonal and

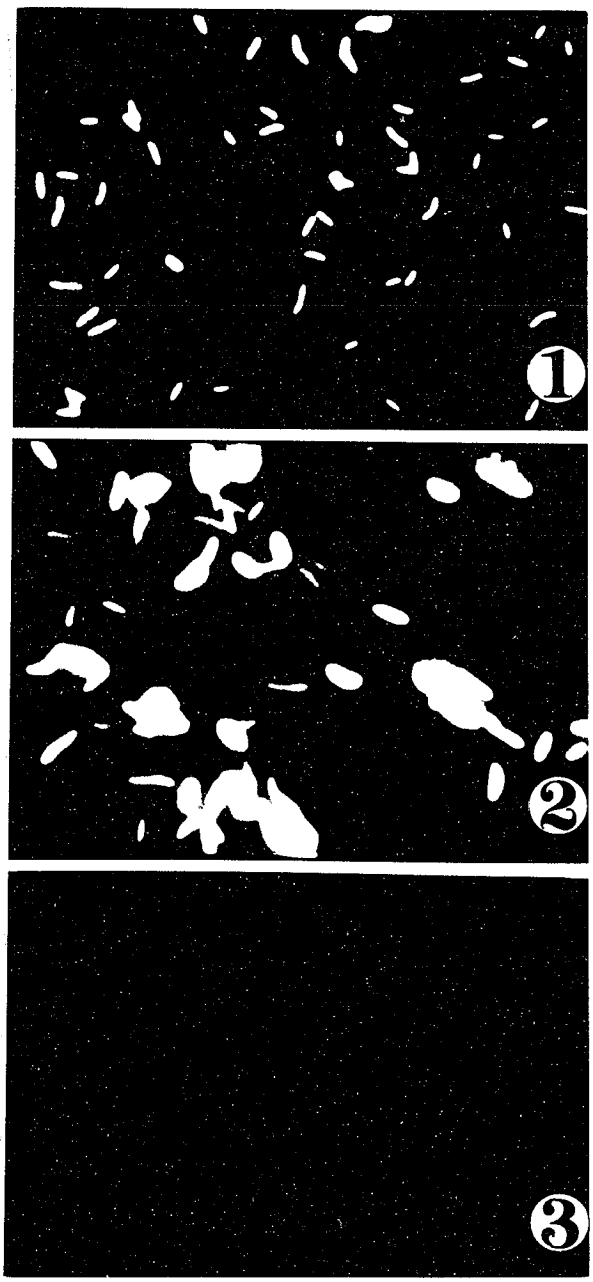


Figure 14. Immunofluorescence using a commercially prepared MOMP-specific antibody. The antibody was raised to a common outer membrane protein, the MOMP of *L. pneumophila*. Picture 1: *L. pneumophila* N_7. Picture 2: *E. coli* LP 116. Picture 3: *E. coli* JM 83. The clone LP 116 showed fluorescence comparable to *L. pneumophila* N_7 while the *E. coli* JM 83 parent strain was negative.

GTGATCGAG**TTGACACGGACTTTAAGTCACACACTA**ACCCTACGAAGCAGTTA

CCCCTACGGGCGAGATCAAGGAGACTCTACCC*ATGCTTAAGTCTCGTTTACTCT*
met leu lys ser arg leu leu

*ACGCAACGCTAGCAGCAGTGGCAATCGTAGCAACTTCCGAGACG*TGTGATCGAG
tyr ala thr leu ala ala val ala ile val ala thr ser ^ glu thr ^ cys asp arg

ACTCGAGCCATTGTACGAAATCGATACGATTGATCGCTCAGTACAGTCCTTGGAT
asp ser ser his cys thr lys ser ile arg leu ile ala gln tyr ser pro trp met

GGCAGTCTCAAAGCTACTGAATAACGTGACAATGTCATGTCTCTCTCTAGATCATA
ala val ser lys leu leu asn asn val thr met ser cys leu ser leu asp his

CTGTACCTATTAGATTTATGCTACAGTGTCGAGTCGTTCGTCGTCAGTCAGTGAC
thr val pro ile arg phe met leu gln cys arg val val arg arg gln ser val thr

TATTGAGAGATTGACGTCAGCTGTTGAGTGCCTACTCGAGTGGCTAGATTCGGA
ile glu arg leu thr ser ala val glu cys leu leu glu trp leu asp ser asp

TGTGTCCTGTAGACCTAAACCCACCTTGGGGGCAGCGGGGGGATACGAGGAGG
val ser cys arg pro lys pro thr leu gly ala ala gly gly tyr glu glu

GGTATTCAGGGGGGTTTGCACGTGTTAAACTACTGCTACCCTTCAGTGGGGTTC
gly tyr ser gly gly phe ala arg val lys leu leu leu pro phe ser gly val

GGTATCCTGCAGCAGTCGTACGGGAGTACGCCGATGGTGCTACAGATCTCAGGA
arg tyr pro ala ala val val arg glu tyr ala asp gly ala thr asp leu arg

TCTGTCACGAGACTCTAGGTCTCCACGCGACCGCAGACCACGTTGACAACTATCA
ile cys his glu thr leu gly leu his ala thr ala asp his val asp asn tyr gln

AAGGGACGTAGGGGTCGCATGGGTAGGTATCGGGGTATGCGAGTATCGGGGTG
arg asp val gly val ala trp val gly ile gly val cys glu tyr arg gly

TGTTTCTAGGGGTCTTGCTTATAGCAAGACTTCGCTCACTGCTATGCGTACTCAAC
val phe leu gly val leu leu ile ala arg leu arg ser leu leu cys val leu asn

GACGCACGAGGCCACCGTTACATTGCTCTAAAGGATGCGTCCAAAAGGGAGAAA
asp ala arg gly his arg tyr ile ala leu lys asp ala ser lys arg glu lys

GGATAAGCATGCGTCAAAGGTAGGTGGTGCAACATTTGTCGGTCACTTAG
gly End***

Figure 15. The 25 kDa MOMP DNA sequence.

The -35 and -10 promoter sequences are in bold type. The Shine-Delgarno sequences are underlined. The open reading frame begins with the initating methionine of the deduced amino acids. The region of inferred signal peptide is in italics and potential cleavage sites are noted by arrowheads. This nucleotide seqence appears in GenBank under the accession number LO5595.

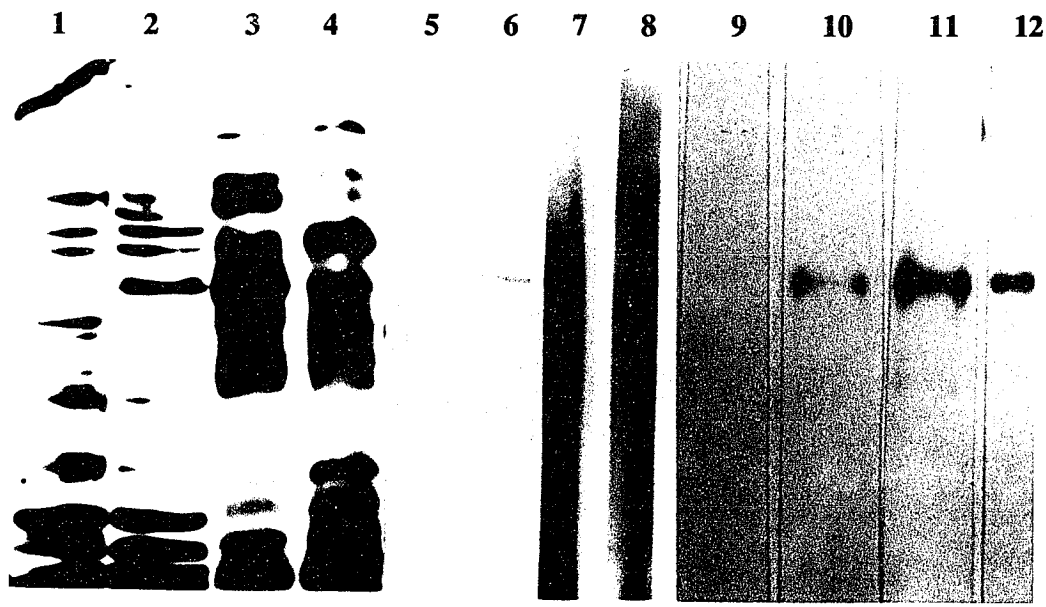


Figure 16. SDS-PAGE and Immunoblots using *L. pneumophila* polyclonal and monoclonal anti-sera. SDS-Page gels were transferred to nitrocellulose filters. Immunoblots were performed using *L. pneumophila* polyclonal rabbit antisera and the MOMP-specific monoclonal antisera. Lane 1: *E. coli* JM 83, Lane 2: *E. coli* LP 116. Lane 3: *L. pneumophila* N_7, virulent. Lane 4: *L. pneumophila* N_7, attenuated. Lanes 5 through 8 and 9-12 correspond to the legend of Lane 1 through 4. Note the additional band at approximately 31 kDa in the attenuated sample (Lane 4) not present in the virulent isolate (Lane 3). Lane 6 and lane 10 showed cross reaction of the *E. coli* LP 116 OMP with the *L. pneumophila* poly clonal antibody and the MOMP-specific monoclonal antibody, respectively.

monoclonal immunoblots whereas the *E. coli* JM 83 parent strain was negative (Fig. 16, Lane 5 and 9).

2.10 Fertile hens' egg assay:

LD_{50} data derived from the chicken embryo lethality assay showed that the *E. coli* JM 83 parent strain was of low virulence while that of the derived clones was much enhanced. The results of the assay are shown in Table 10.

2.11 Adherence of *E. coli* LP 116:

L. pneumophila possessed the ability to adhere to U937 cells (Fig. 17). *E. coli* LP 116 and *E. coli* JM 83 were evaluated for adherence in immunofluorescence studies (IF) and viable bacterial colony counts (VBCC). The data are shown in Table 11.

3. MOMP blocking studies:

Embryonated chicken eggs injected with 10-fold dilutions of *L. pneumophila* N_7 and the *E. coli* LP 116 clone treated with a 1/500 dilution of the MOMP-specific monoclonal antibody showed no deaths up to 8 days post-inoculation (Table 10). *L. pneumophila* N_7 and *E. coli* LP 116 coated with MOMP-specific monoclonal antibody at the 1/500 dilution, when assayed for binding properties with U937 cells, were found to be non-adherent (Table11).

4. Comparative study of Electroporated *L. pneumophila*:

4.1 Detection of the *L. pneumophila* transformants:

As a result of transformation, after 10 days on the BCYE medium containing 50 μg/ml of ampicillin, pinpoint colonies appeared. The control samples did not show signs of growth. The frequency of transformation was 10^{-6} as determined by the number of viable colonies derived from platings of 10^8 cfu/ml. Initial screening of the colonies in each trial showed that pLP 116 and pUC 19 remained autonomous in the cell (Fig. 18, Lanes 3 and 4). Restriction of the plasmids demonstrated that the recombinant plasmid still

Table 10 Lethal dose $_{50}$ values for *E. coli* JM 83, *L. pneumophila* N_7 and the *E. coli* recombinants inoculated into chicken embryo yolk sac

Strains and designations	LD_{50} Endpoints*
E. coli JM 83 with pUC 19	1.7×10^9
†*E. coli* JM 83 with pUC 19 antibody-treated	3.3×10^9
L. pneumophila N_7	4.6×10^2
†*L. pneumophila* N_7 antibody-treated	No death
E. coli LP 20	2.9×10^5
E. coli LP 21	3.3×10^6
E. coli LP 112	3.0×10^3
E. coli* LP 116*	**2.5×10^4**
†***E. coli* LP 116** antibody-treated	No death
E. coli LP 117	9.4×10^4
E. coli LP 119	8.9×10^5
E. coli LP 120	7.0×10^4
E. coli LP 121	4.5×10^4

Plasmid-bearing cells were recovered up to 72 h post inoculation.
*Ten eggs were inoculated per dilution with 0.1 ml samples and LD_{50} data were calculated from three separate experiments. Control eggs were injected with PBS and in this group no deaths occurred up to eight days post inoculation.
**Recombinant used in this study.
† Blocking study. Organisms were treated with *L. pneumophila* MOMP-specfic monoclonal antibody.

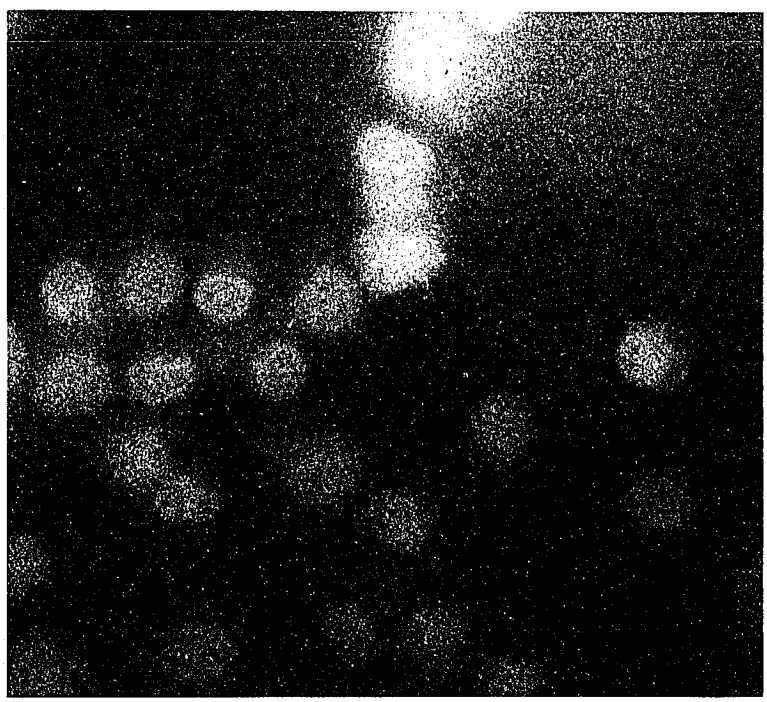
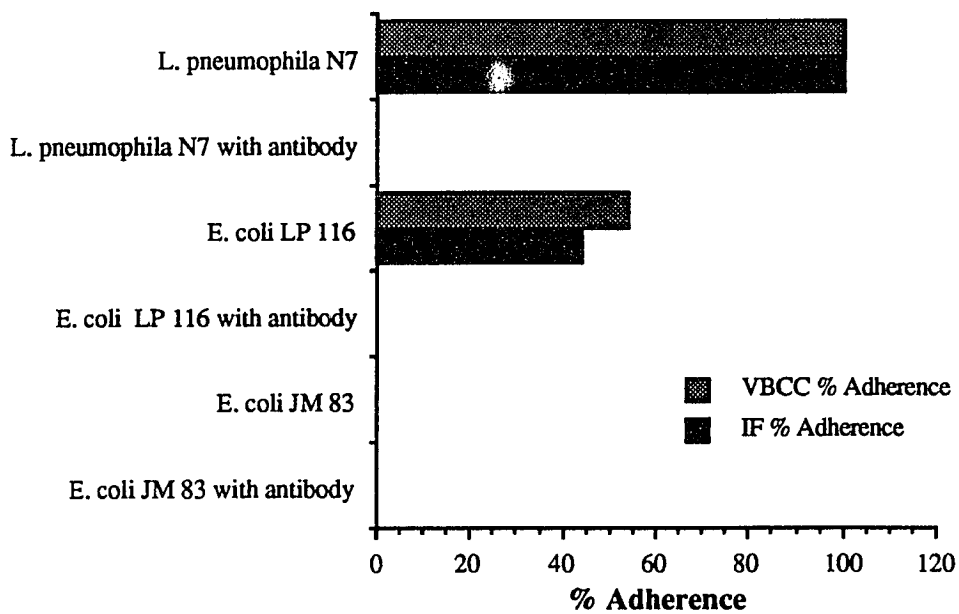


Figure 17. U937 cell culture.
L. pneumophila organisms (green or grey) attached to the U937 cells (red or white). These cells do not form monolayers but can adhere to glass or plastic after treatment with PMA. Cytochalasin D was used to inhibit uptake of the organisms. Reproduced with permission from F. G. Rodgers.

Table 11 % Adherence for *E. coli* JM 83 and *E. coli* LP 116



Data was calculated by the number of organisms attached to 200 U937 cells and accessed by immunofluorescence (IF) and viable bacterial colony counts (VBCC). As noted between washings, not all organisms fluoresce with *E. coli* clone LP 116 due to production of the pUC plasmid without inserts. For VBCC, cells were lysed by addition of sterile deionized water to each of the wells. Nutrient agar containing 50 μg/ml ampicillin were seeded with 0.1 ml of sample per dilution of *E. coli*. *L. pneumophila* organisms were treated similarly but inoculated onto BCYE agar. The organisms were coated with a 1/500 dilution of the MOMP-specific monoclonal antibody in the blocking study.

contained the fragment (Fig. 18, Lane 3). The pLP 116 and pUC 19 plasmids were cured spontaneously with a frequency of 96% when grown on BCYE-α agar without ampicillin present. This was determined by the number of colonies that survived transfer via replica plating onto BCYE-α with ampicillin (on the average of approximately 5/125).

4.2 Examination of the OMP profiles:

SDS-PAGE analysis showed a decrease in the amount of the 25 kDa protein present in the *L. pneumophila* N_7 attenuated strain and the presence of a 31 kDa protein not found in the virulent isolate. The transformant containing the plasmid pLP 116 with the *L. pneumophila* DNA insert exhibited an increase in the 25 kDa protein. The 31 kDa protein which appears only in the attenuated strain of *L. pneumophila* could not be detected (Fig. 19, Lane 5). In the absence of the plasmid, pLP 116 in the cells following growth on non-ampicillin media the concentration of the 25 kDa protein decreased while the 31 kDa protein returned to the profiles (Fig 19, Lane 6). Omp preparations of the attenuated sample electroporated with pUC 19 only, remained unchanged. There was also no apparent change in the OMP profiles of the virulent and attenuated strains subjected to high-voltage with no DNA present.

4.3 Fertile hens' egg assay:

LD_{50} values for the virulent, attenuated and electroporated transformants are listed in Table 12. There were marked decreases in the LD_{50} of the transformant containing the pLP 116 plasmid indicating an increase in virulence for the chicken embryo while the LD_{50} of the cured sample experienced an increase. LD_{50} values for the pUC 19-containing transformant showed no apparent changes. The *L. pneumophila* samples, whether virulent or attenuated and electroporated without DNA present significant changes in the LD_{50} values.

4.4 Adherence studies:

Organisms of *L. pneumophila* were all adherent when assayed in U937 cells. The attenuated isolate and its virulent counterpart are shown in IF and VBCC data in Tables 13 and 14. All samples tested did not reflect any appreciative differences in the ability to adhere in this assay.

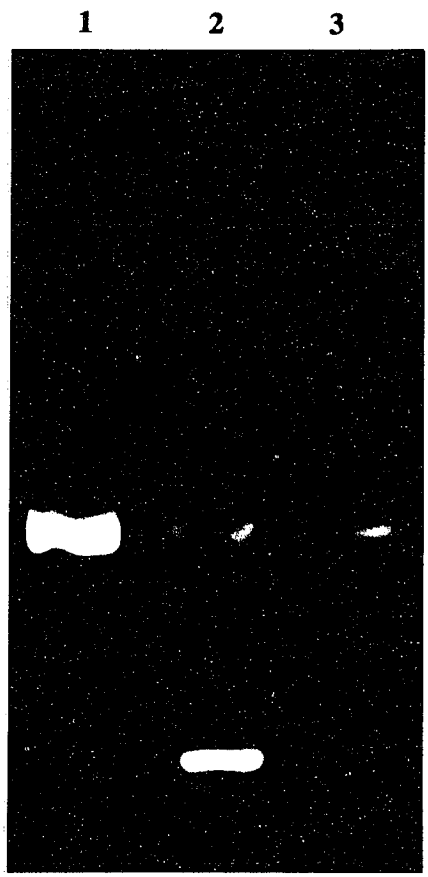


Figure 18 Attenuated *L. pneumophila* N_7 electroporated with pUC 19 and pLP 116. Electroporated transformants grown on BCYE-α containing 50 μg/ml of ampicillin were screened by gel electrophoresis. Lane 1: pUC 19 only. Lane 2: pLP 116 *L. pnuemophila* transformant. Lane 3: pUC 19 *L. pneumophila* transformant. Both Lanes 3 and 4 were digested with *Pst* I.

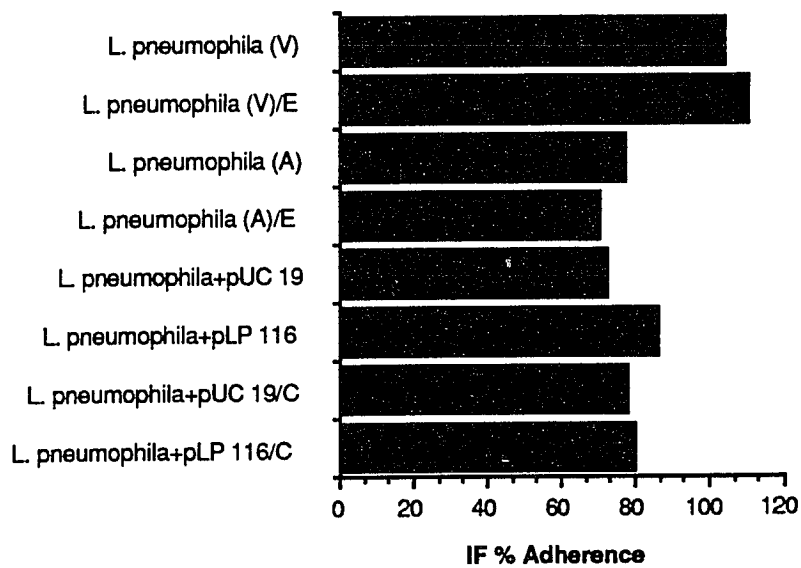
Figure 19. Outer membrane protein profiles of electroporated, attenuated *L. pnemophila.* The OMP of the attenuated, virulent and transformed *L. pneumophila* samples were compared by SDS-PAGE. Lane 1: Molecular weight standard. Lane 2: attenuated *L. pneumophila* N_7. Lane 3: virulent *L. pneumophila* N_7. Lane 4: electroporated attenuated *L. pneumophila* N_7 with pUC 19 only. Lane 5: electroporated *L. pneumophila* N_7 with pLP 116. Lane 6: cured electroporated *L. pneumophila* N_7 pLP 116 transformant.

Table 12 Lethal dose $_{50}$ values for the *L. pneumophila* electroporated transformants inoculated into chicken embryo yolk sac

Strains and designations	LD_{50} Endpoint*
L. pneumophila N_7 (virulent)	4.6×10^2
L. pneuomphila (attenuated)	1.1×10^6
L. pneumophila (pUC 19)	5.2×10^6
L. pneumophila (LP 116)	9.2×10^2
L. pnemophila (pUC 19, (cured)	9.3×10^6
L. pneumophila (LP 116, (cured)	8.1×10^4
L. pneuomphila N_7 (virulent, no insert present)	3.3×10^2
L.pneumophila (attenuated, no insert present)	3.0×10^6

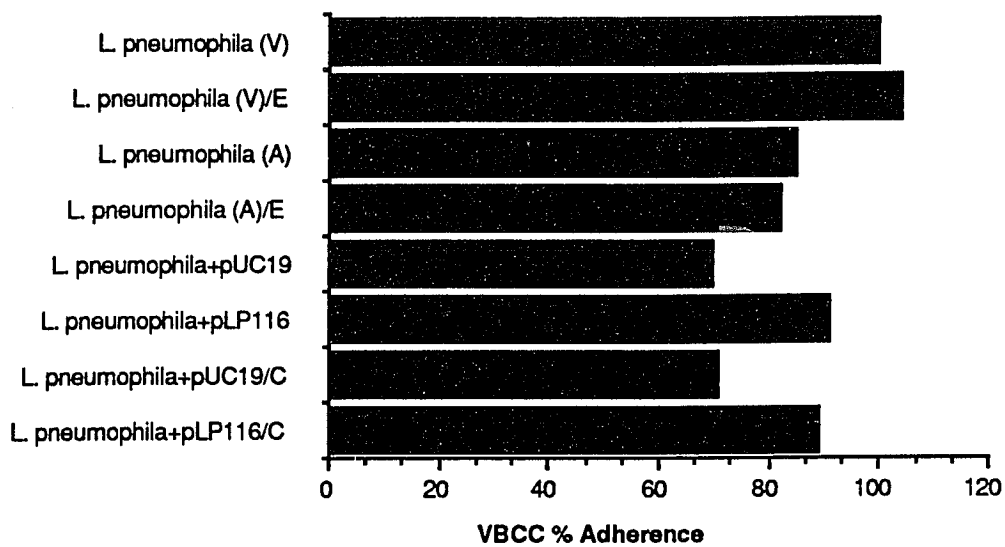
*Ten eggs were inoculated per dilution with 0.1 ml samples and LD_{50} data were calculated from three separate experiments. Control eggs were injected with PBS and in this group no deaths occured up to eight days post inoculation.

Table 13 Immunofluorescence (IF) % adherence for the electrotransformants



Data was calculated by the number of organisms attached to 200 U937 cells.
V=virulent, E=electroporation, A=attenuated and C=cured.

Table 14 Viable Bacterial Colony Counts (VBCC) % adherence for the electrotransformants



U937 cells were lysed by addition of sterile deionized water to each of the wells. BCYE-α agar plates containing 50 μg/ml ampicillin were inoculated with 0.1 ml of sample per dilution.
V=virulent, E=electroporated, A=attenuated and C=cured.

DISCUSSION

1. Plasmids and the pathogenicity of *Legionella*:

The possibility that plasmids contribute to the pathogenicity of *Legionella* was investigated for 32 species and strains. Of these, six isolates possessed plasmids. *L. pneumophila*, strain Heysham 2 and *L. hackeliae*, strain Lansing 2 possessed a common 80 mDa plasmid solely while *L. dumoffii*, strain Tex-KL and *L. bozemanii*, strain WIGA had the 80 mDa plasmid as well as other plasmids of lower molecular mass. The 80 mDa plasmid could not be collected from any of these six isolates using the techniques devised for plasmid recovery. The large size of the 80 mDa plasmid coupled with low copy number was more than likely responsible for the failure to retrieve plasmid. All of these procedures are usually employed with multicopy-number plasmids that are considerably smaller than 80 mDa. No literature could be found using these recovery methods with plasmids as large as 80 mDa. It would have been possible to collect the single 80 mDa plasmid of the *L. hackeliae* species or the Heysham 2 strain of *L. pneumophila* using plasmid purification techniques that eliminate the chromosomal debris. These plasmids could have been digested using a restriction endonuclease and the resultant fragments transformed into a host/vector system such as pUC 19 and *E. coli* JM 83 or into *L. pneumophila* but there were no obvious markers to identify the plasmid fragments. In order to obtain direct evidence that the plasmid influenced the pathogenicity of *Legionella*, LD_{50} studies were performed to see if the presence or absence of the plasmids affected virulence. Trials using the chicken embryonated egg lethality assay (Rodgers, 1973; Tzianabos and Rodgers, 1987) showed that the virulence of the organism did not appear to be related to plasmid content. The results of these trials, albeit using small numbers of

plasmid-bearing strains corresponded with the results of several research groups (Knudson and Mikesell, 1980; Garrity, *et al.*, 1982; Brown, *et al.*, 1982; Nolte, *et al.*, 1984). It became apparent that pathogenic aspects of *L. pneumophila* were not correlated with the presence of plasmids.

2. **The *L. pneumophila* genomic library**:

Successful cloning of *Legionella* proteins in *E. coli* (Engleberg, *et al.*, 1984) prompted the present study on surface-expressed antigens of *L. pneumophila*. Since the surface of the *L. pneumophila* bacterium initially comes into contact with the host cell prior to phagocytosis, the components of the outer membrane may be involved with the adherent properties of the organism. Because of the difficulties encountered when attempting to physically and chemically separate the outer surface of *L pneumophila* (Ehret, *et al.*, 1984; Butler, *et al.*, 1985; Gabay, *et al.*, 1986; Hindahl and Iglewski, 1986) it was necessary to use recombinant DNA techniques to facilitate this study.

The electrophoretic profiles of *L. pneumophila* genomic DNA demonstrated that *Hae* III yielded the smallest base pair fragments, so these were used in the ligation procedure with pUC 19. The gene library generated from the *Hae* III digestions of *L. pneumophila* genomic DNA resulted in eight *E. coli* clones that were able to express *L. pneumophila* proteins on their surface as shown by colony transfers and the dot blot assay using *L. pneumophila* polyclonal rabbit antisera (Rodgers and High, 1991). These clones were also positive for *L. pneumophila* antigens in indirect immunofluorescence trials. The *Hae III* digestions of *L. pneumophila* DNA did not yield many surviving clones and the low frequency of survival of these clones may have resulted from exceeding several limitations of the host/vector system. The size of the fragments in the "shot-gun" cloning strategy may have contributed to the initial loss of the clones which failed to survive either transfer from VRB media to broth containing 50 μg/ml ampicillin or storage at -20°C in the presence of the antibiotic. A consideration in choosing the pUC 19 plasmid was that it

could accept fragments of 10 kilobase pairs or greater. Failure to transfer many of the colonies may have been due to inserts that exceeded this limitation. However, size alone may not explain all the losses. Further investigation of the *L. pneumophila* surface antigen-expressing clones indicated that alternatively the failures may have been the result of the difficulties that the *E. coli* host underwent in order to survive the material encoded for by the insert. The DNA fragment contained in the eight clones did not exceed the size limitations of the vector and although these clones appeared similar, differences were noted in the stability of the vectors by variations in their ability to grow on media containing ampicillin. These differences were also reflected in LD_{50} studies in that lethality for the chicken embryo varied. This may have been the result of the organism attempting to eliminate the plasmid and suggested an apparent intolerance by metabolism of the *E. coli* strain to the *L. pneumophila* protein. This has been found in other studies where *L. pneumophila* OMP sequences have been cloned in *E. coli* (Hoffman, *et al.*, 1991). A 900 base pair fragment containing the sequences encoding for the 28 kDa OMP of the *L. pneumophila* Philadelphia 1 strain appears to be lethal to the host organism. In the present study, fewer organisims were required to kill the host for all the clones when compared to the parent *E. coli* JM 83; however, the clone, LP 116 was used for subsequent studies because of its continued stability when grown on ampicillin-containing media.

Restriction analysis of the eight clones in 3 separate trials revealed the presence of a band averaging 750 base pairs when measured against the *Pst* I digested lambda standard. It was not possible to determine from restriction studies alone whether this 750 base pair piece of DNA which was released after digestion with *Sma* I was the entire fragment originally ligated to the vector. Since there was a *Hae* III-digested *L. pneumophila* DNA fragment, the blunt-end ligation with *Sma* I should have caused a loss of the original restriction site. Nevertheless, the recombinant plasmids still continued to release a fragment. This was caused by a rearrangement of the palindrome that occurred during

ligation as later revealed in the sequencing data. The ^{32}P-labeled *L. pneumophila* DNA probe derived from the genome of *L. pneumophila* N_7 was used in Southern hybridization and showed that the fragments were *L. pneumophila* DNA. A *Pst* I digest of the pLP 116 plasmid was used to release the fragment which was the same size as the fragments from the *Sma* I digests. This fragment after being eluted from the agarose gel was ligated into pUC 19 and transformed into *E. coli* JM 83. The recloned transformants continued to express an *L. pneumophila* antigen on their surface as shown by the colony transfer immunoassay. Attempts to subclone the fragment were unsuccessful in obtaining clones that had antigenic activity detectable on the bacterial surface, a necessary feature for the virulence assays.

PCR was employed to amplify regions of the pLP 116 plasmid in order to obtain sufficient amounts of DNA for the sequencing procedure as it was found that the clone LP 116 existed as a mixed colony. Sequencing data of the pLP 116 plasmid showed that the fragment was 810 base pairs in length and that the open reading frame was 682 base pairs to the stop codon. The protein was in possesion of its own *E. coli*-like promoter. There were 226 amino acids deduced from the DNA sequence of which the first 20-22 amino acids following the ribosomal binding site infered a signal sequence. The total molecular weight determined by the amino acid content was approximately 25, 110 or 25, 400 depending on the cleavage site of the signal peptide. It was also determined from the amino acid content that there were 9 cysteine residues present as predicted by the behavior of the protein in OMP preparations. Base pair and peptide comparisons showed that the protein had no homolgy with any other prokaryotic proteins previously reported, therefore, the sequence was registered with GenBank.

Although the protein expressed on the surface of *E. coli* was that derived from *L. pneumophila*, it was not clear that it was actually a protein normally located on the surface of *L. pneumophila*. Electrophoresis of outer membrane proteins revealed an additional

band of 25 kDa in the *E. coli* clones that was not present in the *E. coli* JM 83 parent. A band also appeared at the same location in the *L. pneumophila* OMP profile. Immunoblots of the OMP profiles of the eight *E. coli* clones using the *L. pneumophila* rabbit antisera demonstrated that there was no crossreactivity other than at the 25 kDa region in the clone LP 116 nor were there any reactions with the *E. coli* parent. That the protein expressed on the surface was the the same 25 kDa MOMP was demonstrated by using the MOMP-specific monoclonal antibody. To establish if the presence of this 25 kDa protein could influence virulence, LD_{50} data derived from the chicken embryo lethality assay showed that the *E. coli* JM 83 parent strain was of low virulence while that of the derived clone was much enhanced. Due to the complex nature of the infectious process in whole animals, data from the fertile chicken egg virulence assay must be interpreted with caution. Nonetheless, results suggested that the presence of the 25 kDa protein in the outer membrane of *L. pneumophila* was involved in pathogenic expression in *E. coli*. Comparative IF and VBCC studies using U937 cell culture to assay for adherence showed that the *E. coli* parent strain was a non-adherent organism and that the *E. coli* clone containing this 25 kDa protein possessed the ability to bind to the U937 cells. The evidence from blocking studies using the MOMP-specific monoclonal antibody-coated organisms showed that when the MOMP is not available, the organism loses its ability to infect chicken embryos and to bind to U937 cells. The recombinant clone resulting from this study was shown to have a surface-expressed *L. pneumophila* protein and furthermore, it was demonstrated that this was an influential factor in the pathogenicity of the organism, in particular, the ability to adhere to cells prior to infection.

3. ***L. pneumophila* "MOMP"** :

Although the molecular weight of this protein has been reported to range between 24 and 29 kDa, variations in reported molecular weight would be dependent on both the methods used to isolate the outer membrane proteins and on how much LPS and

peptidoglycan remained attached to the MOMP of the *L. pneumophila* outer membrane protein sample. Indeed, like the MOMP of *Neisseria gonorrhoeae* (Hitchcock, 1984), the MOMP of *L. pneumophila* is known to be tightly bound to LPS (Gabay *et al.*, 1985; Hindahl & Iglewski, 1986) and resists complete dissociation in SDS at 100°C. When attempting to make a determination for antigenicity and expression of virulence, complications occur if LPS contaminates the outer membrane preparations. This is exacerbated due to the difficulies in the separation of OMP components. For this reason recombinant DNA techniques were used to obtain surface antigens. The polyclonal immunoblot analysis of *L. pneumophila* demonstrated the typical laddering pattern from the smooth LPS that remains in the OMP preparations. The LPS of *L. pneumophila* and of other gram-negative bacteria has long been associated with antigenic activity and could be considered a potential virulence factor *per se*. The polyclonal rabbit antisera raised to the entire *L. pneumophila* organism was inclusive of LPS antibodies. In addition, the outer membrane preparations of *E. coli* JM 83 and the clone contained contaminating amounts of *E. coli* LPS; however, the polyclonal immunoblot revealed only one band that was located at the 25 kDa region of the gel. There was a lack of evidence of antibody binding other than this single band, therefore, it appeared that there was no antigenic relationship between the LPS of *L. pneumophila* and *E. coli*. Polyacrylamide gels of OMP samples of the clone LP 116, the *L. pneumophila* DNA-contributing strain and the *E. coli* parent strain showed dark LPS banding patterns in all three samples tested using periodic acid prior to silver staining. There was no apparent changes in LPS patterns of the *E. coli* clone when compared to the parent strain that may have contributed to the band at 25 kDa in the cross reaction of the *E. coli* clone in the immunoblots using polyclonal antisera. But failure to stain the 25 kDa region for *L. pneumophila* and the clone but not *E. coli* JM 83 indicated that this proteinaceous substance was in common with the MOMP complex of *L. pneumophila* (Hindahl and Iglewski, 1986). The 25 kDa protein did not enter the gel

during electrophoresis experiments using non-reducing buffers. This suggested that the protein formed an aggregate with apparent similarity to the 95 kDa protein reported by Butler *et al.*, (1985). Under non-reduced conditions the OMP profile of the *E. coli* parent strain appeared similar in its migration pattern to clone LP 116 but this similarity was destroyed by the addition of 2-mercaptoethanol and subsequent boiling. When reduced, the 25 kDa protein was detected in both the *E. coli* clone and the *L. pneumophila* OMP profiles which strongly suggested the presence of the MOMP of *L. pneumophila* (Ehret *et al.*, 1984; Butler *et al.*, 1985; Gabay *et al.*, 1985; Hindahl & Iglewski, 1986). This finding was confirmed by direct immunofluorescence using an *L. pneumophila* MOMP-specific monoclonal antibody as the probe. The quality of fluorescence of the *L. pneumophila* N_7 DNA-contributing strain and the clone LP 116 was comparable while the *E. coli* JM 83 parent strain did not fluorescence. Immunoblots showed that the OMP samples of *L. pneumophila* N_7, and the clone LP 116 had reacted specifically with this same monoclonal antiserum at the 25 kDa region. As this band reacted with the monoclonal antibody specific to the MOMP of *L. pneumophila* and did not stain with periodic acid, it appeared that the increased virulence of clone LP 116 was not related to the presence of LPS but to the MOMP of *L. pneumophila.*

The MOMP of *L. pneumophila* was reported to be a porin (Gabay *et al.*, 1985). The role of porin-like structures in the pathogenicity of *L. pneumophila* remains to be elucidated. Bacterial porins have been implicated in the pathogenicity of other gram-negative bacteria by acting as colicins, by influencing the uptake of molecules which may either benefit the organism or destroy the host cell, or by inhibiting phagocytosis (Misra & Benson, 1988; Smarda, 1988; Tufano *et al.*, 1988). Althought the composition of the MOMP has been found to differ from other porins (Hoffman, *et al.*, 1992a), it is possible that the virulence of *L. pneumophila* and the *E. coli* clone associated with the expression of the porin-like MOMP of *L. pneumophila* functions in a manner similar to other gram-

negative bacteria. However, there is no evidence at present that this 25 kDa MOMP of *L. pneumophila* functions as a porin in the clone.

4. Transformation of *Legionella pneumophila*:

Electroporation has been used for many years as a method of transfer of genetic material to eukaryotic cells (Fromm, *et al.*, 1982; Potter, *et al.*, 1984; Sowers, *et al.*, 1986; Chu, *et al.*, 1987). The theory behind electroporation is that under conditions of high electric charge, channels can be opened in membranes. The charge across the surface of the cell during these brief instances allow entry of foreign DNA. Given that the nucleases of the cell do not destroy the incoming DNA, it may become a functional part of the genetic material of the cell. Studies have shown that cells will open to accept DNA in this manner (Sowers, *et al.*, 1986) but the mechanism by which this process takes place is not fully understood. This technology has recently been applied to bacteria. Several bacteria such as *Pseudomonas, Campylobacter, Yersinia* and several *E. coli* K12 strains have been successfully electroporated with variable differences in transformation results depending on the buffers, voltage and time constants used as well as differences in the acceptibility of the genetic material among the species and strains tested (Dower, *et al.*,1988; Miller, *et al.*, 1988; Wirth, *et al.*, 1989). Successful electroporation of eukaryotic and prokaryotic cells may differ in concept. Linearized plasmids yield higher, stable transformants in eukaryotic cells (Chu, *et al.*, 1987) whereas in bacteria, the contrary has been found (Miller, *et al.*, 1988). But all studies agree that the voltage used to transform the cells must be high enough to generate a substantial kill curve to improve efficiencies of transformation and that the amount of DNA is inversely proportional to the efficiencies. Variation in the acceptance of DNA by this technique has also been shown within species and strains of the same genus (Miller, *et al.*, 1988; Wirth, *et al.*, 1989).

Introduction of genetic material into *Legionella* spp has proved difficult. However, studies have shown that *Legionella* can accept broad host range plasmids through conjugal

transfer. This DNA can either remain in the cytoplasm or in some instances be delivered totally or in part into the chromosome (Chen, *et al.*, 1984; Dreyfus and Iglewski, 1985; Keen, *et al.*, 1985; Mintz and Shuman, 1987). Successful triparental matings using plasmids constructed with pBR322 and *L. pneumophila* DNA (Engleberg, *et al.*, 1988) have revealed important genetic mechanisms which greatly affect the pathogenic behavior of members of the genus *Legionella* (Cianciotto, *et al.*, 1989b). But to date, no transformation of *Legionella* has been reported.

During this study it was discovered that the attenuated *L. pneumophila* N_7 derivative, when compared to its virulent counterpart, was in possession of a 31 kDa protein not previously reported in *L. pneumophila*. It was also observed that the 25 kDa protein was greatly diminished in the OMP profiles of the attenuated strain. The ability of the fragment to encode for the 25 kDa MOMP of *L. pneumophila* in *E. coli* was evident. Electroporation was employed to deliver the recombinant plasmid into the MOMP-deficient, attenuated *L. pneumophila* N_7 derivative to determine its influence in *L. pneumophila*. There was an increase in the 25 kDa protein present in the cell as a result of electroporation using pLP 116 and a concomitant elimination of the 31 kDa protein. These changes were not apparent in the OMP profile of the transformant containing pUC 19 alone. Curing of the *L. pneumophila* N_7 of the pLP 116 plasmid returned the OMP profile of the strain to its original state with the predominance of the 31 kDa protein. The OMP of the cured sample containing pUC 19 remained the same. In studies of OMP profiles of the electroporated strains of virulent and attenuated *L. pneumophila* N_7 in the absence of DNA there were no changes reflected in the banding patterns. LD_{50} studies demonstrated that the introduction of pLP 116 increased virulence of the attenuated strain but there were no appreciable changes in the transformant containing pUC 19 alone. In addition the attenuated samples that were electroporated without DNA present did not appear to be affected indicating that

the act of electroporation did not influence virulence. The cell culture adherence assays by IF or VBCC showed that the attenuated *L. pneumophila* N_7 strain did not bind to cells at the same level of adherence as the virulent isolate. However, since the MOMP had not been entirely eliminated from the attenuated strain, the adherence of the electroporated transformants to the host cells was not abolished but was less than fully virulent controls possessing the full complement of MOMP.

BCYE-α broth-grown *L. pneumophila* yielded no transformants due to insufficient numbers of cells. However; electroporation of the attenuated *L. pneumophila* N_7 using an adapted protocol with BCYE-α agar was successful in obtaining transformants containing the pUC 19 vector or the pLP 116 recombinant. Unlike organisms grown in broth culture even for a 24 h period, the use of overnight platings on BCYE-α agar resulted in sufficient numbers of bacteria to produce transformants for transfer to BCYE-α agar containing ampicillin. It was clear that the bacterial cell density was critical to the success of the electropration procedures. Electroporation did not yield transformants when less than 10^8 cfu/ml were present in the original suspension while those suspensions exceeding 10^9 cfu/ml also failed to give transformants because the additional cells in the suspension caused a decrease in the time constant. The length of time in which the cell is subjected to the charge was a critical factor in electroporation.

Selection after electroporation was made at 100 times the MIC normally tolerated by the original *L. pneumophila* N_7 strain. Ampicillin has been used as a selective marker in *L. pneumophila* conjugative experiments (Keen, *et al.*, 1985) while other workers have found the Tn3 transposon tended to be too cumbersome for selection in *Legionella* because it delayed the growth of this slow-growing organism. This was true in this study as well. It was likely that transformation frequencies for *L. pneumophila* N_7 may have been greater had lower concentrations of ampicillin been used. There was no growth on the media containing ampicillin until 10 days post inoculation. However, once the colonies were

established, the growth rate was comparable to the *L. pneumophila* derivative. Previous studies established MIC levels and test performance conditions for ampicillin and *L. pneumophila* (Elliott and Rodgers, 1985). Using data from those studies together with the present investigations, it was clear with the higher antibiotic dosage used that ampicillin resistance was due to the introduction of the plasmid rather than the possibility of creating ampicillin-resistant mutants.

The size of the plasmid appeared be a factor in these experiments because in earlier trials with RP1 (RP4), a broad host range plasmid, electroporation into *L. pneumophila* N_7 did not produce transformants. *L. pneumophila* Knoxville I strain, which has been previously reported to accept the conjugative RP1 or RP4 plasmid (Nolte, *et al.*, 1984, Keen, *et al.*, 1985) also failed to accept the plasmid under electroporation conditions. This plasmid of approximately 40-45 mDa, may have exceeded the size limit for this technique. The plasmid pBR322 was also not suitable for electroporation in this study although the pBR322 plasmid was not more than 1700 base pairs larger than pUC 19. Other successful, high-efficency studies have shown that it is possible to electroporate DNA up to 26.5 kilobases in size into bacterial cells but also showed differences in the acceptance of DNA from species to species (Wirth, *et al.*, 1989). The plasmids pUC 19 and pLP 116 were accepted and remained autonomous at a frequency of 10^{-6}. Therefore, it appeared that this procedure selected for a small number of restriction and/or modification mutants that may have existed in the population of cells of an altered laboratory strain. The efficiency of transformation achieved through these electroporation experiments did not indicate that this was an efficient method of transfer but that it was sufficient to transfer genetic material and is comparable with other methods of genetic transfer used for *Legionella* (Dreyfus and Iglewski, 1984; Nolte, *et al.*, 1984; Keen, *et al.*, 1985). The 96% frequency of curing the plasmids also corresponded with other *L. pneumophila* recombinant studies (Engleberg, *et al.*, 1988).

CONCLUSIONS

In this study of the pathogenic mechanisms of *L. pneumophila,* a recombinant clone responsible for the production of a 25 kDa MOMP was created. The *E. coli* clone LP 116 containing the recombinant plasmid, pLP116 experienced a 40 and 55% increase in adherence when compared to its parent in IF and VBCC adherence trials using U937 cell culture. However, it was also observed that not all "cloned" organisms fluoresced indicating that there were organisms within the population that either did not possess the pLP 116 plasmid or, due to physical limitations, did not expose the protein on the surface of the host bacterial cell. The use of the blocking study demonstrated a complete inhibition of bacterial adherence to U937 cells when these cells were treated with *E. coli* LP 116 or *L. pneumphila* organisms previously incubated with MOMP-specific monoclonal antibody. LD_{50} trials for *E. coli* LP 116 and *L. pnemophila* N_7 coated with the monoclonal antibody showed no lethality for chicken embryos incubated for up to 8 days post inoculation.

The observation of a 31 kDa protein in the attenuated strain was the first evidence of an obvious difference between virulent and attenuated strains not previously reported. The 31 kDa protein was not found in the virulent isolate. More striking in this particular case was that the two isolates were phenotypically and genotypically identical as the attenuated strain was a multiple passage derivative of the virulent isolate. Although there seems to be a relationship between these two proteins, it is unknown why the production of one should decrease the expression of the other. The activity of the virulent and attenuated strains during OMP isolation conditions may reflect changes in the nature of the MOMP itself and under identical conditions this protein may behave differently when in an attenuated strain causing variations in the electrophoretic migration patterns of the OMP. While attempting

to purify *L. pneumophila* MOMP, Butler and Hoffman (1990) first reported a 31 kDa protein found in *L. pneumophila,* Philadelphia I strain that was uncovered unexpectedly when the OMP was isolated using either *N*-acetylmuramidase from the fungus *Chalaropsis* or with mutanolysin from *Streptomyces globisporus*. In these experiments the appearance of the 31 kDa protein led the investigators to examine its function and it was concluded that it was part of the MOMP complex responsible for attachment to the peptidoglycan layer within the cell (Hoffman, *et al.*, 1992a). The genes responsible for the 31 kDa protein are reported to be the same as those used to encode for a 28 kDa OMP (Hoffman, *et al.*, 1992b). The monoclonal immunoblots in this present study have shown faint crossreaction in the 31 kDa region. This phenonenon had been shown earlier when Nolte, *et al.* (1986) determined that the monoclonal antibody developed by Gosting, *et al.* (1984) clearly detected the higher molecular weight protein in the blot. Although the extra band that appeared in the blot was noted by the authors, no explanation for the occurrence was offered. The work of Butler and Hoffman (1990) and Hoffman, *et al.* (1992a,b) did not state the condition of the organism concerning its pathogenic characteristics as no virulence assays were performed. The present study is in agreement with that of these workers in that the expression of a 31 kDa protein by *L. pneumophila* is atypical. However, the present work which suggests that the protein is related to expression of virulence has not been reported by others.

The 25 kDa MOMP of *L. pneumophila* appears to be an important feature in microbial adherence as shown in blocking studies using the MOMP-specific monoclonal antibody. The organisms coated with MOMP-specific monoclonal antibody lost the ability to bind to host cells in culture and were unable to infect embyonated chicken eggs. Bellinger-Kawahara and Horwitz (1990) reported that the MOMP complex of *L. pneumophila* was necessary for the initiation of phagocytosis by C3 binding of the MOMP prior to engulfment. This adherence study shows that the 25 kDa MOMP was responsible

for attachment to the cells. This was apparently independent of phagocytosis as the U937 cells were treated with cytochalasin D to inhibit the uptake of organisms. Attachment to cells prior to phagocytosis may be the first step in the engulfment process indicating that there is a site-specific mechanism located within the MOMP complex of the bacteria. Recently similar results in blocking studies using HeLa cells have been noted (Hoffman, *et al.*, 1992a)

Because the LD_{50} of the transformed attenuated *L. pneumophila* containing the 25 kDa MOMP sequences showed a decrease in the number of organisms necessary to be lethal as compared to the original attenuated sample, the production of this protein may be considered a primary virulence factor. Whether this protein plays any part in the protection of the organism after engulfment is not clear. The attenuated strain produced less of the 25 kDa protein but greater amounts of the 31 kDa protein. This latter material is involved with attachment of the outer membrane to the peptidoglycan. The relative distribution of each may affect the ability to adhere and infect host cells. It has also been shown that strains of environmental origin may depend on different mechanisms for survival than do their clinical counterparts in that some have failed the test using the same monoclonal antibody as used in this study raised to the common antigen of the outer membrane (Vickers, et al., 1990).

It is interesting that the production of a 28 kDa OMP and a 31 kDa OMP of the *L. pneumophila* strain, Philadelphia 1, appear to be encoded for by the same set of genes (Hoffman, *et al.*, 1992a,b). The sequences found in the present study differ from those found for the 28 and 31 kDa OMP indicating that a separate set of genes are responsible for the 25 kDa protein of the MOMP complex. Given that this is the case, any of the genes responsible for the MOMP complex may be under the control of an insertion sequence (IS) element that can either encode for different products by a "switching" mechanism or cause deletions in the adjacent regions as reported in pathogenic strains of *E. coli* (Eisenstein,

1989). Although no IS elements have been reported for the 28/31 kDa proteins and none were found within the sequence of this study, it is possible that IS elements located outside this region may influence the production of these proteins.

The amount of the 25 kDa MOMP produced by *L. pneumophila* strains may influence the outcome of the disease state; however, it must be stressed that these observations are based on an attenuated laboratory strain and may reflect other additional factors arising due to the process of attenuation.

The relationship of the 25 kDa member of the MOMP complex to virulence and what role it serves regarding survival mechanisms for this organism appeared to be important features in the investigation of pathogenic mechanisms. These warrant further investigation.

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APPENDICES

APPENDIX 1

1. Media used for bacterial cultures

All recombinant organisms used in this study were cultured in disposable plasticware. Materials were autoclaved at 250°C for 30 min according to the standards set by the Institutional Biohazard Committee (IBC).

1.1 *Legionella* media or buffered charcoal yeast extract supplemented with α-ketoglutarate (BCYE-α) was obtained from Gibco Laboratories (Madison, Wisconsin) and prepared according to the manufacturers instructions. This medium contains:

yeast extract	10.0 g/l
ACES buffer	10.0 g/l
ferric pyrophosphate	0.25 g/l
α-ketoglutarate	1.0 g/l
agar	15.0 g/l
activated charcoal	2.0 g/l

The pH of this medium is 6.9 which is obtained by addition of 10 M KOH (dropwise). Four ml of a filter-sterilized solution of 10% L-cysteine HCl was added to one liter after autoclaving.

L. pneumophila N_7 strains containing the pUC 19 plasmid or the pLP recombinants were maintained on BCYE-α supplemented with 50 μg/ml ampicillin.

1.2 Nutrient broth or nutrient agar from DIFCO Laboratories was used to maintain the *E. coli* strains used in these experiments. This was prepared according to manufacturers instructions. This medium contains:

	Beef extract	3.0 g/l
	Peptone	5.0 g/l
(Nutrient agar only)	Agar	15.0 g/l

E. coli strains containing the pUC 19 plasmid or the pLP recombinants were maintained with either nutrient broth or nutrient agar supplemented with 50 μg/ml ampicillin.

1.3 Luria-Bertani, Miller (LB broth) was used for the incubation of the transformed *E. coli*. This medium contains:

Tryptone	10.0 g/l
Yeast extract	5.0 g/l
NaCl	10.0 g/l

1.4 Violet red bile (VRB) agar was the media used for the selection of transformed *E. coli* to detect colonies that could not ferment lactose indicating there was an insert interuption in the β-galactosidase region of the pUC 19 vector. This medium contains:

Yeast extract	3.0 g/l
Peptone	7.0 g/l
Bile salts	1.5 g/l
Lactose	10.0 g/l
NaCl	5.0 g/l
Agar	15.0 g/l
Neutral Red	0.03 g/l
Crystal Violet	0.02 g/l

Ampicillin was added to the VRB agar at 50 μg/ml to select for the pUC 19 or recombinant plasmids.

1.5 Phosphate Buffered Saline (PBS) was used as the suspension solution for *L. pneumophila* N_7. Solutions of PBS were made in deionized water and sterilized by autoclaving. These were stored in 100 ml aliquots at 4°C. Ingredients were as follows:

NaCl	8.0 g/l
KCl	0.2 g/l
KH_2PO_4	0.2 g/l
Na_2HPO_4	0.15 g/l

1.6 Frozen cultures of *L. pneumophila* N_7 and *L. pneumophila* recombinants were maintained in 1% serum, 10% sorbitol at -70°C in 1.5 ml sterile plastic freezer tubes.

1.7 Frozen cultures of *E. coli* JM 83 and *E. coli* recombinants were maintained in nutrient broth and 0.8% DMSO at -70°C in 1.5 ml sterile plastic freezer tubes.

APPENDIX 2

2. Cell cultures

All experiments using cell culturewere conducted in a containment hood designated for cell culture work only. The cells were maintained in a humid atmosphere at 37°C in 5% CO_2.

2.1 Deionized water

Water used in all experiments were obtained from a Millipore Milli Q filtration system. Water was collected with a 10 megaholm reading, filter sterilized through a 0.2 μm filter and stored at 4°C in one liter aliquots in sterile glass bottles.

2.2 Hanks' Balanced Salt solution (HBSS)

A concentrated 10 X sterile solution of HBSS with 0.35 g/l sodium bicarbonate and phenol red was obtained from Irvine Scientific. The stock was diluted ten-fold using aseptic techniques with sterile deionized water. The stock and working strength solutions were stored at 4°C in sterile glass bottles.

2.3 Sodium bicarbonate solution ($NaHCO_3$)

A stock of 7.5% solution of sodium bicarbonate was made by dissolving 75 g in one liter of deionized water which was then sterilized by filtration. One hundred ml aliquots were stored at 4°C in sterile glass bottles.

2..4 Bovine Calf Serum

Sterile bovine calf serum was obtained from Hyclone Laboratories, distributed in

50 ml volumes and stored at -20°C in sterile plastic freezer tubes.

2.5 Glutamine

Glutamine was obtained in powder form (Sigma Chemicals, St. Louis, MO), dissolved in deionized water to make a 100X stock (300 mM) solution and filter sterilized. Aliquots were stored at -20°C in sterile plastic freezer tubes.

2.6 Minimal Essential Medium with Earles salts and non-essential amino acids (MEM)

MEM was obtained as sterile 10 X concentrated solution (Irvine Scientific) with added phenol red. For use as stock diutions, ten-fold dilutions of sterile dionized water were added in an aseptic manner. Stock and working strenghths were stored at 4°C in sterile glass bottles.

MEM with Earles salts was prepared as follows:

MEM with Earles salts	450 ml
Fetal bovine calf serum	50 ml
Sodium bicarbonate (7.5%)	10 ml
Glutamine	

APPENDIX 3

3. Reagents for DNA Treatments

Gloves should be worn to avoid contact with DNA. Nucleases present on the skin surface can cause degradation of DNA.

The isolation of chromosomal DNA was performed as described by Marmur. The reagents used in this study were as follows:

3.1 NaCl-EDTA solution

NaCl (0.15 M)	8.8 g/l
EDTA (0.1 M), disodium	37.2 g/l

The pH was adjusted to 8.0 and the solution was stored at room temperature in an Erlenmeyer flask.

3.2 SDS (25%)

A soution of 25% SDS was made by dissolving 25 g of SDS in 100 ml of distilled water. This solution was stored at room temperature in a sterile glass bottle.

3.3 Sodium perchlorate (5 M)

A solution of 5M sodium perchlorate was made by dissolving 61.2 g in 100 ml of distilled water. This solution was stored at room temperature in a sterile glass bottle. Caution should be used in handling sodium perchlorate as it causes severe burns.

3.4 Chloroform/Isoamyl alcohol

The choloroform/isoamyl alcohol mixture was prepared in a 24:1 ratio of

chloroform to alcohol. This solution was stored at room temperature in an Erlenmeyer flask. Caution should be exercised when using chloroform and isoamyl alcohol. The solution was kept in a chemical fume hood at all times.

3.5 Concentrated saline citrate

NaCl (1.5 M)	8.7 g
Sodium citrate (0.15 M)	4.4 g
Distilled water	100 ml

Concentrated saline citrate was stored at room temerature in a sterile glass bottle.

3.6 Diluted saline citrate

NaCl (0.015 M)	88 mg
Sodium citrate (0.0015 M)	44 mg
Distilled water	100 ml

Dilute saline citrate wasstored at room temperature in a sterile glass bottle.

3.7 Ribonuclease A (RNAse)

RNAse was prepared in 10 ml aliquots at 1 mg/ml and stored at -20°C in a sterile plastic freezer tube. RNAse was boiled for 5 minutes prior to each use to inactivate contaminating DNAse.

Two protocols were used for the isolation of plasmid DNA; the alkaline lysis procedure and the phenol/chloroform extraction method. Reagents used in the alkaline lysis method were as follows:

3.8 Solution I

Sucrose (50 mM)	171.17 g/l
EDTA, disodium (10 mM)	37.22 g/l
Tris (25 mM)	30.27 g/l

Solution I was adjusted to pH 8.8 using HCl and stored at room temperature in an Erlenmeyer flask. Lysozyme was added to Solution I as needed and stored at -20°C in 10 mg/ml aliquots in a plastic freezer tube.

3.9 Solution II

NaOH (0.2 N)	8 mg/ml
SDS (1%)	10 mg/ml

Solution II was made fresh and stored at room temperature up to one week in a capped 5 ml glass test tube.

3.10 Solution III

Solution III was prepared using 60 ml of 5 M potassium acetate to which 11.5 ml of glacial acetic acid was added. The pH 4.8 was achieved with HCl. The total volume was adjusted to 100 ml with distilled water. Solution III was stored at room temperature in a sterile glass bottle.

Reagents used in the phenol extraction method were as follows:

3.9 Lysing buffer

Tris (50 mM)	6.10 g/ml
SDS (3%)	3.00 g/ml

Lysing buffer was adjusted to pH 12.6 with NaOH. The buffer was prepared in 100 ml aliquots and was stored at room temperature in a sterile glass bottle.

3.10 Phenol/chloroform mixture

Phenol/chloroform/isoamyl alcohol consisted of water-equilabrated, double-distilled phenol

(IBI) and equal volume of chloroform/isoamyl alcohol prior to use. Caution should be used when handling phenol/chloroform/ isoamyl. These agents must be used in a chemical

fume hood and contact with skin must be avoided.

3.11a Electrophoresis buffer

Tris (40 mM)	48.44 g/l
Sodium acetate (20 mM)	16.40 g/l
EDTA, disodium (2 mM)	7.44 g/l

The electrophoresis (BRL) buffer used for agarose gel electrophoresis was adjusted to pH 7.9 with glacial acetic acid and the volume adjusted to one liter with distilled water.

3.11b Tris EDTA buffer (TE) can be used in DNA electrophoresis for phenol/cholorform extracted plasmids by using the above components less the sodium acetate. Both electrophoresis buffers were stored in glass flasks and stored at room temperature.

3.12 Agarose gels

Agarose and distilled water were boiled in a microwave oven on high power to dissove all traces of solid material. BRL buffer was added after boiling and swirled. Agarose was slightly cooled prior to casting the gel.

Agarose (0.8%)	0.8 g
Distilled water	90 ml
10 X BRL buffer	10 ml

3.12 Tracking dye

Bromophenol blue tracking dye was prepared in 50 ml aliquots and stored in eye dropper bottles at room temperature.

Bromophenol blue (0.7%)	0 .35 g
SDS (0.7%)	0.35 g
Glycerol (33%)	16.5 ml
Distilled water	to 50 ml

3.13 Ethidium bromide stain (for agarose gels)

Ethidium bromide (EtBr) was prepared in 10 ml aliquots using 0.5 μg of EtBr in distilled water and stored at room temperature in an Erlenmeyr flask covered with aluminum foil (light-sensitive). Care should be taken when handling EtBr as it is a carcinogen. Gloves should be worn at all times when handling EtBr or any substance that has been in contact with EtBr.

3.14 High-salt buffer (for electroelution)

3.0 M Sodium acetate, pH 5.2
Distilled water to 1000 ml

The treatment of agarose gels prior to Southern transfer to nitrocellulose consisted of denaturing the DNA within the agarose gel followed by neutralization of the agarose gel.

3.15a Solution 1

NaCl (1.5 M)	87.66 g/l
NaOH (0.5 M)	20.00 g/l

3.15b Solution 2

Tris (1.0 M), pH 8.0	121.1 g/l
NaCl (1.5)	87.66 g/l

Both solutions were stored at room temperature and maintained in an Erlenmeyer flask.

3.16 Standard sodium citrate (20X)

Sodium citrate	88.32 g/l
NaCl	175.32 g/l

Standard sodium citrate for the treatment of nitrocellulose filters throughout the Southern hybridiation procedures was adjusted to pH 7.0 with HCl and the volume adjusted to 1 liter with distilled water. The solution was stored at room temperature in an Erlenmeyer flask.

Reagents used for DNA probe purification for random labeling:

3.17 STE buffer

Tris (10 mM)	12.12 g
EDTA, disodium (1mM)	3.72 g
NaCl (100mM)	5.84 g

The pH of the STE buffer was adjusted to 8.0 with HCl and the volume adjusted to one liter with distilled water and stored at room temperature in an Erlenmeyer flask.

The treatment of nitrocellulose filters after DNA transfer:

3.18 Prehybridization fluid

SSC (6X)
SDS (0.5%)
Denhart's solution (5X)
Denatured salmon sperm DNA (100 μg/ml)

Prehybridization fluids are adjusted in volume according to the dimensions of nitrocellulose (200 μl/cm^2) used in the agarose gel transfers.

3.19 Hybridization fluid

SSC (6X)
Disodium EDTA (0.01 M)
^{32}P-labled probe DNA
Denhart's solution
SDS (0.5%)
Denatured salmon sperm DNA (100 μl/ml)

Hybridization fluids are adjusted in volume according to the dimensions of the nitrocellulose (50μl/cm^2) used in the agarose gel transfers.

3.20 Denhart's solution (50X)

Ficol	5 g
polyvinyl pyrolidine	5 g
BSA (pentax Fraction V)	5 g

The volume of the Denhart's solution was adjusted to 500 ml using distilled water. Denhart's solution was stored at -20°C in 50 ml sterile plastic freezer tubes.

Solutions used for transformation procedures:

3.21 Ligation solution contained 0.1 M Tris, pH 7.2 with HCl and was stored in 100 ml aliquots at 4°C in sterile glass bottles.

3.22 Calcium chloride (50 mM) was used to make *E. coli* cells competent prior to transformation. This solution was prepared in one liter aliquots using distilled water and stored at room temperature in Erlenmeyer flasks.

3.23 Electroporation buffer (electric transformation)

Sucrose (270 mM)	92.42 g
$MgCl_2$ (1 mM)	0.203 g
$NaHPO_4$ (7 mM)	1.88 g

The electroporation buffer was adjusted to pH 7.4 and filtered through a 0.2 µm filter. Electroporation buffer was stored in 100 ml aliquots at 4°C in sterile glass bottles.

APPENDIX 4

4. Reagents used in polyacrylamide gel elctrophoresis and Immunoblotting.

Acrylamide is a neurotoxic substance therefore gloves should be worn at all times when handling polyacrylamide gels.

4.1 Laemmli acrylamide solution

Acrylamide (30%)	60 g
Bis (0.8%)	1.6 g
Deionized water	200 ml

This solution was filtered through a 0.45 μm filter and stored in a sterile glassbottle wrapped with aluminum foil (light-sensitive) at 4°C. While acrylamide is in powdered form wear goggles and mask as well as gloves to avoid contact.

4.2 1 M Tris (pH 6.8)

A one molar solution of tris was made by dissolving 121.1 grams of Tris in 800 ml of deionized water. The pH was adjusted to 6.8 with HCl and the volume adjusted to one liter with deionized water. This was filtered through a 0.45 μm filter and stored at room temperature in sterile glass bottles.

4.3 1 M Tris (pH 8.8)

A one molar solution of Tris was made by dissolving 121.1 grams of tris in 800 ml of deionized water. The pH was adjusted to 8.8 with HCl and the volume adjusted to 1000 ml with deionized water. This was filtered through a 0.45 μm filter and stored at room temperature in sterile glass bottles.

4.4 SDS (20%)

A solution of 20% SDS was made by dissolving 50 grams of SDS in 250 ml of deionized water. This was filtered through a 0.45 µm filter and stored at room temperature in sterile glass bottles.

4.5 Ammonium persulfate (10%).

This solution was made by dissolving 50 mg of ammonium persulfate in 500 µl of deionized water. Ammonium persulfate (dry) was kept desicated in a cool location. This solution, once mixed, could only be stored for up to a week at 4°C in an 1.5 ml Eppendorf tube.

4.6 Separating gel (12%)

1 M Tris (pH8.8)	11.2 g
20% SDS	150 µl
Acrylamide	12.0 g
Deionized water	6.7 ml
Ammonium persulfate (10%)	100 µl
TEMED (N,N,N',N'-Tetra-methylethylenediamine)	20 µl

This mixture was degassed for 30 minutes prior to adding the ammonium sulfate and the TEMED.

4.7 Stacking gel (5%)

1 M Tris (pH 6.8)	1.25 ml
20% SDS	50 µl
Acrylamide	1.67 ml
Deionized water	7.03 ml
Ammonium persulfate (10%)	50 µl
TEMED (N,N, N',N'-Tetra-methylethylenediamine)	10 µl

This mixture was degassed for 30 minutes prior to adding the ammonium persulfate and the TEMED.

4.8 Non-reducing sample buffer

1 M Tris (pH 6.8)	8.0 ml
Glycerol	10.0 ml
20 % SDS	10.0 ml
Deionized water	72.0 ml

Non-reducing sample buffer was stored at 4°C in a sterile glass bottle.

4.9 Reducing buffer

1 M Tris (pH 6.8)	8.0 ml
Glycerol	10.0 ml
20 % SDS	10.0 ml
2-mercaptoethanol	4.8 ml
Deionized water	67.2 ml

Addition of 2-mercaptoethanol to the buffer made it necessary to use a chemical fume hood (use caution). This solution was stored at 4°C in a brown glass bottle.

4.10 Bromophenol blue tracking dye (0.5% in 10% ethanol)

Bromophenol blue	50 mg
10% ethanol	to 10 ml

Bromophenol blue tracking dye was stored at room temperature in 1.5 ml Eppendorf tubes.

4.11 Phenol red tracking dye (0.1%)

Phenol red	0.1 mg
Deionized water	1.0 ml

Phenol red tracking dye was stored at room temperature in 1.5 ml Eppendorf tubes.

4.12 Laemmli Running Buffer (10X)

Tris	30 g
Glycine	144.2 g
SDS	10.0 g
Deionized water	1000 ml

The buffer was stored at room temperature in glass bottles and diluted ten-fold with

deionized water before use.

4.13 Acrylamide gel fixative:

Methanol	50 ml
Acetic Acid	7 ml
Deionized water	43 ml

This solution was stored at room temperature in a brown glass bottle.

4.14 19.5 % silver stain

conc. Ammonium hydroxide	1.4 ml
10 M sodium hydroxide	210 µl
Silver nitrate (19.5 %, in deionized water)	4.0 ml
Deionized water	to 100 ml

This solution was prepared immediately prior to use.

4.15 silver developer

Citric acid	25 mg
Formaldehyde (37%)	0.26 ml
Deionized water	to 500 ml

This solution was prepared immediately prior to use.

4.16 stop bath

Sodium thiosulfate	2 g
Deionized water	100 ml

This solution was prepared immediately prior to use.

Oxidation of acrylamide gels for the detection of lipopolysaccharides:

4.17 Acrylamide gel fixative

Ethanol	40 ml
Acetic acid	5 ml
Deionized water	55 ml

This solution was stored at room temperature in a brown glass bottle.

To oxidize the acrylamide gel 0.7% periodic acid was added to the above mixture.

4.18 20% silver stain

conc. Ammonium hydroxide	2 ml
10 M sodium hydroxide	280 µl
Silver nitrate (20%, in deionized water)	5 ml
Deionized water	to 150 ml

This solution was prepared immediately prior to use.

4.19 Silver developer

Citric acid	50 mg
Formaldehyde (37%)	500 µl
Deionized water	200 ml

This solution was prepared immediately prior to use.

4.20 Transfer buffer (Immunoblotting)

Tris (0.025 M)	12.12 g
Glycine (0.192 M)	57.60 g
Methanol (20%)	800 ml

The pH for the transfer buffer was adjusted to 8.3 with glacial acetic acid and the volume was adjusted to 4 liters. Transfer buffer was stored at 4°C in Erlenmeyer flasks.

4.21 10X Tris buffered saline (TBS)

Tris (20 mM)	24.2 g
NaCl (500 mM)	292.4 g
Deionized water	800 ml

The pH of the TBS buffer was adjusted to 7.5 with HCl and the volume adjusted to 1000 ml with deionized water. This solution was stored at room temperature in a sterile glass bottle. The stock solution was diluted ten-fold with deionized water as needed.

4.22 Blocking solution

Gelatin	3.0 g
1 X TBS	100 ml

4.23 Antibody buffer

Gelatin	1.5 g
1 X TBS	100 ml

4.24 HRP color developer

The HRP color developer (BIO-RAD) was made immediately prior to use. Sixty mg of HRP color developer (4-chloro-1-napthol;) was added to 20 ml of ice cold methanol. Prepared separately, 60 µl of 30% hydrogen peroxide and 100 ml of 1 X TBS were combined in a graduated cylinder. These two solutions were added to a glass baking dish to develop the nitrocellulose sheets.

APPENDIX 5

5. Glass Bead Disruption Procedure.

The disruption of cell membranes should be performed in a containment hood to avoid possible contact with aerosols.

a. Wash the glass beads to be used with approximately 0.01 N HCl and rinse with deionized water.

b. Rinse beads again in PBS (or buffer of choice).

c. A ratio of 17 ml of buffer (10^8 CFU/ml suspension) and 17 g of glass beads will yield sufficient protein for one outer membrane preparation. All proceedures should be performed in a laminar flow hood.

d. Place the mixture in an appropriate sized Omni-mixer blender cup, connect to the power head and immerse the blender cup in an ice bath.

e. Turn the power dial to the highest setting and run for 10-15 min.

f. Let the beads settle and decant the disruptate. Material can be recovered from beads by resuspending the beads in buffer, letting them settle and redecanting. The disruptate is ready for further processing.

g. The glass beads can be used again by autoclaving them after adding enough HCl to make the suspension acid. The beads should be washed in 0.1 N HCl, autoclaved for 15 min at 121°C and the surface and dry at 105-110°C. The beads can then be returned to stock.

h. The blender cup can be autoclaved or immersed in Roccal or another cationic disenfectant (do not use iodine or chlorine as these agents are corrosive). Be sure to disessemble the blender cup cover as this has a tendency to collect glass beads at high speed.