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Investigations into the Intracellular Pathogenesis of *Legionella pneumophila*

by

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for my parents

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ABSTRACT

Legionella pneumophila is the facultatively intracellular, Gram negative bacterium that causes Legionnaires' disease. Within its host cell, the alveolar macrophage, L.pneumophila survives and grows because it is able to resist fusion of its phagosome with lysosomes. The mechanism for this is unknown. Intracellular pathogenesis of L.pneumophila was studied 1) by examining L.pneumophila infection in various cell lines, 2) by comparing a virulent (Lp2064) and an isogenic Mueller-Hinton-selected avirulent (Lp2064M) organism for their respective abilities to survive intracellularly, and 3) by examining the protein profiles of intracellular bacteria and comparing them to extracellular responses. Despite a significant range in relative susceptibility, all of the cell lines examined supported L.pneumophila infection. L929 cells were most sensitive, yielding plaques at low multiplicities of infection. Infection of L929 cells (or monocytes) with Lp2064 resulted in the host cells being lysed due to the growth of the virulent organism within phagosomes. In contrast, Lp2064M was killed within phagolysosomes and consequently produced no cytopathic effect. The major difference between the protein profiles of intracellularly-derived Lp2064 or Lp2064M was the overwhelming expression in Lp2064 of the L.pneumophila heat shock protein, Hsp 60. Also characteristic, was the smearing pattern in lanes containing Lp2064M samples. Lp2064 and Lp2064M were found to respond to tissue culture medium (MEM) by differentially synthesizing many proteins including the major outer membrane protein (MOMP). Since the profiles of broth-grown, or agar-grown organisms were identical, it is postulated that the MEM-induced extracellular changes, or alternatively, the intracellularly-induced changes (possibly mediated through Hsp 60) are responsible for affecting the fusion of phagosomes with lysosomes.

ABBREVIATIONS

ATR	Acidification Tolerance Response
BCYE	Buffered charcoal yeast extract
BYE	Buffered yeast extract
cfu	Colony-forming units
CPE	Cytopathic effect
CR	Complement receptor
Cyt D	Cytochalasin D
FBS	Fetal bovine serum
Gm	Gentamicin
HBSS	Hanks balanced salt solution
Hsp	Heat shock protein
IFN-γ	Interferon-gamma
kDa	KiloDaltons
LD50	Fifty percent lethal dose
M-H	Mueller-Hinton
mAb	Monoclonal antibody
Mip	Macrophage infectivity potentiator
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MSP	Major secretory protein
PBS	Phosphate buffered saline
pfu	Plaque-forming units
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid

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INTRODUCTION

Legionella pneumophila is a Gram negative, rod-shaped bacterium, which in 1977 was identified as the agent causing Legionnaires' disease (McDade et al, 1977). Although the organism is ubiquitous, disease seems to be limited to those individuals who are in some manner, immunocompromised.

L.pneumophila is a facultatively intracellular bacterium that resides in the alveolar macrophages of infected individuals (Nash et al, 1984). Its ability to survive the harsh environment of a cell which normally functions to kill bacteria, relies primarily on the failure of the Legionella-bearing phagosome to fuse with lysosomes (Horwitz, 1983). Within the protective environment of the phagosome, Legionella grows unchecked. The mechanisms underlying such membrane fusion events (or lack thereof) remain largely unknown.

The following literature review focuses almost entirely on L.pneumophila and consists of two parts. The first part provides a general history and background of Legionnaires' disease. It includes a description of Legionnaires' disease and L.pneumophila. It also examines the host (immune) response to the organism. The second part of the review discusses the attempts to identify virulence factors of L.pneumophila, including the ability of the organism to multiply within macrophages. In this regard, several selected intracellular pathogens are compared with respect to the strategies involved

in intracellular invasion.

LITERATURE REVIEW

The year 1976 featured celebrations of the United States Bicentennial, the perceived threat of swine flu, the Summer Olympics in Montreal, the late Billy Martin as manager of the New York Yankees -for the first time, and, according to the August 16th cover of Newsweek magazine, the "mystery of the killer fever."

Sensationalism aside, this part of the review serves to provide a brief background and history of Legionnaires' disease, encompassing aspects of epidemiology, clinical features, pathology, laboratory diagnosis and finally, treatment.

LEGIONNAIRES'DISEASE

History and background

During July and August of 1976, a severe, explosive outbreak of pneumonia was associated with persons attending the 58th annual convention of the Pennsylvania department of the American Legion which was held July 21-24 at the Bellevue-Stratford hotel in Philadelphia (Fraser et al., 1977). Dubbed by the press as "Legionnaires' disease" (Fraser and McDade, 1979), the then mysterious illness afflicted 182 conventioneers, resulting in 29 deaths (Fraser et al., 1977). Additional cases of pneumonia, termed Broad Street pneumonia, were also seen in individuals who had not entered but had been within one block of the hotel. Except for the link with the

Bellevue-Stratford hotel, the exact source of the outbreak was never established although airborne transmission was suspected.

Through the efforts of the investigating team from the Centers for Disease Control (CDC) and the Pennsylvania health department and in particular, the persistence of J. McDade of the CDC, the etiological agent of Legionnaires' disease (and Broad Street pneumonia) was identified in January 1977, as being a Gram negative rod-shaped bacterium (McDade et al., 1977, Astor, 1984). The fastidious nature of the organism had made its isolation difficult, and it was only by using techniques developed for isolating Rickettsia that success was achieved.

Isolation of the bacterium subsequently called Legionella pneumophila (Brenner et al., 1979), enabled the CDC to perform serological tests using sera saved from previous outbreaks of disease for which there was no known cause. These sero-epidemiological studies revealed that Legionnaires' disease was not a 'new' disease. In fact, outbreaks of Legionnaires' disease had previously occurred in a Washington, D.C. hospital in 1965, and in a building of the county health department in Pontiac, Michigan in 1967 (McDade et al., 1977). While the 1965 outbreak was clinically similar to the one in Philadelphia and resulted in 81 cases with 12 deaths (Thacker et al., 1978), the outbreak in Pontiac, called Pontiac fever, consisted of a mainly febrile illness and resulted in no

deaths among the 144 cases (Glick et al., 1978). These retrospective studies also indicated that the earliest known specimen of L.pneumophila appears to be one isolated by E.B. Jackson in 1947 (McDade et al., 1979) while the earliest Legionella species (L.micdadei) was isolated by H. Tatlock in 1943 (Hebert et al., 1980).

Since the Philadelphia epidemic, there have been several outbreaks of Legionnaires' disease throughout the world (Meyer, 1983, Kirby et al., 1980), most notably in Burlington, Vermont (Broome et al., 1979), Memphis, Tennessee (Dondero et al., 1980), Los Angeles, California (Haley et al., 1979) and Stafford, England (Rashed et al., 1986). In Canada, the first recognized case of Legionnaires' disease occurred in a Saint John, New Brunswick man (Bennett, 1978). There have been several sporadic cases throughout the country with the first clustering of cases being reported in Toronto in 1979 (Jessamine, 1979). In Nova Scotia, an outbreak was recorded at Camp Hill hospital in Halifax (Martin, et al.,1988). In addition, a five-year prospective study has identified L.pneumophila as the cause of approximately 2% of the cases of community-acquired pneumonia seen at the Victoria General Hospital in Halifax (Marrie et al.,1989). Results of a one-year study at the same hospital also showed that a significant number of cases (30%) of "atypical" pneumonia were caused by L.pneumophila (Marrie et al., 1981).

Epidemiology

Legionnaires' disease can occur as sporadic cases or as epidemics (England et al., 1981). Both nosocomial (Broome et al., 1979, Kirby et al., 1980) as well as community-acquired pneumonia have been described. And as mentioned above, both a pneumonic and a non-pneumonic (Pontiac fever) disease exist. The reported incidence of Legionnaires' disease among cases of pneumonia varies widely, generally accounting for 1-13% of all pneumonias in North America and Europe (Broome, 1984). Furthermore, it should be noted that serological studies of "control" populations i.e. individuals with no apparent signs of Legionnaires' disease, have also produced varied results which have ranged from 1.3% to almost 25% seropositivity (Broome and Fraser, 1979). Differences in methodology or inclusion criteria however, as well as the small sample size of many of the studies make it difficult to compare the various reports and to arrive at an accurate figure (Reingold, 1988).

The earlier epidemics of Legionnaires' disease suggested an airborne mode of transmission. The bacterium was identified in the lung tissue of sentinel guinea pigs that had succumbed following exposure to the air conditioning system during the Pontiac outbreak. Under laboratory conditions, guinea pigs exposed to aerosols of water from the condenser of the same air conditioners also died, whereas exposure to the same water that was filtered or autoclaved had no effect (Kaufmann et

al., 1981). Air-conditioning units and cooling towers have also been implicated in several other outbreaks (Meyer, 1983, O'Mahony, 1990).

Despite the acceptance that the principal mode of transmission is airborne, there seems to be no person-to-person transmission (Fraser et al., 1977). This has been shown in studies with family members and hospital workers exposed to patients with Legionnaires' disease (Broome and Fraser, 1979).

In most cases of Legionnaires' disease, the actual source of the pathogen appears to be water. In addition to the heat-exchange apparatuses, the bacterium has been isolated from potable water outlets such as showers/taps, humidifiers and whirlpool baths, municipal reservoir water, rain water, and even from a supermarket vegetable sprayer (Meyer, 1983, Lee and West, 1991). Moreover, in potable water systems the organism seems to be isolated more frequently from warm (25-42°C) water sources than cold systems (Wadowsky et al., 1982, Wadowsky et al., 1985). Legionnaires' disease is also associated with excavation projects (Thacker, et al., 1978, Martin, et al., 1988) and with potting soil (Steele et al., 1990).

Since the majority of both clinical and environmental isolates of L.pneumophila belong to serogroup 1 (Reingold et al., 1984, Fliermans et al., 1981), investigators have resorted to using a variety of epidemiological markers in

order to establish whether specific serogroup 1 environmental isolates are (in)distinguishable from those isolated from patients. These markers, whose use has been quite successful, include plasmid profiles (Brown, et al., 1982), reactions with panels of monoclonal antibodies (Watkins et al., 1985, Stout et al., 1988), multilocus enzyme analysis (Edelstein et al., 1986), and more recently, restriction fragment length polymorphism (RFLP) profiles (Saunders et al., 1990).

In the natural environment Legionellae are ubiquitous. As summarized by Lee and West (1991), Legionellae are basically inhabitants of moist natural areas. For example, L.pneumophila has been found at temperatures ranging from 5.7°C to 63°C in various lakes and rivers in the U.S.A. which were not associated with any outbreaks of Legionnaires disease (Fliermans et al., 1981).

Ecologically, Legionellae have been postulated to co-exist with other bacteria, algae, and protozoa (Spriggs, 1987), and it is likely that some protozoa may actually function as amplifiers or reservoirs for the bacteria. Rowbotham (1980) was the first to show that Legionellae can infect Acanthamoeba and Naeglaria, two types of amoeba that are found in fresh water. This observation was confirmed (Holden, et al., 1984) and extended to include Hartmannella (Fields et al., 1989) and the ciliated protozoan, Tetrahymena (Fields et al., 1984). The implications of such studies are underscored with the isolation of Tetrahymena from water

obtained from a cooling tower identified as the source of an outbreak of Legionnaires' disease (Barbaree et al., 1986). In co-culture experiments, the same protozoa were shown to support the intracellular growth of isolates of L.pneumophila which were also obtained from the cooling towers. The ability of amoeba to form cysts under some conditions is an added advantage for both the amoeba and Legionellae. It has recently been shown that cysts of Acanthamoeba polyphaga trophozoites infected with L.pneumophila are able to withstand concentrations of free chlorine up to 50mg/L which is more than 100 times the level that will kill free L.pneumophila (Kilvington and Price, 1990).

In the United States there seems to be a clustering of both sporadic and outbreak-associated cases of Legionnaires' disease in the summer months, with a slight preponderance towards the States east of the Mississippi (Broome and Fraser, 1979, England et al., 1981). Coupled with the ecological niche of the organisms, probably contributing to this finding are factors such as the association of Legionellae with air-conditioning/cooling systems and the link with excavation both of which are more likely to occur in the summer.

Other aspects of the epidemiology of Legionnaires' disease that should be considered are the predisposing host factors which are summarized by Broome and Fraser (1979), England et al. (1981), Kirby et al., 1980), and Meyer (1983). Various studies have supported the initial observations that

although Legionnaires' disease has afflicted both children and adults, it is far more likely to occur in individuals who are over fifty years old, except for Pontiac fever which seems to affect those in their thirties. Legionnaires' disease occurs more frequently in men than in women, at a ratio of approximately 2.6 to 1. Cigarette smoking and alcohol consumption have also been implicated as risk factors, as has treatment with immunosuppressive drugs. Underlying conditions such as neoplasms, renal transplants, pulmonary (eg. chronic bronchitis or emphysema), cardiac and renal diseases, and diabetes further increase the risk of acquiring Legionnaires' disease. It is of note that a study of healthy individuals exposed to L.pneumophila in their hot-tap water systems failed to show any cases of pneumonia, although antibody levels in some individuals suggested evidence of sub-clinical infection (Arnow, et al., 1985).

Clinical features

As mentioned above, there are two clinically distinct forms of Legionnaires' disease: the more familiar pneumonic form, and the non-pneumonic, primarily febrile, Pontiac fever. Compared to the pneumonic form which has an incubation period of 2-10 days and an attack rate of about 0.2-6.8% (Broome and Fraser, 1979), Pontiac fever has an incubation period of about 36 hours and an extremely high attack rate of more than 95 percent (Glick et al.,1978, Broome and Fraser, 1979). Furthermore, in addition to the conspicuous lack of pneumonia,

Pontiac fever affects mostly younger persons and is usually self-limiting (Edelstein and Meyer, 1984, Glick et al., 1978). The exact reasons or mechanisms contributing to the distinction between the pneumonic and non-pneumonic forms of Legionnaires' disease remain unclear. However, it has recently been postulated that Pontiac fever might result from infection with Legionellae (e.g. L.anisa) which are incapable of intracellular multiplication (Fields et al., 1990).

Before considering the clinical presentation of Legionnaires' disease, it should be noted that based on seroconversion data, it does appear that some individuals exposed to the bacterium are afflicted with just a mild, asymptomatic type of illness (Lattimer et al., 1979, Meyer, 1983).

The following then are the signs and symptoms associated with Legionnaires' disease as summarized by Kirby et al. (1980) and Edelstein and Meyer (1984): During the pneumonic disease, a prodrome consisting of a general malaise, anorexia and weakness is followed by a (usually) non-productive cough which may become productive, an unremitting high fever which in many cases can be greater than or equal to 40°C, chills, dyspnea, headache coupled with confusion and/or lethargy, myalgia, diarrhea, relative bradycardia, leukocytosis and to a lesser extent, chest pain, nausea and vomiting. Radiographic evidence frequently reveals patchy alveolar infiltrates which result in various degrees of consolidation.

Pontiac fever has very similar signs and symptoms as the pneumonic disease, except as previously noted, there is no pneumonia. The disease usually resolves itself, generally lasting 2-5 days.

Laboratory diagnosis

Laboratory diagnosis of Legionnaires' disease routinely relies on evidence of seroconversion or if possible, direct demonstration either by immunofluorescence or by culture, of Legionellae in sputum samples (Edelstein et al., 1980). Unfortunately, these techniques have their drawbacks. Seroconversion can take weeks to develop and interpretations are only as good as the antigen preparation used. Immunofluorescence tests depend on the availability of a reasonably extensive panel of antibodies and are also dependent upon the antigen preparation. Moreover, some non-Legionellae tend to react with some of the polyclonal reagents thus resulting in false positives (Edelstein and Edelstein, 1989a). Direct culture while being indisputable when positive, takes several days. Furthermore, it has recently been shown that some viable organisms are not always culturable (Hussong et al., 1987), suggesting a need for alternative approaches.

Lambert and Moss (1989) have proposed the use of capillary gas-liquid chromatography and reverse-phase high-performance liquid chromatography to detect the presence of fatty acid and ubiquinone profiles which are characteristic of Legionella. These tests can be done quickly and the

identity of the organisms can be subsequently confirmed using other methods.

Tests for the detection of Legionella antigen in urine have existed for sometime (Kohler, 1990) but are not in routine use. This may be because antigen can persist in some patients for a long period of time, and because of the short shelf-life and limited specificity of some of the earlier reagents (Kohler, 1990).

DNA probes recognizing sequences coding for the macrophage infectivity potentiating (mip) protein (Mahbubani et al., 1990) and ribosomal RNA sequences (Grimont et al., 1985, Saunders et al., 1988, Kohne et al., 1984) have recently been developed, with one of these being commercially available from Gen-Probe (Edelstein et al., 1986). Early optimism derived from using the Gen-Probe reagent with pure cultures (Edelstein et al., 1986, Wilkinson et al., 1986) or frozen clinical specimens (Edelstein et al., 1987) has been dampened with a recent report that false positive results are found when the probe is used with clinical specimens such as sputum or serum (Laussucq et al., 1988).

It is evident that due to the limitations of the above techniques, a combination of tests would be needed to ensure proper laboratory diagnosis.

Treatment

Fortunately, if recognized early, Legionnaires' disease can be treated with antibiotics. The most effective antibiotic

appears to be erythromycin (Fraser et al., 1977, Broome et al., 1979, Kirby et al., 1980), with rifampin and to a lesser extent, tetracycline also having some success (Kirby et al., 1980). More recently, quinolones such as WIN57273 (Edelstein and Edelstein, 1989b) and sparfloxacin (Edelstein et al., 1990), and other new macrolides eg.clarythromycin (Kirst and Sides, 1989, Fernandes et al., 1986) have been touted as having potential based on laboratory studies, however their large-scale effectiveness awaits confirmation in clinical trials. It should be noted that the ß-lactam antibiotics are particularly ineffective in treating Legionnaires' disease (Kirby et al., 1980).

Pathology

The overwhelming pathology associated with Legionnaires' disease is manifested in the lungs (Blackmon, et al., 1978, Winn et al., 1978, Kirby et al., 1980). Macroscopic examination at autopsy showed that the lungs were heavily consolidated and that the pneumonia was not restricted to any one lobe. Further examination revealed a multi-focal lobular, or bronchopneumonia which has also been referred to as alveolitis. There was some evidence of hepatization and the small amounts of pleural effusions that were seen were serous or serosanguineous in nature.

Microscopically, the distal air spaces, i.e. the alveoli, the alveolar ducts, and the small bronchioles were filled with an inflammatory exudate which was comprised of mononuclear and

polymorphonuclear cells, along with copious amounts of fibrin. Frequently associated with this exudate were lysed cells and the resultant nuclear debris. Bacteria were visible predominantly within or adjacent to the cells in the exudate. The use of the direct fluorescent antibody technique enhanced the visualization of L.pneumophila not only in the air spaces, but also in the pulmonary interstitium including the alveolar and interlobular septa and the peribronchiolar and periarteriolar connective tissue (Hicklin et al., 1980). This study of autopsy material suggested the retrograde spread of the organism from the alveoli to the bronchioles, with the upper airways being less severely affected. It also demonstrated the presence of the bacteria in the blood and lymphatics including the spleen (Hicklin, et al., 1980).

Ultrastructural examination of lung tissue further attested to the intracellular niche of the bacteria. Indeed, bacteria were actually found to be undergoing cell division within the phagocytes comprising the inflammatory exudate (Winn and Meyerowitz, 1981).

In terms of the overall gross pathology of the lungs and the radiological signs, the effects that have been described are not necessarily unique to L.pneumophila. However, the feature which permits distinction between pneumonia caused by Legionella and other bacillary pneumonias is the multiplication of L.pneumophila within macrophages.

ETIOLOGY

Classification

After having isolated the bacterium causing the Philadelphia outbreak of Legionnaires' disease, the task was to identify and classify it. Because of the way it was isolated it was thought that the organism might be related to the Rickettsia. However, an estimate of the genome size showed it to be similar to that of most bacteria and about twice the size of the Rickettsia. The use of DNA hybridization as a measure of relatedness indicated that all isolates of bacteria causing Legionnaires' disease were indeed highly related, and that the organism was unrelated to any of the other bacteria tested that were similar in guanosine-plus-cytosine (G + C) content. The Legionnaires' disease bacterium as it was called was thus classified as Legionella pneumophila, belonging to the family Legionellaceae (Brenner et al., 1979). Currently there are 30 species of Legionellae making up almost 50 serogroups (Thacker et al., 1989, Bornstein et al., 1989). Fourteen of these serogroups belong to L.pneumophila. Although not closely related to other bacteria, a phylogenetic study using 16S ribosomal RNA has placed the Legionellaceae closest to the purple sulfur bacteria (Ludwig and Stackebrandt, 1983). Affiliated with this group are the Enterobacteriaceae and the Vibrionaceae.

Microbiology

As outlined by Brenner (1986) Legionella pneumophila is

an aerobic, nonspore-forming, Gram negative bacillus that is 0.3 to 0.9μm in width and 2 to more than 20μm in length. The bacteria possess flagella and are thus considered motile. They do not appear to ferment or oxidize carbohydrates nor do they reduce nitrate or degrade urea. They are weakly positive, and positive for oxidase and gelatinase respectively. While initially reported as being positive for catalase, Pine et al. (1984) have shown that unlike other species of Legionellae, L.pneumophila is catalase negative, but peroxidase positive. The hydrolysis of hippurate to yield benzoic acid and glycine has been used as a biochemical basis to differentiate amongst the Legionellae (Hebert, 1981, Marmet et al., 1990).

Characteristics of the cell wall include an unusual branched-chain fatty acid profile as opposed to the hydroxy-fatty acids which are seen with most other Gram negative bacteria (Moss et al., 1977). The peptidoglycan layer is composed of muramic acid, glucosamine, glutamic acid, alanine, and *meso*-diaminopimelic acid (m-DAP), and while it was thought to be highly cross-linked most likely through m-DAP and alanine residues (Amano and Williams, 1983a), recent studies have suggested otherwise (Butler and Hoffman, 1990). In addition, the proteins associated with the peptidoglycan layer seem to be highly resistant to proteolysis (Amano and Williams, 1983b). The lipopolysaccharide (LPS) is unusual in that it partitions in the phenol rather than aqueous phase during extraction (Gabay and Horwitz, 1985), and its pattern

as revealed by silver stain, is quite distinctive, forming a much tighter banding pattern than is seen with Salmonella (Ciesielski et al., 1986, Nolte et al., 1986). In addition, the lipid A moiety which in most Gram negative organisms is considerably pyrogenic, is reported to be weakly endotoxic (Wong et al., 1979). Outer membrane proteins include a 47-kiloDalton (kDa) flagellin protein (Elliott and Johnson, 1981), the 29-kDa major outer membrane protein (MOMP) which acts as a porin (Ehret et al., 1984, Hindahl and Iglewski, 1984, Butler et al., 1985, Gabay and Horwitz, 1985, Gabay et al., 1985), a 24-kDa protein called macrophage infectivity potentiating protein (mip; Cianciotto et al., 1989b) and a 19-kDa protein (Engleberg et al., 1986, Hindahl and Iglewski, 1987) which is believed to be a peptidoglycan-associated lipoprotein (Ludwig et al., 1991, Engleberg et al., 1991).

Several extra-cellular enzymes are produced by L.pneumophila: cytotoxins (Friedman et al., 1980, Baine, 1985), proteases (Conlan et al., 1986, Thompson, et al., 1981, Muller, 1980, Gul'nik et al., 1986, Rosenfeld et al., 1986), hemolysins (Bornstein et al., 1988, Thorpe and Miller, 1984, Wintermeyer et al., 1991), phospholipases (Baine 1985 and 1988), DNases, RNases, phosphatases, esterases, and a ß-lactamase (Thorpe and Miller, 1981, Nolte et al., 1982) have all been described. Also present are peroxidase and superoxide dismutase activities (Pine et al., 1984). Some of these enzymes have been purported to be virulence factors and as

such will be discussed in a future section.

In terms of their laboratory growth requirements, the organisms are fastidious. They do not grow on blood agar or other standard laboratory media. Their source of carbon and energy is derived from amino acids (George et al., 1980). They rely on cysteine and iron, and an optimal pH of between 6 and 6.9 for growth (Feeley et al., 1979, Edelstein, 1981). The growth medium that is currently used is buffered charcoal yeast extract (BCYE) agar which is supplemented with cysteine and α-ketoglutarate, and has a pH of 6.9 (Feeley et al., 1979, Edelstein, 1981). The charcoal in the media tends to neutralize toxic compounds such as hydrogen peroxide and superoxide radicals which are found upon exposure of the media to fluorescent light, and whose levels are accelerated upon autoclaving (Hoffman et al., 1983). The organisms are generally grown at 35 to 37°C in a CO_2 incubator. Legionella colonies which are bluish-grey in colour, usually take three to four days to grow on BCYE agar.

HOST RESPONSE TO L. PNEUMOPHILA

Since pathogenesis is the culmination of events resulting from the interplay between host and microorganism, it is necessary to consider how the host reacts to the invasion. This is of considerable importance since many victims of Legionnaires' disease appear to be compromised in some manner prior to the infection.

Genetic susceptibility

In animals, there appears to be a genetic basis for the susceptiblity to Legionella infections, with some species being prone to a more severe infection than others (Yoshida and Mizuguchi, 1986). Differences in susceptibility to infection have also been noted between strains of a given species. For example, the mouse strain A/J, is more susceptible to infection than are BALB/c (Yamamoto et al., 1991) or C57BL/6 (Yoshida et al., 1991) mice as determined by LD_{50} values and the ability of the respective macrophages to support the multiplication of L.pneumophila (Yamamoto et al., 1988, Yamamoto et al., 1991, Yoshida et al., 1991). Through a series of crosses and back-crosses with BALB/c and A/J mice, Yamamoto et al. (1991) have established that at least in mice, there is a genetic basis for susceptibility to L.pneumophila infections and that this trait is recessive. Yoshida et al. (1991) have confirmed these results and have suggested that the susceptibility/resistance gene lies on the proximal part of mouse chromosome 15. This however, has not been proven in human Legionella infections.

Immunity

a) Non-specific immunity:

In general, some of the effectors of non-specific immunity include complement (via the alternate pathway), and substances such as myeloperoxidase and lactoferrin which are found within phagocytes. There are however, conflicting

reports regarding the sensitivity of L.pneumophila to serum, i.e. complement (Horwitz and Silverstein, 1980a, Caparon and Johnson, 1988, Quinn and Weinberg, 1988). Lactoferrin, which is also found in mucosal secretions, does have a bactericidal effect on L.pneumophila, albeit only in its iron-free state (Bortner et al., 1986, and 1989). Myeloperoxidase exerts its effect in the intracellular environment of the neutrophil. Myeloperoxidase, by combining with hydrogen peroxide and various halides, can form toxic compounds which are detrimental to bacteria (Hassett and Cohen, 1989). L.pneumophila is most sensitive to this system (Locksley et al., 1982, Jepras and Fitzgeorge, 1986). Hydrogen peroxide, itself a product of the oxidative burst accompanying phagocytosis, together with superoxide anion (O_2^-), and the resulting hydroxyl radicals, can also be toxic unless neutralized. Jepras and Fitzgeorge (1986), and Summersgill et al., (1990) have demonstrated that different isolates of L.pneumophila exhibit varying degrees of susceptibility to this system.

b) Humoral and cell-mediated immunity:

Both humoral and cell mediated responses are evoked in Legionnaires' disease (Horwitz, 1983). Protective immunity can be induced with (live) Mueller-Hinton agar-passaged avirulent organisms (Blander et al., 1989), or with Legionella membranes (Blander et al., 1991). Furthermore, various L.pneumophila components have also been shown to be immunogenic in guinea

pigs, including the major secretory protein/protease (Blander and Horwitz, 1989 and 1991), and the major outer membrane protein (Zubashev et al., 1990, P. Hoffman personal communication). In addition, L.pneumophila like other intracellular bacteria, induces a significant humoral (Sampson et al., 1986) and cellular response to its 60-kilodalton heat shock protein (Hoffman et al., 1990).

Antibody production may not be entirely protective and may in fact, via Fc or complement receptors, promote the uptake of Legionella into its target cell, the alveolar macrophage (Horwitz and Silverstein, 1981a). Horwitz (1983a) was the first to recognize the importance of cell-mediated immunity in Legionella infections. Cell-mediated immunity (CMI)involves both effector cells, and the production of cytokines which regulate the effector cells. Effector cells believed to be of consequence include PMNs (Fitzgeorge et al., 1988), activated macrophages (Horwitz and Silverstein, 1981a, Bhardwaj et al., 1986), natural killer (NK) cells (Blanchard et al., 1988), lymphokine activated killer (LAK) cells (Blanchard et al., 1987b), and by implication, T cells (Friedman, 1988). These same authors have shown that interferon-γ (IFN-γ), interleukins 1 (Klein et al., 1988) and 2 (IL-1, IL-2), tumour necrosis factor (TNF; Blanchard et al.,, 1987a), and to a lesser extent IFN-α (Blanchard et al., 1985), are some of the cytokines produced in a Legionella infection. The following is an interpretation of the CMI using

the above observations. Central to the control of the infection are 1) the PMNs, since their elimination with anti-PMN serum has proven to be fatal to infected guinea pigs (Fitzgeorge et al., 1988), and 2) macrophages activated by IFN-τ (Bhardwaj et al., 1986, Nash et al., 1988). Thus during an infection, Legionella infects macrophages where it grows and results in the lysis of the host cell. T cells by interacting with macrophages and L.pneumophila (antigen), are stimulated to produce IL-2 and IFN-τ. IL-2 can activate NK cells to become LAK cells (Blanchard et al., 1987b). Both NK cells and LAK cells can then lyse infected-macrophage targets (Blanchard et al., 1987b) causing the release of the bacteria. IFN-τ, which is also produced by NK cells, can augment NK activity (Blanchard et al., 1985 and 1988). Equally or perhaps more importantly, IFN-τ by activating macrophages restricts the growth of the bacteria growing within (Bhardwaj et al., 1986, Nash et al., 1988). Macrophages themselves when stimulated with antigen, produce an array of cytokines which include IL-1, TNF, and IFN-α (Klein et al., 1988, Blanchard et al., 1987a, Blanchard et al., 1985). These cytokines further augment the bactericidal capacity of the PMN. Any perturbation in this network could conceivably contribute to increased intracellular growth and survival of L.pneumophila, thus resulting in a more severe disease.

VIRULENCE

Although there are at least 30 species of Legionella,

comprising almost 50 serogroups (Thacker et al., 1989, Bornstein et al., 1989), the majority of the cases of Legionnaires' disease are caused by L.pneumophila serogroup 1 (Reingold et al., 1984). Legionellae are facultative intracellular parasites that reside within the phagosomes of alveolar macrophages. Therefore candidate virulence factors would include not only factors which might allow the bacteria to directly cause the damage that is seen in the lungs, but also those factors which would permit the bacteria to invade, survive and grow intracellularly. Thus, since virulence is likely to be multifactorial, an organism lacking any one, or a combination of the factors, should be compromised in its capacity to cause disease.

In the laboratory, the virulence of an isolate of L.pneumophila is often measured by determining the 50 percent lethal dose (LD_{50}) in guinea pigs, the animal model of Legionnaires' disease. Embryonated eggs, tissue culture techniques, and to a lesser extent, growth in protozoa have also substituted for the animals. What follows is a brief description of the model systems of virulence and a discussion of the virulence factors themselves. Virulence factors pertaining to intracellular survival strategies will be examined in greater detail in a subsequent section.

Virulence models

Legionellae can infect numerous animal species with varying degrees of severity. These include hamsters, some

strains of mice, rats and monkeys (Baskerville et al., 1983, Collins, 1986, Katz and Poropatich, 1986). The most accepted and widely used animal model is the guinea pig. Usually male Hartley, or female Dunkin-Hartley guinea pigs are used. The inoculum is administered either intraperitoneally, intratracheally, or by aerosol exposure (Fitzgeorge et al., 1983, Katz and Hashemi, 1982, Davis et al., 1983, Winn et al., 1982), with the latter being most effective as demonstrated by comparisons of LD_{50} values (Fitzgeorge et al., 1983). The susceptible animals display such signs as high fever, ruffled fur, laboured respirations and diarrhea (Davis et al., 1982). In the respiratory models, the resultant pathology is quite similar to that seen in humans (Baskerville et al., 1983, Davis et al., 1983, Friedman et al., 1988). Animals inoculated intraperitoneally however, develop peritonitis (Chandler et al., 1979, Katz and Hashemi, 1982), which leads to a disseminated pulmonary infection. As well, the pulmonary lesions themselves appear to originate in the interstitium before spreading to the alveolar spaces (Chandler et al., 1979). The route of infection may also dictate the immune response of the animals (Eisenstein et al., 1984). In this regard, immunization of animals with killed organisms via the intraperitoneal route failed to protect animals against aerosol infections.

While the use of the animal model is paramount in the study of pathogenesis, the costs and availability of suitable

facilities has made alternatives more attractive, especially when trying to assess the "virulence" (i.e. LD_{50}) of a strain. Alternatives to the use of animals are embryonated chicken eggs, either freshly isolated monocytes or tissue culture cells, and protozoan cultures.

With respect to the use of eggs (McDade and Shepard, 1979), usually the yolk sacs of 6-day old embryos are infected and the eggs are candled daily for 12 days for signs of inactivity (death). As is the case with the animals, compared with avirulent or less virulent organisms, virulent organisms have a lower fifty percent infectivity dose (EID_{50}).

Confirming the observations made upon autopsy of both human and guinea pig victims of Legionnaires' disease, Horwitz and Silverstein (1980) demonstrated that Legionellae can infect, and multiply within monocytes isolated from human peripheral blood. These studies have been extended to include explanted alveolar macrophages from humans (Nash et al., 1984), monkeys (Kishimoto et al., 1979, Jacobs et al., 1984), and guinea pigs (Yoshida et al., 1987), as well as the U937 (Pearlman et al., 1988) and HL60 (Marra et al., 1990) monocyte/macrophage cell lines. Non-professional-phagocyte cell lines also support the growth of Legionellae. There have been several studies showing that L.pneumophila can infect tissue culture cells such as HeLa (Daisy et al., 1981, Dreyfus, 1987), human embryonic lung fibroblasts (Wong et al., 1981), primary chick embryo cells (Ormsbee et al., 1981),

McCoy, MRC-5, HEp-2 and Vero cells (Daisy et al., 1981, Oldham and Rodgers, 1985), rat alveolar type II cells (Mody et al., 1989) and what will be discussed later in this thesis, L929 cells (Fernandez et al., 1989). It should be noted that L.pneumophila does not multiply in tissue culture media (Horwitz and Silverstein, 1980). In the cell culture models, virulence is assessed by enumerating the intracellular bacteria released at various time points usually but not always following the deliberate lysis of the infected host cells. Alternatively, host cells are examined for signs of focal or generalized cytopathic effects which are manifested by the intracellular growth of the bacteria. In general, the greater the degree of host cell destruction, and by corollary, the greater the number of bacteria in the lysate, the more virulent the organism.

The protozoan model for virulence is quite similar to the cellular models described above, except in this case a measure of the intracellular growth is obtained by daily plating out aliquots of the Legionella-protozoa co-culture to determine the number of colony forming units (Fields et al., 1986).

Virulence differences amongst isolates

An organism is considered virulent if it elicits disease in humans or in the animal counterpart(s). The predominance of one strain of the Legionellae, specifically L.pneumophila serogroup 1 in causing disease despite the ubiquity of the Legionellae, suggests that some environmental isolates are

more virulent than others. Indeed, reports by Brown et al. (1982) and Plouffe et al. (1983) support this contention. Brown et al.(1982) separated organisms obtained from hospital plumbing fixtures into two groups, one harbouring an 80-megadalton plasmid and the other being plasmidless. When the plasmid profiles of the clinical isolates were determined, they were all found to be plasmidless. Plouffe et al. (1983) discovered that isolates UH-1 and RH-1 from 2 different hospital buildings were associated with very different attack rates despite being isolated in equal numbers. The higher-attack-rate UH-1 strain also had a lower LD_{50} (Bollin et al., 1985) and was more resistant to the killing effects of serum (Plouffe et al., 1985).

The virulence of an isolate can also be affected by serial passage on Mueller-Hinton (M-H) agar that has been supplemented with hemoglobin and Isovitale X (McDade and Shepard, 1979). As few as 5 passages of a virulent organism on M-H agar (supplemented with ferric pyrophosphate and cysteine) is sufficient to render it avirulent (Elliott and Johnson, 1982). The mechanism for this attenuation, molecular or otherwise, is not completely clear but it probably involves sodium chloride which is present in relatively high amounts in M-H agar (Catrenich and Johnson, 1989, Feeley et al., 1978). Avirulent organisms appear to be more resistant to the inhibitory effects of the salt. Contrary to initial reports that the conversion of virulent bacteria to avirulent forms

was reversible by passaging in guinea pigs (Elliott and Johnson, 1982), eggs (Lochner et al., 1983), or tissue culture cells (Wong et al., 1981), Catrenich and Johnson (1988) have argued that the virulence to avirulence conversion is a one-way phenomenon and that the M-H agar selects for pre-existing avirulent forms. Others (Blander et al., 1989) have also failed in their attempts to recover virulent organisms by passaging avirulent ones in guinea pigs.

Virulence markers

Except for the ability to multiply intracellularly, there has been little success in ascribing a conveniently monitored phenotype to virulent strains. For example, colony morphology, lipopolysaccharide patterns, and the presence of certain plasmids including an 80-megadalton plasmid, have not discriminated between virulent and avirulent organisms (Horwitz, 1987, Cianciotto et al., 1989a).

Nowicki et al. (1987) have suggested that virulence is proportional to the length of the organisms, with shorter rods being more virulent than the longer filamentous forms. However, in this study the changes in the lengths of the organisms were imparted by the various types of media used and may not necessarily correlate with alternate methods of producing avirulent mutants.

There have been encouraging reports which suggest that reactivity with the monoclonal antibody mAb2 is a useful virulence marker (Joly and Winn, 1984, Watkins et al., 1985,

Dournon et al., 1988, Stout et al., 1988). However, despite the epidemiological correlation of mAb2 reactivity with virulence, these results should be interpreted with caution. First, even in the above studies, not all clinical isolates react with mAb2. Secondly, acid and heat treatment which are sometimes used when isolating Legionellae have been shown to affect the numbers of organisms isolated belonging to subgroups Philadelphia and OLDA (Harrison et al., 1990). Acid treatment results in increased numbers of the mAb2 positive Philadelphia subgroup, whereas heat treatment results in increased numbers of the mAb2 negative OLDA isolates. Temperature can also affect reactivity with other mAbs. Edelstein et al. (1987) have shown that a serogroup 1 isolate when grown at 25°C lacks reactivity with mAbs W29 and JR5. The same isolate grown at 41°C not only reacts well with mAbs W29 and JR5, it is also found to be less virulent than the 25°C-grown organisms.

Although there is some preliminary evidence that compared to mAb2 negative isolates, mAb2 positive strains are more virulent -by virtue of their lower LD_{50} values (Molina et al., 1987), their ability to resist serum killing and by their capacity to grow intracellularly (Dournon and Rajagopalan, 1987), these experiments have been done with only a few isolates. Furthermore, and perhaps most importantly, the concomitant loss of the mAb2 phenotype with a loss of virulence has not been demonstrated.

Possible virulence factors

Infection with L.pneumophila most likely initiates with the inhalation of infectious particles. A particle size of approximately 5 μm (Fitzgeorge et al., 1983) is important, as is a relative humidity of 60% (Hambleton, et al., 1983). Dennis and Lee (1988) have also suggested that the more virulent organisms are better able to survive in aerosols.

L.pneumophila produces various extracellular enzymes (Thorpe and Miller, 1981, Baine et al., 1979), and it has been hypothesized that some of these may be responsible for the cytopathology resulting from the lysis of the inflammatory cells infiltrating the lungs of individuals suffering from Legionnaires' disease (Baine et al.,1979). Of the various extracellular enzymes, the most extensively studied are the cytotoxin(s), the hemolysin(s) and the protease(s). It should be noted that it is the activity of a protein that defines its identity and as such, different proteins may have the same name. And by corollary, different names may be used for the same protein.

Cytotoxin(s)

Cytotoxic activity as distinct from hemolysis, is usually defined by the ability of the toxin to damage indicator cells such as Chinese Hamster Ovary (CHO), or VERO (African green monkey kidney) cells. Friedman et al. (1980), demonstrated that culture filtrates of L.pneumophila grown in a complex liquid medium were cytotoxic for CHO cells. This cytotoxin was

found to be a heat stable, methanol soluble protein having a molecular weight of approximately 1,300 Daltons and being stable over a pH range of 5 to 8 (Friedman et al., 1980, Friedman et al., 1982). Hexose monophosphate shunt activity and oxygen consumption, both indicators of the respiratory burst associated with phagocyte activation, were reduced in toxin-treated PMNs suggesting that the proposed action of the toxin is to impair the function of PMNs in terms of their ability to kill bacteria (Friedman et al., 1982).

As will be discussed below, cytotoxic activity can also be manifested by the main protease produced by L.pneumophila.

Protease(s)

As evidenced by its various names the main protease is perhaps the most extensively studied protein secreted by L.pneumophila. It is called extracellular protease (Thompson, et al., 1981, Dreyfus and Iglewski, 1986), tissue destructive protease or TDP (Baskerville et al.,1986), phenylalanineaminopeptidase (Gul'nik et al., 1986), major secretory protein or MSP (Blander and Horwitz, 1989), zinc metalloprotease (Quinn and Tompkins, 1989) and cytolysin (Belyi, 1990).

The protease, in a crude i.e. culture filtrate, or purified form has activity against casein, gelatin, and collagen (Thompson, et al., 1981, Conlan, et al., 1986). In addition, suspensions of L.pneumophila as well as purified TDP have been shown to degrade some serum proteins including the

serine protease inhibitors α_1-antichymotrypsin (Müller, 1980) and α_1-antitrypsin (Conlan, et al., 1988).

The purified protease also has a cytotoxic effect on CHO cells (Keen and Hoffman, 1989, Rosenfeld et al., 1986) and dermal ulcerative activity in guinea pigs (Rosenfeld et al., 1986). In addition, it is reported to have hemolytic activity on canine and guinea pig erythrocytes (Keen and Hoffman, 1989).

Purified protease has a molecular weight of 38 kilodaltons (kDa; Dreyfus and Iglewski, 1986, Conlan et al., 1986). Its activity depends on the presence of several metal ions including Zn^{++}, and is not inhibited by serine protease inhibitors such phenylmethylsulfonyl fluoride (PMSF, Dreyfus and Iglewski, 1986, Thompson, et al., 1981). It is sensitive to temperatures above 40°C and is effective over a pH range of between 5 and 8.5 although optimal activity was observed between pH 6 and 6.5 (Dreyfus and Iglewski, 1986). The gene for the protease has recently been cloned and sequenced (Quinn and Tompkins, 1989, Black et al., 1990). Based on the sequence data, it has been shown to be structurally and functionally homologous to the elastase protein of Pseudomonas aeruginosa (Black et al., 1990) and these authors have suggested that the two proteins may have a similar mechanism of action. It should be noted however, that other investigators have failed to show elastase activity with the Legionella protease (Thompson, et al., 1981, Conlan, et al., 1986, Baskerville et al., 1986).

The role for the protease in pathogenesis is subject to debate. Baskerville and co-workers (1986) demonstrated that intranasal or intratracheal administration of purified TDP to guinea pigs resulted in the production of lesions similar to, if not slightly more severe than those observed in experimental Legionnaires' disease; heat-inactivated protease had no effect. In another experiment, equivalent amounts of TDP were observed in the lungs of animals infected with L.pneumophila as detected by enzyme immunoassay and immunoblots of supernatant material from macerated lung tissue (Conlan et al., 1988). Furthermore, immunocytochemical techniques used at the light and electron microscopic level have revealed the presence of large amounts of the protease in conjunction with L.pneumophila in or near the lesions in the lungs of infected animals (Williams et al., 1987). Contrary to these findings, two recent lines of evidence have indicated that the protease is not required for virulence. First, avirulent organisms obtained by serial passage on M-H agar were found to produce, as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), sufficient amounts of the protease despite being unable to replicate in monocytes (Horwitz, 1987) and cause disease in guinea pigs (Blander et al., 1989). And second, mutant organisms incapable of producing the protease because of the insertion of a transposon (Tn9) in the structural gene for the protease, were found to be just as capable of intracellular

growth as were virulent organisms (Szeto and Shuman, 1990). Blander et al. (1990), by examining LD_{50} values and pulmonary pathology confirmed these results in guinea pigs.

From the host's perspective, the protease is immunogenic and Blander and Horwitz (1989) have demonstrated that it can be used to induce cell-mediated and protective immunity.

Finally, as evaluated by the actions of products from wild-type and protease-deficient mutants, the protease may have a role in inhibiting both chemotaxis and superoxide anion generation in PMNs (Sahney et al., 1990).

Notwithstanding its uncertain role in virulence, the protease might simply function to provide the organism with a source of amino acids which it requires for growth (Müller, 1980, George et al., 1980).

Hemolysin

Hemolytic activity is usually measured by the ability of the toxin to lyse erythrocytes (Baine et al.,1979). It appears that the species and age of the erythrocytes can influence the reaction (Baine et al., 1979, Thorpe and Miller, 1984, Keen and Hoffman, 1989). It was generally accepted that the hemolysis that is observed on canine erythrocytes is due to the effects of the above mentioned protease (Keen and Hoffman, 1989, Quinn and Tompkins, 1989, Szeto and Shuman, 1990). However, it is of interest that a recently-identified protein of similar molecular weight called legiolysin, has been found to have hemolytic but not proteolytic activity (Wintermeyer

et al., 1991). The gene encoding legiolysin has been cloned (Wintermeyer et al., 1991). According to these authors, based on the restriction map and the as yet unpublished sequence, legiolysin is genotypically and phenotypically distinct from the protease (Rdest et al., 1991).

A different cytolytic toxin has been identified and characterized by Baine (1985 and 1988). It was isolated from L.pneumophila serogroup 5 and has phospholipase C activity (Baine, 1985). This 50 to 55 kDa protein hydrolyzes the eukaryotic membrane component phosphotidylcholine/lecithin, to diacylglycerol and phosphoryl choline thus serving to increase the fragility of the target cells (Baine, 1988). The higher the phosphotidylcholine content, the more susceptible the cell is to lysis (Baine, 1985). Activity was found at pH values ranging from 6.5 to 8.5 with the greatest activity at the latter pH. And unlike cations such as Mg^{2+} and Ca^{2+} which are required for activity, Zn^{2+}, Fe^{2+}, and Co^{2+} were found to be inhibitory (Baine, 1988).

Surface proteins possibly involved in virulence

Aside from the elaboration of toxins, factors which allow organisms to grow within and colonize the host are of equal or greater importance. Many of these factors usually include surface proteins or structural appendages. Although Legionellae have flagella (Rodgers et al., 1979, Chandler et al., 1980), they have not been implicated in pathogenesis. Legionella surface proteins which have been identified and

associated with virulence are the major outer membrane protein (MOMP; Bellinger-Kawahara and Horwitz, 1990) and the macrophage infectivity potentiating (mip) protein (Cianciotto et al., 1989b).

Two other proteins whose roles in virulence are yet to be fully understood, are the 60-kDa heat shock protein (Hoffman et al., 1989, Hoffman et al., 1990, Steinmetz et al., 1991) and a 19-kDa peptidoglycan-associated protein (Engleberg et al., 1986, Hindahl and Iglewski, 1987, and Ludwig et al., 1991, Engleberg et al., 1991).

Major outer membrane protein

The Legionella major outer membrane protein, MOMP is a 29-kDa protein (Ehret et al., 1984, Hindahl and Iglewski, 1984, Gabay and Horwitz, 1985) that functions as a porin (Gabay et al., 1985). The protein forms large oligomers (Hindahl and Iglewski, 1984, Gabay et al., 1985) of approximately 100 kDa (Butler et al., 1985, Butler and Hoffman, 1990) under non-reducing conditions. The porin tightly associates with peptidoglycan (Hindahl and Iglewski, 1984) by means of an anchor protein (Butler and Hoffman, 1990) which has since been identified as a MOMP subunit (Hoffman et al., 1991b). The Legionella MOMP is unusual in that it contains cysteine and is highly cross-linked by di-sulfide bonds (Butler et al., 1985), a feature that is shared with another intracellular organism, Chlamydia (Newhall and Jones, 1983, Hatch et al., 1984), but is uncommon in most other Gram

negative bacteria. Despite considerable effort (Hindahl and Iglewski, 1987, Ludwig et al., 1991), the gene encoding MOMP has only recently been cloned and sequenced (Hoffman et al., 1991a).

As a virulence factor, it has been suggested that L.pneumophila uses its MOMP to gain entry into its target cell, the alveolar macrophage (Bellinger-Kawahara and Horwitz, 1990, Payne and Horwitz, 1987). These authors have shown by immunoblot techniques using anti-complement component C3 antibodies to probe blots sometimes pre-incubated with fresh serum, and containing electrophoresed samples of either purified MOMP, pre-opsonized whole bacteria or MOMP-liposomes, that MOMP can bind serum complement component C3 (Bellinger-Kawahara and Horwitz, 1990). In separate experiments performed using fresh, non-immune serum, it had been shown that the attachment to and subsequent uptake of L.pneumophila by monocytes pre-treated with monoclonal antibodies to complement receptors CR1 and CR3, was diminished (Payne and Horwitz, 1987). CR1 and CR3 are receptors for complement components C3b and C3bi respectively, and are members of the integrin family (Hynes, 1987).

Thus, it appears that the attachment of L.pneumophila to macrophages is mediated by the binding of C3 to MOMP followed by the binding of the L.pneumophila-C3 complex to macrophage complement receptors, most probably by arginine, glycine, aspartic acid (RGD) sequences on the ligand (Wright et al.,

1987). It is noteworthy that phagocytosis occuring by the binding of ligands to complement receptors seems to circumvent the activation of macrophages that leads to the production of toxic oxygen intermediates (Wright and Silverstein, 1983).

Macrophage infectivity potentiating protein

In an effort to apply a molecular approach to studying Legionella virulence, Engleberg and co-workers identified and cloned in Escherichia coli several proteins which are expressed on the surface of L.pneumophila (Engleberg et al., 1984, Pearlman et al., 1985, Engleberg et al., 1986). One of these proteins has since been identified as macrophage infectivity potentiating, or Mip protein (Cianciotto et al., 1989b). The name was derived from observations that a *mip*- mutant compared to the isogenic parent organism, was less able to survive the early stages of infection following uptake into the macrophage-like U937 cell line (Cianciotto et al., 1989b). The *mip* mutant, constructed by allelic exchange where a functional mip gene was replaced by a disrupted form, was found to be 80-fold less efficient in its ability to initiate infection of U937 cells. In other words, the inoculum containing the mutant had to be increased 80-fold in order to achieve the same degree of infectivity as the parent strain. However once intracellular, the mutant displayed similar growth kinetics as the isogenic parent organism. The virulence of the *mip* mutant was also found to be attenuated in guinea pigs infected intratracheally (Cianciotto et al., 1990b).

Complementation of the mutant organism with a functional *mip* gene successfully restored the virulent phenotype (Cianciotto et al., 1989b and 1990b).

The *mip* gene has been sequenced (Engleberg et al., 1989). DNA hybridization using high stringency has revealed its presence in all L.pneumophila serogroups; reducing the stringency resulted in the hybridization of all other species of Legionella, suggesting the presence of *mip*-like genes in the genus (Cianciotto et al., 1990a). Although at sufficiently high stringencies *mip* does not hybridize to DNA from several other bacteria (Cianciotto et al., 1990a), a similar gene (protein) has recently been identified in Chlamydia trachomatis (Lundemose et al., 1991).

The Mip protein has a molecular weight of about 24 kDa as determined by SDS-PAGE and as inferred by the nucleotide sequence (Engleberg et al., 1989). Despite being similar in molecular weight, the protein is distinct from MOMP (Engleberg et al., 1989, Hoffman et al., 1991a). Mip is composed of 233 amino acids, does not have any cysteines and is a very basic protein with an isoelectric point (pI) of 9.8 (Engleberg et al., 1989).

Interestingly, the carboxy-terminal (residues 120-233) of Mip has 39% identity with the FK506 (an immunosuppressive drug)-binding protein of Neurospora crassa (Tropschug et al., 1990). Human FK506-binding protein has 56% identity at its carboxy-end with the inferred amino acid sequence of an open

reading frame in the genome of Neisseria meningitidis (Standaert et al., 1990). The FK506-binding protein has been shown to have peptidyl-prolyl isomerase activity (Tropschug et al., 1990). Peptidyl-prolyl-*cis-trans*-isomerases, also known as rotamases, are involved in catalyzing slow protein folding reactions (Liu and Walsh, 1990, Tropschug et al.,1990). In E.coli, a similar rotamase is found in the periplasm and it has been postulated that rotamases may be involved in the refolding of secreted proteins (Liu and Walsh, 1990). Whether Mip functions as a rotamase to affect virulence is not known.

Heat shock or stress proteins

Convalescent serum obtained from patients recovering from Legionnaires' disease identified amongst other proteins, a 58-60-kDa protein that was common to all Legionella species as well as several other bacteria (Sampson et al., 1986, Plikaytis et al., 1987). The purified protein had a molecular weight of 650 kDa based on size exclusion chromatography and it could be resolved by SDS-PAGE into a single band with a molecular weight of 60 kDa (Pau et al., 1988). The protein was identified as a heat shock protein (Hsp; Lema et al., 1988), and its gene was subsequently cloned (Hoffman et al., 1989) and sequenced (Hoffman et al., 1990, Sampson, et al., 1990). The gene has been designated *htpB* (Hoffman et al., 1989) and together with another gene *htpA*, forms the heat shock operon *htpAB* of the groEL class of heat shock proteins

(Hoffman et al., 1990). Based on the deduced amino acid sequence, the 60-kDa Hsp shows a high degree of identity to other Hsp's including the E.coli groEL protein (85%), the 65-kDa Mycobacterium tuberculosis and M.leprae proteins (76%), the HtpB of Coxiella burnetii (85%) and to a lesser extent (50%), the 58-kDa protein from Chlamydia (Hoffman et al., 1990).

As reviewed by Lindquist and Craig (1988), the Hsp's are families of highly conserved proteins which are present in eukaryotes and bacteria. Although present constitutively, they can be induced to be expressed at high levels upon various stresses which include heat and anoxia. GroEL forms one of the families of Hsp's. GroEL is said to be a molecular chaperone or chaperonin because it seems to be involved in the assembly of oligomers, and in directing protein folding and protein secretion -all functions that rely on protein-protein interactions (Rothman, 1989). Induction of Hsp's upon stress, may be a response to increased levels of denatured or improperly folded proteins resulting from the stress. The targetting sequences or motifs recognized by GroEL appear to be linear sequences of amino acids that have a tendency to form α-helices (Landry and Gierasch, 1991). Release of GroEL from target proteins involves another Hsp GroES, and the hydrolysis of ATP (Rothman, 1989). Hsp's, especially the GroEL-equivalent, Mycobacterium 65-kDa protein have also been shown to be immunodominant antigens capable of eliciting

strong humoral and cell-mediated immune (CMI) responses (Shinnick, 1991).

Even under non-induced conditions, the Legionella 60-kDa Hsp is present abundantly (Hoffman et al., 1989). It is located primarily in the cytoplasm (Steinmetz et al., 1991), and possibly in the periplasm (Hoffman et al., 1990). Steinmetz et al. (1991) have further demonstrated that the 60-kDa Hsp protein can also be found on the bacterial surface, albeit at much lower levels.

So far, there has been no direct evidence implicating the Legionella 60-kDa Hsp as a virulence factor. However, Hoffman et al. (1990) have detected by immunofluorescence, increased levels of the 60-kDa Hsp in virulent L.pneumophila infecting HeLa cells suggesting that the intracellular environment was somehow contributing to this response; avirulent bacteria showed reduced levels of this protein. Since a similar response was also seen in bacteria simply placed in Dulbecco modified Eagle medium (DMEM), it was hypothesized that the avirulent strain was less capable of sensing and responding to its environment.

Peptidoglycan-associated protein

First recognized as a 19-kDa surface-expressed protein by Engleberg et al. (1986), the gene encoding this protein, termed peptidoglycan-associated protein of L.pneumophila or Ppl, has been cloned (Hindahl and Iglewski, 1987, Ludwig et al., 1991, Engleberg et al., 1991), and sequenced (Ludwig et

al., 1991, Engleberg et al., 1991). Based on the predicted amino acid sequence, Ppl shows a high degree of identity to the peptidogylcan-associated lipoproteins (Pal) of E.coli and H.influenzae (Ludwig et al., 1991, Engleberg et al., 1991). Its role in virulence has not been established.

Other proteins

Other proteins which may act as virulence factors include a 68-kDa acid phosphatase identified in L.micdadei which may act by interfering with neutrophil activation through the hydrolysis of phosphotidylinositol 4,5 bisphosphate (Saha et al., 1985 and 1988), a yet to be characterized putative iron reductase which would facilitate the necessary acquisition of iron (Johnson et al., 1991) in lieu of siderophores which are absent in Legionella (Reeves et al., 1983), and enzymes such as peroxidase, superoxide dismutase and in non-pneumophila species, catalase (Pine et al., 1984) which may function to somewhat neutralize the damaging effects of the microbicidal arsenal of the phagocytic cells.

INTRACELLULAR GROWTH

As outlined, the results from the investigations seeking to identify virulence factors associated with Legionnaires' disease have met with limited success. Several potential candidates have been suggested, but in many cases, experiments using mutant organisms lacking the trait in question have either not been done or have yielded less than favourable results implicating the factor as being essential for

virulence. For some putative factors such as the 60-kDa heat shock protein, the results are less clear and must await further experimentation. What is implicated as a virulence factor is the ability of the organism to gain access into and survive and multiply within the alveolar macrophages. In this regard, both the MOMP and Mip proteins appear to contribute to some degree, to the entry and initial survival of L.pneumophila.

The evidence depicting L.pneumophila as an intracellular pathogen of monocyte/macrophages was basically demonstrated by studies by Horwitz and co-investigators. Extending the previously discussed observations that the inflammatory exudate in the alveoli of lung material obtained from cases of Legionnaires' disease contained both PMNs and macrophages that were filled with L.pneumophila, Horwitz in a series of communications reported that peripheral blood monocytes i.e. adherent cells (Horwitz and Silverstein, 1980) and not PMNs (Horwitz and Silverstein, 1981c) supported the growth of L.pneumophila. The organisms did not multiply in tissue culture medium, nor did they multiply in the presence of sonicated monocytes, indicating the requirement for intact cells (Horwitz and Silverstein, 1980). The capacity of L.pneumophila to multiply intracellularly has since been confirmed by several workers using various types of host cells (see previous section).

Intracellular parasitism is not unique to Legionella.

Despite what is most likely to be an inhospitable environment, several bacteria including Salmonella, Shigella, Yersinia, Mycobacteria, Chlamydia, Coxiella and Listeria all prefer, or seem to have adapted to intracellular lifestyles (Moulder, 1985, Finlay and Falkow, 1989). The following section will consider the various strategies that are used by L.pneumophila and other organisms to survive and grow intracellularly. The mechanisms as they are known will be considered in the context of the specific steps involved in the invasion process.

Entry

In general, as summarized by Finlay and Falkow (1989), Isberg (1991) and Falkow (1991), while some pathogens attach to the surfaces of cells where they proceed with the colonization process, others invade and establish themselves intracellularly. Although the mechanisms are not fully understood, initial studies with Yersinia pseudotuberculosis (Isberg et al., 1987) and most recently with Listeria monocytogenes (Gaillard et al., 1991) have indicated that these organisms use specific but unrelated surface proteins for invasion. These proteins have been named invasin and internalin respectively. As outlined by Isberg (1991), genes encoding an invasion phenotype have also been identified in Yersinia entercolitica, Salmonella, enteropathogenic Escherichia coli and Shigella. The most thoroughly studied of the invasion proteins is invasin. The mammalian cell surface receptors for invasin are integrins in the β_1 family (Isberg

and Leong, 1990). Integrins as mentioned previously, are cell surface proteins which are involved in adhesion (Hynes, 1987). It has been proposed that their function is to integrate or communicate with both extracellular and intracellular proteins, hence the name (Hynes, 1987).

Another mode of entry exploits the host cell's complement receptors (Falkow, 1991). This is a particularly cunning strategy, since the potentially lethal oxidative burst is not triggered when complement receptors are ligated (Wright and Silverstein, 1983). While some pathogens such Leishmania bind complement receptor CR3 directly (Russell and Talamas-Rohana, 1989), others like the Mycobacteria bind to complement receptors only after first binding complement component C3bi (Schlesinger et al., 1990). Studies by Horwitz suggest that L.pneumophila falls in the latter category. As previously discussed, L.pneumophila apparently binds C3b and C3bi to its major outer membrane protein (MOMP) and then uses CR1 and CR3, both $ß_2$-type integrins (Hynes, 1987), to enter macrophages (Payne and Horwitz, 1987, Bellinger-Kawahara and Horwitz, 1990). However it should be noted that even in the presence of fresh non-immune serum (a source of complement), pretreating monocytes with monoclonal antibodies to complement receptors CR1 and CR3, failed to completely inhibit binding of L.pneumophila. The inhibition of adherence was at best, 75% (Payne and Horwitz, 1987). Of course, in the presence of specific antibody, uptake occurs (phagocytosis) and is assumed

to be mediated by antibody-Fc receptors (Payne and Horwitz, 1987) as well as by complement receptors.

In HeLa cells infected with L.pneumophila, uptake apparently can occur in the absence of immune serum or complement (Dreyfus, 1987). Similar observations were made using other non-professional phagocyte cell lines (Daisy et al., 1981, Oldham and Rodgers, 1985), and with guinea pig and rat alveolar macrophages (Elliot and Winn, 1986), human phagocytes (Rechnitzer and Blom, 1989), and the U937 monocyte cell line (King et al., 1991). In these instances neither the bacterial ligands nor the host cell surface receptors have been identified.

As exemplified by Yersinia and discussed by Isberg (1990), pathogens are likely to have multiple routes of entry into host cells. L.pneumophila is probably no exception.

Associated with invasion is the importance of actin polymerization; pretreating host cells with cytochalasin D, a potent inhibitor of actin polymerization and phagocytosis, abrogates the invasion of many bacteria in both professional and non-professional phagocytes (Clerc and Sansonetti, 1987, Falkow, 1991, Finlay and Falkow, 1988, Finlay and Falkow, 1989). Pretreating macrophages or U937 cells with cytochalasin D also inhibits uptake of L.pneumophila (Elliott and Winn, 1986, King et al., 1991). Methylamine, an inhibitor of receptor-mediated pinocytosis was similarly found to affect uptake (King et al., 1991).

Intracellular survival

Following entry, many organisms are found within a phagosome (Moulder, 1985). Actin filaments have been observed to surround Salmonella (Finlay et al., 1989), and Shigella (Clerc and Sansonetti, 1987). While actin may be involved in propelling Shigella about the cell (Bernardini et al., 1989), the significance of this with respect to Salmonella, is presently unknown.

Upon entry into a host cell, an invading organism might encounter intracellular defences which could be detrimental. Aside from the products of the oxidative burst, the organism could face the acid hydrolases/proteases sequestered within the confines of the lysosome (Moulder, 1985). Intracellular pathogens counter and survive by either escaping from the phagosome, by surviving within the phagolysosome, or by preventing fusion of phagosomes with lysosomes (Moulder, 1985).

Organisms such as Listeria and Shigella produce thiol-activated toxins which are homologous to streptolysin O and pneumolysin (Mengaud et al., 1987). While not required for entry, these toxins function to lyse the phagocytic membrane thereby causing the liberation of the organisms to the cytoplasm (Sansonetti et al., 1986, Gaillard et al., 1987). Once in the cytoplasm, the bacteria multiply, unimpeded.

Organisms which are capable of surviving within phagolysosomes include Coxiella and Leishmania (Moulder, 1985,

Hall and Joiner, 1991). These organisms have either adapted metabolically to the microenvironment of the lysosome or have developed mechanisms to resist the powerful enzymes of the lysosomes (Moulder, 1985, Hall and Joiner, 1991).

Examples of pathogens shown to inhibit the fusion of the pathogen-bearing phagosome with lysosomes include Chlamydia, Toxoplasma, Mycobacteria, and Salmonella (Moulder, 1985, Buchmeier and Heffron, 1991). Horwitz (1983) showed by electron microscopy that in contrast to formalin-killed organisms, live L.pneumophila could also inhibit phagosome-lysosome fusion. The inhibition of fusion was overcome somewhat by either pretreating organisms with antibody or by activating monocytes with concanavalin A (Horwitz, 1983). By using fluorescein conjugated bacteria, Horwitz and Maxfield (1984) further demonstrated that phagosomes containing live L.pneumophila had a higher pH than those containing killed bacteria (6.1 versus 5.4 respectively). Although other organisms such as Toxoplasma also fail to lower the pH of their vacuoles (Hall and Joiner, 1991), it is unclear as to whether phagosome acidification is a prerequisite for fusion with lysosomes (Finlay and Falkow, 1988, Melman et al., 1986). As with the above organisms, the phagosome provides L.pneumophila with a haven for unrestricted growth.

Despite the recent report by Joiner et al. (1990), which demonstrated fusion may be dependent on the route of entry of T.gondii, the actual mechanisms enabling organisms to inhibit

the fusion of phagosomes with lysosomes are unknown.

OBJECTIVE OF THE THESIS

The objective of this thesis is to investigate the intracellular pathogenesis of Legionella pneumophila by:

1) examining L.pneumophila infection in various cell lines
2) comparing virulent and avirulent organisms for their respective abilities to survive intracellularly
3) examining the protein profiles of virulent and avirulent organisms before and after entry into host cells.

MATERIALS AND METHODS

Media

Minimum essential medium (MEM) and RPMI 1640 medium (RPMI) were obtained from Gibco BRL (Burlington, Ontario) and were prepared according to the manufacturers' instructions. MEM and RPMI lacking methionine were purchased from Sigma Chemical Company (St. Louis, Mo.) and were similarly prepared. Buffered charcoal-yeast extract (BCYE) agar plates and buffered yeast extract (BYE) broth were generously provided by the Department of Microbiology at the Victoria General Hospital, in Halifax. The plates were made from a commercial preparation of a Legionella agar base (Difco, Baxter/Canlab, Mississauga, Ontario) which contained yeast extract, activated charcoal, N(2-acetoamido)-2 aminoethane sulfonic acid (ACES) buffer, α-ketoglutarate and Bacto agar, and to which triple distilled water, potassium hydroxide, L-cysteine-HCl and ferric pyrophosphate were added. BYE broth was made in-house using similar reagents except charcoal and agar were omitted from the preparation. Phosphate buffered saline (PBS) lacking calcium and magnesium, and Hank's balanced salt solution (HBSS) were made routinely by the media department in the Microbiology department at Dalhousie University. The components of the various media are listed in the Appendix.

Organisms

L.pneumophila isolate number 2064 (Lp2064) was obtained

from the Department of Microbiology at the Victoria General Hospital. It was originally isolated from the sputum of a patient with Legionnaires' disease. It was serologically typed as L.pneumophila serogroup 1 (Oxford) and was found to have a 20 megaDalton plasmid (G. Bezanson, personal communication). It was passaged twice on BCYE agar and stored at -70°C in 20% glycerol.

To obtain an avirulent mutant, Lp2064 was serially passaged five times on Mueller-Hinton agar supplemented with hemoglobin and IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) as described by Dreyfus (1987). At this point, a single colony designated as Lp2064M, was picked and a stock culture was prepared on BCYE agar. This was stored at -70°C as described above. (Both Lp2064 and Lp2064M were also maintained on BCYE agar.) When needed, the organisms were grown for 48 hours on BCYE agar at 37°C in a humidified CO_2 incubator.

All other L.pneumophila isolates used were also obtained from the Department of Microbiology at the Victoria General Hospital. They are listed in Table A1 in the Appendix.

Cell lines

Mouse fibroblast-like cells (L929; ATCC clone CCL1), Chinese hamster ovary cells (CHO-K1; ATCC clone CCL 61, and human monocyte-like cells (U937; ATCC number CRL 1593) were obtained from the American Type Culture Collection (Rockville, Md.). MRC-5 (human fibroblast), HEp-2 (human epithelial), and

Vero (African green monkey kidney) cells were obtained from the Department of Virology at the Victoria General Hospital. Human A549 cells (lung carcinoma) were generously provided by F. Jay, Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba. The L929, MRC-5, HEp2, and Vero cells were maintained in MEM supplemented with 10% fetal bovine serum (FBS; Flow Laboratories/ICN Biomedicals Inc., Mississauga, Ontario) that was heat-inactivated for 30 minutes at 56°C, 100 units of penicillin per mL, and 50μg of streptomycin per mL. The A549 and U937 cells were maintained under similar conditions but with RPMI 1640 medium. All cell lines were cultured in plastic flasks (Falcon, Becton Dickinson, Lincoln Park, NJ, or Costar, Cambridge, Ma.) in a 37°C, humidified incubator containing 5% CO_2. With the exception of the U937 cells, all cell lines were passaged weekly after trypsinizing the monolayers with a solution of calcium and magnesium-free PBS containing 0.25% trypsin (Gibco) and 0.25% ethylene diamine tetraacetic acid (EDTA). The U937 cells were passaged weekly by diluting the cell suspension 1:40 in fresh media.

Isolation of human monocytes

Human monocytes were isolated from the peripheral blood of consenting donors with no known history of Legionnaires' disease. As previously described (Fernandez et al., 1986), peripheral blood, collected in heparinized Vacutainer tubes (Becton-Dickinson) was diluted 1:1 in HBSS prior to layering

it on a cushion of Lymphoprep (Accurate Chemical and Scientific Corporation, Hicksville, NY). The blood was then centrifuged at room temperature at 400*g* for 30 minutes. The resultant band of mononuclear cells was aspirated, washed twice with HBSS and resuspended to a concentration of $\approx 1X10^7$ cells per mL in RPMI containing 20% heat-inactivated human AB serum. Approximately 1.5 mL of the cell suspension was then placed in each well of a 6-well plastic plate (Costar 3406). Following an overnight incubation at 37°C in a CO_2 incubator, the non-adherent cells were removed by aspiration and the remaining adherent cells representing monocytes, were washed several times, and maintained in RPMI containing 10% heat-inactivated FBS until needed.

Antibodies

Polyclonal antibodies to formalin-killed L.pneumophila were prepared in New Zealand White rabbits (obtained from a local distributor) according to methods described by McKinney et al. (1979) and Pearlman et al. (1985). The bacteria were initially killed by exposing them for a few hours to 0.5% formaldehyde (BDH, Dartmouth, Nova Scotia) in PBS. The bacteria were then pelleted, washed several times in PBS to remove the formaldehyde, and resuspended in saline to a concentration of 7×10^9 organisms per mL. To ensure that no viable organisms remained, the suspension was streaked on BCYE plates, and the plates examined for growth.

Following the procedure of McKinney et al. (1979), 2 mL

of the bacterial suspension were mixed with 2 mL of complete Freunds adjuvant (Gibco) and inoculated subcutaneously into 20 sites on the shaved back of a rabbit. After 3 weeks, a further 2 mL of the bacterial suspension was mixed with incomplete Freunds adjuvant and injected intramuscularly into the hind quarters of the same rabbit. One week later, the rabbit was given a final intravenous booster containing 2 mL of the bacterial suspension. The rabbit was bled the following week and the serum was collected and stored at -20°C. In the second procedure (Pearlman et al., 1985), 2 mL of the bacterial suspension were injected subcutaneously into the shaved back of another rabbit. This was repeated bi-weekly for 6 weeks after which the rabbit was bled and the serum collected and stored as above.

To determine the titre of the sera, a suspension of a colony of L.pneumophila was made in 1 mL of PBS and spotted onto acetone-methanol-treated epoxy-coated 10-well slides (Cell Line Associates, Newfield, NJ) for about 5 seconds and then removed. The slides were then air-dried and fixed for 10 minutes in -20°C acetone. After air drying, the slides were stored at 4°C. When needed, the fixed L.pneumophila suspensions were exposed to serial two-fold dilutions of the rabbit sera that were made in PBS. After a 30 minute incubation in a humidified chamber at 37°C, the slides were washed in PBS and exposed to a 1/20 dilution of a fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibody

(Dako, Dimension Laboratories, Mississauga, Ontario). After further incubating as described, the slides were washed in PBS, mounted in buffered glycerol (Syva Co., Palo Alto, Ca.) and viewed microscopically using epifluorescence. The anti-L.pneumophila titre, established as the reciprocal of highest dilution of serum still displaying strong fluorescence, was 2048 and 1024 for the first and second rabbit respectively.

Ammonium sulfate saturated at 33% (19.6 g per 100 mL of serum), was used to precipitate immunoglobulin G (IgG) from a portion of the serum obtained from the second rabbit. After a 2 hour precipitation at 4°C, the precipitate was pelleted and redissolved in a minimal volume of PBS. It was then dialysed first against PBS, then against distilled water, and finally against acetate buffer (4 L of distilled water containing 17.2 g sodium acetate and 84 mL of 1 M acetic acid) with a pH of 5. The contents of the dialysis tubing were then pelleted to remove the precipitated lipoproteins, and the supernatant dialysed against several changes of PBS before aliquotting and storing at -20°C.

Other antibodies used were obtained as follows. A hybridoma cell culture supernatant containing monoclonal antibody GW2X4B8B2H6 which recognizes the L.pneumophila 60-kDa heat shock protein (Helsel et al., 1988, Hoffman et al., 1989) was a generous gift from P. Hoffman, Dalhousie University. Affinity-purified, biotinylated, rabbit anti-mouse IgG antibodies, affinity-purified, biotinylated goat anti-

rabbit IgG antibodies and horse radish peroxidase (HRP)-conjugated streptavidin were purchased from Jackson Immunoresearch Laboratories (Bio/Can Scientific Inc., Mississauga, Ontario). Freeze-dried reagents were reconstituted as directed by the manufacturer, aliquotted, and stored at -20°C.

Virulence determination of Lp2064 and Lp2064M

Forty-eight-hour-cultures of L.pneumophila were scraped off BCYE plates with a bent Pasteur pipette and resuspended in either MEM, RPMI or saline to an OD_{660} of about 0.75. Unless otherwise indicated, ten-fold dilutions of Lp2064 or Lp2064M ranging from approximately 10^9 to 10^3 organisms per mL were used to inoculate tissue culture cells, fertile chicken eggs, and guinea pigs as described below. In every case, the organisms were further diluted and aliquots of each dilution were plated on BCYE agar to determine the initial size of the inoculum.

(a) Cell culture

A549 or L929 cells were seeded in 96-well plates (Costar 3596) at a concentration of 10^5 cells per well in 0.1 mL of culture medium consisting of antibiotic-free RPMI or MEM respectively, with added 1% heat-inactivated FBS. The cells were incubated for 3 hours to allow a monolayer to form. Duplicate monolayers were then inoculated with either Lp2064 or Lp2064M in 0.1 mL of culture medium. The monolayers were then examined daily under an inverted microscope for any

discernable cytopathic effect. Three days after the infection, the monolayers were stained by adding to each well, 50 μL of culture medium containing 0.01% neutral red (Fisher Scientific, Dartmouth, N.S.), a dye ordinarily taken up only by viable cells (Katz et al., 1974). The cells were then incubated for 2 hours at 37°C after which they were washed twice with PBS. The dye was subsequently eluted into the wells by adding 100 μL of a 1:1 solution of ethanol and 0.1 M NaH_2PO_4 to each well (Katz et al., 1974). The colour in the plates was measured in a Microelisa reader at 570 nm, and the resulting optical density readings were plotted against the number of organisms. From this, the number of organisms causing a 50% reduction of neutral red uptake by control (i.e. uninfected) cells was determined.

(b) Embryonated eggs

The yolk sacs of 8-day-old embryonated White Leghorn hen eggs were infected as described by McDade et al. (1977). The eggs were purchased from Cook's Hatchery (Truro, N.S.), and after a short (24 to 48 h) recovery period in a 37°C incubator, the eggs were candled to locate the yolk sacs. Using 6 eggs per dilution, the yolk sacs were infected with 0.2 mL of the organisms or with saline alone. The eggs were returned to the incubator and candled daily for 8 days to determine the number of embryos killed. After excluding from the assay any embryos killed after the first day, the 50% egg infectivity dose (EID_{50}) was calculated according to the method

of Karber (1931).

(c) Guinea pigs

Male Hartley guinea pigs each weighing 250 to 300 g were obtained from High Oak Ranch (Goodwood, Ontario). After a one week recovery period, and using 4 animals per dilution, the guinea pigs were infected intraperitoneally with 1 mL of the organisms in saline (Fields et al., 1986). Control animals were inoculated with saline alone. The guinea pigs were examined daily for at least one week for symptoms such as ruffled fur, watery eyes, and fever. The number of animals succumbing to the infection was recorded, and the 50% lethal dose (LD_{50}) was determined as described above.

Assessment of the cytopathic effect of L.pneumophila in various host cells

MRC-5, Vero, HEp2, A549, CHO, and L929 cells in antibiotic-free culture media were seeded separately into 96-well plates and infected with serial dilutions of various isolates of L.pneumophila as described above. The infected monolayers were monitored daily for cytopathic effect (CPE) and after 3 days, the cells were fixed with 10% formalin in HBSS prior to staining them with a solution of 20% ethanol containing 1% crystal violet. The degree of CPE was then scored qualitatively for the different dilutions of organisms used.

L929 Plaque Assay

The L929 cells were plated in 24-well tissue culture

plates (Costar 3524) in antibiotic-free culture medium (MEM supplemented with 10% heat-inactivated FBS) at a concentration of 5 X 10^5 cells per well. After overnight incubation, or alternatively, after allowing the cells to adhere for 2 to 3 hours, the culture medium was removed and the resulting confluent monolayers were infected in duplicate with, per well, 0.5 mL containing organisms serially diluted in culture medium. After a usual 60-minute adsorption period at 37°C, the inoculum was removed by aspiration, and the monolayers were washed three times with culture medium containing 50 μg of gentamicin sulfate (Sigma Chemical Co.) per mL (Dreyfus, 1987, Isberg and Falkow, 1985). The cultures were then incubated for an additional 60 minutes in 1 mL per well of the gentamicin wash medium, after which the monolayers were washed three times with culture medium alone prior to adding a 1 mL per well overlay of 0.6% agarose (Type IIA, Medium EEO, Sigma Chemical Co.) in antibiotic-free MEM containing 1% heat-inactivated FBS.

The monolayers were incubated for four days at 37°C and then fixed with 10% formalin in HBSS. After several hours, the agarose overlay was removed, and the fixed cells were stained with 1% crystal violet in 20% ethanol. The resulting plaques were macroscopically enumerated.

Growth and survival of L.pneumophila in L929 cells

L929 cells were infected with either Lp2064 or Lp2064M at a dilution of organisms corresponding to a ratio of

approximately 10 host cells for each bacterium. All the steps of the plaque assay were followed except that the agarose overlay was omitted. Instead, 0.5 mL of the culture medium was added to the infected cells. To establish the initial number of organisms that had penetrated the monolayers, the culture medium was collected and replaced with 0.5 mL of 0.1% Triton-X 100 in PBS (Jacobs et al., 1984). Ten minutes later, the lysate was harvested, added to the previously collected culture medium and 0.1 mL of this mixture was plated on BCYE agar plates. The infected monolayers were then lysed as described at 24, 48, 72, and 96 hours postinfection. After incubating the BCYE plates for 4 days at 37°C, the resulting colonies were enumerated and recorded.

Infectivity index as determined by plaque assay

The plaque assay was used to screen various isolates of L.pneumophila which are listed in the Appendix. Forty-eight hour-old cultures of the isolates to be tested were scraped off BCYE plates and resuspended in antibiotic-free MEM containing 10% heat-inactivated FBS. Serial ten-fold dilutions were made in the same culture medium, and as described above, these were used to infect the L929 monolayers. To ensure consistency, the monolayers were exposed to the inoculum containing the organisms for exactly 60 minutes. Dilutions of the organisms were also plated in duplicate on BCYE agar so that the initial number of viable colony forming units (cfu) could be established. The infectivity index was then

calculated as a ratio of the initial number of cfu to the number of plaque forming units (pfu) at a given dilution of organisms. For convenience, the resulting ratio was divided by 1000. For example, if the initial number of cfu per mL for a given isolate was 2 X 10^9, and the average number of pfu at the 10^{-5} dilution was 20, then the infectivity index would be 1.

Interaction of L.pneumophila with host cells

Immunofluorescence

Eight-well Lab Tek chamber/slides (CanLab, Mississauga, Ontario) were seeded with 0.3 mL of antibiotic-free culture medium containing approximately 3 X 10^5 L929 cells or monocytes. The monolayers were infected at 37°C, with dilutions of bacteria for time points ranging from 5 to 60 minutes. Thereafter, the monolayers were washed three to five times, and fixed either immediately, or after incubating the cells for an additional time period which could range from 10 minutes to usually 24 hours, or up to as long as 96 hours. The cells were fixed using one of two methods. In the first method (Edelstein et al., 1987), the cells were fixed with 10% formaldehyde in doubled distilled water for 5 minutes. The cells were then rinsed several times with PBS, and further incubated in water for 5 minutes. After air drying, the slides were ready to be stained. In the second method (Mounier et al., 1990), the cells were first washed three times with PBS, next with PHEM buffer (10 mM ethylene glycol-bis(ß-amino-ethyl

ether)-N,N,N',N'-tetraacetic acid [EGTA], 1 mM $MgCl_2$, 60 mM piperazine-N,N'-bis(2-ethanesulfonic acid) [PIPES], and 23 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES] pH 6.9), and then permeabilized with 0.5% Triton X-100 in PHEM for 1 minute. At this point the plastic chamber was removed from the slide, leaving the gasket, and the slide was immersed in -20°C acetone for 3 minutes. After air drying, the slides were stored until needed.

The slides were stained by indirect immunofluorescence as follows: To each section of the chamber slides, 100 μL of PBS containing 0.5% bovine serum albumin (BSA; Calbiochem, La Jolla, Ca.) and a 1/500 dilution of the polyclonal rabbit antibody against L.pneumophila (described above) was added. After incubating the slides for 30 minutes at 37°C in a humidified container, they were washed with PBS before adding to each chamber, 75 μL of a 1/30 dilution of an FITC-conjugated swine anti-rabbit IgG antibody (Dako). The slides were then incubated and washed as described prior to mounting them in buffered glycerol. Slides fixed using the method described by Mounier et al. (1990), were stained similarly, except the cells were first washed with 0.5% gelatin in PBS before adding the primary antibody.

The slides were viewed using a Nikon microphot FX microscope equipped for epi-fluorescence. Photographs were taken using Kodak EL 400 slide film or T MAX 400 print film.

Electron microscopy

Six-well plates containing approximately 2 to 3 X 10^6 L929 cells or monocytes per well in antibiotic-free culture media containing 10% heat-inactivated FBS were infected for 4, 6 or 18 hours at 37°C in a CO_2 incubator with either Lp2064 or Lp2064M at a multiplicity of infection (m.o.i.) of about 10 bacteria per host cell. The monolayers were washed several times with culture medium and incubated for a further 60 minutes. After removing the culture medium, the monolayers were rinsed with 0.1 M sodium cacodylate (pH 7.3) prior to fixing them at 4°C for 90 minutes with 2.5% glutaraldehyde in cacodylate buffer. The glutaraldehyde was removed with three 10-minute washes in cacodylate buffer, and (for convenience) the fixed cells were left overnight at 4°C in the same buffer.

The next day, the cells were scraped off the plates with a rubber policeman, collected into glass test tubes and taken to the Electron Microscopy (EM) Unit in the Department of Microbiology, for further processing. Details of their standard EM protocol are provided in the Appendix. In brief, the samples were post-fixed first with osmium tetroxide and then uranyl acetate. This was followed by a dehydration step involving increasing concentrations of acetone. The fixed and dehydrated samples were then infiltrated and embedded with TAAB resin using TAAB embedding kit TK3 (Marivac Ltd., Halifax, N.S).

Silver grey sections were made and floated onto 300-mesh

copper grids. The sections on the grids were viewed using a Philips 200 electron microscope.

Assessment of phagosome-lysosome fusion

(a) Thorium/ferritin method

In a modification of methods described by Horwitz (1983) and Mor and Goren (1987), L929 cells or monocytes seeded in 6-well plates as outlined above, were pre-treated with either 20 μL or 10 μL of Thoria solution (# 2684, Polysciences Inc., Warrington, Pa) in 3 mL of culture media, or with ferritin (# 8689, Polysciences Inc.) at a concentration of 0.33 mg per mL. The plates were incubated for 20 hours at 37°C in a CO_2 incubator. They were then washed, and incubated for a further ? hours in culture medium. Next, as described above, the cells were infected with Lp2064 or Lp2064M for 4 to 6 hours and processed for electron microscopy. The fusion of phagosomes with lysosomes was assessed by looking for ferritin or thorium within vacuoles containing L.pneumophila. This could be visualized by electron-dense zones found surrounding the bacteria within a vacuole. The numbers of fused vacuoles were counted and compared.

(b) Acid phosphatase method

After infecting L929 cells or monocytes with Lp2064 or Lp2064M for 4 or 6 hours (see above), the inoculum was washed off and replaced with culture medium for one hour. Then, following the method of Robinson and Karnovsky (1983), the culture medium was removed and the cells washed three times

with HBSS or saline to remove the FBS. The cells were pre-fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.3, for 30 minutes at 4°C. After washing off the glutaraldehyde with 3 saline or HBSS washes, the cells were briefly treated with 0.1M acetate buffer (70 mL 0.2M sodium acetate, 30 mL 0.2M acetic acid, 100 mL distilled water) having a pH of 5. The substrate solution which was made up just prior to use was pre-filtered using a 0.45 μm filter (Millipore (Canada) Ltd., Malton, Ontario), and pre-warmed to 37°C. It consisted of 10mM cerium chloride (Sigma Chemical Co.) and 1mM sodium ß-glycerophosphate (Marivac Ltd., Halifax, N.S.) in the acetate buffer. The cerium chloride and ß-glycerophosphate were initially made up respectively as 200mM and 100mM solutions in distilled water before diluting them 100-fold in the acetate buffer. For some experiments, the ß-glycerophosphate was omitted from the "substrate buffer".

Following two 30-minute incubations at 37°C in substrate solution (prepared freshly each time), the cells were washed twice with the acetate buffer. After two additional washes with cacodylate buffer, the cells were fixed at 4°C for 1 hour in cacodylate buffer containing 2.5% glutaraldehyde. The glutaraldehyde was then removed, and the cells washed three times before collecting them and taking them to the EM Unit for further processing as described above. Phagosome-lysosome fusion was assessed by examining vacuoles containing L.pneumophila for electron-dense zones resulting from the

"capture" of cerium due to the acid phosphatase activity found within lysosomes. Electron dense vacuoles containing organisms were counted and compared.

Metabolic labeling of virulent and avirulent L.pneumophila under intracellular conditions

(a) General procedure in L929 cells

Approximately 2 X 10^6 L929 cells in culture medium (antibiotic-free MEM containing 10% heat-inactivated FBS) were seeded into 6-well plates (Costar) and placed in a 37°C CO_2 incubator. The next day, the cells were prepared as follows prior to infection. The culture medium was first replaced with deficient medium and the cells returned to the incubator. Deficient medium consisted of MEM lacking antibiotics and methionine (MEM$^-$), but containing 10% heat-inactivated FBS which had previously been dialysed against HBSS. After 2 hours, the deficient medium was discarded and the cells were then exposed for an hour to deficient medium containing cycloheximide (Sigma Chemical Co.) at a concentration of 10 μg per mL.

Lp2064 or Lp2064M which had been growing on BCYE plates for 48 hours were scraped off the plates using a bent Pasteur pipette and resuspended in deficient media to an OD_{660} of approximately 0.75. A portion of the bacterial suspension (0.5 mL) was placed in a 1.5 mL Eppendorf centrifuge tube (Brinkmann Instruments, Inc., Westbury, NY) to serve as the tissue culture medium control.

The L929 monolayers which had been prepared as described were either mock-infected with deficient medium , or infected with 1 mL of the bacterial suspension. After 5 minutes, the inoculum was removed and the cells washed 3 to 5 times with deficient medium and incubated with 1 mL of the same medium. At this point "Time 0" (T=0) was established. At T=0 and at various time points thereafter (i.e. T=10, 30, or 60 minutes), the plates were removed from the incubator and Tran^{35}S-label (ICN) containing 75 to 125 μCi of [^{35}S] Methionine was added to each well and to the Eppendorf tube containing the medium control (5 to 10 μCi). Tran^{35}S-label has a specific activity $\geq$ 1000Ci/mmol and consists of $\geq$ 70% [^{35}S] L-Methionine, $\leq$ 15% [^{35}S] L-Cysteine, $\leq$ 7% [^{35}S] L-Methionine sulfone, $\leq$ 3% [^{35}S] L-cysteic acid, and $\leq$ 5% other ^{35}S compounds. The infected cells and the bacteria were exposed to the radiolabel for 10 minutes at 37°C in a plastic container.

The infected L929 cells were then processed as follows. The label was carefully aspirated and the monolayer washed 3 to 5 times with culture medium. After removing the last wash, the monolayer was lysed by adding 1 mL of PBS containing 0.1% Triton X-100 (Sigma), 0.3% methionine, and freshly prepared 100 μM phenyl methyl sulfonyl fluoride (PMSF, Sigma) for 10 minutes. To recover the intracellular bacteria, the lysate was collected and centrifuged at 250*g* for 10 minutes to remove host cell debris. The supernatant was collected, pipetted into 1.5-mL Eppendorf centrifuge tubes and spun at 14,000*g* for 5

minutes to pellet the bacteria. The bacteria were washed 3 times in PBS and on some occasions a 10 μL aliquot was removed, serially diluted in PBS and plated onto BCYE agar plates to determine the number of cfu of bacteria. Following the last PBS wash, the bacteria were pelleted, resuspended in 60 μL of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and stored at -20°C. The labeled proteins were analysed by initially determining the number of TCA-precipitable counts and then loading equal numbers of counts of the various samples into each well of an SDS polyacrylamide gel. After electrophoresis (SDS-PAGE), the proteins were visualized by autoradiography or fluorography.

With respect to the bacteria being exposed to the tissue culture medium, the radiolabeling was halted by placing the bacteria on ice and then adding 0.5 mL of cold culture medium containing 0.3% methionine. The bacteria were then pelleted and washed 3 times in PBS before resuspending them in a small volume of PBS and storing them at -20°C. The labeled proteins were analysed as described above.

(b) Gentamicin treatment

In some experiments, the initial inoculum was washed off with deficient medium containing gentamicin sulfate at a concentration of 50 μg per mL and the monolayers were incubated in the presence of gentamicin. At T=0 or T=30, the radiolabel was added as described, and the infected cells incubated for a further 30 minutes. The label was removed and

the monolayers washed 3 times (with culture medium lacking antibiotics) before lysing them as outlined above. Intracellular bacteria were then isolated and analysed as described.

(c) Cytochalasin D treatment

Host cells were prepared as outlined except that the monolayers were exposed to cytochalasin D (Sigma) at a concentration of 1 μg per mL in deficient medium for 1 hour prior to infecting them. The cells were infected and labeled in the presence of cytochalasin D using the standard protocol. Infection of monolayers that were not exposed to cytochalasin D was used as a control. Bacteria interacting with host cells were recovered and analysed as above.

(d) Monocyte infection

Monocytes were isolated in 6-well plates as outlined. To the monocytes was added either antibiotic-free RPMI containing heat-inactivated FBS (culture medium) or culture medium supplemented with per mL, 1000 units (U) of recombinant interferon gamma (IFN-τ; Boehringer-Mannheim, Laval, Quebec). After 24 hours, the culture medium and the IFN were removed and replaced for 1 hour with RPMI deficient medium (antibiotic-free RPMI lacking methionine and supplemented with 10% heat-inactivated serum that had been dialysed against HBSS) containing cycloheximide (1 μg/mL). Monocytes so treated were infected for 30 minutes with the bacterial suspension obtained as described except that Lp2064 was diluted 100-fold

prior to use. After washing off the inoculum, 0.5 mL of deficient medium containing gentamicin was added to each well and the plates were incubated for 30 minutes. Approximately 125 μCi of [^{35}S] methionine (Tran^{35}S-label) was added to each well for an additional 30 minutes after which the label was removed and the infected cells processed as outlined.

Metabolic labeling of BCYE agar-grown organisms expcsed to various conditions

Lp2064 or Lp2064M growing on BCYE agar were scraped off the plates and resuspended in PBS to an OD_{660} of 5. A ten-fold dilution (50 μL into 450 μL) was then made in the following: PBS, MEM, MEM pH 6.3, MEM pH 5.5, MEM pH 4.0, double distilled water, double distilled water containing 0.3% NaCl, double distilled water containing 0.6% NaCl, and RPMI. Immediately thereafter, 10 μCi of [^{35}S] methionine (Tran^{35}S-label) was added. After allowing the labeling to proceed for 10 minutes, the reaction was stopped by adding an equal volume of ice-cold 20% trichloroacetic acid (TCA, Sigma Chemical Co.) according to the method of Keen and Hoffman (1989). After 1 hour, the precipitates were pelleted and washed twice with acetone. The acetone was removed, the precipitates resuspended in SDS-PAGE sample buffer, and stored at -20°C until analysed by SDS-PAGE-autoradiography/fluorography.

For some experiments, the bacteria were labeled for 10 minutes at 30, 60, 120, or 240 minutes after the initial exposure to MEM.

Protease production by virulent and avirulent L.pneumophila

Five millilitres of BYE broth were inoculated with Lp2064 or Lp2064M which had been growing on BCYE plates. To the cultures was added [^{35}S]-methionine (Specific activity 1230Ci/mmol, Amersham Canada, Oakville, Ontario; or Tran^{35}S-label) at a concentration of 0.5 μCi per mL. After growing the organisms at 37°C in a roller drum for about 48 hours, the bacterial cultures were centrifuged at 7,000*g* for 10 minutes. The supernatants were collected and the pelleted bacteria were resuspended in PBS and stored at -20°C.

The supernatants that were collected were filtered using a 0.45 μm low protein binding Millex GV filter (Millipore). Then according to the method of Keen and Hoffman (1989), an equal volume of cold 20% trichloroacetic acid (TCA) was added to the filtered supernatants before leaving them at 4°C for 1 hour. The precipitated proteins were pelleted by centrifuging for 10 minutes at 14,000*g* before washing them twice with cold (-20°C) acetone. The acetone was removed, and the washed precipitates were resuspended in a small (50 to 100 μL) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and stored at -20°C until analysed by SDS-PAGE, followed by autoradiography/fluorography.

Evaluation of the heat shock response of virulent and avirulent organisms

BYE broth was inoculated with Lp2064 or Lp2064M as

described above except the organisms were grown at 23°C. Once an OD_{660} of about 0.75 was reached, the heat shock response was examined according to the procedure of Hoffman et al. (1989). As such, 0.5-mL aliquots of the broth cultures were placed into 1.5-mL Eppendorf centrifuge tubes and 10 μCi of [^{35}S]-methionine was added to each sample. The samples were immediately placed for 10 minutes, into temperature blocks or water baths set at 23, 37, or 42°C. The reaction was stopped by adding 0.5 mL of cold 20% TCA. After allowing the proteins to precipitate at 4°C, the precipitates were pelleted, washed twice with cold acetone, resuspended in 50 to 100 μL of SDS-PAGE sample buffer and stored at -20°C.

Samples containing equal numbers of TCA-precipitable counts were analysed by SDS-PAGE fluorography.

Determination of TCA-precipitable counts

The amount of radioactivity incorporated into proteins labeled with [^{35}S]-methionine or Tran^{35}S-label was determined by using a modified version of the method described by Johnston et al. (1977). Performed in duplicate, a 5 μL aliquot of each sample was placed in a glass test tube containing 5 mL of 5% ice-cold TCA, 10 μg BSA, and 10-fold excess unlabeled methionine. After allowing the samples to precipitate on ice for 30 minutes, each sample was filtered through a Whatman GF/C glass filter (Whatman International, Maidstone, England) that was pre-wetted with the TCA-methionine solution. Using 5 mL of the same solution, the precipitates were carefully

washed 3 times. The filters were then rinsed with 95% ethanol before putting them into plastic scintillation vials. After adding the universal scintillation cocktail Aquasol-2 (NEN, Dupont (Canada), Mississauga, Ontario), the samples were counted in an Isocap/300 liquid scintillation counter to establish the number of counts per minute.

SDS-PAGE

SDS-PAGE was performed by the system of Laemmli (1970) in an LKB (Fisher) vertical slab gel apparatus using stacking and separating gels of 3 and 11% acrylamide respectively, unless otherwise noted. Except for the Tris (Tris[hydroxymethyl]aminomethane) buffer which was from the Sigma Chemical Co., all electrophoresis reagents were purchased from BioRad (Mississauga, Ontario). The protein molecular weight markers (phosphorylase B 94 kDa, BSA 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, soybean trypsin inhibitor 20.1 kDa, α-lactalbumin 14.4 kDa) were obtained as a kit from Pharmacia (Dorval, Quebec).

The separating gel was made with 12.6 mL of a 30:0.8% stock solution of acrylamide:bisacrylamide, 8.75 mL of 1.5M Tris buffer (pH 8.8), 35 μL of N,N,N',N'-tetramethylenediamine (TEMED), 75 μL of 20% SDS, 13.1 mL double distilled water, and 225 μL of freshly prepared 10% ammonium persulfate. The reagents were carefully but briefly mixed and about 30 mL were poured into the gel assembly. Immediately thereafter, a solution of 0.1% SDS was carefully layered on top of the

acrylamide and the acylamide was allowed to polymerize for 1 hour. Next, after removing all of the SDS overlay, the stacking gel solution consisting of 1 mL of the acrylamide stock, 2.5 mL of 0.5M Tris buffer (pH 6.8), 10 μL TEMED, 50 μL of 20% SDS, 6.3 mL double distilled water, and 100 μL of 10% ammonium persulfate was added, along with a teflon comb. After a further 60 minutes, the comb was removed and the resulting wells washed with electrode buffer (24 g Tris, 115.2 g glycine and 4 g SDS in 4 L double distilled water). Samples to be electrophoresed including molecular weight markers were diluted in SDS-PAGE sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue in 0.25M Tris having a pH of 6.8) and were heated to 100°C for 5 minutes prior to loading them into the wells.

Electrophoresis was performed in a tank containing electrode buffer at 15°C, using 10 Watts constant power for about 3 hours or until the dye front reached the bottom of the gel. The gel was removed and stained for 1 hour at 50°C (or overnight at room temperature) with 0.04% Coomassie blue R-250 in 25% isopropanol and 10% acetic acid in double distilled water. The gel was then destained at 50°C for 12 to 24 hours using several changes of 7% acetic acid. The gel was subsequently photographed or dried under vacuum on filter paper and the stained proteins examined.

Autoradiography/Fluorography

Following the separation of radioactive samples by SDS-

PAGE, the gels were stained/destained and then either dried directly onto filter paper or pretreated with the fluor solution "En^{3}Hance" (NEN, Dupont) for 1 hour at room temperature. Gels that were soaking in En^{3}Hance were then placed in cold distilled water for 30 minutes to precipitate the fluors, and then dried. The dried gels were subsequently exposed o Kodak X Omat AR X-ray film at -80°C. The exposure times varied from 12 hours to 3-4 weeks depending on the amount of radioactivity loaded. The films were developed manually according to the manufacturer's directions or by using an automatic X-ray film processor.

Immunoblotting

Using modifications of the technique originally described by Towbin et al. (1979), each gel containing samples separated by SDS-PAGE was placed for 30 minutes in a transfer buffer solution consisting of 20mM Tris and 150mM glycine in double distilled water containing 20% methanol. A pre-wetted piece of nitrocellulose membrane (pore size of 0.2μm, Hoefer Scientific Instruments, San Francisco, Ca. or BioRad) was placed on top of the equilibrated gel taking care to remove air bubbles. The gel and nitrocellulose were then sandwiched between pre-wetted filter paper and sponges and the sandwich was placed in a TransBlot apparatus (BioRad) containing transfer buffer. Electroblotting was performed at 4°C for 3 hours at 70 volts. The success of the blotting was confirmed by the transfer of pre-stained molecular weight markers

(Gibco/BRL) to the nitrocellulose membrane. The prestained markers and their approximate molecular weights were as follows: myosin 200 kDa, phosphorylase B 97.4 kDa, BSA 68 kDa, ovalbumin 43 kDa, α-chymotrypsinogen 25.7 kDa, ß-lactoglobulin 18.4 kDa, and lysozyme 14.3 kDa.

The transferred proteins (e.g. the 60-kDa heat shock protein, hsp 60) were visualized using the procedure of Hoffman et al. (1989) as a guideline. The nitrocellulose membrane (blot) was placed in PBS containing 3% gelatin for 30 minutes at room temperature and then overnight at 4°C. The next day, the blot was warmed to 37°C on a rotating platform before placing it in a plastic bag containing the anti-hsp 60 monoclonal antibody diluted 1:1 in a pH 7.2 tris saline buffer (10mM Tris and 0.9% sodium chloride) with added 0.3% gelatin (TS-G). The blot was rotated gently with the antibody for 2 hours in a 37°C incubator. Three 10-minute washes ensued at room temperature using the above buffer lacking gelatin but containing 0.01% tween 20 (TS-T). The biotinylated rabbit anti-mouse antibody was diluted 1:1000 in TS-G, added to the blot, and incubated as above. After three TS-T washes, a 1:1000 dilution of the peroxidase-conjugated streptavidin in TS-G was added and the blot incubated as described for 1 hour. The blot was washed three times and then exposed to the substrate solution (60 μL of hydrogen peroxide added just prior to use to TS containing 30 mg of 4-chloro-1-naphthol [Sigma Chemical Co.]). The reaction was stopped within minutes

by replacing the substrate solution with 2 to 3 changes of distilled water. Protein bands reacting with the antibody were easily visualized.

Isolation of lipopolysaccharide (LPS)

LPS was isolated from Lp2064 or Lp2064M by the proteinase K digestion method of Hitchcock and Brown (1983). Bacteria were collected in PBS, pelletted, and the pellets resuspended in 50μL lysing buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol and bromophenol blue in 1M Tris pH 6.8). The samples were boiled for 10 minutes after which proteinase K (25 μg in 10 μL lysing buffer) was added and the reaction continued at 60°C. The samples were run on SDS-polyacrylamide gels and visualized by silver stain.

Silver staining was performed exactly as outlined by Tsai and Frasch (1982). The LPS was fixed overnight with a solution containing 40% ethanol and 5% acetic acid. The solution was replaced with an oxidizing solution (0.7% periodic acid in 40% ethanol-5% acetic acid) for 5 minutes. Three 15-minute washes with double distilled water ensued. Next, the freshly-prepared staining agent was added. It was made by first adding 2 mL of ammonium hydroxide to 28 mL 0.1N sodium hydroxide. While stirring, 5 mL of a 20% solution of silver nitrate was slowly added followed by 115 mL of water. After 10 minutes, the stain was removed and the gel was washed three times (10 minutes each). Then the formaldehyde developer (50 mg citric acid, 0.5 mL of 37% formaldehyde per litre of

water) was added. After the desired staining intensity was achieved, the developing was stopped by washing with water.

Isolation of L.pneumophila RNA

Lp2064 or Lp2064M were scraped off BCYE agar plates and resuspended in PBS to an OD_{660} of 5. In an effort to reproduce the conditions of the ^{35}S-labeling experiments, the bacterial suspensions were diluted 10 fold in either MEM lacking methionine (MEM$^-$) or in BYE broth, both prewarmed to 37°C and the cultures were incubated in a 37°C water bath. At the same time, the bacteria were also diluted 10-fold in pre-warmed PBS. This was considered to be the zero time control (T=0). The PBS cultures (10 mL each) were immediately centrifuged in polypropylene tubes at 7,000*g* for 10 minutes at 4°C. The supernatants were discarded, and the pellets placed on ice. Then, at 10, 30, and 60 minutes after exposure to MEM or BYE, a 10-mL sample was withdrawn from each culture, centrifuged as described above, and the pellets placed on ice.

In collaboration with P. Hoffman and M. Ripley, RNA was extracted using the hot SDS/acid-phenol method outlined by Hoffman et al. (1991b). From this point forward, all solutions that were used were made with sterile water treated with 0.1% diethyl pyrocarbonate (DEPC, Sigma Chemical Co.). The bacteria were lysed by adding to each pellet 3 mL of boiling TES buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl) containing 1% SDS. The pellets were vortexed briefly before adding 3 mL of acid phenol (pH 5) heated to 65°C. The acid phenol was made

previously by equilibrating phenol in a separatory funnel, with equal volumes of first 500 mM sodium acetate pH 4.8, and then 2 to 3 changes of 50 mM sodium acetate pH 4.8. After adding the acid phenol, the pellets were vortexed and placed in a 65°C water bath for 5 minutes. The tubes were removed from the water bath and centrifuged for 5 minutes at 7,000*g*. From each tube, the aqueous layer, presumably lacking in contaminating proteins and DNA, was carefully removed and transferred to a fresh tube. After repeating the phenol extraction, the aqueous layer was further extracted with an equal volume of a 24:1 ratio of Chloroform:Isoamyl alcohol to remove the phenol. The aqueous phase was removed after centrifuging and the RNA was precipitated by adding 10% sodium acetate (3M stock) and 2.5 volumes of cold (-20°C) 95% ethanol. The samples were placed at -70°C for 30 minutes after which they were centrifuged at 4°C for 10 minutes at 13,000*g*. The supernatants were discarded and 1 mL of cold 70% ethanol made in DEPC-treated water was added to each tube. After transferring to a sterile 1.5-mL Eppendorf tube, each sample was centrifuged for 10 minutes and then dried under vacuum. Each pellet was resuspended in 50 μL of DEPC-treated water by repeated pipetting and placed on ice. An aliquot of each sample was diluted (e.g. 500-fold) and the absorbance at 260 nm was determined. From this, the amount of RNA was calculated. For each sample, a volume of RNA corresponding to 20 μg was placed into an Eppendorf tube and the RNA dried

under vacuum. The RNA was stored at -20°C. Northern analysis of the RNA was subsequently performed essentially by the method of Kroczek and Siebert (1990).

Northern analysis of RNA

Formaldehyde-agarose gels (10.5 X 14 cm) were made by dissolving 0.5 g of agarose (SeaKem, FMC BioProducts, Rockland, Me.) in 36.7 mL distilled water and 5 mL of ten times concentrated (10X) 4-morpholinepropanesulfonic acid (MOPS)/EDTA buffer (per 300 mL: 31.4 g MOPS, 6 mL 0.5M EDTA, pH 7). After melting the agarose, and cooling it to 60°C, 8.3 mL of 37% formaldehyde was added and the gel was cast. One hour later, the gel was pre-electrophoresed in 1X MOPS/EDTA for 30 minutes at 60 V.

Modifying the procedure of Kroczek and Siebert (1990), the RNA samples were each resuspended in 11 μL of gel loading buffer which consisted of 2.2 μL of Buffer A (294 μL 10X MOPS/EDTA, 706 μL distilled water), 4.8 μL formaldehyde/formamide (89 μL formaldehyde, 250 μL formamide), 2 μL gel loading buffer (50 mg ficol, 5 mg xylene cyanol, 5 mg bromocresol green, 322 μL buffer A, 178 μL 37% formaldehyde, 500 μL formamide) and 2 μL of ethidium bromide (0.5 mg/mL stock). The samples were then heated to 55°C for 15 minutes and quenched on ice before loading. A 0.24-9.5 kilobase (kb) RNA ladder (Gibco/BRL) was included amongst the samples. The samples were electrophoresed at 60 V for about 6 hours. The gel was viewed with the aid of a transilluminator

and photographed. The gel was transferred to the VacuGene XL vacuum blotting apparatus (Pharmacia) where it was placed above a piece of nylon membrane (Nytran, Schleicher and Schuell, Spectrex, Montreal, Quebec) which was pre-wetted with 2X saline/sodium citrate (SSC: 20X has per litre, 3M NaCl, 0.3M sodium citrate, pH 7). Vacuum blotting was performed exactly as outlined by the manufacturer. First the formaldehyde was removed with distilled water for a period of 5 minutes. Next, an alkaline solution (50mM NaOH, 10mM NaCl) was passed through the gel for 5 minutes. The alkaline solution was neutralised by adding a solution of 0.1M Tris-HCl, pH 7.4 for an additional 5 minutes, before commencing the 30-minute transfer in 20X SSC. The filter containing the RNA was briefly rinsed in 20X SSC and covered with a plastic wrap. The filter was placed RNA side down, on a transilluminator and exposed to UV light at the high setting for 5 minutes. The filter was removed from the wrap, briefly air-dried and then baked for 2 hours at 80°C under vacuum.

The filter (i.e. blot) was prehybridized and probed with fragments of L.pneumophila *htpB* and *omp28* genes as follows. The blot was placed in a plastic bag and hydrated with 2X SSC. The SSC was poured off and replaced with the prehybridization solution consisting of 2 mL of 50X Denhardts (1% BSA, 1% polyvinylpyrrolidone-360 and 1% Ficoll-400), 5 mL of 20X SSC, 0.2 mL of 10% SDS, 0.4 mL of single stranded DNA (10 mg/mL) and 12.6 mL distilled water. After removing air bubbles, the

bag was sealed, and incubated overnight in a 42°C water bath.

The L.pneumophila probes were made by M. Ripley and P. Hoffman. They represented segments of the ≈2 kb *htpB* gene encoding the 60-kDa heat shock protein (Hoffman et al., 1990) and the ≈0.9kb *omp28* gene encoding the 29-kDa major outer membrane protein (MOMP, Hoffman et al., 1991a). For each gene, two synthetic oligonucleotides hybridizing to internal sequences more than 500 base pairs (bp) apart were used as primers. The DNA spanning the primers was amplified and labeled by the polymerase chain reaction (PCR) by replacing some of the deoxycytidine triphosphates (dCTP) in the reaction mix with [^{32}P] dCTP as described (Hoffman et al., 1991a). The amplified fragments were partially purified through a mini-Sephadex G50 column (Silhavy et al., 1984) and the amount of radioactivity was determined by scintillation counting.

The probes were boiled for 5 minutes to denature them, quenched on ice, and 6 to 8 X 10^6 counts per minute of each probe was then added directly to prehybridization mix in the bag containing the blot. After re-sealing the bag, the hybridization was continued for 24 hours under the same conditions.

After removing the blot from the bag, it was washed as will be described, in various concentrations of SSC all containing 0.1% SDS. After each wash, the level of radioactivity at the edges and centre of the blot was monitored with a survey meter. The blot was first washed for

20 minutes at room temperature with 5X SSC, then in order, 5 minutes at 55°C with 5X SSC, two separate 10 minute washes at 55°C with 2X SSC, 1 to 2 minutes at room temperature in 0.2X SSC and finally, 1 to 2 minutes at 55°C with 0.2X SSC. Having satisfied that the background radioactivity was at a minimum, the blot was wrapped in plastic, placed in a casette containing intensifying screens, and exposed to Kodak X-Omat AR X-ray film at -70°C. The film was developed 12 to 24 hours later.

RESULTS

Development of an *in vitro* infectivity assay

Preliminary Observations

The infection of MRC-5, A549, HEp2, and Vero cells with serial dilutions of various *L.pneumophila* isolates gave rise to a generalized form of cell destruction. This cytopathic effect (CPE) could be scored either qualitatively or quantitatively. The qualitative assessment of CPE, visualized by crystal violet staining, was achieved by noting the highest dilution of organisms causing complete CPE in all of the wells that were inoculated. As shown in Figure 1 and Table 1, a comparison of the different cell lines revealed varying degrees of sensitivity to infection. In general, for any given isolate, the MRC-5 cells were most susceptible to infection, requiring fewer organisms to produce complete CPE. This was followed in order by the A549, HEp2, and Vero cells; the latter being relatively impervious to infection. In addition, the individual cell lines were able to distinguish amongst the different *L.pneumophila* isolates. For example, CDC and LEG, the laboratory strains with unknown passage history caused significantly less CPE than did the clinical (1473 and 2755) and environmental (E72) isolates. Furthermore, the ranking of *L.pneumophila* isolates with respect to infectivity did not vary from one cell line to the next.

Figure 1. Cytopathic effect caused by various L.pneumophila isolates in MRC-5, HEp2, A549, and Vero cells.
The cell lines were seeded into 96-well plates and were either mock-infected (columns labeled C) or infected with serial dilutions of L.pneumophila isolates 1473, 2755, CDC, Leg, and E72. Three days later the cells were fixed with formalin and stained with crystal violet.

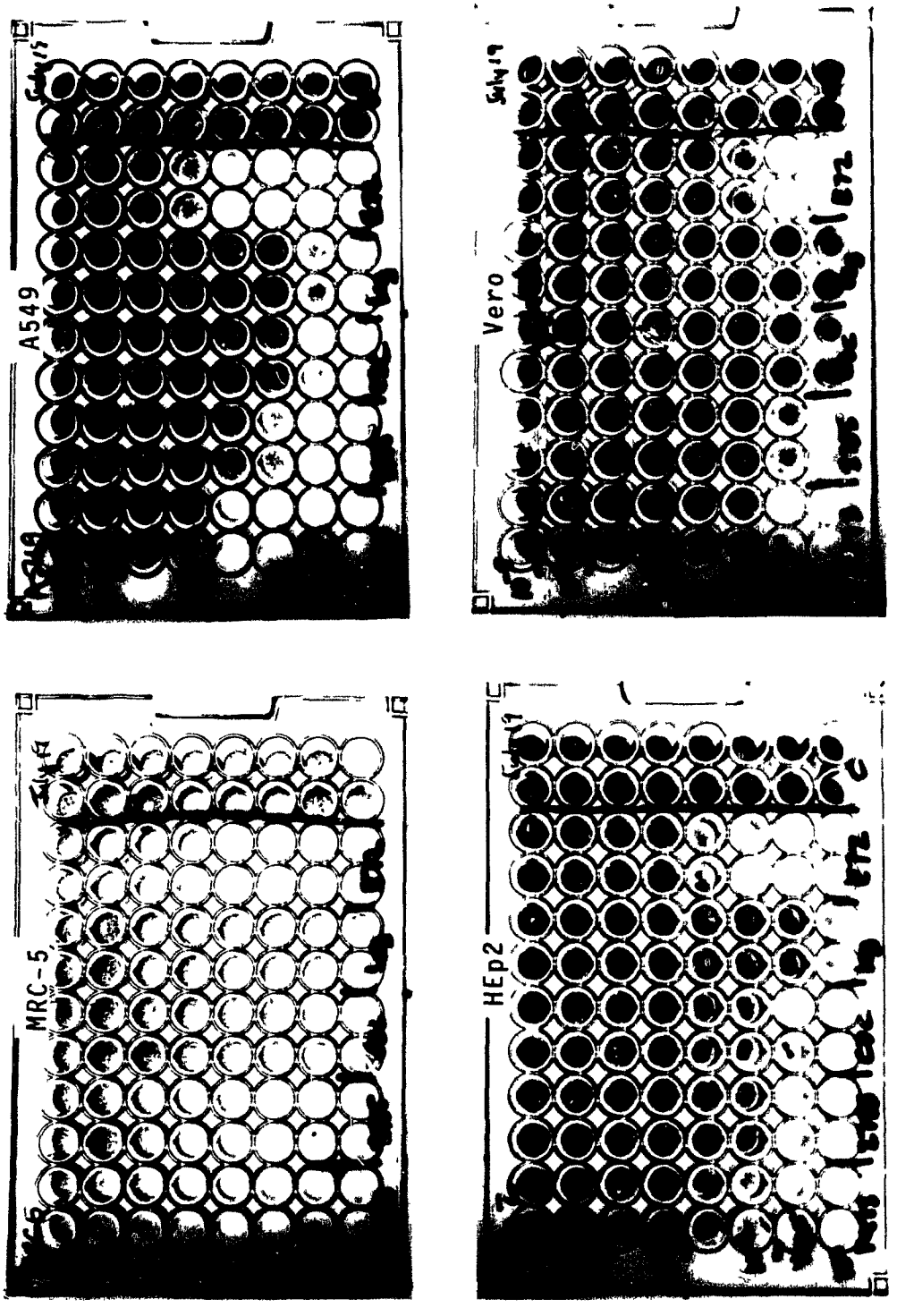


Figure 1

Table 1. The fewest number of L.pneumophila needed to cause a complete cytopathic effect (CPE)* in various mammalian cell lines.

Mammalian Cell lines	Isolates of L.pneumophila+				
	1473	E72	2755	CDC	LEG
MRC-5	$1.4X10^5$	$2X10^5$	$5X10^5$	$>2X10^7$	$>1.3X10^7$
A549	$7X10^5$	$1X10^6$	$5X10^6$	$2X10^7$	$1.3X10^7$
HEp2	$1.4X10^6$	$2X10^6$	$5X10^6$	$1X10^7$	$>1.3X10^7$
Vero	$7X10^6$	$1X10^7$	$1X10^7$	$>2X10^7$	$>1.3X10^7$

*Three days post-infection. Revealed by crystal violet staining of the formalin-fixed monolayers.

+Isolates 1473 and 2755 were isolated from patients, isolate E72 was obtained from a hospital water supply, and isolates CDC and LEG were laboratory strains which had been passaged numerous times. All belonged to L.pneumophila serogroup 1.

Although the MRC-5 cells were the most sensitive to infection, they stained faintly with crystal violet. For this reason A549 cells were used in attempts to quantify the CPE. The CPE was quantified by staining the monolayers with the vital dye, neutral red. Uptake of neutral red into viable cells was monitored by measuring the absorbance of the eluted dye at 570nm. A plot of the absorbance (in arbitrary units) against the actual number of organisms in the inoculum is illustrated in Figure 2. From this, the 50% cell culture infectivity dose CID_{50}, which is defined as the number of organisms per mL causing a 50% reduction in neutral red uptake compared with uptake by uninfected cells, was calculated. The results (Table 2, Figure 2) essentially paralleled those in Table 1.

Figure 2. Neutral red uptake of L.pneumophila-infected A549 cells. A549 cells were seeded into 96-well plates and infected with serial dilutions of L.pneumophila isolates 1473 (open triangles), 2755 (squares), CDC (filled circles), Leg (open circles), and E72 (filled triangles). Three days later, the cells were stained with neutral red. After washing the monolayers, the dye was eluted into each well with a 1:1 solution of ethanol:NaH_2PO_4. The absorbance of the eluted dye was measured at 570nm, converted into arbitrary units and plotted against the corresponding numbers of colony forming units (CFU) of bacteria. The 50% uptake cut-off was established by dividing the absorbance of uninfected cells by 2.

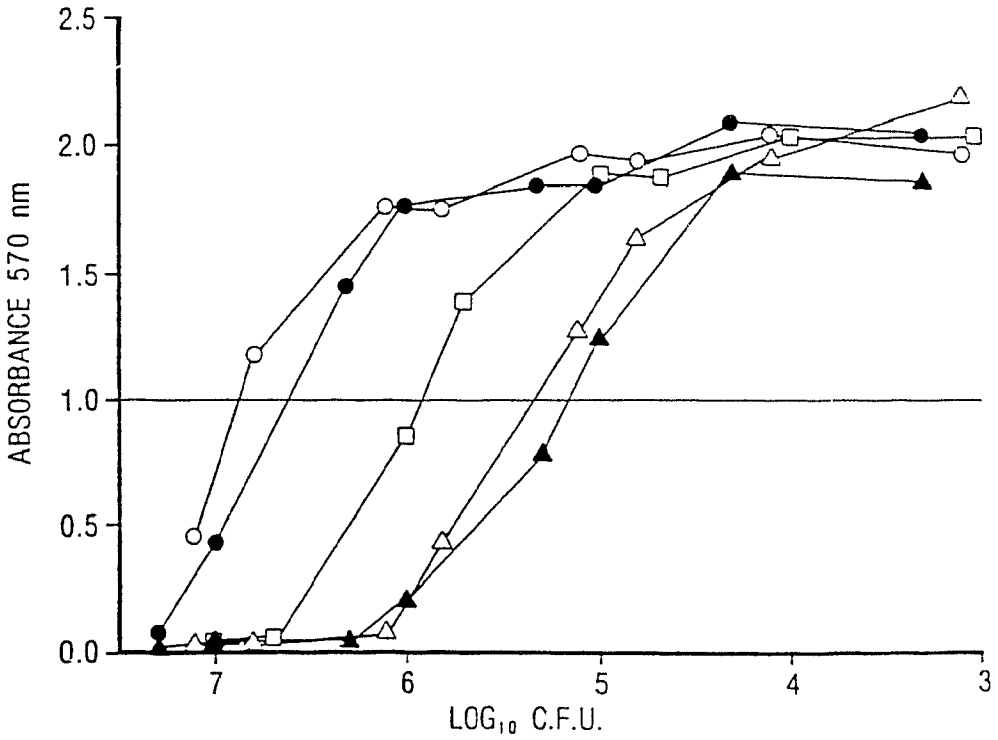


Figure 2

Table 2. A comparison of the crystal violet assay with the neutral red uptake assay in A549 cells.

L. pneumophila isolate	Crystal violet assay*	Neutral red uptake assay+
1473	7×10^5	2×10^5
E72	1×10^6	1.3×10^5
2755	5×10^6	1×10^6
CDC	2×10^7	4.0×10^6
LEG	1.3×10^7	7.9×10^6

*See Table 1

+Deduced from Figure 2. Represents the 50% cell culture infectivity dose (CID_{50}). It refers to the number of organisms needed to cause a 50% reduction (relative to uninfected controls) in the uptake of neutral red.

L929 plaque assay

A routine search for other cell lines more susceptible to L.pneumophila infection resulted in a fortuitous observation. At low multiplicities of infection (MOI) corresponding to bacterium:host cell ratios less than 1, small "holes" in the infected monolayer of L929 cells were noticed (Figure 3). There was a direct correlation between the number of foci which resembled virus-like plaques, and the dilution of the organisms. Furthermore, these plaques were detectable at concentrations of organisms far below those causing visible CPE in A549, MRC-5, HEp2 or Vero cells (Figures 1 and 3).

In order to accurately measure the number of plaques produced by a given isolate of L.pneumophila, the L.pneumophila plaque assay was developed. It combined the features of virus plaque assays first described by Dulbecco (1952), and a gentamicin step to kill extracellular bacteria not involved in the initial invasion (Dreyfus, 1987). Typical L.pneumophila plaques are shown in Figure 4.

The number of bacteria necessary to produce a single plaque was determined by adapting the method of Dulbecco (1952). The plaque assay was thus performed using serial two-fold dilutions of organisms starting with a bacterium:host cell ratio of approximately 0.01. The resulting numbers of plaques or plaque-forming units (pfu) when plotted against the dilutions, rendered a linear relationship (Figure 5). This indicated that the initial infection of a cell with a single

Figure 3. Discovery of L.pneumophila "plaques" in L929 cells. A549 and L929 cells were infected, fixed, and stained as described in the legend to Figure 1. Virus-like plaques were visible in L929 cells but not in A549 cells.

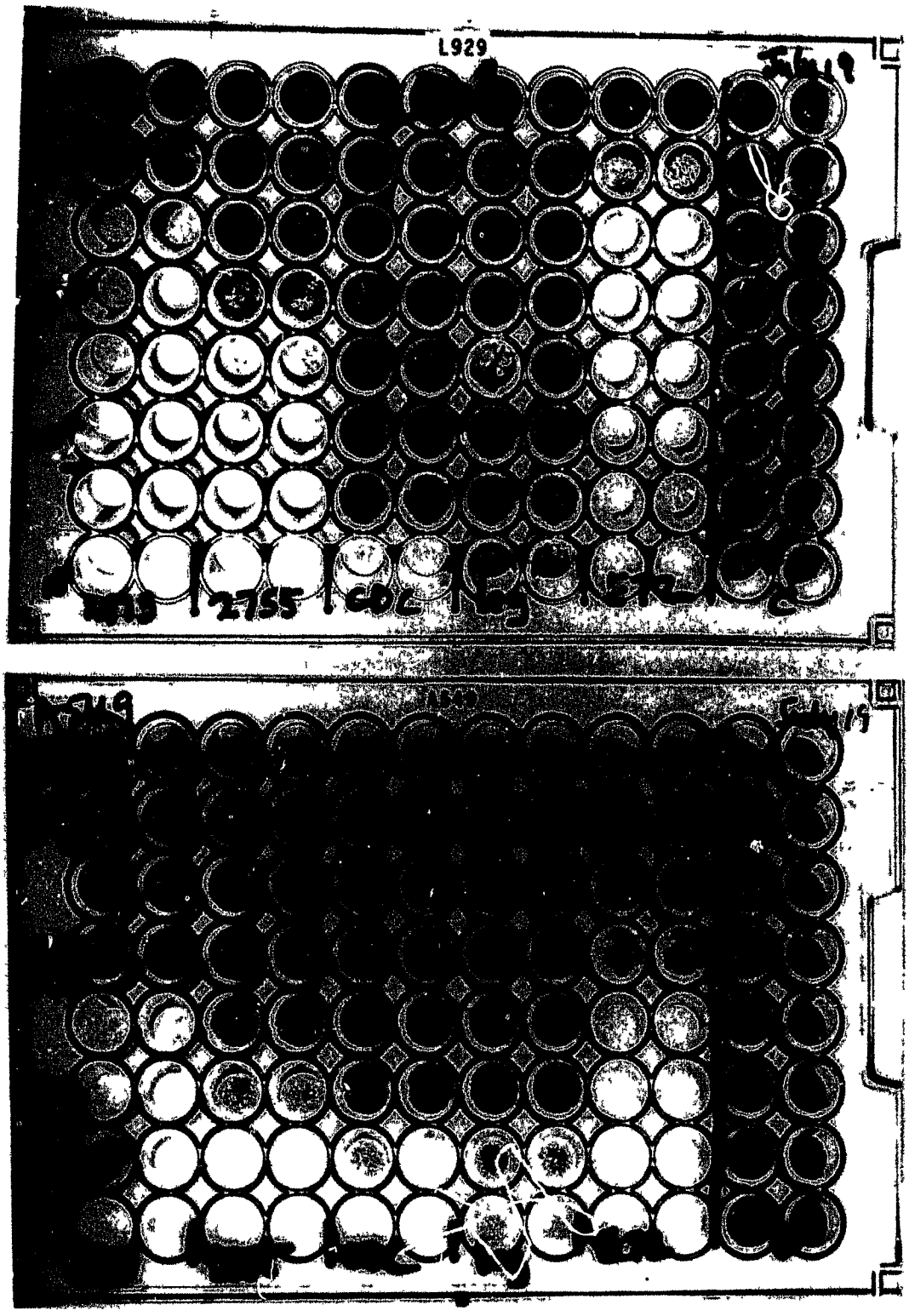


Figure 3

Figure 4. Typical L.pneumophila plaques in L929 cells. (A) Monolayers of L929 cells were infected with L.pneumophila for 60 minutes. The inoculum was removed, washed, and replaced with gentamicin for an additional 60 minutes. After the gentamicin was washed away, the monolayers were overlaid with MEM containing agarose and were incubated for 4 days. The monolayers were then fixed with formalin. After removing the agarose overlay, the cells were stained with crystal violet. (B) Mock-infected L929 cells.

Figure 4

bacterium was sufficient to produce a single plaque (Dulbecco, 1952, Dulbecco and Vogt, 1954).

To examine the kinetics of plaque production, serial ten-fold dilutions of the organisms were plated onto L929 cells. After various time periods, the inoculum was removed, replaced with gentamicin, and the plaque assay was continued precisely as described. Figure 6 compares the number of plaques produced at 5, 30, 60, 120, 180 and 240 minutes for two different strains of L.pneumophila; namely LEG, the lab-adapted organism, and 1473, the clinical isolate. Focusing on any one curve (either organism; any MOI), it is apparent that the longer the initial inoculum is exposed to the monolayer prior to the gentamicin step, the greater the number of plaques. Secondly, for either organism, at a given time point the number of plaques was proportional to the bacterial concentration or MOI. Thus even a 5 minute exposure was sufficient to produce plaques at high MOIs. When LEG and 1473 are compared, it is evident that it either took a longer period of time, or increased numbers of organisms, for LEG to produce equivalent numbers of plaques. Interestingly, within the first 2 hours of infection, the two organisms displayed comparable curves at MOIs which differed 2000-fold (MOI=13 for LEG and 0.007 for 1473). The divergence after 2 hours is likely due to a second round of infection initiated by bacteria emerging from previously infected cells.

The plaque assay was also compared with the Dreyfus

Figure 5. Relationship between plaque number and L.pneumophila concentration. L929 cells were infected in duplicate with two-fold dilutions of L.pneumophila 1473, starting with a concentration of 10^4 organisms per mL as described in the legend to Figure 4. Each point is the mean of duplicate cultures with the error bars representing the standard error.

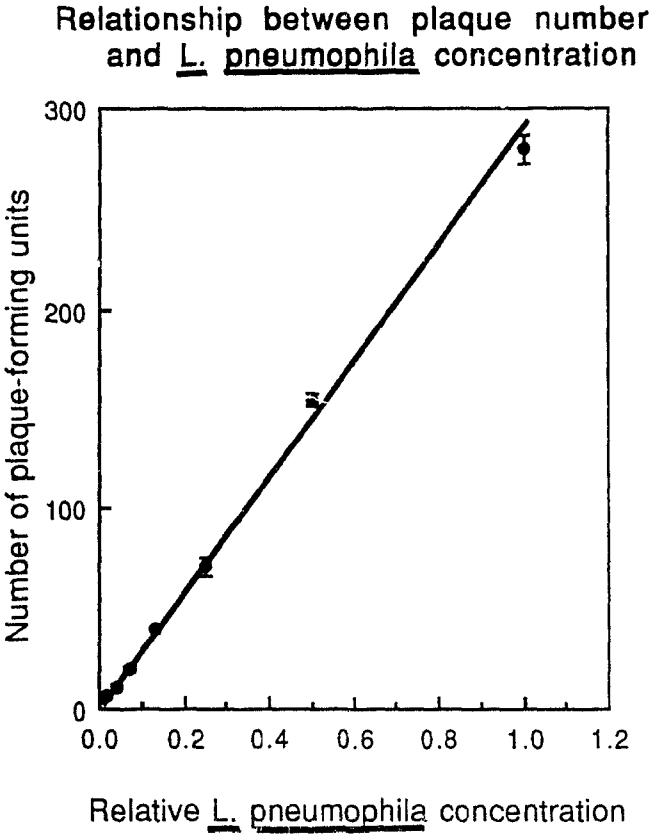

Relationship between plaque number and L. pneumophila concentration
Number of plaque-forming units
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Relative L. pneumophila concentration

Figure 5

Figure 6. Kinetics of plaque production. L929 cells were infected with serial dilutions of L.pneumophila isolates 1473 (filled symbols, solid lines) or Leg (open symbols, dashed lines). After either 5, 30, 60, 120, 180, or 240 minutes, the inoculum was removed, replaced with gentamicin, and the rest of the plaque assay was performed as described in the legend to Figure 4. TMTC refers to "too many to count" at the next time point. MOI refers to the multiplicity of infection which in this case represents the ratio of the number of bacteria to the number of host cells. Each point is the mean of duplicate cultures with the error bars representing the standard error.

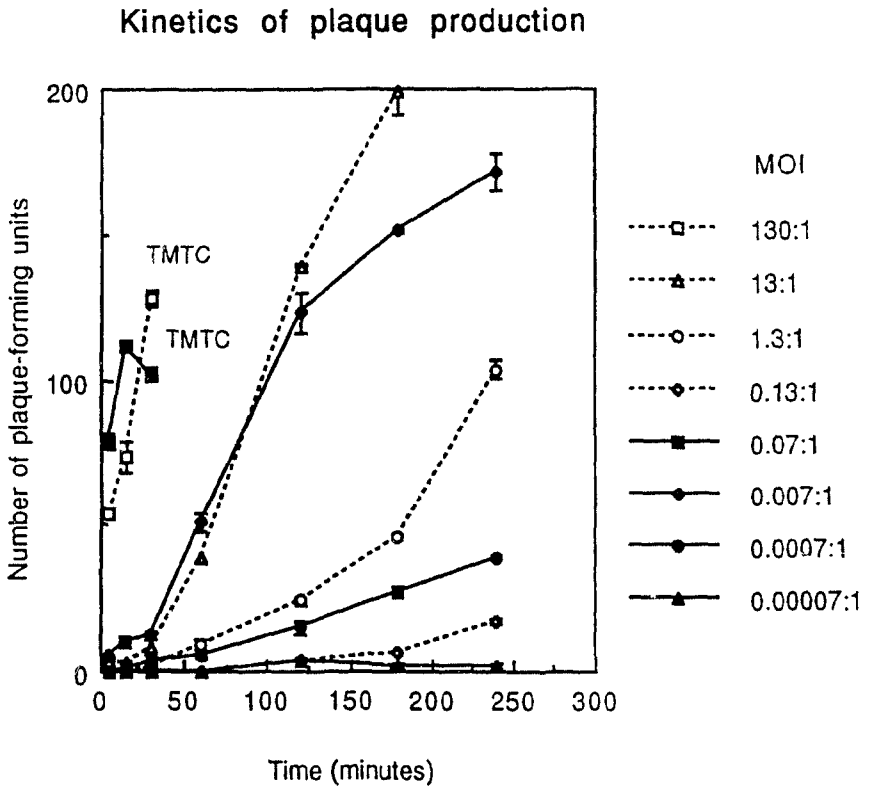

Kinetics of plaque production
200
100
0
Number of plaque-forming units
TMTC
TMTC
0
50
100
150
200
250
300
Time (minutes)
MOI
130:1
13:1
1.3:1
0.13:1
0.07:1
0.007:1
0.0007:1
0.00007:1

Figure 6

(1987) HeLa cell invasion assay. Two approaches were used. First, the kinetic data from the Dreyfus study was plotted (Figure 7). The curves generated from the virulent organism (solid lines) displayed remarkable similarity to the curves in Figure 6. Secondly, HeLa cells and L929 cells were infected identically with serial two-fold dilutions of L.pneumophila as described for the plaque assay. However instead of adding agarose to the HeLa cells, molten BCYE agar was used (Dreyfus, 1987). After 4 days, colonies appearing on the surface of and throughout the agar were counted and the numbers compared to the numbers of L929 plaques. The results are shown in Figure 8. There was a linear dose-response relationship in both cell lines, with the plaque assay being exactly twice as sensitive as the HeLa cell invasion assay.

Figure 7. HeLa invasion data from Dreyfus (1987). The data from Table 1 in Dreyfus, (1987) [Microbial Pathogenesis **3**:45-52] was plotted. The solid lines represent the invasion kinetics of Dreyfus' virulent L.pneumophila isolate; the dashed lines, the avirulent mutant. In the Dreyfus assay, HeLa cells were infected with serial dilutions of L.pneumophila for various time periods. After removing the inoculum and treating the monolayers with gentamicin, molten BCYE agar was placed on the infected HeLa cells. L.pneumophila colonies that appeared 4 days later were enumerated and plotted.

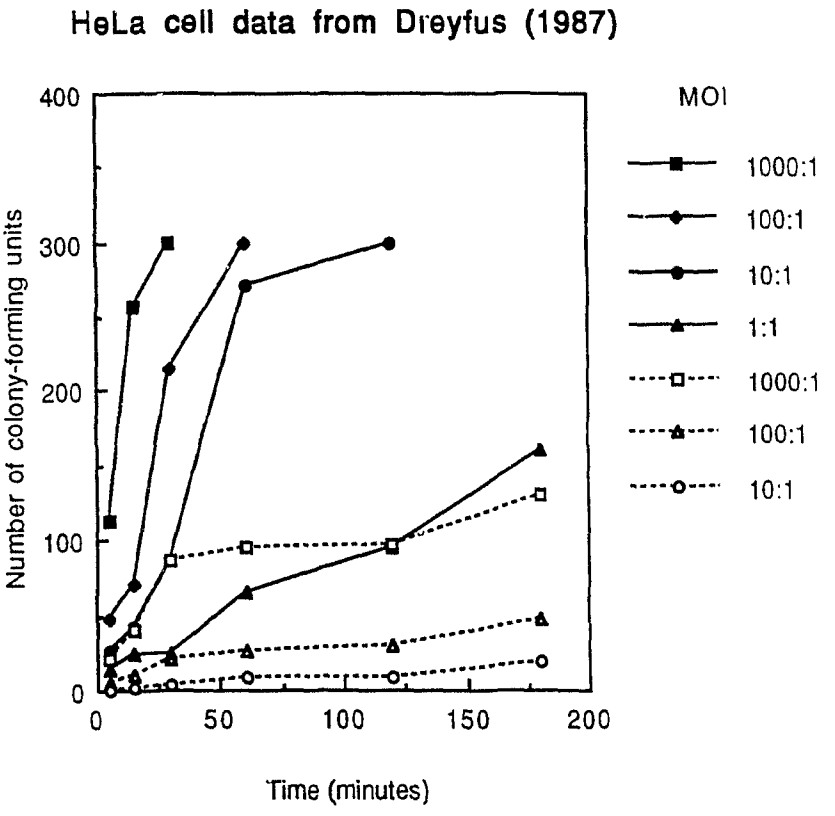

HeLa cell data from Dreyfus (1987)
Number of colony-forming units
400
300
200
100
0
0
50
100
150
200
Time (minutes)
MOI
1000:1
100:1
10:1
1:1
1000:1
100:1
10:1

Figure 7

Figure 8. Comparison of the L929 plaque assay with the HeLa cell invasion assay. HeLa cells (open circles) or L929 cells (filled squares) were infected identically with serial two-fold dilutions of L.pneumophila isolate 1473 as described in the legends to Figures 5 and 7. The resulting plaques (L929 cells) or colonies (HeLa cells) were enumerated and plotted.

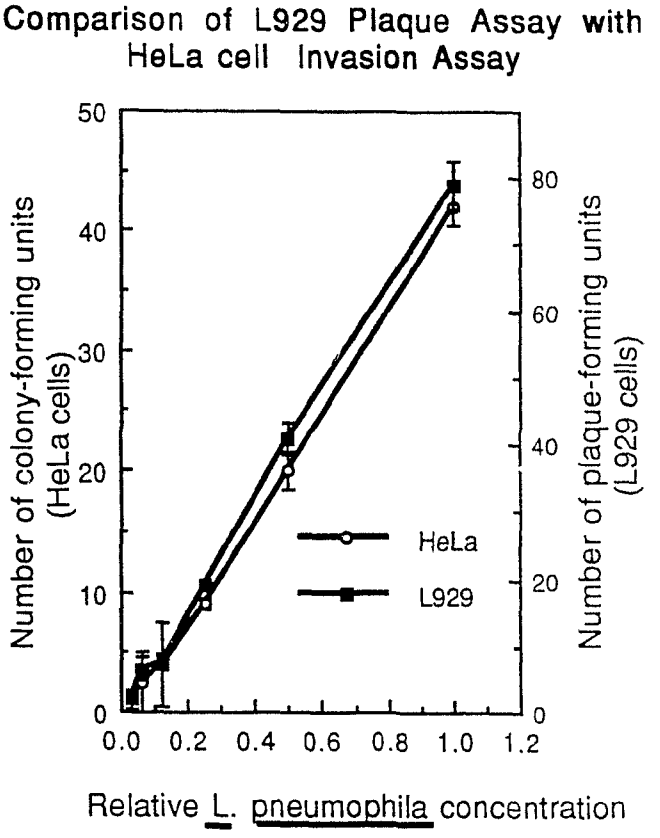

Comparison of L929 Plaque Assay with HeLa cell Invasion Assay
Number of colony-forming units (HeLa cells)
Number of plaque-forming units (L929 cells)
HeLa
L929
0
10
20
30
40
50
0
20
40
60
80
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Relative L. pneumophila concentration

Figure 8

L929 infectivity index

In order to quantify the plaque assay, an arbitrary index was created. The L929 infectivity index is defined as the ratio of the number of bacteria in the inoculum (colony forming units or cfu) to the plaque titre (pfu) divided by 1000. It is an estimate of the proportion of organisms within a population which are capable of infecting and producing visible plaques in L929 cells. For example, using the numbers from the 1473 data at the 60-minute time point (Figure 6), the dilution containing $7X10^3$ bacteria produced 51 plaques, implying that 51 out of 7000, or 1 out of 137 bacteria were plaque producers. Similar calculations showed that only 1 in 333,333 LEG organisms (39 plaques produced by $1.3X10^7$ bacteria) were capable of producing plaques. For convenience, this number was arbitrarily divided by 1000 to arrive at an index of infectivity.

In order to assess the reproducibility of the assay and to enable comparisons amongst isolates, it was important to ensure uniformity by adhering to a common exposure time. Thus, a one hour adsorption period was used for all assays. The plaque assay was found to be highly reproducible. One isolate (Lp2064) has been assayed more than 12 times over a three year period yielding an index of about 1.8 consistently. The average infectivity indexes from 6 separate experiments for isolates 1473 and LEG were calculated as being 0.16±0.02 and 464±168 respectively; about a 2000-fold difference which was

reflected in Figure 6. [For comparison, the differences between 1473 and LEG in the A549 cell crystal violet and neutral red assays were 20 and 40-fold respectively (Tables 1 and 2)].

Thus far in "blinded" tests, numerous isolates, both clinical and environmental, have been evaluated in the plaque infectivity assay. Table 3 provides a record of the results. The indexes ranged from 0.28 to 2000 with the overall majority being under 5. To evaluate whether there was a correlation between particular characteristics (where known) and the infectivity index, the organisms were grouped by G. Bezanson (Department of Microbiology, Victoria General Hospital) according to plasmid profiles (0, II, III, VI), restriction enzyme fragmentation patterns (b, c, d), monoclonal antibody reactivity (OLDA, France, Oxford), and source (patient or environmental). Plasmid profiles were defined as follows: Type 0 isolates lacked plasmids, Type II had a 20-megaDalton (MDa) plasmid, Type III carried both a 96 and a 72-MDa plasmid, and Type VI had a 100 MDa plasmid. Restriction enzyme fragmentation patterns b, c, and d refer to particular electrophoretic patterns generated by subjecting the bacterial DNA to Eco-R1 or Bgl-II restriction enzymes prior to electrophoresis. The results are presented in Table 4.

Organisms of the 0/c/France type were found to be the least effective in the plaque assay. Indeed, unlike those produced by the other organisms, the plaques produced by many

Table 3. L929 infectivity index* for various L.pneumophila isolates.

L.pneumophila Isolate	Infectivity Index(n)+	L.pneumophila Isolate	Infectivity Index(n)+
87-1405	1	87-1512	0.45
87-1638	0.94	87-1519	0.8
87-1727	0.33	87-1811	1.07
87-1819	1	87-1814	1.35
87-1250	1.9	87-1317	1.7
87-1394	2.16	87-1209	1.1
87-1238	0.98	87-1242	3.5
87-1240	3.08	87-1721	3.08
87-1718	4.11	87-1398	1.7
87-100	1.6 (3)	87-778	1.6
87-1095	168 (3)	88-2064	1.8 (12)
87-261	0.7	87-1140	0.9
88-2751	3.85 (2)	85-578	6.3
87-185	0.8	87-1109	3.3 (4)
88-106	2.3	87-256	2.3
87-1045	1.4	87-589	2.3
87-588	1.1	84-219	2.2
88-2280	49 (2)	88-2673	82.6 (3)
88-2478	980	89-2579	1.2 (6)
89-313	2.7 (2)	89-1122	3.3 (3)
89-2704	9 (3)	89-1470	1.4
88-1375	20	88-1451	5
89-2590	5	89-1567	245 (2)
89-1604	2000	90-4269	1.2 (2)
90-4527	2.5 (2)	89-341	0.9
90-4264	300	91-236161	10.2
91-194977	0.59	91-4831	1
90-136787	0.28		

*Refers to the colony forming unit:plaque forming unit ratio divided by 1000; the smaller the number, the greater the proportion of organisms that are capable of producing plaques in L929 cells.

+ Tested in duplicate, the average of (n) if more than 1, tests

Table 4. Relationship between L929 infectivity index and genotypic/phenotypic characteristics of L.pneumophila isolates.

Properties Pp/Rfp/mAb/S*	Isolates	Average Infectivity Index (n) (range)
0/c/France/P	88-2673, 88-2478, 87-1095, 89-1567	246.8 (9) (13-980)
0/c/France/E	88-2280, 89-4264 89-1604	599.2 (4) (34-2000)
II/b/OLDA/P	87-256	2.3
II/b/OLDA/E	89-4527, 87-1109 88-2751	3.6 (7) (2.4-4)
II/d/OLDA/P	88-106	2.3
II/b/Oxford/P	88-2064	1.8 (12) (0.5-5)
II/b/Oxford/E	87-261	0.7
II/d/Oxford/E	87-1140, 90-4269 89-341	1.1 (4) (0.7-2)
III/d/OLDA/P	87-100	1.6 (3) (2.1-1.2)
III/a/Oxford/E III/d/Oxford/E	89-1470 87-589	1.9 (2) (1.4-2.3)
VI/b/OLDA/P	87-2579, 89-313	1.8 (7) (0.4-3.8)
VI/b/Oxford/E	89-1122	3.3 (3) (2.1-5.4)
VI/d/Oxford/E	87-588	1.1

*Represents: plasmid profile (0= no plasmid, II= 20 megaDalton (MDa) plasmid, III = 96 and 72 MDa plasmids, VI= 100 MDa plasmid)/restriction enzyme fragmentation pattern using EcoRI or BglII/monoclonal antibody reactivity/environmental or patient source.

of the 0/c/France isolates were difficult to count because of their small size. The infectivity index could not be used adequately to distinguish further amongst the other isolates.

The significance of the index with respect to virulence is currently being evaluated in guinea pigs as part of an on-going study conducted by T. Marrie, G. Bezanson and colleagues at the Victoria General Hospital. Preliminary findings, have resulted in the grouping of the organisms into 4 categories of pathogenicity (G. Bezanson, personal communication). Each category describes the organisms with respect to infectivity in L929 cells (high infectivity refers to a low index), and mortality in guinea pigs. Organisms in Category 1 had high infectivity in L929 cells, produced high mortality in guinea pigs ($LD50=10^6 - 10^7$), and were represented by organisms of type II/b/OLDA or Oxford; organisms in Category 2 had high infectivity, moderate mortality ($LD50=10^8$) and were of type VI/b/OLDA or Oxford; organisms in Category 3 had high infectivity, low mortality ($LD50=10^9$) represented by type III/b/OLDA; and, organisms in Category 4 had low infectivity, moderate mortality and were of type 0/c/France. Based on these preliminary results, it appears that while the L929 assay can effectively identify Category 1 organisms, it is unable to predict the (a)virulence of Category 3 organisms. It is also less able to discriminate between Category 2 and Category 4 organisms which differ in the L929 assay but not with respect to guinea pig mortality.

Use of L929 cells to study the intracellular pathogenesis of L.pneumophila

Although L929 cells may not be able to adequately discriminate amongst all categories of L.pneumophila in terms of predicting overall virulence, they can be utilized to study the events leading to intracellular pathogenesis; an important virulence factor in Legionnaires' disease. Certainly, all categories of L.pneumophila serogroup 1 tested to date have produced plaques, even the O/c/France isolates.

The aim of this study was to examine intracellular pathogenesis; comparing virulent and avirulent organisms. Since L929 plaques are the manifestation of L.pneumophila invasion and intracellular growth, the initial task was to find an isolate that failed to produce plaques. To avoid potential problems in trying to compare organisms that were not isogenic, it was decided to exploit growth on Mueller-Hinton (M-H) medium (supplemented with hemoglobin and IsoVitale X) as a means of selecting avirulent mutants (McDade and Shepard, 1979).

The L.pneumophila serogroup 1 strain that was chosen for these studies was originally isolated from a patient with Legionnaires' Disease. Catalogued by the Victoria General Hospital as being isolate 88-2064, it was characterized as a type II/b/Oxford strain. For convenience it will be referred to as Lp2064. A colony of Lp2064 was streaked onto M-H agar to obtain an avirulent mutant. Twelve days later, tiny,

pinpoint colonies appeared. A single colony was picked and used to inoculate another M-H plate. This procedure was repeated 5 times resulting in the isolation of Lp2064M.

Characterization of Lp2064 and Lp2064M

The growth of Lp2064 and Lp2064M on BCYE agar yielded colonies which were quite similar in size, shape, and colour. Lp2064 sometimes but not always formed colonies which were slightly less compact than the Lp2064M colonies (Figure 9A). The size of the colonies did not vary significantly.

The protein profiles of BYE broth-grown organisms and BCYE agar-grown organisms are depicted in Figure 9B and 9C respectively. There appeared to be no difference between Lp2064 and Lp2064M with respect to proteins, as analyzed by SDS-PAGE. When lipopolysaccharide patterns were compared, both organisms displayed similar banding patterns (not shown).

Furthermore both Lp2064 and Lp2064M were equally capable of producing a 38-kDa protein which was found in the supernatant fractions of broth-grown organisms. This was assumed to be the L.pneumophila 38-kDa protease (Figure 10).

Figure 9. Colony morphology and protein profiles of Lp2064 and Lp2064M. (A) Colonies on BCYE agar. Visualized 4 days after inoculation. (B) Coomassie blue-stained protein profiles of 18h cultures of BYE broth-grown organisms. Aliquots of the cultures were pelleted and resuspended in SDS-PAGE sample buffer. The samples were boiled for 5 minutes and loaded onto a discontinuous SDS polyacrylamide gel (11% separating gel). After electrophoresis, the gel was fixed, and stained with Coomassie blue. 2 and M refer to Lp2064 and Lp2064M respectively. (C) Coomassie blue-stained protein profiles of 2-day cultures of BCYE agar-grown Lp2064 (2) and Lp2064M (M). Molecular weight markers ranging from 94 to 14.4 kDa are indicated.

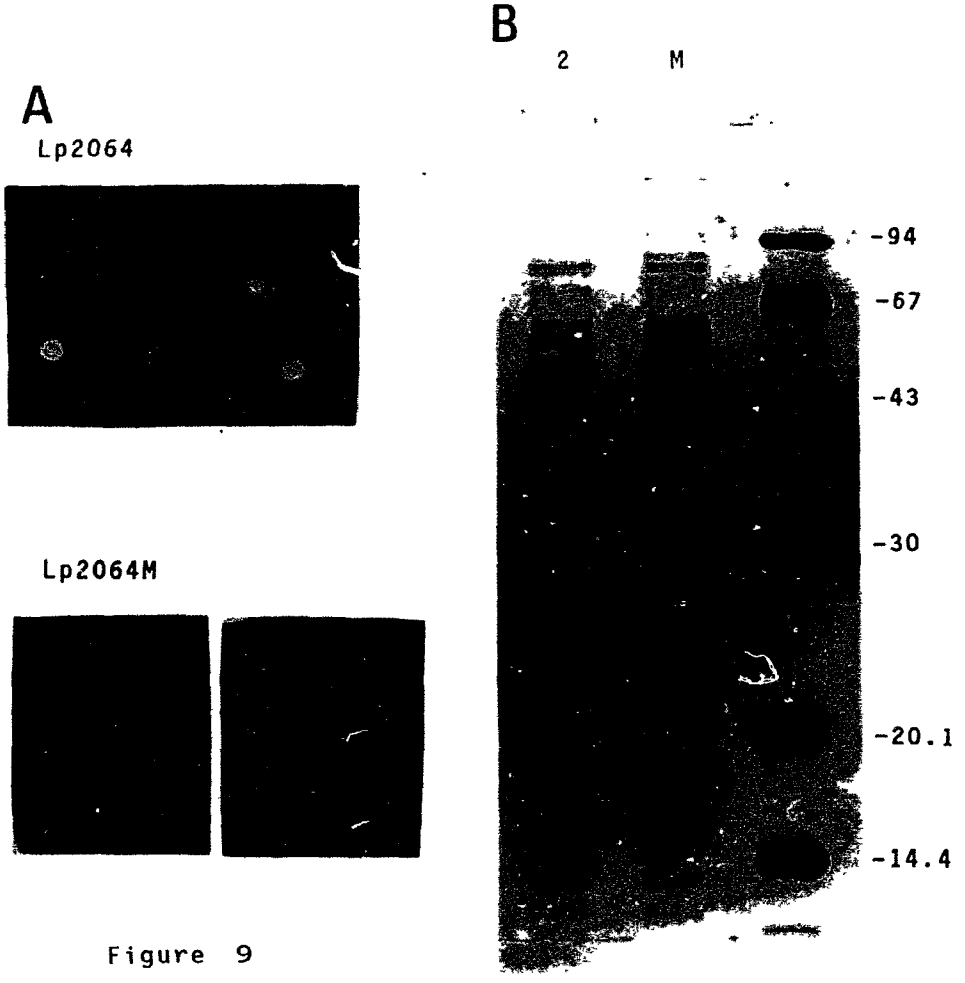


Figure 9

Figure 10. Production of a 38-kDa secretory protein by Lp2064 and Lp2064M as detected by autoradiography. [^{35}S]-methionine was immediately added to BYE broth inoculated with either Lp2064 or Lp2064M. Forty-eight hours later, the bacteria were pelleted and the supernatants passed through a 0.45μm filter. Proteins in the supernatant fractions were precipitated with TCA, washed with acetone, and resuspended in SDS-PAGE sample buffer. Twenty-five μL of each sample was boiled and run on an SDS polyacrylamide gel. The gel was stained, destained, dried and exposed to Xray film. P refers to the bacterial pellet (see legend to Figure 9) and S to the filtered supernatant fractions. 2 and M are Lp2064 and Lp2064M respectively.

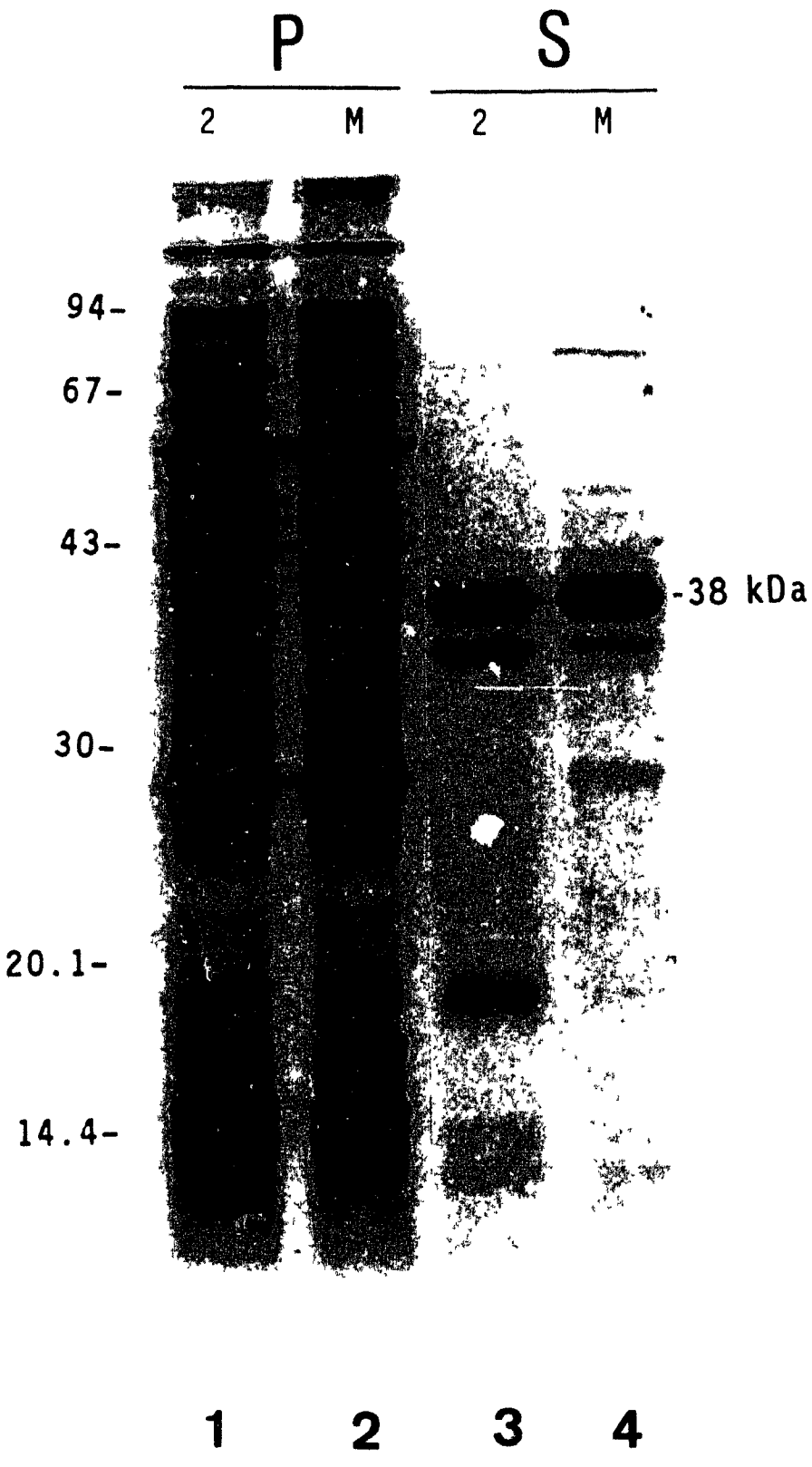


Figure 10

Virulence determination of Lp2064 and Lp2064M

The virulence of Lp2064 and Lp2064M was ascertained in A549 cells, in embryonated hen eggs and in guinea pigs. On the basis of these assays, the results of which are recorded in Table 5, Lp 2064M was deemed avirulent. Particularly noteworthy is the guinea pig data. There was a greater than 1000-fold difference in the LD50 values between the two organisms. In fact, even at $1X10^9$ organisms per mL, none of the guinea pigs inoculated with Lp2064M succumbed.

In addition, unlike Lp2064M, Lp2064 was able to ellicit damage in monocytes and in the U937 monocyte-like cell line.

Interactions of Lp2064 and Lp2064M with L929 cells

The capacity of Lp2064 and Lp2064M to infect L929 cells was examined. Lp2064 produced plaques in L929 cells and, as illustrated in Figure 11, with kinetics similar to those previously shown (Figure 6). While the infectivity index for Lp2064 was calculated as being 2, not a single plaque was produced by Lp2064M, even at an MOI of more than 1000. Incidently, although plaque production was abolished after 2 passages on M-H, passaging Lp2064 once on M-H still yielded some plaques (infectivity index of 50,000).

Possible reasons for the failure of Lp2064M to produce plaques were explored. These included the potential inability of Lp2064M to 1) bind to L929 cells; 2) to invade, if binding was not impaired; 3) to survive, and 4) to multiply intracellularly.

Table 5. Comparison of virulence of L. pneumophila strains Lp2064 and Lp2064M in tissue culture, eggs and guinea pigs.

	Assays of Virulence		
L. pneumophila Strain	Tissue[a] Culture	Eggs[b] EID_{50}	Guinea pigs[c] LD_{50}
Lp2064	3×10^6	1×10^6	1×10^6
Lp2064M	$>6 \times 10^8$	1×10^8	$>1 \times 10^9$

[a]The number of organisms/ml causing a 50% reduction in neutral red uptake by uninfected A549 cells.

[b]Assayed by the inoculation of yolk sacs of 8 day old chick eggs.

[c]Assayed by intraperitoneal inoculation of male guinea pigs weighing 250-300g. The results from 3 experiments were pooled.

Figure 11. Kinetics of Lp2064 plaque production. L929 cells were infected with Lp2064 as described in the legend to Figure 6 except that the 180 and 240 minute time points were omitted. TMTC refers to "too many to count" at the next time point. MOI refers to the multiplicity of infection.

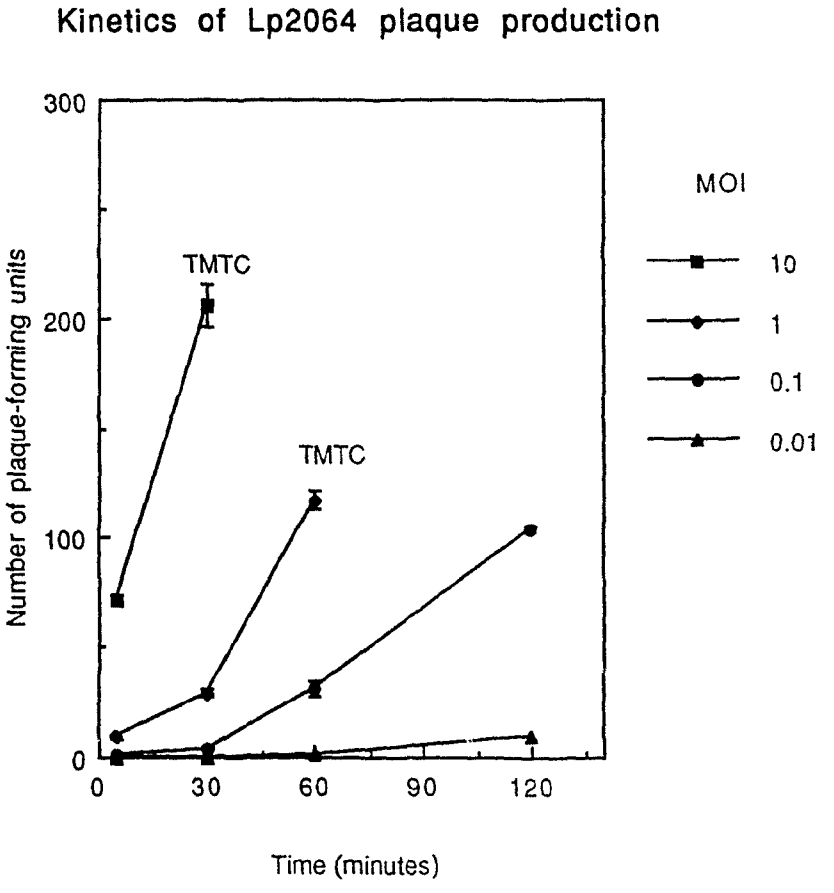

Kinetics of Lp2064 plaque production
300
200
100
0
Number of plaque-forming units
TMTC
TMTC
0
30
60
90
120
Time (minutes)
MOI
10
1
0.1
0.01

Figure 11

To investigate the first possibility, L929 cells were infected with equal numbers of Lp2064 or Lp2064M for 60 minutes after which they were washed, fixed, and stained by immunofluorescence. Figure 12 shows no appreciable difference in the ability of either organism to bind L929 cells.

Confirmation that Lp2064M was indeed capable of invading was provided by three lines of evidence. First, monolayers were infected for 5 minutes, washed to remove nonadherent bacteria, and then returned to the incubator. The next day, the cells were once again washed extensively, permeabilized with 0.5% Triton X-100, and stained by immunofluorescence. Figure 13 compares uninfected L929 cells with L929 cells infected with either Lp2064 or Lp2064M. These pictures suggest that Lp2064M is capable of penetrating L929 cells (Panel B); however the possibility that extracellular bacteria (i.e. bacteria adhering to the cell surface) were stained could not be ruled out. What is obvious from Figure 13 (Panel C), is the graphic illustration of the intracellular multiplication of Lp2064.

The second piece of evidence that Lp2064M is capable of invading is much more convincing. L929 cells were infected with either organism for 6 hours or 18 hours. After washing the monolayers, they were fixed with glutaraldehyde and processed for electron microscopy. Thin sections of uninfected and infected cells are shown in Figure 14. As exhibited in Panel B, individual Lp2064M are found within vacuoles in the

Figure 12. Immunofluorescence staining of L.pneumophila showing the binding of Lp2064 (A) and Lp2064M (B) to L929 cells. L929 cells were infected with equal numbers (MOI of approximately 100) of Lp2064 or Lp2064M for 60 minutes. After the inocula were removed, the cells were washed, fixed with formalin, and stained by indirect immunofluorescence.

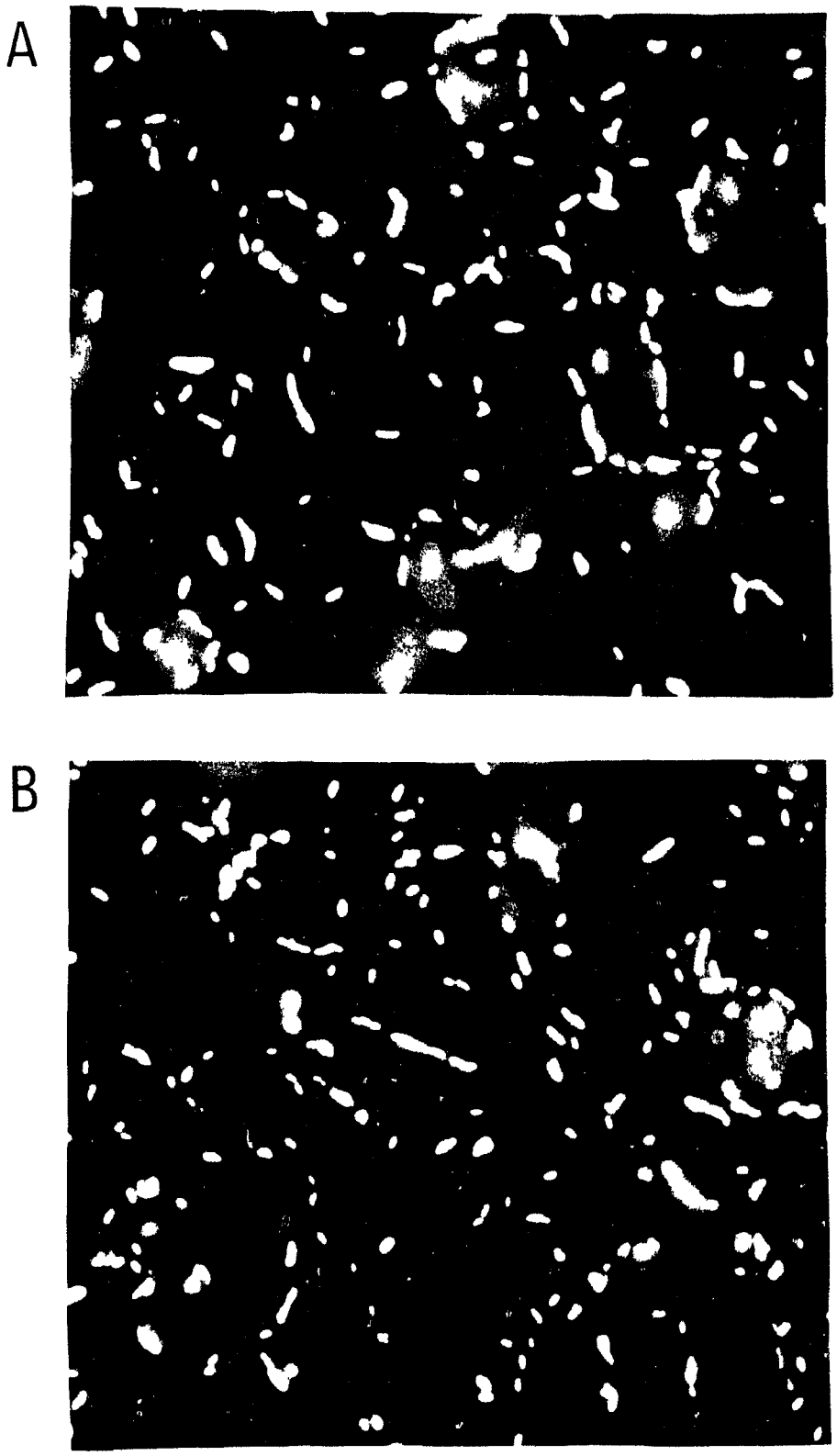


Figure 12

Figure 13. Immunofluorescence staining of L.pneumophila showing the intracellular location of Lp2064 and Lp2064M 24h following the initial infection. L929 cells were infected with Lp2064 or Lp2064M for 5 minutes. The infected monolayers were washed and incubated. Twenty-four hours later, the cells were washed, briefly permeabilized with 0.5% Triton X-100 and fixed with acetone. The bacteria were visualized by indirect immunofluorescence. (A) Uninfected L929 cells. (B) L929 cells infected with Lp2064M. (C) L929 cells infected with Lp2064.

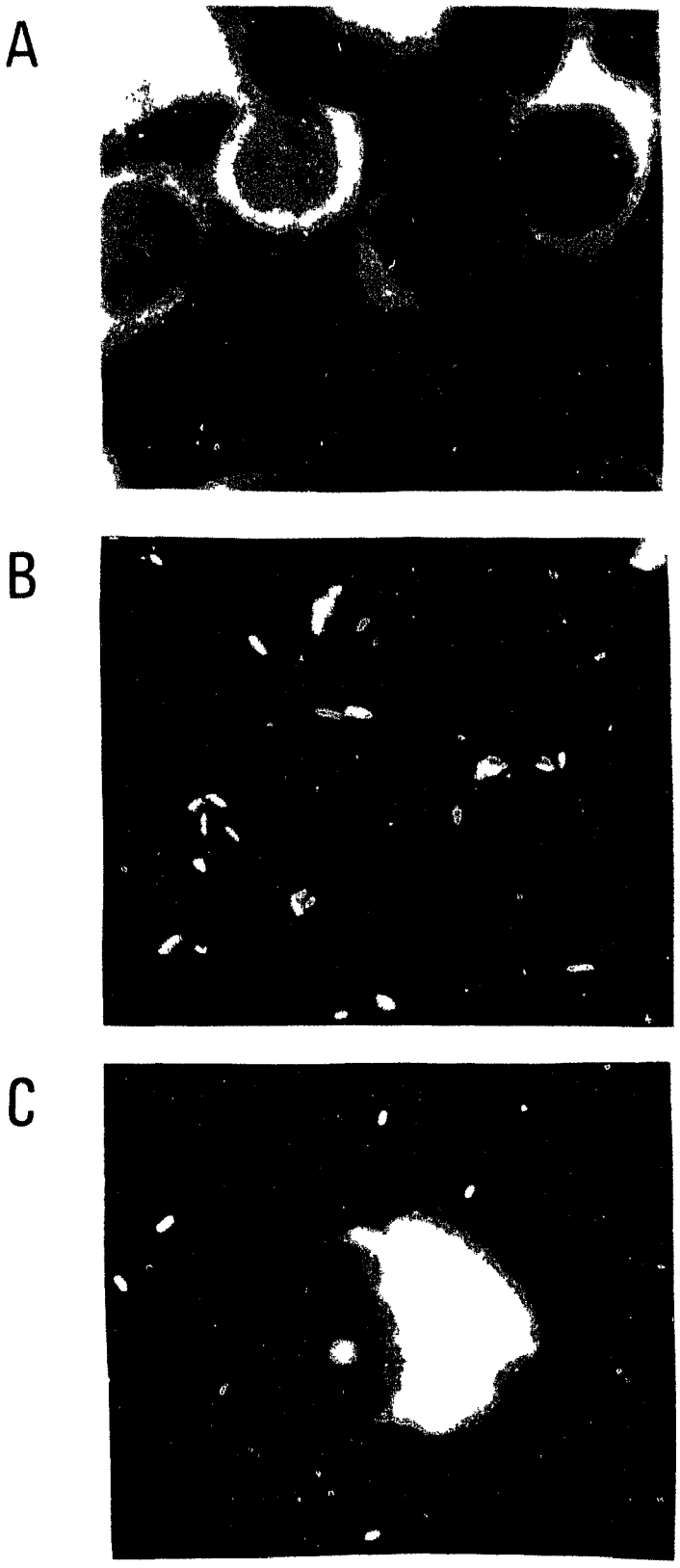


Figure 13

L929 cell, establishing that this organism can in fact, invade.

Panel C of Figure 14 further attests to the intracellular growth of Lp2064 as numerous bacteria enclosed within (a) vacuole(s) can be seen clustered to one end of the cell. A closer inspection of Lp2064 and Lp2064M at 18 hours (Panels D and E respectively) shows that 1) vacuoles of cells infected with Lp2064 contained multiple organisms, compared with only 1 bacterium per vacuole in Lp2064M-infected cells; and 2) compared with Lp2064, the morphological integrity of Lp2064M was compromised.

Finally, that invasion of viable Lp2064M organisms occured was supported by the following experiment. L929 cells were infected for 1 hour with Lp2064M after which the inoculum was removed, and replaced with gentamicin to kill extracellular bacteria. After washing off the gentamicin, the L929 cells were lysed with 0.1% Triton and the contents plated on BCYE plates. Viable organisms, corresponding to the number of Lp2064 plaques attained at the same dilution, were recovered when the plates were examined 4 days later.

While it is apparent that Lp2064 is able to survive and multiply within L929 cells (Figure 15A), the fate of Lp2064M following invasion was the subject of the next experiment. L929 cells were infected for 1 hour with Lp2064M, subjected to gentamicin as described and lysed either immediately after the gentamicin step, or at 24, 48, or 72 hours after

Figure 14. Electron micrographs depicting intracellular L.pneumophila. L929 cells were infected for 6 or 18 hours with Lp2064 or Lp2064M. The monolayers were washed, fixed with glutaraldehyde and processed for electron microscopy. Thin sections of (A) uninfected [X43,200], or (B to E) infected cells are illustrated. (B) Lp2064M 6 hours after infection [X24,000]. (C) Lp2064, 18 hours (D) Lp2064, 18 hours (E) Lp2064M, 18 hours.

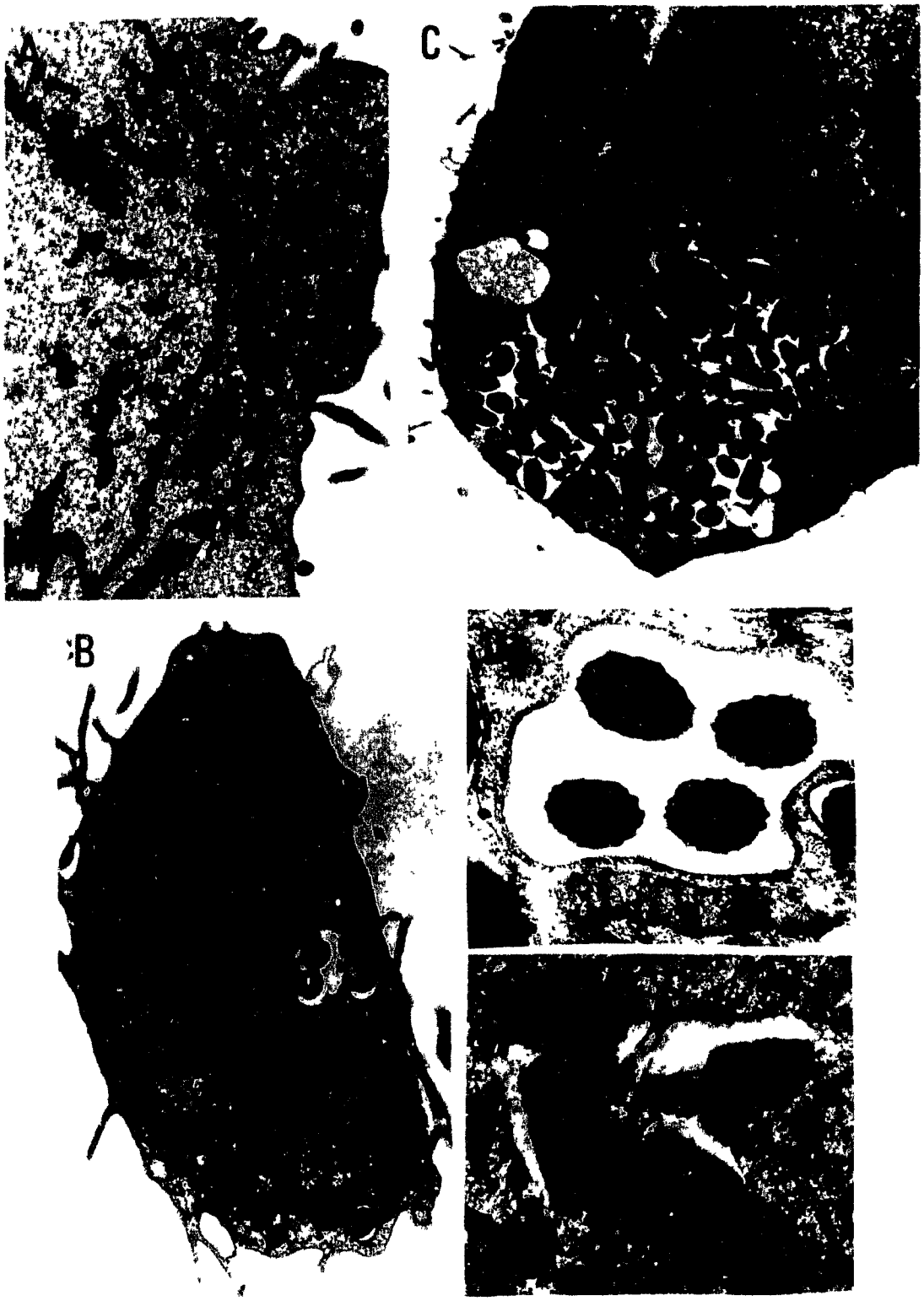


Figure 14

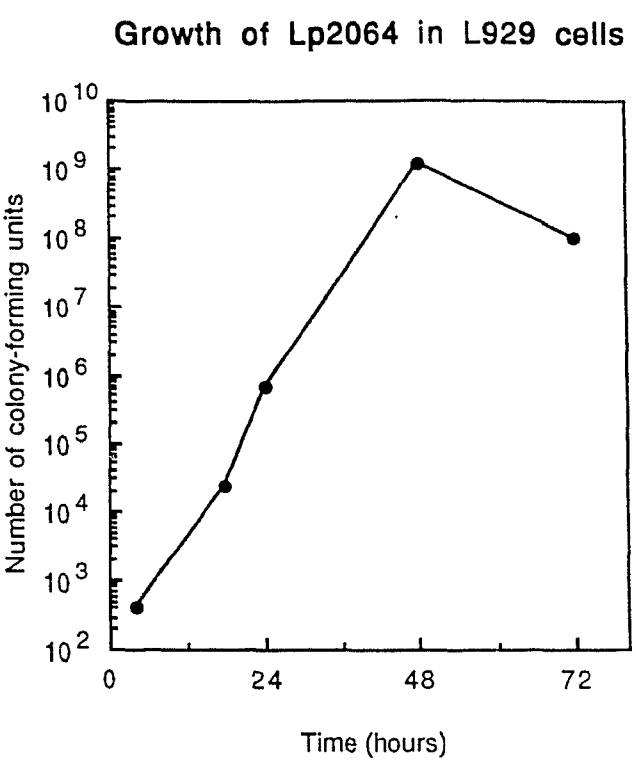
infection. The resulting yield of colony forming units was plotted and is shown in Figure 15B. There was a precipitous drop in the number of colonies recovered 24 hours after infection, and by 72 hours no bacteria were observed. Thus, Lp2064M fails to produce any plaques because it is killed intracellularly.

Mechanism of intracellular survival

It is evident that unlike Lp2064M, Lp2064 is able to survive and multiply intracellularly. Previously, Horwitz (1983b) had shown that L.pneumophila survives within macrophages by inhibiting the fusion of L.pneumophila-bearing phagosomes with lysosomes. Within this privileged site, the bacteria grow unchecked until they eventually lyse the cell. The role of phagosome-lysosome fusion in affecting the fates of Lp2064 and Lp2064M was thus investigated. Two approaches were used. The first, relied on assessing phagosome-lysosome fusion by looking for L.pneumophila in secondary lysosomes. This was achieved by treating L929 cells prior to infection with either ferritin or Thorotrast, both of which accumulate in lysosomes and are electron dense. Thin sections of infected cells were then examined by electron microscopy. Fusion was scored by the presence of the organisms within vacuoles containing ferritin or Thorotrast. The second method was more direct. Lysosomes were identified cytochemically by taking advantage of their acid phosphatase activity. L929 cells or monocytes were infected with either Lp2064 or Lp2064M for 4

Figure 15. Growth and survival of L.pneumophila in L929 cells. L929 cells were infected with either Lp2064 or Lp2064M (MOI of 0.1) as described in the legend to Figure 4 except that culture medium was added instead of the agarose overlay. At various time points after the infection, the monolayers were lysed with 0.1% Triton X-100 and the contents plated on BCYE plates. The resulting number of colony-forming units were counted and plotted. (A) Growth of Lp2064 in L929 cells. (B) Survival of Lp2064M in L929 cells.

A


Growth of Lp2064 in L929 cells
Number of colony-forming units
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
0
24
48
72
Time (hours)

B

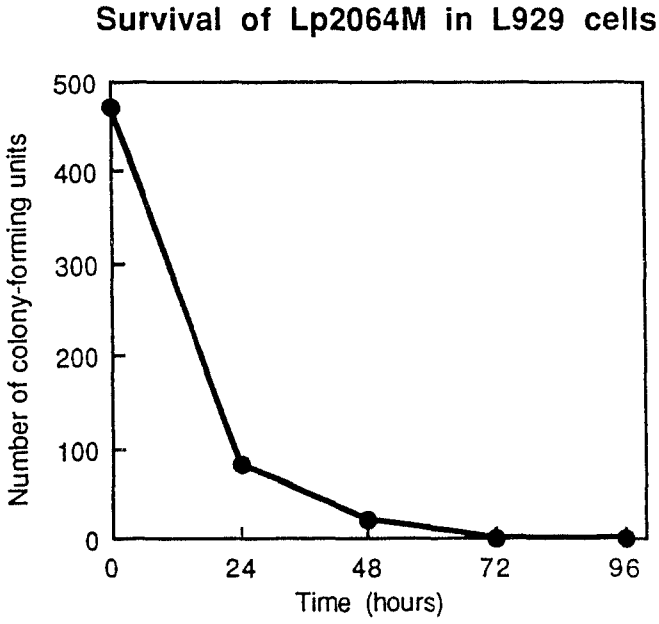

Survival of Lp2064M in L929 cells
Number of colony-forming units
500
400
300
200
100
0
0
24
48
72
96
Time (hours)

Figure 15

to 6 hours. The cells were briefly fixed and then exposed to a buffered solution (pH 5) containing the substrate ß-glycerophosphate, and cerium chloride. In control preparations, the ß-glycerophosphate was omitted from the buffer. The presence of acid phosphatase activity, as visualized in thin sections, was indicated by a membrane-bound electron-dense area resulting from the hydrolysis of phosphate and the ensuing capture of cerium. Fusion was thus scored by the presence of an organism within a vacuole displaying an intense staining reaction.

Regardless of the method used, Lp2064 was observed to resist the fusion of its phagosome with lysosomes. Lp2064M however, was unable to do the same. The results of the cytochemical experiments are illustrated in Figure 16 and are tabulated in Table 6. Whereas panel A of Figure 16 shows Lp2064 within a phagosome, Panels B and C show Lp2064M within phagolysosomes in L929 cells and monocytes respectively. Table 6 indicates that 81.3% of Lp2064M were found in vacuoles with acid phosphatase activity, compared with only 3.2% of Lp2064. No acid phosphatase activity was seen when the substrate was omitted.

Figure 16. Demonstration of phagosome-lysosome fusion in thin sections of Lp2064M-infected cells. L929 cells or monocytes were infected with Lp2064 or Lp2064M as described in the legend to Figure 14. Following infection, the cells were briefly fixed with glutaraldehyde and exposed to a cytochemical solution (pH5) containing ß-glycerophosphate and cerium chloride. After further fixing the cells, they were processed for electron microscopy. Electron-dense areas found within vacuoles containing L.pneumophila are due to the "capture" of cerium (Ce) and are indicative of acid phosphatase activity. (A) and (B) Lp2064 and Lp2064M respectively in L929 cells [X57,000]. (C) Monocyte infected with Lp2064M [X24,000]. Arrows point out Cerium (Ce) capture; L is Lp2064M.

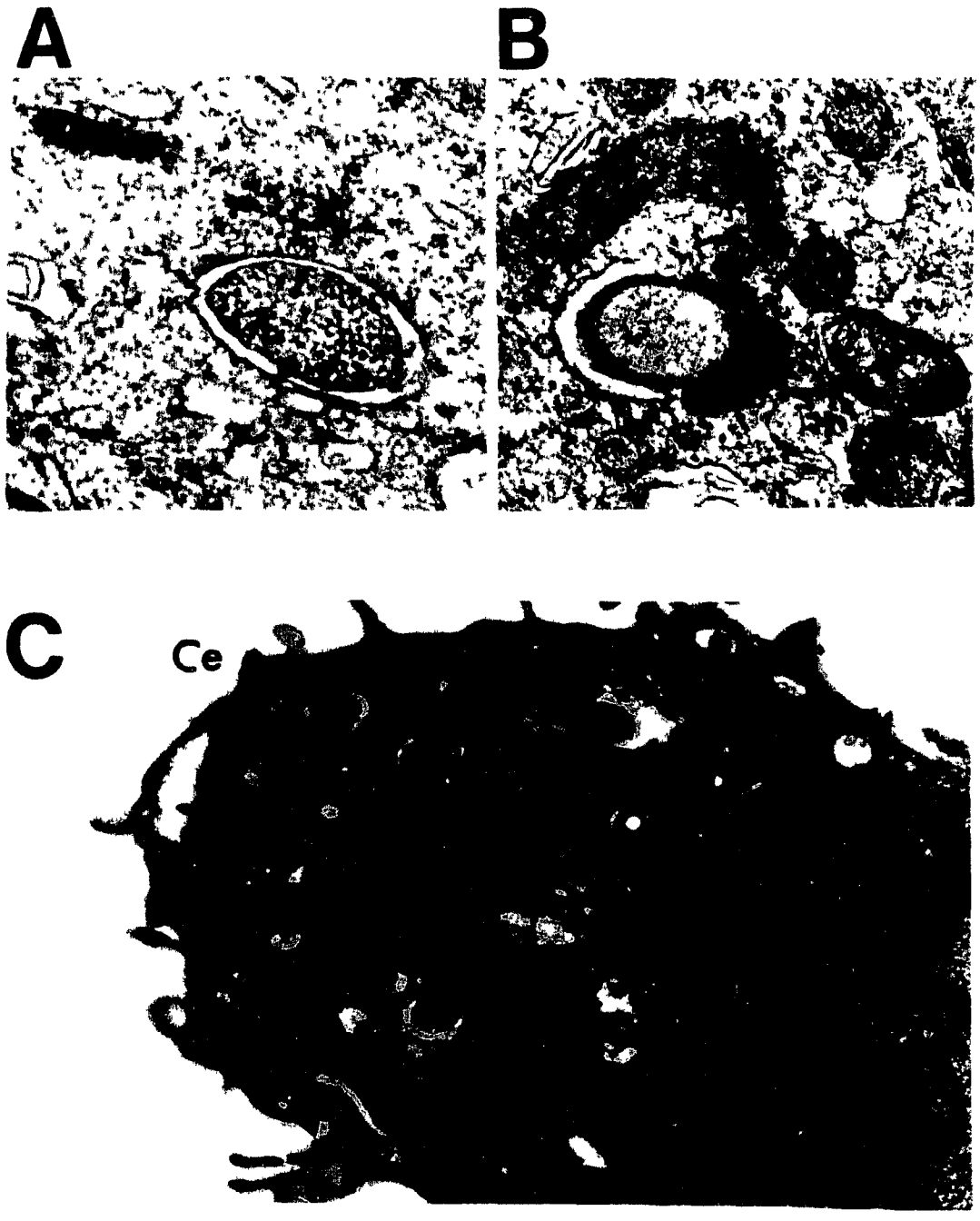


Figure 16

Table 6. Assessment of the ability of Lp2064 and Lp2064M to inhibit phagosome-lysosome fusion.

Isolate	# of L.pneumophila vacuoles with AcPase[§] activity	# of L.pneumophila vacuoles with NO AcPase[§] activity	Percent Fusion Inhibition
Lp2064 (31)*	2	61	96.8
Lp2064M (38)*	87	20	18.7

[§]Acid phosphatase
*Refers to the number of monocytes examined

Influence of host cells in the expression of Lp2064 and Lp2064M proteins

a) General observations

As previously demonstrated (Figure 9), Lp2064 and Lp2064M appeared to be similar in terms of their colony morphology, and protein profiles, regardless of whether the organisms were grown in BYE broth or on BCYE agar. Yet, when these bacteria were intracellular, they experienced drastically different fates. The purpose of the present series of experiments was to examine the influence, if any, of the interaction between BCYE agar grown-organisms and host cells, on bacterial protein synthesis; reasoning that any differences in the responses between Lp2064 or Lp2064M might ultimately be involved in affecting phagosome-lysosome fusion.

L929 cells were prepared as follows prior to infection. They were first placed in medium lacking methionine (MEM$^-$) and then exposed to cycloheximide. The objective was to enhance the labeling of the bacteria while minimizing host cell protein synthesis. The cycloheximide treatment did not affect infectivity. L929 cells prepared as described were infected, for 5 minutes with either Lp2064 or Lp2064M at an MOI of 1000. The short exposure period was chosen so that responses pertinent to the early stages of infection could be analyzed. Therefore, to compensate for this short infection period, a high MOI was selected. (Recall from Figure 11 that at a high MOI, a 5 minute exposure was sufficient to produce plaques.)

Following infection, the inoculum was removed, the monolayers washed 3 to 5 times to remove non-adherent bacteria, and the last wash replaced with 1 mL of MEM. At this point, Time 0 (T=0) was established. At T=0, T=30 minutes (T=30) or T=60 minutes (T=60), the [^{35}S]-methionine label was added for either 10 or 30 minutes. The label was then removed and the monolayers washed extensively. This second wash step was essential. It served to remove any extracellular non-adherent bacteria which might have incorporated the label because they survived the first series of washes. The washed monolayers were then lysed with 0.1% Triton X-100 and the bacteria collected by differential centrifugation. Viability of the bacteria was unaffected by Triton X-100. After determining the number of trichloroacetic acid (TCA)-precipitable counts, equal numbers of counts of each sample were analysed by SDS-PAGE fluorography. In this manner, the relative levels of specific proteins could be compared.

Panel A of Figure 17 provides a comparison of Lp2064 (Lanes 1, 3, and 5) and Lp2064M (Lanes 2, 4, and 6) at T=0, T=30 and T=60. It is evident that Lp2064 and Lp2064M differed in their respective protein profiles at each of the time points. In examining Lanes 1, 3, and 5, the most prevalent of the Lp2064 proteins was one migrating at 60 kDa. Although Lp2064M also appeared to produce a 60-kDa protein, it was not as dominant and it usually but not always seemed to migrate at a position slightly below 60 kDa. The increased expression

Figure 17. Protein profiles of Lp2064 and Lp2064M exposed to L929 cells. L929 cells were initially placed in medium lacking methionine before treating them with cycloheximide. The cells were then infected for 5 minutes with either Lp2064 or Lp2064M at an MOI of 1000. After washing the infected cells, the wash medium was replaced with culture medium (T=0). At T=0, T=30 minutes, and at T=60 minutes the cells were pulsed for 10 minutes with the [^{35}S] label. The culture medium was removed, the monolayers washed and then lysed with Triton X-100. The bacteria in the lysate were collected by differential centrifugation. The numbers of TCA-precipitable counts were determined and equal numbers of counts of each sample were compared by SDS-PAGE followed by autoradiography/fluorography. (A) 0, 30 and 60 refer to the post-infection time points; 2 and M to Lp2064 and Lp2064M respectively. The large and small arrowheads represent the 60 and 29-kDa proteins, respectively. The small arrows from top to bottom, refer to proteins having the following molecular weights in kDa: 32, 31, 26, 23, 17, 16, 15, and 13. Molecular weight markers in kDa are also indicated. (B) Instead of equivalent counts, amount loaded corresponded to the number of bacteria (cfu) loaded in Lane 6.

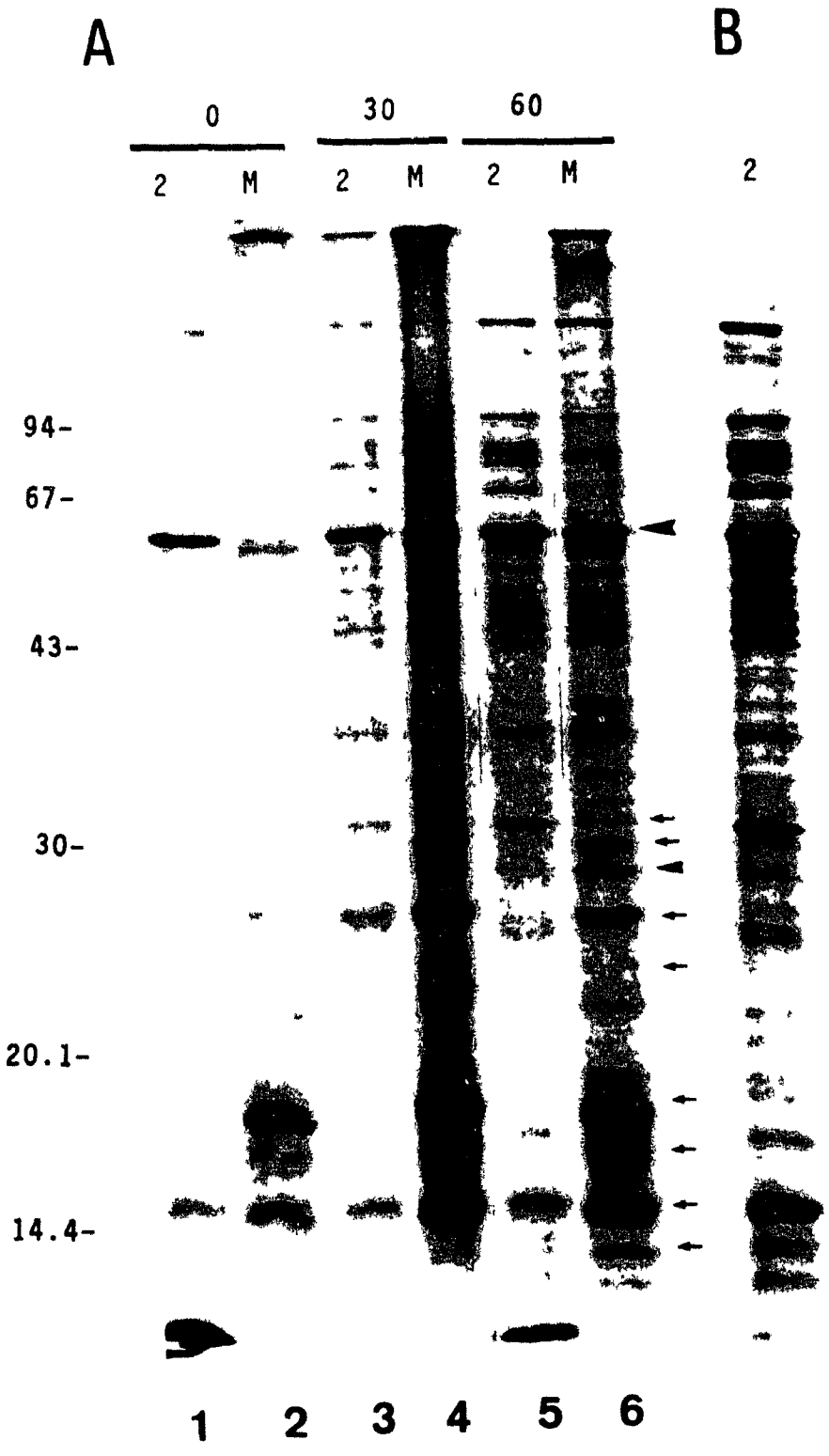


Figure 17

of the 60-kDa protein in Lp2064 was also evident when comparisons were made using equal numbers of bacteria per lane instead of equal counts (Figure 17, Panel B, and Lane 6 in Panel A).

There were other differences between Lp2064 and Lp2064M. Amongst these were changes in the expression of proteins having approximate molecular weights of 32, 31, 29, 26, 23, 17, 16, 15, and 13 kDa. Most but not all of these proteins were synthesized at much higher levels in Lp2064M. This was especially true of the 15 and 17-kDa proteins which appeared as diffuse bands. While there were changes in high molecular weight proteins, these changes were not always consistent.

Labeling of mock-infected monolayers always yielded counts that were only slightly above background levels and were thus insufficient for analysis.

b) Effect of cytochalasin D treatment of L929 cells on Lp2064 and Lp2064M protein synthesis

Although differences in the protein profiles of Lp2064 and Lp2064M were visible, it was not known whether these changes were imparted by the intracellular environment or whether they had occurred at a step prior to entry. In order to address this issue, host cells prepared as described were either pretreated with cytochalasin D for 1 hour or left untreated. Cytochalasin D inhibits actin polymerization and phagocytosis, and is known to inhibit uptake of L.pneumophila (Elliott and Winn, 1986, King et al., 1991). Thus pretreating the L929 cells with cytochalasin D would effectively ensure that only extracellular bacteria (either free-floating, or attached to the host cells) were being labeled. L929 cells in the presence or absence of cytochalasin D were subsequently infected, and the bacteria labeled as described above. At the end of the labeling period, the culture medium was aspirated to collect bacteria not interacting with the host cells. The monolayers were then washed carefully to remove non-adherent bacteria. The washed L929 cells were lysed, and the bacteria harvested and analyzed as outlined above.

The results in Figure 18, (Panels A and B) indicate that with respect to Lp2064, the protein profiles remained essentially the same regardless of whether the bacteria were obtained from untreated monolayers or from the monolayers pretreated with cytochalasin D.

Figure 18. Effect of cytochalasin D treatment of L929 cells on Lp2064 or Lp2064M protein synthesis. L929 cells were prepared, infected and labeled as described in the legend to Figure 17, except that prior to infection some monolayers were pretreated with cytochalasin D for 1 hour. Panels A and B represent bacteria interacting with L929 cells. Panel C refers to bacteria exposed to cytochalasin D but not to L929 cells. Cyt D refers to cytochalasin D; large arrowhead, 60-kDa protein; small arrowhead, 29-kDa protein. Arrows in Panel A, top to bottom represent proteins (in kDa) of the following molecular weights: 26, 23, 21, 19, 17, 16, 15, and 13; Panel B, arrows: 21, 19, 17, and 16 kDa; Panel C, arrow: 13 kDa. Molecular weight markers for each panel are indicated.

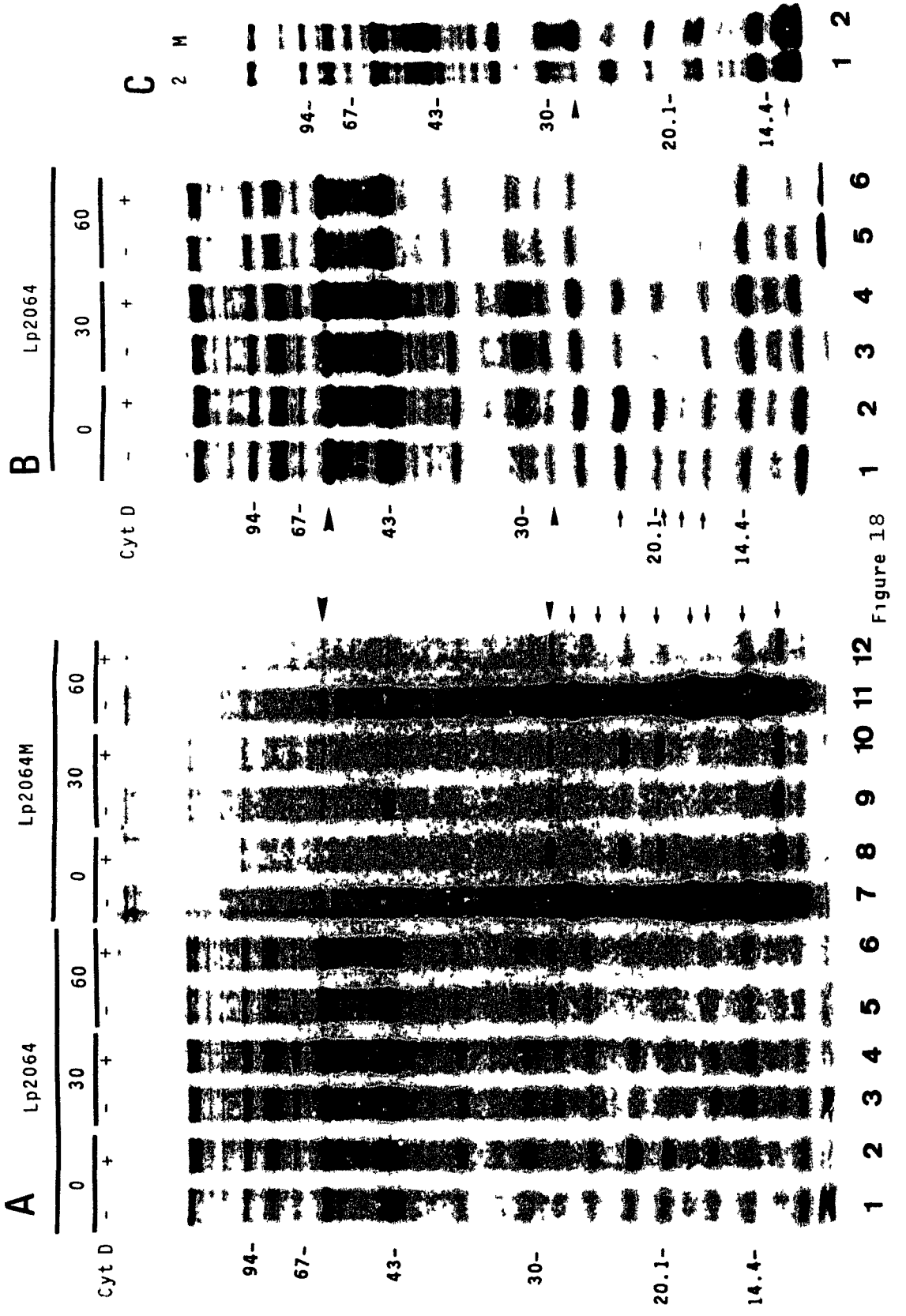

A
Lp2064
Lp2064M
0
30
60
Cyt D
94-
67-
43-
30-
20.1-
14.4-
1 2 3 4 5 6 7 8 9 10 11 12
B
Lp2064
1 2 3 4 5 6
C
2 M
1 2

Figure 18

However, when Lp2064M profiles were compared, the profiles of the bacteria obtained from treated versus untreated monolayers were different. Bacteria obtained from cells pretreated with cytochalasin D (i.e. extracellular or adherent bacteria) did not exhibit the smearing pattern observed in the bacteria from cells not treated with cytochalasin D (Figure 18, Panel A, lanes 7 to 12). Also absent, or present in reduced amounts in the "extracellular" organisms were the 15, 17, 23 and 26-kDa proteins. This suggested that these proteins were possibly either induced by the intracellular environment, or were formed as a consequence of the bacteria being intracellular. Although Lanes 9 and 10 (T=30, +/- cytochalasin D) exhibited identical profiles, this was not the case in other experiments.

A comparison of "extracellular/adherent" Lp2064 and Lp2064M (Lanes 2 and 8, 4 and 10, and 6 and 12 in Panel A, Figure 18) indicated that there were differences in the expression of the 29, 26, 23, 17, 16, 15, and 13-kDa proteins. Furthermore, the synthesis of the 16 and 17-kDa proteins in Lp2064, and of the 19 and 21-kDa proteins in both organisms diminished at T=60.

Panel C of Figure 18 compares the profiles of cytochalasin D-treated Lp2064 and Lp2064M which were not in contact with L929 cells. The respective profiles essentially mirrored those of the "extracellular/adherent" bacteria represented in Lanes 4 and 10 in Panel A (Figure 18). Both

organisms in Panel C displayed similar profiles of the higher molecular weight proteins (i.e. those >30 kDa). Although not as readily apparent, the same appears true in Panel A. Thus, the changes in the protein profiles of Lp2064 and Lp2064M witnessed in Panel A could not be attributed to cytochalasin D itself.

Subsequently it was found that simply placing the organisms in tissue culture medium (MEM) in the absence of host cells was sufficient to elicit the different profiles seen in Lp2064 and Lp2064M (Figure 19). Most obvious was the increased expression of the 29-kDa protein in Lp2064M.

Thus, the results from this series of experiments seem to indicate that many of the observed changes in the protein profiles of Lp2064 and Lp2064M were imparted prior to the organisms' entry into the host cell, and were in fact, directly attributable to the tissue culture medium. Furthermore, some of these proteins were synthesized transiently, appearing at T=0 and T=30, but not at T=60.

Figure 19. Response of Lp2064 and Lp2064M to tissue culture medium (MEM). Two-day old cultures of Lp2064 and Lp2064M were scraped off BCYE plates and placed in MEM. The bacteria were pulsed for 10 minutes with the [^{35}S] label. The reaction was stopped by adding an equal volume of cold 20% TCA. The precipitates were washed and resuspended in SDS-PAGE sample buffer. Equal numbers of counts were compared by SDS-PAGE autoradiography/fluorography. 2 and M refer to Lp2064 and Lp2064M respectively. Arrowhead is the 29-kDa protein; arrow is the 13-kDa protein. Molecular weight markers are indicated.

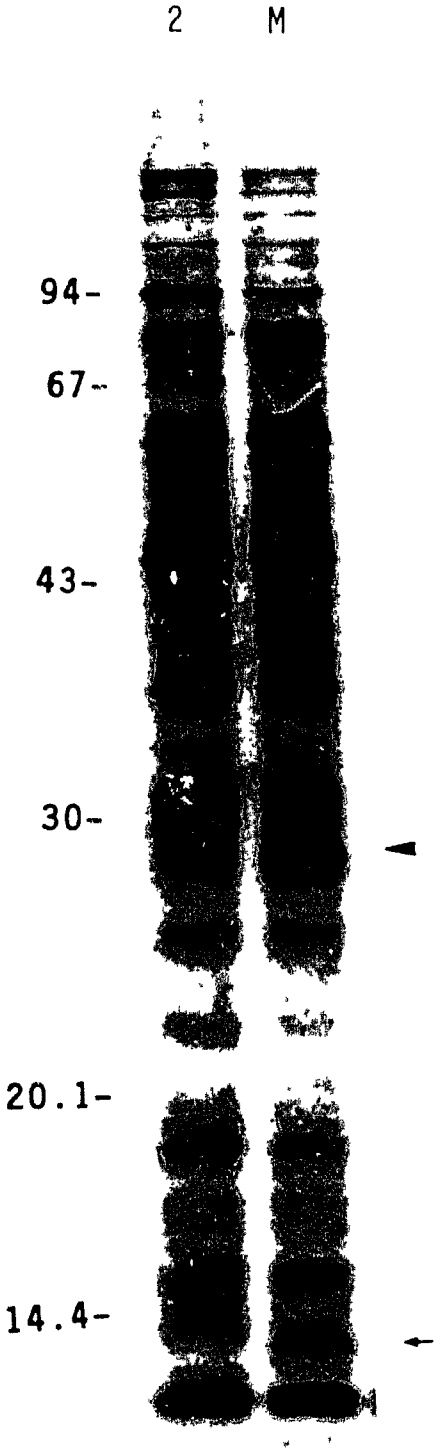


Figure 19

c) Use of gentamicin to ensure intracellular labeling of Lp2064 and Lp2064M

Since it was apparent that the extracellular environment was influencing the induction of many proteins and since the experimental procedure used thus far was unable to adequately discriminate between intracellular bacteria and those adhering to the surface of the host cell, the original protocol was modified by adding gentamicin to the wash medium following infection. It was hoped that the gentamicin would act to either kill extracellular bacteria, or to inhibit their protein synthesis, thus providing a means of augmenting analysis of proteins induced by the intracellular environment. To this end, L929 cells were prepared and infected as initially described. Following infection, the inoculum was removed and the infected monolayers washed with medium containing gentamicin. At T=0, T=30, or T=60, the bacteria were labeled in the presence of gentamicin for a period of 30 minutes. After washing off the gentamicin, the host cells were lysed, and the bacteria recovered and analyzed as outlined.

Figure 20 illustrates the results from 2 separate experiments. In general, compared with the profiles of organisms isolated from monolayers not exposed to gentamicin, the profiles of bacteria isolated from the post-infection, gentamicin-treated monolayers displayed fewer proteins. With respect to Lp2064, the dominance of the 60-kDa protein as reflected in Figure 17, was clearly augmented in the bacteria

Figure 20. Use of gentamicin to ensure labeling of intracellular bacteria. L929 cells were prepared, and infected as described in the legend to Figure 17. Following infection, the cells were washed and labeled for 30 minutes in the presence or absence of gentamicin. The label was removed and the gentamicin washed off before lysing the L929 cells. The bacteria were collected and analyzed as described in the legend to Figure 17. Gm refers to gentamicin; 2 and M to Lp2064 and Lp2064M respectively; large arrowhead to the 60-kDa protein; small arrowhead to the 29-kDa protein. Arrows in (A) from top to bottom in kDa: 17, 15, and 13; arrows in (B): 17 and 15 kDa.

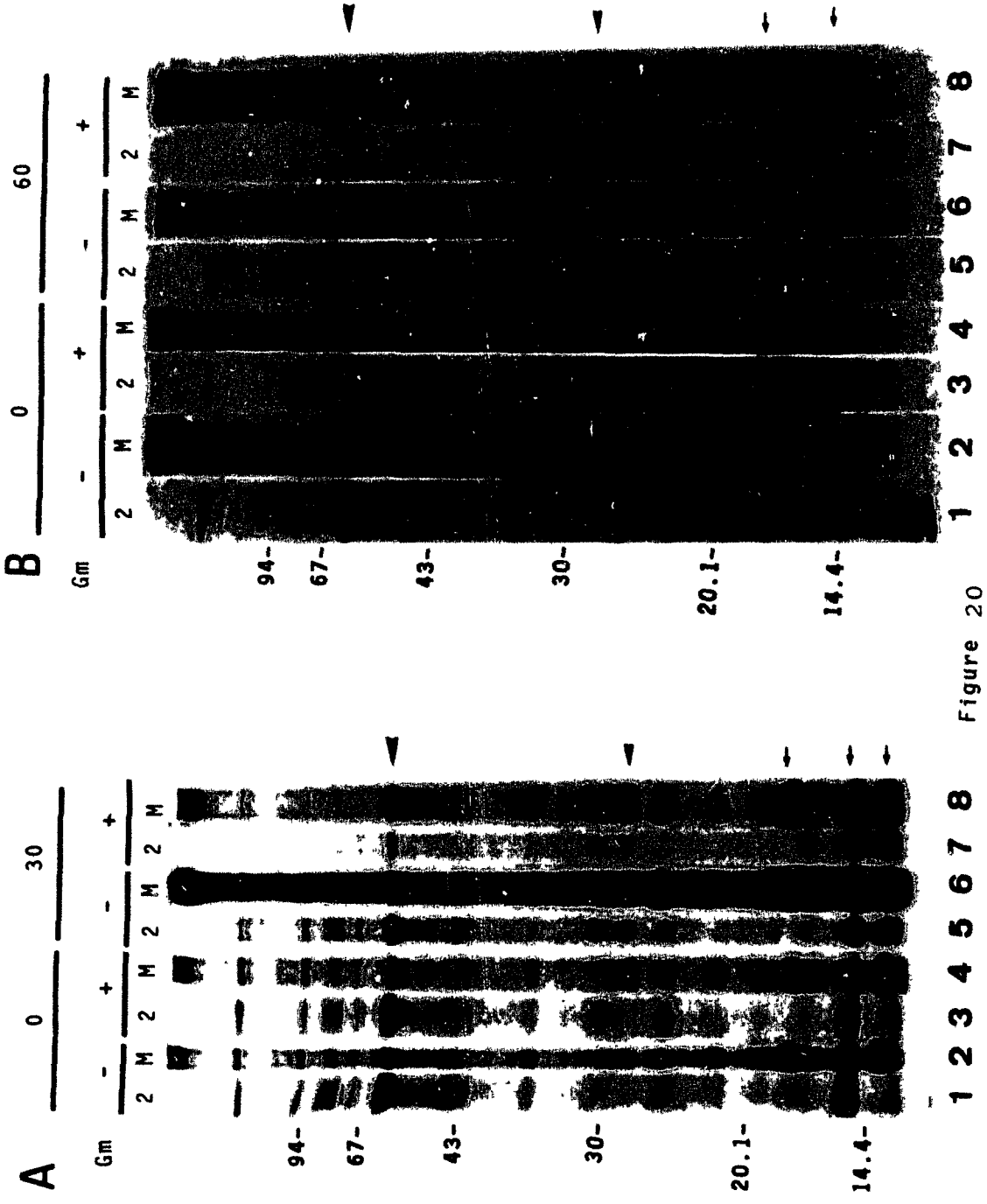


Figure 20

recovered from gentamicin-treated monolayers (both Panels, Lanes 3 and 7). Although some low molecular weight proteins were also evident (e.g. with molecular weights of 12 and 15, and 17 kDa), their expression was less apparent by 60 minutes.

The smearing pattern observed for Lp2064M in Figure 17 was also conspicuous in Figure 20. Treating Lp2064M-infected monolayers with gentamicin did not appear to change the outcome, suggesting that the smearing was a consequence of the bacteria being intracellular. In agreement with the observations of Figure 17, Lp2064M was able to produce a 60-kDa protein, but relative to the other proteins, it was not the most dominant product. Furthermore, in some samples (Panel B, lanes 2, 4, 6, and 8), what appeared to be the 60-kDa protein seemed to migrate at a slightly lower position relative to the Lp2064 counterpart.

Although increased levels of low molecular weight proteins (13, 15 and 17 kDa) were present in Lp2064M lanes, the increased synthesis of the 13-kDa protein seemed to reflect the response of the organisms to the tissue culture medium (Figure 19). The levels of three proteins were enhanced when the organisms were intracellular. Furthermore, the 15 and 17-kDa Lp2064M proteins which were initially induced by the medium continued to be synthesized even at 60 minutes.

In essence, the results of the labeling experiments can be summarized as follows. Lp2064 and Lp2064M responded to tissue culture medium by displaying different protein

profiles. Upon exposure to host cells, the profiles of the extracellular bacteria (free-floating or adhering to host cells) were similar to what was seen when the organisms were in tissue culture medium alone. Notwithstanding the MEM-induced changes, once intracellular, the respective profiles differed from those observed when the organisms were extracellular. With respect to Lp2064, the predominant protein was the 60-kDa protein, especially at T=60. With respect to Lp2064M, the profile was smeared and the dominant proteins were the 15 and 17-kDa proteins which also presented as diffuse bands. The Lp2064M 60-kDa protein (if it was indeed the same protein) frequently appeared to migrate slightly more quickly in bacteria that were intracellular. Furthermore, unlike what was seen in the Lp2064 profile, the 60-kDa Lp2064M protein was not the predominant protein synthesized.

d) Use of monocytes as host cells in the labeling experiments

Alveolar macrophages are the usual host cells within which L.pneumophila multiply. Activation of these cells with interferon gamma (IFN-γ) results in the inhibition of the intracellular growth of L.pneumophila (Bhardwaj et al., 1986). Because it is not always convenient or possible to obtain them to study host-bacteria interactions, an alternate choice has been to use peripheral blood monocytes. To see if the results of the above metabolic labeling experiments could be reproduced in monocytes, monocytes were isolated from the peripheral blood of normal healthy volunteers. After allowing the monocytes to adhere overnight, the cells were split into two groups. To one group was added IFN-γ. The other was left untreated. Twenty-four hours later, the monocytes were washed. One hour prior to infection, the cells were placed in methionine-free RPMI containing 10% heat-inactivated, dialyzed FBS and 1 μg/mL cycloheximide. The monocytes were then infected for 30 minutes with Lp2064M and 100-fold fewer Lp2064. Because fewer numbers of monocytes were used compared to the numbers of L929 cells, it was necessary to improve the yield of intracellular bacteria; hence the longer infection period. However since this resulted in many of the Lp2064-infected monocytes being rendered non-adherent, the lower MOI had to be used for these organisms. Following adherence, the bacteria were washed with media containing gentamicin. The infected cells were then labeled in the presence of gentamicin

for 30 minutes. Next, as previously described, the monocytes were washed and the bacteria collected. For comparison, the bacteria were also labeled in RPMI (using the same time frame, i.e. T=30) in the absence of monocytes.

The results of two separate experiments are presented in Figure 21. Looking at Lanes 5 and 6 in both Panels, it is obvious that the exposure of Lp2064 and Lp2064M to RPMI resulted in a different pattern of induced proteins. Lp2064M (Lane 6) expressed increased amounts of proteins having approximate molecular weights of 29, 21, 19, 15, and 13 kDa, and decreased levels of the 26-kDa protein. When the bacteria were intracellular, Lp2064M (Lane 2) maintained the RPMI-induced profile and showed enhanced expression of the 17 and 15-kDa proteins. The tendency to produce a "smeared" pattern was also evident as was a 57-kDa protein which may or may not be an altered form of the 60-kDa protein (Panel B). In support of previous observations, the Lp2064M 60-kDa protein was not preeminent, relative to the other proteins. As in the L929 experiments, the most striking feature of the Lp2064 profile (Lane 1) was once again the sheer abundance of the 60-kDa protein.

Compared to Lanes 1 and 2, activating the monocytes with IFN-γ did not alter the respective protein patterns significantly (Lanes 3 and 4).

Except for the more pronounced smearing exhibited in the Lp2064M samples derived from L929 cells, the results from the

Figure 21. Labeling of Lp2064 and Lp2064M in unactivated and IFN-τ-activated monocytes. Monocytes were isolated from human peripheral blood and either pretreated with IFN-τ or left untreated. Twenty-four hours later, the cells were washed, exposed to methionine-free RPMI containing cycloheximide for 1 hour, and infected with Lp2064 (MOI of 10) or Lp2064M (1000) for 30 minutes. The infected cells were then washed and labeled for 30 minutes in the presence of gentamicin. Bacteria were also labeled in the absence of monocytes (i.e. in RPMI + 10% dialyzed FBS). Intracellular bacteria were collected and analyzed as described. Bacteria labeled in RPMI were pelleted, and washed in cold PBS. The labeled bacteria were analyzed as above. 2 and M refer to Lp2064 and Lp2064M respectively. Large arrowhead is the 60-kDa protein; small arrowhead the 29-kDa protein. In kDa, from top to bottom, the arrows identify proteins of the following molecular weight: 26, 21, 19, 17, 15, and 13. Molecular weight markers are also indicated.

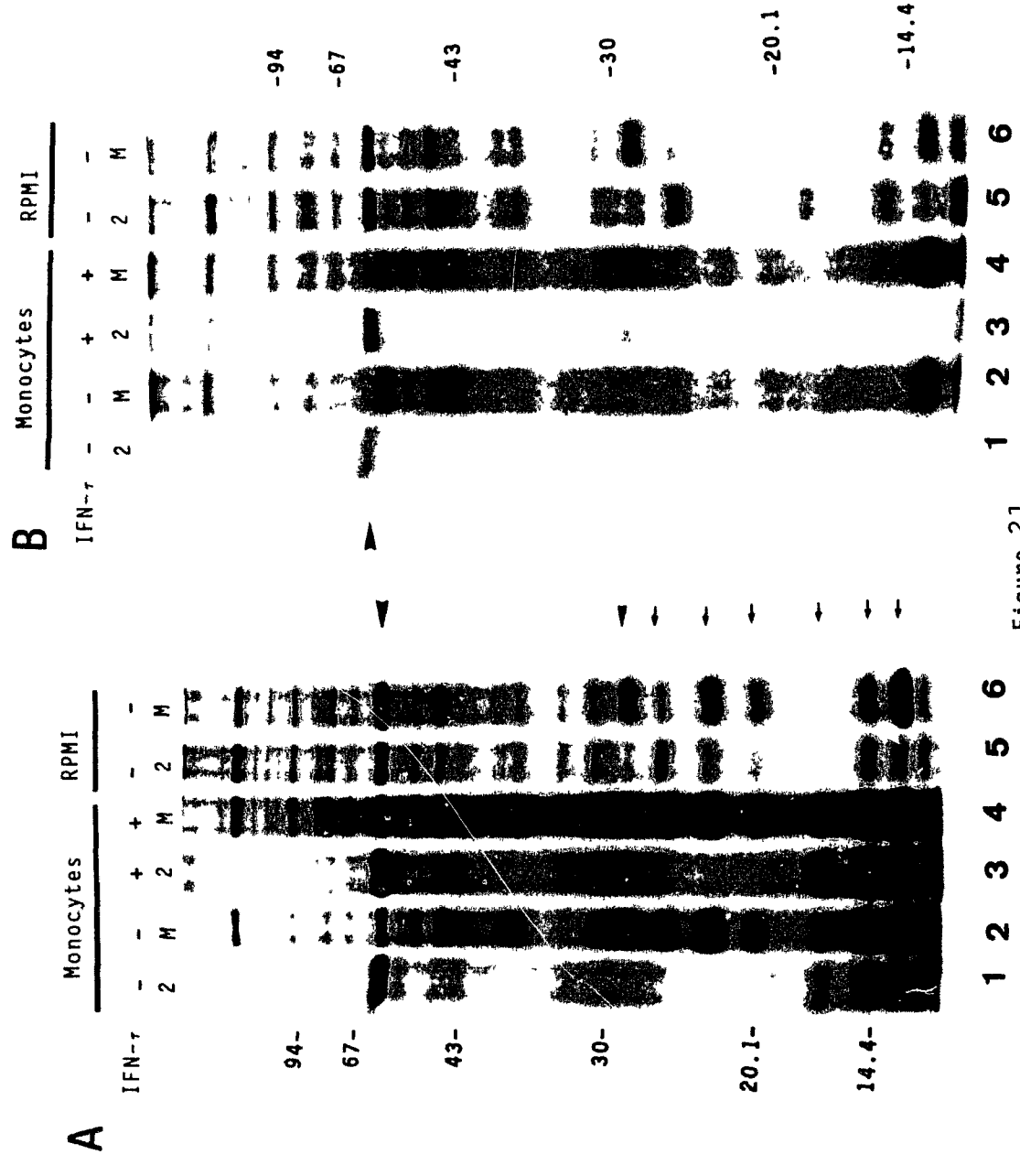


Figure 21

monocyte experiments generally compared favourably with the L929 cell data.

Identification of the 60-kDa protein

The molecular weight of the 60-kDa protein suggested that it might be the L.pneumophila groEL-equivalent heat shock protein (HSP). This was indirectly confirmed by immunoblotting using a monoclonal anti-hsp 60 antibody (Helsel et al., 1988, Hoffman et al., 1989) as the probe. Due to the paucity of available sample and the detection limit of the system, a complete comparison of Lp2064 and Lp2064M under intracellular conditions was not done. Nevertheless, using samples from a previous series of experiments it was possible to identify the 60-kDa protein observed on autoradiograms, as the hsp 60 protein (Figure 22, Panel A). It should be noted and emphasized that these particular samples were from an early experiment where gentamicin was not used and the host cells not washed extensively to ensure removal of non-adherent bacteria. As a result, both extracellular and intracellular bacteria were represented (with the majority being extracellular). Under these conditions, it thus appeared that Lp2064M (and not Lp2064) was producing increased levels of the 60-kDa protein. Regardless, the protein was identified as hsp 60 by immunoblot (Figure 22, Panel B).

The heat shock response of Lp2064 and Lp2064M

Based on the analysis of intracellular bacteria (Figures 20 and 21) it was apparent that Lp2064 produced vastly

Figure 22. Identification of the 60-kDa protein. L929 cells were infected with Lp2064 and Lp2064M for 5 minutes and labeled as described in the legend to Figure 17, except that the wash step following labeling was omitted. Labeled bacteria were subjected to SDS-PAGE. (A) Protein profiles examined by autoradiography/fluorography. Molecular weight markers (in kDa) are indicated. (B) Proteins separated by SDS-PAGE were electroblotted to nitrocellulose and the blot was probed with a monoclonal antibody to the L.pneumophila 60-kDa heat shock protein (anti hsp-60 antibody). The reaction was visualized using a biotinylated secondary antibody, followed by peroxidase-conjugated streptavidin, and the substrate solution containing peroxide and chloronaphthol. Prestained molecular weight markers in kDa are from top to bottom: 200, 97.4, 68, 43, 25.7, 18.4, and 14.3. 2 and M are Lp2064 and Lp2064M respectively.

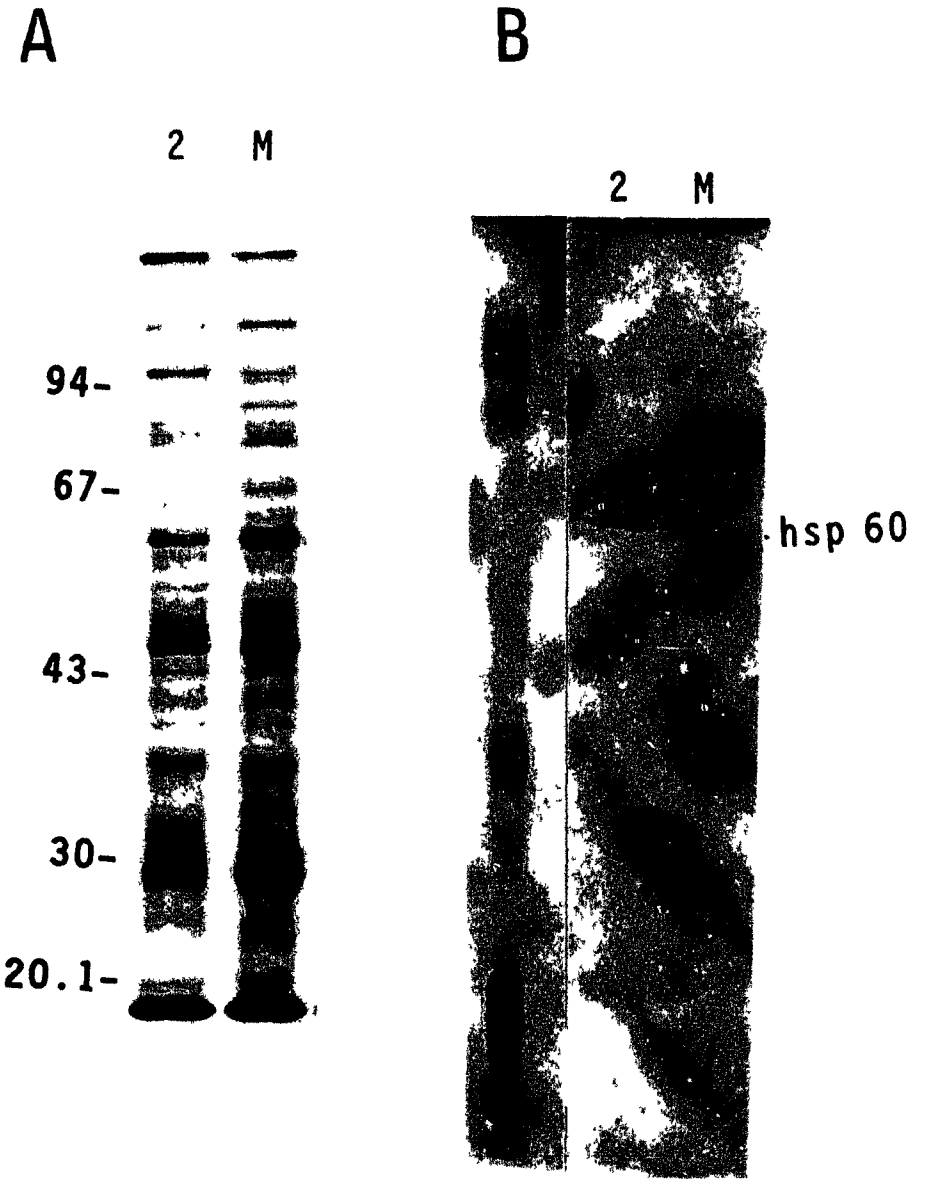


Figure 22

greater amounts of the 60-kDa protein compared with Lp2064M. This raised the question of the actual capability of Lp2064M to mount an adequate heat shock response. The heat shock response of Lp2064 and Lp2064M was thus evaluated using the method of Hoffman et al. (1989). Organisms were grown in BYE broth at 23°C, and then transferred to 23, 37, or 42°C where they were labeled for 10 minutes with [^{35}S]. The labeling was halted by the addition of cold TCA. SDS-PAGE was performed using equal numbers of TCA-precipitable counts for each sample. Figure 23 (Panel A) illustrates that indeed both Lp2064 and Lp2064M were equally capable of producing a heat shock response as indicated by the increased levels of the 60-kDa protein at 37 and 42°C. This was verified by immunoblot (Figure 23, Panel B). Increased synthesis was also observed with a >94-kDa protein as well as a protein migrating at the bottom of the gel. Inadequate separation of the low molecular weight proteins on this gel precluded a proper estimation of the molecular weight.

Thus, these results indicate that the disparate hsp 60 levels observed in intracellular Lp2064 and Lp2064M cannot be attributed to a potentially innately aberrant ability of Lp2064M to produce a heat shock response.

Figure 23. Heat shock response of Lp2064 and Lp2064M. Organisms were grown in BYE broth at 23°C and transferred to 23, 37, or 42°C where they were immediately labeled for 10 minutes. The reaction was stopped by adding cold TCA. The resulting precipitates were washed and resuspended in SDS-PAGE sample buffer. Equal numbers of TCA-precipitable counts were analyzed by SDS-PAGE. (A) Autoradiogram of labeled proteins. (B) Immunoblot using anti-hsp 60 as described in the legend to Figure 22. Molecular weight markers are indicated.

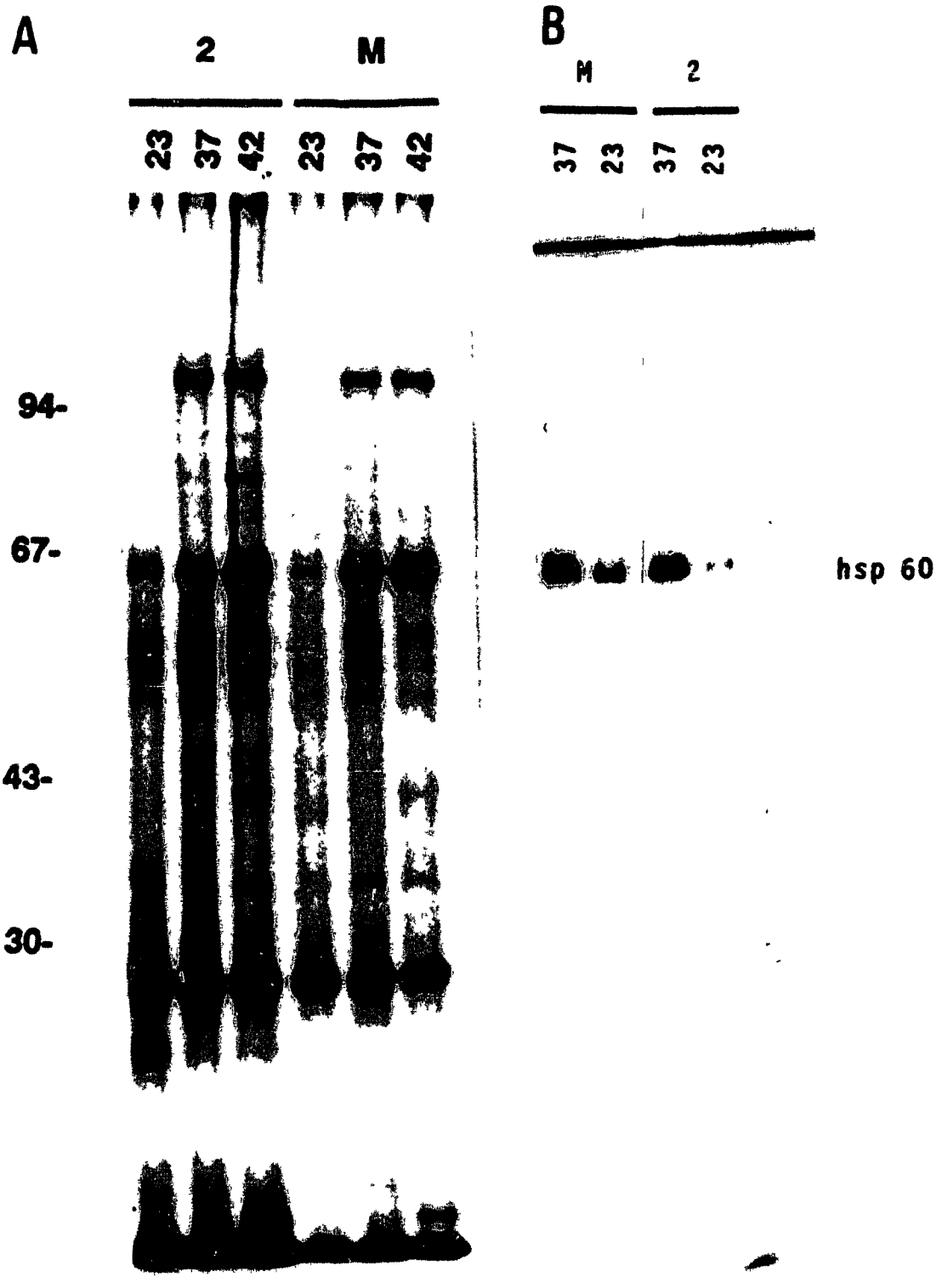


Figure 23

Further investigation into the response of Lp2064 and Lp2064M to tissue culture medium

As illustrated in Figure 9, the protein profiles of BCYE-grown Lp2064 and Lp2064M were similar. Yet, when the organisms were placed in tissue culture medium they exhibited two different patterns of induced proteins. To further explore these observations and to see if the "intracellular " patterns could be reproduced in tissue culture medium, the following experiments were done. Lp2064 and Lp2064M were scraped off BCYE plates and suspended in PBS. A ten-fold dilution was then made in each of the following: PBS, MEM, RPMI, MEM pH 6.3, MEM pH 5.5, and MEM pH 4.0. As well, organisms were similarly placed in water, and in water containing 0.3%, and 0.6% NaCl. The low pH values were chosen in order to mimic the pH found within phagolysosomes or lysosomes. The effect of NaCl was investigated because it has been shown that growth of virulent, but not Mueller-Hinton agar-selected avirulent L.pneumophila, is completely inhibited at concentrations of NaCl which are greater than 0.4% (Catrenich and Johnson, 1989). Immediately after exposing the organisms to the above conditions, the [^{35}S] label was added. Ten minutes later, the labeling was stopped by adding cold TCA. Samples containing equal numbers of TCA-precipitable counts were analyzed by SDS-PAGE. For some experiments, the bacteria were labeled for 10 minutes at either 30, 60, 120, or 240 minutes after the initial exposure to MEM.

Figure 24 compares the protein profiles of Lp2064 and Lp2064M exposed to PBS, MEM and RPMI. The major differences in the PBS-exposed organisms (Lanes 1 and 2) were in the level of expression of both the 13, and 29-kDa proteins; increased levels were seen in Lp2064M. When the organisms were exposed to tissue culture medium (Lanes 3 to 6), the increased level of expression of the 13 and 29-kDa proteins in Lp2064M was enhanced. In addition, proteins of 60, 33, 22 and 19 kDa were induced in both Lp2064 and Lp2064M. With respect to the 22 and 19-kDa proteins, higher levels were seen in Lp2064M.

As demonstrated in Figure 25, except for the possible induction in both organisms of a protein of approximately 17 kDa, lowering the pH did not affect the pattern just described. Because apparently more counts were (unintentionally) loaded in Lanes 6-10 (low pH), it is difficult to assess whether the 17-kDa protein truly was induced to the slightly higher levels. Furthermore, synthesis of the 60-kDa protein was not affected by the low pH.

Figure 25 also illustrates that the 22 and 19-kDa proteins were no longer synthesized one hour after the initial exposure to MEM (Lanes 11 and 12).

Diluting Lp2064 or Lp2064M in water, or water supplemented with NaCl, resulted in the induction of proteins of approximately 17, 16, and 15 kDa (Figure 26, Lanes 7 to 12). However, the most striking result of placing the organisms in these conditions, was the lack of induction of

Figure 24. Response of Lp2064 and Lp2064M to tissue culture medium. Two-day old cultures of Lp2064 and Lp2064M were scraped off BCYE plates and resuspended into PBS. A ten-fold dilution was made in PBS, MEM or RPMI and the bacteria were labeled for 10 minutes. The reaction was stopped with cold TCA as described in the legend to Figure 23. The proteins were analyzed by SDS-PAGE. 2 and M refer to Lp2064 and Lp2064M respectively. Large and small arrowheads are the 60 and 29-kDa proteins respectively. The arrows from top to bottom are proteins of the following molecular weights (in kDa): 33, 22, 19 and 13. Molecular weight markers (in kDa) are indicated.

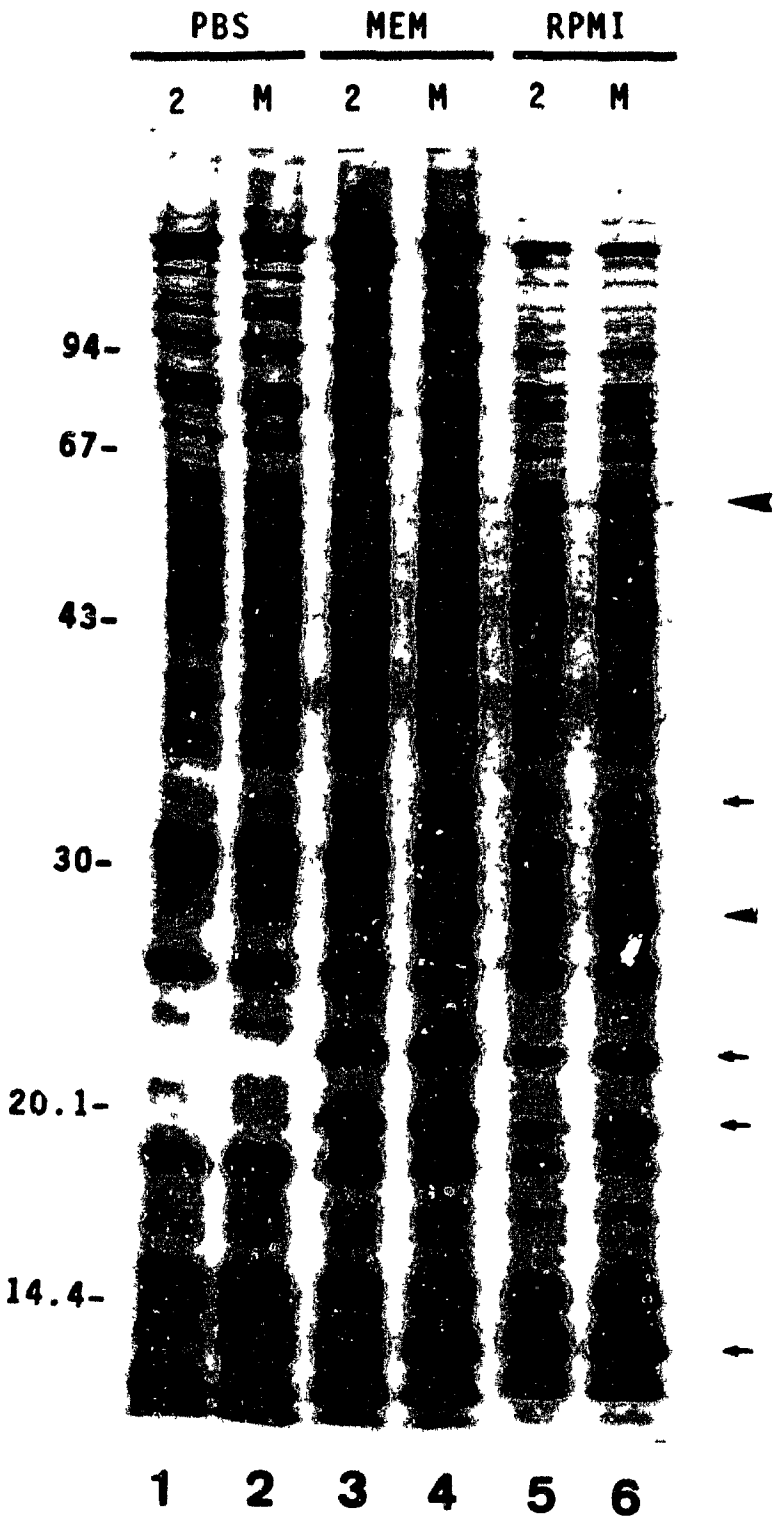


Figure 24

Figure 25. Response of Lp2064 and Lp2064M to low pH. The experiment was carried out as described in the legend to Figure 24 except that additional dilutions were made in MEM pH 6.3, MEM pH 5.5, and MEM pH 4.0. Bacteria were also labeled as described, 60 minutes after exposure to MEM. 2 and M are Lp2064 and Lp2064M respectively. 6.3, 5.5 and 4 refer to the pH. The large and small arrowheads refer to the 60 and 29-kDa proteins respectively. The arrows point out proteins of 33, 22, 19, and 13-kDa in molecular weight. Molecular weight markers (in kDa) are also indicated.

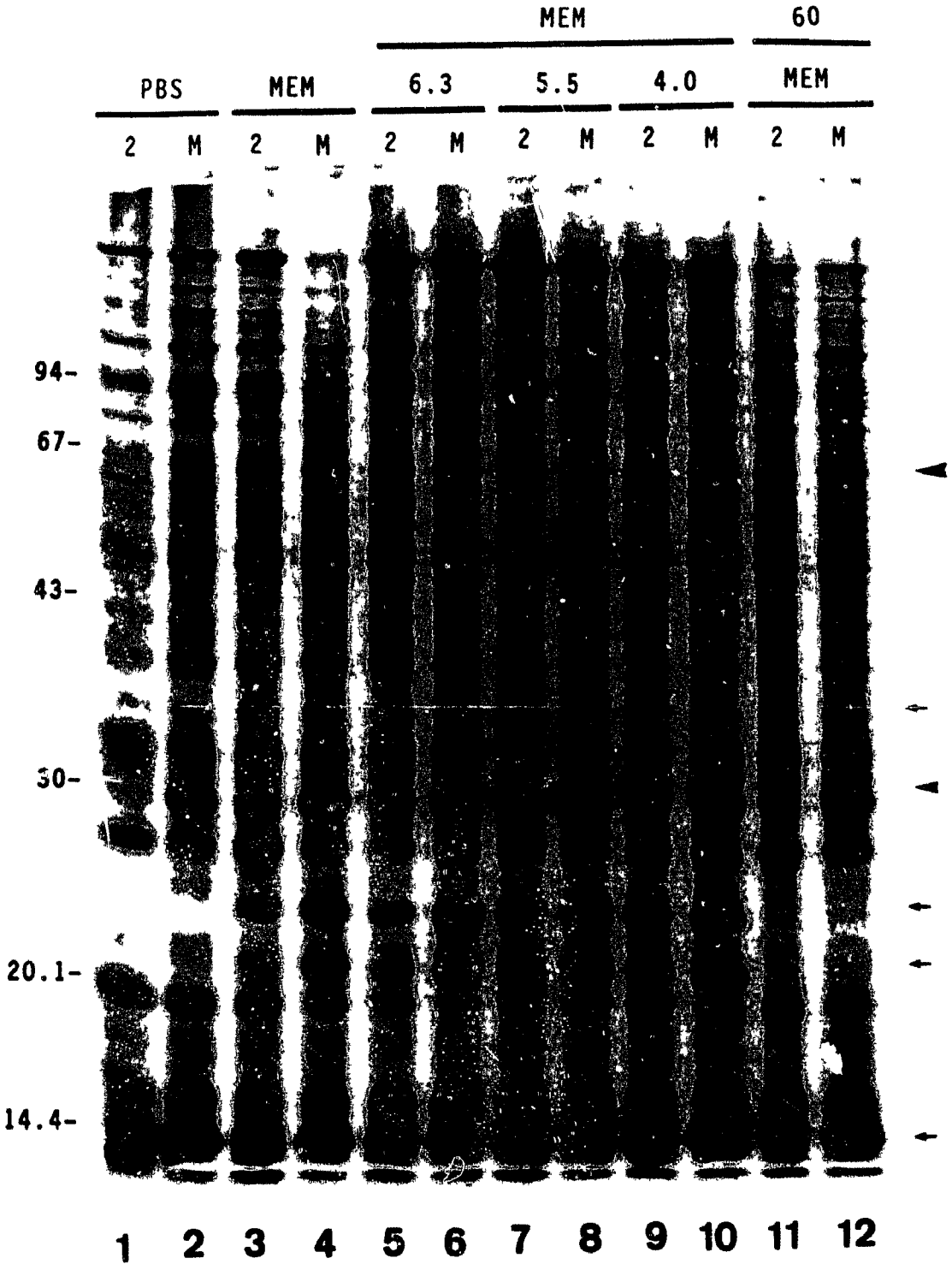

MEM
60
PBS
MEM
6.3
5.5
4.0
MEM
2
M
2
M
2
M
2
M
2
M
2
M
94-
67-
43-
30-
20.1-
14.4-
1
2
3
4
5
6
7
8
9
10
11
12

Figure 25

Figure 26. Response of Lp2064 and Lp2064M to water and water containing 0.3% and 0.6% NaCl. The experiment was performed as described in Figure 24, except that additional dilutions were made in double distilled water, or water supplemented with 0.3% or 0.6% NaCl. 2 and M are Lp2064 and Lp2064M respectively. The large and small arrowheads are the 60 and 29-kDa proteins respectively. The arrows from top to bottom are 33, 22, 19, 17, 16, 15, and 13-kDa proteins. Molecular weight markers are also indicated.

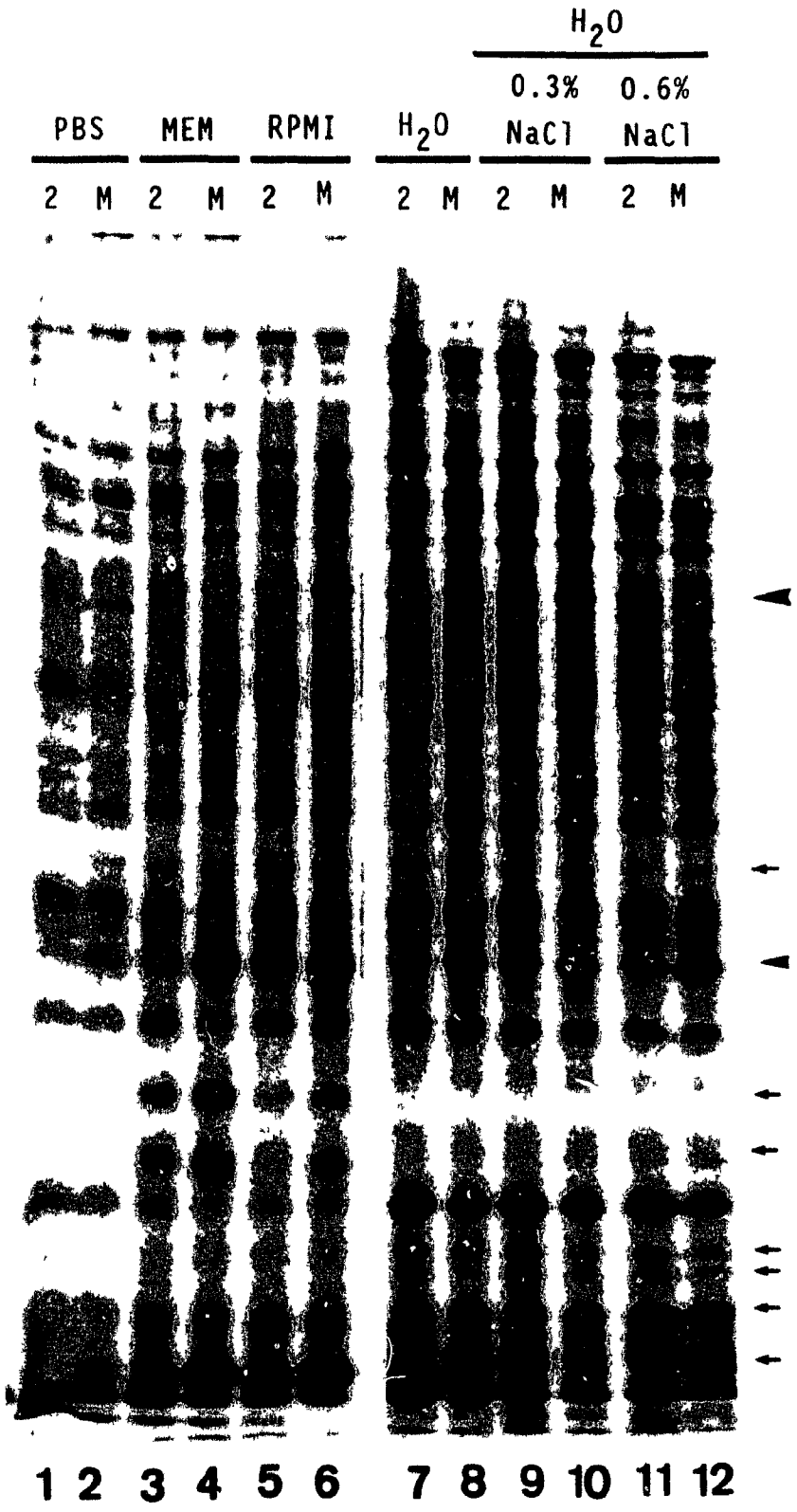

H2O
0.3% NaCl
0.6% NaCl
PBS
MEM
RPMI
H2O
2 M 2 M 2 M 2 M 2 M 2 M
1 2 3 4 5 6 7 8 9 10 11 12

Figure 26

the 22 and 19-kDa proteins. Also present in reduced amounts was the 33-kDa protein in samples exposed to 0.6% NaCl (Lanes 11 and 12). In addition, compared to MEM/RPMI-induced levels (Lanes 3 to 6), there were much lower levels of the 13-kDa protein in Lp2064M. Otherwise, the patterns were similar to what was seen in the MEM/RPMI-induced lanes.

The kinetics of the MEM response is demonstrated in Figure 27. From this, it can be seen that the levels of induction of the 19 and 22-kDa proteins were greatly diminished by 30 minutes (Lanes 3 and 4), and were not at all visible by 60 minutes (Lanes 7 and 8). The levels of several proteins increased, and then decreased with time. Among these the most obvious were the 60, 29, and 13-kDa proteins. Levels of the 60 kDa-protein appeared to peak at 10 to 30 minutes, with some synthesis occurring at 60 minutes. The 29-kDa protein peaked at 30 minutes, with a significant amount of synthesis occurring at 60 minutes before diminishing by 120 minutes. Although a similar result was attained with Lp2064, this was most apparent with Lp2064M. Finally, peak amounts of the Lp2064M 13-kDa protein were visible at 30 and 60 minutes before waning between 120 and 240 minutes.

Northern analysis of RNA isolated from a different, yet similarly conducted experiment supported some of these observations. Total RNA was isolated and separated on a formaldehyde-agarose gel. Following transfer to a nylon membrane, the RNA was probed with internal fragments of *htpB*

Figure 27. Kinetics of the MEM response. The experiment was performed as described in the legend to Figure 24, except that the bacteria were labeled (for 10 minutes) at 10, 30, 60, 120, and 240 minutes after exposure to MEM. 2 and M are Lp2064 and Lp2064M respectively. The large and small arrowheads are the 60 and 29-kDa proteins respectively. The arrows refer to the 22, 19, and 13-kDa proteins. Molecular weight markers in kDa are also indicated.

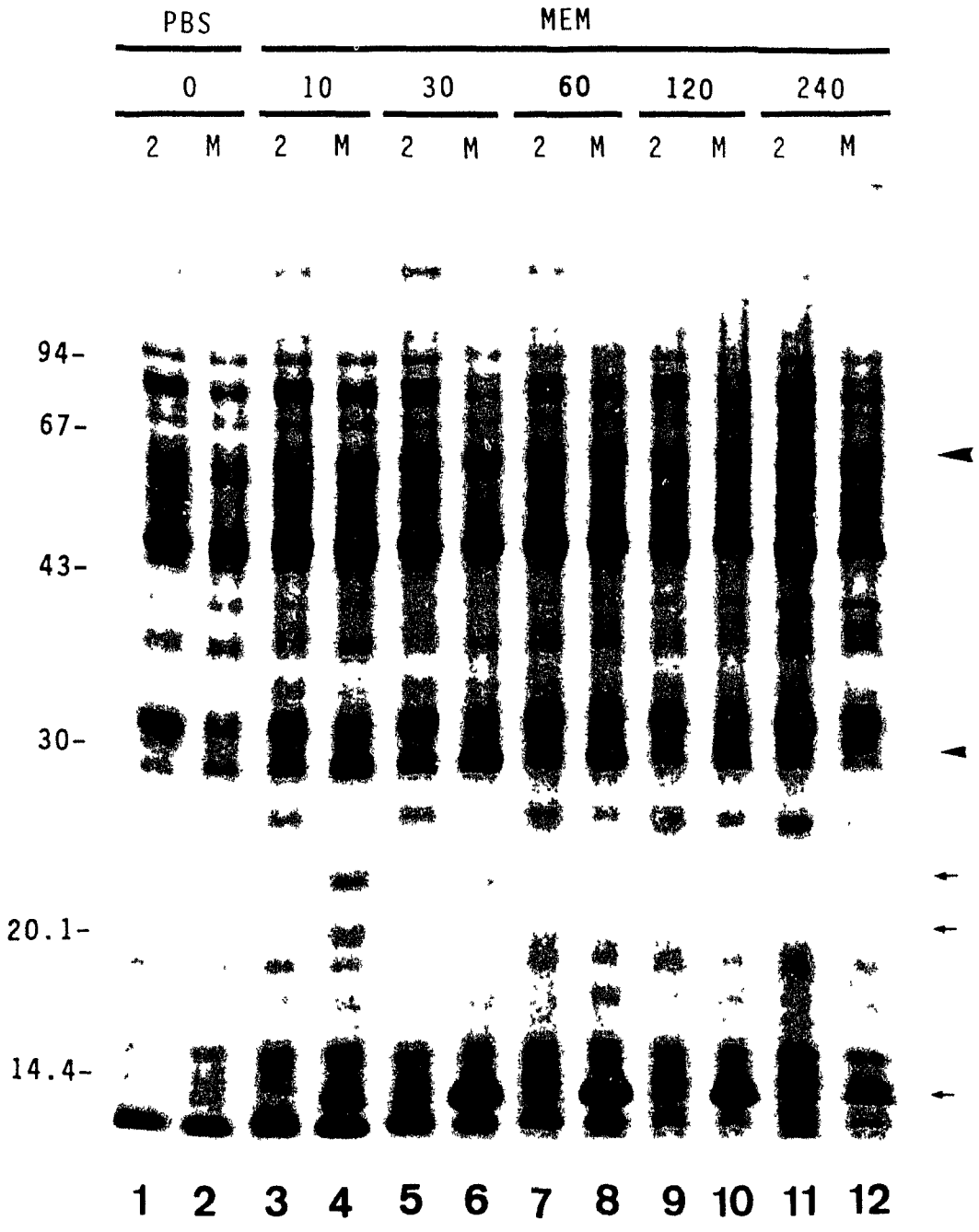

PBS
MEM
0
10
30
60
120
240
2 M 2 M 2 M 2 M 2 M 2 M
94-
67-
43-
30-
20.1-
14.4-
1 2 3 4 5 6 7 8 9 10 11 12

Figure 27

and *omp28*. These genes encode the 60-kDa heat shock protein, and the 29(28)-kDa major outer membrane protein (MOMP) respectively (Hoffman et al., 1990, Hoffman et al., 1991a). In the absence of a suitable control for demonstrating equal amounts of RNA in each lane, the ethidium bromide stained gel is shown in Panel A of Figure 28. Based on the staining intensity of the ribosomal RNA bands, it appeared that virtually equal amounts of RNA were loaded in all lanes except in Lanes 7 and 8, which seemed to have slightly less RNA. The results in Panel B indicate that more of the hsp 60 transcript was present in Lp2064 than in Lp2064M, whereas more MOMP transcripts were present in Lp2064M. Furthermore, MOMP transcript levels appeared to peak at 10 minutes while continuing to be present at 30 minutes. The levels of the hsp 60 transcripts peaked at 30 minutes. By 60 minutes, the levels of both transcripts were diminished. However this conclusion should be tempered somewhat due to the appearance of seemingly less RNA in these lanes. Nevertheless, these results appeared to parallel the induction patterns of the respective proteins in Figure 27.

Despite being unable to completely reproduce the induction profiles seen when the organisms were intracellular, the differential responses of these organisms to tissue culture medium (e.g. the 29-kDa MOMP) as indicated in Figures 27 and 28, could exploited to study the regulation of L.pneumophila genes/proteins.

Figure 28. Northern blot of the MEM response. The experiment was performed as described in the legend to Figure 27 except instead of labeling the bacteria, total RNA was isolated from it. For each sample, 20 μg of RNA was separated on a formaldehyde-agarose gel. (A) Ethidium bromide stained gel. 23S and 16S refer to ribosomal RNA bands. RNA markers are to the left of the gel and are as follows: (from top to bottom in kb) (9.49, 7.46, 4.40, 2.37, 1.35, 0.24) Lanes 1 to 8 correspond to Lanes 1 to 8 in Panel B. (B) Northern blot (autoradiogram) of the samples in Panel A. Separated RNA was transferred to a nylon membrane and probed with internal fragments of *htpB* and *omp28* which are the genes encoding the 60-kDa hsp 60 protein (HSP) and the 29-kDa outer membrane protein (OMP), respectively. 2 and M are Lp2064 and Lp2064M, respectively.

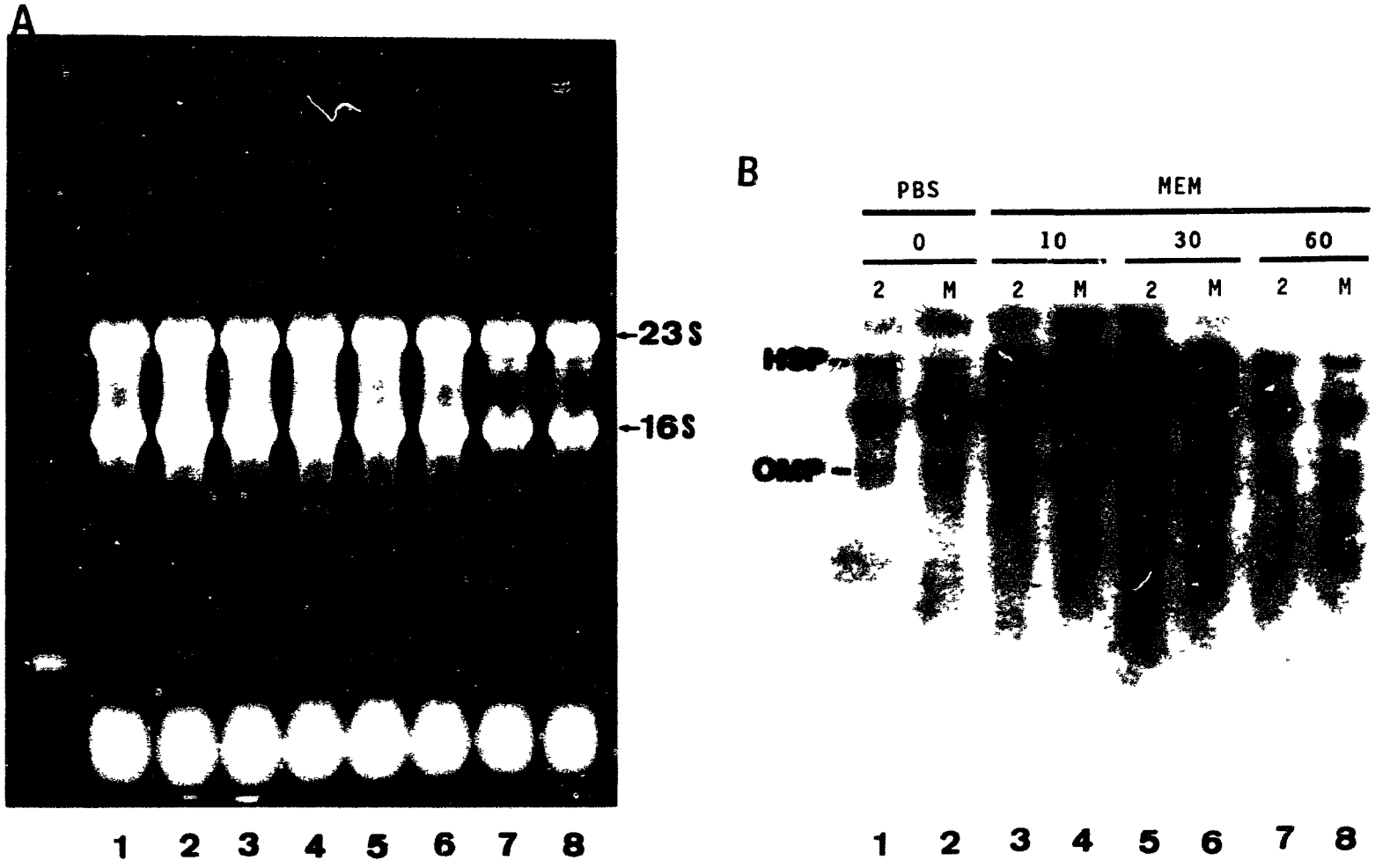


Figure 28

DISCUSSION

The objective of this thesis was to examine the intracellular pathogenesis of L.pneumophila infections. Comparisons were made between a virulent clinical isolate (Lp2064) and an isogenic avirulent mutant (Lp2064M) which was selected by passage on Mueller-Hinton media. As a prelude to studying intracellular pathogenesis, the interactions between L.pneumophila and several cell lines were examined. This process led to the discovery of "plaques" in L929 cells and the creation of the L929 infectivity index. The first part of the discussion will focus on the in vitro infectivity assays and the L929 infectivity index. The next part of the discussion will examine and attempt to interpret the results of the interactions of Lp2064 and Lp2064M with L929 cells (and monocytes). In this context, the discussion will also assess the response of the two organisms to different environments. Suggestions as to how these responses might influence virulence will be offered.

In vitro infectivity assays

a) General observations

The infection (in the presence of heat-inactivated serum) of MRC-5, A549, HEp2, Vero and L929 cells, all non-professional phagocytes, with various isolates of L.pneumophila serogroup 1 revealed that for a given isolate, L929 cells were most susceptible to infection, followed in

order by MRC-5, A549, HEp2 and Vero cells (Figures 1 and 3). Although CPE was not directly measured in HeLa cells, about half as many L.pneumophila invaded HeLa cells compared to the numbers invading L929 cells (Figure 8). These results in part, support the observations of Oldham and Rodgers (1985) who found 1) that infection of MRC-5 cells produced a rapidly lytic infection, 2) that uptake of L.pneumophila was about 10-fold less in Vero cells compared to HEp2 cells, and 3) that there was a more gradual release of organisms from infected HEp2 cells compared with MRC-5 and Vero cells which were infected with the same organism.

The potential mechanisms accounting for the range in the sensitivity of the different cell lines to infection were not investigated. However, it is possible that the degree of CPE is a reflection of both the amount, or the type of hitherto unidentified L.pneumophila "receptors" which may be present on the different host cells, as well as the intracellular growth rate of the organisms. If invasion of L.pneumophila occurs by a single mechanism, then the range of CPE might reflect a potential difference in the absolute numbers of receptors on the different cell lines. If L.pneumophila has multiple modes of entry, then the degree of CPE might be a consequence of not only the number of receptors but the type of receptors present on the host cells. That bacteria may have multiple pathways of entry which may confer a cell line specificity with respect to invasion has been postulated by

Isberg (1990). For example, Yersinia enterocolitica can use the products of at least two genes (*inv* and *ail*) to enter host cells. When normally non-invasive E.coli were engineered to express either *inv* or *ail*, it was found that bacteria expressing the *inv* gene were able to invade both HEp2 and CHO cells; those bacteria expressing the *ail* gene alone were able to invade only CHO cells (Miller and Falkow, 1988, Isberg, 1990), thus suggesting that HEp2 cells lack a receptor present on CHO cells.

The intracellular growth rate of the bacteria may also influence the amount of CPE generated. L.pneumophila requires iron and cysteine for growth (Feeley et al., 1978). It has been shown that under conditions where the availability of iron is limited (eg. either during IFN-γ-activation [Byrd and Horwitz, 1989], or chloroquine treatment [Byrd and Horwitz, 1991] of monocytes), growth of L.pneumophila is restricted. So, should the innate host cell metabolism affect the amount of iron (or another essential nutrient) available to L.pneumophila, then the growth rate of the organism may be altered, thus affecting the amount of CPE produced.

Another possible reason for the variation in CPE in the different cell lines may be due to the production of a toxic factor by L.pneumophila to which only certain cell lines are sensitive.

Despite the variation in demonstrable CPE, the ranking with respect to infectivity, of the limited number of

L.pneumophila isolates tested, did not vary among the cell lines.

b. L929 plaque assay

The infection of L929 cells with sufficiently high dilutions of L.pneumophila resulted in the production of a CPE which resembled virus-like plaques (Figure 3). The number of plaques produced was directly proportional to the concentration of bacteria in the inoculum. To more accurately analyze plaque production, the L929 plaque assay was developed. The salient features of the assay were the inclusion of a gentamicin step, and the use of agarose in the overlay following infection. Gentamicin being an aminoglycoside, penetrates mammalian cells poorly. It is therefore used to kill extracellular bacteria while sparing those organisms which have escaped by invading the host cell. The use of the agarose overlay serves to localize the infection to neighbouring cells. Each L.pneumophila plaque then is 1) the result of the initial infection of a single L929 cell with a single bacterium (Figure 5), and 2) is manifested by the intracellular multiplication of the bacteria. In addition, the number of plaques produced is dependent upon the length of time that the inoculum is exposed to the monolayer; the longer the exposure, the greater the number of plaques (Figure 6).

Plaque formation as a consequence of L.pneumophila infection is not unique to L929 cells. Indeed, Ormsbee et al.

(1981) noticed plaques in L.pneumophila-infected primary chick embryo cells. And although not shown, infected Chinese hamster ovary (CHO) cells also produce plaques. However, during the initial screening, no plaques were seen in A549, HEp2, MRC-5, or Vero cells. The reasons why L.pneumophila infection results in a generalized form of cell destruction in some cell lines while producing a more focal lesion in other cells, remain obscure.

c) L929 infectivity index

Preliminary results indicated that L.pneumophila isolates could be discriminated based on the numbers of plaques they produced. To enable comparisons amongst isolates, the L929 infectivity index was created. Operationally defined as the ratio of the inoculum size to the plaque yield, divided by 1000, it estimates the number of organisms within a population which are capable of producing visible plaques. An index of 2 means that 1 out of every 2000 bacteria in a population (i.e. in the inoculum) is a plaque producer. Thus, the higher the index, the fewer the number of plaque producers in the inoculum. In preliminary studies, the repeated assay of several isolates showed that the infectivity index was reproducible and therefore amenable for routine evaluation of the infectivity potential of the numerous clinical and environmental organisms isolated from local hospitals. Because the index can vary with the length of time that the inoculum is exposed to the monolayer, it was necessary to adhere to a

common exposure time to ensure valid comparisons amongst isolates.

The results of "blinded" tests coupled with the phenotypic/genotypic groupings of the organisms were presented in Tables 3 and 4. It was obvious that the type 0/c/France organisms were markedly distinct from the others by virtue of their high L929 index. Furthermore, not only were the numbers of visible plaques reduced, but the plaques were also much smaller and very difficult to count. Comparisons of L929 indexes with guinea pig mortality (using a peritonitis model) resulted in defining 4 categories of pathogenesis (G. Bezanson, personal communication). While the L929 assay was able to effectively identify Category 1 organisms (II/b/OLDA or Oxford) which were highly virulent in both assays, it did not predict the low guinea pig mortality characteristic of infections with organisms in Category 3 (type III/b/OLDA). These results underscore the multifactorial nature of the pathogenesis of L.pneumophila infections and suggest that the organisms in Category 3 are susceptible to extracellular factors, such as complement (perhaps). Interestingly, organisms in Categories 2 (VI/b/OLDA or Oxford) and 4 (0/c/France) differed in the L929 assay, but not with respect to guinea pig mortality which was moderate.

It is noteworthy that in a study that compared the ability of several species of Legionellae to multiply within Tetrahymena, with the ability to infect and kill guinea pigs,

similar categories of virulence were defined (Fields et al., 1986). The L.pneumophila isolate in the Fields study behaved like the organisms in Category 1. Both studies used the guinea pig peritoneal model to assess virulence. However, the guinea pig respiratory model (aerosol or intratracheal) is a more sensitive and appropriate barometer of virulence than is the peritoneal model. It better approximates the disease in terms of pulmonary pathology (see Introduction) and as such, it may be of interest to see whether its use influences the categories of virulence which have already been defined.

Except for Category 4 organisms which were unique with respect to their genotypic/phenotypic profiles, the isolates in Categories 1, 2, and 3 differed only with respect to plasmid content. In the literature there has been no clear correlation with plasmid carriage and the ability to cause disease. In one study, plasmidless isolates were found to be more virulent (Brown et al., 1982). Of the 4 Categories, only organisms in Category 4 lacked a plasmid. Interestingly, the most virulent organisms with respect to guinea pig LD50's and L929 infectivity possessed a 20-MDa plasmid (Type II profile). Key to the understanding of the role of plasmids in L.pneumophila virulence would be the ability to effectively (and easily) manipulate and mobilize the plasmids between the L.pneumophila isolates. Unfortunately this is currently a difficult task.

The marked difference in the number and type of plaques

produced by O/c/France organisms in Category 4 is intriguing. Type O/c/France organisms differ from the others in three ways: they lack plasmids, they have a distinct restriction enzyme fragmentation pattern, and they are $mAb2^{+}$ based on their reactivity with a monoclonal antibody panel (Joly et al., 1986). "OLDA" and "Oxford" organisms are $mAb2^{-}$ (Joly et al., 1986). The epitopes recognized by the mAbs are likely to be found on the LPS moiety of the bacterial surface (Ciesielski et al., 1986). It is not known how L.pneumophila enter non-professional phagocytes. However, it is possible that uptake of the bacteria may occur by a lectin-mediated process (Ofek and Sharon, 1988). As such, differences in LPS recognized by the mAbs may affect binding/uptake and ultimately the number of plaques. The initial sluggishness in uptake (reflected by the reduced numbers of plaques) may also contribute to the small plaque size. Newly emerging bacteria from infected cells, like the parent organism, may be impaired in their ability to invade neighbouring cells. In addition, the size of the plaques may simply reflect the intracellular growth rate of the organism. Whether O/c/France organisms are compromised in their ability to bind and invade, or whether they simply grow at a slower rate within L929 cells can easily be tested. Differences in binding can be monitored by immunofluorescence. Invasion, as distinct from intracellular multiplication, can be studied using the Dreyfus invasion assay (Dreyfus, 1987) substituting L929 cells for HeLa cells.

Intracellular growth rates can be measured by lysing infected cells at various time points after infection and enumerating the organisms in the lysates. It would also be interesting to determine the expression of the *mip* gene in these bacteria. The product of the *mip* gene is involved in some early (as yet undefined) phase of intracellular infection (Cianciotto et al., 1989b). These authors showed that a *mip*⁻ mutant was 80-fold less efficient in its ability to infect U937 cells.

In studies of Shigella infections, a mutant has been generated that fails to form plaques in HeLa cells. The gene responsible for intercellular spread (assayed by plaque formation) has been identified and mapped to the Shigella virulence plasmid (Bernardini et al., 1989). Upon entry, Shigella (unlike L.pneumophila which are found in phagosomes) lyse the phagosome membrane and are released into the cytoplasm where they multiply. In the cytoplasm, actin filaments are polymerized at one end of the bacteria and these serve to propel the organisms to the edge of the host cell where they then protrude into and invade apposing cells (Bernardini et al., 1989). No actin is seen surrounding mutant organisms which fail to form plaques. Whether an analogous gene exits in L.pneumophila, and if so, whether its function is altered in O/c/France isolates, is not known. However, since the different subcellular locations of Shigella and Legionella may evoke different methods of intercellular spread, the nature of the two disparate infections argues

against the need for such a gene in L.pneumophila.

Interaction of Lp2064 and Lp2064M with L929 cells

As judged by the infection of A549 cells, embryonated eggs and guinea pigs, Lp2064 was regarded as being virulent. By the same criteria Lp2064M was found to be avirulent. However, by their colony morphology, their BYE-grown or BCYE-grown protein profiles, their LPS profiles, and their ability to produce the 38-kDa L.pneumophila protease, both organisms were virtually indistinguishable. In terms of intracellular pathogenesis, Lp2064 successfully and productively infected monocytes and produced plaques in L929 cells with an infectivity index of about 2. On the other hand, Lp2064M failed to produce any overt cytopathology in either monocytes or L929 cells even at MOI's of 1000. Although Lp2064M was able to bind to and invade L929 cells, more than 80% of the organisms were killed within the first 24 hours most probably because the Lp2064M bearing phagosome cannot resist fusion with lysosomes (Figure 16, Table 6). This inability of Lp2064M to survive intracellularly is in contrast to the published results describing other Mueller-Hinton-generated mutants which were found to survive (but not multiply) within phagolysosomes (Horwitz, 1987). Except for their intracellular fates, like Lp2064 and Lp2064M, the mutants described by Horwitz (1987) also did not differ from the wild type counterpart in terms of the phenotypic characteristics

outlined above.

Despite the similarity between the protein profiles of Lp2064 and Lp2064M grown in either BYE broth or on BCYE agar (Figures 9 and 10), when the organisms were exposed to L929 cells, monocytes, or monocytes activated with IFN-γ, their respective profiles changed. Several of these changes were directly due to the tissue culture media (MEM or RPMI) and will be discussed in that context later. An examination of the protein profiles of extracellular and intracellular organisms proved to be quite revealing (Figures 18 and 20). A summary of the results using Lp2064 at T=30 as an example, are presented in Figure 29. In the absence of host cells (Lane 1) several proteins (including Hsp 60) were synthesized and for the most part, continued to be expressed when the organisms were in contact with (but not within) the L929 cells (Lane 2). When gentamicin was added to suppress the labeling of extracellular bacteria while still allowing intracellular organisms to be labeled (Lane 4), the 60-kDa heat shock protein (identified by immunoblot) was one of the most dominant proteins synthesized. This summary figure also serves to illustrate that unless precautions are taken, the labeling of extracellular bacteria will occur, thus contributing to the protein profile of organisms which may be assumed to be intracellular. Such was the case in Lane 3.

Whether in monocytes, in activated monocytes, or in L929 cells, the protein profile of intracellular Lp2064 was

Figure 29. Response of Lp2064 to extracellular and intracellular conditions. L929 cells were infected and labeled as described in the legends to Figures 18 (Lanes 1 and 2) and 20 (Lanes 3 and 4). Bacteria were labeled in the absence of L929 cells in Lane 1. Gm and Cyt D refer to gentamicin and cytochalasin D, respectively. Arrowhead indicates the 60-kDa protein. Molecular weight markers (in kDa) are also indicated.

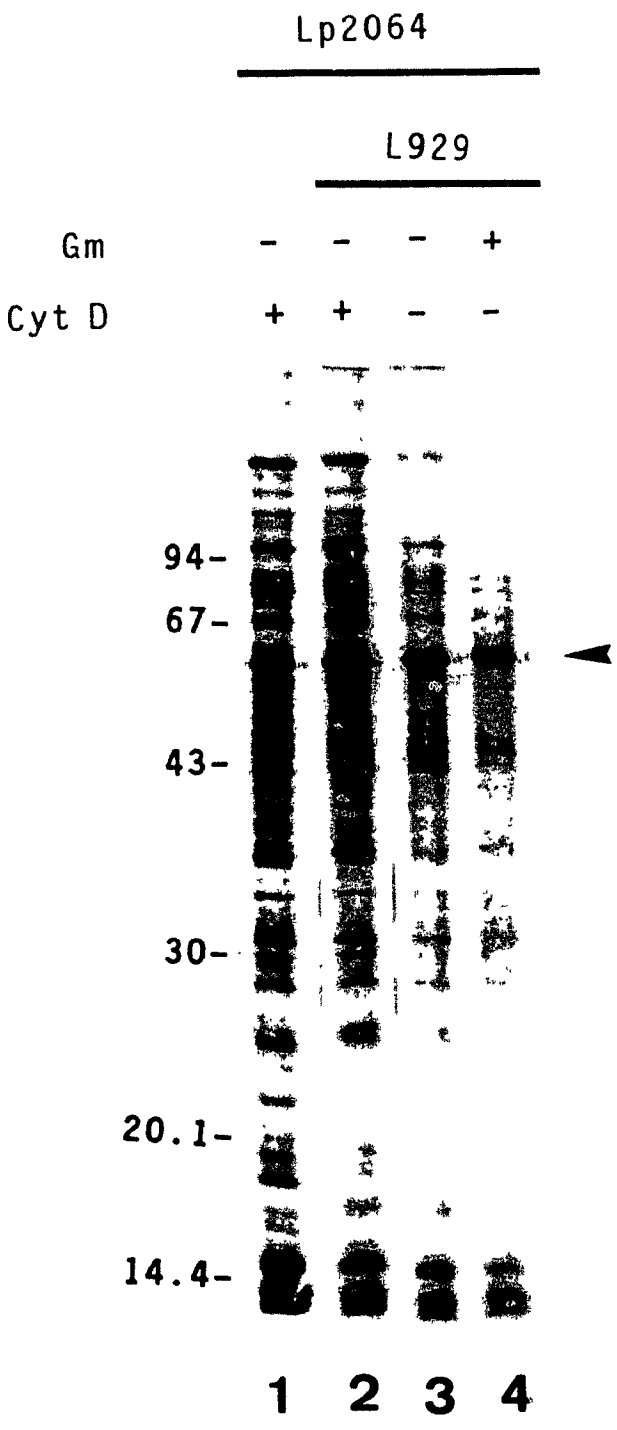


Figure 29

dramatically different from the Lp2064M profile. There was a remarkable consistency however in the response of a particular isolate to each of the three types of cells. It is perhaps not surprising that pretreating the monocytes with IFN-τ had little effect on the resulting profiles (i.e. compared to non-activated cells). Although the block in phagosome-lysosome fusion is relieved slightly in activated monocytes (Horwitz, 1983), the primary affect of interferon treatment is to limit the amount of iron that is available to L.pneumophila, thus impinging on its ability to grow (Byrd and Horwitz, 1989). The results (Figure 21) suggest that Lp2064 and Lp2064M are found in the same respective intracellular compartments in both activated and non-activated monocytes. A similar interpretation may be offered regarding L929 cells.

In Figures 20 and 21 the perceptible amount of radioactivity per lane suggested that perhaps significantly more of the Lp2064M proteins were loaded. This was at odds with the fact that equal numbers of TCA-precipitable counts were actually loaded. Although the result is very reproducible, the reasons for the apparent discrepancy are not known. Nevertheless, in relative terms, the 60-kDa protein was one of the most (if not the most) abundant protein synthesized in the Lp2064 samples. If for some inexplicable reason, fewer counts were loaded (in every Lp2064 lane), then at the very least, the increased 60-kDa response seen in Figures 20 and 21 is an underestimation of the actual response.

A 60-kDa protein was also seen in the Lp2064M lanes. However, it was not as prominent as the Lp2064 60-kDa protein. Both Lp2064 and Lp2064M were found to produce similar amounts of this protein upon exposure to MEM or to increased temperature suggesting that the differential expression of the protein under intracellular conditions might either be due to the organisms being exposed to different stresses or that Lp2064M is somehow impaired in its ability to sense a particular stress. Not only was the 60-kDa protein not the most prominent one synthesized by the avirulent bacterium, it also frequently appeared to migrate at a slightly lower position on the gels. Insufficient sample material precluded confirmation by immunoblot that the aberrantly migrating protein was indeed the 60-kDa heat shock protein. In addition to the extensive smearing that characterized the Lp2064M lanes, there was a significant increase in the level of expression of the 17, 15, and 13-kDa proteins. In fact, these bands were frequently found to be quite diffuse. In the absence of specific probes, the identity of these proteins cannot be determined. However, based solely on molecular weight, it is conceivable that any one of these proteins may represent the stress protein which is the product of the GroES-equivalent, *htpA* gene (Lema et al., 1988, Hoffman et al., 1989, and Hoffman et al., 1990).

As suggested above, the diverse intracellular responses exhibited by Lp2064 and Lp2064M may be a reflection of the

different microenvironments within which each organism is found. Lp2064 resides within a phagosome, whereas Lp2064M is forced to encounter the more harsh environment of the phagolysosome. In this regard, the two organisms would be exposed to different stresses which might result in the induction of a variety of responses. One such stress might be the low pH of the (phago)lysosome. An acid shock response has recently been described in E.coli (Heyde and Portalier, 1990, Hickey and Hirshfield, 1990) and in S.typhimurium (Foster, 1991). Although the exposure to the stress was brief, the results in Figure 25 suggest that exposure to a low pH, such as that found within lysosomes, may not account for the intracellularly-induced protein pattern displayed by Lp2064M.

It is also possible that the smearing pattern displayed by Lp2064M may be the result of exposure of the organism to proteolytic enzymes such as the acid hydrolases found within lysosomes. As such, the increased levels of lower-molecular-weight-proteins may represent the proteolytic breakdown products of higher-molecular-weight-proteins.

Regardless of the identity of the specific proteins, it is reasonable to assume that the intracellularly-ellicited protein profiles of Lp2064M are a reflection of the consequences of the organisms being in a lysosome. It may be useful to compare this response with that of other M-H-selected organisms, such as those described by Horwitz (1987).

In general, following uptake into either professional or

non-professional phagocytes bacteria are frequently found in phagosomes. [For many bacteria, these phagosomes eventually fuse with lysosomes and the bacteria are digested]. Acidification of the phagosome is believed to occur within the first 5 minutes after internalization (Mellman et al., 1986). Although L.pneumophila somehow resist significant acidification of their phagosome, the intraphagosomal pH has been found to be 6.1 (Horwitz and Maxfield, 1984). The acidification tolerance response (ATR) in Salmonella has recently been identified by Foster and Hall (1990). Distinct from the acid shock response, it is induced under mild pH (5.5 to 6) and protects the organisms when the pH is lowered to less than 4.5. One of the ATR proteins is GroEL. Compared to the responses of Lp2064 (or Lp2064M) exposed to tissue culture medium (MEM or RPMI) at pH 7.2, lowering the pH (in MEM) to 6.3, 5.5, or 4.0 did not appear to significantly increase the GroEL-equivalent, Hsp 60 response (Figure 25). It should be noted however that the responses in Figure 25 were measured within the first 10 minutes of exposure to the various conditions, whereas the ATR proteins in the Foster study were detected between 15 and 30 minutes following exposure to the low pH. The results in Figure 28 indicated that peak Hsp 60 transcripts are evident between 10 and 30 minutes following exposure to the MEM stress. Thus before assessing whether the increased intracellular Hsp 60 response is similar to the ATR in Salmonella, it would be necessary to repeat these pH

experiments, incorporating the revised time frame.

The enhanced expression of the Hsp 60 protein in Lp2064 found within L929 cells or monocytes is similar but not identical to the S.typhimurium GroEL response to the intracellular milieu of the J774 macrophage cell line (Buchmeier and Heffron, 1990). The dominance of the 60-kDa protein in L.pneumophila is much more impressive. And, if as suggested by Buchmeier and Heffron (1990), the intracellular environment causes the repression of Salmonella proteins, then the L.pneumophila response is greater by this criterion as well. It is interesting to note that unlike the L.pneumophila response which was seen in both monocytes and L929 cells, the intracellular induction of the Salmonella GroEL protein occurred only when the organisms were in macrophages and not when they were in epithelial cell lines.

The significance of the Hsp 60 response as a virulence factor can only be speculated upon. As reviewed by Lindquist and Craig (1988) and Rothman (1989), the heat shock or stress response is in evolutionary terms, a highly conserved response that all organisms have to adverse conditions. Although present constitutively, under conditions of stress, the various proteins can be induced to high levels. The synthesis of stress proteins is considered to be a protective response that shields the organisms against further, more harsher stresses. The GroEL family of stress proteins, of which Hsp 60 is a member, normally function in processes which involve

protein-protein interactions. Also known as a molecular chaperone, Hsp 60 is believed to assist in the assembly of macromolecules and to facilitate in the translocation of proteins across membranes.

As a protective mechanism, the L.pneumophila Hsp 60 may provide an "acidification tolerance" response to the slightly acidic interior of the phagosome.

As a chaperonin, Hsp 60 may function as a virulence factor by possibly interfering with signals necessary for fusion of phagosomes with lysosomes. That this may be so is based on the following premises: 1) membrane fusion is a directed process involving specific proteins present on both the docking and target vesicles (Lingappa, 1989, Wilson et al., 1991), 2) Hsp's function through protein-protein interactions (Rothman, 1989), and as such could interfere with the targeting/docking protein, and 3) virulent L.pneumophila that are stressed express Hsp 60 on their surface (as demonstrated by immunoelectron microscopy, P. Hoffman, personal communication), thus making it accessible for the interactions just described. An anti-idiotype antibody made to the monoclonal anti-Hsp 60 antibody could be used as a probe to test this hypothesis. Such an approach was recently used to study virus assembly (Vaux et al., 1988). Before embarking on such studies, it would be useful to know if Hsp 60 was surfaced expressed on Lp2064M.

In its capacity as a chaperonin, Hsp 60 may also aid in

the secretion (translocation) of an unknown factor which might ultimately interfere with the fusion of phagosomes with lysosomes.

It is tempting to speculate that the different intracellularly-derived protein profiles of Lp2064 and Lp2064M may correlate with their respective abilities to inhibit phagosome-lysosome fusion, possibly in the manner just described. However, despite this association, the possibility that the fates of the two organisms were determined either prior to or upon entry, cannot be discounted. If so, the protein profiles may indeed merely reflect the consequences of being in separate environments. In this vein, Joiner et al. (1990) have shown that in infected fibroblasts, the fusion competence of Toxoplasma-bearing vacuoles was directly associated with the route of uptake. Normally, during the infection of CHO cells, T.gondii is found within a parasitophorous vacuole which resists fusion with lysosomes irreversibly. However, in CHO cells transfected to express Fc receptors, antibody-coated T.gondii were found to reside within phagolysosomes. The fusion block was thus circumvented by forcing the organisms to enter via an Fc receptor-mediated route. The mechanism of uptake into non-transfected CHO cells has not been identified, but it may involve laminin and an integrin on CHO cells (K. Joiner, personal communication).

In L.pneumophila infections, entry into monocytes in the absence of immune serum occurs via complement receptor (CR)-

mediated uptake. The organisms first bind complement components C3b and C3bi to their major outer membrane protein (MOMP), and then utilize the bound complement as ligands for CR1 and CR3 (Payne and Horwitz, 1987, Bellinger-Kawahara and Horwitz, 1990). It is possible that other routes of uptake (in the absence of antibody) exist since in these same experiments, antibodies to CR1 and CR3 at best blocked uptake of L.pneumophila by 75%. L.pneumophila can also invade a number of non-professional phagocytes. While these cells do not possess complement receptors *per se* (Morgan, 1990), they do express integrins (Albelda and Buck, 1990). Complement receptors belong to the integrin family of cell surface proteins and there is a significant degree of homology (at the amino acid level) amongst the various classes of integrins (Albelda and Buck, 1990). Thus it is conceivable that uptake into monocytes and L929 cells occurs by analogous mechanisms. Uptake via complement receptors is advantageous to the invading pathogen since ligation of complement receptors circumvents the oxidative burst and the ensuing deleterious effects (Wright and Silverstein, 1983). It is not known however whether Lp2064 and Lp2064M differ in their respective entry pathways. An examination of their phagosome membranes (for the presence of complement receptors, in monocytes for example) might provide the answer.

Response of Lp2064 and Lp2064M to tissue culture medium

Exposure of Lp2064 and Lp2064M to MEM or RPMI resulted

1) in the induction of Hsp 60 and a 33-kDa protein in both organisms, 2) in the differential expression of some proteins; most obvious were those having relative molecular weights of 29 (MOMP), 21, 19, and 13 kDa, and 3) in the induction in both organisms of proteins (21 and 19 kDa) whose expression was transient. If, as suggested, the intracellular fate of the organisms is determined prior to entry, and since the protein profiles of extracellularly growing organisms (i.e. on BCYE or in BYE) are similar (Figures 9 and 10), then it is possible that the different responses of Lp2064 and Lp2064M elicited by the tissue culture medium (Figures 19, 24 and 27), may ultimately determine the mode of entry used by the organisms. Since virtually nothing is known about the specific identities of most of the induced proteins, the discussion will focus on general concepts.

The ability to sense and respond to environmental cues is an important virulence mechanism of pathogenic bacteria (Miller, J.F. et al., 1989, Miller, S., 1991). Characterized as a "two-component regulatory system", the mechanism consists of the interaction between a membrane-spanning "sensor" protein which senses changes in the environment, and a "regulator" protein which is involved in effecting the transcription of a set of (usually unlinked) genes in a coordinate manner (Ronson et al., 1987). As summarized by Mizuno and Mizushima (1990), exposure to the appropriate stimulus results in the transmembrane sensor being

autophosphorylated. The phosphate is then transfered to the regulator (or activator) protein. Once phosphorylated, the regulator activates transcription by binding to DNA sequences in the promotor regions of the appropriate genes (Mizuno and Mizushima, 1990). It has been suggested that changes in DNA supercoiling (which is also responsive to environmental stimuli) may also play a role (Dorman, 1991). Recent studies with Salmonella illustrate the importance of the two-component system in pathogenesis (reviewed by Miller, 1991). The locus coding for a two-component system in Salmonella is called *phoP/phoQ*. Mutations in this locus result in the diminished capacity of the mutant strains to kill mice and to survive in macrophages. One of the genes regulated by PhoQ is *pagC*. Mutations in *pagC* also result in attenuation with respect to survival in macrophages. The product of the *pagC* gene is a membrane protein which (based on nucleotide sequence) is similar to other outer membrane proteins including the invasion-associated product of the Y.enterocolitica *ail* gene. The function of the 18-kDa PagC protein is unknown. Although it is necessary for intracellular survival, it does not seem to affect invasion. As a result, Pulkkinen and Miller (1991) have suggested that protein-protein interactions mediated by the PhoQ-regulated PagC protein might be involved in virulent organisms selecting a pathway of invasion that ultimately allows them to survive intracellularly. It would be interesting to see if there is a PagC homologue in

L.pneumophila, and if present, whether it is somehow altered in Lp2064M. If so, the possibility exists that its (PagC) expression might be influenced by some component of the tissue culture medium. In this regard, PagC-type candidates may include the 19 and 21-kDa proteins. In the Salmonella studies, the actual environmental stimulus effecting *pagC* transcription was not mentioned.

Two-component regulatory mechanisms have yet to be identified in Legionellae. However the differential expression of several proteins especially MOMP, upon exposure to tissue culture media, implies the existence of such a system. The Legionella MOMP is a porin (Gabay et al., 1985). In E.coli, the expression of outer membrane proteins, many of which are porins, is influenced by the osmolarity of the surrounding medium. Porin expression may be enhanced or repressed (Forst and Inouye, 1988). The stimulus to which Lp2064 and Lp2064M are responding is not known. In terms of total protein synthesis, Lp2064 is much more sensitive to 0.3 and 0.6% NaCl (data not shown) than is Lp2064M. In fact. Mueller-Hinton selection of avirulent mutants is based on the ability of the avirulent organisms to grow at high salt concentrations (Catrenich and Johnson, 1989). However as demonstrated in Figure 26, changing the NaCl concentration did not affect the differential expression of MOMP, suggesting that the stimulus is not NaCl.

Although the existence of a two-component regulatory

system is inferred, studies utilizing the cloned MOMP gene (Hoffman et al., 1991) might help define regulation of this response. Since the levels (protein and mRNA) of MOMP are clearly different in Lp2064 and Lp2064M, it would be interesting to compare Lp2064 and Lp2064M with respect to their promotors and the sequences flanking them.

It has been suggested (Hoffman et al., 1991) that the differential responses of virulent and Mueller-Hinton-selected avirulent organisms (e.g. Lp2064 and Lp2064M) to MEM as illustrated by Northern analysis (e.g. Figure 28), may reflect an impaired capacity of the avirulent organism to sense and/or respond to (certain) changes in its environment; Lp2064 respond by down-regulating their production of MOMP, whereas Lp2064M are unable to do so. Extending this hypothesis, virulent (but not avirulent) organisms would produce a factor (akin to PagC, perhaps) elaborated by a putative two-component regulatory system, that would promote intracellular survival. The construction of Tn*phoA* mutants by allelic exchange might aid in identifying such a protein. The mutants could be tested for the concomitant inability to regulate their synthesis of MOMP with the ability to survive in mammalian cells. Two-dimensional gel electrophoresis could then be used to analyze the proteins from wild-type and mutant organisms. This procedure could also be used to clone the two-component system.

Although the manifestation of Legionnaires' disease is a result of a multifactorial process which includes, but is not restricted to intracellular multiplication (Cianciotto et al., 1989a), the ability of the pathogen to survive and multiply within macrophages is an important virulence factor. While intracellular pathogenesis is best studied in alveolar macrophages (the target host cell), they are difficult to obtain -especially in large quantities, and their susceptibility to infection may be influenced by the immune status of the host. While monocytes are more readily available, in addition to sharing the above mentioned drawbacks, they must be isolated from peripheral blood each time an experiment is performed. L929 cells on the other hand, are plentiful and are easy to grow in large quantities. These cells, while not being professional phagocytes, are infected productively with L.pneumophila, possibly via a mechanism that involves integrins which share homology with complement receptors (discussed above). As they do in monocytes, virulent L.pneumophila (Lp2064) inhibit phagosome-lysosome fusion in L929 cells, and similar intracellular protein profiles (Lp2064 or Lp2064M) were seen in the two types of cells, suggesting that L.pneumophila utilize analogous strategies to survive within both professional and non-professional phagocytic cells. In addition, since it is unlikely that L929 cells possess complement receptors, and since it appears that antibodies to complement receptors fail to completely block

uptake into monocytes (Payne and Horwitz, 1987), the use of non-professional phagocytic cells such as L929 cells may aid in identifying alternate routes/mechanisms of uptake of L.pneumophila into host cells. Such studies may also provide some insight as to how other intracellular organisms such as Chlamydia and Toxoplasma survive within non-professional phagocytes.

SUMMARY

L.pneumophila can infect a variety of professional and non-professional cells to various degrees. The most sensitive of the non-professional cell lines tested were the L929 cells. In fact, at low multiplicities of infection the bacteria produced plaques in the L929 monolayer. Each plaque was the result of the initial infection of an L929 cell with a single bacterium. Macroscopic visualization of the plaques necessarily depended upon the intracellular multiplication of the organisms and the subsequent infection of neighbouring cells. The number of plaques was a function of the inoculum size as well as the length of time the inoculum was exposed to the monolayer (prior to the gentamicin step); increasing the MOI or increasing the exposure time resulted in a concomitant increase in plaque number.

Numerous L.pneumophila isolates were assessed for their ability to infect L929 cells, using for comparison, the L929 infectivity index. The index, which was found to be highly reproducible, was defined as the ratio of the number of colony-forming units in the inoculum to the plaque titre, divided by 1000 (for convenience). The larger the index, the fewer the number of plaque producers.

A comparison of the index with guinea pig mortality resulted in the defining of 4 Categories of pathogenesis. Underscoring the multifactorial nature of Legionnaires'

disease, only organisms in Category 1 were highly virulent in both assays. Organisms in Category 4 were unusual in that they produced plaques which were small both in number and size. With the exception of a laboratory-generated avirulent mutant which produced no plaques, all of the isolates tested were capable of producing plaques.

The interaction of a Category 1 isolates with L929 cells was examined. Growth on Mueller-Hinton agar was employed to isolate avirulent isogenic organisms. The virulent isolate was designated Lp2064; the avirulent, Lp2064M. The two organisms were similar in colony morphology, LPS patterns, ability to produce the 38-kDa protease and in their BYE or BCYE-grown protein profiles. They differed with respect to guinea pig mortality, egg infectivity, and the ability to produce plaques. While both organisms attached to and invaded L929 cells, Lp2064 successfully inhibited fusion of its phagosome with lysosomes and was thus able to proliferate unhindered. In contrast, Lp2064M was unable to block fusion of its phagosome, and was killed within the confines of a phagolysosome.

Whether derived from L929 cells or monocytes, the protein profiles of intracellular Lp2064 were characterized by the predominance of the stress protein, Hsp 60. On the other hand, lanes containing Lp2064M typically displayed a smeared pattern with the heightened expression of some lower molecular weight proteins. In addition, the tissue culture medium (MEM or RPMI)

itself was responsible for the differential expression of several proteins including the 29-kDa major outer membrane protein (MOMP).

The relationship between the MEM/intracellularly-induced proteins with the ability to inhibit phagosome-lysosome fusion was discussed in the context of the route of entry into host cells, two-component regulatory systems, and the possibility that Lp2064 and Lp2064M differ in their respective capacities to sense and respond to changes in their environment. Since Hsp 60 was so dominant in its expression, a possible role in inhibiting fusion of phagosomes with lysosomes was suggested.

It has now been 15 years since the "mystery of the killer fever" has been solved. The Olympic Stadium in Montreal is literally crumbling (rumours of its debt were not exaggerated). In the United States, Bicentennial celebrations have given way to the impending festivities that will surround the 500th anniversary of Columbus' crossing. The Yankees have fired, and hired, yet another manager (some things never change). And, the disease which was once described as "one of the most dangerous things in the world" (Astor, 1983), has surrendered to more insidious threats like AIDS. Despite Astor's contention in 1983, that "research on the Legionnaires' disease front is quiet now", many questions regarding gene regulation and intracellular parasitism remain.

APPENDIX

MEDIA

a) BCYE agar

per L distilled water:

Legionella Agar base	37 g*
1N KOH	25 mL+
4% L-cysteine-HCl (sterile)	10 mL§
2.5% Ferric pyrophosphate (sterile)	10 mL§
Triple distilled water	955 mL

*from DIFCO:

Bacto Yeast Extract	10 g
Activated Charcoal	1.5 g
ACES Buffer	6 g
α-ketoglutarate	1 g
Potassium Hydroxide	1.5 g
Bacto Agar	17 g

+Amount may vary between lot numbers of media.

§added aseptically after autoclaving rest of media.

final pH 6.90 ± 0.05 at 25°C.

b) BYE broth

per L distilled water:

A)	ACES Buffer	10 g
	Double distilled water	500 mL
B)	1N KOH	40 mL
	Double distilled water	440 mL
C)	Yeast Extract	10 g

Solutions A and B are mixed and added to C.

After adjusting the pH to 6.9, the media is autoclaved.

D)	4% L-cysteine	10 mL
E)	2.5% Ferric pyrophosphate	10 mL

Solutions D and E are added once the autoclaved media has cooled.

c) MEM Eagles Medium (deficient) Sigma (catalogue # M 7270)

Contains Earle's salts

Component	g/L	Component	g/L
L-Arginine.HCl	0.126	L-Cystine.2HCl	0.0313
L-Glutamine	0.292	L-Histidine.HCl.H_2O	0.042
L-Isoleucine	0.052	L-Phenylalanine	0.032
L-Threonine	0.048	L-Tryptophan	0.010
L-Tyrosine 2Na	0.0519	L-Valine	0.046
Choline chloride	0.001	Folic acid	0.001
Myo-inositol	0.002	Niacinamide	0.001
D-Pantothenic acid Ca	0.001	Pyridoxal HCl	0.001
Riboflavin	0.0001	Thiamine HCl	0.001
Calcium chloride.2H_2O	0.265	Magnesium sulfate	
Potassium chloride	0.400	(anhydrous)	0.100
Sodium phosphate monobasic		Sodium chloride	6.800
(anhydrous)	0.125	D-Glucose	1.000
Phenol Red Na	0.011		

To this is added (g/L):

L-Leucine	0.052
L-Lysine.HCl	0.0725
Sodium bicarbonate	2.2

The prepared medium is filtered through a 0.45μm filter.

d) RPMI 1640 (deficient) Sigma (Catalogue # R 7130)

Component	g/L	Component	g/L
L-Arginine	0.200	L-Asparagine	0.050
L-Aspartic acid	0.020	L-Cystine.2HCl	0.0652
L-Glutamic acid	0.020	Glycine	0.010
L-Histidine	0.015	L-Hydroxyproline	0.020
L-Isoleucine	0.050	L-Phenylalanine	0.015
L-Proline	0.020	L-Serine	0.030
L-Threonine	0.020	L-Tryptophan	0.005
L-Tyrosine.2Na	0.02883	L-Valine	0.020
Biotin	0.0002	Choline chloride	0.003
Folic acid	0.001	Myo-Inositol	0.035
Niacinamide	0.001	PABA	0.001
D-Pantothenic acid Ca	0.00025	Pyridoxine HCl	0.001
Riboflavin	0.0002	Thiamine HCl	0.001
Vitamin B12	0.000005	Calcium nitrate.$4H_2O$	0.100
Magnesium sulfate	0.04884	Potassium chloride	0.400
Sodium chloride	6.000	Sodium phosphate,	
D-Glucose	2.000	dibasic	0.800
Glutathione,reduced	0.001	Phenol red, Na	0.0053

To this is added (mM):

L-Glutamine	2.05	Sodium bicarbonate	2.0g
L-Leucine	0.38		
L-Lysine	0.22		

The prepared medium is filtered through a 0.45μm filter.

e) PBS (calcium and magnesium-free)

Component	Amount
NaCl	8 g
KCl	0.2 g
$Na_2HPO_4.12H_2O$	2.8 g
KH_2PO_4	0.2 g
double distilled H_2O	1 L

This solution is autoclaved for 35 minutes.

f) HBSS

	Component	Amount
Solution 1	NaCl	80 g
	KCl	4 g
	$CaCl_2$	1.4 g
	$MgSO_4.7H_2O$	2.0 g
	double distilled H_2O	9 L
Solution 2	$Na_2HPO_4.12H_2O$	1.52 g
	KH_2PO_4	0.60 g
	Dextrose	10 g
	double distilled H_2O	1 L

The two solutions are combined and 45 mL of 2.8% $NaHCO_3$ are added to adjust the pH to 7.2 to 7.3.

The final solution is filtered through a 0.22 μm filter.

Table A1. Characteristics of L.pneumophila isolates (where known) obtained from the Microbiology department at the Victoria General Hospital

Isolate[a]	Source[b]	mAb Type[c]	Plasmid Carriage[d]	Fragmentation Pattern[e]
87-778	Env	Oxford	VII	a
87-100	Patient	OLDA	III	d
88-2064	Patient	Oxford	II	b
87-1095	Pt	France	0	c
87-261	Env	Oxford	II	b
87-1140	Env	Oxford	II	d
88-2751	Env	Oxford	II	b
85-578	Pt	untypable	II	a
87-185	Env	OLDA	V	d
87-1109	Env	OLDA	II/I	b
88-106	Pt	OLDA	II	d
87-256	Pt	OLDA	II	b
87-1045	Pt	France	0	c
87-589	Env	Oxford	III	a
87-588	Env	Oxford	VI	d
84-219	Pt	OLDA	IV	d
88-2280	Env		0	
88-2673	Pt	France	0	c
88-2478	Pt		0	
89-2579	Pt	OLDA	VI	b
89-313	Pt	OLDA	VI	b
89-1122	Env	Oxford	VI	b
89-2704	Env	untypable	0	b
89-1470	Env		III	
88-1375	Patient	OLDA	II	d
88-1451	Env		II	b
89-2590	Env	Oxford	VI	b
89-1567	Patient	France	0	c
89-1604	Env	France	0	
90-4269	Env		II	
90-4527	Env		II	

Table A1 (continued)

Isolate[a]	Source[b]	mAb Type[c]	Plasmid Carriage[d]	Fragmentation Pattern[e]
89-341	Env	Oxford	II	b
90-4264	Env		0	
91-236161	Env		0	c
91-194977	Patient		III	
91-4831	Env		III	
90-136787	Patient		III	
1473	Pt			
Leg				
CDC				
2755	Pt			
E72	Env			

a. Refers to isolate number at the Victoria General Hospital
b. Environmental (i.e. water), or patient source
c. Monoclonal antibody type
d. Plasmid profile (0= no plasmid, II= 20 megadalton (MDa) plasmid, III= 96 and 72 MDa plasmids, VI= 100 MDa plasmid.
e. Restriction enzyme fragmentation pattern using Eco-R1 or Bgl-II

STANDARD EM PREPARATION PROTOCOL (from G. Faulkner, EM UNIT)

1) 2.5% glutaraldehyde in buffer (0.1M sodium cacodylate pH 7.3)	2 h
2) Rinse with glutaraldehyde in buffer	10 min (X3)
3) 1% Osmium tetroxide in glutaraldehyde buffer	2 h
4) Rinse out Osmium tetroxide with dist. water	5 min (X2)
5) 0.25 -0.5% Uranyl acetate	2 h or O/N
6) Quick rinse in dist. water	
7) 50% Acetone	10 min (X1)
8) 70% Acetone	10 min (X2)
9) 95% Acetone	10 min (X2)
10) 100% Acetone	10 min (X2)
11) 100% Acetone (dried)	10 min (X1)

-steps 1 to 8 are done at 4°C; steps 9 onward at Rm temp

Infiltration carried out using TAAB embedding kit TK3

Resin made up as follows:

TAAB Resin: DDSA: MNA 20mL: 10mL: 10mL

-add 1 drop DMP-30 for each mL of complete resin to be made.

Infiltration Steps:

Acetone (dried): complete resin

4	:	1	2 h
1	:	1	2 h
1	:	5	2 h

Remove specimen from vial with toothpick, transport to capsule containing complete resin, add label, centrifuge if necessary. Leave at Rm temp 12-13 h. Cure at 70°C 24 to 48 h.

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