Molecular analysis of *Legionella longbeachae* serogroup 1 virulence

Robyn Michelle Doyle

Infectious Diseases Laboratories
Institute of Medical and Veterinary Science

and

Department of Microbiology and Immunology
University of Adelaide.

Adelaide, South Australia.

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Abbreviations

The following abbreviations have been used throughout this thesis and follow the style recommended by the American Society for Microbiology for journals and books.

aka	also known as
aa	amino acid
Ap^r	ampicillin resistance
ATCC	American type culture collection
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
BYE	Buffered Yeast Extract Broth
C-terminal	Carboxyl-terminal of protein sequence
CA	columbia agar
CDC	Centers for Disease Control and Prevention
CFU	colony forming units
Cm^r	chloramphenicol resistance
CR	complement receptor
CYE	Charcoal Yeast Extract agar
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dTTP	2'-deoxy-thymidine-5'-triphosphate
dUTP	2'-deoxy-uridine-5'-triphosphate
DMEM	Dulbecco Modified Eagle Medium
dot	defective in organelle trafficking
EDTA	ethylenediaminetetra acetic acid
Em	electron microscopy
ER	endoplasmic reticulum
HBA	horse blood agar
icm	intracellular multiplication
IPTG	isopropyl-α-D-thiogalactopyranoside
Km^r	kanamycin resistance
KDa	kilodalton
LD_{50}	50% lethal dose
LLAP	*Legionella*-like ameobal pathogen
LPS	lipopolysaccharide
MH	Meuller-Hinton agar
Mip	macrophage infectivity potentiator protein
MOMP	major outer membrane protein
MSP	major secretory protein
MW	molecular weight
NBT	nitroblue tetrazolium chloride
NCBI	National Center for Biotechnology Information
N-terminal	Amino-terminal of a protein sequence
nt	nucleotide
OMP	outer membrane protein

Abbreviations

O/N	over night
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMNL	polymorphonuclear neutrophilic leukocytes
PPIase	peptidyl prolyl cis/trans isomerase
RBS	ribosome binding site
RFLP	Restriction Fragment Length Polymorphism
sg	serogroup
SDS	sodium dodecyl sulphate
SSC	sodium citrate-sodium chloride
TAE	Tris-Acetate-EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N,N,-tetramethylethylenediamine
Tris	Tris([hydroxymethyl]aminomethane)
TTBS	Tris buffered saline with Tween-20
U-937	human histiocytic lymphoma cell line
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-α-D-galactopyranoside
X-P	5-bromo-4-chloro-3-indoyl-phosphate toluidine salt

Declaration

I declare that the work described herein contains no material that has been previously submitted for the award of any degree or diploma in any university and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Summary

In Australia, *Legionella longbeachae* sg 1 is an important human pathogen, responsible for nearly half of all clinical cases of *Legionella* related pneumonia reported each year. Few studies have been undertaken with *L. longbeachae* sg 1, and little is known about the pathogenesis of this species since most virulence studies have been conducted with *L. pneumophila* sg 1 strains. The aim of this project was to characterise potential virulence factors of *L. longbeachae* sg 1.

An aerosol model of experimental legionellosis was established for assessment of virulence of a panel of *L. longbeachae* isolates in guinea pigs. The results showed that there were distinct, statistically significant, virulence groupings of *L. longbeachae* sg 1 strains based on the severity of disease. Additionally, infection studies confirmed that *L. longbeachae* sg 1 was able to multiply in U937 cells a hallmark of virulence.

Examination of cell surface components of the bacteria showed that protein and lipopolysaccharide profiles of *L. longbeachae* sg 1 strains were very similar to each other but distinct from *L. pneumophila*. *L. longbeachae* sg 1 strains did not appear to express a MOMP-like protein, characteristic of *L. pneumophila*. However, a MOMP-like gene did appear to exist in the species. *L. longbeachae* sg 1 strains also did not possess a *flaA* gene.

The role of the outer membrane Mip protein on the pathogenesis of *L. longbeachae* sg 1 was studied. The *L. longbeachae* sg 1 *mip* gene showed strong identity with *mip* from *L. pneumophila* sg 1 (76%) and the inferred protein was 87% identical at the amino acid level. Primer extension analysis also determined that the start site of transcription was the same for both species, with some differences observed for the -10 and -35 promoter regions.

Allelic exchange mutagenesis, using the suicide vector pCACTUS-*mob*, was used to generate isogenic *mip* mutants in ATCC 33462 and strain A5H5. The ATCC 33462 *mip*

mutant was unable to infect a strain of *Acanthamoeba* spp. in liquid or potting soil co-culture while the A5H5 *mip* mutant behaved in a manner similar to an *L. pneumophila* sg 1 *mip* mutant, ie.it had a reduced capacity to infect and multiply within *Acanthamoeba*. The *mip* mutant was also attenuated in an animal model of infection and did not kill any animals under two different dose regimes.

To determine if plasmid-encoded virulence factors may play a role in pathogenesis of *L. longbeachae* sg 1 strains, a highly virulent Australian isolate, A5H5, that contained a large plasmid, designated pA5H5, was examined. Random cloning of the plasmid and DNA sequencing revealed the plasmid contained a two-component regulatory system with inferred homology to the OmpR family of two-component transcriptional regulatory proteins and EnvZ sensor kinases. An isogenic mutant was constructed in the transcriptional regulatory gene, designated *lrpR* (*L. longbeachae* sg 1 regulatory protein) and this strain was tested in *Acanthamoeba*, U937 cells and in the animal model. The mutant was attenuated for intracellular multiplication within *Acanthamoeba,* but not U937 macrophage-like cells. However, the *lrpR* mutant did appear attenuated for attachment and uptake in the early stages of infection of U937 cells. The *lrpR* mutant was also attenuated in an animal model of infection.

Publications

Doyle, R. M., T. W. Steele, A. M. McLennan, I. H. Parkinson, P. A. Manning, and M. W. Heuzenroeder. 1998. Sequence analysis of the *mip* gene of the soilborne pathogen *Legionella longbeachae*. Infect Immun. 66:1492-1499.

Manuscripts in preparation

Doyle, R. M., P. A. Manning, N. P. Cianciotto, and M. W. Heuzenroeder. 2000. Analysis of virulence of *Legionella longbeachae* serogroup 1 strains in U937 cells and a guinea pig model of infection.

Doyle, R. M., P. A. Manning, and M. W. Heuzenroder. 2000. A putative two-component regulatory element on a *Legionella longbeachae* serogroup 1 plasmid attenuates virulence in a guinea pig animal model.

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Chapter 1

Introduction

1.1 History

A "new" bacterial pathogen was identified in the summer of 1976 in Philadelphia, USA. It was responsible for an explosive outbreak of pneumonia that received worldwide attention due to the severity of the illness, the fatality rate and the perplexing nature of the infectious agent. The outbreak was associated with the American Legion Convention and was centred on the Bellevue-Stratford Hotel. Following the convention, an outbreak of pneumonia occurred involving 182 delegates, 147 of whom were hospitalised with 29 deaths (Fraser, *et al.*, 1977). The outbreak also included people who had not attended the convention but had visited the hotel or had walked within a block of it. Epidemiological studies determined that food, alcohol, tobacco, drinking water, animals and person to person spread were not likely factors in the transmission of the infectious agent and that the spread appeared to be airborne (Fraser, *et al.*, 1977).

The cause of this pneumonic illness, termed Legionnaires' disease, was found to be a gram negative bacillus that was isolated subsequently from the lung tissues of fatal Legionnaires' disease cases by culture in guinea pigs and yolk sacs of embryonated eggs (McDade, *et al.*, 1977). The etiological role of the bacterium, whose classification was unknown, was demonstrated by indirect fluorescent-antibody testing of convalescent serum samples from surviving patients. Diagnostic increases in antibody titres were shown for 91% of cases of Legionnaires' disease (McDade, *et al.*, 1977). DNA relatedness studies undertaken in conjunction with biochemical reactions and growth patterns determined that the unclassified gram negative bacillus responsible for Legionnaires' disease was sufficiently unique to warrant placing it in a new genus, *Legionella*; as a new species *Legionella*

pneumophila (Brenner, *et al.*, 1979). Shortly after the Philadelphia incident, a second species of *Legionella* was isolated and identified as the cause of a pneumonic disease in Pittsburg, Pa. (Myerowitz, *et al.*, 1979) and Charlottesville, Va. (Rogers, *et al.*, 1979) and was called *Legionella micdadei* (Hebert, *et al.*, 1980). *Legionella pneumophila* was to become the first recognised member of what would become a very large family of ubiquitous bacteria (Benson and Fields, 1998, Benson, *et al.*, 1996). Since then numerous additional species of the genus have been identified.

Studies later showed that Legionnaires' disease was not a "new" disease and that the agent *L. pneumophila* had been around in the environment for some time. The earliest retrospectively identified outbreaks had occurred in 1957 (McDade, *et al.*, 1977, Osterholm, *et al.*, 1983) and isolated cases as far back as 1947 (McDade, *et al.*, 1979).

1.2 Taxonomy of *Legionella*

The family *Legionellaceae* form a subgroup within the purple bacteria (Ludwig and Stackebrandt, 1983). DNA-relatedness studies based on homology and G + C content defined by Brenner *et al.* (1979) indicated that the family *Legionellaceae* should consist of a single genus with a number of species. It had been suggested that the family should be subdivided on the basis of DNA relatedness studies to include two additional genera, *Fluoribacter* and *Tatlockia* (Garrity, *et al.*, 1980). Despite the controversies regarding the taxonomy of the *Legionellaceae,* it is commonly accepted that there is a single genus within the family supported by two main methods of analysis. Firstly, analysis of 16S ribosomal RNA of different *Legionella* strains has determined that the *Legionellaceae* form a monophyletic subgroup within a subdivision of the protobacteria (Fry, *et al.*, 1991). Secondly, it has been proposed that all *Legionella* are sufficiently similar and unusual in phenotype to be classified as a single genus (Brenner, 1987).

Since the identification of the Legionnaires' bacterium in 1976, membership of the *Legionella* family has increased considerably. Presently there are 41 published species of

Legionella and 59 serogroups (Benson, *et al.*, 1996, Ratcliff, *et al.*, 1998) (Table 1-1 and 1-2). Many species have been documented to cause disease in humans (Benson, *et al.*, 1991, O'Connell, *et al.*, 1996b, Yu, 1995) (Table 1-1). Although most disease caused by *Legionella* species is in human hosts, a few publications have reported disease caused by this organism in animals (Collins, 1986, Fabbi, *et al.*, 1998).

1.3 Description of *Legionella*

Members of the genus are gram negative bacilli observed as blunt or tapering rods of 0.3-0.9μm in width and $\geq$5μm in length (Chandler, *et al.*, 1979a, Winn, 1999, Yu, 1995). The morphology of *L. pneumophila* is typical of gram negative bacteria with a rigid cell wall (peptidoglycan) surrounded on each side by a distinct tri-laminar unit membrane (Chandler, *et al.*, 1979a, Flesher, *et al.*, 1979). Electron microscopy of *L. pneumophila* has revealed a ruffled surface with a single polar flagellum and multiple pili (Rodgers, *et al.*, 1980, Yu, 1995). There is no clearly defined capsule layer although an acid polysaccharide capsule surrounding occasional cells from agar grown preparations has been reported for some *Legionella* species (Hebert, *et al.*, 1984).

Legionellaceae are facultative intracellular bacteria that are nutritionally fastidious and do not grow on standard bacteriological media (Yu, 1995). Charcoal Yeast Extract agar (CYE) supplemented with cysteine and ferric pyrophosphate (ferric irons) (Feeley, *et al.*, 1979), ACES buffer (N-2-acetamido-2-aminoethane-sulphonic acid) (Pasculle, *et al.*, 1980) and alpha-ketoglutarate (Edelstein, 1981) is the medium of choice for *in vitro* cultivation of *Legionella* species. The activated charcoal absorbs and detoxifies fatty acids and oxygen radicals as well as preventing the oxidation of cysteine, critical for the growth of *Legionella* (Yu, 1995). Members of the genus are aerobic (stimulated by 5% CO_2) (Winn, 1999) and when suitable medium is used *L. pneumophila* growth is evident in 2-3 days after incubation at 37°C. Some species of *Legionella* may require up to 10 days incubation. Colonies of *L. pneumophila* are circular and convex with a slightly irregular edge and a smooth shiny

surface, with an opalescent "cut glass" appearance, and range in colour from grey-white to blue, green or pink (Fig 1.1a and b) (Winn, 1999). Colonies of some *Legionella* species fluoresce blue-white or red under ultraviolet light which can be a useful feature for preliminary identification to species level (Winn, 1999, Yu, 1995) (Fig 1.1c).

Biochemically, *Legionella* species are relatively inert but all species are catalase positive and negative for urease, nitrate reduction and fermentative activity (Yu, 1995). Oxidase activity and the ability to hydrolyse hippurate varies with individual species (Yu, 1995). *L. pneumophila* can hydrolyse starch and can oxidise certain sugars although its energy metabolism is based on oxidation rather than fermentation (Dowling, *et al.*, 1992). *Legionella* species derive most of their energy from the oxidation of amino acids by means of the Krebs cycle (Dowling, *et al.*, 1992, Yu, 1995). *L. pneumophila* lacks the enzymes necessary to convert serine to O-acetyl serine and ultimately cysteine, consequently cysteine is a vital constituent in the extracellular growth medium and its requirement in extracellular growth medium is a phenotypic characteristic of the genus (Yu, 1995).

1.4 Legionellosis

Legionellosis is the term used to refer to all clinical syndromes produced by the genus *Legionella* (Dowling, *et al.*, 1992, Winn, 1999, Yu, 1995). Legionnaires' disease was a term used to refer to the 1976 Philadelphia epidemic and now the more general term legionellosis is used (Winn, 1999). The most common clinical manifestation of legionellosis is a pneumonic disease with an incubation period of 2-10 days, with symptoms ranging from a mild cough with fever to multi organ involvement (Winn, 1999, Yu, 1995). Typically the patient has a high fever at the onset of the disease with a non-productive cough, chills, myalgia and headache that can lead to a severe pneumonia. Hepatic, renal and cerebral involvement are also possible (Yu, 1995). Other symptoms include nausea, abdominal pain, headache, confusion and diarrhoea (Davis and Winn, 1987, Johnson, *et al.*, 1984, Yu, 1995). Bacteremia occurs in at least 20% of patients with the disease (Edelstein, 1993). Pneumonic

legionellosis is difficult to diagnose due to this broad range of clinical symptoms and radiographic and biochemical features of the disease are not sufficiently specific to differentiate it from other causes of atypical pneumonia (Edelstein, 1993, Helms, *et al.*, 1979, Yu, 1995).

Another manifestation of legionellosis is Pontiac fever, an acute, self limiting, non-pneumonic form of disease (Glick, *et al.*, 1978, Kaufmann, *et al.*, 1981, Winn, 1999). The infection is characterised by a very high attack rate (>95%) among those exposed (Winn, 1999). The incubation period is 24-48 hours and flu-like symptoms include fever, chills, headache, myalgia, a non productive cough and occasionally nausea (Yu, 1995). The illness is difficult to diagnose as it is a culture-negative infection and also because the symptoms are often indistinguishable from a variety of viral respiratory syndromes. The number of cases of Pontiac fever may be underestimated due to the self-limiting nature of this form of legionellosis.

Why there are two clinical respiratory presentations of legionellosis is not known. It has been proposed that inoculum size (Girod, *et al.*, 1982), mode of transmission (Yu, 1995), susceptibility of the individual (Thomas, *et al.*, 1993), inhalation of dead bacteria (Fields, *et al.*, 1990) or inhalation of amoebae filled with *Legionella* (Rowbotham, 1986) are possible explanations.

1.5 Ecology

Legionella are ubiquitous organisms within the environment (Fields, 1996). The natural habitat for *L. pneumophila* is water and it has been isolated from shower heads, potable water, cooling towers, evaporative condensers, ponds, rivers, lakes, streams, thermally polluted waters and marine waters (Fliermans, *et al.*, 1981, Rowbotham, 1980, Yu, 1995). *L. pneumophila* is able to colonise man-made habitats such as cooling towers as they are resistant to chlorine (Muraca, *et al.*, 1987) and hence survive the water treatment process (Yu, 1995). Once an artificial water system has been colonised, the organism is very difficult to

eradicate (Lin, *et al.*, 1998). The habitat of other species of *Legionella* is largely unknown and their ecology is poorly understood. *L. longbeachae* sg 1 has been shown to reside in potting soil rather than water (Steele, *et al.*, 1990a). Interestingly, the organism was found in potting mixes, and the waste wood products of Australian samples, but was not isolated from potting mixes obtained from Europe (Steele, *et al.*, 1990b). *L. longbeachae* sg 1 and other *Legionella* species have been found in potting mixes, in composted plant matter from home gardens, and bulk composting depots (Hughes and Steele, 1994). Temperatures in the composting heaps, even in winter, are within the optimal range (25-35^{0}C) for the multiplication of soil *Legionella.* Hence composting may be an important step in the amplification of *Legionella* species in the environment (Hughes and Steele, 1994, Ross, *et al.*, 1997). *L. longbeachae* organisms have been detected in water (Saint and Ho, 1999), however, this may not be the preferred ecological niche for the organism.

The ubiquitous distribution of *Legionella* in the environment is paradoxical given their fastidious laboratory growth requirements and it has been proposed that they are not free living in the environment (Rowbotham, 1980). Early evidence to support this view was the demonstration of a relationship between *L. pneumophila* and *Fischerella* sp., a Cyanobacterium, showing that growth of *L. pneumophila* was dependent on photosynthesis (Tison, *et al.*, 1980). Rowbotham demonstrated that *Legionella* are ingested by free living amoebae such as *Acanthamoeba* and *Naegleria,* ubiquitous in fresh water and soil, and proposed that they are the natural host for *Legionella* in the environment (Henke and Seidel, 1986, Rowbotham, 1980, Rowbotham, 1986).

L. pneumophila can multiply in a large variety of amoebae including *Acanthmoeba*, *Echinamoeba*, *Hartmannella*, *Naegleria*, and *Valkampfia* as well as the ciliated protozoan *Tetrahymena* (Anand, *et al.*, 1983, Fields, 1996, Fields, *et al.*, 1984, Newsome, *et al.*, 1985). Amoebae grow over a wide range of temperatures and have an intracellular pH that is compatible for growth of *Legionella* (Anand, *et al.*, 1983). Other species of *Legionella* have been shown to survive and replicate in amoebae and ciliated protozoans (Fields, 1996, Fields,

et al., 1986, Fields, *et al.*, 1990, Moffat and Tompkins, 1992, Neumeister, *et al.*, 1997, Steele and McLennan, 1996) isolated from water samples implicated as potential sources of infection in outbreaks (Barbaree, *et al.*, 1986, Holden, *et al.*, 1984). Amoebae are also essential for the growth of *Legionella* in potting mix (Steele, 1996). The ability of *Legionella* to multiply intracellularly in protozoa is central to the ecology of *Legionella* (Fields, 1996).

The multiplication of *Legionella* in amoebae *in vitro* has enabled the identification of an additional 12 phylogenetic groups in the family *Legionellaceae.* Designated LLAPs (*Legionella*-like amoebal pathogens) (Adeleke, *et al.*, 1996), these organisms can only be cultured intra-cellularly within protozoa. The first known LLAP was isolated from soil in Poland (1954) by Drozanski and until 1991 was named *Sarcobium lyticum* (Adeleke, *et al.*, 1996). Genetic analysis of this organism revealed an extremely close relationship to the legionellae, and that it should be included in the genus *Legionella* (Springer, *et al.*, 1992). Many LLAPs have been identified and they infect a variety of amoebae (Adeleke, *et al.*, 1996). LLAPs have been shown to be responsible for some cases of pneumonia in the United States with previously unknown etiology (Abu Kwaik, *et al.*, 1998b). Recently the name *L. lytica* has been proposed for *S. lyticum* (Adeleke, *et al.*, 1996, Birtles, *et al.*, 1996, Winn, 1999).

Amoebae are likely to control the number and type of *Legionella* in the environment and significantly influence the epidemiology of the disease. Amoebae may also provide a means by which intracellular organisms survive chlorine treatment (King, *et al.*, 1988, Kuchta, *et al.*, 1993). The survival of *Legionella* in amoebic cysts would also enable them to be more resistant to adverse environmental conditions than free living *Legionella* (Anand, *et al.*, 1983, Rowbotham, 1986). While viable *Legionella* have been observed within protozoan cysts (Adeleke, *et al.*, 1996), attempts to isolate amoebic cysts containing *L. longbeachae* from soil amoebae were unsuccessful (Steele, 1996). It has also been shown that *Acanthamoeba* spp. expel vesicles, prior to encystment, that may contain high numbers of *Legionella* (Berk, *et al.*, 1998, Rowbotham, 1986). These vesicles are resistant to biocides,

and some environmental conditions, and therefore may allow survival of the bacteria prior to encountering a susceptible human host. Hence, the ability of *L. pneumophila* to multiply within protozoan hosts plays a critical role in environmental survival and pathogenesis (Berk, *et al.*, 1998).

1.6 Epidemiology

1.6.1 Incidence

Legionellosis occurs worldwide with numerous studies reporting *L. pneumophila* to be one of the top three bacterial causes of community acquired pneumonia (Bates, *et al.*, 1992, Stout and Yu, 1997, Yu, 1995). The true incidence of legionellosis world wide is difficult to determine as epidemiological studies are often based on serological evidence rather than organism isolation, resulting in an over estimation of disease frequency (Helms, *et al.*, 1979, Nichol, *et al.*, 1991). Many healthy individuals show elevated antibody titres to *Legionella* species without evidence of the disease (Cameron, *et al.*, 1991, Pitt, *et al.*, 1980, Winn, 1999). In the United States, the incidence of *Legionella* pneumonia may be as high as 23,000 cases per year (Winn, 1999). *L. pneumophila,* predominantly sg 1, is responsible for about 85% of reported cases of *Legionella* infection, with the remaining percentage of infections due to other species of *Legionella,* in particular *L. micdadei* (Pasculle, *et al.*, 1980, Reingold, *et al.*, 1984, Winn, 1999, Yu, 1995). There have been clusters of nosocomial pneumonia due to *L. micdadei, L. bozemanii* and *L. dumoffii* and non-pneumonic outbreaks involving *L. micdadei, L. anisa* and *L. feeleii.* However, reports of community based outbreaks of pneumonia due to species other than *L. pneumophila* are rare (Yu, 1995). One exception was an outbreak of *L. longbeachae* pneumonia in Australia (Cameron, *et al.*, 1991).

In Australia, 160 notifications of disease due to *Legionella* infection were reported in 1995, constituting a rate of 0.9 cases per 100,000 population, with similar numbers noted in the previous 4 years (Herceg, *et al.*, 1996). Since 1995, 255 notifications of disease have been reported per year (Saint and Ho, 1999). *L. longbeachae* sg 1 is responsible for almost half of

all cases of legionellosis reported in Australia (Doyle, *et al.*, 1998, Gabbay, *et al.*, 1996). *L. longbeachae* sg 1 was first recognised as a cause of pneumonia in 1980 (McKinney, *et al.*, 1981). A second serogroup was described in 1981 and is the only published report of isolation of this serogroup from a patient (Bibb, *et al.*, 1981). Infections due to *L. longbeachae* sg 1 have been reported in countries such as New Zealand, Sweden, Germany, Denmark, Canada and the Netherlands (Cameron, *et al.*, 1991, van't Hullenaar, *et al.*, 1996). In Australia, it was first isolated from a patient in 1987 (Lim, *et al.*, 1989) with numerous cases of infection now reported (Cameron, *et al.*, 1991, Gabbay, *et al.*, 1996, Steele, 1989, Steele, *et al.*, 1990a).

1.6.2 Risk factors

There appear to be several risk factors that determine susceptibility of an individual to infection with *Legionella,* including cigarette smoking, chronic lung disease, chronic cardiovascular disease and immunosuppression (including HIV patients) (Blatt, *et al.*, 1994, Eickhoff, 1979, Stout and Yu, 1997, Winn, 1999). Surgery is also a major predisposing factor in nosocomially acquired *Legionella* infection with transplant recipients at the highest risk, most likely due to immunosuppressant drugs (Stout and Yu, 1997, Yu, 1995). Legionnaires' disease generally occurs in middle-aged or older persons, with the mean age in most outbreaks between 50-60 years (Eickhoff, 1979). However, legionellosis has been reported in young children, generally in those who are immunosuppressed or have underlying pulmonary disease (Stout and Yu, 1997, Yu, 1995). In neonates, most cases are associated with hospital ventilators (Stout and Yu, 1997).

1.6.3 Transmission

Legionnaires' disease is acquired by the inhalation of aerosols containing *Legionella* or by micro-aspiration of water contaminated with *Legionella* (Blatt, *et al.*, 1993, Edelstein, 1993, Stout and Yu, 1997, Yu, 1995). Strong evidence for the aerosol route of transmission came from the Pontiac fever outbreak in a Health department building in Pontiac, Michigan (Glick, *et al.*, 1978). Guinea pigs placed in the building in cages developed pneumonia and *L.*

pneumophila was recovered subsequently from their lungs. Environmental reservoirs linked with disease include water cooling towers, evaporative condensers, spa baths and respiratory therapy equipment (Stout and Yu, 1997, Winn, 1999). Potable water is an identified source for community acquired and nosocomial infection (Edelstein, 1993, Joseph, *et al.*, 1994, Stout and Yu, 1997). Water distribution systems have also been linked with outbreaks (Lin, *et al.*, 1998). The low concentration of *Legionella* generally found in natural aquatic bodies can be significantly amplified in water distribution systems as they provide the organism with optimum growth conditions such as warm temperatures and nutrients.

Aerosol inhalation of soil particles has also been proposed as a mode of transmission (Thacker, *et al.*, 1978). *Legionella* species have been found in potting mixes and in composted plant matter from home gardeners and bulk composting depots (Hughes and Steele, 1994). There is no evidence to suggest that inhalation of dust particles is a means of transmission although *L. longbeachae* can survive on the hands for up to 30 minutes after soil handling suggesting that aspiration of the organism could be a possible means of infection (Steele, 1996). Aspiration of water contaminated with *Legionella* is now thought to be a major mode of transmission of *Legionella* (Stout and Yu, 1997, Yu, 1993). There is evidence that some cases of nosocomial Legionnaires' disease are due to micro-aspiration of contaminated water in association with nasogastric tube use (Blatt, *et al.*, 1993, Edelstein, 1993, Johnson, *et al.*, 1985).

Ingestion, followed by bacteremic spread from the gastrointestinal tract has also been proposed as a mode of transmission of *Legionella* because of the incidence of diarrhoea in patients with Legionnaires' disease. However, there is little clinical support for the theory (Yu, 1995). Human to human spread of the disease has not been documented (Winn, 1999).

1.7 Pathology

Legionellosis is a disease of the lower respiratory tract. Lesions are found in distal terminal bronchioles, respiratory bronchioles, alveolar ducts and alveoli, while the remainder

of the respiratory tract appears normal (Winn and Myerowitz, 1981). Infection was probably caused by inhalation of *L. pneumophila* in an aerosol of small particle size that could penetrate directly into the respiratory bronchioles and alveoli of the lower respiratory tract (Baskerville, *et al.*, 1981). Particles less than 5μm in size generated by air conditioning units, showers and cooling towers have been implicated as a source of infection (Stout and Yu, 1997).

Microscopic examination of infected lungs shows lobar segmental or patchy pulmonary infiltration described as an acute fibrinopurulent pneumonia, sometimes associated with acute diffuse alveolar damage and bronchiolitis (Winn and Myerowitz, 1981, Yu, 1995). The fibrinopurulent pneumonia is characterised by a dense, intra-alveolar infiltrate of neutrophils, macrophages and fibrin (Carrington, 1979, Yu, 1995). Lytic destruction of inflammatory cells, a process termed leukocytoclasis, is a dramatic microscopic feature observed in the exudate seen in many cases (Carrington, 1979, Dowling, *et al.*, 1992). The most significant observation is large numbers of bacteria phagocytosed by inflammatory cells, in particular macrophages (Chandler, *et al.*, 1979b). Upon inhalation, *L. pneumophila* is phagocytosed by polymorphonuclear neutrophilic leukocytes (PMNLs) and alveolar macrophages that arrive in the alveoli and within 24 hours bacteria are found in both cell types. The bacteria in the PMNLs appear damaged whereas macrophages contained large numbers of bacteria (Davis, *et al.*, 1983). Studies using pulmonary lavages from infected guinea pigs have shown that PMNs actually phagocytose a small portion of the bacteria though they appear to be efficient at killing those that are ingested (Jepras and Fitzgeorge, 1986).

Studies of the *in vitro* interaction of *L. pneumophila* polymorphonuclear neutrophils, alveolar macrophages and blood monocyte-macrophages have determined that *L. pneumophila* can multiply intracellularly in human monocytes and alveolar macrophages, an ability central to the pathogenesis of disease (Dowling, *et al.*, 1992). In experimental models of disease, the majority of viable *L. pneumophila* are associated with mononuclear phagocytes

24 hours after infection (Davis, *et al.*, 1983). Additionally, there is an association between the susceptibility of an animal species to the disease with the capacity of *L. pneumophila* to multiply within explanted macrophages from that species (Yamamoto, *et al.*, 1988, Yoshida and Mizuguchi, 1986). Mutants of *L. pneumophila* that are unable to multiply *in vitro* in macrophages are avirulent in an animal model of infection (Cianciotto, *et al.*, 1989a, Horwitz, 1987).

Therefore disease due to *L. pneumophila* is a complex interaction with the host that is dependent on intracellular replication of the bacteria, the immune system of the host, and virulence of the infecting strain (Cianciotto, *et al.*, 1989a).

1.8 Host defence against *Legionella* infection

The role of the immune system for defence against *Legionella* is important. Antibiotics do not kill the organism once it has gained access to the intracellular environment (Horwitz and Silverstein, 1983). Rifampin and erythromycin are the two antibiotics of choice to treat cases of Legionnaires' disease (Horwitz and Silverstein, 1983, Thornsberry, *et al.*, 1978). These antibiotics inhibit the extracellular growth of *L. pneumophila in vitro* and kill *L. pneumophila* at relatively low concentrations. However, they are unable to kill *L. pneumophila* within human monocytes although they prevent the organism from multiplying. Removal of the antibiotic from the cell culture medium restores the ability to multiply (Horwitz and Silverstein, 1983). Therefore patients with disease that undergo antibiotic treatment also require host defences to eliminate *L. pneumophila* (Horwitz and Silverstein, 1983).

The interaction between *Legionella* and the immune system of the infected host is a major determinant in the development of legionellosis. Host defence is primarily handled by the phagocytic cell system.

1.8.1 Humoral immunity

Patients with legionellosis respond by producing antibodies against *L. pneumophila* (Horwitz and Silverstein, 1980a, Nash, *et al.*, 1984). *In vitro* studies, however, have shown that humoral immunity may not be an effective host defence against *Legionella* infection (Horwitz and Silverstein, 1980a, Horwitz and Silverstein, 1980b). Antibody is unable to promote killing of *L. pneumophila* by complement, and virulent *L. pneumophila* is completely resistant to complement even in the presence of high titer anti-*L. pneumophila* antibody (Horwitz and Silverstein, 1980a). Antibody is able to promote the phagocytosis of *L. pneumophila* by PMN and monocytes in the presence of complement although phagocytosis is inefficient with either of these opsonins acting alone (Horwitz and Silverstein, 1980a, Horwitz and Silverstein, 1980b). In the presence of complement, antibody enhances phagocytosis but promotes only a modest degree of killing by polymorphonuclear leukocytes and monocytes. Antibody also does not significantly influence the rate of intracellular multiplication of *L. pneumophila* in human monocytes (Horwitz and Silverstein, 1980b). However, PMNs are unable to support the intracellular growth of *L. pneumophila* (Horwitz and Silverstein, 1980a, Horwitz and Silverstein, 1980c). As antibody and complement fail to promote active killing of *L. pneumophila* by PMNs and monocytes *in vitro,* humoral immunity appears an ineffective host defence against *L. pneumophila* and may actually enhance uptake of the organism by phagocytes where they can then multiply (Horwitz, 1983a).

1.8.2 Cell mediated immunity

Cell mediated immunity (CMI) plays an important role in defence against *Legionella* (Horwitz, 1983a). Patients with legionellosis develop cell mediated immunity, since their mononuclear cells respond specifically to *L. pneumophila* antigens by proliferation and by production of monocyte-activating cytokines (Horwitz, 1983a, Horwitz and Silverstein, 1981). Convalescent patients have circulating peripheral blood mononuclear cells sensitized to *L. pneumophila* antigens which persist for many months (Horwitz, 1983a). CMI has also

been induced in animals following sublethal infection and contributes to protection against subsequent lethal doses (Breiman and Horwitz, 1987).

Activated human monocytes inhibit growth of *L. pneumophila in vitro* (Bhardwaj, *et al.*, 1986, Horwitz and Silverstein, 1981). Fewer organisms are phagocytosed by activated monocytes than non-activated monocytes thus restricting access to the intracellular niche required for multiplication. In addition, activated monocytes slow the multiplication rate of those bacteria that are internalised (Horwitz and Silverstein, 1981). However, activated monocytes do not kill *L. pneumophila* more efficiently than non-activated monocytes, which require antibody and complement and even then only kill a very small number. Inhibition of multiplication is also independent of antibody and complement, indicating a major role for CMI in host defence against *Legionella*. Immunocompromised patients are at risk of infection with *L. pneumophila* and a defect in CMI would render these patients incapable of limiting organism multiplication. Humoral immunity without CMI could endanger these patients by enhancing uptake of the bacteria by monocytes where they can then multiply (Horwitz and Silverstein, 1981). In the normal host, humoral immunity and cell mediated immunity most likely work together to eliminate *L. pneumophila*.

Legionella antigens also induce enhanced natural killer cell activity and the production of interferon-gamma (Blanchard, *et al.*, 1985), interleukin 1 and tissue necrosis factor (TNF) (Blanchard, *et al.*, 1987a). Cytokine activated natural killer cells are capable of killing *Legionella* infected macrophages (Blanchard, *et al.*, 1987b).

1.9 Models of infection and intracellular life cycle

To study the intracellular life of Legionella and the pathogenesis of infection a number of biological models have been established.

1.9.1 Animal models

L. pneumophila has been shown to infect a variety of animals but the guinea pig is the most susceptible species (Collins, 1986). Rats and hamsters can be infected with *L.*

pneumophila but in contrast to guinea pigs they seldom die from the infection (Collins, 1986, Winn, *et al.*, 1982). Other animals with low susceptibility to infection include mice, rats, rabbits, rhesus monkeys and marmosets (Baskerville, *et al.*, 1983a, Collins, 1986).

Initial experiments relied on intraperitoneal infection of *L. pneumophila* in guinea pigs (Baskerville, *et al.*, 1981, Chandler, *et al.*, 1979b, Katz and Hashemi, 1982, McDade, *et al.*, 1977). Although the infection was fatal, with dissemination of the organism to the lungs, the model bore little similarity to the pneumonic form of the disease seen in humans. Baskerville *et al* (1981) demonstrated successful transmission of pneumonic legionellosis to guinea pigs and monkeys by exposure to aerosols containing *L. pneumophila.* Aerosol infection was produced by administration of the bacteria using a Collison spray nebulizer, in a modified Henderson apparatus, which allowed particles of the correct size to be deposited directly into the respiratory tract. The pneumonic lesions produced were similar to those seen in humans demonstrating the usefulness of the model for studying pathogenesis. Infection of guinea pigs by the aerosol route had been attempted previously, however, despite fever and death, no lung lesions characteristic of the disease in humans were produced (Berendt, *et al.*, 1980).

Subsequent studies showed that a fatal pneumonia could be caused in guinea pigs by aerosol and intratracheal inoculation methods (Baskerville, *et al.*, 1983a, Blander, *et al.*, 1990, Breiman and Horwitz, 1987, Eisenstein, *et al.*, 1984, Fitzgeorge, *et al.*, 1983, Winn, *et al.*, 1982). However, intranasal inoculation of guinea pigs is not a consistent model as the disease outcome is variable (Fitzgeorge, *et al.*, 1983, Katz and Hashemi, 1982).

Mice are very resistant to infection with *L. pneumophila* (Collins, 1986, Fitzgeorge, *et al.*, 1983). However, immuno-compromised mice are susceptible and can be used as a model of infection to study the immune response to *Legionella* (Blanchard, *et al.*, 1988). Treatment of susceptible A/J mice with antibody to α-inteferon, normally induced at 24 hours after intratracheal inoculation of *Legionella,* enhances bacterial replication and disease progression (Brieland, *et al.*, 1994). Recently a CD1 suckling mouse model has been described for the

assesment of virulence of strains of *Legionella* inoculated via the intraperitoneal route (Pastoris, *et al.*, 1997).

The guinea pig model has also been a useful tool to assess the efficacy of antibiotics in treatment of the disease (Edelstein, *et al.*, 1984, Gibson, *et al.*, 1983, Pasculle, *et al.*, 1985) and in vaccine development (Blander, *et al.*, 1989, Blander and Horwitz, 1991).

Animal models of infection are important for assessment of molecular aspects of pathogenesis, including analysis of isogenic mutants of virulence factors (Cianciotto, *et al.*, 1989a). Recently the guinea pig model was used to discover new virulence associated genes in *L. pneumophila* by screening of signature tagged mutant strains *in vivo* (Edelstein, *et al.*, 1999).

1.9.2 *In vitro* cell models

A key feature of *Legionella* virulence is their intracellular lifestyle. These organisms are capable of infecting and multiplying within a variety of mammalian and protozoan cell lines (Fields, 1996).

L. pneumophila can multiply in explanted human blood monocytes and alveolar macrophages (Horwitz and Silverstein, 1980c, Nash, *et al.*, 1984). Multiplication has also been observed in macrophages derived from monkeys, guinea pigs and mice (Cianciotto, *et al.*, 1989a, Daisy, *et al.*, 1981, Yoshida and Mizuguchi, 1986). Cell lines resembling macrophages that support the intracellular growth of *L. pneumophila* include U937 cells (Pearlman, *et al.*, 1988) and HL-60 cells (Marra, *et al.*, 1990). However, *L. pneumophila* replicate in non-phagocytic cells including HeLa cells (human cervical carcinoma) (Daisy, *et al.*, 1981), rat alveolar type II cells (Mody, *et al.*, 1993), type I and type II alveolar epithelial cells (Abu Kwaik, *et al.*, 1998a, Cianciotto, *et al.*, 1995a, Fields, 1996), Vero cells (Oldham and Rodgers, 1985), MRC-5 cells (human embryonic lung fibroblast) (Oldham and Rodgers, 1985, Wong, *et al.*, 1980), HEp-2 cells (human epithelial laryngeal carcinoma) (Daisy, *et al.*, 1981, Oldham and Rodgers, 1985), McCoy cells (mouse synovium) (Daisy, *et al.*, 1981), chicken embryo fibroblasts and mouse L929 cells(Cianciotto, *et al.*, 1989a). It is not

surprising that *L. pneumophila* can multiply in alveolar epithelial cells as they make up over 95% of the alveolar surface (Abu Kwaik, *et al.*, 1998a).

Legionella have been shown to multiply in as many as 13 species of amoebae and in two species of ciliated protozoan (Abu Kwaik, *et al.*, 1998b, Fields, 1996). These naturally occurring single cell microorganisms have been used in recent years to study the intracellular life cycle of *Legionella* and to predict pathogenic potential.

1.10 Intracellular lifecycle of *Legionella* in mammalian and protozoan cells

Attachment to host cell surfaces is generally the first event in bacterial infection and for intracellular pathogens such as *Legionella,* it is a crucial pre-requisite to cellular infection and establishment of disease.

1.10.1 Attachment/Uptake

L. pneumophila is taken up into phagocytic cell by an unusual process known as coiling phagocytosis, where long phagocyte psuedopods coil around the bacterium as it is internalised (Horwitz, 1984). Uptake of *L. pneumophila* into human monocytes, alveolar macrophages, polymorphonuclear leukocytes and amoebae occurs by this mechanism (Abu Kwaik, 1996, Abu Kwaik, *et al.*, 1998a, Abu Kwaik, *et al.*, 1998b, Bozue and Johnson, 1996, Gao, *et al.*, 1997, Horwitz, 1984). Treatment of *L. pneumophila* with specific antibody, in the presence or absence of complement, abolishes coiling phagocytosis and instead the bacteria are taken up by conventional phagocytosis (Horwitz, 1984). Studies with other strains of *L. pneumophila* sg 1 and *L. micdadei* have determined that uptake can also occur by conventional phagocytosis, however, coiling phagocytosis appears to be specific to the Philadelphia strain (Elliott and Winn, 1986, Oldham and Rodgers, 1985, Rechnitzer and Blom, 1989).

Conventional phagocytosis occurs by a "zipper mechanism" whereby sequential interactions between receptors and ligands on the surface of the phagocyte and the bacterium pull the plasma membrane around the bacteria like a zipper until it is completely enclosed in a

vacuole (Fig 1.1a) (Rittig, *et al.*, 1998c). In contrast, "coiling" phagocytosis is a process whereby unilateral pseudopods do not fuse and continue to wrap around the bacterium in multiple turns (pseudopod whorls) (Fig 1.1b) (Rittig, *et al.*, 1998a, Rittig, *et al.*, 1999,). Coiling phagocytosis in *Legionella* is distinguished from conventional phagocytosis by unilateral pseudopods and absence of pseudopod fusion resulting in self apposed pseudopod layers (stacking of pseudopods) (Rittig, *et al.*, 1998a, Rittig, *et al.*, 1998b, Rittig, *et al.*, 1998c). This phenomenon, originally proposed to be unique to *L. pneumophila* (Horwitz, 1984), has been observed for numerous organisms including, bacteria, protozoa and fungi (Rittig, *et al.*, 1998c) and evidence suggests that it is actively induced (Rittig, *et al.*, 1998a). It is not induced by motility as the process is observed for live (motile) and killed (non-motile) *L. pneumophila* and other microorganisms (Horwitz, 1984, Rittig, *et al.*, 1998a, Rittig, *et al.*, 1998b) suggesting that the process is phagocyte driven (Rittig, *et al.*, 1999, Rittig, *et al.*, 1998b). This distinguishes coiling phagocytosis from other non-classical uptake systems as they are generally microbial invasion strategies (Rittig, *et al.*, 1999, Rittig, *et al.*, 1998a). It is unlikely that the diverse array of microorganisms shown to exhibit coiling phagocytosis share epitopes that bind to the same phagocytic cell receptor (Rittig, *et al.*, 1998a), therefore, it is likely that coiling phagocytosis involves several phagocytosis promoting receptors (Rittig, *et al.*, 1998a). Inhibitors of classical phagocytosis also inhibit coiling phagocytosis, indicating that the same receptors are able to trigger both events rather than there being a specific receptor for this phenomenon (eg complement receptors) (Rittig, *et al.*, 1998a). Blocking complement receptors (CR) inhibits, almost completely, *L. pneumophila* uptake (Marra, *et al.*, 1990, Payne and Horwitz, 1987). Fragments of complement component C3 are the primary ligands recognised by CR1 and CR3 and these ligands mediate adherence to of *L. pneumophila* to the monocyte (Payne and Horwitz, 1987).

In summary, coiling phagocytosis in not a specific bacterial strategy or a phagocyte mechanism but reflects a disturbance in the course of conventional zipper type phagocytosis, leading to characteristic pseudopod coils (Rittig, *et al.*, 1998a, Rittig, *et al.*, 1998c).

Asymmetrical receptor clustering has been proposed as an initial trigger in both CR and non-CR cases of coiling phagocytosis (Rittig, *et al.*, 1998a).

Attachment to mammalian cells by *L. pneumophila* is mediated by both complement and non-complement receptors (Gibson, *et al.*, 1994, Husmann and Johnson, 1992, Payne and Horwitz, 1987). The complement receptor pathway may be a preferred route of entry for intracellular pathogens as entry via these receptors may allow them to avoid the adverse consequences of the metabolic oxidative burst (Payne and Horwitz, 1987).

Uptake of *L. pneumophila* into phagocytic cells can also occur by opsonin independent methods that do not require the presence of antibody or complement (Gibson, *et al.*, 1994, Rodgers and Gibson, 1993). Bacterial surface factors proposed to be potential ligands include flagella, fimbriae, lipopolysaccharide and outer membrane proteins (Rodgers, 1983). Studies suggest that the ligand is a protein structure with lectin-like properties associated with carbohydrate or lipid structures located on the bacterial cell surface. The receptor present on the host cell has properties consistent with a carbohydrate or complex saccharide structure (Gibson, *et al.*, 1994). It is proposed that during the early stages of infection in the lung where opsonins and complement are limited that opsonin independent binding of the bacterium to the host cell may be more significant (Gibson, *et al.*, 1994). Such a process would potentially mimic the attachment of this organism to protozoans in the environment (Gibson, *et al.*, 1994). Similarly non-opsonic mechanisms of attachment may occur during early stages of infection prior to an immune response eliciting specific antibody that may act alone or in conjunction with complement components to enhance uptake of *Legionella* (Husmann and Johnson, 1992). The ligand involved in the non-opsonic process may be the recently identified type IV pili of *L. pneumophila* (Stone and Abu Kwaik, 1998). Mutants defective in expression of the pili show reduced attachment to protozoan cells although they are not altered in their ability to multiply within them (Stone and Abu Kwaik, 1998). The host receptor is proposed to be the newly described lectin receptor of *Hartmannella vermiformis* (Abu Kwaik, *et al.*, 1998b, Venkataraman, *et al.*, 1997). This protozoan receptor

is a galactose/N-acetylgalactosamine (Gal/GalNAc) lectin with similarity to the ß2 integrin like Gal/GalNAc lectin of the pathogenic *Entamoeba histolytica*. *L. micdadei* has also been shown to use the Gal//GalNAc lectin for attachment and uptake into the protozoan host *Hartmannella vermiformis* (Abu Kwaik, *et al.*, 1998c).

Integrins are heterodimeric protein tyrosine kinase receptors that undergo tyrosine phosphorylation upon ligand binding resulting in recruitment and rearrangement of the cytoskeletion. In the normal state, this lectin is tyrosine phosphorylated and is associated with several other phosphorylated proteins including several cytoskeletal proteins which can potentially interact with the lectin receptor. Attachment of *L. pneumophila* to the lectin and invasion of the host cell generates an increase in bacterium induced tyrosine phosphatase activity in the amoebae resulting in dephosphorylation of several host proteins including the lectin receptor and cytoskeletal proteins (Venkataraman, *et al.*, 1997, Venkataraman, *et al.*, 1998). This observation has also been documented for another species of *Legionella, L. micdadei*, upon invasion of *Hartmannella vermiformis* (Abu Kwaik, *et al.*, 1998c).

Following attachment of the organism to the protozoan host, uptake occurs by both coiling phagocytosis and classical phagocytosis (Abu Kwaik, 1996, Bozue and Johnson, 1996). There appears to be heterogeneity of uptake mechanisms for *L. pneumophila* in different amoeboid hosts indicating that other receptors, in addition to the Gal/GalNac lectin, are involved (Harb, *et al.*, 1998).

1.10.2 Intracellular survival and multiplication

In mammalian macrophages, *L. pneumophila* multiplies within a phagosome that is surrounded by rough endoplasmic reticulum and this phagosome does not fuse with host cell primary or secondary lysosomes (Horwitz, 1983b, Horwitz, 1983c) or undergo normal acidification (Horwitz and Maxfield, 1984). Formation of the phagosome entails a complex sequence of cytoplasmic events that take place during the first 4-8 hours after phagocytosis (Horwitz, 1983b). Initially the vacuole is surrounded by smooth vesicles and mitochondria (1 hour) but these are gradually replaced by ribosomes and rough vesicles (4 – 6 hours) and by

approximately 8 hours after infection, the vacuole is "studded" with ribosomes. The vacuole also appears surrounded by rough endoplasmic reticulum indicated by the presence of the BiP protein, an endoplasmic reticulum luminal marker (Horwitz, 1983b, Swanson and Isberg, 1995). *L. pneumophila* begin multiplying in the phagosome 4 - 10 hours after phagocytosis (Horwitz, 1983b, Swanson and Isberg, 1995). Eventually the specialised phagosome lyses releasing large numbers of bacteria that can begin subsequent rounds of infection. Production of this novel phagosome is observed in alveolar macrophages and monocytes *in vitro* and also in alveolar macrophages in lung biopsy specimens obtained from patients with Legionnaires' disease (Glavin, *et al.*, 1979). In contrast, formalin killed *L. pneumophila* enter a membrane bound vacuole that does not undergo organelle recruitment and the organism is readily degraded after fusion with host cell lysosomes (Horwitz, 1983b, Horwitz, 1983c). This suggests that a surface structure is unlikely to play a role in the process of inhibition of fusion, that intracellular fate is determined by factors other than those that determine mode of entry, and that phagocytosis and formation of the replicative phagosome are independent phenomena (Rittig, *et al.*, 1999).

Within protozoa, *L. pneumophila* also replicates in a phagosome surrounded by rough endoplasmic reticulum that does not acidify or fuse with lysosomes (Abu Kwaik, 1996, Bozue and Johnson, 1996, Swanson and Isberg, 1993). This specialised phagosome has been referred to as the EMB (endosomal maturation blocked) phagosome (Abu Kwaik, *et al.*, 1998b), and its formation is believed essential for the intracellular survival and replication of *L. pneumophila* in both mammalian and protozoan cells. Mutants that do not establish the replicative phagosome are unable to multiply intracellularly (Abu Kwaik, 1996, Berger and Isberg, 1993, Marra, *et al.*, 1992, Swanson and Isberg, 1993).

Other intracellular pathogens also able to inhibit phagosome-lysosome fusion include *Mycobacterium tuberculosis*, *Toxoplasma gondii* and *Chlamydia psittaci* (Horwitz, 1983c). Phagosomes containing *C. psittaci* and *T. gondii* also share some of the unusual morphological features of the *L. pneumophila* phagosome including ribosome studding

suggesting a common mechanism may underly this survival strategy (Horwitz, 1983b, Horwitz, 1983c). The mechanism by which *L. pneumophila* and other pathogens inhibit fusion is unknown. Perhaps they lack an intrinsic fusion factor or possess a factor that inhibits fusion. In *Mycobacterium tuberculosis,* sulfatide (anionic trehalose glycolipids) and ammonia produced by the organism have been suggested to play an antifusion role (Horwitz, 1983c). Inhibition of fusion is important to allow *L. pneumophila* to resist microbicidal effects generated by phagocytic cells (Horwitz, 1983c).

These observations can not be generalised to include all species of *Legionella* as *L. pneumophila* sg 1 (strain Knoxville) and *Legionella micdadei* do not inhibit fusion of lysosomes with phagosomes that contain them (Rechnitzer and Blom, 1989). Infection of protozoa by *L. micdadei* results in formation of a phagosome that is not surrounded by rough endoplasmic reticulum (Abu Kwaik, *et al.*, 1998c). Similarly in macrophages the phagosome containing *L. micdadei* does not appear to be surrounded with host ER (Weinbaum, *et al.*, 1984). The intracellular locations of *L. micdadei* and *L. pneumophila* differ, suggesting different mechanisms for the survival of these two organisms in the monocyte (Weinbaum, *et al.*, 1984). However, the strategy that *L. micdadei* uses to survive the killing mechanism of the monocyte remains to be determined.

How does *L. pneumophila* bring about this complex series of events? Treatment of *L. pneumophila* with erythromycin, an antibiotic known to block bacterial protein synthesis, does not prevent formation of the novel vacuole indicating that ongoing protein synthesis is not required (Horwitz, 1983b, Horwitz, 1983c). Similarly, inhibition of protein synthesis in the host cell does not prevent formation of the novel vacuole suggesting that formation is dictated at the biochemical level of signal transduction (Abu Kwaik, 1998b, Abu Kwaik, *et al.*, 1998a, Venkataraman, *et al.*, 1998).

It has been hypothesised that *L. pneumophila* exploits the autophagy machinery in macrophages directing the phagosome to avoid fusion with the lysosome possibly by

releasing a soluble factor (Swanson and Isberg, 1995). By exploiting the autophagy host cell process, *L. pneumophila* would also be able to obtain metabolites required for multiplication. Invasion of human monocytes by *L. pneumophila* activates phosphorylation signals necessary to induce cytoskeletal re-arrangement required for uptake (Coxon, *et al.*, 1998). Entry of *L. pneumophila* activates tyrosine kinase (TK) and protein kinase C (PKC) and induces actin polymerisation at the site of entry. Upon entry, six major cellular monocyte proteins were tyrosine phosphorylated. However, an avirulent isolate of *L. pneumophila* has been shown to induce the same pattern of phosphorylated proteins upon uptake into monocytes, indicating that TK phosphorylation and perhaps PKC phosphorylation is not a virulence-associated event. A signal may still occur during binding of the organism to the host cell that may lead to the modification of the endocytic pathway. Dephosphorylation of multiple host proteins including the lectin receptor is known to occur when *L. pneumophila* invades the protozoan *H. vermiformis* (Venkataraman, *et al.*, 1997) and virulent *L. pneumophila,* but not avirulent strains, induce phosphorylation of a 76-kDa protein upon binding to murine peritoneal macrophages (Yamamoto, *et al.*, 1992).

Calcium may play a role in the intracellular trafficking of the *L. pneumophila* phagosome and involve an unidentified receptor (Coxon, *et al.*, 1998). Non-opsonic uptake of *L. pneumophila* can occur (Pearlman, *et al.*, 1988). This receptor may be responsible for the uptake and subsequent replication of this pathogen in non-professional phagocytes such as HeLa cells, embryonic lung fibroblasts and human epithelial cells (Coxon, *et al.*, 1998) and may be analogous to the Gal/GalNAc receptor lectin mentioned previously (Venkataraman, *et al.*, 1997).

In summary, infection of host cells results in the formation of a specialised compartment that is essential for the growth and intracellular survival of *L. pneumophila.* Lack of fusion between this phagosome and host cell lysosomal bodies is critical for survival of *L. pneumophila* as mutants that are unable to avoid doing so are incapable of intracellular growth, are unable to kill the host cell, and are avirulent in an animal model of disease.

1.10.3 Genetic studies of the intracellular life cycle

Similarities of intracellular multiplication of *L. pneumophila* in both mammalian cells and protozoa, and manipulation of the host cell processes have lead to studies aimed at determining if the same genetic loci mediate infection for both cell types. Analysis of mutants for their ability to replicate in protozoa and mammalian cells has identified *pmi* (protozoa and macrophage infectivity) loci (Gao, *et al.*, 1997). Some of the genes identified in these loci have homology with the *dot/icm* genes of *L. pneumophila* (Berger and Isberg, 1993, Marra, *et al.*, 1992) known to be important for the intracellular survival of the organism in macrophages. A class of mutants defective only in their ability to survival in macrophages but unaffected in protozoa has enabled the identification of a loci designated *mil* (macrophage-specific infectivity loci) (Gao, *et al.*, 1998a). Southern hybridisation and sequence analysis determined that the *mil* loci identified do not have homology with the *dot/icm* genes (Gao, *et al.*, 1998a) and that many of the genes have no homologues in the databases. It has been postulated that *L. pneumophila* evolved in the protozoan host, later acquiring the *mil* loci that has allowed adaptation to the intracellular niche of the human phagocytic cells. This form of specific pathogenic evolution is supported by the observation that *L. pneumophila* is competent for transformation (Stone and Abu Kwaik, 1999) and conjugation of DNA (Vogel, *et al.*, 1998). Some of the *pmi* and *mil* loci are also required for replication in alveolar epithelial cells as 30 of 121 mutants defective for growth in macrophages were also defective in epithelial cells (Gao, *et al.*, 1998b). Type I and II alveolar epithelial cells constitute more than 95% of the alveolar surface area and *L. pneumophila* can replicate in both cells *in vitro* (Abu Kwaik, *et al.*, 1998a, Cianciotto, *et al.*, 1995a, Mody, *et al.*, 1993). Most genetic loci in *L. pneumophila* are therefore required for the survival of the bacteria in phagocytic cells. However, there may be loci that are exclusively required for replication in epithelial cells. At the ultrastructural level, the infection of mammalian and protozoan cells appears highly similar however, and the biochemical and molecular mechanisms involved in their survival in both, are distinct (Gao, *et al.*, 1998a).

1.10.4 Destruction of the host cell

The mechanisms by which *L. pneumophila* kill the host cell are largely unknown, however, studies with mammalian cells are beginning to clarify the process. The ability to kill macrophages is determined by a number of genetic loci in *L. pneumophila* (Sadosky, *et al.*, 1993). The potential role of a cytotoxin in infection and host cell killing is unclear (Friedman, *et al.*, 1980, Keen and Hoffman, 1989, Quinn, *et al.*, 1989, Quinn and Tompkins, 1989), although such a cytotoxin would have to have a broad host range since *Legionella* can infect both mammalian cells and protozoa (Hagele, *et al.*, 1998).

Legionella bacteria may be able to induce apoptosis or programmed cell death in the host, documented for a number of intracellular bacterial pathogens (Chen and Zychlinsky, 1994). Muller *et al* (1996) were the first to report that *L. pneumophila* could induce apoptosis in the human macrophage like cell line HL-60. Infected cells show DNA fragmentation, chromatin condensation and segmentation of the nucleus which are characteristic of apoptosis (Salvesen and Dixit, 1998). This phenomenon has been observed only for viable organisms. Cell cytotoxity of *L. pneumophila* is mediated in part by apoptosis although whether it activates the suicide program in the host cell or produces a factor that is able to bypass this normal pathway is not known (Muller, *et al.*, 1996). Apoptotic death has also been observed with *L. pneumophila* in human monocytes and is dependent on multiplicity of infection, incubation time and intracellular location of the bacteria (Hagele, *et al.*, 1998). *L. pneumophila* must be intracellular in order to induce apoptosis efficiently. *L. pneumophila* does not induce programmed cell death in *Acanthamoeba castellanii* therefore, different mechanisms are probably responsible for host cell killing in protozoan and mammalian cells (Hagele, *et al.*, 1998). Failure to detect apoptosis in amoebae may be due to the inability of *L. pneumophila* to induce it in this host, presence of a different killing mechanism or because amoebae do not possess the genetic programme for cell death.

Cytotoxic activity of *L. pneumophila* in the absence of intracellular uptake and replication is also observed suggesting that lung lesions occurring as a result of infection with

L. pneumophila may not be due to intracellular infection (Husmann and Johnson, 1994). *L. pneumophila* opsonised with specific antibody and incubated with guinea pig peritoneal macrophages for up to 6 hours at a bacterium-to-macrophage ratio of 1000:1 destroy the majority of host cells with few bacteria observed internally. Extracellular *L. pneumophila* are capable of mediating cytotoxicity but this requires close proximity of the organism with the mononuclear cell surface suggesting that an intracellular environment is not required to induce cytotoxic activity. Cytochalasin D, which blocks phagocytosis, has no effect on the degree of cytotoxicity observed for both untreated and treated cells, however, cytotoxicity is enhanced in both cases with the addition of specific antibody. Therefore, the toxic activity shown by *L. pneumophila* appears to require close contact with the macrophage surface. No specific toxin has been identified (Husmann and Johnson, 1994).

Further work by Kirby *et al* (1998) has demonstrated that cytotoxicity of *L. pneumophila* for macrophages and red blood cells at high multiplicity of infection and close association with the host cell occurrs rapidly (1 hour) with cell death due to osmotic lysis. This is probably the same cytotoxic activity described by Husmann *et al* (1994) and is mediated by a pore-forming toxin. At low multiplicities of infection, host cell killing occurs by apoptosis. It has been proposed that cell death due to *L. pneumophila* may be biphasic with rapid necrotic death occurring when bacterial numbers are high followed by apoptopic death with low multiplicity of infection. Low levels of infecting bacteria would favour the outcome of disease for the organism and is consistent with the numbers of bacteria that are likely to infect amoebae or humans in the natural state.

Apoptosis is induced in *L. pneumophila* infected mammalian host cells at low multiplicities of infection within 2-3 hours of infection (Gao and Abu Kwaik, 1999b). This precedes intracellular replication of the bacteria as mutants, defective for intracellular replication in macrophages and amoebae (Gao, *et al.*, 1997), induce host cell killing. *L. pneumophila* is able to induce apoptosis in alveolar epithelial also, although its significance is unknown. Gao and Abu Kwaik (1999b) proposed that when numbers of bacteria are low,

apoptosis is induced in the host cell leading to eventual release of large numbers of bacteria into the surrounding tissues, thereby inducing necrotic cell death. This is consistent with the model proposed by Kirby *et al.*(1998) and is also supported by the studies showing that *L. pneumophila* becomes cytotoxic due to necrosis when high numbers of bacteria are present in the late stage of infection (Byrne and Swanson, 1998). It is likely that this second phase of induced necrotic host cell death is mediated by a toxin of the type mentioned previously (Husmann and Johnson, 1994, Kirby, *et al.*, 1998).

L. pneumophila induces apoptosis in mammalian cells by activating the caspase cascade (Gao and Abu Kwaik, 1999a, Gao and Abu Kwaik, 1999b). Caspases are a family of cysteine proteases that specifically cleave proteins after aspartate residues, and are required for induction of apoptosis (Salvesen and Dixit, 1998). Eleven caspase proteins have been identified with caspase 3 playing a central role in the apoptopic induction pathway, and *L. pneumophila* induced cell death is mediated through activation of this protein (Gao and Abu Kwaik, 1999a).

Contact mediated export of a bacterial factor/s resulting in the activation of this cascade leading to apoptopic death has been proposed (Gao and Abu Kwaik, 1999b). This factor/s may directly interact with the caspase cascade similar to the apoptosis induction mechanism described for *Shigella flexneri* (Hagele, *et al.*, 1998). Apoptosis induced prior to intracellular multiplication may be required for alteration of the host endocytic pathway and formation of replicative phagosome (Gao and Abu Kwaik, 1999b). Induction of apoptosis may play an important role in subsequent release of intracellular bacteria from the host cell (Gao and Abu Kwaik, 1999a). The role of a putative cytotoxin in *L. pneumophila* has not been determined. However, it may mediate escape from the host cell as cytotoxicity is associated with stationary phase of growth (Byrne and Swanson, 1998). Alternatively, it may also be required during uptake of the organism to direct formation of the novel phagosome. Contact dependent cytotoxicity, a stationary phase activity, is thought to be important for *L. pneumophila* to establish and/or escape from, its replicative phagosome within amoebae and

macrophages (Byrne and Swanson, 1998, Husmann and Johnson, 1994, Kirby and Isberg, 1998, Kirby, *et al.*, 1998).

1.11 Genetic studies of *Legionella*

The establishment of reliable molecular genetic techniques is important for detecting and analysing factors in *Legionella* that play a role in pathogenesis, epidemiology, diagnosis and prevention of disease, and in vaccine development.

Comparison of spontaneously occuring avirulent strains of *Legionella* with parent strains has been one method used to understand pathogenesis of this organism (Hacker, *et al.*, 1993). *Legionella* can spontaneously convert to avirulent status at a relatively high frequency (Catrenich and Johnson, 1988). Avirulent strains can differ from the parent strain in their colonial morphology (Nowicki, *et al.*, 1987), serum resistance (Caparon and Johnson, 1988) and ability to replicate in macrophages and protozoans (Hacker, *et al.*, 1993).

Genetic methods have also been attempted to clone and characterise putative virulence factors of *Legionella* such as screening *L. pneumophila* genomic libraries with immune serum to identify antigens (Engleberg, *et al.*, 1984a, Engleberg, *et al.*, 1984b), complementation of defined *E. coli* mutations (Dreyfus, 1989) and cloning *L. pneumophila* genes that can complement mutants (Marra and Shuman, 1992).

Strategies for gene transfer and genetic exchange in *Legionella* are limited. Bacteriophages and transduction have not been reported for *Legionella* (Mintz and Shuman, 1988). Conjugation has been demonstrated for *L. pneumophila* and wide host range plasmids from incompatability groups IncP, IncW, IncC, IncN and IncQ have been transferred by conjugation from *E. coli* to *L. pneumophila* (Chen, *et al.*, 1984, Dreyfus and Iglewski, 1985, Keen, *et al.*, 1985, Marra and Shuman, 1989, Mintz and Shuman, 1987, Tully, *et al.*, 1992a). Restriction enzymes (*Lpn*I, *Lpn*II) present in some strains of *L. pneumophila* limit the conjugal transfer of foreign plasmid DNA into *L. pneumophila* (Chen, *et al.*, 1986). Restriction deficient mutants that have helped overcome poor mating frequencies have been

identified (Cianciotto, *et al.*, 1988, Marra and Shuman, 1989, Marra and Shuman, 1992, Ott, 1994). Most wide host range plasmids are stably maintained in *L. pneumophila* without selection and can be mated back into *E. coli* or other strains of *L. pneumophila* (Chen, *et al.*, 1984, Dreyfus and Iglewski, 1985, Keen, *et al.*, 1985, Marra and Shuman, 1989). Wide host range plasmids can also be transferred into other species of *Legionella* including *L. micdadei* and *L. bozemanii* (Chen, *et al.*, 1984).

Transformation of *Legionella* using traditional methods has not been documented (Cianciotto, *et al.*, 1989a), although a recent report demonstrated natural competence of *L. pneumophila* for transformation of *L. pneumophila* chromosomal DNA or plasmid DNA containing *L. pneumophila* DNA and this correlated with expression of type IV pili (Stone and Abu Kwaik, 1999). Electroporation, however, has been used successfully to introduce plasmid DNA into *Legionella* (Cianciotto and Fields, 1992, Doyle, *et al.*, 1998, Marra and Shuman, 1992, Ott, 1994).

Although gene delivery methods are now well established for *Legionella,* identification of suitable vectors for use in genetic exchange has been hampered by vector size, copy number, incompatibility group, multiple drug resistance and variable antibiotic expression in *Legionella* (Dreyfus and Iglewski, 1985, Mintz and Shuman, 1988, Wiater, *et al.*, 1994a). Kanamycin, chloramphenicol, and gentamycin have been used successfully as selective agents although concentrations can be critical due to the level of expression in the strain of interest (Marra, *et al.*, 1992, Mintz and Shuman, 1988, Ott, 1994). Ampicillin resistance in *L. pneumophila* is variable but success with this antibiotic as a selective marker has been achieved (Jain, *et al.*, 1992). Tetracycline is poorly expressed in *Legionella* (Mintz and Shuman, 1988).

Several vectors have been developed and used successfully, including mobilizable *pir*-dependent plasmids from the incX group, unable to replicate in *L. pneumophila* due to lack of the Pir protein (Szeto and Shuman, 1990), and unstable narrow host range plasmids such as ColE1 (Cianciotto, *et al.*, 1989b, Engleberg, *et al.*, 1988, Ott, 1994). A potential system for

gene delivery into *L. pneumophila* using the F plasmid of *E. coli* has also been developed (Wiater, *et al.*, 1994a). The F plasmid can be introduced into *L. pneumophila* by conjugation and since it is only maintained with selection and is devoid of antibiotic markers, it is a suitable vector to mediate allelic exchange or donate transposons like the ColE1 system. Site directed allelic exchange has now been used successfully by many workers to generate isogenic mutants in many *Legionella* genes of interest (Cianciotto, *et al.*, 1989b, Cianciotto, *et al.*, 1988, Doyle, *et al.*, 1998, Ott, 1994, Szeto and Shuman, 1990, Wintermeyer, *et al.*, 1994).

Transposon and chemical mutagenesis have also been used to isolate avirulent mutants and hence identify genes involved in pathogenesis (Cianciotto, *et al.*, 1989a). Random chemical mutagenesis has been used successfully by several workers to generate mutants (Andrews, *et al.*, 1998, Berger and Isberg, 1993, Mintz, *et al.*, 1988). Several transposons have been delivered into *L. pneumophila,* however, the generation of mutants is dependent on the transposon used (Cianciotto, *et al.*, 1989a, Engleberg and Eisenstein, 1991, Keen, *et al.*, 1985, Marra and Shuman, 1992, Mintz and Shuman, 1987, Pope, *et al.*, 1994). Transposon, Tn5, has a low transposition frequency in *L. pneumophila* (Keen, *et al.*, 1985, Marra and Shuman, 1992). The plasmid pSUP1021 was used to generate Tn5 insertions in *L. pneumophila* and workers identified three mutants with reduced ability to multiply in macrophages and cause disease (Tully, *et al.*, 1992a, Tully, *et al.*, 1992b). Transposon Tn*phoA* has been used to mutagenise *L. pneumophila* (Albano, *et al.*, 1992, Arroyo, *et al.*, 1994, Cianciotto, *et al.*, 1989a, Engleberg and Eisenstein, 1991). The use of *phoA* containing transposons has allowed screening for mutations occurring in genes encoding secreted or membrane proteins. A Tn903 derivative carrying a truncated *lacZ* gene, Tn903dII*lacZ*, has been shown to create translational gene fusions in *L. pneumophila* and mutants have been isolated that have a reduced ability to kill HL-60 cells (Marra and Shuman, 1992, Sadosky, *et al.*, 1993).

Physical mapping using pulse field electrophoresis has allowed size determination of the *L. pneumophila* genome (Cianciotto, *et al.*, 1989a) which is estimated to be approximately

4 MD in size (Bender, *et al.*, 1990). However, a genetic map of the genome of *L. pneumophila* is currently unavailable (Cianciotto, *et al.*, 1989a, Ott, 1994). Genetic markers, such as auxotrophic growth requirements, have been difficult to obtain in *Legionella* (Mintz and Shuman, 1988, Mintz, *et al.*, 1988,) and attempts to mobilise the genome of *L. pneumophila* have been largely unsuccessful as the organism lacks an endogenous chromosome transfer system (Dreyfus and Iglewski, 1985, Mintz and Shuman, 1988).

1.12 Virulence factors

1.12.1 Lipopolysacharide (LPS)

The LPS of *Legionella* is the dominant serogroup specific antigen and the main antigen responsible for the reactivity of patient serum in fluorescent antibody assays (Conlan and Ashworth, 1986, Dowling, *et al.*, 1992). Up to 98% of the antigens present in the sera of patients with past evidence of infection are directed against the LPS (Gabay and Horwitz, 1985). The LPS is serogroup and species specific and is determined by the O-antigen (Conlan and Ashworth, 1986, Nolte, *et al.*, 1986). *L. pneumophila* LPS is smooth with an atypical pattern compared with other gram-negative bacilli (Dowling, *et al.*, 1992, Gabay and Horwitz, 1985, Nolte, *et al.*, 1986) and also, atypically, only weak pyrogenicity in rabbits and low toxicity in mice (Wong, *et al.*, 1979). *Legionella* LPS is atypical as it consists of branched chain fatty acids while the hydroxy fatty acids generally associated with classical LPS in other bacteria are not present or are at very low levels (Mayberry, 1981, Moss, *et al.*, 1977). *Legionella* species possess an individual and characteristic banding pattern unique for each species (Jurgens and Fehrenbach, 1997).

The role of LPS in the pathogenesis of *Legionella* is uncertain. It is likely that it participates in a number of essential capacities such as adaptation to various environmental conditions due to its unique chemical structure (Zähringer, *et al.*, 1995). *L. pneumophila* has a hydrophobic cell surface that may support concentration of the bacterium in aerosols as well as adherence to host cells (Lüneberg, *et al.*, 1998, Zähringer, *et al.*, 1995). LPS of *L.*

pneumophila has also been shown to interact with the complement system suggesting a role in uptake of the bacterium by mononuclear phagocytes (Mintz, *et al.*, 1992b). LPS profiles of virulent and avirulent *L. pneumophila* strains, however, are very similar suggesting that it is unlikely to have a significant effect on virulence (Conlan and Ashworth, 1986).

A mutant strain of *L. pneumophila,* unable to react with a monoclonal antibody specific for wild type LPS, was unaltered in its ability to multiply in U937 cells (Mintz and Zou, 1992). In contrast, a naturally occurring LPS mutant unable to bind a wild type specific monoclonal antibody has reduced ability to cause disease in guinea pigs and to replicate intracellularly in HL-60 cells (Lüneberg, *et al.*, 1998). The mutant has an unstable phenotype *in vivo,* reverting to wild type LPS, indicating a selective advantage of the wild type phenotype over the mutant one.

Identification of the genes involved in LPS biosynthesis will facilitate construction of defined genetic mutants to assess the role of LPS in *Legionella* pathogenesis. Recently a locus responsible for O-acetylation of the O-polysaccharide of *L. pneumophila* has been cloned and characterised (Zou, *et al.*, 1999).

1.12.2 Lipoprotein

Two groups have independently cloned and sequenced the gene encoding a 19kDa lipoprotein from *L. pneumophila* (Engleberg, *et al.*, 1991, Ludwig, *et al.*, 1991). The protein, termed PplA or PAL, is a highly immunogenic outer membrane protein associated with the peptidoglycan layer of the cell wall (Hindahl and Iglewski, 1987). A 19 kDa antigenic protein has also been reported for *L. micdadei* (Bangsborg, 1997).

The lipoprotein has homology to the peptidoglycan associated Pal lipoproteins of *Escherichia coli* K-12 (59.6% similarity) and *Haemophilus influenzae* (60.8% similarity) (Engleberg, *et al.*, 1991, Ludwig, *et al.*, 1991). It also has a ß-turn structure and signal sequence cleavage site specific for signal peptidase II, features shared by other bacterial lipoproteins (Ludwig, *et al.*, 1991). The protein is distinct from other bacterial lipoproteins,

as determined by Southern hybridisation and Western blot, but is conserved in *Legionella*, although not all species appear to express the protein (Engleberg, *et al.*, 1986, Ott, *et al.*, 1991a).

The role of the lipoprotein in pathogenesis of *Legionella* is unknown and no isogenic mutant has been constructed. The protein may play a role in the ability of *Legionella* to inhibit phagosome-lysosome fusion (Ludwig, *et al.*, 1991). The lipoprotein may play a role in modulating the immune response of the host as it is highly immunogenic, or it may play a role in serum resistance (Engleberg, *et al.*, 1991). The Braun lipoprotein of *E. coli* is known to be a B-cell mitogen (Melchers, *et al.*, 1975). Recently it has been reported that lipoprotein can be released from growing or lysed bacteria of the *Enterobacteriaceae* and that this may play a role in pathogenesis (Zhang, *et al.*, 1998).

1.12.3 Major Outer Membrane Protein (MOMP)

The most prominent feature common to the protein profiles of *L. pneumophila* strains is the major outer membrane protein (MOMP) (Butler, *et al.*, 1985, Ehret and Ruckdeschel, 1983, Hindahl and Iglewski, 1986, Hoffman, *et al.*, 1992b). MOMP is associated with the peptidoglycan and has a molecular mass ranging between 24 and 29 kDa (Butler, *et al.*, 1985, Gabay, *et al.*, 1985, Hindahl and Iglewski, 1984, Hindahl and Iglewski, 1986). MOMP appears to be specific to strains of *L. pneumophila* (Ehret and Ruckdeschel, 1983, Hindahl and Iglewski, 1986). Butler *et al* (1985) showed immunological detection of MOMP in all but one species of *Legionella* although this finding has not been confirmed independently. A specific monoclonal antibody (Genetic Systems Corp., Seattle, Wash.) used diagnostically for the detection of *L. pneumophila* strains recognises an epitope on MOMP, further suggesting that this protein is specific to *L. pneumophila* (Nolte and Conlin, 1986).

MOMP is a large aggregate stabilised by disulphide bonds composed of 28kDa and 31kDa subunits anchored to the cell wall by the 31kDa subunit (Butler and Hoffman, 1990, Butler, *et al.*, 1985, Gabay, *et al.*, 1985, Hindahl and Iglewski, 1984, Hoffman, *et al.*, 1992b).

The 31kDa subunit has been confirmed as a modified 28kDa subunit bound to a fragment of peptidoglycan, and a single gene encodes both sub-units (Hoffman, *et al.*, 1992b). The structural gene for both subunits, *ompS,* encodes a protein of 297 amino acids that is rich in glycine and aromatic amino acids, and contains four cysteine residues (Hoffman, *et al.*, 1992a). The current model for MOMP is that of an aggregate structure covalently attached to the peptidoglycan via a modified 28kDa subunit (31kDa anchor protein) cross linked to other 28kDa subunits by interchain disulphide bonds (Hoffman, *et al.*, 1992b).

MOMP binds complement component C3 (Bellinger-Kawahara and Horwitz, 1990) thus mediating phagocytosis of *L. pneumophila* via the human monocyte complement receptors CR1 and CR2 (Payne and Horwitz, 1987) suggesting an important role in pathogenesis. MOMP may even mediate uptake into He-La cells directly (Quinn, *et al.*, 1987). MOMP is an important antigen associated with the development of protective immunity in guinea pigs (Blander and Horwitz, 1991, Quinn, *et al.*, 1987, Weeratna, *et al.*, 1994). MOMP is also a cation selective porin with properties similar to *E. coli* porins (Gabay, *et al.*, 1985). However, *L. pneumophila* MOMP has disulphide bonds which are not a common feature of outer membrane proteins of other gram-negative bacteria. The disulphide bonds may play a role in influencing pore size hence effecting tolerance of *L. pneumophila* to sodium ions, hydrophobicity and sensitivity to reducing agents (Hoffman, *et al.*, 1992b). A MOMP mutant has not been generated at this time and may be a lethal mutation.

High *et al* (1993) have cloned in *E. coli* a gene *ompM* encoding a surface protein of *L. pneumophila* with properties similar to MOMP. DNA sequencing revealed that the protein cloned was distinct from *ompS* encoding MOMP. The protein was cysteine rich with a signal sequence and a deduced molecular mass of approx. 25kDa. The *E. coli* clone expressing this MOMP showed enhanced virulence in a fertile chicken egg assay by comparison to the parent *E. coli* strain (High, *et al.*, 1993) and in the absence of opsonins was five times more adherent to U937 cells than the parent *E. coli* strain (Krinos, *et al.*, 1999). The results suggest that the

25 kDa MOMP of *L. pneumophila* may be a ligand for attachment to host cells and may have a role in virulence of the organism. An isogenic mutant has not been constructed.

1.12.4 Macrophage infectivity potentiator (Mip) protein

Mip was the first virulence factor described for the genus *Legionella* (Cianciotto, *et al.*, 1989b). The 24kDa protein was originally targeted for analysis as it was highly immunogenic and surface located (Cianciotto, *et al.*, 1989b, Engleberg, *et al.*, 1984a, Engleberg, *et al.*, 1984b, Pearlman, *et al.*, 1985). An isogenic *mip* mutant, constructed by allelic exchange, was shown to require an 80-fold greater inoculum to initiate infection of U937 cells and explanted alveolar macrophages compared with the parent strain (Cianciotto, *et al.*, 1989b). The mutant was impaired in its ability to initiate infection, as the growth rate after uptake was similar to the parent strain once infection was established. The Mip protein was not simply a ligand involved in uptake as opsonisation studies determined that the mutant associated equally as well with the cells as did the parent. When inoculated into guinea pigs, the *mip* mutant was attenuated by comparison with the parent strain, with slower onset of disease and reduced recovery from the lungs (Cianciotto, *et al.*, 1990b). The Mip protein is important for the early events in infection of protozoan hosts (Cianciotto and Fields, 1992), and is involved in the invasion of alveolar epithelial type I and type II cells (Cianciotto, *et al.*, 1995a). The *mip* gene is observed in all *Legionella* species known to date (Bangsborg, *et al.*, 1991, Cianciotto, *et al.*, 1990a, Doyle, *et al.*, 1998, Ratcliff, *et al.*, 1998) with protein expression determined for the majority of species (Cianciotto, *et al.*, 1990a).

L. pneumophila Mip is a highly basic protein of 233 amino acids with a pI of 9.8. The protein has a typical signal sequence followed by a hydrophilic amino terminal region consisting of alpha helixes and a carboxy terminal region consisting of beta sheets (Engleberg, *et al.*, 1989). Mip has homology with members of a family of immunophilins that bind the immunosuppressive drug FK506, known as FK506 binding proteins (FKBPs) (Bangsborg, *et al.*, 1991) that are house keeping proteins present in a wide variety of

organisms (Hacker and Fischer, 1993, Schreiber, 1991). Immunophilins possess peptidyl prolyl *cis-trans* isomerase (PPIase) activity enabling them to accelerate the rate of conformational events in proline containing polypeptides (Hacker and Fischer, 1993). Thus they may have an active role in protein folding, membrane channelling and protein trafficking. The Mip protein of *L. pneumophila* (Philadelphia) possesses PPIase activity (Fischer, *et al.*, 1992). It is not known if all Mip proteins have PPIase activity.

Cloning of *mip* from *L. pneumophila* (Engleberg, *et al.*, 1989, Fischer, *et al.*, 1992, Ludwig, *et al.*, 1994), *L. longbeachae* (Doyle, *et al.*, 1998) and *L. micdadei* (Bangsborg, *et al.*, 1991) and characterisation of the protein for each species has revealed a high degree of conservation of the structural features of Mip. This includes predicted secondary structures and conservation of the amino acid residues critical for PPIase activity (Hacker and Fischer, 1993, Ludwig, *et al.*, 1994), confirmed among the majority of species by the work of Ratcliff *et al* (1997).

Native *L. pneumophila* Mip exists in a dimeric state on the surface of the bacterium (Schmidt, *et al.*, 1994). The dimeric state consists of a C-terminal domain homologous with the FKBP family of proteins (Fischer, *et al.*, 1992, Harrison and Stein, 1990), and an N-terminal domain containing a long alpha helix with no homology to any known protein. It is proposed that dimerization involves the N-terminal domain for each monomer.

The exact structure and function of Mip is still unclear. It may function as a surface poly-cation, forming an elongated structure projecting from the cell surface due to the alpha helix region, thereby enhancing phagocytosis or preventing phagosome fusion or acidification (Engleberg, *et al.*, 1989). This may allow the C-terminus, containing the PPIase activity, to interact with a target membrane structure/s or putative partner molecule/s (Bangsborg, *et al.*, 1991, Hacker and Fischer, 1993). It could therefore modify bacterial cell structures or surface proteins of phagocytic cells, enhancing phagocytosis or possibly effecting some role in intracellular survival once inside the phagocytic cell.

Mip appears to be important in *Legionella* and in initiating infection by other pathogenic species as *mip* mutant strains all exhibit similar defects (Cianciotto and Fields, 1992, Doyle, *et al.*, 1998, O'Connell, *et al.*, 1995). Mip-like proteins have been detected in other intracellular pathogens suggesting that the protein may be important in intracellular lifestyle. *Chlamydia trachomatis,* an obligate intracellular pathogen has a Mip-like protein important in initiation of productive infection that also has significant homology with FKBPs and has been shown to have PPIase activity (Lundemose, *et al.*, 1991, Lundemose, *et al.*, 1992, Lundemose, *et al.*, 1993,). Mip-like proteins are highly conserved in other *Chlamydia* serovars suggesting that it may have an important role in the life cycle of the organism. *Coxiella burnetii* an intracellular pathogen of alveolar macrophages expresses a Mip-like protein highly homologous with the Mip from *Legionella* and *Chlamydia* and with the FKBP family of immunophilins and has PPIase activity when overproduced in *E. coli* (Mo, *et al.*, 1995). Mip-related proteins and *mip*-like sequences have also been detected within the *Rickettsiaceae* family (Cianciotto, *et al.*, 1995b). FKBPs have also been described for enteric organisms (Horne and Young, 1995). The FkpA protein in *E. coli* is homologous to the Mip protein of *Legionella* and *C. trachomatis* but does not appear essential for laboratory growth of the organism although it may have a role in the intracellular lifecycle of some members of *E. coli* and in the *Enterobacteriaceae.* A protein with homology to the FkpA protein and the Mip protein of *Legionella* has also been described in *Aeromonas* (Wong, *et al.*, 1997). However, it does not appear to be essential for virulence, as an isogenic mutant was not attenuated for virulence in a suckling mouse model. An *fpkA* gene has been identified in *Salmonella typhimurium* that encodes a protein with homology to Mip from *Legionella* and *Chlamydia* and appears important for intracellular survival of the organism (Horne, *et al.*, 1997).

Mip may play an important role in a process not strictly involved in pathogenesis. It may have an important housekeeping function consistent with its homology to FKBPs as the gene is conserved in both pathogenic and non-pathogenic species of *Legionella* (Ratcliff, *et*

al., 1997). However, PPIase activity does not contribute to intracellular survival of *L. pneumophila*, as a site specific altered Mip protein, with reduced levels of PPIase activity, can still support intracellular survival (Wintermeyer, *et al.*, 1995).

Recently *L. pneumophila* has been shown to possess additional PPIase activity due to a cytoplasmic protein that belongs to the cyclophillin members of the immunophilin family of proteins (Schmidt, *et al.*, 1996). The gene *lcy* (*Legionella* cyclophilin) has been cloned and sequenced, and encodes a protein of 17.9kDa with strong homology with the cyclophilins of *E. coli*. The *lcy* negative mutant constructed in *L. pneumophila* was shown to be 10-fold less infective for *Acanthamoeba castellanii* suggesting that these proteins may have an important role in *Legionella*. Few cyclophilins have been described for bacteria and their role is unknown.

1.12.5 Heat shock proteins

The 60kDa heat shock protein is the best characterised of five heat shock proteins identified in *L. pneumophila* (Lema, *et al.*, 1988). The protein cross reacts with polyclonal antisera to the GroEL heat shock protein of *E. coli* (Lema, *et al.*, 1988) and has been detected in all *Legionella* species (Helsel, *et al.*, 1988, Steinmetz, *et al.*, 1991). It is highly immunogenic and is the predominant *Legionella* protein reactive with human convalescent sera from confirmed cases (Sampson, *et al.*, 1986).

The gene encoding the protein has been cloned and a DNA probe generated hybridises with chromosomal DNA from all *Legionella* species tested, suggesting a high degree of conservation (Hoffman, *et al.*, 1989). Maxicell analysis of the clone has determined that two polypeptides are translated, 15kDa and 60kDa in size, products of the *htpA* and *htpB* genes respectively. Independent cloning of *htpB* by Sampson *et al.*(1990) suggests that the size of the protein encoded by *htpB* is 58kDa. HtpA and HtpB are expressed from an operon similar to *E. coli GroE*, with a consensus heat shock promoter sequence found upstream of *htpA* (Hoffman, *et al.*, 1990). HtpB is highly homologous with the 65kDa protein antigen of *M.*

tuberculosis (76%), GroEL from *E. coli* (85%) and HtpB from *C. burnetti* (85%) and cross reacts immunologically with the *Mycobacterium* antigen and *E. coli* GroEL. Indirect fluorescent antibody studies indicate that the 60kDa protein may be located in the periplasm or cell surface of intracellular bacteria suggesting a stress related mechanism may be involved in the expression of this protein (Hoffman, *et al.*, 1990, Steinmetz, *et al.*, 1991). Expression of *htpB* in *Legionella* is regulated by thermal stress in a transient manner, indicative of heat shock genes, and requires a sigma-32 factor or a factor whose synthesis is dependent on sigma-32 (Hoffman, *et al.*, 1989, Hoffman, *et al.*, 1990) .

The 60-kDa protein is important in the development of cellular immunity and is antigenic for T-lymphocytes (Hoffman, *et al.*, 1990). However, Weeratna *et al* (1994) found that it was not immunoprotective in guinea pigs in contrast to Blander and Horwitz (1993), who demonstrated protective immunity in guinea pigs vaccinated on three occasions with the 60kDa protein prior to lethal challenge.

The role of heat shock proteins in the pathogenesis of *Legionella* is unclear and there is no evidence to establish them as virulence factors, although their expression may be coordinate with genes associated with virulence (Buchmeier and Heffron, 1990). They may play a role upon release from the cell after interaction with the host phagosome, and may play a role in prevention of phagolysosome fusion (Hoffman, *et al.*, 1990). The heat shock protein may form a complex tertiary structure like the GroEL protein and that it may play a role in attachment of proteins for membrane associated processes such as DNA synthesis (Lema, *et al.*, 1988). The heat shock protein may function as a molecular chaperone playing a role in folding and export of proteins (Ellis, 1987, Hemmingsen, *et al.*, 1988). Recently it has been shown that the surface associated HtpB promotes attachment and invasion of *L. pneumophila* in a HeLa cell model (Garduno, *et al.*, 1998, Hoffman and Garduno, 1999) and that elevated levels of HtpB early in infection of human monocytes and L929 cells correlates with virulence (Fernandez, *et al.*, 1996).

1.12.6 Legiolysin and pigment production

Legiolysin (Lly) is a 39kDa protein that confers haemolysis, fluorescence and the phenotypic characteristic of browning of culture medium in *L. pneumophila* (Wintermeyer, *et al.*, 1991). The gene for legiolysin, *lly,* has been cloned (Rdest, *et al.*, 1991, Wintermeyer, *et al.*, 1994). The Lly protein has strong homology with the MelA protein of *Shewanella colwelliana,* involved in melanin synthesis, and also with the enzyme 4-hydroxyphenylpyruvate dioxygenase of a *Pseudomonas* spp. involved in degradation of aromatic amino acids (Wintermeyer, *et al.*, 1994). The *lly* negative mutant was unaffected for survival in *A. castellanii* and U937 cells (Wintermeyer, *et al.*, 1994) and for replication in *H. vermiformis* (Steinert, *et al.*, 1995) indicating that the protein is not required for multiplication in these cell types. However, the mutant strain showed decreased survival, in comparison with the parent strain, when exposed to light (halogen lamp: 12V, 75W) suggesting that the Lly associated pigment production appears important for the survival of *L. pneumophila* stressed by light (Steinert, *et al.*, 1995). The protein may have a role in metabolism of *L. pneumophila* and in ecological adaptation of the organism (Wintermeyer, *et al.*, 1994).

Southern hybridisation has determined that a *lly* gene is present in all *Legionella* species although anti-legiolysin monospecific antibodies only cross react with *L. pneumophila* in Western blots (Bender, *et al.*, 1991). This suggests that the phenotype of browning of culture medium, observed for many species of *Legionella* may not be associated with Legiolysin (Bender, *et al.*, 1991, Vickers and Yu, 1984). Recently a growth phase regulated pigment gene has been identified in *L. pneumophila* (Wiater, *et al.*, 1994b). The pigment gene, *pig*, is upregulated during intracellular growth in macrophages and a *pig* mutant does not produce the brown pigment characteristic of *Legionella.* It is thought that the *pig* gene is a separate locus from *lly* as restriction analysis of the cloned DNA shows no similarity. Sequencing of the *pig* gene will clarify this issue.

The role that pigment plays in the biology of *L. pneumophila* is unknown, although for some organisms it has a protective effect against oxidative damage. A *pig* mutant, however,

shows no increased sensitivity to oxidizing compounds compared with wild type *L. pneumophila* (Wiater, *et al.*, 1994b). The mutant is still able to multiply in macrophages suggesting that the pigment may not be required for virulence and therefore may play a protective role for the organism in the environment. The *rpoS* sigma factor is not required for stationary phase induction of the brown pigment (Hales and Shuman, 1999b).

1.12.7 Oxygen scavenging enzymes

Superoxide dismutase (SOD) is observed in all *Legionella* while catalase and peroxidase activity is variable (Dowling, *et al.*, 1992, Pine, *et al.*, 1984). The variable distribution of these enzymes suggests that they are unlikely to play a role in pathogenesis. However, *L. pneumophila* and *L. micdadei* are susceptible to oxygen-dependent microbiocidal systems (Dowling, *et al.*, 1992, Jepras and Fitzgeorge, 1986, Lochner, *et al.*, 1983, Locksley, *et al.*, 1982, Rechnitzer, 1994). Therefore, suppression of the respiratory burst of phagocytes may contribute to the intracellular survival of *Legionella*. Sonic extracts of *L. pneumophila*, in low concentration stimulate neutrophils and monocytes to produce toxic oxygen radicals while high concentrations suppress the oxidative burst of both types of cells (Rechnitzer, 1994, Rechnitzer, *et al.*, 1987).

L. pneumophila contains two bi-functional catalase-peroxidase enzymes KatA and KatB (Bandyopadhyay and Steinman, 1998) which is in contrast to a previous report of mono-functional catalases or peroxidases for the species (Pine, *et al.*, 1984). The *katB* gene of *L. pneumophila* has been cloned and sequenced and the inferred protein is highly homologous to other bacterial catalase-peroxidases (Bandyopadhyay and Steinman, 1998). KatB is also functionally similar to the *E. coli* KatG catalase-peroxidase as it is induced during exponential growth and appears to play a role in resistance to H_2O_2 (Bandyopadhyay and Steinman, 1998). A mutation generated in *katB* did not affect exponential growth and stationary phase survival, however, the mutant was delayed in infection and lysis of macrophage-like cell lines. Induction of peroxidase activity that occurs in stationary phase is

attributable to KatA, and increases of peroxidase activity due to this protein may be a useful marker for the stationary-phase gene expression that precedes induction of virulence in *L. pneumophila* (Byrne and Swanson, 1998).

L. pneumophila has two SODs, a cytosolic iron superoxide dismutase, SodB, and a periplasmic copper-zinc superoxide dismutase, SodC (Sadosky, *et al.*, 1994, St. John and Steinman, 1996, Steinman, 1992). Mutations generated in the genes encoding these proteins have determined that *sodB* is essential for viability of *L. pneumophila* (Sadosky, *et al.*, 1994) while *sodC* is important for stationary phase growth of *L. pneumophila* but not essential for growth within and the killing of macrophages (St. John and Steinman, 1996). The role of these enzymes in pathogenesis is unknown but they may play a role in stationary phase or in antioxidant defence against the phagosome by decomposing superoxide generated by phagocytic cells.

1.12.8 Plasmid encoded virulence genes

The first report of a plasmid in *Legionella* was a cryptic plasmid, of approx. 30MDa, detected in two of sixteen strains of *L. pneumophila* representing six serogroups (Knudson and Mikesell, 1980). Cryptic plasmids have been identified in other strains and serogroups of *L. pneumophila,* in other *Legionella* species (*L. bozemanii, L. dumoffii, L. micdadei, L. gormanii* and *L. longbeachae* and in some *Legionella*-like unclassified organisms (Aye, *et al.*, 1981, Brown, *et al.*, 1982, Edelstein, *et al.*, 1986, Johnson and Schalla, 1982, Knudson and Mikesell, 1980, Maher, *et al.*, 1983, Mikesell, *et al.*, 1981, Nolte, *et al.*, 1984, Tompkins, *et al.*, 1987). Plasmids observed in strains of *L. pneumophila* have been found in both clinical and environmental isolates (Johnson and Schalla, 1982, Knudson and Mikesell, 1980, Maher, *et al.*, 1983, Mikesell, *et al.*, 1981). Clinical isolates generally lack plasmids while a high proportion of environmental isolates appear to have plasmids (Brown, *et al.*, 1982, Maher, *et al.*, 1983). *L. pneumophila* strains with plasmids appear to persist in the environment longer than strains lacking plasmids (Brown, *et al.*, 1982). Similar plasmids have been detected in

strains from very different geographical locations suggesting that plasmids in *Legionella* may be self-transmissible by conjugation (Johnson and Schalla, 1982, Maher, *et al.*, 1987).

The cryptic plasmids detected in *Legionella* are generally stable upon passage on laboratory media or in animals (Brown, *et al.*, 1982, Edelstein, *et al.*, 1986, Maher, *et al.*, 1987). They have not been demonstrated to encode a particular function and plasmid content has been used predominantly as a tool in epidemiological investigations of legionellosis (Brown, *et al.*, 1982, Cianciotto, *et al.*, 1989a, Edelstein, *et al.*, 1986, Maher, *et al.*, 1983, Maher, *et al.*, 1987, Nolte, *et al.*, 1984). Plasmid typing methods are generally used in conjunction with other established typing techniques (Edelstein, *et al.*, 1986).

Tully *et al* (1991) were the first to report a potential role of a 36MDa plasmid in a strain of *L. pneumophila*. The plasmid conferred resistance to low levels of short wave UV light, possibly by means of an error prone UV repair system, suggesting that it may be important for survival of the organism in natural waters. The plasmid was self-transmissible to other strains of *L. pneumophila* confirming earlier reports of potential conjugal plasmid transfer in *Legionella* (Johnson and Schalla, 1982, Maher, *et al.*, 1987). An 85MDa plasmid from a strain of *L. pneumophila* was also self transmissible to other strains of *L. pneumophila* but did not appear to contribute to the ability of the organism to enter and replicate within eukaryotic cells (Mintz, *et al.*, 1992a).

The first evidence for the possible role of a plasmid in virulence of *L. pneumophila* was the correlation of growth of *Legionella* strains on CYE medium and supplemented Meuller-Hinton plates with plasmid content and the ability to invade mouse A-J macrophage cell lines (Daaka, *et al.*, 1994). The study found that in general, highly virulent strains of *L. pneumophila* were infectious for mouse macrophages and grew better than the avirulent strains and also appeared to contain a single plasmid lacking in avirulent isolates.

To date no specific gene that may play a role in pathogenesis has been identified or sequenced from a plasmid in *Legionella*.

1.12.9 Dot/Icm secretion system

Complementation of an avirulent mutant strain of *L. pneumophila* that was unable to multiply in human monocytes (Horwitz, 1987) identified a genetic locus specifically required for intracellular growth in macrophages (Marra, *et al.*, 1992). The *icm* (intracellular multiplication) locus restored the capacity of the mutant to modify the endosomal pathway and to cause disease in guinea pigs (Marra, *et al.*, 1992). The locus contained four unique genes, *icmWXYZ*, transcribed from a single promoter (Brand, *et al.*, 1994). A similar study complementing mutants defective for intracellular multiplication identified the *dotA* locus (defective for organelle trafficking), another region on the *L. pneumophila* chromosome also required for intracellular multiplication (Berger and Isberg, 1993, Berger, *et al.*, 1994). DotA was shown to be a unique inner membrane protein of approx. 1048 amino acids in size (Roy and Isberg, 1997). The fact that mutations in either the *dot* or *icm* locus produced similar growth defects suggested that the products of these independently identified genes may be coordinately regulated and encode a multi-protein system required for the establishment of intracellular growth of *Legionella* (Berger, *et al.*, 1994, Roy and Isberg, 1997).

Since the initial discovery of these loci, more *dot/icm* genes have been identified that are involved in host cell growth and killing by *L. pneumophila* (Andrews, *et al.*, 1998, Berger and Isberg, 1993, Purcell and Shuman, 1998, Sadosky, *et al.*, 1993, Segal and Shuman, 1997, Segal, *et al.*, 1998, Vogel, *et al.*, 1998). Identification of these genes by two independent research labs has led to the generation of two nomenclatures for identical genes (Fig 1.3a). The *dot/icm* genes are located on two unlinked regions of the *L. pneumophila* chromosome and probably account for most of the genes required for growth within and killing of macrophages by *L. pneumophila.* Several phenotypes are associated with mutations in *dot/icm* genes (Segal and Shuman, 1998a). Mutations in all the genes tested resulted in strains completely or partly defective for the following phenotypes: intracellular multiplication, host killing, LAMP-1 co-localisation and immediate cytotoxicity (Segal and Shuman, 1998a). Additionally, most of the *dot/icm* genes are also required for growth in the

protozoan host *A. castellanii* (Gao, *et al.*, 1997, Segal and Shuman, 1999a) and some are also required for infection and growth within alveolar epithelial cells (Gao, *et al.*, 1998b).

Most of the proteins encoded by the *dot/icm* genes are predicted to be membrane associated and most are unique with no known homologues in the database. Four proteins (DotG, DotM, DotL and DotB) have significant homology with components of bacterial conjugation systems (Segal and Shuman, 1997, Segal, *et al.*, 1998, Vogel, *et al.*, 1998). The *dotG* and *dotB* genes were required for mobilisation of an IncQ plasmid, RSF1010, unable to code for its own transfer although mobilisation is also dependent on *dot/icm* genes that do not have homology to known conjugation genes (*dotA, dotE* and i*cmWXYZ*) (Vogel, *et al.*, 1998). However, not all of the *dot/icm* genes are required for conjugation of RSF1010 (Segal and Shuman, 1998b). It has been proposed that as the Dot proteins were able to mobilise RSF1010, they are likely to form a secretion system in the membrane of *L. pneumophila* capable of transferring a substrate across the outer membrane (Vogel, *et al.*, 1998). The secretion system may have evolved from a DNA transfer system of an integrated plasmid and may represent a pathogenicity island (Vogel, *et al.*, 1998). The secretion system of *L. pneumophila* may be related to the type IV systems observed in other pathogens (Winans, *et al.*, 1996). The plant pathogen *Agrobacterium tumefaciens* has an operon of genes, *virB*, thought to form a channel in the bacterial membrane through which oncogenic DNA is transferred into the plant cell. Two Dot proteins (DotB and DotG) of *L. pneumophila* have homology with Vir proteins encoded by the *virB* operon of *A. tumefaciens* (Vogel, *et al.*, 1998). This pathogen is also capable of mobilising plasmid RSF1010, suggesting that the two systems share functional similarities.

Type IV secretion systems contain two proteins with nucleotide binding motifs that are potential candidates for the motor behind the transport process or for signalling opening of the gate/channel (Burns, 1999). DotB is homologous to a large family of nucleotide binding proteins that include members of various conjugal transfer systems (Vogel, *et al.*, 1998).

The *dot/icm* genes have homology with *tra* and *trb* genes in the transfer region of the *Shigella sonnei* collb-P9 IncI plasmid suggesting that the *dot/icm* genes have the same origin and perform their function together (Segal and Shuman, 1999b). Homologues of some of the *dot* genes have also been identified in the intracellular pathogen *Coxiella burnetii* suggesting that *L. pneumophila* and *C. burnetii* have incorporated an IncI-plasmid conjugation system to export effectors into host cells. However, the two pathogens have different intracellular fates suggesting that factors unique to each correspond to the effectors that determine the intracellular fate of each (Segal and Shuman, 1999b).

Additional support for the model of the *dot/icm* genes forming a secretion apparatus that transfers effector molecules to the host cell was from studies demonstrating that the presence of a functional mobilisation system inhibits intracellular growth and killing of HL-60-derived macrophages in *L. pneumophila* (Segal and Shuman, 1998b). *L. pneumophila* containing RSF1010 derived plasmids that conjugate at high frequency are unable to multiply within and kill HL-60 cells probably because conjugation of RSF1010 competes with the natural substrate/s of the secretory apparatus. Conjugation has been found to be dependent on a functional MobA protein suggesting that the conjugation substrate is probably a nucleoprotein complex (MobA-ss DNA) (Segal and Shuman, 1998b).

The actual substrate/s transferred by the *dot/icm* secretion system is unknown. Based on homology to type IV secretion systems, it is possible that *L. pneumophila* exports either DNA (*A. tumefaciens*) or a protein toxin (*Bordella pertussis*) (Vogel and Isberg, 1999). It is unlikely to be a DNA molecule as modification of the endocytic pathway by *L. pneumophila* once inside the macrophage occurs very rapidly (Roy, *et al.*, 1998, Wiater, *et al.*, 1998). It is likely that an effector protein is transferred to the host cell where it may act by inhibiting or modifying the endocytic pathway (Segal and Shuman, 1998a). Some of the *dot/icm* proteins may be potential effector proteins, including DotI which has two amphipathic ß-sheet regions, a structural feature found in pore forming toxins (Segal and Shuman, 1998a). *L. pneumophila* has a rapid cytotoxic activity resulting from insertion of a pore upon contact with eukaryotic

membranes at high multiplicities of infection (Kirby, *et al.*, 1998). Lysis of macrophages and erythrocytes has been demonstrated by direct contact of bacteria with the cell and the observed cytotoxicity is dependent on *dotA*, *dotB*, *dotE*, *dotG*, *dotH*, *dotI* and *dotO*. The *dot* mutants are unable to insert a pore into the mammalian cell membrane indicating that pore formation is necessary for intracellular growth and may influence intracellular trafficking. The pore may allow bacterial proteins to enter the mammalian cytoplasm where they alter the endocytic machinery, analogous to YopB/YopD of *Yersinia pseudotuberculosis* a type III secretion system (Kirby and Isberg, 1998, Kirby, *et al.*, 1998, Segal and Shuman, 1998a). As *L. pneumophila* is able to insert pores into different host cell types it is likely that a relatively non-specific receptor-ligand interaction triggers pore formation by the *dot/icm* system possibly by recognition of a common component in eukaryotic membranes such as a lipid (Kirby and Isberg, 1998). The rapid kinetics of the cytotoxicity suggested that pore formation might be the first event that initiates altering of phagosome trafficking.

A study by Zuckman *et al* (1999) showed that pore forming activity of *L. pneumophila* is not sufficient for phagosome trafficking and intracellular growth (Zuckman, *et al.*, 1999). Zuckman's study focused on IcmW, a potentially soluble *dot/icm* protein that may be distinct from the membrane bound components and hence may have a role in intracellular growth. This study showed that IcmW was required for intracellular growth and survival in macrophages but was not required for cytotoxicity to macrophages at high multiplicity of infection. This suggests that *L. pneumophila* require functions, in addition to pore formation, to alter phagosome trafficking and establish a replicative vacuole. Initial contact with the host cell may lead to pore formation requiring *dot/icm,* then a signal event requiring IcmW that alters phagosome trafficking (Zuckman, *et al.*, 1999). IcmW was shown to be a cytosolic protein sharing features with cytoplasmic chaperone proteins required for the translocation of effector protein by type III secretion systems (Zuckman, *et al.*, 1999). It may alter phagosome trafficking by presenting effectors of phagosome trafficking to the membrane bound *dot/icm*

translocation apparatus and as such would not be required for pore formation (Zuckman, *et al.*, 1999).

The *dot/icm* complex is predominantly required for trafficking of the *L. pneumophila* phagosome and once the replicative niche is established, the system is not required for growth (Roy, 1999). The transport system must be produced by the bacterial progeny prior to transmission to the next host cell. Conjugation of RSF1010 plasmids occurs during lag phase suggesting that stationary phase *L. pneumophila* already contain a functional *icm/dot* system that can probably initiate infection (Segal and Shuman, 1998b). Wild type *L. pneumophila* can rescue intracellular growth of a *dotA* mutant within the same phagosome suggesting that the system is dispensable, at least temporarily, after establishment of the replicative vacuole (Coers, *et al.*, 1999). The wild type *L. pneumophila* in the phagosome determine formation of the replicative niche, despite containing the *dot* mutant bacteria, which suggests the signal involved is *cis* acting (Roy, 1999). Factors transported by the *dot/icm* system may remodel the phagosome into a specialised organelle after which nutrients and additional membrane supporting bacterial growth inside the phagosome are delivered by the host cell (Coers, *et al.*, 1999). The transported factors may inhibit phagosome maturation by interfering with the normal cellular machinery involved in the endocytic pathway. Alternatively, the system may translocate a factor that mimics a normal host protein effectively camouflaging the vacuole within which the bacteria reside thus protecting it from routing through the normal endocytic pathway (Roy, 1999).

Two models have been proposed to describe how the *dot/icm* genes function in *L. pneumophila*. In a concerted model proposed by Wiater *et al* (1998), the *dot/icm* genes would be expressed prior to infection allowing the bacterium to form a specialised phagosome whose features would determine subsequent intracellular events. This is consistent with the rapid kinetics of phagosome fusion that occur within minutes after uptake of *dot/icm* mutants (Roy, *et al.*, 1998, Wiater, *et al.*, 1998) and with the observation that conjugation of RSF1010 occurs during lag phase suggesting that stationary phase bacteria already contains a functional

dot/icm complex (Segal and Shuman, 1998b). Sequential *dot/icm* gene activation has also been proposed based on the observation that mutants defective for intracellular growth have distinct intracellular fates suggesting that the products of these genes are likely to affect different bacterial pathways (Swanson and Isberg, 1996). However, the complex phenotypes may be a result of partial gene products due to the mutagenesis process (Wiater, *et al.*, 1998).

Recently, a second distinct type IV secretion system has been described in *L. pneumophila* (Segal, *et al.*, 1999). The genes were not required for growth in macrophages and amoebae but were partially required for RSF1010 conjugation. The *lvh* genes could substitute for some components of the *dot/icm* secretion system, for conjugation of RSF1010, and it may be that components of the two systems interact with one another (Segal, *et al.*, 1999).

1.12.10 Flagella

Both pili (fimbriae) and flagella have been observed on *L. pneumophila* (Chandler, *et al.*, 1980, Elliott and Johnson, 1981, Rodgers, *et al.*, 1980). Expression of flagella in *L. pneumophila* is temperature-regulated (Elliott and Johnson, 1981, Ott, *et al.*, 1991b) and is also affected by growth phase, medium viscosity, osmolarity, and by amino acid concentration (Heuner, *et al.*, 1999).

L. pneumophila has a single monopolar flagellum composed of one major subunit, a 47kDa protein (Heuner, *et al.*, 1999, Ott, *et al.*, 1991b) encoded by the gene *flaA* (Heuner, *et al.*, 1995). The inferred protein, FlaA, has strong homology with flagellin proteins of other bacteria. Temperature dependent expression of FlaA is regulated at the transcriptional level by a sigma 28-like RpoF-FilA factor from a monocistronic message (Heuner, *et al.*, 1995). The alternate sigma factor 28 of *L. pneumophila* restores flagellin production and motility in a *fliA* mutant of *E. coli* (Heuner, *et al.*, 1997).

To determine association of flagella expression and intracellular growth of *L. pneumophila* mutants that no longer expressed flagella have been generated by transposon

mutagenesis. These were assessed for their ability to infect *H. vermiformis* and macrophage-like U937 cells (Pruckler, *et al.*, 1995). Seven of ten flagella minus (F^-) mutants were attenuated in both models while ten randomly selected flagella positive (F^+) mutants were unaffected for growth in either model. This suggests that the flagella structure is not essential for virulence in *L. pneumophila*. However, the ability of the organism to infect amoebae and human phagocytic cells appears linked with flagella expression suggesting that virulence factors may be co-regulated with flagella expression. The attenuated mutants may have mutations in regulatory genes associated with the flagellum system (Pruckler, *et al.*, 1995). Recent studies have shown a direct association of flagellar phenotype and virulence in *H. vermiformis* in a majority of *L. pneumophila* isolates and in other species of *Legionella* (Bosshardt, *et al.*, 1997).

A defined mutation has been constructed in a gene known to be involved in flagellum biosynthesis in *L. pneumophila* and assessed for intracellular growth (Merriam, *et al.*, 1997). The inferred protein product of *fliI* has strong homology with nucleotide binding proteins that are components of type III secretion systems (Salmond and Reeves, 1993). Many organisms directly transfer factors to the host cell in a signal sequence independent manner by a process involving approximately twenty conserved proteins that promote protein translocation. This apparatus is referred to as the Type III secretion system. Several of the components of this system have homologs found in the biosynthesis apparatus of flagella such as the products of the *fliI* and *fliA* genes (Dreyfus, *et al.*, 1993). The *L. pneumophila fliI* mutant does not express flagella and is not affected for growth in cultured U937 cells indicating that assembly of flagella is not required for intracellular growth in mammalian cells (Merriam, *et al.*, 1997).

The importance of flagella in *Legionella* is unknown. A physiological link between biosynthesis of flagella and growth in macrophages implies that genes involved in the biosynthesis of flagella may be needed for the export of other factors involved in intracellular growth (Merriam, *et al.*, 1997). Motility appears important for survival of *Legionella* in the environment as survival is dependent on the bacteria actively finding an amoeba host

(Rowbotham, 1986). Consistent with a role in survival in the environment is the observation that expression of flagella is repressed at 37°C (Ott, *et al.*, 1991b). Flagella appear to be found in other species of *Legionella* as determined by Southern hybridisation with a *flaA* probe (Heuner, *et al.*, 1995) and detection of a cross reacting protein of similar size using polyclonal antibodies specific for the 47kDa subunit of *L. pneumophila* in a Western blot (Ott, *et al.*, 1991b). Additionally, a rapid latex agglutination test developed for the detection of all *Legionella* has detected the presence of a common flagellum antigen in most species (Bornstein, *et al.*, 1991). Therefore flagella may be conserved throughout the genus. The presence of flagella may serve as a genus-wide marker for predicting strain virulence (Bosshardt, *et al.*, 1997).

1.12.11 Pili

Pili have been described for *Legionella* (Ott, *et al.*, 1991b, Rodgers, *et al.*, 1980). Recently, a long (0.8-1.5μm) and short (0.1-0.6μm) pilus have been observed on a strain of *L. pneumophila* possibly representing two sets of pilin genes (Stone and Abu Kwaik, 1998). The cloned gene for one of these pili, $pilE_L$, encodes an inferred protein product of approximately 16.5 kDa. $PilE_L$ has homology with the type IV pilin family of proteins, described for many pathogenic bacteria including *Neisseria* and *Pseudomonas* species, and has conservation of a number of characteristic features of this family (Stone and Abu Kwaik, 1998). $PilE_L$ is proposed to be a group A type IV pre-pilin based on the site of cleavage to generate the mature protein (Strom and Lory, 1993). Protein components involved in type IV pilis biogenesis have been implicated in protein secretion, twitching motility and filamentous phage assembly (Hobbs and Mattick, 1993, Strom and Lory, 1993). An insertion mutation in $pilE_L$, introduced by allelic exchange in *L. pneumophila,* causes loss of expression of long pili and the mutant is defective in attachment to epithelial cells, macrophages and amoebae but replicates as well as the parent once inside the host cell (Stone and Abu Kwaik, 1998). $PilE_L$ pili may represent an adherence factor that binds a common host cell component and may

play a role in enhanced survival of *L. pneumophila* in the environment. Southern hybridisation has determined that $pilE_L$ is conserved in *L. pneumophila* although some other species hybridise at low stringency suggesting heterogeneity in the gene sequence of $pilE_L$ in *Legionella* (Stone and Abu Kwaik, 1998).

The ability of *L. pneumophila* to become competent for DNA transformation is dependent on $pilE_L$ (Stone and Abu Kwaik, 1999). The type IV pilin structural protein gene *pilE* and pilin biogenesis gene *pilC* of *N. gonorrhoeae* are required for natural DNA transformation competence (Rudel, *et al.*, 1995). Natural transformation requires uptake of extracellular DNA prior to incorporation of that DNA into the bacterial chromosome and this can occur in a sequence dependent or independent manner (Lorenz and Wackernagel, 1994). Processing of DNA prior to transformation allows entry of linear DNA into the bacterium in single stranded form prior to recombination into the chromosome. The ability of *L. pneumophila* to become competent for DNA transformation is dependent on growth conditions and is induced under conditions similar to those required for expression of type IV pili (Stone and Abu Kwaik, 1999). Disruption of $pilE_L$ in *L. pneumophila* results in loss of pili production and competence for DNA transformation which can both be restored by reintroduction of the wild type $pilE_L$ locus on a cosmid. Therefore, type IV pilus expression is associated with competence for DNA transformation in *L. pneumophila.* Since these pili are also involved in adherence of *L. pneumophila* to protozoa and mammalian cells, they have been designated CAP (competence-and adherence-associated pili) (Stone and Abu Kwaik, 1999). Natural competence for DNA transformation in *Legionella* may allow incorporation into the chromosome of genes that improve survival in the environment or exploit all possible ecological niches available to it (Stone and Abu Kwaik, 1999).

An operon, *pilBCD*, encoding proteins with homology to the *P. aeruginosa* PilB, PilC and PilD proteins, required for type IV pilin production and type II protein secretion, has also been identified in *L. pneumophila* (Liles, *et al.*, 1998). The *pilBCD*, is distinct from $pilE_L$, determined by Southern hybridisation, suggesting two regions on the chromosome are

involved in type IV pilin biosynthesis (Liles, *et al.*, 1998) although it remains to be determined whether the *pilE*$_L$ and *pilBCD* loci act together to assemble a pilus structure. Expression of the *pilBCD* operon is enhanced at 30°C and appears to be regulated by transcriptional control by the alternative σ^{28}-like RpoF factor similar to the flagellin gene of *L. pneumophila* (Heuner, *et al.*, 1995). In correlation with this, pili were not observed on *L. pneumophila* cells grown at 37°C. The PilB and PilC proteins are required for translocation of type IV prepilin across the inner membrane where it is cleaved by pre-pilin peptidase, PilD (Liles, *et al.*, 1999). The pre-pilin peptidase can also cleave proteins required for type II secretion in Gram negative bacteria therefore a *pilD* mutant was constructed to assess importance of type II secretion in *L. pneumophila* (Liles, *et al.*, 1999). Three proteins were absent from the supernatant of the mutant compared with the wild type parent strain indicating that *pilD* is necessary for the secretion of *Legionella* proteins. The mutant was also impaired for the ability to replicate in amoebae and macrophages and was attenuated in a guinea pig model of infection. Therefore, it appears that factors secreted by the *L. pneumophila* type II secretion system contribute to virulence (Liles, *et al.*, 1999).

1.12.12 Zinc-Metalloprotease / Major Secretory Protein (MSP)

The most abundant protein found in the culture supernatant of *L. pneumophila* is the zinc-metalloprotease or major secretory protein (MSP) (Dowling, *et al.*, 1992). Initial studies determined that it was an extracellular protein of 38 kDa, with the capacity to digest collagen, casein and gelatin and was a zinc metalloprotein (Conlan, *et al.*, 1986, Dreyfus and Iglewski, 1986). The protein also had cytotoxic and hemolytic properties with a phenotypic correlation between the two functions (Dowling, *et al.*, 1992, Keen and Hoffman, 1989). Cloning and transposon inactivation analysis of the *L. pneumophila* metalloprotease gene has now determined that a single polypeptide encoded on a 1.2 kb fragment of DNA is responsible for all three properties (Quinn, *et al.*, 1989, Quinn and Tompkins, 1989). The gene, *proA*, encodes a protein of 543 amino acids with a calculated molecular mass of 60,775 Da (Black,

et al., 1990). The metalloprotein has extensive homology with the elastase of *Pseudomonas aeruginosa* particularly in regions forming the active site of the protein and may share a similar molecular mechanism of function (Black, *et al.*, 1990). The *L. pneumophila* metalloprotease gene has been cloned independently and designated *mspA* and the protein termed MSP (Szeto and Shuman, 1990). The protein encoded by *mspA* is 543 amino acids in size with a mass of 60,775 Da which is in agreement with that previously determined (Quinn and Tompkins, 1989). The predicted size of the metalloprotease is larger than the observed 38 kDa, however, cleavage of a long leader sequence occurs to derive the mature protein in a manner similar to that observed in other zinc metalloproteases such as the elastase from *P. aeruginosa* (Moffat, *et al.*, 1994a).

MSP is likely to have an important role in pathogenesis. It is immunogenic, inducing protective humoral and cell mediated immunity (Blander and Horwitz, 1989, Breiman and Horwitz, 1987, Quinn, *et al.*, 1989). It is cytotoxic and haemolytic *in vitro* (Quinn and Tompkins, 1989), and when purified, causes lesions in guinea pig lungs that are indistinguishable from those produced during natural infection (Baskerville, *et al.*, 1986, Conlan, *et al.*, 1986). Construction of mutants in *L. pneumophila* that do not produce the protease has allowed the assessment of these strains in macrophages, amoebae and in guinea pigs (Blander, *et al.*, 1990, Moffat, *et al.*, 1994b, Szeto and Shuman, 1990). An MSP mutant has been shown to be unaffected in its capacity to infect and kill human macrophages and to infect protozoans (Szeto and Shuman, 1990) and was comparable to wild type *L. pneumophila* in an animal model of infection (Blander, *et al.*, 1990). Similarly, an isogenic *proA* mutant was not affected in its ability to infect macrophages or *Acanthomoeba.* However, it did show some attenuation in a guinea pig model of infection (Moffat, *et al.*, 1994b). Subtle effects of attenuation in the animal model observed for this mutant, which contrasts with the results of Blander *et al* (Blander, *et al.*, 1990), are likely to be due to dose differences. Recent studies using signature tagged mutagenesis have confirmed that the protease is a virulence factor of *L. pneumophila* (Edelstein, *et al.*, 1999), thus helping to clear the controversy regarding the role

of this protein in pathogenesis. The metalloprotease has been shown to activate α_1–antitrypsin, an important blood proteinase inhibitor (Conlan, *et al.*, 1988). It can inhibit natural killer cell cytolytic activity, possibly effecting recruitment of phagocytic and NKC function to the site of infection (Rechnitzer, *et al.*, 1989), and has been shown to cleave TNF-alpha produced by a macrophage cell line (Hell, *et al.*, 1993).

MSP is produced by all serogroups of *L. pneumophila* as determined by Southern hybridisation and Western blot (Quinn, *et al.*, 1989). Hybridisation and a cross reactive protein have not been observed for any other *Legionella* species tested although they have been shown to have proteolytic and haemolytic activity. This suggests that MSP may be present in other *Legionella* species but it is genetically and immunologically distinct from that of *L. pneumophila.* A protease has been cloned from *L. longbeachae* sg 1 that has haemolytic activity and the protein sequence shows 78% homology with the MSP of *L. pneumophila,* including sites critical for enzymatic action of the protein (Lim and Heuzenroeder – personal communication). Additionally, unpublished PCR and sequence data shows that a MSP gene has been found in many species of *Legionella* suggesting conservation throughout the genes (R. Ratcliff – personal communication, 2000).

The MSP protein is secreted from *L. pneumophila* but when cloned in *E .coli,* the protein is contained within the peri-plasm (Moffat, *et al.*, 1994a) implying that additional gene products are required in *Legionella* for secretion of the protein. The pre-pilin peptidase, PilD, required for type IV pili production and type II protein secretion in *L. pneumophila* is necessary for secretion of MSP (Liles, *et al.*, 1999). *L. pneumophila* also contains a type II general secretion pathway that is required for secretion of MSP (Hales and Shuman, 1999a).

1.12.13 Type II secretion system

The *lspFGHIJK* (*Legionella* secretion pathway) genes of *L. pneumophila* (Hales and Shuman, 1999a) encode inferred proteins with homology to the general secretion pathway (GSP) family of proteins exemplified by the pullinase secretion system of *Klebsiella oxytoca*

(Pugsley, 1993). The GSP is a type II protein secretion pathway that appears to be highly conserved in gram-negative bacteria (Pugsley, 1993). In type II protein secretion, exoproteins are transported across the cell membrane in a sec-dependent manner and the mature protein reaches the outer membrane by the combined action of some 14 proteins (Pugsley, 1993, Russel, 1998).

A null mutation has been generated in the *L. pneumophila* type II secretion system as a result of the polar insertion of a gentamycin resistance cassette in *lspGH* and allelic exchange into the chromosome. The mutant strain was unable to hydrolise casein and lacked significant MSP activity in the culture supernatant by comparison with wild type *L. pneumophila* confirming that MSP is a substrate for the type II secretion pathway. The analysis also identified three other protein species that are likely to be secreted by the *L. pneumophila* GSP although they are unknown at this time.

Studies of the mutant strain in eukaryotic cells has determined that the type II secretion system, or a secreted protein factor, are not required for killing of a macrophage like cell line. The mutant was defective for replication within *A. castellanii* suggesting that the GSP or a secreted protein is required for replication within protozoa. The MSP protein has been shown not to be that factor.

Multiple secretion systems are not a unique occurrence in bacterial pathogens and *L. pneumophila* appears to have acquired representatives of several systems summarised in Table 1-3.

1.12.14 *Legionella* toxins

A peptide toxin was the first toxin described in *Legionella* (Friedman, *et al.*, 1980). The 1.3 kDa toxin of *L. pneumophila* was methanol soluble, heat and acid stable, cytotoxic and was shown to inhibit neutrophil activation (Friedman, *et al.*, 1980, Friedman, *et al.*, 1982). Another toxin purified from *L. pneumophila* was also shown to depress neutrophil activity (Lochner, *et al.*, 1985). *L. micdadei* was shown to have a heat stable toxin that could inhibit the oxidative response of stimulated neutrophils (Dowling, *et al.*, 1992). An acid

soluble toxin of approximately 3.4 kDa in size has been demonstrated in crude extracts from *L. pneumophila, L. micdadei, L. bozemanii, L. gormanii* and *L. dumoffii* and is capable of killing mouse macrophages (Hedlund, 1981). It is likely to be the same toxin described by others (Dowling, *et al.*, 1992).

The role of this toxin in pathogenesis is unclear. However, in *L. micdadei* the toxin is the prime candidate responsible for inhibition of neutrophil activation, following ingestion of heat-killed organisms, and hence it is considered to be a virulence factor (Dowling, *et al.*, 1992). Studies on immunisation of mice with toxin containing material shows that it protects against lethal challenge of *Legionella* (Hedlund, 1981).

Legionella is known to be cytotoxic for macrophages at high multiplicity. However, the cytotoxin has not been identified, although it is thought to be different from those reported previously (Husmann and Johnson, 1994, Kirby, *et al.*, 1998). Evidence for a toxin is suggested by the fact that a mutant unable to produce MSP, known to have a cytotoxic function, is still cytotoxic for HL-60 cells, suggesting that there are other factors in *L. pneumophila* responsible for this phenotype (Byrne and Swanson, 1998, Szeto and Shuman, 1990).

1.12.15 Iron-related proteins of *Legionella*

Iron is essential for growth of *Legionella* both intracellularly and extracellularly (Byrd and Horwitz, 1989, Feeley, *et al.*, 1978, Feeley, *et al.*, 1979, James, *et al.*, 1995, Pope, *et al.*, 1996). The ability of *Legionella* to survive in the low iron environment of the phagosome suggests that the availability of intracellular iron *in vivo* is an important factor influencing pathogenicity (Cianciotto, *et al.*, 1989a).

Mononuclear cells activated with IFN-γ or treated with an iron chelator to decrease intracellular iron levels are unable to support the intracellular replication of *L. pneumophila* (Bhardwaj, *et al.*, 1986, Byrd and Horwitz, 1989). Additionally, mutants of *L. pneumophila* defective in iron acquisition are defective in intracellular infection (Pope, *et al.*, 1996). Iron

scavenging systems such as high affinity siderophores common in other bacteria are absent in *L. pneumophila* (Cianciotto, *et al.*, 1989a, Liles and Cianciotto, 1996, Reeves, *et al.*, 1983). Similarly, *L. pneumophila* does not bind or utilise transferrin or lactoferrin (Bortner, *et al.*, 1989, Byrd and Horwitz, 1989, Johnson, *et al.*, 1991). *L. pneumophila* has ferric reductase enzymes that may play a role in acquisition and processing of internalised iron (Johnson, *et al.*, 1991, Poch and Johnson, 1993). *L. pneumophila* can bind and utilize hemin as an iron source (O'Connell, *et al.*, 1996a).

In *L. pneumophila,* iron is found predominantly in major iron-containing proteins (MICP) (Mengaud and Horwitz, 1993). The MICP protein has homology with the *E. coli* aconitase protein, an enzyme of the tricarboxic acid cycle, and with the human iron-responsive element binding protein. Abundance of MICP in *L. pneumophila* may play a role in the physiology of the bacterium. Other enzymes of *Legionella* require iron as a co-factor and these include a superoxide dismutase (Hickey and Cianciotto, 1997, Steinman, 1992).

A *fur* gene, encoding a ferric uptake regulation protein, has been demonstrated in all *Legionella* (Hickey and Cianciotto, 1994). The gene for *fur* was cloned from *L. pneumophila* and the inferred protein had strong homology with Fur proteins from other bacteria (Hickey and Cianciotto, 1994). Fur regulates the expression of iron-uptake systems such as siderophores and virulence determinants such as toxins, and hemolysins (Bagg and Neilands, 1987, Guerinot, 1994, Hickey and Cianciotto, 1997, Litwin and Calderwood, 1993). When Fur is complexed with iron, it represses the transcription of iron-regulated genes and it is likely that the Fur protein from *L. pneumophila* is active when complexed with iron (Hickey and Cianciotto, 1994, Hickey and Cianciotto, 1997). The *fur* gene of *L. pneumophila* is essential for viability and hence Manganese resistant isolates, that are spontaneous *fur* mutants, have allowed identification of Fur-regulated proteins (Hickey and Cianciotto, 1997).

A gene, *frgA*, has been identified that is modulated by Fur and encodes a protein with homology to two proteins, IucA and IucC, of the *E. coli* aerobactin operon involved in siderophore biosynthesis and iron uptake (Martinez, *et al.*, 1994). Siderophore activity has

previously not been demonstrated in *Legionella* (Liles and Cianciotto, 1996, Reeves, *et al.*, 1983) and this was an unexpected result. A *frgA* mutant strain of *L. pneumophila* was significantly impaired in intracellular growth within U937 cells compared to the wild type parent strain. The importance of *frgA* for growth within macrophages, an iron-limiting environment, and its iron-dependent regulation by Fur suggests a role in iron acquisition in *L. pneumophila.*

1.13 Gene expression by intracellular *L. pneumophila*

Invasion of host cells by *L. pneumophila* results in rapid environmental changes within the phagosome and this process is likely to be a multi-step process requiring coordinate regulation of different genes in order for the organism to survive and multiply. Many phenotypic changes have been observed in *L. pneumophila* once they infect host cells. How these genes are regulated is largely unknown.

L. pneumophila grown in amoebae are small and highly motile while their *in vitro* grown counterparts appear to be non-motile and are often filamentous (Barker, *et al.*, 1986, Barker, *et al.*, 1993, Rowbotham, 1986). Within protozoa *L. pneumophila* also undergoes phenotypic changes: alterations to fatty acid profiles, and an amoebic protein of 15 kDa is incorporated in the outer membrane (Barker, *et al.*, 1993), and they exhibit increased resistance to anti-microbial agents and biocide inactivation (Barker, *et al.*, 1992, Barker, *et al.*, 1995). Cirillo *et al* also reported dramatic changes in the proteins expressed by *L. pneumophila* grown in *Acanthamoeba castellanii* in comparison with agar grown bacteria (Cirillo, *et al.*, 1994). These changes in protein expression correlated with an increased ability to invade mammalian cells *in vitro* suggesting that prior growth in protozoa pre-adapts *Legionella* to the intracellular niche provided by the macrophage.

L. pneumophila grown in eukaryotic cells are shorter, thicker and highly motile and have a smooth thick cell wall and a higher proportion of β-hydroxybutyrate content as well as different staining properties and protein expression in comparison with broth grown *L.*

pneumophila (Abu Kwaik, *et al.*, 1993, Cirillo, *et al.*, 1994, Rowbotham, 1986). Modulation of protein expression occurs in *L. pneumophila* after growth in macrophages (Abu Kwaik, 1998b, Abu Kwaik, *et al.*, 1993). At least 23 proteins are induced and 32 proteins repressed during exponential growth of *L. pneumophila* in U937 cells. Many of the macrophage induced proteins (MI) are also induced in response to stress conditions *in vitro* suggesting the response pattern seen is due to a global stress response perhaps due to the action of various stress response regulons (Abu Kwaik, *et al.*, 1993). The study identified a protein induced in all stress conditions analysed, UspA (universal stress protein). The protein showed no homology to similar universal stress proteins found in *E.coli* and has since been renamed global stress protein, GspA, upon complete cloning and sequencing (Abu Kwaik and Engleberg, 1994). GspA has homology with the LbpA and LbpB heat shock proteins of *E. coli* believed to have a chaperone-like function. A null mutation introduced into the gene has shown that *gspA* is not required for intracellular infection of mammalian and protozoan host cells *in vitro* (Abu Kwaik, *et al.*, 1997).

Regulation of several proteins occurs in *L. pneumophila* upon infection of susceptable host animal macrophages. Four novel proteins have been shown to be induced in *L. pneumophila* grown intracellularly compared to agar grown bacteria (Miyamoto, *et al.*, 1993). Studies by Susa *et al* (1996) also identified protein antigens induced during early infection of host cells. Intracellular growth of *L. pneumophila* is accompanied by a significant change in metabolic pathways with some changes occurring within the first 4-8 hours of infection indicating rapid adaption of the organism to the intracellular niche. Two antigens were upregulated including Mip and a 44 kDa unknown protein, three antigens were downregulated and three novel proteins were induced that were lacking in the agar grown isolate. The 44kDa protein antigen was one of the proteins produced exclusively in the intracellular phase of growth and was designated *Legionella* intracellular growth antigen (LIGA). It is possible that some of the proteins identified by these authors may be identical due to slight differences in technique leading to sizing inaccuracies.

One of the 23 macrophage induced (MI) genes of *L. pneumophila* specifically induced during the first 2 hours post-infection has been cloned (Abu Kwaik, 1998b). The gene encodes a 20kDa protein with strong homology (80%) to inorganic pyrophosphatase (PPase) of *E. coli* including conservation of functionally important residues. The highly conserved nature of this protein underlines its vital house keeping function in macromolecular biosynthesis, and attempts to generate a null mutation in the gene were unsuccessful suggesting this gene is vital in *L. pneumophila.* The gene was designated *ppa* and was shown to be induced four fold during intracellular growth as compared to extracellularly grown bacteria. This was the first example of a regulated *ppa* gene that is selectively induced during intracellular infection.

Differential display PCR has identified a locus in *L. pneumophila* that is induced during the first few hours of infection (Abu Kwaik and Pederson, 1996). The locus, designated *eml* (early stage macrophage-induced locus), is transiently induced during the first few hours of intracellular infection indicating that the proteins that it encodes are required during early stages of infection. Mutants generated in the *eml* locus by transposon mutagenesis were defective in the early stages of intracellular survival in macrophages in protozoa. Similarities of the mutant phenotypes suggested that the transposon insertions were located in a single gene or an operon. The 3.7 kb DNA fragment containing *eml* shows no homology with sequences in the genetic data base and the proteins encoded on the locus are unknown but are involved in the adaption of *L. pneumophila* to survival within host cells (Abu Kwaik and Pederson, 1996).

In addition to induction of gene expression in host cells, many virulence related phenotypes are repressed in *L. pneumophila* during intracellular exponential growth and then induced when the bacteria enter the post exponential phase (Byrne and Swanson, 1998). Post-exponential phase *L. pneumophila* are more infectious for macrophages than the same strain tested in exponential phase of growth. Post-exponential phase *L. pneumophila* are sodium sensitive, flagellated, cytotoxic, resistant to osmotic shock, and able to resist phagosome

lysosome fusion. *L. pneumophila* undergo the same phenotypic alterations in macrophages upon transition of growth phase: they produced flagella, are motile in the final phase of intracellular life and also become sodium sensitive. This suggests that in both broth and macrophages, *L. pneumophila* regulates the expression of virulence traits in response to environmental conditions. When nutrients are limiting, *L. pneumophila* exits exponential phase growth and expresses the virulent phenotype associated with post exponential phase growth producing factors that enable them to lyse the host cell (ie. cytotoxin), survive osmotic stress, disperse into the environment (ie. flagella), and re-establish infection in a protected phagosome. When nutrient levels and other condtions are favourable in the host cell, *L. pneumophila* expresses functions to replicate maximally (Byrne and Swanson, 1998).

Sodium sensitivity is a characteristic of virulent isolates of *Legionella* (Catrenich and Johnson, 1989, Sadosky, *et al.*, 1993, Vogel, *et al.*, 1996). The biological mechanism behind the link between sodium sensitivity and virulence in *L. pneumophila* is not known. The *dot/icm* secretion complex may allow inhibitory levels of sodium chloride to leak into the cell (Vogel, *et al.*, 1996). This is possible since cytotoxic activity linked with these genes (Kirby, *et al.*, 1998) is also induced in the stationary phase of growth of *L. pneumophila* as well as sodium sensitivity (Byrne and Swanson, 1998).

The response of *L. pneumophila* to amino acid starvation is mediated by ppGpp (guanosine 3',5'-bispyrophosphate) the product of the enzyme RelA a ppGpp synthetase (Hammer and Swanson, 1999). PpGpp plays a crucial role in the co-ordinate adaptive response of many microorganism in response to amino acid starvation (stringent response) (Cashel, *et al.*, 1996). In *E. coli,* accumulation of ppGpp triggers the adaptive response of the organism which includes the production of stationary phase sigma factor σ^s (rpoS). *L. pneumophila* also accumulates ppGpp in response to amino acid starvation that initiates both entry into stationary phase and expression of virulence phenotypes including motility, cytotoxity and enhanced infectivity.

To identify a protein that may play a role in regulating virulence expression, a mutant strain of *L. pneumophila* (Swanson and Isberg, 1996) that had an observed heterogeneity in the ability to survive and multiply in macrophages was identified for further study (Hammer and Swanson, 1999). It was reasoned that a mutation in a global regulator of virulence might account for the phenotype of the mutant. The mutant was able to accumulate ppGpp in response to amino acid starvation but failed to switch to a virulent phenotype upon exiting exponential phase growth, implying that it lacked a factor that co-ordinates virulence expression in response to ppGpp, the intracellular signal for amino acid starvation. It was proposed that ppGpp acts as an "alarmone" requiring an additional factor, missing in the mutant strain, to co-ordinate the adaptive response of the *L. pneumophila* to starvation (Hammer and Swanson, 1999).

The mutant may have a defect in the stationary phase sigma factor RpoS function since induction of RpoS by ppGpp is thought to elicit much of the adaptive response that occurs upon starvation in *E. coli* (Cashel, *et al.*, 1996). It is not known if RpoS is required in *L. pneumophila* for expression of some or all of the virulence phenotypes that are induced upon amino acid starvation. However, RpoS is required for maximal expression of virulence in several pathogens, suggesting it likely to play a role in *L. pneumophila* (Hammer and Swanson, 1999). Recently the *rpoS* gene of *L. pneumophila* has been cloned and characterised (Hales and Shuman, 1999b). The inferred protein has homology (60%) with RpoS of *E. coli* and is induced in stationary phase cultures of *L. pneumophila* as observed for *E.coli.* Insertional inactivation of *rpoS* determined that unlike *E. coli* where RpoS is required for control of resistance to stress of stationary phase cells the *L. pneumophila* RpoS is not required for this growth phase dependent resistance. Therefore, another unidentified sigma factor or other global regulatory protein may play a role in this resistance.

The gene, *rpoS,* is not required for *L. pneumophila* to multiply in and kill human macrophages (Hales and Shuman, 1999b). In contrast, it is absolutely required for growth

within *A. castellanii* suggesting that entry into protozoans requires *RpoS* regulated proteins that may be fundamentally different from those required in eukaryotic host cells.

In summary, *L. pneumophila* probably regulates gene expression in response to changes in the environment such as those encountered during growth in eukaryotic host cells where it encounters adverse conditions such as nutrient deprivation and oxidative stress. How it co-ordinately regulates these genes is still not fully known but ppGpp appears to play an important role. The RpoS sigma factor is known to be involved in the regulation of expression of genes induced in stationary phase and during stress conditions such as nutrient starvation.

1.14 Comparison of *L. pneumophila* with other *Legionella* species

Nearly all of the studies undertaken to understand the importance of *Legionella* have focussed on *L. pneumophila* sg 1, in particular strain Philadelphia. A few studies have been undertaken with *L. micdadei,* the second most common etiologic agent of Legionnaires' disease in the Unites States (Joshi and Swanson, 1999, Reingold, *et al.*, 1984). *L. micdadei* infects immunocompromised hosts primarily but the number of infections caused by this pathogen are substantially less than those caused by *L. pneumophila.* It has been proposed that the relative rarity of infection due to *L. micdadei,* in comparison with *L. pneumophila* may be due, in part, to differences in growth kinetics in the environment rather than to a genuine difference between the two species (Best, *et al.*, 1985).

L. micdadei shares some virulence features in common with *L. pneumophila* such as Mip (O'Connell, *et al.*, 1995) and flagella (Bangsborg, *et al.*, 1995), but they are not taken up by coiling phagocytosis (Rechnitzer and Blom, 1989) and do not appear to replicate in a specialised phagosome associated with the host ER (Weinbaum, *et al.*, 1984). A comparative study of virulence traits of *L. pneumophila* and *L. micdadei* determined that the two species appear to use distinct mechanisms to parasitise macrophages (Joshi and Swanson, 1999).

L. longbeachae sg 1 was recognised as a pathogenic species of *Legionella* capable of causing pneumonia in 1987 (McKinney, *et al.*, 1981). In the United States, it is associated with sporadic disease and is represented in the 10-15% of cases of pneumonia caused by species of *Legionella* other than *L. pneumophila* (Reingold, *et al.*, 1984). In Australia, there have been numerous cases of pneumonia caused by *L. longbeachae* sg 1, many clustered in time (Cameron, *et al.*, 1991, Lanser, *et al.*, 1990, Lim, *et al.*, 1989, Walker and Weinstein, 1992). Nearly half of all cases of pneumonia, caused by *Legionella*, in South Australia are due to *L. longbeachae* sg 1 (Cameron, *et al.*, 1991, Walker and Weinstein, 1992) which reflects the national trend (Gabbay, *et al.*, 1996).

Few virulence studies have been undertaken with this organism (Neumeister, *et al.*, 1997, O'Connell, *et al.*, 1996b) and little is known of the intracellular life cycle of the organism and what factors may contribute to pathogenicity. This thesis will attempt to answer some of the questions relating to the virulence of this organism.

1.15 Aims and Objectives

- To develop an animal model to quantify virulence of strains of *Legionella*.
- To evaluate virulence of strains of *L. longbeachae* from diverse geographical locations in an animal model.
- To determine potential virulence factors of *L. longbeachae* sg 1
- To construct isogenic mutants of identified virulence factors and assess them in biological models.

Figure 1.1

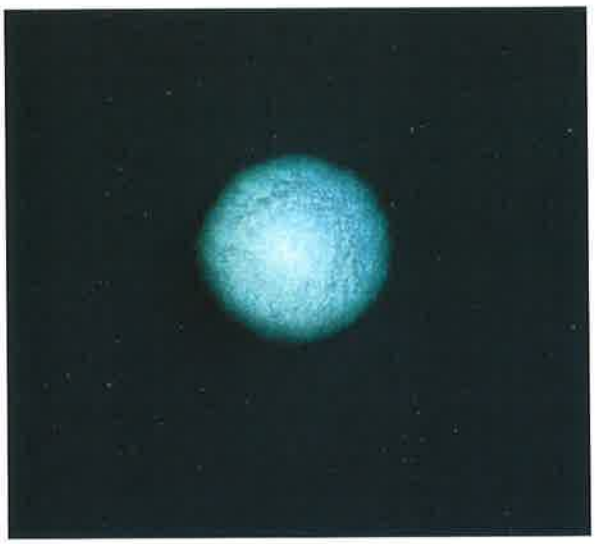
a: Growth of *Legionella pneumophila* sg 1 on Charcoal Yeast Extract (CYE) agar. The "cut glass" appearance of the colonies is evident under reflected light using a dissecting microscope.

b: Growth of *Legionella longbeachae* sg 1 on CYE agar. A "cut glass" appearance is also observed, however, the colony often has a bulls-eye appearance due to heavier growth in the centre of the colony.

c: Autofluorescence of some *Legionella* species is observed under long wave UV light. Blue-white fluorescence is observed for several species of *Legionella* while red fluorescence is noted for only two species, *L. rubrilucens* and *L. erythra.*

a

b



c

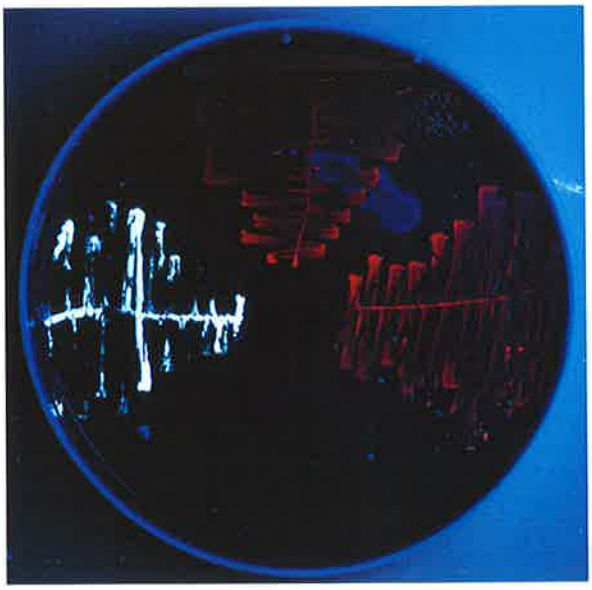


Figure 1.2

a: Classical phagocytosis occurs by a zipper mechanism whereby particles are engulfed by a cup-like pseudopod extending from the phagocytic cell around the circumference of the particle. Fusion of the approaching pseudopod extensions seals the cup forming around the particle thus allowing the sealed phagosome to bud off from the plasma membrane.

b: Coiling phagocytosis occurs with several intracellular pathogens and is characterised by a unilateral pseudopod extending from the phagocytic cell that rolls into itself rather than fusing with its stem. It is known that for some pathogens the resulting pseudopod whorls fuse with a confluent phagosome wall (*Leishmania* promastigotes) or break down the internalised membranes resulting in release into the cytosol (*B. burgdorferi*). *Legionella pneumophila,* however, ends up in a ribosome studded replicative vacuole although how this occurs is not fully known.

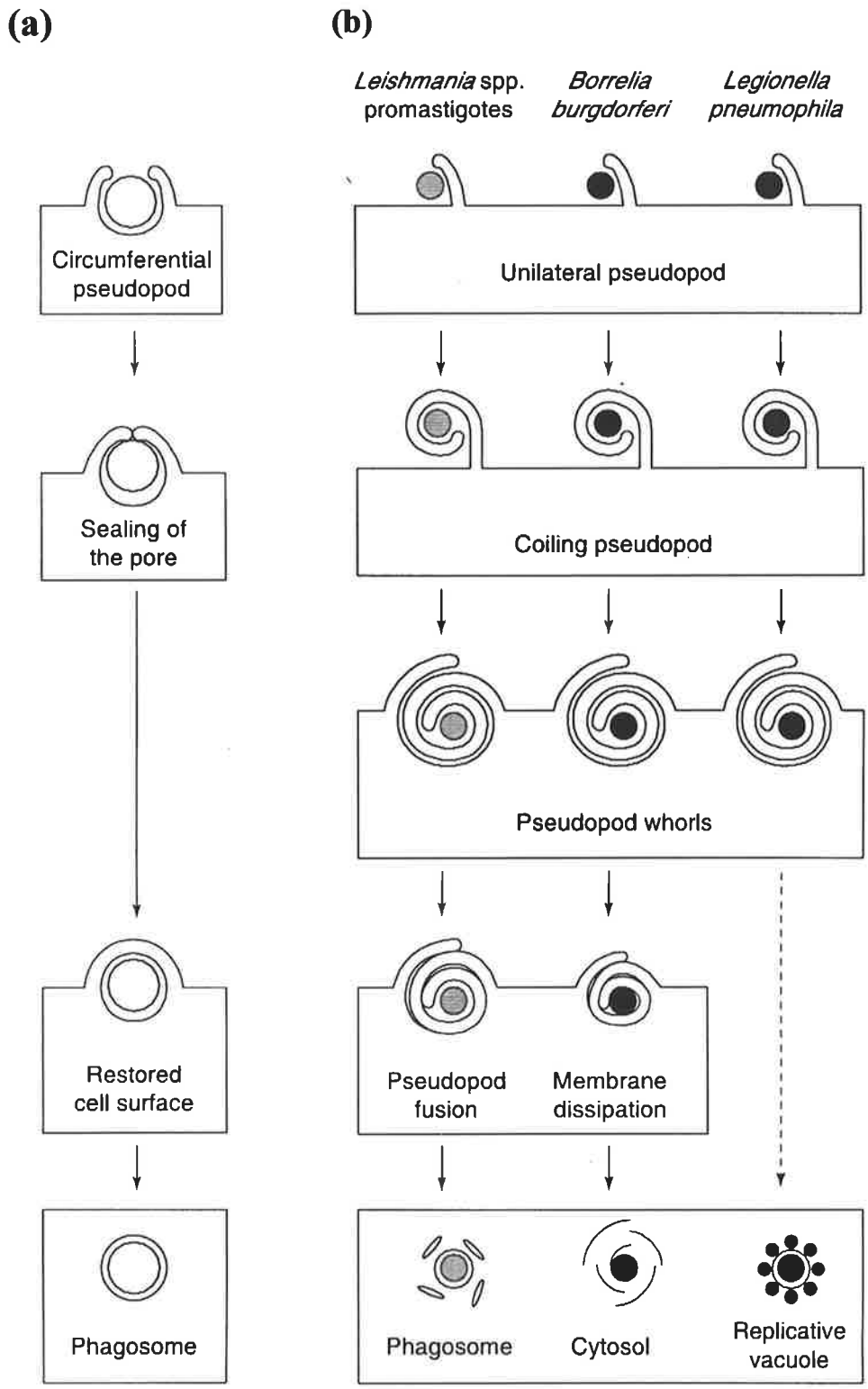
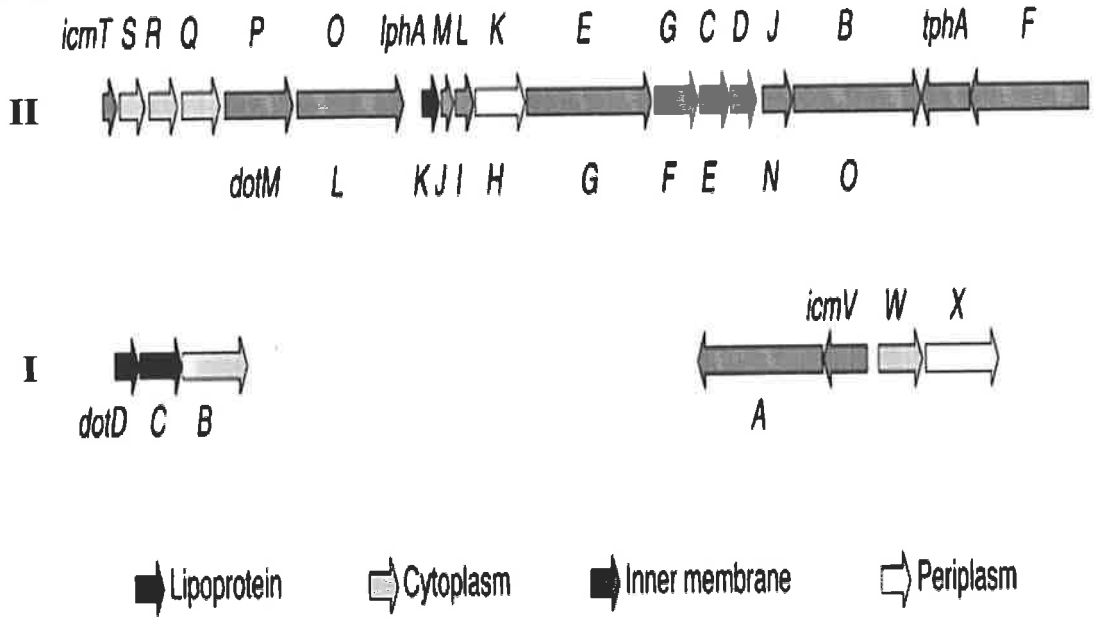

(a)
(b)
Leishmania spp. promastigotes
Borrelia burgdorferi
Legionella pneumophila
Circumferential pseudopod
Unilateral pseudopod
Sealing of the pore
Coiling pseudopod
Pseudopod whorls
Restored cell surface
Pseudopod fusion
Membrane dissipation
Phagosome
Phagosome
Cytosol
Replicative vacuole

Figure 1.3

(a): Map showing the two unlinked regions (I and II) on *L. pneumophila* containing the *dot/icm* genes involved in intracellular survival and killing of macrophages. The two nomenclatures that exist for identical genes are shown as *icm* above the gene arrows and *dot* below. Arrows indicate coding regions for the genes and direction of transcription while the colour shading indicates the predicted cellular location of the protein in *L. pneumophila.*

(b): Proposed model for the function of the Dot/Icm secretory apparatus. Proteins encoded by the *dot/icm* genes assemble to form a secretory apparatus that is required for formation of a pore in the membrane of the eukaryotic host cell upon initial contact. Effector molecules are predicted to be transported by the apparatus into the host cell where the main function is to alter intracellular trafficking of the phagosome containing *L. pneumophila.* This allows establishment of a replicative niche within the host cell that is not degraded by the normal endocytic pathway.

(a)


icmT S R Q P O lphA M L K E G C D J B tphA F
II
dotM L K J I H G F E N O
I
dotD C B
icmV W X
A
Lipoprotein
Cytoplasm
Inner membrane
Periplasm

(b)

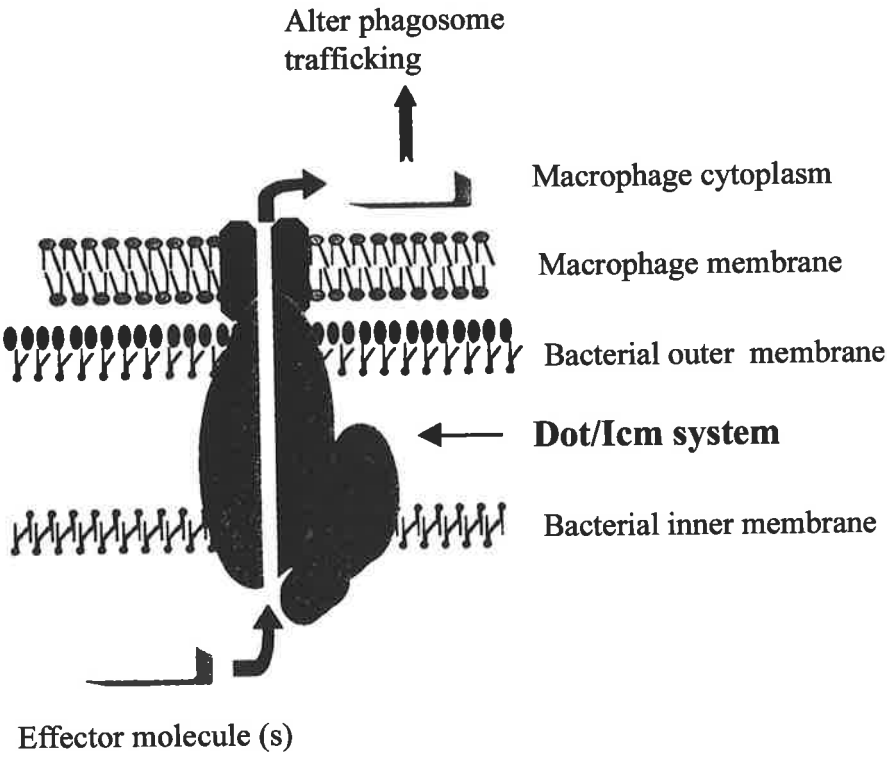

Alter phagosome trafficking
Macrophage cytoplasm
Macrophage membrane
Bacterial outer membrane
Dot/Icm system
Bacterial inner membrane
Effector molecule (s)

Table 1-1 *Legionella* species associated with disease

Legionella species	Reference
L. anisa	(Gorman, *et al.*, 1985, Thacker, *et al.*, 1990)
L. birminghamensis	(Wilkinson, *et al.*, 1987)
L. bozemanii sg 1 & 2	(Brenner, *et al.*, 1980, Harris, *et al.*, 1998)
L. cherrii	(Brenner, *et al.*, 1985, Fang, *et al.*, 1989)
L. cincinnatiensis	(Jernigan, *et al.*, 1994, Thacker, *et al.*, 1988a)
L. dumoffii	(Brenner, *et al.*, 1980)
L. feeleii sg 1 & 2	(Herwaldt, *et al.*, 1984, Thacker, *et al.*, 1983)
L. gormanii	(Griffith, *et al.*, 1988, Morris, *et al.*, 1980)
L. hackeliae sg 1 & 2	(Brenner, *et al.*, 1985, Wilkinson, *et al.*, 1985b)
L. jordanis	(Thacker, *et al.*, 1983, Thacker, *et al.*, 1988b)
L. lansingensis	(Thacker, *et al.*, 1992)
L. longbeachae sg 1 & 2	(Lim, *et al.*, 1989, McKinney, *et al.*, 1981)
L. lytica	(Hookey, *et al.*, 1996)
L. maceachernii	(Brenner, *et al.*, 1985, Wilkinson, *et al.*, 1985a)
L. micdadei	(Hebert, *et al.*, 1980)
L. oakridgensis	(Orrison, *et al.*, 1983)
L. parisiensis	(Lo Presti, *et al.*, 1997)
L. pneumophila sg 1 -16	(Brenner, *et al.*, 1979)
L. sainthelensi sg 1 & 2	(Benson, *et al.*, 1990, Campbell, *et al.*, 1984)
L. tucsonensis	(Thacker, *et al.*, 1989)
L. wadsworthii	(Edelstein, *et al.*, 1982)

Table 1-2 *Legionella* species not associated with disease

Legionella species	Reference
L. adelaidensis	(Benson, et al., 1991)
L. brunensis	(Wilkinson, et al., 1988)
L. erythra	(Brenner, et al., 1985)
L. fairfieldensis	(Thacker, et al., 1991)
L. geestiana	(Dennis, et al., 1993)
L. gratiana	(Bornstein, et al., 1989)
L. israelensis	(Bercovier, et al., 1986)
L. jamestowniensis	(Brenner, et al., 1985)
L. londiniensis	(Dennis, et al., 1993)
L. moravica	(Wilkinson, et al., 1988)
L. nautarum	(Dennis, et al., 1993)
L. quateirensis	(Dennis, et al., 1993)
L. quinlivanii	(Benson, et al., 1989, Birtles, et al., 1991)
L. rubrilucens	(Brenner, et al., 1985)
L. santicrucis	(Brenner, et al., 1985)
L. shakespearei	(Verma, et al., 1992)
L. spiritensis	(Brenner, et al., 1985)
L. steigerwaltii	(Brenner, et al., 1985)
L. taurinensis	(Lo Presti, et al., 1999)
L. waltersii	(Benson, et al., 1996)
L. worsleiensis	(Dennis, et al., 1993)

Table 1-3 Secretion systems of *Legionella pneumophila*

Secretion System	Type II	Type II	Type III
Encoded by genes:	*lsp*FGHIJK (Hales and Shuman, 1999a)	*pil*BCD (Liles, *et al.*, 1999, Liles, *et al.*, 1998)	*fli*I (Merriam, *et al.*, 1997)
Virulence factor/s associated with:	- MSP	- Type IV pili	- Flagella
Affect on virulence:	- Unable to multiply in amoebae - Efficient at killing macrophages	- Impaired for growth in macrophages and amoebae - Attenuated in an animal model of infection.	- Not required for growth in macrophages.
Required for:	- Secretion of MSP	- Expression of pili - Protein secretion	- Expression of flagella
Products secreted:	- MSP - Three other unidentified proteins	- Unknown, although MSP has been suggested as one of the likely proteins.	- Unknown.

Secretion System	Type IV	Type IV
Encoded by genes:	*lvh* genes (Segal, et al., 1999)	*dot/icm* genes (Segal, *et al.*, 1998, Vogel, *et al.*, 1998).
Virulence factor/s associated with:		- Cytotoxin
Effect on virulence:	- Not required for growth in macrophages - Not required for growth in amoebae - Partly required for conjugation of plasmid RSF1010	- Unable to replicate in macrophages. - Unable to replicate in amoebae.
Required for:		-Some required for conjugation of plasmid RSF1010 -Cytotoxicity to macrophages -Pore forming capacity
Products secreted:	- Unknown	- Cytotoxin

Chapter 2

General methods and materials

2.1 Media

2.1.1 Solid media

The following solid media were used for bacterial cultivation. Columbia blood agar base (CA) (1% Bacto pantone, 1% Bacto Bitone, 0.3% tryptic digest of beef heart, 0.1% corn starch, 0.5% NaCl and 1.5% Bacto agar), Horse blood agar (HBA) (CA with 5% defibrinated horse blood) and Meuller-Hinton agar (MH) (30% beef infusion, 1.75% casamino acids, 0.15 % starch and 1.7% Bacto agar) were the general solid media used for culture of *E. coli* strains and were purchased from Medvet Science Pty. Ltd., Adelaide, South Australia. Selection of plasmid pGEM-7Zf(-) containing *Legionella* DNA inserts transformed into in *E. coli* cells was achieved on CA containing 0.007 mM X-gal and 0.1 mM IPTG.

Charcoal yeast extract (CYE) agar (and base) (1.7% Bacto agar, 1.0% yeast extract, 0.2% activated charcoal and 0.025% soluble ferric pyrophosphate supplemented with 0.1% bovine serum albumin, 0.04% L-cysteine and 0.1% α-ketoglutarate) was used for cultivation of *Legionella* strains. For some experiments *Legionella* isolates were plated on CYE plates containing Pimafucin (250mg/liter), Polymixin B (80,000 IU/liter) and vancomycin (2mg/liter), (CYE-VPP).

Plates were incubated at 30°C or 37°C where appropriate.

2.1.2 Liquid media

The following liquid media were used for bacterial cultivation. Luria-broth (L-broth) (1% Oxoid tryptone L42, 0.5% Oxoid yeast L21, and 0.5% NaCl) was the general growth medium for *E. coli* strains. SOC medium containing 2% Bacto tryptone, 0.5% Bacto yeast, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose was used to maximise recovery of *E. coli* transformants following electroporation (Dower, *et al.*, 1988). Super broth consisting of 1.2% Bacto tryptone, 2.4% Bacto yeast extract, 0.5% glycerol, 1.15% KH_2PO_4 and 6.25% K_2HPO_4 was used for growth of bacterial strains of *E. coli* containing plasmids for extraction (Sambrook, *et al.*, 1989).

Legionella isolates were cultured in buffered yeast extract (BYE) broth containing 1% yeast extract, 1% *N*-(2-acetamido)-2-aminoethanesulfonic acid buffer, 0.1% α-ketoglutarate, 0.04% L-cysteine-HCl and 0.025% ferric pyrophosphate (Steinmetz, *et al.*, 1991). Broths were incubated at 30°C or 37°C where appropriate. A purity check of the broth cultures was performed by plating an aliquot on HBA.

Tryptic soy broth with glycerol (snap freeze medium) (1.7% Bacto tryptone, 0.3% Bacto soyatone, 0.25% Bacto dextrose, 0.5% NaCl, 0.25% K_2HPO_4 and 10% glycerol) was purchased from Medvet Science, Pty. Ltd., Adelaide, South Australia and was used as a freezing medium for maintenance of all bacterial stocks.

2.1.3 Antibiotics

Antibiotics were added where appropriate to broth or solid media as follows. For *E. coli* strains, the following final concentrations were used: ampicillin (Ap) 100 μg/ml, kanamycin (Km) 50 μg/ml and chloramphenicol (Cm) 25 μg/ml. For *Legionella* strains the following final concentrations were used: kanamycin 10 or 25 μg/ml, chloramphenicol 5 μg/ml and aztreonam (Az) 4μg/ml.

2.2 Chemicals and reagents

The following AnalaR grade chemicals were used. Calcium chloride was purchased from Ajax Chemicals, NSW, Australia. Butanol, caesium chloride (CsCl), chloroform, ethanol, ethidium bromide, ethylene-diamine-tetra-acetic-acid (EDTA) disodium salt, glacial acetic acid, formamide, glucose, glycerol, glycine, hydrogen peroxide, hydrochloric acid (HCL), iso-amyl alcohol, magnesium chloride ($MgCl_2$), methanol, magnesium sulphate (Mg_2SO_4), β-mercaptoethanol, phenol, potasium acetate, periodic acid, potassium chloride (KCl) di-potassium hydrogen orthophosphate (K_2HPO_4), potassium di-hydrogen orthophosphate (KH_2PO_4), propoan-2-ol (isopropanol), sucrose, sodium citrate, sodium dodecyl sulphate (SDS), silver nitrate ($AgNO_3$), di-sodium hydrogen orthophosphate (Na_2HPO_4), sodium chloride (NaCl), 4-chloro-1-naphthol, sodium lauroylsarcosine (sarkosyl), Tris(hydroxymethyl)aminomethane and trichloroacetic acid were purchased from BDH Chemicals (Victoria, Australia) or Sigma Chemical Company (St. Louis, MO, US).

Guanidinium isothiocyanate was purchased from Gibco BRL (Gaithersburg, MD, US). Skim milk powder was from Carnation, Australia. Bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. Antibiotics were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) (ampicillin, kanamycin sulphate and chloramphenicol), Sigma (streptomycin sulphate) or Gibco BRL (gentamycin). Aztreonam was purchased from Bristol-Meyers Squibb Pharmaceuticals (Noble Park, Victoria, Australia). Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), Digoxigenin labelling mix, 10 × hexanucleotides, nitroblue tetrazolium chloride (NBT), and brom-4-chlor-3-indolyl-phosphate toluidine salt (X-P) were purchased from Boehringer Mannheim.

Electrophoresis grade reagents were obtained from the following companies as indicated: acrylamide (National Diagnostics, Atlanta, Georgia, US), ammonium persulphate (Bio-Rad, Hercules, California) and N,N,N,N,-tetramethylethylenediamine

(TEMED) (Sigma), agarose (Bio-Rad or Progen Industries, Qld, Australia). Low melt point preparative agarose and bromophenol blue were obtained from Bio Rad. Coomassie brilliant blue R-250 for staining PAGE gels was purchased from Sigma.

For PCR, the four deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP) were purchased from Pharmacia biotech (Quarry Bay, Hong Kong). Adenosine-5'-triphosphate sodium salt (ATP) was purchased from Boehringer Mannheim.

2.2.1 Enzymes

Lysozyme, pronase and Rnase A were obtained from Boehringer Mannheim. Klenow enzyme and T_4 DNA ligase was obtained from Pharmacia or New England Biolabs (Beverly, MA). Proteinase K was obtained from Merck chemicals (Darmstadt, Germany). Restriction endonucleases *Acc*I, *Apa*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sma*I and *Xba*I were purchased from Amersham, Boehringher Mannheim, New England Biolabs or Pharmacia and used with the supplied appropriate buffer. AmpliTaq DNA polymerase and its supplied buffers: Buffer II (100 mM Tris and 100 mM KCl) and 25 mM $MgCl_2$ were purchased from Roche (Mannheim, Germany).

2.2.2 Oligonucleotide primers

Synthetic oligonucleotides (primers) were synthesised using reagents purchased from Applied Biosystems or Ajax Chemicals (acetonitrile). Synthesis was performed on an Applied Biosystems 381A DNA synthesiser by the Division of Molecular Pathology, IMVS, Adelaide. To prepare oligonucleotides for experimental use, an aliquot (100 µl) was extracted with butanol (1 ml) to remove residual salts, centrifuged (14,000 × g) for 10 minutes, dried *in vacuo* and resuspended in sterile purified water (100 µl) (Sawadogo and Van Dyke, 1990). All primer stocks were stored at –20°C.

2.3 Bacterial strains and plasmids

General bacterial strains used in this thesis are listed in Table 2-1. Plasmids used in this thesis and their relevant characteristics are listed in Table 2-2.

All bacterial strains were maintained in snap freeze medium at minus 70°C. Fresh cultures were prepared by removal of a small portion of the frozen stock using a sterile glass paster pipette and inoculating onto the appropriate solid media with or without antibiotic selection as required.

2.4 Extraction of chromosomal DNA

Chromosomal DNA was extracted using one of the following two procedures:

Method 1: Genomic DNA extraction by the method of Manning *et al* (1986) yielded high quality chromosomal DNA from *Legionella* strains and was used for cloning. *Legionella* cells were gently resuspended from two plates of growth on CYE (72 hours), in sterile water and pooled into a sterile 10ml centrifuge tube (Disposable Products Co., SA. Australia). Bacteria were pelleted at 3500 rpm in a Heraeus Christ (model GL) centrifuge for 10 min. The bacterial pellet was washed once in TES buffer (50 mM Tris, 5 mM EDTA, 50 mM NaCl, pH 8.0) and centrifuged as above. The pellet was resuspended in 2 ml of Sucrose-Tris solution (50 mM Tris, pH 8.0, 25% sucrose) and 1 ml of 10 mg/ml lysozyme in 250 mM EDTA, pH 8.0, and incubated for 60 min on ice. Then 750 µl of TE solution (100 mM Tris, pH 8.0, 10 mM EDTA), 250 µl of lysis solution (1 M Tris, pH 8.0, 10% sarkosyl, 250 mM EDTA) and 40 mg of solid Pronase was added, mixed gently and the solution then incubated for 2 h at 56°C. The lysed suspension was extracted three times with Tris-saturated phenol to remove proteins associated with DNA and then 2-3 times with chloroform-isoamylalcohol (24:1) to remove any residual phenol. In between extractions, the phases were separated by centrifugation as above. The aqueous phase from the last extraction was dialysed against 1 x TE buffer overnight at 4°C. The chromosomal DNA was stored at 4°C.

Method 2: Alternatively, genomic DNA was prepared by the rapid extraction method of Saunders *et al* (1990). *Legionella* bacterial growth having an approximate volume of 50-100µl was removed from CYE plates, grown as above, using a sterile disposable 10µl inoculating loop (Disposable Products Co., SA. Australia) and placed into a sterile eppendorf tube containing 1 ml of sterile water. The cells were pelleted 17,000 x g for 3 min, resuspended in 200 µl of 2 mg/ml of lysozyme (made up in water) then incubated for 15 min at room temperature. The suspension was lysed by the addition of 400 µl of 5 M guanidinium isothiocyanate in 100 mM EDTA, pH 7.0 by gentle mixing. Proteins were removed from the suspension by extraction with an equal volume of phenol, chloroform, isoamylalcohol mixture (25:24:1). The suspension was mixed vigorously for 30 sec to emulsify and centrifuged at 17,000 x g for 5 min to separate the phases. Chromosomal DNA in the aqueous phase was precipitated by the addition of 0.56 vol of isopropanol. The precipitated DNA was apparent as a white cloud-like suspension in the isopropanol and was removed using a sterile pipette tip into an eppendorf tube containing 80% ethanol. The DNA was washed twice in 80% ethanol by gentle mixing and aspiration. The DNA was dried *in vacuo*, resuspended in TE solution and stored at 4°C.

2.5 Extraction of Plasmid DNA

Plasmid DNA was isolated by one of the two following procedures:

Method 1: Large-scale plasmid purification was performed by the three step alkali lysis method (Garger, *et al.*, 1983). Cells from a 500 ml culture of *E. coli* (Superbroth) or *Legionella* (BYE broth) were harvested at 4,500 x g for 15 min at 4°C and resuspended in 12 ml of solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). Freshly prepared lysozyme (4 ml of 20 mg/ml in solution 1) was mixed with the cell suspension and incubated at room temperature for 10 min. Addition of 27.6 ml of solution 2 (0.2 M NaOH, 1% (w/v) SDS), followed by a 5 min incubation on ice slurry resulted in total lysis of the cells. After the addition of 14 ml solution 3 (5 M potassium acetate, pH 4.8) and

incubation on ice for 15 min, protein, chromosomal DNA and high MW RNA were removed by centrifugation (4,500 x g for 15 min at 4°C). The supernatant was then extracted with an equal volume of a TE saturated phenol, chloroform, isoamylalcohol mixture (25:24:1) at 4,500 x g for 15 min at 4°C. Plasmid DNA from the aqueous phase was precipitated with an equal volume of 100% isopropanol at room temperature for 20 min and collected by centrifugation (11,000 x g for 30 min at 4°C). After washing in 70% (v/v) ethanol (11,000 x g for 20 min at 4°C), the pellet was dried *in vacuo* and resuspended in 1.6 ml of TE. Plasmid DNA was purified from contaminating protein and RNA by centrifugation on a two step CsCl ethidium bromide gradient according to Garger *et al* (1983). The DNA, CsCl, ethidium bromide mixture was prepared by mixing the 1.6 ml DNA in TE with 2.910 g of solid CsCl and 300 µl of 10 mg/ml of ethidium bromide. Final volume of the mixture was made up to approximately 3 ml with TE solution. The mixture was divided between two 4.2 ml polyallomer tubes filled with 3.2 ml of less dense CsCl in TE solution (1.470 g/ml, Refractive index 1.3780). The tube was filled to the top with the less dense CsCl solution then centrifuged at 372, 000 x g for 3 h at 25°C. The plasmid DNA band was removed by side puncture of the tube with a 19-gauge needle attached to a 1 ml syringe. The ethidium bromide was extracted using isoamylalcohol. CsCl was then removed by dialysis overnight against 5 litres of 1x TE at 4°C. Plasmid DNA was stored at 4°C.

Method 2 (mini prep): Small-scale plasmid purification was performed by the three step alkali lysis method using a modification of Garger *et al* (1983). Overnight bacterial cultures (4ml for *E.coli* and 5ml for *Legionella*) were transferred to a sterile 10ml centrifuge tube (Disposable Products Co., SA. Australia) and harvested by centrifugation at 3500 rpm for 10 min in a Heraeus Christ Labfuge (model GL). The pellet was resuspended in 200 µl of solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) for *E. coli,* or 200 µl solution 1 containing 2mg/ml lysosyme for *Legionella*. The cells were lysed by the addition of 400 µl of solution 2 (0.2 M NaOH, 1% (w/v) SDS) with

gentle mixing followed by a 10 min incubation on ice to lyse the cells. After the addition of 300 µl of solution 3 (5 M potassium acetate, pH 4.8) the suspension was incubated on ice for 5 min. Protein, chromosomal DNA and high MW RNA were then collected by centrifugation (17,000 x g for 5 min). The supernatant was transferred to a fresh tube and extracted once with an equal volume of a Tris saturated phenol, chloroform, isoamylalcohol mixture (25:24:1). Plasmid DNA from the aqueous phase was precipitated with an equal volume of 100% isopropanol at room temperature for 20 min and collected by centrifugation 17, 000 x g for 20 min. After washing in 70% (v/v) ethanol (17,000 x g for 5 min), the pellet was dried *in vacuo* and resuspended in 40 µl of sterile purified water.

2.6 Analysis and manipulation of DNA

2.6.1 DNA quantitation

The concentration of DNA in solutions was determined by measurement of absorption at 260 nm. The concentration was calculated using the following standards: absorption of 1.0 at A_{260} is equal to 50 µg of double stranded DNA/ml or 20µg/ml for oligonucleotides (Sambrook, *et al.*, 1989).

2.6.2 Restriction endonuclease digestion of DNA

Digestion of DNA with restriction enzymes was performed using the supplied buffer for the enzyme with or without the addition of BSA as specified by the manufacturer's instructions. For restriction digestion of chromosomal DNA, 5-10 µg was incubated with the appropriate enzyme buffer and 20-40 U of each restriction enzyme in a final volume of 50 - 100 µl at 37°C, 30°C or 25^0C, as specified for the particular enzyme, and incubated overnight. For digestion of plasmid DNA, an aliquot of 0.5-1 µg (unless specified otherwise) was incubated with the appropriate buffer and 4 U of enzyme and incubated for 1-2 hours at the appropriate temperature. For some methods, the restriction digest was terminated by heating at 85°C for 10 min.

Prior to loading onto a gel, a one tenth volume of tracking dye (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 0.1 mg/ml RNase A) was added to the sample or aliquot.

2.6.3 Agarose gel electrophoresis of DNA

Electrophoresis of DNA samples was performed at room temperature on horizontal, 1% or 2% (w/v) agarose gels. Gels were elecrophoresed in a Pharmacia model GNA-200 tank (Pharmacia Biotech Asia Pacific, Ltd., Quarry Bay, HK.) or a BRL model H5 tank (BRL, Gaithersburg, MD, USA). Gels were run in 1x TAE buffer (40 mM sodium acetate, 40 mM Tris and 2 mM EDTA). After electrophoresis the gels were stained in distilled water containing approx. 2 mg/ml ethidium bromide and DNA was visualised with ultraviolet light on a Model TM-36 transilluminator (UVP, Inc., San Gabriel, CA, USA) and photographed using Polaroid 667 positive film.

The sizes of restriction enzyme fragments, PCR products or plasmid bands were estimated by comparing their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriophage SPP-1 DNA (Geneworks, Thebarton, SA, Australia). The sizes of the fragments of DNA in this commercially available preparation used were: 8.557, 7.427, 6.106, 4.899, 3.639, 2.799, 1.953, 1.882, 1.515, 1.412, 1.164, 0.992, 0.710, 0.492, 0.359 and 0.081 kilobases (kb). In some applications, the molecular weight marker pUC19 was used and was obtained from Geneworks or New England Biolabs. The sizes of the fragments of DNA in this preparation were: 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, 26 bp.

2.6.4 Purification of DNA fragments

Bio-Rad low-gelling-temperature agarose at a concentration of 1.0% (w/v) was used for separation of restriction fragments required for cloning or labelling purposes. The specific fragment/s were recovered by the following method. The DNA band of interest was excised from the gel and the agarose melted at 65°C for 10 min. The sample

was then vortexed vigorously with equal volume of Tris saturated phenol. Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one-tenth volume of 3 M sodium acetate, pH 5.2. DNA was collected by centrifugation (17, 000 x g for 25 min), washed once with 70% (v/v) ethanol and dried *in vacuo* before being resuspended in purified water. All purified DNA was stored at minus 20°C until required.

2.6.5 *In vitro* cloning

DNA fragments to be cloned were combined with appropriately digested vector DNA in a ratio of approximately 3-1 or 2-1. The DNA fragments were ligated with 5-10 U of T_4 DNA ligase in ligase buffer (20mM Tris-HCL, pH 7.5, 10mm $MgCl_2$, 10mM dithiothreitol and 0.6 mM ATP) in a final volume of 20 µl an incubated overnight at 4°C. The ligated DNA was purified prior to transformation of *E. coli* stains as follows. An equal volume of Tris saturated phenol, chloroform, isoamyl alcohol (25.24.1) was mixed with the ligation reaction then centrifuged at 17, 000 × g for 5 min. The aqueous phase was precipitated in two volumes of ethanol and 1/10 vol of 3 M sodium acetate, pH 5.2. DNA was collected by centrifugation (17,000 × g for 25 min), washed once with 70% (v/v) ethanol and dried *in vacuo* before being resuspended in 10 µl of purified water.

2.7 Genetic transfer methods

2.7.1 Transformation

Competent cells were prepared for *E. coli* strains for transformation with plasmid DNA as follows. A culture incubated overnight with aeration (in L-broth) was diluted 1:20 into L-broth and incubated with shaking for 3 h at 37°C (A_{600} of 0.6, ca. 4 x 10^8 cells/ml). The cells (approx. 20 ml total volume) were chilled on ice for 30 min, pelleted at 4,500 x g at 4°C and resuspended in 10 ml of ice cold 100 mM $MgCl_2$. The cells were re-centrifuged and resuspended in a final volume of 2 ml of ice cold 100 mM $CaCl_2$. The

cells were allowed to stand for 60 min on ice before addition of DNA. Alternatively, 200 µl of sterile glycerol was added to the suspension (10% final concentration), mixed gently, and stored at minus 70°C in 100 µl aliquots for further use.

Transformation was performed as according to Sambrook *et al* (1989). Competent cells (100 µl) were mixed with DNA (volume made to 100 µl with 1x TE buffer (TE buffer is 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated on ice for 30 min. The cell-DNA mixture was heated at 42°C for 2 min and then rapidly chilled on ice for 1-2 min prior to the addition of 800 µl of L-broth. The suspension was mixed gently and incubated with shaking at 37°C for 45 min - hour. The culture was then plated onto selection plates directly or concentrated by centrifugation and plated.

2.7.2 Electroporation

Electrocompetent *E. coli* cells were prepared using the method of Dower *et al* (1988). *E. coli* DH5α or S1-7 was made competent for electroporation with plasmid DNA as follows: an overnight shaken culture (in L-broth) was diluted 1:20 into L-broth (250ml) and incubated for 3 h at 37°C with gentle agitation at 150 rpm (A_{600} of 0.6, ca. 4 x 10^8 cells/ml). The log phase culture was chilled on ice for one hour and then harvested at 4,500 x g for 20 min at 4°C. The pellet was washed twice in 250 ml of ice-cold purified water and once in 5 ml of ice-cold 10% glycerol. The final pellet was resuspended in 800 to 1000 µl of 10% glycerol and stored at minus 70°C in 40 µl aliquots for further use.

Electroporation was performed according to the method described by Dower *et al* (1988). Purified ligated DNA or CsCl gradient prepared plasmid DNA (total volume 10 µl) was added to the bottom of a chilled 0.2 cm electroporation cuvette (Bio Rad) containing 40 µl of ice-thawed electrocompetent cells. Electroporation of the cells/DNA suspension was performed using the Bio-Rad Gene Pulser with capacitance extender (Model No. 1652078) using the following settings: 200 ohms resistance, 25 µF capacitance and 2.5 kV pulse strength. Following electroporation, approx. 1 ml of SOC

medium was added immediately and the cells gently resuspended and transferred to a sterile universal container (Disposable Products Co., SA, Australia). The cell suspension was incubated for 1- 2 h at 30°C or 37°C with shaking. The culture was then plated onto selection plates directly or concentrated by centrifugation and plated.

For *Legionella,* cells were prepared for electroporation as follows. Bacterial cells were harvested from 100 ml of exponential phase culture (A_{600} of 0.6) by centrifugation at 4 500 × g for 15 min. The pellet was resuspended in an equal volume of ice cold PBS pH 7.4, and centrifuged as before. The pellet was then washed a second time with ice-cold 10% glycerol and finally resuspended in 300μl of 10% ice-cold glycerol. Aliquots of 40µl were stored frozen at -70°C. Electroporation of glycerol treated *Legionella* with plasmid DNA was performed at 100 ohms resistance and 2.3kV as outlined above. The pulsed cells were gently resuspended in approx. 1 ml of BYE broth and incubated at 30°C or 37°C as required for 4-6 hours, before plating on CYE plates containing the appropriate antibiotics.

2.7.3 Bacterial conjugation

Vector constructs generated using plasmid pCACTUS were introduced in to *Legionella longbeachae* sg 1 strains by a modification of the method of Bradley *et al* (1980). Briefly, growth of *Legionella* was harvested from a 48-hour plate and suspended in 1 × PBS to give approx. 1×10^9 cells per ml (OD_{550} of approximately 0.65). An overnight unshaken culture of *E. coli* S17-1 (containing the suicide vector pCACTUS-*mob* with *Legionella* insert DNA) was grown without antibiotics to give approx. 5×10^8 cells/ml (OD550 of ca 0.7). For mating, 100 µl of donor (*E. coli* strain) was mixed with 200 µl of *Legionella* suspension. A 300 µl aliquot of the suspension of cells was then plated onto CYE agar, dried for 10 min and incubated for 6 hours at 30°C. The cells were then scraped from the agar surface into 3 ml of 1 × PBS, vortexed, to dissociate the mating pairs and serially diluted 10 fold in PBS. Aliquots of diluted mating mix (100 µl) were

then plated onto CYE plates containing 5μg/ml Cm and 4μg/ml aztreonam. Aztreonam was used to select against the donor cells by exploiting the natural resistance of *Legionella longbeachae* species to this antibiotic. All plates were incubated at 30°C.

2.8 Polymerase chain reaction

Each PCR reaction mix contained 200 μM of each nucleotide dATP, dGTP, dTTP, and dCTP, 20 pmol of each primers, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 2 U of AmpliTaq DNA polymerase and test DNA (50-100 ng) in a total reaction volume of 50 μl. The amplification protocol consisted of 35 cycles of 1 min at 94°C, 1 min at the specific annealing temperature optimised for each primer pair used, and 1 min at 72°C unless specified otherwise. Each thermocycle was preceded with a 3 min denaturation cycle 94°C and completed with a 7 min extension file at 72°C. A soak file of 4°C maintained PCR products after cycling. Reactions were amplified in a Corbett Research Thermal Sequencer Model FTS-960 (Sydney, Australia). Reactions were analysed by electrophoresis on 1% or 2% (v/w) agarose gels and the size of the products were estimated by comparing with lanes containing either SPP-1 DNA marker or pUC19 marker.

2.9 Southern hybridisation

2.9.1 Preparation of Digoxigenin labelled DNA

Labelling of DNA fragments with Digoxigenin-dUTP was performed according to the manufacturers instructions (Boehringer Mannheim). PCR products were purified using a QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions and eluted in 30μl of elution buffer prior to labelling. Purified DNA fragments, restriction digests (10-15 μl) or SPP1 molecular weight marker (1 μg) (up to 3 μg DNA total for each) were labelled in a total volume of 20 μl. Mineral oil was layered over the reaction mix and incubated overnight at 37°C. The labelled DNA was

added to 10 ml of hybridisation fluid with 5 µl of labelled SPP1 marker and stored at minus 20°C. High stringency hybridisation fluid consists of 50% (v/v) formamide, 7% (w/v) SDS , 1% (w/v) skim milk powder, 5 x SSPE, [1 x SSPE is 0.18M NaCl, 10mM sodium phosphate, 1mM EDTA] and 2.5 mg/ml salmon sperm DNA. Low stringency hybridisation fluid consisted of 18% (v/v) formamide, 5 × SSC [1 x times SSC is 0.15M sodium chloride plus 0.015M sodium citrate pH 7.0], 1% (w/v) skim milk powder, 7% (w/v) SDS and 2.5 mg/ml salmon sperm DNA (Cianciotto, *et al.*, 1990a).

2.9.2 Southern blot

Southern blot analysis was performed according to the method of Southern (1975). Briefly, ethidium bromide stained agarose gel was soaked for 8 to 10 min in 250 mM HCl. The gel was then washed twice in 500 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH) and twice in 500 ml of neutralising solution (0.5 M Tris, pH 7.4: 1.5 M NaCl). DNA bands from the gel was allowed to transfer by capillary action onto Hybond N^+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 18 h at 25°C in 10 x SSC.

2.9.3 Hybridisation and development

Nylon membrane filters were soaked in 5 x SSC solution after transfer and the DNA cross-linked onto nylon under ultraviolet illumination (wavelength approximately 302 nm) for 2 min. The nylon filter was then probed with the appropriate low or high stringency hybridisation fluid, containing the Digoxigenin labelled probe, and incubated at 37°C or 42°C respectively for 18 hours. For high stringency conditions, unbound Digoxigenin labelled DNA was removed by washing the nylon membrane filter twice in 2 x SSC plus 0.1% (v/v) SDS for 10 minutes at room temperature, followed by 2 washes in 1 x SSC plus 0.1% (v/v) SDS for 25 minutes at 68°C. For low stringency conditions, the membrane was washed twice in 5.3 × SSC with 0.1% SDS for 25 minutes at 50°C. The

low stringency conditions used allowed approx. 30% base-pair mismatching (Cianciotto, *et al.*, 1990a).

The filters were then developed according to the manufactures protocol with the exception that the filter was blocked with 5% skim milk in Buffer 1 (100 mM Tris HCl, pH 7.5, 150 mM NaCl) for 90 min at room temperature.

2.9.4 Colony hybridisation

Colony blot hybridisation was performed essentially using the method of Paton *et al* (1992). Briefly, colonies to be screened were grown in a 96-well microtitre tray overnight and then centrifuged at 1500 rpm for 15 minutes in a Hermle Z300 centrifuge. The supernatant was discarded and the pellet resuspended in 10µl of TE buffer. After emulsifying the pellet, 5µl of 10% SDS was added followed by 50µl of a solution containing 0.5 M NaOH and 1.5 M NaCl. The lysate was transferred to a nylon membrane (Hybond N+) for hybridisation.

2.10 DNA sequencing and analysis

Sequencing of PCR products and plasmid constructs was performed using ABI sequencing chemistry kits, as per the manufacturers instructions. Details of the specific sequencing strategies are outlined in the chapter specific methods and materials section relevant to the gene sequence determined. DNA sequencing was performed on an ABI 373A DNA sequence analyser (Applied Biosystems, USA) in the Division of Molecular Pathology, IMVS, Adelaide. Sequence reactions were prepared for analysis according to the manufacturer's protocol. The assembly of contiguous nucleotide sequence, analysis of open reading frames and comparison of amino acid sequence data were performed using Gene Compar 2.0 (Applied Maths, Kotrijk, Belgium). The BLASTX program was used to identify potential protein coding regions (Altschul, *et al.*, 1990). DNASIS was used in some cases to identify potential open reading frames and transcriptional attenuators

(Hitachi Software Engineering Co. Ltd). Hydropathy plots were produced by the program OMIGA 1.1, using the method of Kyte-Doolittle (Kyte and Doolittle, 1982).

2.11 Protein analysis

2.11.1 Whole cell protein preparation

Whole proteins extracts were prepared using a modification of the method of Pearlman *et al* (1985). Bacterial cells were harvested from a 5 ml overnight culture at 3,500 rpm for 15 min. The pellet was resuspended in 5ml 10% trichloroacetic acid and proteins allowed to precipitate for 4-5 h at 4°C. The protein precipitate was harvested at 29,000 x g for 20 min at 4°C and washed three times with phosphate buffered saline, pH 7.4. The final pellet was dissolved and stored at minus 20°C in 500 µl of sample buffer (6.25 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) (Lugtenberg, *et al.*, 1975).

2.11.2 Whole membrane preparation

The following method was used to isolate the outer membrane fraction from *Legionella* and *E. coli* strains. Whole bacterial cells were grown as a 10 ml overnight culture and harvested at 6,000 × g for 15 min at 4°C. The cells were washed once in 30 mM Tris-HCl, pH 8.1 and re-pelleted as above. The bacterial pellet was resuspended in 200 µl of 20% surose in 30 mM Tris-HCl, pH 8.1 and 20 µl of 1 mg/ml of lysozyme in 100 mM EDTA, pH 7.3 and incubated on ice for 1 hour to generate sphaeroplasts. After the addition of 3 ml of 3 mM EDTA, pH7.3, the suspension was sonicated on ice for 2 × 1 min at 65% output using a Micro Ultrasonic Cell Disrupter Model KT-50 (AdeLab, Adelaide, SA, Australia). Unlysed cells were removed by centrifugation at 3, 000 × g for 7 min. The supernatant was harvested at 29,000 x g for 60 min at 4°C. The pellet collected was the whole membrane fraction and was resuspended in 100 µl of sample

buffer (Lugtenberg, *et al.*, 1975). The preparation was then stored at minus 20°C until required.

2.11.3 Outer membrane preparation

The outer membrane protein fraction was obtained as outlined for whole membrane preparations with the exception that the pellet was treated as modified by Achtman *et al* (1983). Briefly, protein was harvested at 29,000 × g for 60 min at 4°C then washed three times with 200 µl of 1% sarkosyl at 37,200 × g for 30 min at room temperature. The final pellet was resuspended in 100 µl of purified water and stored frozen at minus 20°C.

2.11.4 SDS polyacrylamide gel electrophoresis (PAGE)

The protocol for SDS PAGE was modified from the method of Laemmli (1970). The stacker gel contained 4% acrylamide in 2 mM Tris, pH 6.8 and 0.001% SDS. The separating gel was either 12 or 15% acrylamide in solution containing 11 mM Tris, pH 8.8, and 0.6% SDS. The gels were run on a Hoeffer Model SE-600 tank (Pharmacia) at 15 mA for 18 to 24 h. All samples were heated at approx. 100°C for 5 min prior to loading.

Pre-stained size marker (Bio-Rad) containing phosphorylase B protein (101 kDa), Bovine serum albumin (83 kDa), ovalbumin (50.6 kDa), carbonic anhydrase (35.5 kDa), soyabean trypsin inhibitor (29.1 kDa) and lysozyme (20.9 kDa) was run on each gel as a marker track for molecular size determinations.

PAGE gels were stained with 0.275% Coomassie brilliant blue R-250 in 10% (v/v) ethanol, 10% (v/v) methanol and 7.5 % glacial acetic acid for a minimum of 30 min. Gels were de-stained to visualize protein bands by gentle agitation in 10% (v/v) ethanol, 10% (v/v) methanol and 7.5 % glacial acetic acid.

2.11.5 Western blot analysis

The method for Western blot analysis was essentially according to Towbin *et al* (1979). Protein bands from SDS PAGE gels were electro-transferred in a Trans-blot Cell aparatus (BioRad) onto supported nitrocellulose membrane, Hibond-C supported nitrocellulose (Amersham Pharmacia biotech) at 30 V overnight in electroblot buffer (25 mM Tris, 192 mM glycine, 5% methanol). The membrane filter was blocked with 5% skim milk in Tris-buffered-saline buffer (TBS) (100 mM Tris, 500 mM NaCl) for 30 min at 37°C. The filter was then reacted with primary antibody in TBS containing 0.5% Tween-20 (TTBS) overnight at room temperature. Unbound antibody was removed using three 10 min washes of TTBS. The membrane filter was then reacted with an anti-species immunoglobulin conjugated with horseradish peroxidase for 4 to 6 h at room temperature. Any unbound antibody was washed off with two 15 min washes in TBS. To detect the presence of the antigen-antibody complexes, peroxidase substrate 30 mg 4-chloro-1-naphthol, dissolved in 10 ml minus 20°C methanol, was added to 49.5 ml TBS with 45 µl 30% hydrogen peroxide and the membrane allowed to incubate for 10 to 15 min as described by Hawkes *et al* (1982). The reaction was stopped by washing the filter in purified water.

2.12 Lipopolysaccharide isolation and silver staining

Lipopolysaccharide (LPS) was extracted from whole membrane extractions of isolates of *Legionella* using the method adapted by Gabay and Horwitz (1985). *Legionella* whole membranes, prepared as described previously, were resuspended in 200µl of 10mM Tris, 0.1 M EDTA tetrasodium salt and 2% SDS, pH 8 (TES buffer). The membrane components in this buffer were completely solubilised by further treatment of the suspension in a sonicating water bath (UniSonics, Pty. Ltd., Sydney, Australia) for 30 mins. The sample was then centrifuged at 14 000 × g to pellet peptidoglycan. The supernatant was then incubated overnight at 37°C with pronase at a final concentration of

200µg/ml to digest proteins. The next day, the sample was precipitated with 2 volumes of 0.375 M $MgCl_2$ in 95% ethanol at –20°C for several hours. After precipitation, the sample was centrifuged at 15 600 × g for 30 mins to pellet the LPS. The pellet was resuspended in 200µl of TES buffer again and incubated at 85°C for 30 mins to denature any pronase resistant proteins. After cooling to room temperature, pronase was added at 200µg/ml to digest remaining contaminant proteins and incubated overnight at 37°C. After incubation, the LPS was precipitated with 2 volumes of 0.375 M $MgCl_2$ in 95% ethanol and pelleted as described above. The final pellet was resuspended in 200µl of H_2O and stored at –20°C.

Silver staining of LPS in polyacrylalmide gels was performed using the method of Tsai and Frasch (1982). After elecrophoresis, the acrylamide gels were fixed overnight in a solution containing 40% ethanol and 10% acetic acid. The gel was then oxidised for 5 mins in a solution containing 7% periodic acid in 40% ethanol and 10% acetic acid followed by three 10 min washes in sterile H_2O. The gel was stained for 10 min in the dark with a solution containing 28 ml of 0.1M NaOH, 2 ml of NH_4OH (30%) and 5 ml of freshly prepared 20% $AgNO_3$ with a final volume of 150 ml made up with sterile H_2O. After a further three 10 min washes in sterile H_2O, the gel was developed in a solution containing 50 mg citric acid and 500µl formaldehyde (33% stock solution) made up to 1 litre in sterile H_2O.

Table 2-1 Bacterial strains used in this thesis

Bacterial isolate	Strain characteristic(s)	Source and/or reference
***Legionella* strains**		
L. pneumophila serogroup 1 (Philadelphia)	ATCC 33152; type strain	ATCC[a]
L. pneumophila serogroup 1 (Corby)		(Jepras, *et al.*, 1985) obtained from J. Helbig
L. micdadei	ATCC 33218; type strain	ATCC
L. longbeachae serogroup 1 Original isolate (obtained 1987)	ATCC 33462; type strain (Long Beach 4)	ATCC
L. longbeachae serogroup 1 Recent isolate (obtained 1997)	ATCC 33462; type strain (Long Beach 4)	ATCC
L. longbeachae serogroup 2	ATCC 33484; type strain (Tucker 1)	ATCC
L. longbeachae serogroup 1 strain A5H5	Clinical isolate QLD, Australia	This study
L. longbeachae serogroup 1 strain A4C5	Clinical isolate SA, Australia	This study
L. longbeachae serogroup 1 strain A5H3	Clinical isolate QLD, Australia	This study
L. longbeachae serogroup 1 strain A5E1	Clinical isolate VIC, Australia	This study
L. longbeachae serogroup 1 strain A4A7	Clinical isolate SA, Australia	This study
L. longbeachae serogroup 1 strain A5E7	Clinical isolate NSW, Australia	This study
L. longbeachae serogroup 1 strain A4G7	Clinical isolate SA, Australia	This study
L. longbeachae serogroup 1 strain A4B3	Clinical isolate SA, Australia	This study
L. longbeachae serogroup 1 strain L6C9	Environmental isolate SA, Australia	This study
L. longbeachae serogroup 1 strain K5H9	Environmental isolate QLD, Australia	This study
L. longbeachae serogroup 1 strain K4A1	Environmental isolate SA, Australia	This study
L. longbeachae serogroup 1 strain K8B9	Environmental isolate WA, Australia	This study
L. longbeachae serogroup 1 strain K4E1	Environmental isolate SA, Australia	This study
L. longbeachae serogroup 1 strain A7C1	Environmental isolate WA, Australia	This study
L. longbeachae serogroup 1 strain K5F1	Environmental isolate QLD, Australia	This study
L. longbeachae serogroup 1 strain K7C6	Environmental isolate NSW, Australia	This study
L. longbeachae serogroup 1 strain K8C7	Environmental isolate VIC, Australia	This study

Table 2-1 Bacterial strains used in this thesis (continued)

Bacterial isolate	Strain characteristic(s)	Source and/or reference
L. longbeachae serogroup 2 strain K4G7	Environmental isolate SA, Australia	This study
L. longbeachae serogroup 1 Strain Atlanta-5	Clinical isolate	CDC[b] (Aye, *et al.*, 1981)
L. longbeachae serogroup 1 Strain LA-24	Clinical isolate	CDC (Aye, *et al.*, 1981)
L. longbeachae serogroup 1 Strain D-63	Ohio, USA	CDC
L. longbeachae serogroup 1 Strain D-493	California, USA	CDC
L. longbeachae serogroup 1 Strain D-880	Massachusetts, USA	CDC
L. longbeachae serogroup 1 Strain D-1028	Massachusetts, USA	CDC
L. longbeachae serogroup 1 Strain D-1056	New Mexico, USA	CDC
L. longbeachae serogroup 1 Strain D-1624	Israel	CDC
L. longbeachae serogroup 2 Strain D-1738	Texas, USA	CDC
L. longbeachae serogroup 1 Strain D-1750	Georgia, USA	CDC
L. longbeachae serogroup 1 Strain D-1751	Ohio, USA	CDC
L. longbeachae Strain D-1820	Florida, USA	CDC
L. longbeachae serogroup 2 Strain D-1959	Ohio, USA	CDC
L. longbeachae serogroup 1 Strain D-1992	Wisconsin, USA	CDC
***E. coli* strains**		
E. coli strain DH5α	F- ϕ80dlacZΔM15, Δ(lacZYA-argF)U169, endA1 recA1 hsdR17(r_K^- m_K^+), deoR, thi-1 supE44, gyrA96, relA1	(Hanahan, 1985)
E. coli Strain S17-1	RecA derivative of E. coli 294 (hsdR Pro) with RP4-2Tc::Mu (Ap Km Nm Tc::Mu) Km::Tn7 in the chromosome	P. Sansonetti (Ménard, *et al.*, 1993)
E. coli Strain DH1	F-, endA1, recA1, hsdR17(r_K^- m_K^+), thi-1, supE44, gyrA96, relA1	(Hanahan, 1983)
E. coli ATCC type strain	ATCC 25922	ATCC

a: ATCC, American Type Culture Collection, Atlanta, Ga.
b: CDC, Centers for Disease Control and Prevention, Atlanta, Ga. Obtained from Mr. Robert Benson.

Table 2-2 General plasmids used in this thesis

Plasmid	Characteristic/s	Source or Reference
pGEM-7Zf(-)	Ap^r cloning vector	Promega
pUC-18K[a]	pUC-18 containing *aphA*-3 Km^r cassette	(Ménard, *et al.*, 1993)
pPCACTUS-*mob*	Cm^r cloning vector containing *sacB* and a temperature sensitive origin of replication	C. Clark *et al* (unpublished observations)
pWKS130	Km^r cloning and sequencing vector	(Wang and Kushner, 1991)

a: This vector was a kind donation from Prof. P.A. Manning, Microbial Pathogenesis Unit, Department of Microbiology and Immunology, University of Adelaide.

Chapter 3

Assessment of virulence of *Legionella longbeachae* using a guinea pig model of infection.

3.1 Introduction

In order to investigate the virulence of *L. longbeachae* isolates, a reliable model of *in vivo* experimental infection was required. The guinea pig is the most susceptible animal to infection with *L. pneumophila* and is the recognised animal model for experimental legionellosis (Collins, 1986). A pneumonia develops in guinea pigs exposed to an aerosol dose of the bacteria that closely resembles the disease produced in humans (Baskerville, *et al.*, 1981, Baskerville, *et al.*, 1983a, Davis, *et al.*, 1983, Eisenstein, *et al.*, 1984, Muller, *et al.*, 1983, Winn, *et al.*, 1982). The aerosol route of infection is the most "natural" route of infection although an intra-peritoneal route has been used to examine virulence (Chandler, *et al.*, 1979b, Fields, *et al.*, 1986, Fitzgeorge, *et al.*, 1983, Katz and Hashemi, 1982). The intra-peritoneal model of infection can be used to compare virulence of *Legionella* isolates (Fields, *et al.*, 1986), although, it is not thought to be a suitable model of infection as the disease process may be significantly different from that induced by pulmonary inoculation (Eisenstein, *et al.*, 1984).

No animal virulence studies have been carried out with *L. longbeachae* sg 1 isolates although *L. longbeachae* sg 2 has been investigated in an intraperitoneal model of infection in guinea pigs (Fields, *et al.*, 1986). Recent publications relating to *in vitro* models for intracellular growth of *Legionella* have shown that *L. longbeachae* sg 1 ATCC 33462 strain,

(aka Long Beach 4), can replicate in U937 macrophage-like cells (O'Connell, *et al.*, 1996b) but is unable to replicate in Mac-6 cells or in *A. castellanii* (Neumeister, *et al.*, 1997).

Disease caused by *L. pneumophila* is due to the ability of the organism to grow within alveolar macrophages (Cianciotto, *et al.*, 1989a, Dowling, *et al.*, 1992, O'Connell, *et al.*, 1996b). It is generally assumed that the ability to replicate within phagocytes is a hallmark of virulent *Legionella* strains. Therefore, several strains of *L. longbeachae* were tested for their ability to infect macrophage-like U937 cells.

Additionally, an intra-peritoneal and an aerosol *in vivo* model of infection of guinea pigs were established and assessed for the study of virulence of *L. longbeachae* isolates with two main aims. The first was to establish a reliable animal model of infection that would allow for differences in virulence to be detected among strains of *L. longbeachae.* This would allow the assessment of Australian and overseas isolates to determine if Australian isolates are inherently more virulent. Secondly, establishment of an animal model would provide a suitable model in which isogenic mutant strains of *L. longbeachae* sg 1 could be assessed by comparison with the virulent parent strain to determine the role of potential virulence factors in pathogenesis.

This chapter describes the development of an animal model of experimental infection with *Legionella* and analysis of virulence of *L. longbeachae* sg 1 isolates. The results were compared with infection studies performed in U937 macrophage like cells.

3.2 Materials and methods specific to this chapter

3.2.1 *Legionella* strains

Strains of *Legionella* used in this chapter are listed in Table 2-1.

3.2.2 Intra-peritoneal inoculation of guinea pigs

Outbred guinea pigs, IMVS coloured stock (Vet Services, IMVS, Gilles Plains, South Australia, Australia), weighing between 200-700g were inoculated intra-peritoneally with a suspension of *Legionella* (5×10^8 – 1×10^{10} total). *Legionella* organisms were prepared by suspending growth from a 48-hour CYE plate in sterile tap water and comparing the suspension with a turbidity standard (McFarland nephelometer barium sulphate standard number four). The suspension turbidity was confirmed spectrophotometrically (OD $\approx$ 1.0 at 550 nm). This method reliably generated a suspension of approximately 10^9 organisms/ml. The final administered dose for intra-peritoneal injection was enumerated accurately using a counting chamber (Hausser Scientific Partnership, Horsham PA) after treatment of an aliquot of the suspensions with formalin to render the organism non-motile. The dose was administered using a 1ml syringe and 19G needle. The actual dose administered was accurately determined retrospectively by ten-fold serial dilution in sterile tap water and plating onto CYE.

3.2.3 Aerosol inoculation

Guinea pigs were infected experimentally by exposure to an aerosolised dose of approximately 10^9 or 10^{10} *Legionella* organisms. The dose of the test strain was prepared in sterile tap water as outlined for the intra-peritoneal model. The actual dose administered was prepared by aliquotting 1ml of the bacterial suspension with 2ml of sterile tap water to give a final volume of 3ml. For preparation of the 10^{10} dose, the bacterial suspension was compared with a McFarland standard number 10 and confirmed spectrophotometrically (OD 1.8 at 550nm). This resulted in a suspension of approximately 3×10^9 organisms/ml. A 3.5 ml aliquot of the bacterial suspension was used to inoculate the guinea pigs and contained approx. 10^{10} *Legionella* bacteria. The actual number of organisms in the test dose was determined retrospectively in each case by serial 10-fold dilution in sterile tap water and plating onto CYE.

A sealed chamber was constructed for containment of the animals during administration of the dose and was made of perspex (Lucite). The chamber measured 220 mm (W) × 220 mm (L) × 240 mm (D), and had a removable lid for entry and exit of the experimental animals (Fig 3.1). A nebuliser pump therapy kit (Ventalair forte II, Allersearch, Australia) was used to generate the aerosol. The average particle size generated by the nebuliser, according to the manufacturer, was 3.9 microns. An inlet was constructed on one side of the chamber through which the nebuliser hose was inserted and sealed in place connecting the nebuliser bowl to the pump unit outside. The nebuliser pump generated positive pressure in the chamber which was vented through a small outlet valve on the opposite side of the box bubbling through a flask containing 70% ethanol as a safety measure. As an additional safety precaution, the chamber was placed in a Type II bio-safety cabinet (model BHA-120, Gelman Sciences) during the administration of the dose. Guinea pigs were placed in the chamber and exposed to an aerosol of the test strain contained in the nebuliser bowl. The pump was operated until the nebuliser reservoir bowl was emptied (approx. 15min) and the guinea pig was held in the chamber for a further 5 minutes to ensure retention of the dose.

After exposure, the animals were removed and placed in cages only with animals exposed to the same *Legionella* strain. The animals were checked two to three times daily for signs of illness and weights were recorded. Animals were monitored for 7 days after exposure and results recorded on a separate work sheet for each animal in the test group. Symptoms noted included: activity (lethargy), signs of laboured breathing, food intake and water consumption. Animals that were very sick, as evidenced by extreme lethargy and laboured breathing, determined as unlikely to survive more than a few hours, were euthanased as a requirement of the IMVS animal ethics committee (approval 27/96).

3.2.4 Determination of retained dose in the lungs

In some aerosol experiments, the actual numbers of *Legionella* organisms that had been retained in the lungs of exposed guinea pigs was determined. One animal in each test group was killed immediately after exposure to the test dose and the lungs removed. The lungs were homogenised in 100ml of sterile tap water using a Waring commercial blender (Waring Products, New Hartford, Conn.). Viable counts were determined by serial dilution of the homogenate in sterile tap water and plating in duplicate onto CYE and CYE-VPP plates.

3.2.5 Histological staining of lung specimens

In some experiments, lungs were taken from animals that died or were euthanased for histological staining and examination to confirm establishment of pneumonia. The lungs removed from infected animals were fixed in 10% neutral buffered formalin for a minimum of 72 hours. Representative slices, 3 mm in thickness, were then processed into paraffin wax and 5μm thick sections were stained with Hematoxylin and Eosin. Section staining was performed by Mr. Ian Parkinson, Division of Tissue Pathology, IMVS.

3.2.6 Infection of macrophage-like U937 cells

To assess the relative infectivities of *L. longbeachae* strains, 50% infective doses (ID_{50}) for macrophage-like U937 cells were determined. The ID_{50} study was kindly performed by Dr. Nicholas Cianciotto and Dr. Shaila Banvi, Northwestern University, Chicago. The infective doses were determined using previously published methods (Cianciotto, *et al.*, 1989b, Pearlman, *et al.*, 1988), described briefly as follows. U937 cells are a human histiocytic lymphoma cell line that differentiate into non dividing, glass-adherent cells with the phenotypic characteristics of macrophages, when treated with phorbol esters (Sundstrom and Nilsson, 1976). Monolayers were prepared in 96 well microtitre trays with approx. 1×10^5 to 2×10^5 transformed U937 cells per well and inoculated separately with 10-fold serial dilutions of each test strain (10^2 to 10^8 bacteria per well). Cells and bacteria were

prepared in Dulbecco modified Eagle medium (DMEM) media. The plates were incubated for 2 hours to allow bacterial uptake and adherent extracellular bacteria remaining after this period were killed by treatment of the monolayers with gentamycin (50 μg/ml). The monolayers were then washed three times to remove traces of gentamycin and finally resuspended in DMEM containing 10% fetal bovine serum. The plates were incubated at 37^0C. At day three the monolayers were lysed with 0.1% Triton X-100, (100μl), and 10μl of the lysate was spotted onto BCYE agar. After 48-72 hours incubation each spot was examined for colonies (CFU). The proportion of spots that contained growth was determined for each inoculum and the ID_{50} was defined as the minimum inoculum that yielded intracellular bacteria in 50% of the inoculated monolayers (ie CFUs in four of eight spots). The ID_{50} was calculated by the method of Reed and Muench (1938).

3.2.7 Restriction fragment length polymorphism (RFLP) analysis

Isolates of *L. longbeachae* received from Mr. R. Benson, (CDC, Atlanta) for virulence studies were typed by using restriction fragment length polymorphism analysis according to the method developed by Lanser *et al* (1990). Briefly, genomic DNA was digested with *Hin*dIII and *Bam*HI prior to Southern transfer (Chapter 2). The probes used to differentiate strains of *L. longbeachae* were clones chosen empirically from two genomic banks constructed independently from *L. longbeachae* sg 1 ATCC 33462 (Lanser, *et al.*, 1990). One bank was constructed in LambdaGEM-11 (Promega Biotec, Madison, Wis.) and the other in cosmid pHC79 (Hohn and Collins, 1980). The clones derived from these banks were chosen as they gave optimal differentiation of a panel of *L. longbeachae* isolates determined by Southern hybridisation (Lanser, *et al.*, 1990). The probes, designated cosmid8 and λclone2, were labelled with Digoxigenin prior to hybridising with the test filters under high stringency conditions (section 2.9.3).

3.2.8 Electron microscopy

L. longbeachae sg 1 ATCC 33462, an avirulent (type 3) strain, and a highly virulent (Type 1) Australian clinical isolate, strain A5H5, were chosen for examination by electron microscopy to determine the nature of intracellular infection of this species. The strains were inoculated into one guinea pig each, using the aerosol model of infection, with a standardised dose of 10^9 organisms as outlined above. At day 3 post-infection, the guinea pigs were euthanased the lungs removed and placed into 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. Sections were prepared by Dr. Peter Sutton-Smith (Division of Tissue Pathology, IMVS) for transmission electron microscopy. Briefly, lungs were post-fixed by immersion in 2% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour, followed by dehydration in methanol and infiltration and embedding in Spurr's epoxy resin. Survey sections (0.5 micron) were stained with toluidine blue and scanned by light microscopy to define areas containing bacteria for ultrastructural examination. Ultra thin sections (0.1 micron) were then cut, stained with uranyl acetate and lead citrate, and examined in a JEOL1200EXII transmission electron microscope.

3.3 Results

3.3.1 Intra-peritoneal model of infection of *Legionella*

The intra-peritoneal model of infection of *Legionella* allowed accurate doses of the test organism to be administered to the experimental animal and compared with other test isolates (Table 3-1). *L. pneumophila* (Philadelphia) was virulent in this model with death occurring in all guinea pigs within 30 hours of administration of the dose. The animals exhibited symptoms of lethargy, fever and weight loss prior to death consistent with previous experiments using this model of infection of guinea pigs with *L. pneumophila* (Fields, *et al.*, 1986). However, strains of *L. longbeachae* rarely caused death by this route of inoculation.

The type strains of *L. longbeachae* sg 1 and sg 2 were avirulent in this model as evidenced by lack of specific symptoms (Table 3-1). The Australian clinical isolate of *L. longbeachae* sg 1 strain A5H5 did produce some symptoms, predominantly weight loss, in test guinea pigs and death occurred in one of these animals four days after exposure (Table 3-1).

The data indicated that, while this model of infection required minimal set up and adaptation of previously established techniques, it was not a suitable model for assessment of virulence of *L. longbeachae* strains. The strains of *L. longbeachae* used in this study were relatively avirulent in comparison with *L. pneumophila*.

3.3.2 Aerosol model of infection of *Legionella*

A nebuliser apparatus allowed administration of a fine aerosol containing *Legionella* bacteria to be administered to guinea pigs in a sealed perspex chamber (Fig 3.1). The chamber was similar to that used previously for aerosol inoculation of *Legionella* (Blander, *et al.*, 1989, Breiman and Horwitz, 1987) and represents a more "natural" route of infection with this organism.

A representative group of *Legionella* strains were tested to determine if virulence of *L. longbeachae* species could be assessed in this model of infection (Table 3-2). *L. pneumophila* sg 1 strains Philadelphia and Corby and *L. longbeachae* sg 1 strain A5H5 were all virulent in this model of infection. After exposure to a test dose of approx. 10^9 organisms *L. pneumophila* (Philadelphia) killed 2/3 animals, *L. pneumophila* (Corby) killed 5/5 animals and *L. longbeachae* 1 (A5H5) killed 3/5 animals (Table 3-2). Animals exposed to an aerosol of the same dose of *L. longbeachae* sg 1 ATCC 33462 and *L. longbeachae* sg 2 ATCC 33484 showed no symptoms and were avirulent in this model despite similar numbers of organisms retained in the lung compared with virulent isolates (Table 3-2). A higher dose of approx. 10^{10} organisms was also used to test the type strain *L. longbeachae* ATCC 33462. However, despite larger numbers of organisms retained in the lungs (approx. 1×10^6) the strain remained avirulent and was unable to kill any animals (Table 3-2). Although slight

fluctuation in weight of guinea pigs was observed no specific symptoms of disease were evident. A passaged stock of the ATCC 33462 strain, obtained from the spleen of a guinea pig inoculated intra-peritoneally, was also avirulent in the aerosol model (Table 3-2).

To ensure that our stock of *L. longbeachae* ATCC 33462 had not become laboratory attenuated since it was obtained in 1987, a fresh isolate was obtained from the American Type Culture Collection (ATCC). The new isolate, obtained in 1997, was also avirulent in this model even with a test dose of 10^{10} organisms (Table 3-2). On post mortem examination of the lungs, however, it was noted that small foci of infection on the surface were evident although this did not appear to manifest itself through other symptoms. Therefore, avirulence of this strain in the animal model was not likelyto be due to attenuation.

The number of CFU retained in the lungs of guinea pigs was always only a small fraction of the number actually aerosolised in the test dose. On average, approx. 10^5 organisms were retained in the lungs using a test dose of 10^9 organisms. Therefore, it was assumed in further experiments that a test dose of 10^9 organisms would deposit approx. 10^5 organisms into the lungs of each animal used to evaluate a particular strain at that dose. This was confirmed further by random measurement of retained dose in experiments conducted at various points in time.

Histological staining of lungs taken from a guinea pig that had died after exposure to *L. pneumophila* (Philadelphia) or *L. longbeachae* (A5H5) showed that the air spaces of the lung parenchyma were filled with a dense cellular infiltrate consisting of neutrophils and monocyte cells (Fig 3.2 a and b). The appearance was consistent with a severe acute pneumonia as observed by other workers for strains of *L. pneumophila* sg 1 (Baskerville, *et al.*, 1983a, Baskerville, *et al.*, 1983b, Eisenstein, *et al.*, 1984). This is in contrast with the open spongy appearance of normal healthy lung tissue when lungs taken from uninfected guinea pigs are stained by the same procedure (Fig 3.2 c).

The results indicated that the aerosol model of infection was a suitable model for assessment of virulence of *L. longbeachae*.

3.3.3 Analysis of overseas strains of *L. longbeachae* by restriction fragment length polymorphism (RFLP)

Using typing methods previously developed in this laboratory (Lanser, *et al.*, 1990), the classification of the species and in particular serogroup of the overseas isolates of *L. longbeachae* were confirmed. The classifications of two isolates, D-1738 and D-1959 were proposed to be sg 2 at the time that they were received and one isolate, D-1820, did not have a suggested serogroup. This was done to ensure the correct classification of the isolates using a molecular method developed in our laboratory, prior to experimental work with the isolates. This was particularly important in the case of the guinea pig model, as it is time consuming and expensive. The RFLP typing result, using λclone2 as a probe, suggested that all of the isolates were likely to fall into the classification of sg 1 (Fig 3.3). This result was similar for the cosmid8 probe (data not shown).

Classification of all of the overseas isolates as *L. longbeachae* sg 1 was also confirmed using the *mip* gene sequencing methodology developed by Mr. R. Ratcliff in this laboratory (data not shown) (Ratcliff, *et al.*, 1998). Additionally, a latex agglutinating reagent specific for *L. longbeachae* sg 1 (Medvet Science Pty. Ltd., Adelaide, S.A., Australia) also reacted with all of the overseas strains including D-1959, D-1738 and D-1820.

3.3.4 Assessment of virulence of *L. longbeachae* serogroup 1 strains in an aerosol model of infection

Strains of *L. longbeachae* sg 1 were assessed for their ability to cause disease in five guinea pigs exposed to a dose (approx. 10^9 organisms) of each test strain. Results for each isolate were plotted as percentage weight gain or loss for each animal against number of days after exposure. The results were compared to each other and to a similarly generated plot for a strain of *L. pneumophila* sg 1 (Corby) (Jepras, *et al.*, 1985) and *L. longbeachae* sg 1 ATCC 33462 (Fig 3.4).

The Corby strain of *L. pneumophila* is a highly virulent isolate that produces a severe disease in guinea pigs. Infection with this strain causes rapid weight loss and death in 3 days or less for all test animals, shown by termination of the ribbon graph well prior to end of the experiment at day 7 (Fig 3.4A part i). A similar severe infection was also observed for *L. pneumophila* (Philadelphia) (Table 3-2). Strains of *L. longbeachae* sg 1 showed distinct differences in their ability to cause disease in this model of infection (Fig 3.4). A general assessment of the data suggested that there appeared to be three virulence groupings based on number of deaths and the severity of the disease as shown by weight loss and time to death. Statistical analysis was used to determine possible groupings using the SAS statistical package version 6 (SAS Institute Inc.). The analysis was performed by Ms. Nicole Chamberlain from the Department of Public Health, University of Adelaide. Cluster analysis was used to place strains into groups according to chosen variables.

3.3.4.1 Weight change

Initially, change in weight (weight difference) was thought to be a good predictor of virulence of a particular strain as it is a symptom that can be easily measured and recorded. However, cluster analysis did not distinguish strains that were avirulent, and did not kill any test animals, from those that were clearly virulent as evidenced by death of more than half of the animals in the test group (data not shown). Strains that killed test animals quickly would not allow time for a large weight loss to occur and hence would not be distinguished from those strains that did not produce severe weight loss symptoms and were avirulent. Similarly, when weight change was taken into account with the number of deaths that occurred for a particular test strain, avirulent and virulent strains could not be distinguised as above (data not shown). The results indicated that weight difference was not a good indicator of overall virulence of a particular strain of *L. longbeachae*.

3.3.4.2 Total number of deaths.

The number of animals killed by each test strain of *L. longbeachae* was then compared by cluster analysis. The analysis defined three statistically significant groupings classified as

Type 1, 2 or 3 (Table 3-3). Type 1 strains killed four or five guinea pigs and contained one overseas isolate (D-493) and an Australian isolate (K8B9). Type 2 strains were of moderate virulence and killed 2-3 animals in each test group. Five Australian isolates clustered in this category (A5E1, A5H5, K5H9, L6C9, and K4A1) including two overseas isolates (Atlanta-5 and D-880). Type 3 strains, were avirulent or killed only one guinea pig and represented the least virulent cluster grouping based on number of animals dead. Interestingly, six overseas strains of *L. longbeachae* sg 1 clustered in this group (LA-24, D-1959, D-1056, D-1624, D-63, D-1750) and two Australian isolates (A5H3 and A4C5), both of which killed one guinea pig each. The type strain *L. longbeachae* sg 1(ATCC 33462) would also be included in the type 3 cluster by default, as it is also avirulent in an aerosol model of infection. Interestingly, all Australian isolates tested were able to cause disease and death in this animal model. A fishers exact test (2-tail) also confirmed that the groups defined by the cluster analysis were statistically different from each other ($p=5.36 \times 10^{-7}$).

3.3.4.3 Mean time to death.

Time to death was also assessed as a variable for grouping of *L. longbeachae* strain virulence. Analysis of the raw data suggested that some virulent strains, defined by the cluster groupings based on number of animals dead in each test group shown in Table 3-3, killed animals more rapidly than others even though the total number of dead animals was the same for each. Mean time to death is a suitable variable of analysis due to the low numbers of guinea pigs (5) exposed in each test group and also this variable captures severity of the outcome of the experiment. Cluster analysis defined four groupings, however, cluster four contained only one strain, D-493, which was included in cluster three for simplicity of comparison. These three groupings were similar to that observed using number of animals killed as a variable. Type I strains were highly virulent and caused death with a mean number of days to death ranging from 4-5.8 (Table 3-4). Type 2 strains caused death of test animals with a mean number of days ranging from 6.4-7.6. The last category, type 3 strains had a mean of 7.8-8 days. Wilcoxons rank sum test determined that the clusters (1, 2 and 3=3/4

combined) were statistically significantly different from each other with p values of 0.0187 (C1 versus C2), p=0.0001, (C1 versus C3) and p=0.0001 (C2 versus C3). The Wilcoxons rank sum test is a non-parametric test and was used because the time to death data was not normally distributed and therefore the assumptions of parametric tests such as the t-test are not met (or are violated). The same groupings were seen using median time to death as a variable for non-normally distributed data such as seen with mean time to death.

Therefore, the 18 strains of *L. longbeachae* tested were classified into three main virulence types based on time to death cluster analysis (Fig 3.4). This data and the aerosol experiment results are summarised in Table 3-5. Type 1 strains killed the majority (>50%) of the animals in the test group and included strains A5H5 (Fig 3.4A part ii), K4A1 (Fig 3.4A part iii), K8B9 (Fig 3.4A part iv), A5E1 (Fig 3.4A part v), D-880 (Fig 3.4A part vi) and D-493 (Fig 3.4A part vii). The onset of death in some animals was very sudden and the severity of disease was similar to that produced by strains of *L. pneumophila* which would also fall into this category based on this criterium (Fig 3.4A part i). Type 2 strains caused death and disease in some animals and included strains K5H9 (Fig 3.4B part i), A5H3 (Fig 3.4B part ii), A4C5 (Fig 3.4B part iii), L6C9 (Fig 3.4B part iv), Atlanta-5 (Fig 3.4B part v), D-63 (Fig 3.4B part vi) and D-1750 (Fig 3.4 part vii). Type 3 strains were generally unable to kill any animals infected although one strain D-1959 killed one animal and also fell in this category (Table 3-5). Type 3 strains were classed as relatively avirulent because symptoms such as weight loss were sometimes observed (Table 3-5). Strains that fell in this class included D-1624 (Fig 3.4C part i), LA-24 (Fig 3.4C part ii), D-1056 (Fig 3.4C part iii) and D-1959 (Fig 3.4C part iv). Strain ATCC 33462, both the original stock obtained in 1987 (Fig 3.4C part v) and the more recent stock obtained in 1997 (Fig3.4C part vi), also belong to this cluster by default. This ATCC strain is unable to establish clinical disease even at doses as high as 10^{10} despite the fact that small foci of disease were evident in the lung post-mortem (Table 3-2). Similarly, the *L. longbeachae* sg 2 type strain would also belong to the type 3 group at it is also avirulent in the animal model (Table 3-2).

In summary, analysis of the results of the aerosol experiments determined that *L. longbeachae* sg 1 strains are virulent in an animal model, therefore justifying further study of this species. Although only a relatively small number of geographically diverse isolates (18) were examined, a few general trends were observed. The incidence of disease due to this species in Australia may in part be due to the inherent virulence of Australian isolates in comparison with those from elsewhere. None of the Australian isolates were avirulent in guinea pigs whereas several of the overseas isolates fell into this category independent of the grouping variable (Table 3-3, 3-4 and 3-5). A large proportion of the Australian isolates also belonged to the highly virulent type 1 strains in comparison with foreign isolates that rarely fell in this category (Table 3-5).

3.3.5 Infectivity of *L. longbeachae* strains in U937 cells

Legionellosis is a disease characterised by the ability of *L. pneumophila* to infect and grow within alveolar macrophages and it is generally assumed that replication in phagocytes is a hallmark of virulent *Legionella* species (Levi, *et al.*, 1987, O'Connell, *et al.*, 1996b). Other pathogenic and non-pathogenic species of *Legionella* have been assessed in this model of infection to correlate pathogenicity with ability to infect U937 cells a macrophage like cell line (O'Connell, *et al.*, 1996b). Some non-pathogenic species were shown to be infective for these cell types indicating that they may cause disease in the human host if opportunity allows contact with a susceptible individual.

Infective doses (ID_{50}) were determined at 72 hours post infection, for selected strains of *L. longbeachae*, in a U937 model of infection (Cianciotto, *et al.*, 1989b). Several strains were chosen empirically from the IMVS culture collection and sent to Dr. Nicholas Cianciotto for assessment of relative infectivities in this cell culture model (Table 3-6). Two infections were set up for each test strain although a result was not achieved in duplicate for two strains, K4A1 and ATCC 33462. A *L. pneumophila* positive control (strain 130b) was also set up in each experiment as it is well established that this strain can replicate efficiently in U937 cells

(Cianciotto, *et al.*, 1989a, Dowling, *et al.*, 1992, O'Connell, *et al.*, 1996b, Pearlman, *et al.*, 1988).

L. pneumophila (130b) was able to infect the U937 cells exhibiting an ID_{50} of less than 1200 bacteria (Table 3-6), consistent with previously published observations (O'Connell, *et al.*, 1996b, Pearlman, *et al.*, 1988). All of the Australian isolates of *L. longbeachae* sg 1 were infective for U937 cells and had an ID_{50}s that were comparable to each other and to *L. pneumophila* (130b), with the exception of strain K5H9 (Table 3-6). Strain K5H9 had a variable infectivity with an ID_{50} of 10^2 bacteria in one infection and 10^4 in a replicate test (Table 3-6). Interestingly, this strain killed 3/5 guinea pigs in the aerosol model but grouped as a type 2 strain based on time to death. This may reflect the slower progressing disease caused by this organism which may be related to its ability to establish infection in macrophages. The *L. longbeachae* ATCC 33462 and 33484 type strains both appeared incapable of intracellular growth (Table 3-6). High ID_{50}s of 10^4 bacteria suggest that the strains are impaired for intracellular replication within U937 cells (O'Connell, *et al.*, 1996b). The result does not preclude the possibility however that these isolates may still be capable of surviving within the U937 cells. The result with our original ATCC 33462 type strain are in contrast with those of O'Connell *et al.* who showed that this same strain (Long Beach 4) was able to infect U937 cells with an ID_{50} of less than 1000 bacteria (O'Connell, *et al.*, 1996b). The result is indicative that the original ATCC 33462 isolate has become attenuated, however, a recently acquired stock of ATCC 33462 is also avirulent in the guinea pig model of infection (Table 3-2). In general, the ability of Australian isolates of *L. longbeachae* to infect U937 cells correlated well with their ability to cause disease in an animal model of infection (Table 3-6).

3.3.6 Transmission electron microscopy analysis of lung tissue infected with *L. longbeachae* serogroup 1

In mammalian and protozoan host cells, *L. pneumophila* sg 1 replicates intracellularly in a phagosome surrounded by host cell rough endoplasmic reticulum (RER) (Abu Kwaik, 1996, Horwitz, 1983b, Swanson and Isberg, 1993). This recruitment of host organelles occurs within the first 2 - 4 hours after uptake. Electron microscopy (Em) of *L. longbeachae* infected lung tissue was therefore undertaken to determine if *L. longbeachae* sg 1 shares any of the features of the intracellular life cycle of *L. pneumophila*. Lungs were taken from guinea pigs three days post-infection with *L. longbeachae* sg 1, strain ATCC 33462 and A5H5 and hence sections were not representative of a particular stage of infection.

Phagosomes containing the virulent A5H5 strain were observed in macrophages by electron microscopy (Fig 3.5A and B). Some phagosomes appeared quite large in relation to the total size of the macrophage and contained several bacteria (Fig 3.5B). Mitochondria and host cell organelles were seen in close proximity to the phagosomes containing the bacteria and the membrane appeared to be surrounded by electron dense structures (ribosomes) (Fig 3.5A). Electron lucent cytoplasmic vacuoles were apparent in the majority of strain A5H5 bacteria. This finding is consistent with the observation of other workers using *L. pneumophila* (Chandler, *et al.*, 1979b, Horwitz, 1983b). Interestingly, macrophages containing the avirulent ATCC 33462 type strain did not appear to have electron lucent vacuoles (Fig 3.5C and D). Phagosomes containing several A5H5 bacteria (Fig 3.5B) may be representative of later stages of infection. The membrane of these potential later stage vacuoles, did not appear to be surrounded with ribosomes but generally appeared to be associated with host cell organelles. Fig 3.5B may also be representative of a phagosome that has fused with lysosomes or a cell that is dying, perhaps due to release of bacteria. The nucleus of the cell still appears intact suggesting the former is more likely. The debris in the phagosome may be indicative of the general tissue destruction occurring within the lung. Phagocytic cells may engulf not only bacteria but also debris occurring as a result of cell

destruction in their vicinity. Lungs taken from animals infected with A5H5 exhibited severe damage and consolidation (Fig 3.2b), suggesting that this is likely. It may be that this strain is taken up by classical phagocytosis (Fig 3.5B), since coiling phagocytosis was not observed in any of the fields examined.

The avirulent ATCC 33462 strain was also found in phagosomes in macrophages (Fig 3.5C and D). Figure (3.5C) may represent an early event just after uptake of the bacteria into the macrophage cell with four phagosomes each containing a single bacterium. The arrow shows a phagosome that appears to have just engulfed a bacterium suggesting classical uptake of this organism by cup-like pseudopod extensions from the phagocyte. Some phagosomes appeared to have fused to form one large phagosome containing a few bacteria and this may represent a later stage of infection (Fig 3.5D). The vacuole containing these bacteria was surrounded by a smooth membrane that did not appear to be associated with any host organelles or ribosomes. Association of phagosomes with host cell organelles and ribosomes was not apparent in any of the fields examined (data not shown). Large vacuoles containing several bacteria observed for A5H5 were not seen with ATCC 33462.

In general, phagosomes containing the ATCC 33462 strain were small and relatively clear in comparison with those containing the virulent A5H5 strain. Strain A5H5 was generally associated with larger phagosomes, containing several bacteria, that also appeared to contain cellular debris.

3.4 Discussion

The aerosol model of infection of guinea pigs was a good model for assessment of virulence of isolates of *L. longbeachae*. This model has been used successfully for infection of guinea pigs with strains of *L. pneumophila* and mimics the "natural" route of infection due to this organism (Baskerville, *et al.*, 1981, Blander, *et al.*, 1990, Breiman and Horwitz, 1987, Eisenstein, *et al.*, 1984, Fitzgeorge, *et al.*, 1983).

Other models of infection have been used and include inoculation into the peritoneal cavity (Fields, *et al.*, 1986, Fitzgeorge, *et al.*, 1983, Katz and Hashemi, 1982), via the trachea through surgery (Cianciotto, *et al.*, 1990b) and intranasally (Fitzgeorge, *et al.*, 1983, Katz and Hashemi, 1982). The intra-peritoneal model of infection has been useful for analysis of virulence of strains of *L. pneumophila,* however, this model was not suitable for assessment of *L. longbeachae* strains as they were relatively avirulent in comparison with *L. pneumophila*. Intranasal inoculation is an unreliable method of inoculation as it can result in flooding of the upper respiratory tract rather than deposition of the organism into the lower respiratory tract where infection is established. Intratracheal inoculation was not considered since surgical trauma of the animal might be a factor affecting the outcome of disease. Original experiments with aerosol inoculation of guinea pigs involved the use of a Henderson-type apparatus (Baskerville, *et al.*, 1981). The disadvantages of this method of inoculation were the wide range of particle sizes generated by the Collison spray (average particle size (< 5μm) and the continuous dosage that may result in the retention of abnormally high numbers of organisms in the lungs.

The model developed in this study allowed test animals to be retained within a chamber and thus exposed to a defined dose of aerosolised *Legionella*. *L. longbeachae* sg 1 strains were shown to be pathogenic by this method of inoculation in similar fashion to *L. pneumophila*. The symptoms produced and the time course of the disease were similar to that observed by other workers for *L. pneumophila* (Cianciotto, *et al.*, 1990b).

A number of *L. longbeachae* isolates were tested in the aerosol model of infection. Strains were classified into three types based on their ability to cause disease in the experimentally infected guinea pigs. Type 1 strains were highly virulent, and rapidly killed most animals in the test group. The majority of the isolates in this category were from Australia. However, two foreign isolates also fell into this group suggesting that type 1 organisms are not unique to Australia. Type 2 strains caused disease and killed animals in the test group, however the time course of the disease was moderate in comparison with type 1

strains. Generally, type 2 strains killed fewer animals and had a slower progressing disease in comparison with type 1 strains. The remaining Australian isolates tested fell into this category although three overseas isolates were also represented in this group. Type 3 strains represented those isolates that were relatively avirulent in an animal model of infection. Most were unable to cause disease although a strain that killed one animal on day 6 also fell into this category. This group contained only overseas isolates. No Australian isolate tested was avirulent in the aerosol model. Whether this is a true reflection of inherent virulence in the Australian isolates is difficult to ascertain. Statistical cluster analysis showed a strong trend for a higher proportion of Australian isolates to fall into virulent groups while the relatively avirulent grouping contained only overseas isolates. The groupings are reflections of trends in the data and are by no means exact due to the small number of animals tested for each strain and the relatively small number (18) of isolates tested. A larger panel of *L. longbeachae* strains would need to be analysed to make any definite conclusions. One factor that may influence groupings may be variations in the test dose administered. The cluster grouping determined for strain D-1056, given the lowest dose of 7.5×10^8 organisms may have changed if a dose closer to 10^9 organisms been administered. Additionally, innate resistance of the out-bred guinea pigs used may influence disease outcome. The data indicated that a more detailed analysis of the isolates is important and may lead to the discovery of virulence factors unique to the Type 1 isolates that may be lacking in type 2 and 3 strains.

The U937 infection studies indicated that the Australian isolates of *L. longbeachae* sg 1 were likely to be pathogenic as they were all capable of infecting these cells at levels comparable to *L. pneumophila* and other virulent species. This result was consistent with the animal model data, as strains that could infect the U937 cells were also capable of causing disease. There was only one inconsistency in this work, and that was the results for the original stock of *L. longbeachae* ATCC 33462 obtained in 1987. Results with this stock in the U937 model showed that it was unable to infect macrophages while this same strain

(ATCC 33462/Long Beach 4) had been shown previously to be able to infect these cells at a level comparable with *L. pneumophila* (O'Connell, *et al.*, 1996b). It is unusual for a strain to be capable of infecting macrophages and unable to cause disease in an animal model of infection (O'Connell, *et al.*, 1996b). It would be interesting to test the O'Connell Long Beach 4 ATCC 33462 strain in the animal model to determine if it is capable of causing disease. The recently obtained stock of ATCC 33462 (1997) was not assessed by Dr. Cianciotto and therefore we do not know if is infective for U937 cells. Pathology of lungs taken from animals infected with this strain (10^{10} CFU) suggest that it is able to set up a minor infection in the lung even though it is unable to establish clinical disease, as reflected in the low ID_{50} result. Virulence is a multi-factorial process and most likely involves more than just the ability to infect macrophage cells, therefore this alone may not necessarily be a good indicator of the pathogenic capability of a particular strain.

As an initial approach to understanding the intracellular nature of pathogenesis of *L. longbeachae* sg 1, transmission electron microscopic examination of infected lung tissue was undertaken. The results were consistent with the organism being engulfed by macrophages, and they appear to be taken up classical phagocytosis. The data is difficult to interpret as the sections were taken from infected lung tissue and not from macrophage cells infected *in vitro*. Under *in vitro* conditions, various stages of infection can be examined and compared with each other. In these experiments lungs were taken from infected animals three days after exposure and hence may reflect all stages of infection together with the immunogenic response of the guinea pig to infection. Relatively few ATCC 33462 bacteria were seen in comparison to A5H5. This may be due to clearance of bacteria from the lung, since this strain is avirulent in an animal model. Strain ATCC 33462 was contained in phagosomes that differed morphologically from those harbouring strain A5H5 since they were small, did not appear associated with host cell organelles and often contained a single bacterium. Strain A5H5, in contrast, may replicate in a phagosome that associates with cellular organelles, and ribosomes, in a manner similar to *L. pneumophila* sg 1 (Abu Kwaik, 1996, Horwitz, 1983b,

Swanson and Isberg, 1993). The layer of electron dense structures around the phagosome was not heavy, as has been observed with *L. pneumophila,* but was observed around the entire perimeter of the phagosome. The large size of the vacuoles observed for A5H5, however, may be indicative of fusion of the phagosome with host cell lysosomes (Horwitz, 1983b, Horwitz and Silverstein, 1983). This is further supported by the observation that the phagosomes appear to contain debris. The bacteria in these phagosomes also do not appear damaged, perhaps suggesting that virulent strains of *L. longbeachae* sg 1 may be able to survive and multiply within phagosomes that have fused with lysosomes. *L. micdadei* can multiply in macrophages but the phagosomes that contain them are not studded with ribosomes (Weinbaum, *et al.*, 1984) and appear to fuse with host cell lysosomes (Rechnitzer and Blom, 1989). How *L. micdadei* survives the killing mechanisms of the host remains unclear. Therefore it is possible that strain A5H5 survives in a phagolysosome in a manner similar to *L. micdadei.* However the phagosome containing A5H5 appeared to be studded with ribosomes, suggesting that this may not be the case. The large size of the vacuole may be due to uptake of cellular debris by the macrophages, indicative of tissue destruction occurring in the lung due to the severity of the infection caused by this strain. *In vitro* models are likely to be a better model to study the intracellular life cycle as the time course of infection can be controlled so that an assessment based on time after infection can be correlated with ultrastructural features.

An analysis of virulence of *L. longbeachae* sg 1 is important for several reasons. It is a virulent species of *Legionella* as determined in an animal model of infection with a high incidence of disease association in Australia. Its increased incidence in Australia may simply be due to differences in the manufacture of potting mix in different countries, or it may be a reflection of the increased surveillance for this strain in Australia. Alternatively, it may be due to virulence factors inherent in Australian isolates. Very little is known about the intracellular lifecycle of this species and what factors contribute to pathogenesis. These

factors may be shared with *L. pneumophila* and other species of *Legionella* or they may be unique to *L. longbeachae*.

3.5 Summary

A guinea pig model of experimental legionellosis was established for assessment of virulence of isolates of *L. longbeachae*. The results showed that there were distinct virulence groupings of *L. longbeachae* sg 1 strains based on the severity of disease that was produced in this model. Infection studies performed with U937 cells also confirmed that this species of *Legionella* was potentially virulent as determined by the ability to multiply in these phagocytic cells. Electron microscopy of infected lung tissue determined that *L. longbeachae* sg 1 appears to be taken up by classical phagocytosis rather than coiling phagocytosis. Possible ribosome studding, apparent around phagosomes containing A5H5 suggest that it may replicate in a specialised compartment in a manner similar to *L. pneumophila* (Philadelphia). However, this feature was not significant and additional features of the vacuole suggest it is likely that phagosomes containing *L. longbeachae* sg 1strains fuse with lysosomes in a manner similar to that suggested for *L. micdadei*.

Figure 3.1

The apparatus used to expose guinea pigs to an aerosol dose of *Legionella* strains. A perspex box, with a removable lid, was constructed to house the guinea pig and measured 220 by 220 by 240 mm. A nebuliser bowl was connected to the pump therapy kit on the outside of the box containing the dose of the test organism. A vent on the opposite side of the box was connected to a tube that bubbled the exhausted aerosol through 70% ethanol as a safety measure. Each guinea pig was exposed to an aerosol of the test organism until the nebuliser bowl was emptied (approx. 15 minutes).

Sealed perspex chamber

Nebuliser bowl

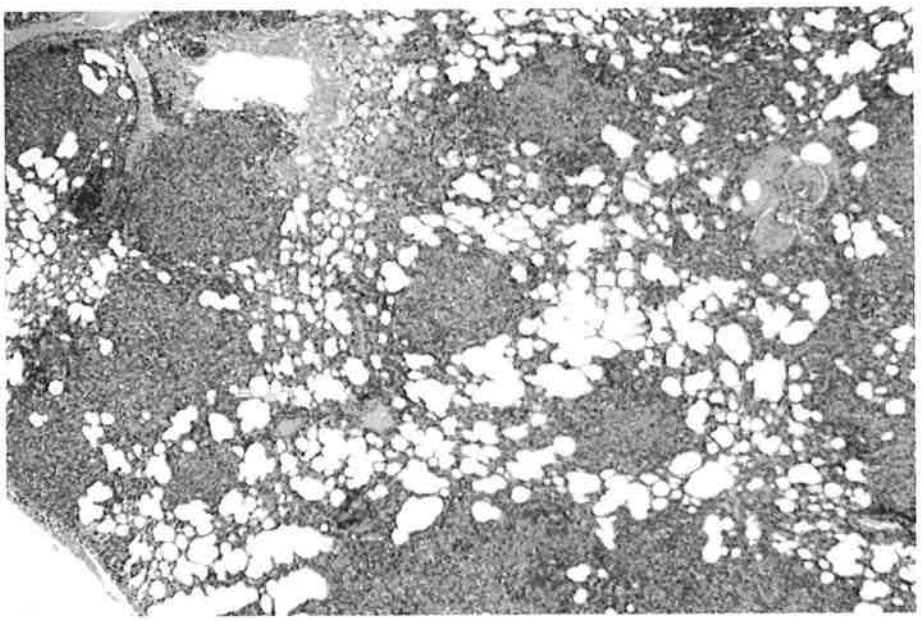
Flask containing 70% ethanol

Nebuliser pump therapy kit

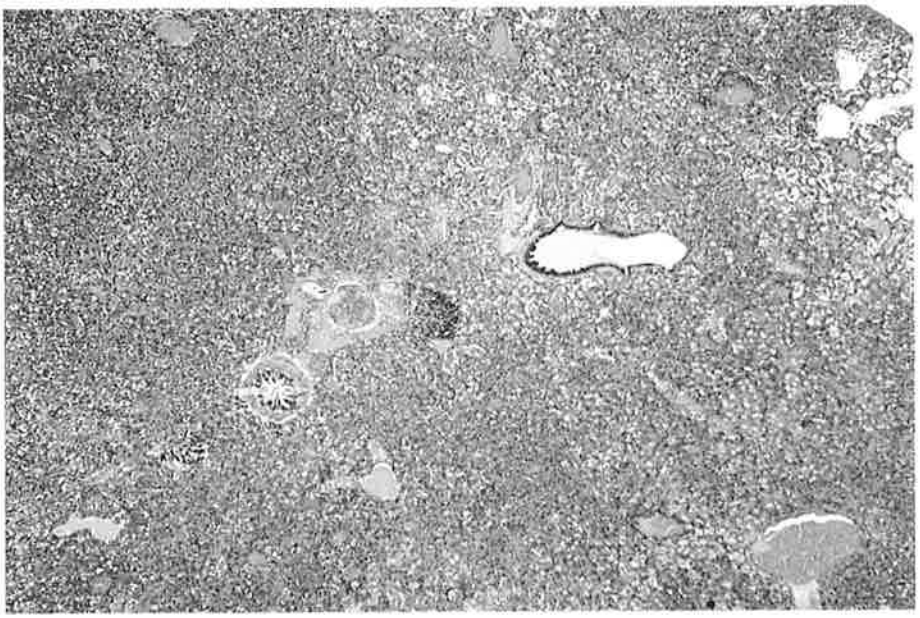
Figure 3.2

Lungs taken from guinea pigs, that died or were euthanased, following infection with *Legionella* strains, were stained for histological examination to confirm pneumonic disease. Lungs were first fixed in 10% neutral buffered formalin and 3 mm slices were processed into paraffin wax. Sections of 5 μm were stained with Hematoxylin and Eosin. Lung photos are shown at 40 × magnification. Staining of lungs infected with (a) *L. pneumophila* (Philadelphia) or (b) *L. longbeachae* sg 1 (A5H5) showed that they contained a dense cellular infiltrate consisting predominantly of neutrophils and monocyte cells, and the appearance was consistent with severe acute pneumonia. In contrast, lungs taken from healthy uninfected guinea pigs (c), stained by the same procedure, show an open spongy appearance and clear air spaces of the parenchyma.

a



b



c

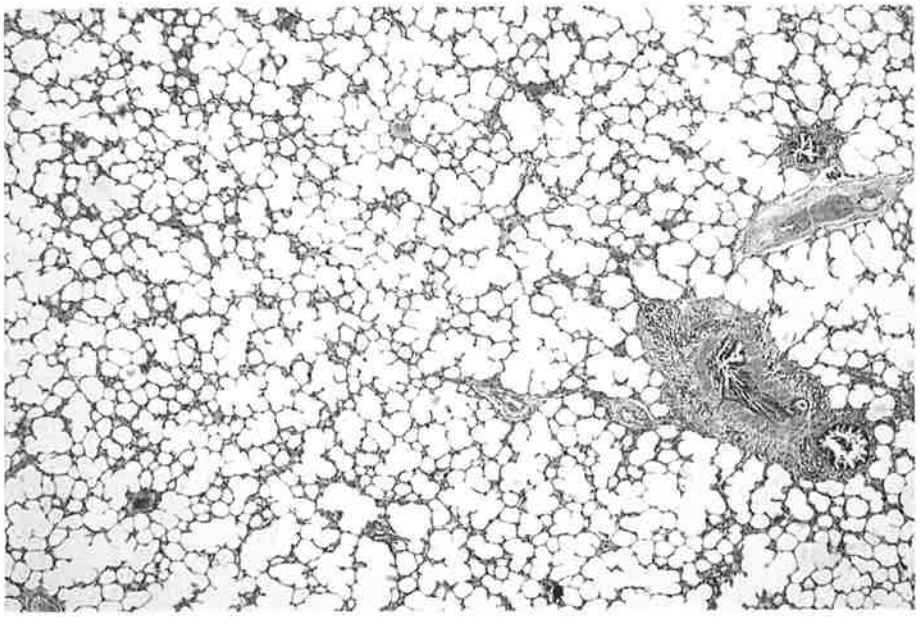
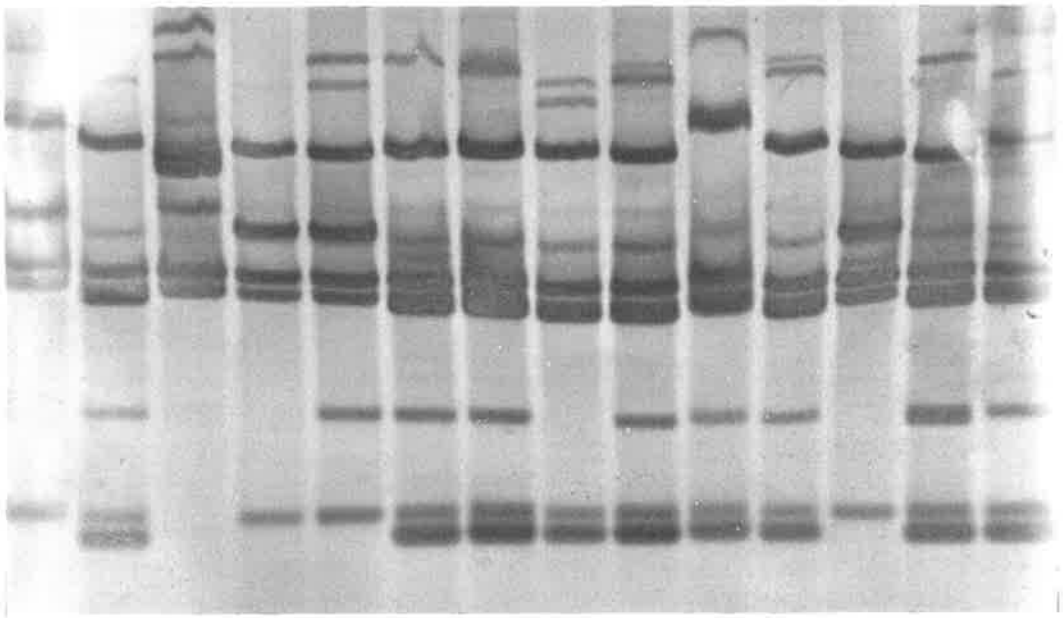


Figure 3.3

Restriction fragment length polymorphism (RFLP) typing analysis of *L. longbeachae* isolates. Chromosomal DNA was digested with *Hind*III and *Bam*HI, separated by gel electrophoresis transferred onto nylon membrane. The filter was probed with a Digoxigenin labelled Lambda clone, λclone2, chosen emperically from a genomic bank constructed from the type strain *L. longbeachae* sg 1 (ATCC 33462). Tracks show overseas isolates, listed as follows. Lane 1: D-1959, Lane 2: D-1738, Lane 3: *L. longbeachae* sg 2 ATCC 33484, Lane 4: D-1820, Lane 5: D-1992, Lane 6: D-1751, Lane 7: D-1750, Lane 8: D-1624, Lane 9: D-1056, Lane 10: D-1028, Lane 11: D-880, Lane 12: D-493, Lane 13: D-63, Lane 14: *L. longbeachae* sg 1 ATCC 33462,



1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.4

Percentage weight gain or loss in guinea pigs exposed to an aerosol of different strains of *Legionella.* Guinea pig death is indicated by termination of the ribbon graph prior to the end of the experiment at day seven. Animals were exposed to a test dose of approx. 10^9 CFU and were classified into virulence groups according to the severity of the disease produced. Groupings were assigned based on time to death using cluster analysis (SAS Institute Inc.).

3.4A - Type 1 strains.

Type 1 strains were highly virulent strains that killed more than 50% of the guinea pigs in the test group. The mean number of days before death occurred ranged from 4 – 5.8. Type 1 *L. longbeachae* sg 1 strains produced a disease in guinea pigs that was similar to that observed for strains of *L. pneumophila.* A representative graph of disease outcome due to *L. pneumophila* strain Corby is shown in 3.4A part i. Strains of *L. longbeachae* sg 1 that clustered in this group include: A5H5 (ii), K4A1 (iii), K8B9 (iv), A5E1 (v), D-880 (vi) and D-493 (vii).

3.4B - Type 2 strains.

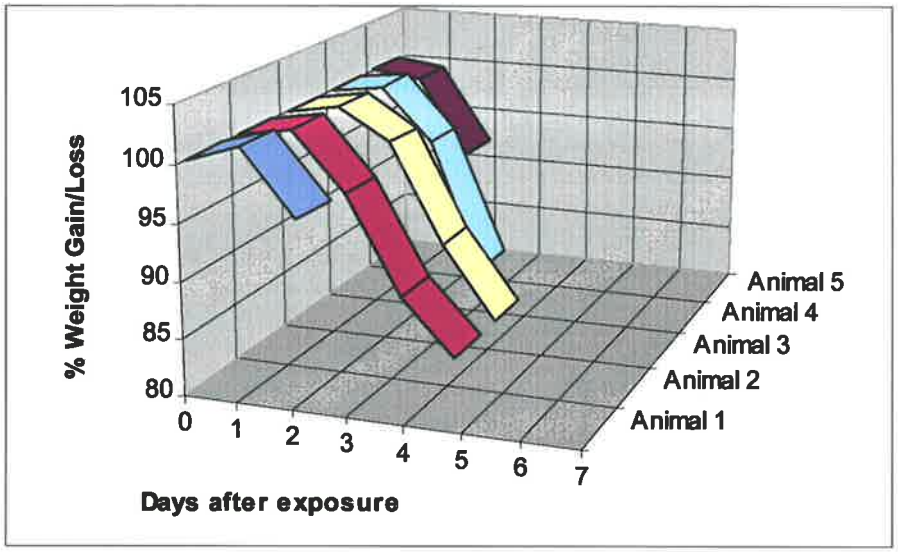
Type 2 classified strains were moderately virulent strains establishing disease and causing death in some animals in the test group. The mean number of days before death occurred ranged from 6.4 – 7.6. Strains of *L. longbeachae* sg 1 that clustered in this group include: K5H9 (i), A5H3 (ii), A4C5 (iii), L6C9 (iv), Atlanta-5 (v), D-63 (vi) and D-1750 (vii).

3.4C - Type 3 strains.

Type 3 classified strains were relatively avirulent strains and generally were unable to kill any animals although one strain killed one animal. The mean number of days before death determined for this group was 7.8 – 8 days. Strains of *L. longbeachae* sg 1 that clustered in this group include: D-1624 (i), LA-24 (ii), D-1056 (iii) and D-1959 (iv). The type strain *L. longbeachae* 1 ATCC 33462, original stock (v) and recent stock (vi), was placed in this group by default as it is also avirulent in guinea pigs at a test dose of 10^{10} CFU.

Figure 3.4A - Type 1 strains

i



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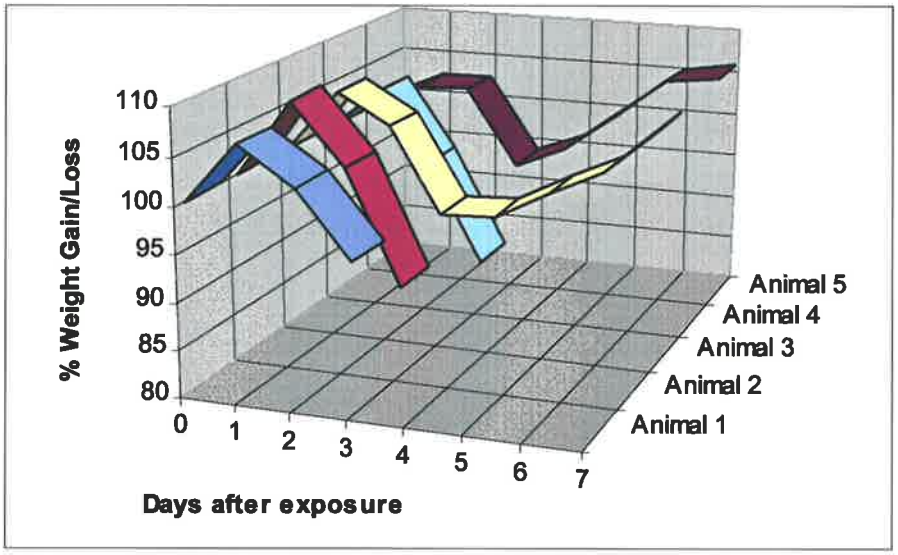

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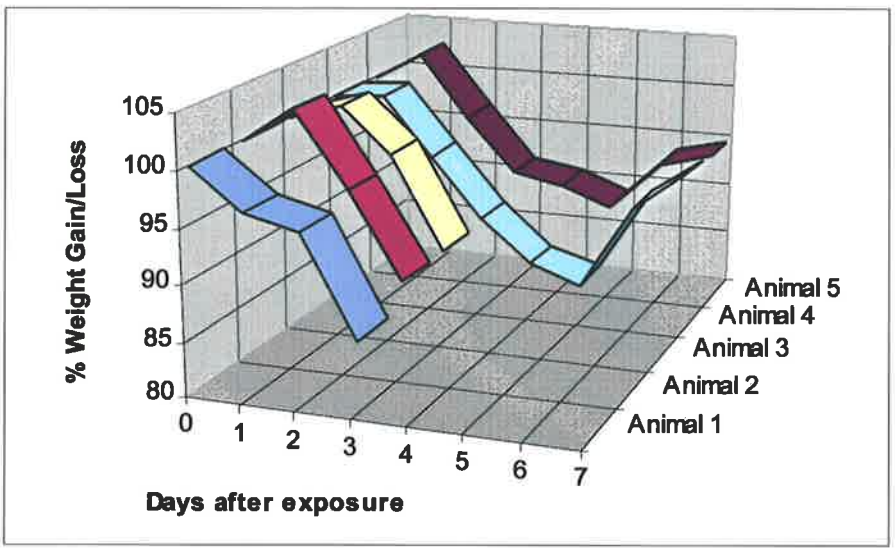

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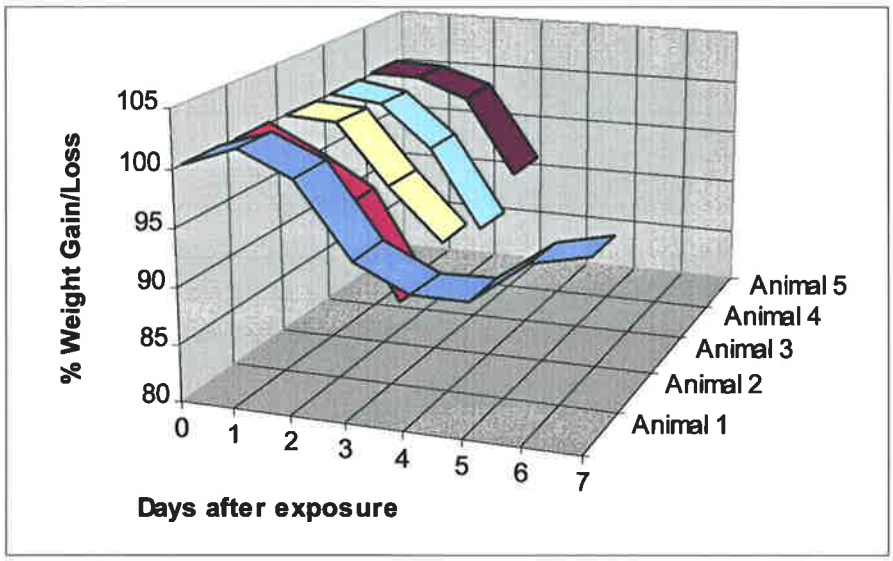
105
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% Weight Gain/Loss
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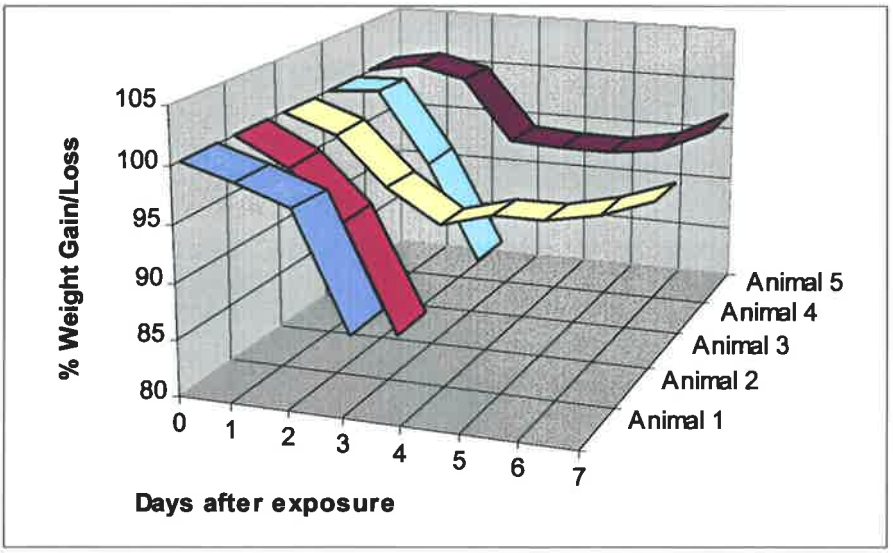
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

v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% Weight Gain/Loss
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Days after exposure
Animal 5
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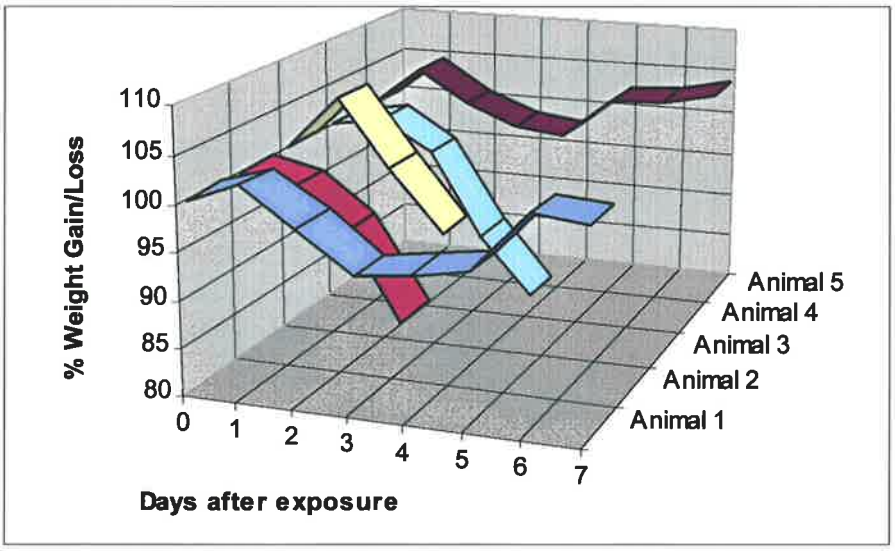
Animal 1

vi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% Weight Gain/Loss
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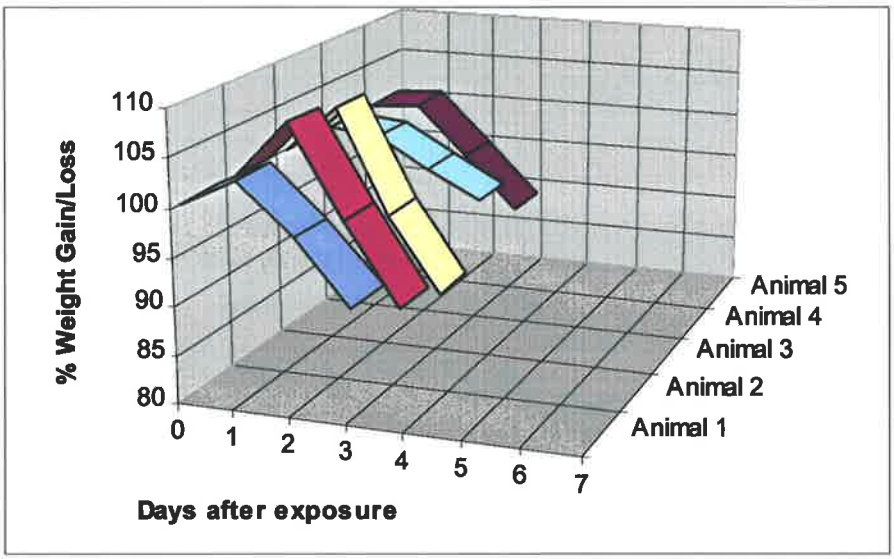
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

vii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Days after exposure
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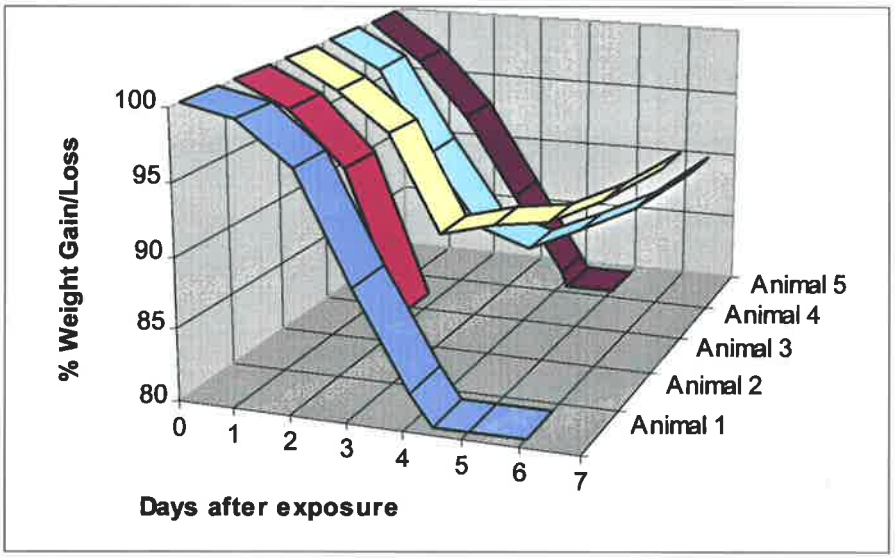
Animal 4
Animal 3
Animal 2
Animal 1

Figure 3.4B - Type 2 strains

i



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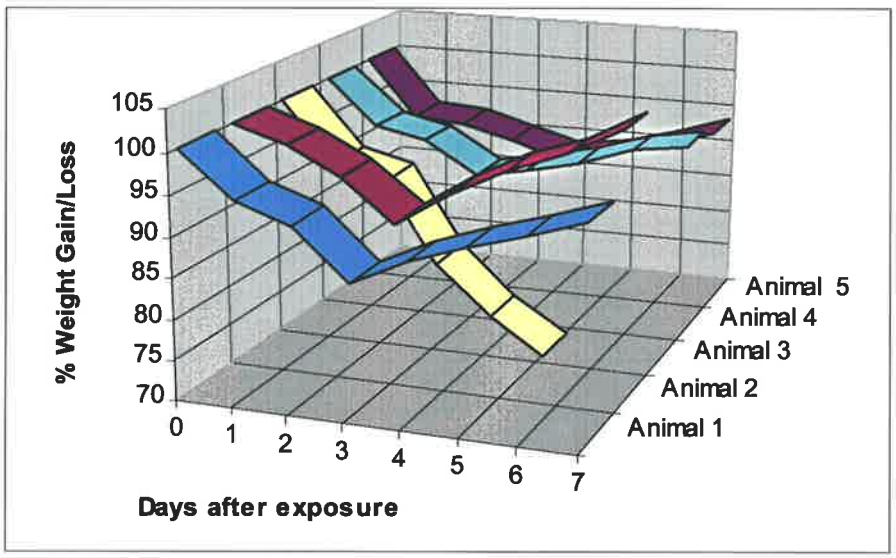

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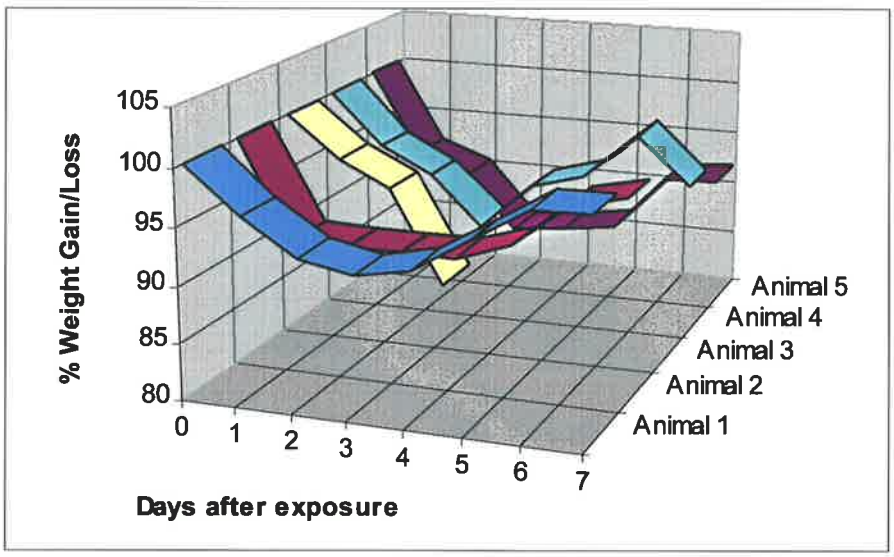

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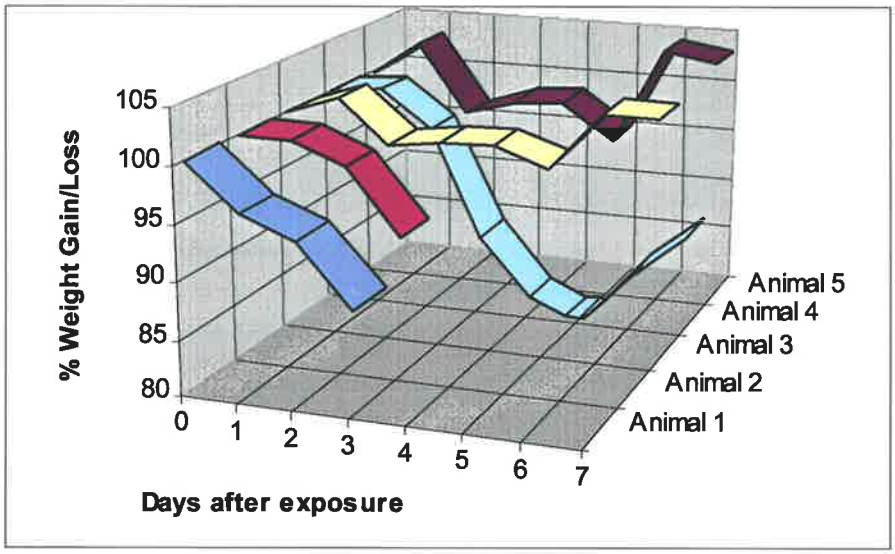
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% Weight Gain/Loss
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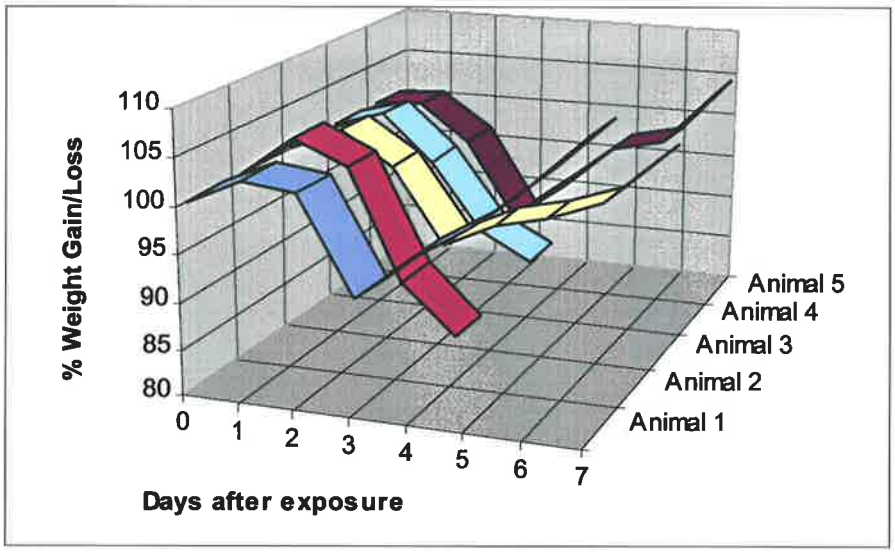
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

v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% Weight Gain/Loss
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Days after exposure
Animal 5
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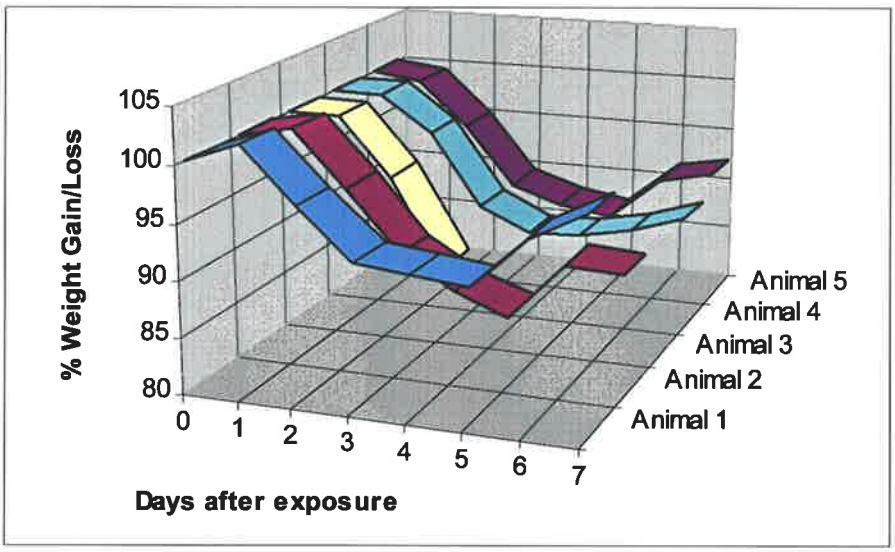
Animal 3
Animal 2
Animal 1

vi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% Weight Gain/Loss
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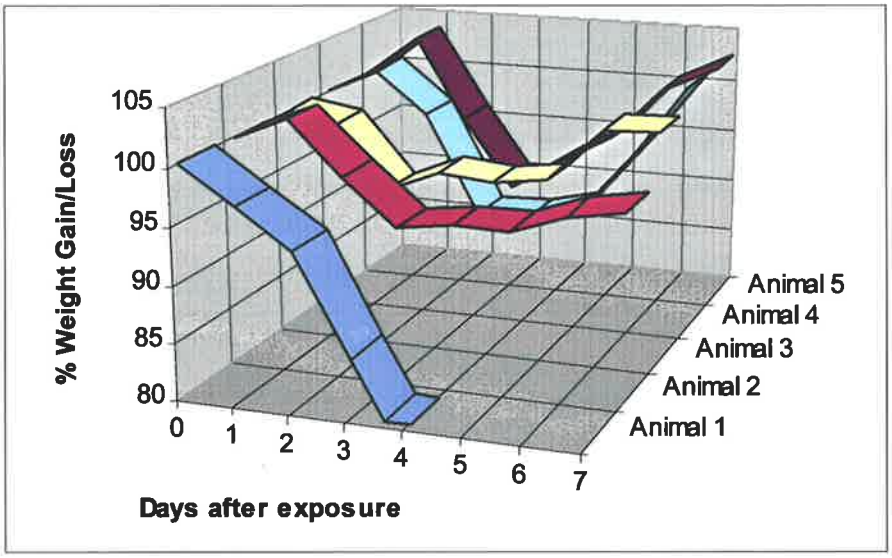
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

vii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% Weight Gain/Loss
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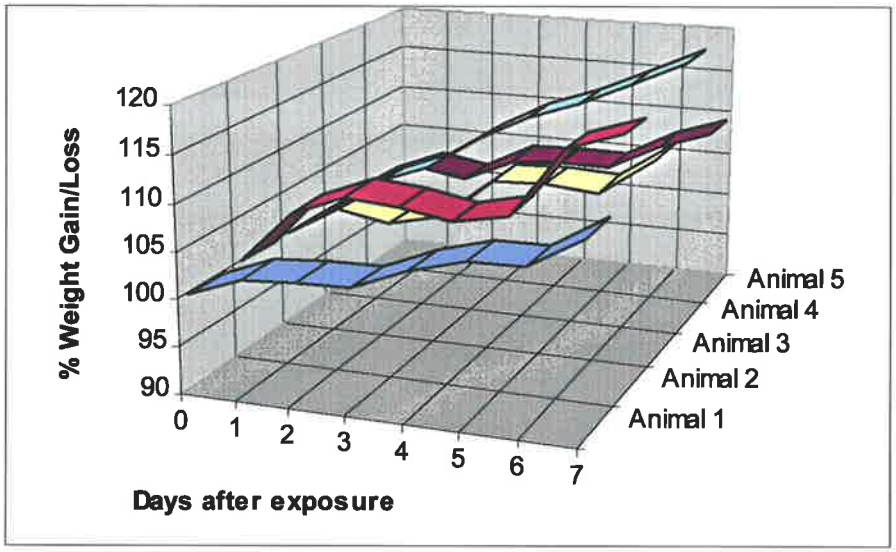
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

Figure 3.4C - Type 3 strains

i



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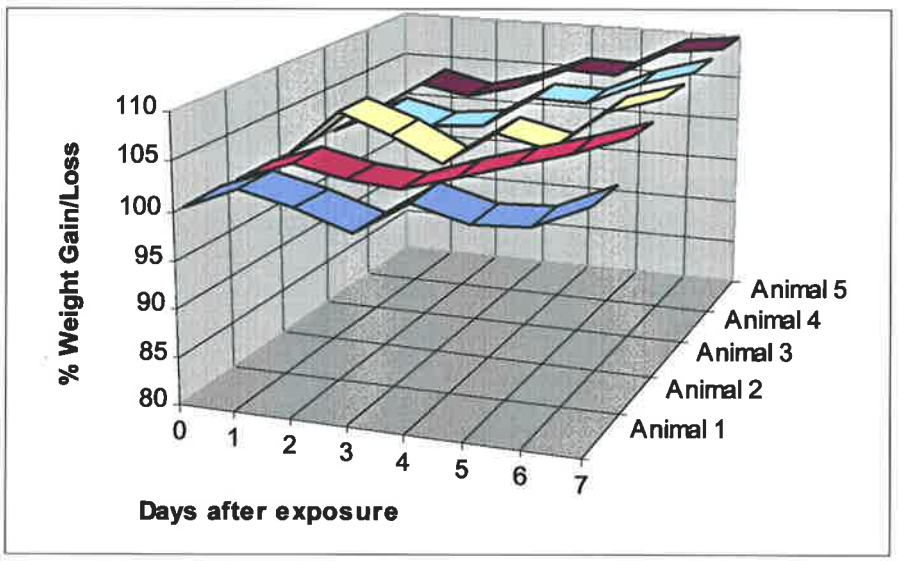

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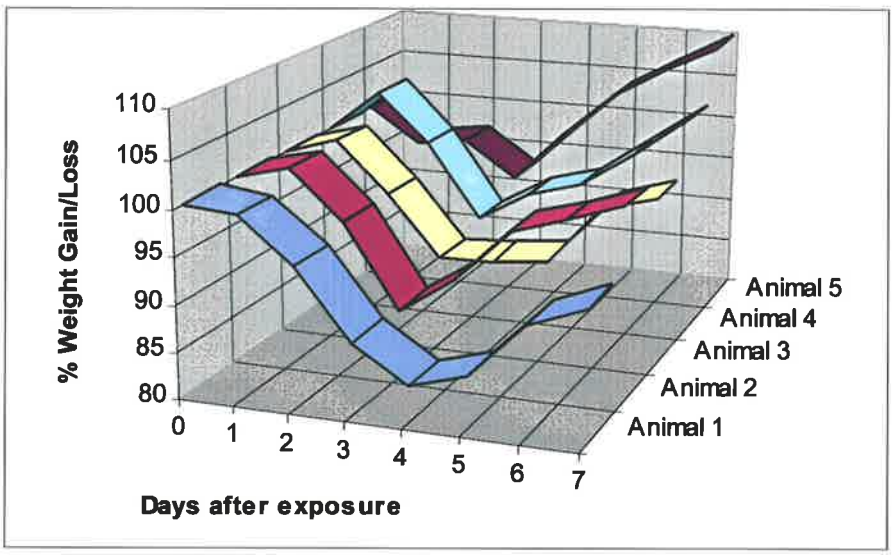

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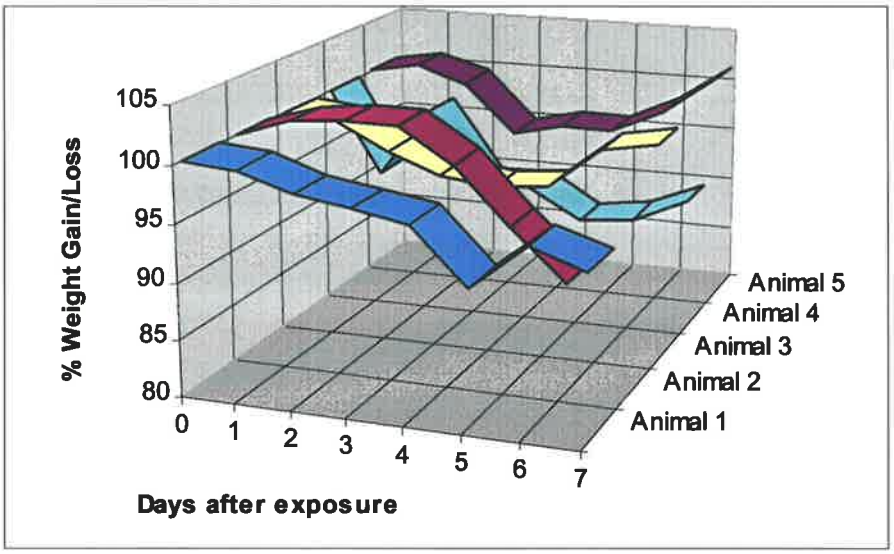
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% Weight Gain/Loss
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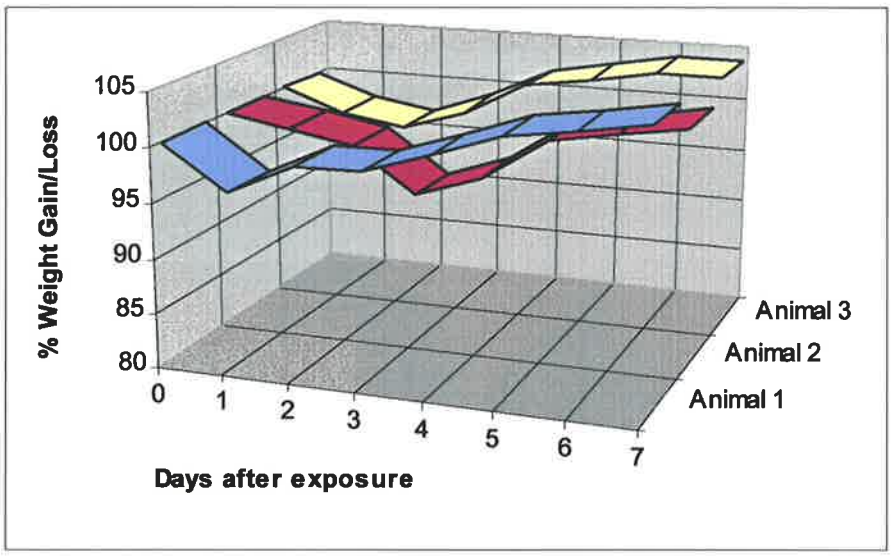
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

v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% Weight Gain/Loss
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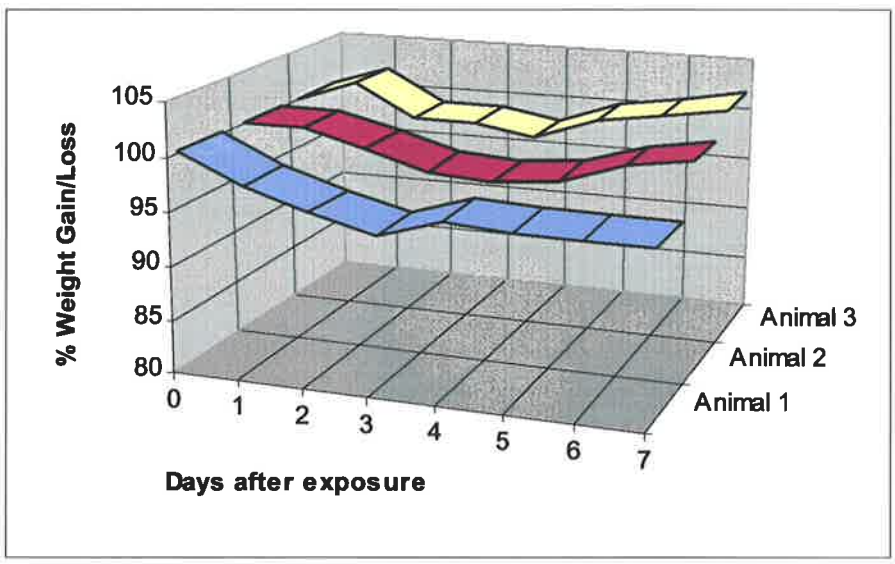
Days after exposure
Animal 3
Animal 2
Animal 1

vi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% Weight Gain/Loss
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Days after exposure
Animal 3
Animal 2
Animal 1

Figure 3.5

Electron micrograph showing the intracellular location of *L. longbeachae* sg 1 strains Lungs were taken from a guinea pig infected with ATCC 33462 avirulent type strain and A5H5, a virulent Australian clinical isolate. Three days after exposure, the animal was euthanased and the lungs prepared for examination by electron microscopy.

A: *L. longbeachae* sg 1 strain A5H5 bacteria (B) observed in a membrane bound vacuole within a macrophage. Potential ribosome studding of the vacuole membrane is shown by an arrow. Host organelles such as mitochondria (**M**) appear associated with the vacuole. Magnification (× 15, 900; bar, 1μm).

B: Ultra-thin sections of a vacuole containing several A5H5 bacteria. The arrow shows a single bacterium in a separate vacuole close to surface of macrophage membrane suggesting classical uptake by phagocytic cells of this strain. Magnification (× 10, 335; bar, 1μm).

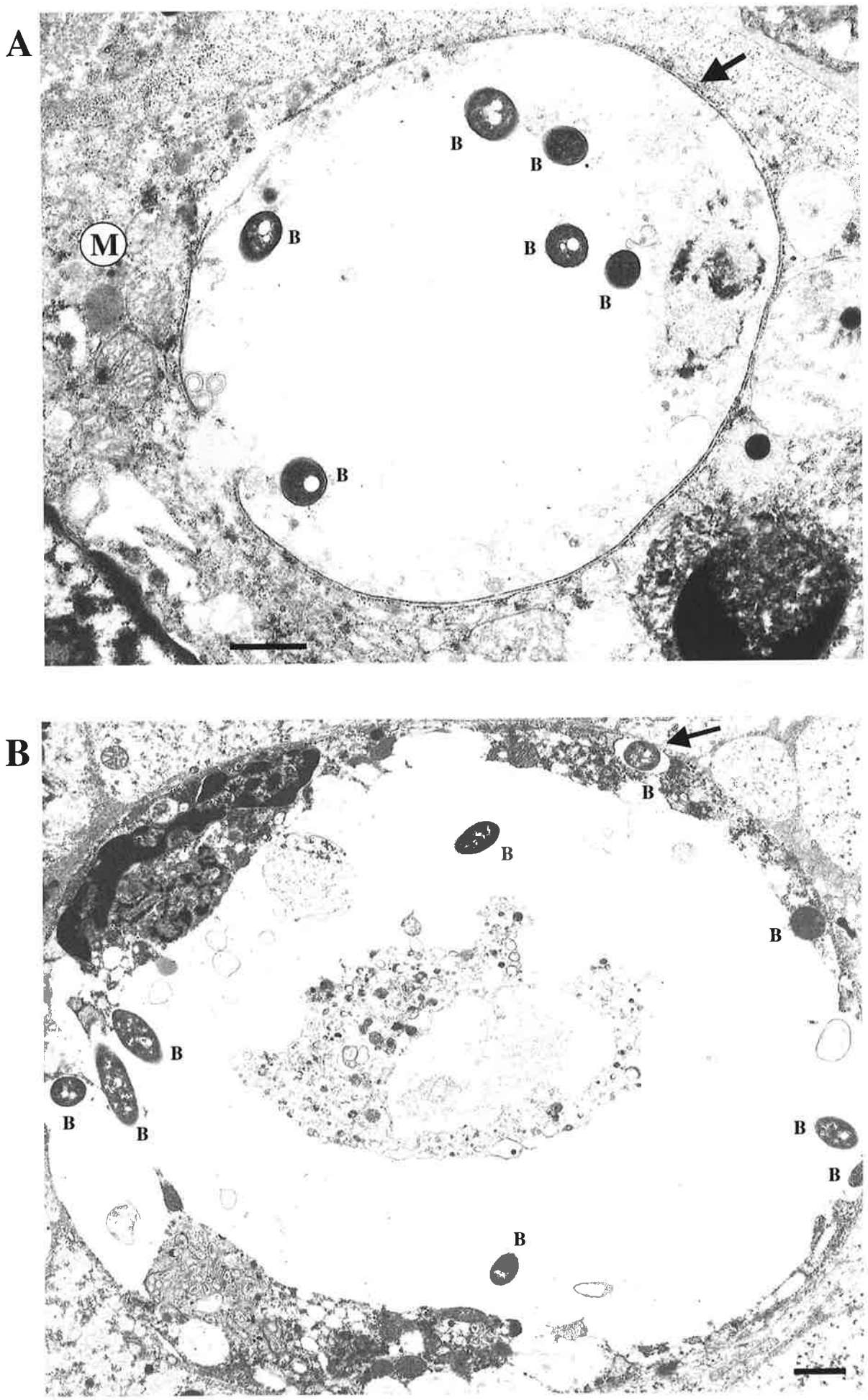
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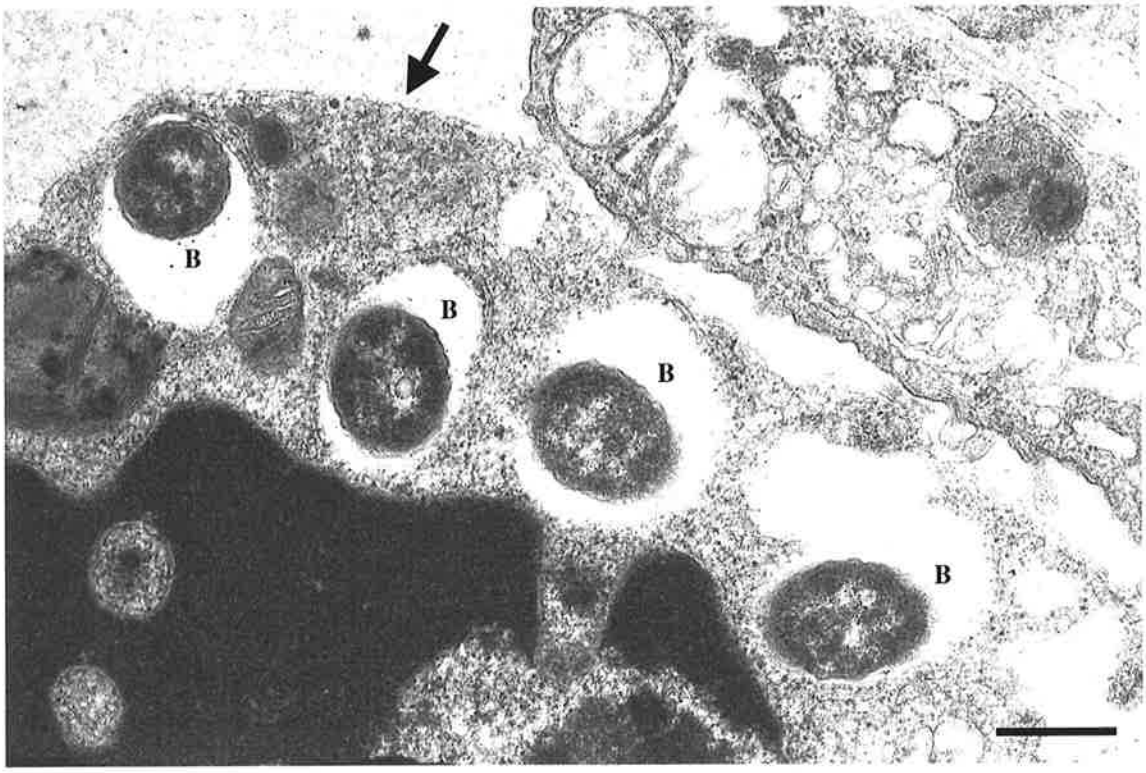
Figure 3.5 (continued)

C: A macrophage with four membrane vacuoles, each containing one *L. longbeachae* sg 1 ATCC 33462 bacterium. Magnification (× 39, 750; bar, 0.5μm). Arrow shows macrophage membrane.

D: A phagosome within a macrophage containing several ATCC 33462 bacteria. The membrane of the vacuole appears smooth walled. Magnification (× 34, 980; bar, 0.5μm). Arrow shows phagosome membrane.

C



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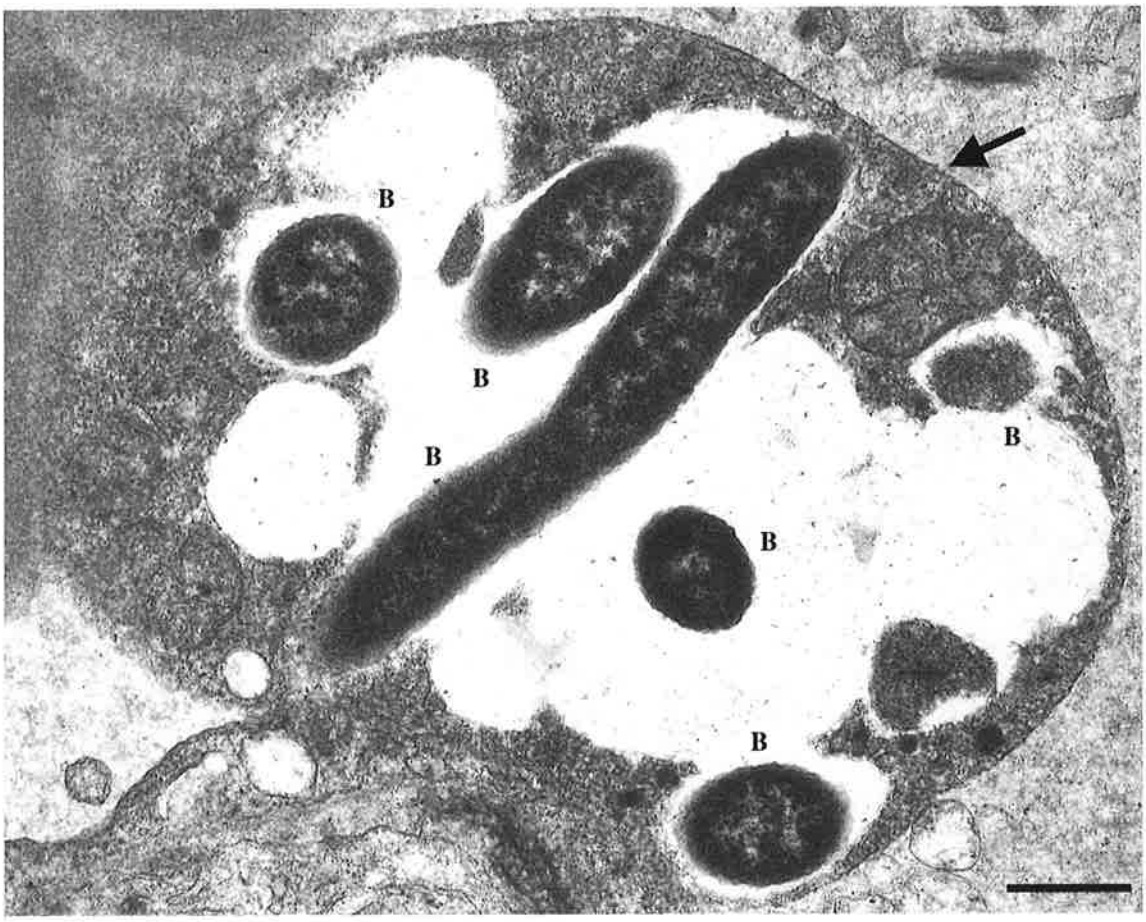


Table 3-1 Intra-peritoneal inoculation of *Legionella* strains

Species	Strain	Dose (CFU total)[a]	Guinea pig deaths	Comments
L. pneumophila serogroup 1	Philadelphia ATCC 33152	5×10^{8}	3/3	- death within 30 hours - spleens contained 10^{7}-10^{8} organisms
L. longbeachae serogroup 2	ATCC 33484	2×10^{9}	0/3	- slight symptoms - fully recovered at day 6
L. longbeachae serogroup 1	ATCC 33462	5×10^{8}	0/3	- slight symptoms - fully recovered at day 6
L. longbeachae serogroup 1	ATCC 33462	1×10^{10}	0/3	- slight symptoms - fully recovered at day 6
L. longbeachae serogroup 1	A5H5	1×10^{9}	1/3	- one death at day 4

Table 3-2 Aerosol inoculation of *Legionella* strains

Species	Strain	Dose (CFU total)[c]	Guinea pig deaths	Comments	Retained dose[d]
L. pneumophila serogroup 1	Philadelphia ATCC 33152	$\approx 1\times10^{9}$	2/3	death within 5 days	$\approx 10^{5}$
L. pneumophila serogroup 1	Corby	$\approx 1\times10^{9}$	5/5	death within 3 days	$\approx 10^{5}$
L. longbeachae serogroup 2	ATCC 33484	$\approx 1\times10^{9}$	0/3	no symptoms	$\approx 10^{5}$
L. longbeachae serogroup 1 (original stock/1987)	ATCC 33462	$\approx 1\times10^{9}$	0/3	no symptoms	2×10^{5}
L. longbeachae serogroup 1 (original stock/1987) - passaged[a]	ATCC 33462	$\approx 1\times10^{9}$	0/3	no symptoms	5×10^{5}
L. longbeachae Serogroup 1 (original stock/1987)	ATCC 33462	$\approx 1\times10^{10}$	0/3	no symptoms	$\approx 10^{6}$
L. longbeachae serogroup 1 (recent stock /1997)	ATCC 33462	$\approx 1\times10^{10}$	0/3	no symptoms[b]	$\approx 10^{6}$
L. longbeachae serogroup 1	A5H5	$\approx 1\times10^{9}$	3/5	death within 5 days (days 2 and 4)	3.5×10^{5}

a: original stock inoculated intra-peritoneally into a guinea pig and harvested from the spleen.

b: evidence of minor lung infection on post mortem examination.

c: Dose in the nebuliser bowl.

d: Determined by homogenisation of the lungs of on of the test guinea pigs immediately after exposure to the dose

Table 3-3 Cluster analysis groupings based on the number of animals dead in each test group.

Cluster	Strain	Dead[a]	STD[b]	Grouping	Origin[c]
1	Atlanta-5	2	0.54772	Type 2	O/S
	D-880	3	0.54772	"	O/S
	A5E1	3	0.54772	"	Aus
	A5H5	3	0.54772	"	Aus
	K5H9	3	0.54772	"	Aus
	L6C9	2	0.54772	"	Aus
	K4A1	3	0.54772	"	Aus
2	LA-24	0	0.00000	Type 3	O/S
	D-1959	1	0.44721	"	O/S
	D-1056	0	0.00000	"	O/S
	D-1624	0	0.00000	"	O/S
	D-63	1	0.44721	"	O/S
	D-1750	1	0.44721	"	O/S
	A5H3	1	0.44721	"	Aus
	A4C5	1	0.44721	"	Aus
3	D-493	5	0.00000	Type 1	O/S
	K8B9	4	0.44721	"	Aus

a: Number of guinea pigs killed out of a total of 5 exposed to the test strain.

b: Standard deviation based on the number of animals dead.

c: The origin of the strain designated as O/S: overseas isolate, or Aus: Australian isolate.

Table 3-4 Cluster analysis groupings based on mean time to death

Cluster	Strain	Mean[a]	Median[b]	STD[c]	Grouping	Origin[d]
1	LA-24	8	8	0.00000	Type 3	O/S
	D-1056	8	8	0.00000	"	O/S
	D-1624	8	8	0.00000	"	O/S
	D-1959	7.8	8	0.44721	"	O/S
2	D-63	7.2	8	1.78885	Type 2	O/S
	D-1750	7.4	8	1.34164	"	O/S
	Atlanta-5	6.8	8	1.64317	"	O/S
	A5H3	7.6	8	0.89443	"	Aus
	A4C5	7.2	8	1.78885	"	Aus
	K5H9	6.6	7	1.67332	"	Aus
	L6C9	6.4	8	2.19089	"	Aus
3	A5E1	5.6	4	2.19089	Type 1	Aus
	A5H5	5.6	4	2.19089	"	Aus
	K8B9	4.8	4	1.78885	"	Aus
	K4A1	5.6	4	2.19089	"	Aus
	D-880	5.8	5	2.04939	"	O/S
					"	
4	D-493	4	4	0.00000	Type 1	O/S

a: The mean number of days before death due to that strain occurred

b: The median number of days before death for that particular strain.

c: Standard deviation based on the mean number of days till death.

d: Strain origin designated as O/S: overseas or Aus: Australian.

Table 3-5 Summary of analysis of virulence of *L. longbeachae* serogroup 1 strains in a guinea pig model of infection

Strain	Origin	Dose (CFU)[a]	Comment[b]	No. of Deaths	Time of death[c]	Classification[d]
A5H5	Australia	1.3×10^{9}	all animals symptomatic	3/5	day 3	Type 1
A4C5	Australia	1.2×10^{9}	all animals symptomatic	1/5	day 3	Type 2
A5H3	Australia	1.4×10^{9}	all animals symptomatic	1/5	day 5	Type 2
A5E1	Australia	1.3×10^{9}	all animals symptomatic	3/5	day 3	Type 1
L6C9	Australia	1.54×10^{9}	some animals symptomatic	2/5	day 3	Type 2
K5H9	Australia	1×10^{9}	all animals symptomatic	3/5	one at day 3, 5 and 6	Type 2
K4A1	Australia	1.6×10^{9}	all animals symptomatic	3/5	day 3	Type 1
K8B9	Australia	2×10^{9}	symptoms only in survivor	4/5	day 3	Type 1
ATCC 33462	USA	1×10^{10}	no symptoms	0/3	-	Type 3
Atlanta-5	USA	1.4×10^{9}	animals that died symptomatic	2/5	day 4	Type 2
LA-24	USA	5.5×10^{9}	no symptoms	0/5	-	Type 3
D-63	USA	1.32×10^{9}	all animals symptomatic	1/5	day 3	Type 2
D-493	USA	1.9×10^{9}	all animals symptomatic	5/5	day 3	Type 1
D-880	USA	1.2×10^{9}	all animals symptomatic	3/5	2 at day 3, 1 at day 4	Type 1
D-1056	USA	7.5×10^{8}	all animals symptomatic	0/5	-	Type 3
D-1624	Israel	1.5×10^{9}	no symptoms	0/5	-	Type 3
D-1750	USA	8.9×10^{8}	some animals symptomatic	1/5	day 4	Type 2
D-1959	USA	3.5×10^{9}	animal that died symptomatic	1/5	day 6	Type 3

a: Dose of test strain (CFU) placed in the nebuliser bowl.
b: Symptoms include change in activity, food or water consumption and laboured breathing. Weight change was observed for every animal irrespective of avirulence and hence is not included.
c: Multiple deaths occurred on same day specified unless stated otherwise.
d: Virulence grouping based on mean time to death cluster analysis data (Table 3-4).

Table 3-6 Infectivities of *Legionella* for U937 cells

Species	Strain	$Log_{10}ID_{50}$[a]	Virulence in guinea pigs	Type[b]
L. pneumophila serogroup 1	130b	2.73 ± 0.13 3.08 ± 0.28 2.04 ± 0.19 2.38 ± 0.18 3.00 ± 0.28 3.10 ± 0.13	virulent	NT
L. longbeachae serogroup 2	ATCC 33484	4.62 ± 0.27 5.60 ± 0.28	avirulent	3
L. longbeachae serogroup 1	ATCC 33462 Long Beach 4	4.55 ± 0.13 -	avirulent	3
L. longbeachae serogroup 1	ATCC 33462 Long Beach 4	1.93 ± 0.13[c]	-	NT
L. longbeachae serogroup 1	A5H5	2.60 ± 0.28 3.14 ± 0.10	3/5 deaths	1
L. longbeachae serogroup 1	A5E1	3.20 ± 0.25 3.30 ± 0.0	3/5 deaths rapid death	1
L. longbeachae serogroup 1	K5H9	2.53 ± 0.13 4.00 ± 0.0	3/5 deaths	2
L. longbeachae serogroup 1	K8B9	2.27 ± 0.30 2.30 ± 0.0	4/5 deaths rapid killer	1
L. longbeachae serogroup 1	K4A1	3.08 ± 0.30 -	3/5 deaths	1
L. longbeachae serogroup 1	A4C5	1.36 ± 0.10 2.70 ± 0.42	1/5 deaths	2
L. longbeachae serogroup 1	A5H3	2.20 ± 0.25 2.88 ± 0.28	1/5 deaths	2
L. longbeachae serogroup 1	L6C9	3.08 ± 0.14 2.85 ± 0.15	2/5 deaths	2

NT: strain not tested in the animal model therefore no virulence group type assigned.

a: The ID_{50} is expressed as a log value ± the standard deviation (expressed as a log value). Values for different infections are shown for each strain if available.

b: Virulence grouping based on mean time to death cluster analysis data (Table 3-4).

c: Value determined in the study by O'Connell *et al* (1996b).

Chapter 4

Examination of cell surface structures of *Legionella longbeachae*

4.1 Introduction

Most studies on the pathogenesis of *Legionella* have been undertaken with *L. pneumophila* sg 1, the major species and serotype globally associated with legionellosis (Dowling, *et al.*, 1992, Reingold, *et al.*, 1984). Some studies have been undertaken with *L. micdadei,* including molecular analysis of virulence factors, as after *L. pneumophila,* it is the second most common species associated with disease in the United States (Reingold, *et al.*, 1984). Studies with other pathogenic species have been limited, and no molecular analysis of virulence factors has been undertaken.

L. longbeachae is differs from *L. pneumophila* in that it has been reported not to possess a major outer membrane protein (MOMP) (Ehret and Ruckdeschel, 1983, Hindahl and Iglewski, 1986, Hindahl and Iglewski, 1987). The MOMP protein of *L. pneumophila* can fix complement component C3 (Bellinger-Kawahara and Horwitz, 1990) thus mediating phagocytosis of the organism via complement receptors CR1 and CR2 (Payne and Horwitz, 1987). The absence of a MOMP-like protein in *L. longbeachae* may suggest that other outer membrane components of this species may have a significant role in pathogenesis. It is not known what factors contribute to pathogenesis, and if these factors are shared with *L. pneumophila.*

This chapter describes the analysis and characterisation of *L. longbeachae* strains to determine the factors that may play a role in their intracellular life cycle. Three strains of *L. longbeachae* sg 1 were chosen for analysis, representing the virulence groupings defined by the animal model experiments, discussed in chapter 3. Strain A5H5, a highly virulent type 1

strain, L6C9, a moderately virulent type 2 strain and the avirulent ATCC 33462 type strain were chosen for examination. Studies were focused on cell surface components of the bacterium as these are often associated with virulence in other bacterial species.

4.2 Materials and methods specific to this chapter

4.2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 2-1.

4.2.2 Detection of MOMP DNA sequences in *Legionella longbeachae*

To screen for the presence of a MOMP-like gene or related sequence in *L. longbeachae,* a probe was generated from the published *ompS,* sequence of *L. pneumophila* (Genbank accession number M76178) (Hoffman, *et al.*, 1992a). Primers MBH1F and MBH1R (Table 4-1) were designed to amplify the entire *ompS* gene from *L. pneumophila* (Philadelphia) and had a *Bam*HI restriction site incorporated in their 5' terminii to aid in cloning if required. This primer pair was designed to regions of DNA flanking *ompS,* taking advantage of areas that closely resembled *Bam*HI recognition sites, in order to minimise base pair mismatch. Additional primers MOMPF and MOMPR (Table 4-1) were designed internal to the coding region of *ompS.*

PCR was performed essentially as outlined in section 2.8 except that a 30 sec annealing time was used at 60°C (MBH1F/MBH1R) and 62°C (MOMPF/MOMPR) respectively, optimised for each primer pair. The $MgCl_2$ concentration was 2.5 mM and extension times were increased to 2 min. Low stringency PCR to detect MOMP-like sequences in *L. longbeachae* was performed at 42°C annealing temperature, for 1 min, with all other conditions as described above.

Genomic DNA from *Legionella* strains was digested with *Bam*HI, transferred to nylon membrane and hybridised with the Digoxigenin labelled *ompS* PCR product, amplified from *L. pneumophila* sg 1, under high and low stringency (section 2.9.3).

4.2.3 Detection of flagellum DNA sequences in *Legionella*

Primers were designed from the published flagellum gene sequence, *flaA,* of *L. pneumophila* (Corby) (Heuner, *et al.*, 1995) to generate a probe to detect DNA sequences related to *flaA* in *L. longbeachae* by Southern hybridisation. Primers were designed to DNA sequences, encoding regions of the *L. pneumophila* FlaA protein that appeared highly conserved in FlaA proteins from other bacteria (Heuner, *et al.*, 1995). The PCR reaction used standard conditions (section 2.8) with an annealing temperature of 65°C. The PCR product generated was purified by agarose gel electrophoresis (section 2.6.4) and labelled with Digoxigenin as per manufacturers instructions (section 2.9.1).

Genomic DNA from *Legionella* strains was digested with *Hind*III, transferred to nylon membrane and hybridised with the Digoxigenin labelled *flaA* PCR product, amplified from *L. pneumophila* sg 1, under high and low stringency conditions (section 2.9.3).

4.3 Results

4.3.1 Analysis of protein profiles of *L. longbeachae* on SDS-PAGE

Whole cell, whole membrane and outer membrane protein extracts were prepared from *L. longbeachae* sg 1 strains ATCC 33462, A5H5 and L6C9, *L. longbeachae* sg 2 ATCC 33484 and *L. pneumophila* 1 (Philadelphia), grown at 30°C or 37°C. The three strains of *L. longbeachae* sg 1 were representative of each of the virulence groupings defined in chapter 3. Strain A5H5, was a highly virulent type 1 strain, L6C9, a moderately virulent type 2 strain and ATCC 33462 type strain was a relatively avirulent type 3 strain. Protein profiles were

examined by SDS-PAGE to detect any potential morphological differences between strains of *L. longbeachae* sg 1 and/or differences between *L. longbeachae* sg 1 and *L. pneumophila* sg 1.

Whole cell protein extracts of *Legionella* strains, prepared at 37°C, were run on 12% SDS-PAGE gels (Fig 4.1). No obvious differences in protein profile were observed when strains of *L. longbeachae* sg 1 were compared with each other and *L. longbeachae* sg 2 ATCC 33484. This result is consistent with that of Ehret and Ruckdeschel (1983) who noted that these two serogroups yielded identical patterns when whole cell wall and outer membrane protein preparations on SDS-PAGE. The most prominent protein band in *L. pneumophila,* appeared to be MOMP (Fig 4.1, lane d), constituting around 30-40% of the total cellular protein for this strain. A similar result was observed for extracts prepared at 30°C (data not shown). An abundant MOMP-like protein was not observed in any of the *L. longbeachae* isolates. No MOMP-like protein in similar quantity to *L. pneumophila* MOMP was observed in *L. longbeachae* isolates irrespective of the protein extraction process (Fig 4.1, Fig 4.2 and Fig 4.3).

Examination of whole membrane protein profiles, prepared at two different growth temperatures, also showed no differences between representative *L. longbeachae* isolates (Fig 4.2). The whole protein profiles for this species, however, were different from *L. pneumophila* and as observed with whole cell extracts, a distinct MOMP-like protein in *L. pneumophila* was the most obvious distinguishing feature between the two species (Fig 4.2 lane a and b). Whole membrane profiles of *L. longbeachae* also differed from *L. pneumophila* (Philadelphia) in that there was a large, densely staining band/s, at the top of the gel (Fig 4.2). All strains of *L. longbeachae* appeared to have this intensely staining membrane material. It is unlikely that this represents an aggregated MOMP-like protein in this species as the preparations were resuspended in ß-mercaptoethanol and boiled prior to electrophoresis to reduce any internal disulphide bonds to allow entry into the gel.

Analysis of preparations enriched for outer membrane proteins revealed MOMP was the most abundant protein in *L. pneumophila* and clearly highlighted the absence of a

similarly abundant protein in *L. longbeachae* species (Fig 4.3). In general, the outer-membrane profiles of *L. pneumophila* showed more protein bands than *L. longbeachae.* Conversely, *L. longbeachae* strains, in general, had fewer protein bands and no major band in similar abundance to MOMP. An intensely staining band of approx. 90 kDa, observed in all strains of *L. longbeachae,* appeared to be absent in *L. pneumophila* (Fig 4.3). There was a band of approx. 50 kDa in all of the *L. longbeachae* strains except ATCC 33462 (Fig. 4.3). The band size suggests that it may be a flagellum sub-unit since it is of similar size to *L. pneumophila* FlaA (Heuner, *et al.*, 1995). Molecular analysis was therefore undertaken to determine if a *flaA* gene homologue was present in *L. longbeachae* sg 1. In addition, the absence of protein species that may be MOMP-like in *L. longbeachae* on SDS-PAGE requires further examination using genetic methods as it is possible that MOMP-like proteins could be repressed under laboratory growth conditions.

4.3.2 Determination of the presence of an *ompS*-like gene sequence in *L. longbeachae*

Two sets of primers were designed from the published *ompS* sequence of *L. pneumophila.* One set of primers (MBH1F and MBH1R) was designed to regions external to the MOMP gene sequence, in order to generate a PCR fragment from *L. pneumophila* for use in Southern hybridisation studies, including the entire coding sequence of *ompS.* Primers MOMPF and MOMPR were designed to bind to internal regions of the *ompS* gene sequence to use primarily in PCR screening for an *ompS*-like gene in *L. longbeachae.*

Primers MBH1F and MBH1R amplified an approx. 1300 bp product from *L. pneumophila* (Philadelphia) DNA as predicted. Similarly, primers MOMPF and MOMPR amplified an approx. 750 bp product. High stringency PCR using either primer pair did not produce a product from *L. longbeachae* sg 1 ATCC 33462, A5H5, L6C9 or serogroup 2 strain ATCC 33484 genomic DNA. Low stringency PCR conditions generated multiple spurious bands with ATCC 33462 template DNA when primers MOMPF and MOMPR were used (data not shown). None of the bands generated in ATCC 33462 were similar in size to the

expected product from *L. pneumophila* sg 1 under the same conditions (data not shown). Primers MBH1F and MBH1R used at low stringency produced a distinct band of approx. 1400 bp in ATCC 33462 (data not shown). When the amplicon was sequenced the inferred protein sequence did not share any identity with MOMP or other outer membrane proteins in a BLASTX analysis.

The 1300 bp product amplified with primers MBH1F and MBH1R from *L. pneumophila* was labelled with Digoxigenin and used as a probe in Southern analysis of a panel of *Legionella* genomic DNA digests (Fig 4.4). Sequencing determined that *ompS* had been correctly amplified (data not shown). Using high stringency hybridisation conditions only the *L. pneumophila* (Philadelphia) strain hybridised with this probe (Fig 4.4 ii). However, when low stringency hybridisation conditions were used, two hybridising bands were seen in *L. pneumophila* (Fig 4.4 i, lane a). Both bands most likely represent the *ompS* gene, encoding MOMP, and possibly the previously described *ompM* gene of *L. pneumophila* (Nottingham N_7) that encodes an outer membrane protein (High, *et al.*, 1993). Analysis of the *ompS* and *ompM* gene sequences showed that they share approximately 55.3% identity at the nucleotide level over the entire gene sequence. However, some small areas of strong identity were observed, which under reduced stringency conditions may have allowed detection of *ompM* in addition to *ompS*. A single band was observed in *L. longbeachae* strains probed under reduced stringency conditions (Fig 4.4 i). No hybridising band was observed in the control lane containing *E. coli* DH5α DNA.

To determine if the band observed in *L. longbeachae* strains was an *ompM*-like gene, PCR analysis was performed using primers designed from the published *ompM* sequence of *L. pneumophila* (GenBank accession number L05595) (High, *et al.*, 1993). Two sets of primers were designed internal and external to the *ompM* coding sequence, however, a product of the correct size could not be amplified from *L. pneumophila, L. longbeachae* sg 1 ATCC 33462 and A5H5 (data not shown). Southern hybridisation was therefore not undertaken, as a probe

could not be generated by PCR. It is possible that ompM may only be present in the *L. pneumophila* Nottingham strain.

4.3.3 Determination of the presence of a *flaA*-like gene sequence in *L. longbeachae*

The probe used in Southern hybridisations to detect flagellum subunit gene sequences was derived from the published sequence of *flaA* of *L. pneumophila* (Corby) (Heuner, *et al.*, 1995). The primers amplified an expected product of approx. 1200 bp in *L. pneumophila* (Philadelphia) but did not amplify any product in *L. longbeachae*. Several attempts at low stringency, to amplify *flaA* related sequences in *L. longbeachae* by PCR were unsuccessful. Partial sequencing of the amplified 1200 bp fragment generated from *L. pneumophila* (Philadephia) confirmed that the correct product had been amplified (data not shown). A BLASTX search of the NCBI database showed that the inferred protein product had the highest level of identity with the published FlaA sequence (Heuner, *et al.*, 1995). When the product was used to probe a panel of *Legionella* genomic digests at high stringency, only *L. pneumophila* (Philadelphia) hybridised with the probe (Fig 4.5 ii). No hybridising band was observed for *L. micdadei*, *L. longbeachae* strain ATCC 33462, A5H5, A5E1, K8B9 and L6C9 or for the *E. coli* DH1 negative control. Two hybridising bands were observed for *L. pneumophila* (Philadelphia), suggesting that internal *Hind*III sites present in *flaA* (Corby strain) are likely to be conserved in *L. pneumophila* strain Philadelphia.

The same panel of *Legionella* genomic digests was hybridised under reduced stringency conditions that allowed approx. 30% mismatch between target and probe sequences (Cianciotto, *et al.*, 1990a). The hybridising pattern observed for *L. pneumophila* (Philadelphia) under these reduced conditions was identical to that seen with high stringency (Fig 4.5 i). In addition, a hybridising band was also observed for *L. micdadei* also known to possess a *fla* gene sequence (Bangsborg, *et al.*, 1995). No hybridising band was observed for *L. longbeachae* sg 1 strains ATCC 33462, A5H5, L6C9, K8B9 or A5E1 (Fig 4.5). *E. coli* DH1 control DNA also did not hybridise with the *flaA* gene probe as expected. *L.*

longbeachae sg 2 ATCC 33484 did not appear to hybridise with the *flaA* probe under low stringency conditions. A repeat filter, hybridised at low stringency, and washed at 45°C instead of 50°C determined that a hybridising band was present in this species (data not shown). An identical panel of *L. longbeachae* sg 1 strains tested under the same conditions did not hybridise with the probe (data not shown). The results suggest that a *flaA*-like gene sequence may be present in *L. longbeachae* sg 2 ATCC 33484.

4.3.4 Analysis of the lipopolysaccharide of *L. longbeachae* on SDS-PAGE

LPS was prepared from several test stains of *L. longbeachae* sg 1 to determine if banding pattern differences on SDS-PAGE might correlate with virulence of the strain as determined in the animal model. *L. pneumophila* (Philadelphia) and *L. longbeachae* sg 2 extractions were also prepared as controls. Visualisation of LPS banding patterns by silver staining determined that there were no differences between the three strains of *L. longbeachae* sg 1 (Fig 4.6). As expected, the LPS banding profile of *L. pneumophila* strain Philadelphia was distinct from that observed for *L. longbeachae* (Jurgens and Fehrenbach, 1997). The LPS banding pattern observed for *L. longbeachae* sg 2 ATCC 33484 was different from *L. longbeachae* sg 1 strains as expected.

4.4 Discussion

Three strains of *L. longbeachae* sg 1 were chosen for analysis of potential virulence factors as each represented one of the virulence groupings defined in chapter 3. Strain A5H5 is a highly virulent type 1 strain, L6C9 a moderately virulent type 2 strain and ATCC 33462 type strain an avirulent isolate. Studies were initially focused on cell surface components since they are often associated with virulence in other bacterial species. *L. pneumophila* (Philadelphia) was used as a control strain as some virulence factors for this species have been well characterised. It was thought that any major differences between this species and *L. longbeachae* might suggest targets for further analysis. In addition, differences observed

between the representative isolates of *L. longbeachae* sg 1 might account for the variability in causing disease in the animal model.

Analysis of *L. longbeachae* protein profiles by SDS-PAGE determined that a highly abundant MOMP-like protein did not appear to be present in this species. This result is in agreement with other workers who reported that MOMP is specific for *L. pneumophila* (Ehret and Ruckdeschel, 1983, Hindahl and Iglewski, 1986). Gosting *et al* (1984) identified a species specific 29 kDa antigen in *L. pneumophila* using a monoclonal antibody in western blots thought to be MOMP. A specific monoclonal antibody (Genetic Systems Corp., Seattle, Washington), used diagnostically for detection of *L. pneumophila* strains, recognises an epitope on MOMP suggesting that this protein is specific to that species (Nolte and Conlin, 1986). Other workers have proposed that a MOMP protein is present in almost all species of *Legionella* (Butler, *et al.*, 1985). A 24 kDa MOMP protein was identified in *L. pneumophila* and all species surveyed, including *L. longbeachae*, contained the 24 kDa MOMP that exhibited immunologic cross-reactivity with the 24 kDa MOMP of *L. pneumophila* (Butler, *et al.*, 1985). Southern hybridisation analysis using the cloned *ompS* gene has shown that under moderate stringency (approx. 15% bp mismatch), hybridisation is observed for several *Legionella* species, suggesting that there are genus common epitopes of MOMP (Hoffman, *et al.*, 1992a). *L. longbeachae* was not tested in that panel of isolates and our data has determined that an *ompS*-like gene sequence is not present in this species when hybridised under high stringency conditions.

Low stringency hybridisation conditions that allowed approx. 30 % bp mismatch did appear to detect MOMP-related DNA sequences in *L. longbeachae* sg 1. Two hybridising bands were observed for *L. pneumophila,* most likely representing *ompS* (Hoffman, *et al.*, 1992a), and perhaps the previously described *ompM* gene of *L. pneumophila* (Nottingham N_7) (High, *et al.*, 1993). Since there were some short areas of strong identity between the two gene sequences, and using reduced stringency conditions (approx 30% bp mismatch), *ompM* may have been detected in addition to *ompS*. Alternatively there may be other *ompS*-like

genes in *L. pneumophila*. A single hybridising band was observed in *L. longbeachae* strains under these same low stringency conditions which may represent a *ompS*-like gene in this species. An abundant MOMP-like protein could not be detected by SDS-PAGE, however, this does not preclude the possibility that one is expressed in *L. longbeachae*. The culture conditions and extraction process used in this study may not have optimised expression of a MOMP-like protein in *L. longbeachae* or alternatively the gene may be present but it does not encode a functional protein.

To determine if the hybridising band observed in *L. longbeachae* strains may be an *ompM*-like gene, PCR analysis was performed using primers designed from the published sequence of *ompM* in *L. pneumophila* (High, *et al.*, 1993). An *ompM*-like sequence could not be amplified from *L. pneumophila* (Philadelphia) so it could not be determined whether a related gene was present in *L. longbeachae*. It may be that *ompM* is only found in Nottingham-N7 strain. Attempts to obtain this strain from the authors describing *ompM* were unsuccessful.

The MOMP protein of *L. pneumophila* can fix complement component C3 (Bellinger-Kawahara and Horwitz, 1990) thus mediating phagocytosis of the organism via complement receptors CR1 and CR2 (Payne and Horwitz, 1987). The absence of a MOMP-like protein in *L. longbeachae* suggests that perhaps other outer membrane components of this species may have a significant role in pathogenesis. In particular the Mip protein of *L. pneumophila,* the first virulence factor described for *Legionella* (Cianciotto, *et al.*, 1990b, Engleberg, *et al.*, 1989), is such a protein and therefore warrants further analysis in *L. longbeachae* sg 1 since it has been shown that a *mip* gene exists in this species (Cianciotto, *et al.*, 1990a).

Flagella have been observed for *L. pneumophila* and are thought to have a role in pathogenesis (Chandler, *et al.*, 1980, Elliott and Johnson, 1981, Heuner, *et al.*, 1999, Ott, *et al.*, 1991b, Rodgers, *et al.*, 1980). Southern analysis to detect a *flaA*-like gene in strains of *L. longbeachae* sg 1 revealed that only *L. longbeachae* sg 2 ATCC 33484 at low stringency had such a sequence. The negative observation for *L. longbeachae* sg 1 ATCC 33462 was

consistent with the observations of Heuner *et al* using the *flaA* gene as a probe (Heuner, *et al.*, 1995). The same workers also determined that flagella were absent in this strain by electron microscopy. These results are surprising since the initial description of *L. longbeachae* as a species suggested that flagella were present on three of the four test case study isolates as determined by direct fluorescence antibody staining (McKinney, *et al.*, 1981). Ott *et al* (1991b) have shown that polyclonal antibodies specific for the flagellum subunit of *L. pneumophila* (Philadelphia) cross react with a similar sized protein in *L. longbeachae* sg 2 but not in *L. longbeachae* sg 1 consistent with the observations made in this chapter. A rapid latex agglutination test, based upon a common flagellum antigen, indicated the presence of this antigen in all *Legionella* species except *L. brunensis*, *L. cincinnatiensis*, *L. oakridgensis* and *L. longbeachae* sg 1 (Bornstein, *et al.*, 1991). The absence of a *flaA* gene in *L. longbeachae* sg 1 strains suggest that this factor may not be important in this species even though the less commonly isolated *L. longbeachae* sg 2 appears to possess a *flaA*-like gene and express flagella.

In *L. pneumophila,* factors such as LPS have been shown to interact with the complement system suggesting LPS may play a role in uptake of this species in addition to MOMP (Mintz, *et al.*, 1992b). The LPS banding profile on SDS-PAGE of *L. longbeachae* is distinct from *L. pneumophila* (Jurgens and Fehrenbach, 1997), consistent with the results in this chapter. As expected, the LPS pattern for *L. longbeachae* sg 2 was distinct from the sg 1 strain pattern and from *L. pneumophila* (Philadelphia). Identical patterns were observed for all isolates of *L. longbeachae* sg 1. Thus, it would appear at this gross phenotypic level that there are no differences between the three virulence categories defined for this serogroup in chapter 3. The possibility that differences in LPS structure, not resulting in an obvious difference by PAGE analysis, could account for the differences in virulence of these isolates cannot be excluded. It has been shown recently that a mutant strain of *L. pneumophila* unable to bind a monoclonal antibody specific for the wild type LPS was unable to replicate intracellularly in HL-60 cell lines and show reduced ability to infect guinea pigs (Lüneberg, *et*

al., 1998). The mutant strain did not show any differences in O-chain structure and length and exhibited the characteristic bimodular distribution of LPS O-chain as the parent strain.

Since *L. longbeachae* appears to lack an abundant MOMP-like protein, perhaps outer membrane proteins such as Mip may play an important role in uptake and pathogenesis. Chapter 5 examines the role of the Mip protein in *L. longbeachae* sg 1 pathogenesis.

4.5 Summary

Analysis of whole protein, whole membrane and outer membrane protein profiles of strains of *L. longbeachae* sg 1 representing three different virulence groups (defined in chapter 3) determined the species to be highly clonal and does not appear to have a MOMP-like protein. Southern hybridisation at low stringency did detect a possible MOMP-related sequence , however, this may not be expressed by this organism under laboratory conditions resulting in the lack of an obvious protein on Coomassie stained SDS-PAGE gels. Alternatively the gene may not encode for a functional protein. A *flaA*-like flagella gene was not detected in *L. longbeachae* sg 1 strains but did appear to exist in *L. longbeachae* sg 2 ATCC 33484, consistent with the literature.

Figure 4.1

12% SDS-Polyacrylamide gel of whole cell protein preparations of *Legionella* strains grown at 37°C. Lane **a**: *L. longbeachae* sg 1, ATCC 33462 (original isolate): lane **b**: *L. longbeachae* sg 1 strain A5H5; lane **c**: *L. longbeachae* sg 1 strain L6C9; lane **d**: *L. pneumophila* sg 1 strain Philadelphia; lane **e**: *L. longbeachae* sg 2, ATCC 33484; lane **f**: *L. longbeachae* sg 1 ATCC 33462 (recent isolate). The arrow in figure indicates the position of the Major Outer Membrane Protein (MOMP) of *L. pneumophila* (Philadelphia).

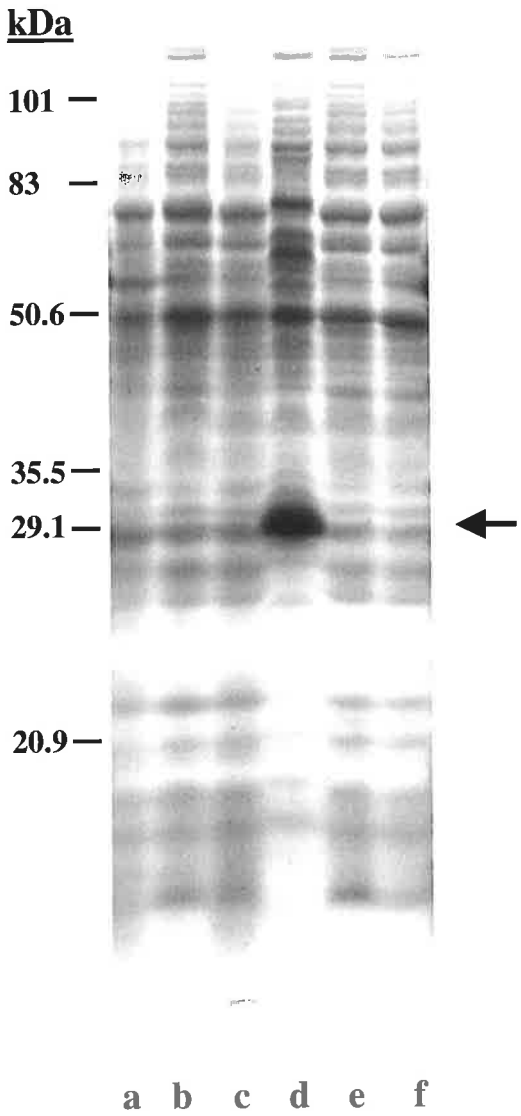

kDa
101
83
50.6
35.5
29.1
20.9
a b c d e f

Figure 4.2

15% SDS-PAGE analysis of whole membrane profiles of *Legionella* strains prepared at 30°C and 37°C. Lane **a**: *L. longbeachae* sg 2 ATCC 33484 (37°C), lane **b**: *L. longbeachae* sg 2 ATCC 33484 (30°C), lane **c**: *L. longbeachae* sg 1 strain L6C9 (37°C), lane **d**: *L. longbeachae* sg 1 strain L6C9 (30°C), lane **e**: *L. longbeachae* sg 1 strain A5H5 (37°C), lane **f**: *L. longbeachae* sg 1 strain A5H5 (30°C), lane **g**: *L. longbeachae* sg 1 ATCC 33462 (37°C); lane **h**: *L. longbeachae* sg 1 ATCC 33462 (30°C), lane **i**: *L. pneumophila* strain Philadelphia (37°C), lane **j**: *L. pneumophila* strain Philadelphia (30°C). The large rightward arrow indicates the prominent MOMP protein of *L. pneumophila* (Philadelphia) strain.

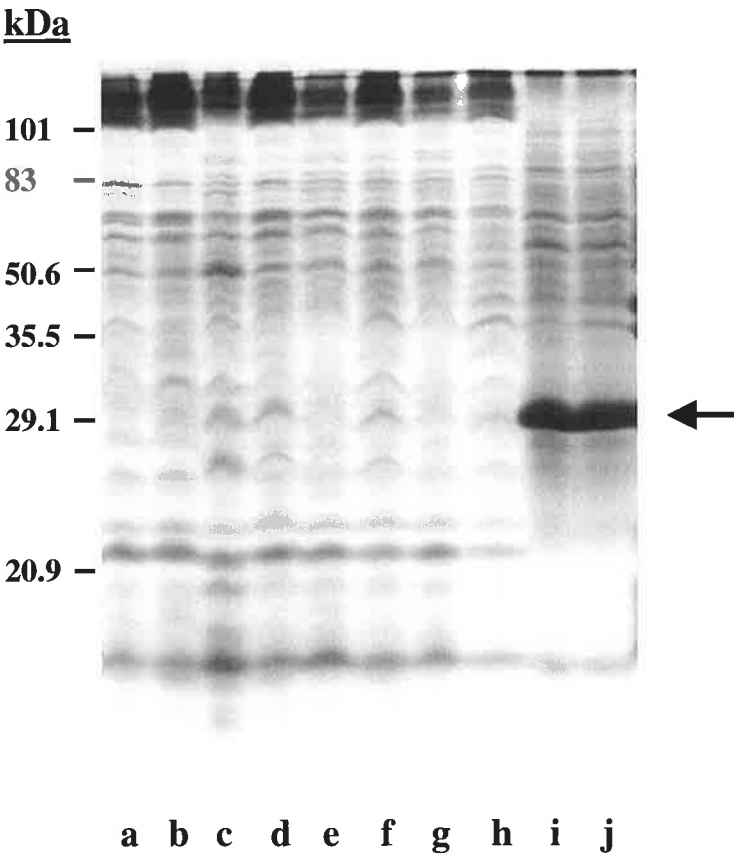

kDa
101
83
50.6
35.5
29.1
20.9
a b c d e f g h i j

Figure 4.3

15% SDS-Polyacrylamide gel analysis of outer membrane profiles of *Legionella* strains prepared at 30°C and 37°C. Lane **a**: *L. pneumophila* strain Philadelphia (30°C); lane **b**: *L. pneumophila* strain Philadelphia (37°C); lane **c**: *L. longbeachae* sg 1 ATCC 33462 (30°C); lane **d**: *L. longbeachae* sg 1 ATCC 33462 (37°C); lane **e**: *L. longbeachae* sg 1 strain A5H5 (30°C); lane **f**: *L. longbeachae* sg 1 strain A5H5 (37°C); lane **g**: *L. longbeachae* sg 1 strain L6C9 (30°C); lane **h**: *L. longbeachae* sg 1 strain L6C9 (37°C); lane **i**: *L. longbeachae* sg 2 ATCC 33484 (30°C); lane **j**: *L. longbeachae* sg 2 ATCC 33484 (37°C). The large rightward arrow indicates the prominent MOMP protein of *L. pneumophila* (Philadelphia) strain. The smaller rightward arrow indicates the cryptic outer membrane protein (approx. 50kDa), absent in the *L. longbeachae* sg 1 ATCC 33462 type strain (lanes a and b).

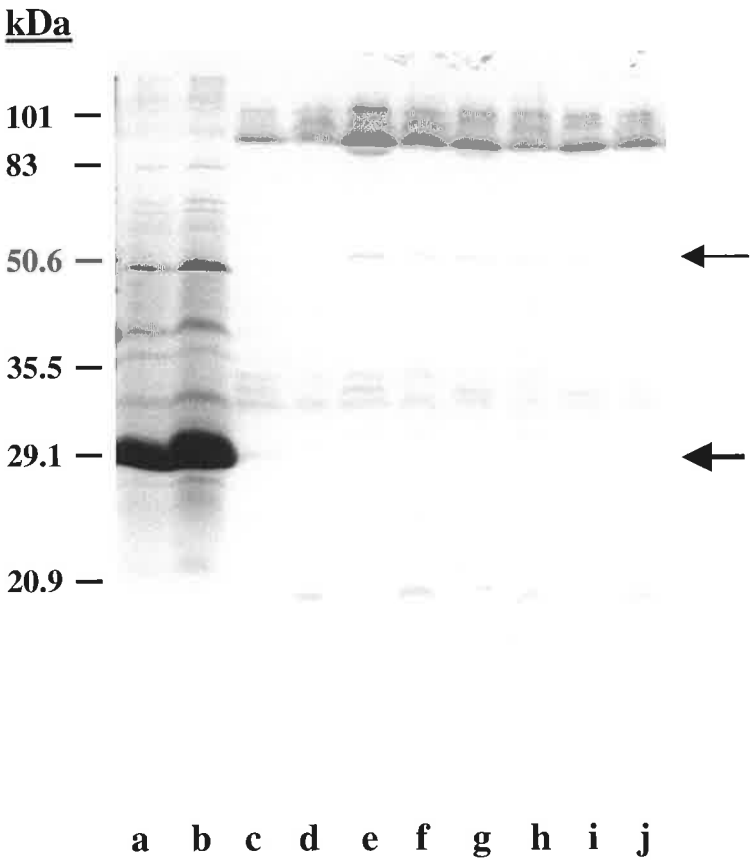
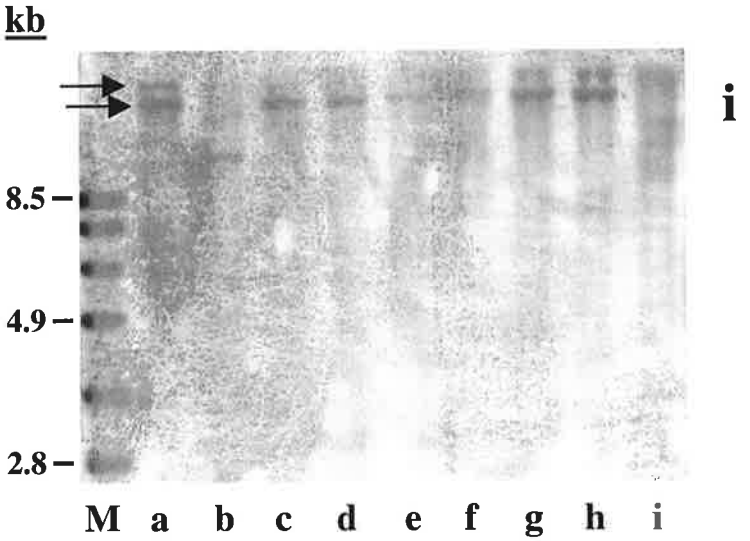

kDa
101
83
50.6
35.5
29.1
20.9
a b c d e f g h i j

Figure 4.4

Southern analysis of *Legionella* DNA probed with the gene sequence of *ompS* encoding the Major Outer Membrane Protein (MOMP) of *L. pneumophila* generated by PCR. Chromosomal DNA was digested with *Bam*HI. Duplicate filters were probed with Digoxigenin-labelled *ompS* PCR product under low (approx. 30% bp mismatch) (i) and high (ii) stringency conditions. Lane **a**: *L. pneumophila* strain Philadelphia; lane **b**: *L. micdadei*; lane **c**: *L. longbeachae* sg 2 ATCC 33484; lane **d**: *L. longbeachae* sg 1 ATCC 33462; lane **e**: *L. longbeachae* sg 1 strain L6C9; lane **f**: *L. longbeachae* sg 1 strain A5H5; lane **g**: *L. longbeachae* sg 1 strain K8B9; lane **h**: *L. longbeachae* sg 1 strain A5E1; lane *i*: *E. coli* strain DH5α. Lane **M**: SPP-1 molecular weight marker track. Arrows show the double bands observed in the case of *L. pneumophila* strain Philadelphia under reduced stringency conditions.


kb
8.5
4.9
2.8
M a b c d e f g h i
i

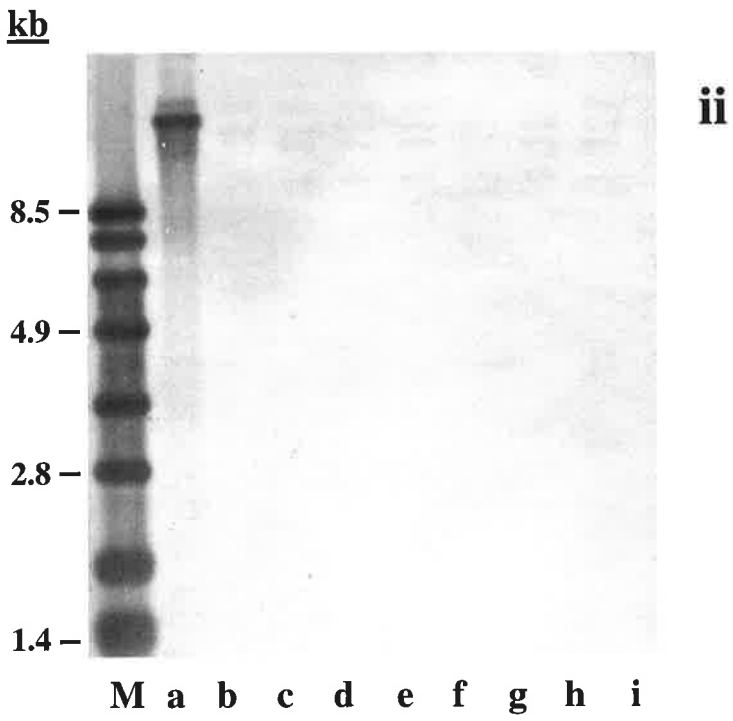
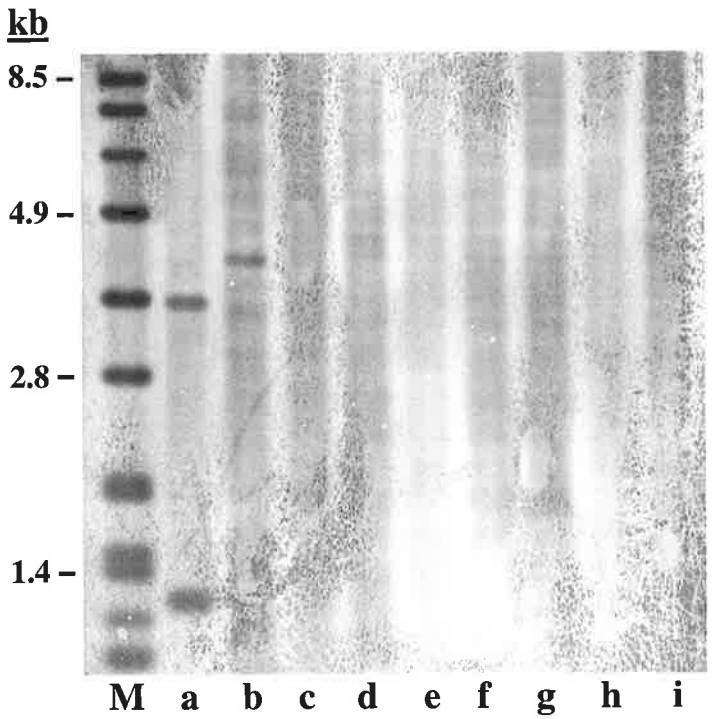
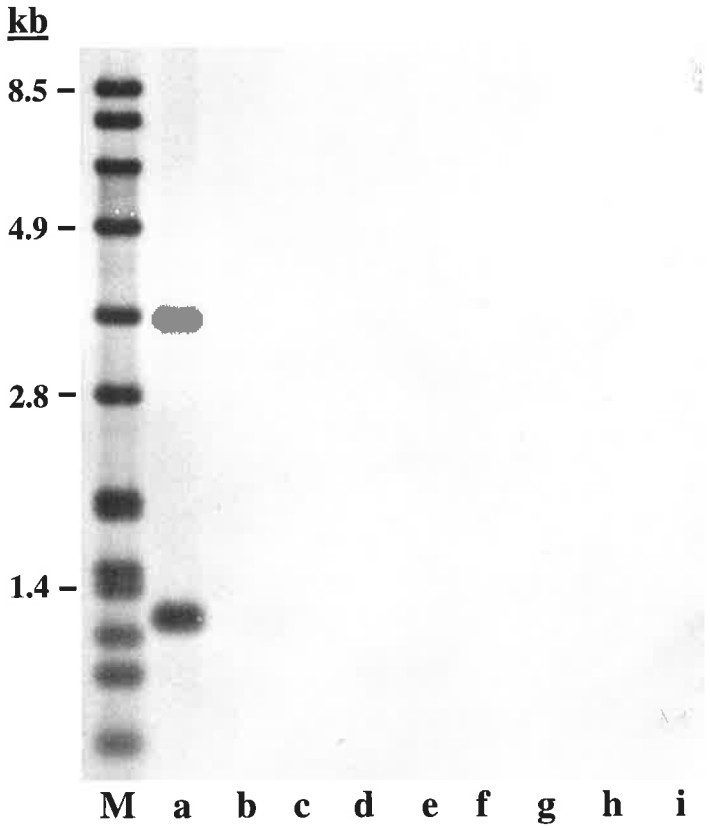

kb
8.5
4.9
2.8
1.4
M a b c d e f g h i
ii

Figure 4.5

Southern analysis of *Legionella* DNA probed with the *flaA* gene sequence from *L. pneumophila* generated by PCR. Chromosomal DNA was digested with *Hin*dIII. Duplicate filters were probed with Digoxigenin-labelled *flaA* PCR product under low (approx. 30% bp mismatch) (i) and high (ii) stringency conditions. Lane **a**: *L. pneumophila* strain Philadelphia; lane **b**: *L. micdadei*; lane **c**: *L. longbeachae* sg 2 ATCC 33484; lane **d**: *L. longbeachae* sg 1 ATCC 33462; lane **e**: *L. longbeachae* sg 1 strain A5H5; lane **f**: *L. longbeachae* sg 1 strain A5E1; lane **g**: *L. longbeachae* sg 1 strain K8B9; lane **h**: *L. longbeachae* sg 1 strain L6C9; lane **i**: *E. coli* strain DH1. Lane **M**: SPP-1 molecular weight marker track.



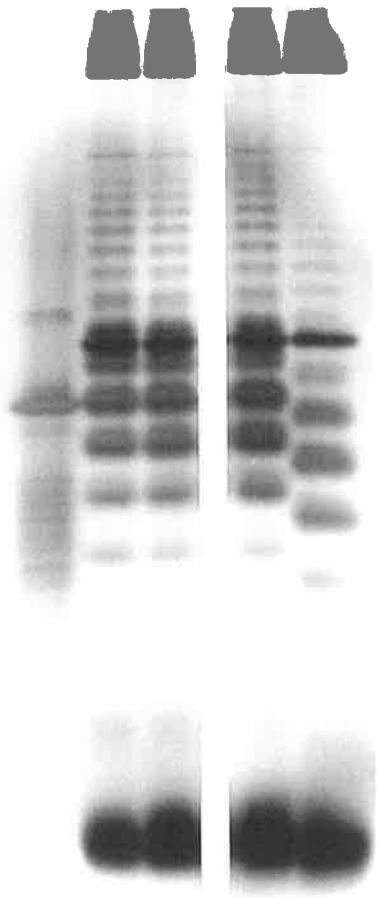
i



ii

Figure 4.6

15% SDS-Polyacrylamide gel analysis of lipopolysaccharide preparations of strains of *Legionella*. Lane **a**: *L. pneumophila* strain Philadelphia; lane **b**: *L. longbeachae* sg 1 ATCC 33462; lane **c**: *L. longbeachae* sg 1 strain A5H5; lane **d**: *L. longbeachae* sg 1 strain L6C9; lane **e**: *L. longbeachae* sg 2 ATCC 33484.



a b c d e

Table 4-1 Oligonucleotide primers relevant to chapter 4

Primer designation	DNA sequence	Experimental application
MBH1F[a]	5' – gat acg gat cca atc tta cat tta atg ttt - 3'	Amplification of MOMP-like gene sequences
MBH1R[b]	5' – cag cgg atc ccg gat gtg att ttt cat - 3'	"
MOMPF[a]	5' – ggt acg atg ggt cca gta tgt - 3'	"
MOMPR[b]	5' – gcc aac att cac ggc agc agt - 3'	"
Fla-7[a]	5' – gtg cca aag atg atg cag cag g - 3'	Amplification of flagella DNA gene sequences
Fla-8[b]	5' – cca aca tcg ctg tac ctg ctt g - 3'	"

a: Forward primer
b: Reverse primer

Chapter 5

Cloning and characterisation of the *mip* gene of *Legionella longbeachae*

5.1 Introduction

The Mip protein is a virulence factor that plays an important role in the intracellular life cycle of *L. pneumophila* 1 (Cianciotto and Fields, 1992, Cianciotto, *et al.*, 1989b, Cianciotto, *et al.*, 1990b, Cianciotto, *et al.*, 1995a, Engleberg, *et al.*, 1989). A mutant strain, lacking Mip, is impaired significantly in its ability to infect alveolar macrophages and protozoa (Cianciotto, *et al.*, 1989b, Cianciotto and Fields, 1992), and is attenuated in its ability to cause disease in guinea pigs (Cianciotto, *et al.*, 1990b). A Mip protein also occurs in *L. micdadei* (Bangsborg, *et al.*, 1991), a species of *Legionella* associated with disease in humans, and a *L. micdadei mip* mutant also has reduced ability to infect macrophages and amoebae (O'Connell, *et al.*, 1995). Mip gene homologues have been detected in all species of *Legionella* including *L. longbeachae* sg 1 (Bangsborg, *et al.*, 1991, Cianciotto, *et al.*, 1990a, Doyle, *et al.*, 1998, Ratcliff, *et al.*, 1998).

This chapter presents the cloning and sequence analysis of the *mip* gene from *L. longbeachae* sg 1 ATCC 33462, and compares it with those from *L. pneumophila* sg 1 (Engleberg, *et al.*, 1989), *L. longbeachae* sg 2 ATCC 33484, *L. micdadei* (Bangsborg, *et al.*, 1991) and an Australian clinical isolate of *L. longbeachae* sg 1 strain A5H5.

5.2 Methods and materials specific for this chapter

5.2.1 Strains and plasmids

Specific bacterial strains and plasmids constructed in this study are listed in Table 5-1.

5.2.2 Synthetic oligonucleotide primers

Synthetic oligonucleotide primers designed specifically for use in this study and their experimental application are described in Table 5-2.

5.2.3 Construction of *L. longbeachae* sg 1 ATCC 33462 plasmid bank

Chromosomal DNA from *L. longbeachae* sg 1 ATCC 33462 was digested with *Bam*HI, *Eco*RI and *Hind*III in all combinations. Restriction fragments were separated by electrophoresis, transferred to a nylon membrane and hybridised with a Digoxigenin labelled probe containing the *mip* gene from *L. pneumophila* (Philadelphia). The probe was prepared as follows. Primers MIPB1 and MIPB2 were designed to amplify the entire coding region of the *L. pneumophila* sg 1 *mip* gene sequence (Engleberg, *et al.*, 1989). The primers amplified an approx. 1200 bp fragment from *L. pneumophila*. The amplicon was gel purified prior to labelling and the filters were hybridised at high stringency (section 2.9). The filters were washed twice at room temperature in 2 × SSC, 0.1 % SDS for 10 min followed by two washes in 5 × SSC, 0.1% SDS at 37°C for 25 min and detected according to manufacturer's instructions.

Southern hybridisation showed that the *L. longbeachae* sg 1 *mip* gene was on a *Bam*HI-*Eco*RI fragment of approx. 8 kb (data not shown). Fragments ranging in size from approximately 7-10 kb were purified from *L. longbeachae* sg 1 ATCC 33462 chromosomal DNA after digestion with *Bam*HI and *Eco*RI by agarose gel electrophoresis (section 2.6). The fragments were ligated into similarly restricted pGEM-7Zf(-) purified by agarose gel electrophoresis. The ligation mix was transformed into competent *E. coli* DH5α (section 2.7).

Transformants were screened by colony immunoblot using polyclonal monospecific anti-*L. pneumophila* Mip serum (section 5.2.4) to isolate a clone that expressed the *L. longbeachae* sg 1 Mip protein. Colonies were incubated overnight and then transferred onto nitrocellulose membrane disc filters by overlaying the membrane onto the agar plate until wetted through. The filter was then placed, colony side up, onto Whatmann 3MM paper soaked in 0.5M HCl and incubated for 30 min at room temperature. Cellular debris was removed by washing the membrane with saline prior to immunoblotting with Mip antiserum. One antibody reactive clone was identified and designated clone DH5α[pIMVS26]. This clone expressed a protein of 27 kDa as determined by western analysis (data not shown). The plasmid construct pIMVS26 contained an approximately 8 kb insert of *L. longbeachae* sg 1 DNA, confirmed by restriction digestion and Southern analysis (data not shown).

Subcloning of pIMVS26 to isolate the *L. longbeachae* sg 1 *mip* gene to a smaller fragment of DNA, suitable for sequencing, was achieved as follows. Random fragments generated by restriction digestion of a CsCl gradient purified preparation of pIMVS26 with enzymes *Sac*I, *Xba*I, *Mlu*I, *Sph*I, *Cla*I, *Hind*III and *Kpn*I were cloned into pGEM-7Zf(-), linearised with the corresponding enzyme and the ligation mix transformed into competent *E. coli* DH5α cells. Transformants were screened for expression of the Mip protein by western analysis (data not shown). A sub-clone, expressing the *L. longbeachae* sg 1 Mip protein, contained a plasmid construct with a 1.3 kb *Sac*I insert. This clone was chosen for subsequent sequence studies and the recombinant plasmid from this clone was designated pIMVS27.

5.2.4 Antisera and antibodies

Polyclonal monospecific anti-Mip antisera used initially in screening the *L. longbeachae* sg 1 plasmid bank for a clone expressing the Mip protein was a kind gift from Dr. Nicholas Cianciotto (Northwestern University, Chigago). Upon isolation of a clone, DH5α[pIMVS26], polyclonal anti-serum was prepared specifically against the *L. longbeachae* sg 1 Mip protein as follows.

The Mip protein expressed in strain DH5α[pIMVS26] was observed as a band of approx. 27 kDa compared to a control track containing clone DH5α[pGEM-7Zf(-)] when whole cell protein extracts were run on a 15% PAGE gel and stained with Coommassie R-250 (data not shown). The Mip protein was purified on a preparative 15% SDS-PAGE after staining (section 2.11). The gel slice was emulsified in phosphate buffered saline pH 7.2 (PBS) by passage through a fine gauge needle, and approximately 100μg of protein was subcutaneously injected into each of two New Zealand white rabbits. A booster injection was given after two weeks and the anti-sera harvested after 6 weeks. The anti-sera was filter sterilised prior to use and extensively adsorbed against *E. coli.* strain DH5α [pGem-7Zf(-)] .

5.2.5 Amino terminal analysis

The Mip protein from ATCC 33462 was purified for N-terminal amino acid analysis as follows. Approximately 0.6 ml of a whole cell protein extraction from clone DH5α [pIMVS26] was electrophoresed on a preparative 15% SDS-PAGE. The gel was stained with Coomassie blue G250 and the protein band corresponding to Mip was excised using a sterile scalpel. The protein extract was run again on another 15% polyacrylamide gel, for further purification, and electro-transferred to polyvinylidenedifluride (PVDF) membrane (Bio Rad) (section 2.11). After transfer the membrane was stained for 10 minutes in 0.025% Coomassie blue R-250 (Bio-Rad) in 40% methanol. The membrane was subsequently de-stained in 50% (v/v) methanol and allowed to air dry. The N-terminal amino acid sequence of the desired band was performed at the Macquarie University Centre for Analytical Biotechnology (Macquarie University, School of Biological Sciences, N.S.W., Australia) on a 470A Applied Biosystems protein sequencer.

5.2.6 Sequencing of insert DNA of pIMVS27

Plasmid pIMVS27 was sequenced in the forward direction using ABI double stranded dye labelled primer protocol (Applied Biosystems, Foster City. CA). The protocol was

applied to templates generated by nested deletion of pIMVS27 using the Erase-a-Base system (Promega) as per manufacturer's instructions. The complementary strand of the clone was determined using ABI dye-deoxy terminator sequencing chemistry and custom designed oligonucleotides. Double stranded pIMVS27 was used as the template and synthetic oligonucleotides were designed complementary to the forward sequence data generated above, and used as sequencing primers (Table 5-2). This strategy allowed sequencing of both strands of insert DNA of pIMVS27. The resulting sequence data was analysed by DNASIS and PROSIS (Hitachi Software).

5.2.7 Sequencing of *mip* genes from other *Legionella longbeachae* strains

Primers were designed from the completed gene sequence of the *mip* gene of *L. longbeachae* sg 1 (ATCC 33462) to amplify the *mip* gene from *L. longbeachae* sg 2 (ATCC 33484) and an Australian clinical isolate of *L. longbeachae* sg 1 (A5H5). Primers 844 and 845 were designed to amplify the entire *mip* gene sequence from purified genomic DNA by PCR (section 2.8). PCR was performed using a standard reagent mix and amplified as follows. Initial denaturation of 94°C for 3 minutes followed by an amplification cycle consisting of denaturation at 94°C for 1 min, 45°C annealing for 30 seconds and 72°C extension for 1 minute. PCR products were identified by agarose gel electrophoresis and the expected 850 bp band was purified for each sample using a Qiagen QIAquick PCR Purification Kit as recommended by the manufacturer. The product was sequenced using ABI dye-terminator sequencing chemistry with primers 844 and 845 as per manufacturers instructions.

5.2.8 Primer extension from total bacterial RNA

To map the 5' end of *mip* mRNA, primer extension analysis was performed, using a synthetic oligonucleotide primer. Primer RT-1 was chosen from a highly conserved region of DNA, 54 bases downstream from the putative start site of translation of the *mip* gene. Total

bacterial RNA was extracted from *L. longbeachae* sg 1 ATCC 33462 and *E. coli* strains DH5α[pIMVS27] and DH5α[pGEM-7Zf(-)] by the hot phenol method of Aiba *et al* (1981). Bacterial cells were harvested from exponential phase broth culture for each strain by centrifugation at 3,500 × g for 15 min at 4°C. The pellet was resuspended in 750 µl of lysis solution (0.02M Sodium acetate pH 5.5, 0.5% sodium dodecyl sulphate and 1mM EDTA) and the contents transferred to an eppendorf microfuge tube. The lysate was mixed with an equal volume of Tris buffer equilibrated phenol (pH 5.5), incubated at 65°C for 5 minutes and centrifuged at 17,000 × g for 5 min. The aqueous phase was re-extracted 4 times in this manner and then precipitated in three volumes of ice-cold ethanol at -20°C overnight. Extracted RNA was treated with RNAse free DNAse 1 (Boehringer Mannheim) prior to setting up the primer extension reaction.

Primer RT-1 was labelled and purified prior to hybridisation with the extracted RNA as follows. Primer (approx. 300 ng) was incubated with 1µl of [^{32}p]-δ-ATP and 4U of T4 polynucleotide kinase (Boehringer Mannheim) in the manufacturer supplied buffer at 37°C for 45 minutes and than stopped with the addition of 0.25M EDTA. The primer was purified from unincorporated label on a 10% acrylamide gel by excision of the labelled band after exposure of the PAGE gel to x-ray film. The labelled primer was eluted from the gel slice in TE at 37°C overnight.

The labelled primer was hybridised to 20μg of total RNA for each test strain and the mix was extended according to the method of Williams and Manning (1991). Briefly, total RNA was hybridised with labelled RT-1 primer (5×10^4 cpm) at 75°C for 3 minutes, then at 37°C for 90 minutes. Complimentary cDNA was synthesised from the RNA-primer mix with 5 U of Moloney murine leukemia virus reverse transcriptase (M-MuLV) with 20mM each of dATP, dGTP, dCTP and dTTP. The reaction mix was incubated at 37°C for 60 min, stopped with 0.25M EDTA, and precipitated with ethanol, 3M sodium acetate pH 5.5 and glycogen at -20°C overnight. Samples were concentrated by centrifugation, resuspended in 10µl of stop

buffer (Boehringer Mannheim) and heated at 75°C for 3 minutes prior to electrophoresis. The reaction was loaded onto a 6% acrylamide-urea sequencing gel and visualised by autoradiography. Plasmid pIMVS27 was sequenced with the DNA Sequencing Kit Version 2 (Amersham Life Science), as per manufacturers instructions, and loaded adjacent to primer extension products as a control.

5.2.9 Nucleotide sequence accession numbers

The *mip* gene sequence data obtained for *L. longbeachae* sg 1 ATCC 33462 and *L. longbeachae* sg 2 ATCC 33484 used in this study are available under GenBank and EMBL accession numbers X83036 and AF000958, respectively.

5.3 Results

5.3.1 Mip expression by strains of *L. longbeachae*

To determine if a Mip protein was conserved and expressed within diverse *L. longbeachae* sg 1 strains, whole protein profiles were analysed by western immunoblot, using anti-serum raised against the purified recombinant Mip protein from ATCC 33462. Each strain tested expressed a single reactive protein, which was approximately 27 kDa in size (Fig 5.1). Previous studies suggested that a Mip-like protein existed in ATCC 33462 (Cianciotto, *et al.*, 1990a) and additionally the same protein appeared to be expressed in all Australian isolates of this species examined. The conservation of Mip within a number of clinical and environmental isolates suggests that like *L. pneumophila* sg 1 (Cianciotto and Fields, 1992), Mip might promote intracellular infection by *L. longbeachae* sg 1, and is present throughout the species, including serogroup 2.

5.3.2 Analysis of *L. longbeachae* Mip

Amino terminal amino acid sequence analysis of the 27 kDa protein expressed by clone DH5α[pIMVS26] was performed to confirm cloning of a gene expressing the Mip-like

protein from *L. longbeachae* sg 1 ATCC 33462 and if it is processed in *E. coli*. The data was unambiguous and demonstrated that the protein was homologous to the first 15/16 amino acids of the processed protein from *L pneumophila* sg 1. However, a threonine residue was found at position 8 rather than an alanine residue (Ala - Thr - Asp - Ala - Thr - Ser - Leu – **Thr** - Thr - Asp - Lys - Asp - Lys- Leu - Ser - Tyr). Therefore, having established the identity of the protein expressed by the recombinant clone, the 1300 bp insert of plasmid pIMVS27 was sequenced.

Only one potential open reading frame was identified and determined to be 699 bp in size. A strong RBS (5'AAGGGG 3') was also found in close proximity to the putative AUG start site for translation. Downstream of the stop codon (UAA), was a region of dyad symmetry, corresponding to a putative transcriptional terminator, similar to that seen for the *L. pneumophila* sg 1 *mip* gene sequence (Engleberg, *et al.*, 1989). This most likely represents a factor independent transcription termination signal as has also been proposed for the *L. micdadei mip* gene (Bangsborg, *et al.*, 1991).

Primers designed from the *mip* gene sequence of ATCC 33462 were used to amplify the *mip* genes from *L. longbeachae* sg 2 ATCC 33484 and an Australian clinical isolate of *L. longbeachae* sg 1 A5H5. The *mip* gene from A5H5 was 100% identical to the ATCC 33462 type strain *mip* DNA sequence. The sg 2 strain of *L. longbeachae* differed by two base pairs (positions 517 and 523) in third codon positions. The inferred amino acid sequence of the Mip proteins from *L. longbeachae* sg 1 strain A5H5 and *L. longbeachae* sg 2 ATCC 33484, were 100% identical with the *L. longbeachae* sg 1 ATCC 33462 Mip protein.

The inferred *L. longbeachae* Mip protein was a polypeptide of 233 amino acids in size with a predicted molecular mass of 24, 661 Da. The difference in size as determined by SDS-PAGE and western immunoblot of approximately 27 kDa (Fig 5.1) may be explained by the difference in net charge between the two proteins. The *L. longbeachae* Mip protein was very similar to *L. pneumophila* sg 1 Mip sharing approximately 85% identity, and also with *L. micdadei* Mip protein sharing 77 % homology (Fig 5.2).

The first 20 amino acids comprise a signal sequence, inferring that the Mip protein in *L. longbeachae* sg 1 is also translocated to the outer membrane, and possesses a typical cleavage site as seen for *L. pneumophila* sg 1 (Engleberg, *et al.*, 1989). This data in conjunction with the N-terminal analysis results suggests the protein is processed in *E. coli* in a similar manner to *L. pneumophila* sg 1 Mip protein. The hydropathy plot of the protein, determined using OMIGA 1.1 and the Kyte-Dolittle constraints, was also similar to that determined for *L. pneumophila* sg 1 (Engleberg, *et al.*, 1989), suggesting that structurally the proteins are very similar (Fig 5.3).

The key sites proposed to be involved in FKBP-506 PPIase activity (Hacker and Fischer, 1993), determined of the *L. pneumophila* sg 1 (Philadelphia) Mip protein (Fischer, *et al.*, 1992), appear to be conserved in the *L. longbeachae* sg 1 Mip protein (Fig 5.2), suggesting that the Mip protein, in *L. longbeachae,* may have a similar function and role in pathogenesis.

5.3.3 Analysis of *L. longbeachae* sg 1 *mip* transcriptional signals

To confirm the start site for transcription of the *mip* gene in ATCC 33462 primer extension analysis was performed. Identification of the 5' ends of the *mip* gene from mRNA isolated from *L. longbeachae* sg 1 ATCC 33462 and *E. coli* strains DH5α[pIMVS27] and DH5α[pGEM-7Zf(-)] was determined by synthesis of cDNA with a primer that was complimentary to a region of DNA 54 bp downstream from the putative AUG start site on the *mip* mRNA. Using this oligonucleotide as a primer we observed identical cDNA bands synthesised from RNA from ATCC 33462 (fig 5.4, lane b) and DH5α[pIMVS27] (Fig 5.4, lane c). No discrete band was seen in the control track where DH5α[pGEM7Zf (-)] was used as a template (fig 5.4, lane a). By comparing these bands with the sequencing reaction of pIMVS27, primed with the same oligonucleotide, and loaded adjacent to the primer extension reactions on the same gel, we mapped the 5' end of the *mip* mRNA of ATCC 33462 to the G residue at nucleotide position 473 of the *L. longbeachae* sg 1 ATCC 33462 *mip* gene

sequence. This result confirmed that the start site for transcription in *L. longbeachae* sg 1 and *L. pneumophila* sg 1 was identical in both species (Fig 5.5). Accepting nucleotide 473 as the start site in ATCC 33462, the probable -10 and -35 promoter consensus sequences were identified and compared with those for *L. pneumophila* sg 1 (Fig. 5.5). The predicted -10 promoter box was identical for *L. longbeachae* sg 1 and *L. pneumophila* sg 1, however a -35 region was identified (Fig 5.5) in *L. longbeachae* sg 1 that is a more likely part of the promoter sequence than that suggested for *L. pneumophila* sg 1 (Engleberg, *et al.*, 1989). The spacing of the -10 and -35 regions for *L. longbeachae* sg 1 is a closer match with the consensus sequences determined for *E. coli* and the spacing between them is optimal (17 ± 1nt).

Analysis of the sequencing reaction for pIMVS27 revealed a strong region of compression, indicating that an area of secondary structure could be present at the start site of translation of the *mip* gene in *L. longbeachae* sg 1 (Fig 5.4). Analysis of this region of DNA upstream of the *mip* gene of *L. longbeachae* sg 1 ATCC 33462, using DNASIS, revealed several potential stem loop structures. No potential stem loop structure was detected in this area of the *mip* gene in *L .pneumophila* 1 (data not shown). Whether this difference plays a role in the control of transcription of *L. longbeachae mip* is unknown.

5.3.4 Cellular location of the Mip protein of *L. longbeachae*

Whole membrane and outer membrane protein profiles were examined from strains of *Legionella* to help determine the cellular location of Mip in *L. longbeachae* since the protein does not conform strictly to the structure that would be expected for outer membrane proteins. Protein extracts were prepared from *E. coli* DH5α[pIMVS27], *L. longbeachae* sg 1 ATCC 33462, A5H5 and L6C9, *L. longbeachae* sg 2 ATCC 33484 and *L. pneumophila* sg 1 (Philadelphia), grown at 30°C or 37°C. Samples were separated by 15% SDS-PAGE, transferred onto nitrocellulose and then probed with anti-*L. longbeachae* Mip polyclonal antiserum (1 in 500 dilution).

Analysis of whole membrane protein profiles by western immunoblot (Fig 5. 6, part A) determined that a Mip protein was present in all preparations including the *E. coli* DH5α containing pIMVS27. Amino terminal analysis suggested that the *L. longbeachae* sg 1 Mip protein was processed in an *E. coli* background and this was consistent with the western immunoblot data. Western immunoblot analysis of outer membrane protein profiles (Fig 5.6, part B) could not detect a Mip protein in any of the *Legionella* isolates. A faint band of the correct size was observed for *E. coli* DH5α containing pIMVS27. This suggests that if Mip is indeed outer membrane located, then it must be expressed in very low amounts or may be redominantly located in the inner membrane or periplasm.

5.4 Discussion

The sequence of the *mip* gene was determined for *L. longbeachae* sg 1 ATCC 33462, *L. longbeachae* sg 2 ATCC 33484 and an Australian clinical isolate A5H5. The *mip* gene sequence was identical for the two isolates of *L. longbeachae* sg 1 while the sequence determined for *L. longbeachae* sg 2 ATCC 33484 differed from this by two bases (positions 517 and 523) which resulted in no change in the protein level due to third base pair redundancy. The nucleotide sequence of the *mip* gene of *L. longbeachae* sg 1 ATCC 33462, A5H5 and *L. longbeachae* sg 2 ATCC 33484 did not reflect any strain virulence variation, in agreement with the observations of Ludwig *et al* (1994) who sequenced the *mip* gene of three *L. pneumophila* isolates of varying virulence. Some variations in the *mip* gene sequence were observed but this resulted in only one amino acid substitution in the Mip proteins, that did not effect PPIase activity.

The inferred Mip protein sequences for all three *L. longbeachae* strains were identical and highly homologous to the Mip protein sequences determined for *L. pneumophila* (Engleberg, *et al.*, 1989, Ludwig, *et al.*, 1994) and *L. micdadei* (Bangsborg, *et al.*, 1991) which were the only sequences available at that time. Infact, the Mip protein is highly

conserved in all species (Ratcliff, *et al.*, 1997). The size of the inferred Mip protein of *L. longbeachae* (24, 661 kDa) was virtually identical to that proposed for the Mip protein of *L. pneumophila* (24, 868 kDa) (Engleberg, *et al.*, 1989). However, the estimated size of the *L. longbeachae* Mip protein, determined by SDS-PAGE was approx. 27 kDa. This may be explained in part by the overall net negative charge of the *L. longbeachae* Mip protein in comparison with *L. pneumophila*. This result is consistent with the observation of Cianciotto *et al* (1990a) who determined that the size of the Mip protein expressed by *L. longbeachae* sg 1 ATCC 33462 was 27 kDa. Immunoblotting in the same study showed that the size of the Mip proteins from *Legionella* ranged from 24 kDa to 31 kDa. Studies by Helbig *et al* (1995) using a panel of monoclonal antibodies (MAb) that react with the Mip protein of *L. pneumophila* and *L. micdadei* also determined that the Mip proteins from *Legionella* range in size from 24-31 kDa.

PPIase activity, determined for *L. pneumophila* (Philadelphia) (Fischer, *et al.*, 1992), was not determined for the Mip protein of *L. longbeachae*. However, complete conservation of the amino acids critical for this enzymatic activity suggest that the *L. longbeachae* Mip protein has a similar mechanism of action. Conservation of these amino acids has subsequently been confirmed for the Mip protein in all species of *Legionella* (Ratcliff, *et al.*, 1997). Amino terminal analysis also determined that the initial amino acids of the recombinant Mip protein of *L. longbeachae* sg 1 were identical to that determined for the Mip protein of *L. pneumophila,* indicating processing at an identical cleavage site (Engleberg, *et al.*, 1989, Ludwig, *et al.*, 1994).

The *L. pneumophila* Mip protein is a surface exposed outer membrane protien (Cianciotto, *et al.*, 1989b, Engleberg, *et al.*, 1989, Pearlman, *et al.*, 1985). A panel of monoclonal antibodies that react with the Mip protein of *L. pneumophila* and *L. micdadei* also suggests that the Mip protein is surface expressed as ELISAs performed with whole cells reacted with a genus specific monoclonal antibody (22/1) (Helbig, *et al.*, 1995). Western immunoblot analysis of preparations enriched for outer membrane proteins, using anti-*L.*

longbeachae sg 1 polyclonal antiserum, did not detect a Mip protein in any strain of *L. longbeachae* or *L. pneumophila*. However, a strongly reacting Mip protein band was observed for whole membrane preparations tested in an identical manner. Attempts to detect Mip on the surface of *L. longbeachae* sg 1 ATCC 33462, *L. longbeachae* sg 1 A5H5 and *L. pneumophila* sg 1 Philadelphia strain by electron microscopy using immunogold labelled anti-*L. longbeachae* sg 1 Mip polyclonal antisera failed to detect Mip on the surface of the organisms (data not shown).

Demonstration of Mip in the outer membrane of *L. pneumophila* has not been shown by immunoblot using preparations enriched for outer membrane proteins. It may be that the method used in this study was not sensitive enough for detection of Mip. The sarkosyl method of enriching for outer membrane proteins relies on the insolubility of these proteins in non-ionic detergents. Mip does not appear to be a typical outer-membrane protein and hence may be soluble in sarkosyl and therefore lost during purification. It is thought that Mip does not exist in large quantities on the cell surface (Helbig, J. H., personal communication). Alternatively, Mip may be anchored in both membrane layers with a larger proportion retained in the whole membrane fraction. It has been proposed that Mip exists as a dimer on the cell surface (Schmidt, *et al.*, 1994, Schmidt, *et al.*, 1995). The polyclonal antiserum used in this study was prepared against denatured Mip purified from an SDS-PAGE gel and therefore may only recognises epitopes of the denatured protein. Most studies undertaken with Mip to determine cellular location have used sera raised against whole cells and undenatured protein.

It is possible that the Mip protein of *Legionella* may be secreted from the membrane to the exterior via a cell surface intermediate. This is observed for some secreted proteins in other gram-negative bacteria (Pugsley, 1993). The proteins present in the supernatant of overnight broth cultures of several *Legionella* strains were concentrated by ammonium sulphate precipitation (data not shown). Western immunoblotting using anti-*L. longbeachae* Mip polyclonal antiserum suggested that a cross reactive protein was present in the culture

supernatant of *L. longbeachae* and *L. pneumophila.* However, as the preparations were from overnight broth cultures, it is possible that the product detected may be protein released into the supernatant due to cell break down. This assay was not repeated and therefore no definite conclusions can be made. It is possible that Mip is secreted from *Legionella* as has been proposed for the Mip like protein of *Chlamydia trachomatis* (Mo, *et al.*, 1995).

The start site for transcription determined for *L. longbeachae* sg 1 ATCC 33462 was identical to that published for *L. pneumophila* Engleberg, 1989 #1661], confirming that the Mip protein in *L. longbeachae* is highly conserved. Putative –10 and –35 transcriptional promotor sequences determined for the *L. longbeachae* sg 1 *mip* gene, however, differed slightly from that determined for *L. pneumophila.* It is possible that the proteins may be regulated differently as a large area of compression upstream of the *L. longbeachae* 1 *mip* gene, indicating secondary structure suggests that this may be likely.

In general, the Mip protein of *L. longbeachae* is highly homologous to other Mip proteins of the genus. Conservation of critical enzymatic sites suggests it is likely to have a similar function and mechanism of action and hence play a significant role in the pathogenesis of this organism or survival in protozoa and the environment. The high degree of conservation of Mip proteins in *Legionella* suggests that the protein may have an important housekeeping function consistent with its homology with FKBPs and determined PPIase activity.

Mip may have a more significant role in pathogenesis of *Legionella* species that do not share all of the proposed virulence factors determined for *L. pneumophila*. A *mip* mutant has been constructed in *L. micdadei,* the second most common *Legionella* species associated with disease in the United States (Reingold, *et al.*, 1984). However, the mutant was not tested in an *in vivo* animal model. The construction of isogenic *mip* mutants of *L. longbeachae* sg 1 for testing in biological models is discussed in the next chapter.

5.5 Summary

Amino terminal analysis of the purified, cloned *L. longbeachae* sg 1 ATCC 33462 Mip protein confirmed that the cloned gene protein was expressed and processed in an *E. coli* background. DNA sequence analysis of plasmid pIMVS27, containing the entire *L. longbeachae* sg 1 ATCC 33462 *mip* gene, revealed a high degree of homology with the *mip* gene of *L. pneumophila* sg 1, showing 76% homology at the DNA level and 87% identity at the amino acid level. Primer extension analysis determined that the start site of transcription was the same for both species, with some differences observed for the -10 and -35 promoter regions. Primers designed from the *mip* gene sequence obtained for *L. longbeachae* sg 1 ATCC 33462 were used to amplify the *mip* gene from *L. longbeachae* sg 2 ATCC 33484 and an Australian clinical isolate of *L. longbeachae* sg 1 strain A5H5. The *mip* gene from A5H5 was 100% identical with the type strain sequence. The sg 2 strain of *L. longbeachae* differed by two base pairs in third codon positions which did not result in a change at the protein level due to third base pair redundancy.

Figure 5.1

Western blot analysis of bacterial whole cell protein extracts with anti-*L. longbeachae* sg 1 polyclonal Mip antiserum. Lane a: *E. coli* clone DH5α[pIMVS26]; lane b: *L. pneumophila* (Philadelphia); lane c: *L. longbeachae* sg 1 (ATCC 33462); lane d: *L. longbeachae* sg 1 (K4E1); lane e: *L. longbeachae* sg 1 (A5E7); lane f: *L. longbeachae* sg 1(A5E1); lane g: *L. longbeachae* sg 1 (K8C7); lane h: *L. longbeachae* sg 1 (A5H5); lane i: *L. longbeachae sg* 1 (K5F1); lane j: *L. longbeachae* sg 1 (A7C1); lane k: *L. longbeachae* sg 1 (A4G7); lane l: *L. longbeachae* sg 1 (K5H9); lane m: *L. longbeachae* sg 1 (K7C6); lane n: *L. longbeachae* sg 1 (A4A7); lane o: *L. longbeachae* sg 1 (A4B3); lane p: *L. longbeachae* sg 2 (ATCC 33484); lane q: *L. longbeachae* sg 2 (K4G7). Solid arrow shows the approx. 24 kDa Mip protein of *L. pneumophila* (Philadelphia). Dashed arrow shows approx. 27 kDa Mip protein detected in all *L. longbeachae* strains.

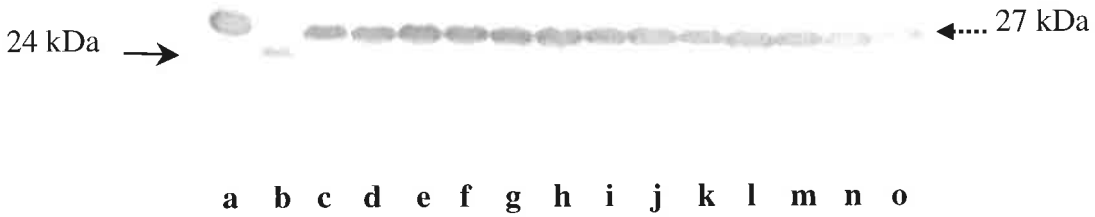

24 kDa
27 kDa
a b c d e f g h i j k l m n o

Figure 5.2

Amino acid comparison of the Mip proteins of *L. longbeachae* sg 1 ATCC 33462 (L.1), *L. longbeachae* sg 1 strain A5H5 (A5H5), *L. longbeachae* sg 2 ATCC 33484 (L.2), *L. pneumophila* sg 1 (L. PNEUM. 1) and *L. micdadei* (L. MIC.). * indicate amino acids identical to *L. longbeachae*, ▼ indicate amino acids predicted to form part of the active site for PPIase activity of Mip. The arrow indicates the site of signal peptidase cleavage.

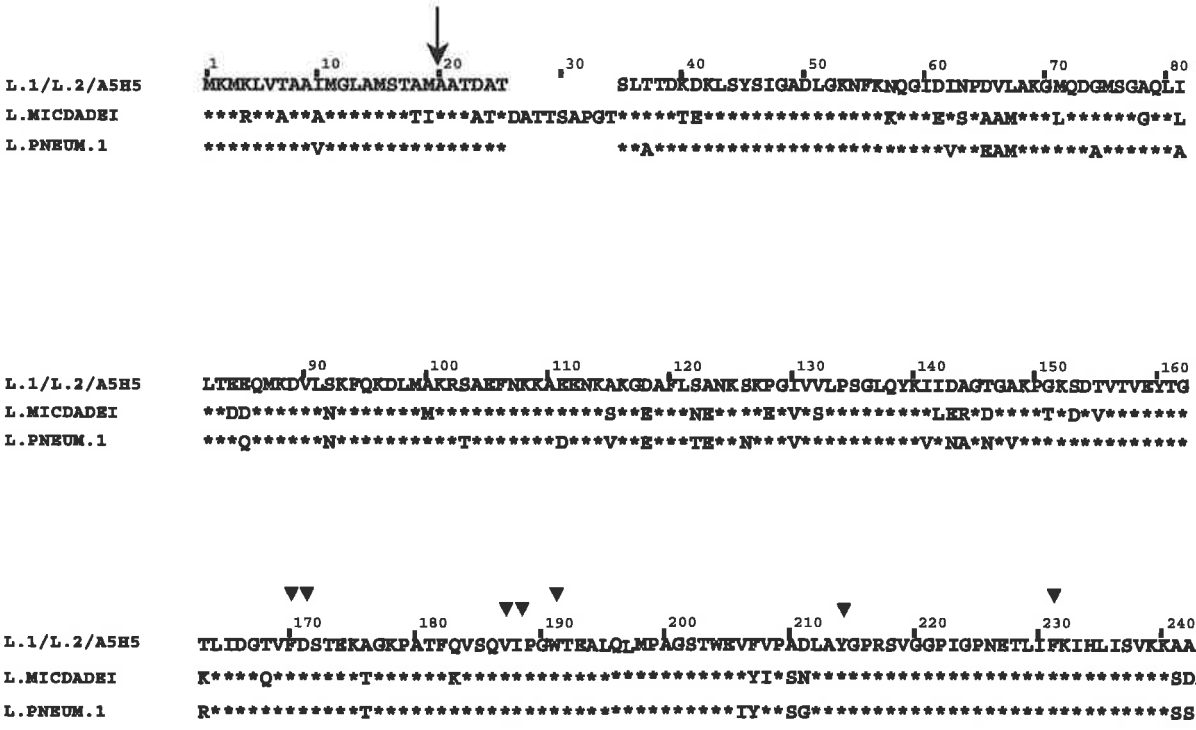
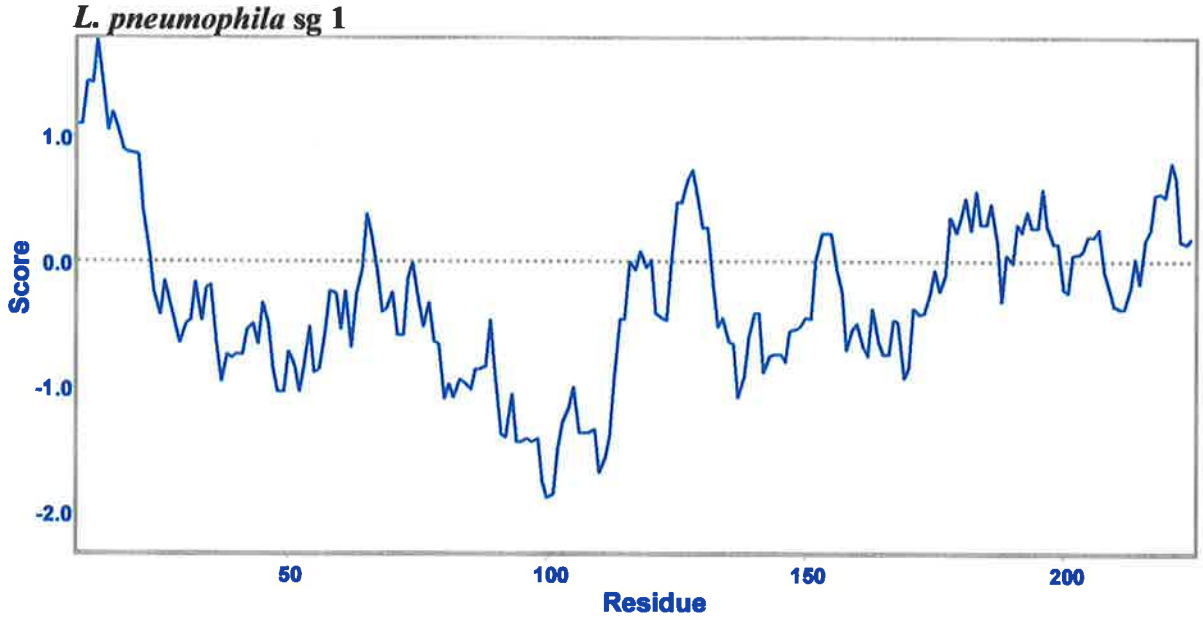

L.1/L.2/A5H5
L.MICDADEI
L.PNEUM.1
MKMKLVTAAIMGLAMSTAMAATDAT
SLTTDKDKLSYSIGADLGKNFKNQGIDINPDVLAKGMQDGMSGAQLI
LTEEQMKDVLSKFQKDLMAKRSAEFNKKAEENKAKGDAFLSANKSKPGIVVLPSGLQYKIIDAGTGAKPGKSDTVTVEYTG
TLIDGTVFDSTEKAGKPATFQVSQVIPGWTEALQLMPAGSTWEVFVPADLAYGPRSVGGPIGPNETLIFKIHLISVKKAA

Figure 5.3

Hydropathy predictions of the Mip protein of *Legionella*. *L. pneumophila sg* 1 (Philadelphia) and *L. longbeachae sg* 1 (ATCC 33462). Plots were produced by OMIGA 1.1 using the method of Kyte-Doolittle (Kyte and Doolittle, 1982).


L. pneumophila sg 1
1.0
0.0
-1.0
-2.0
Score
50
100
150
200
Residue

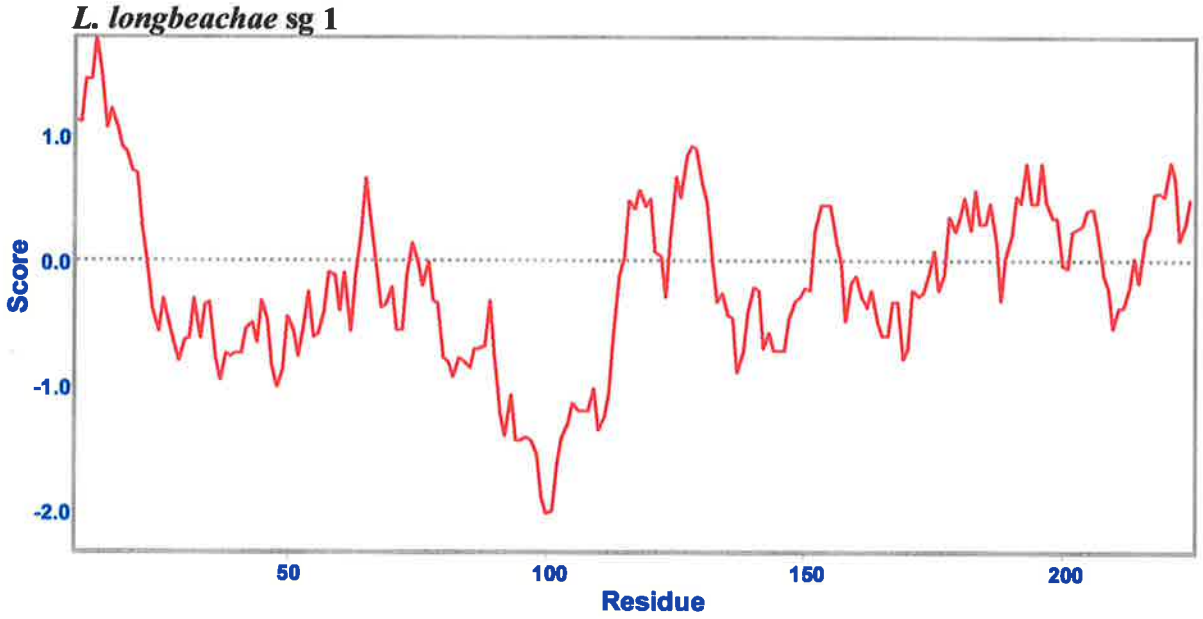

L. longbeachae sg 1
1.0
0.0
-1.0
-2.0
Score
50
100
150
200
Residue

Figure 5.4

Primer extension mapping of the 5′ end of *mip* mRNA from *L. longbeachae* sg 1 ATCC 33462. Autoradiogram of a 6% polyacrylamide-urea sequencing gel, showing chain-termination sequencing reaction (lanes A, C, G, T) using pIMVS27 as a template and oligonucleotide RT-1 as the primer. Total bacterial mRNA was hybridised with [^{32}P]-δ-ATP labelled RT-1 primer and reverse transcribed to generate cDNA. Template RNA was isolated from *E. coli* [pIMVS27] (lane c), *E. coli* [pGEM7-Zf(-)] (lane a) and *L. longbeachae sg* 1 ATCC 33462 (lane b). Dotted arrow shows region of compression. Solid arrow indicates the 5' end of the mRNA species isolated from *E. coli* [pIMVS27] and *L. longbeachae sg* 1 ATCC 33462. The start site was mapped to a G residue at nucleotide position 473 of the *L. longbeachae sg* 1 ATCC 33462 *mip* gene sequence.

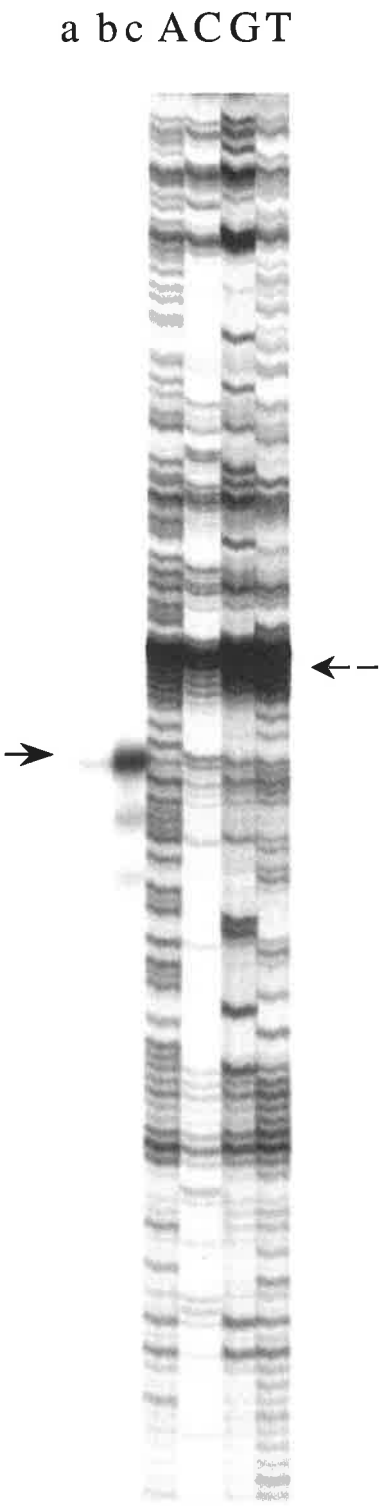

a bc ACGT

Figure 5.5

Line diagram depicts the DNA sequence data determined from sequencing pIMVS27. The solid box shows the *mip* gene from *L. longbeachae* sg 1 ATCC 33462 and selected restriction sites in the *mip* gene and stem loop structure at the end of the ORF. The inset sequence is the DNA sequence upstream of the ATG start site for translation in *L. longbeachae* sg 1 and *L. pneumophila* sg 1, showing the -10 and -35 promoter regions determined by primer extension analysis. The shaded box indicates the proposed -35 promoter region proposed for *L. longbeachae*. The non shaded box is the -35 region proposed in *L. pneumophila* (Engleberg, *et al.*, 1989). The start site for transcription is shown with a solid arrow.

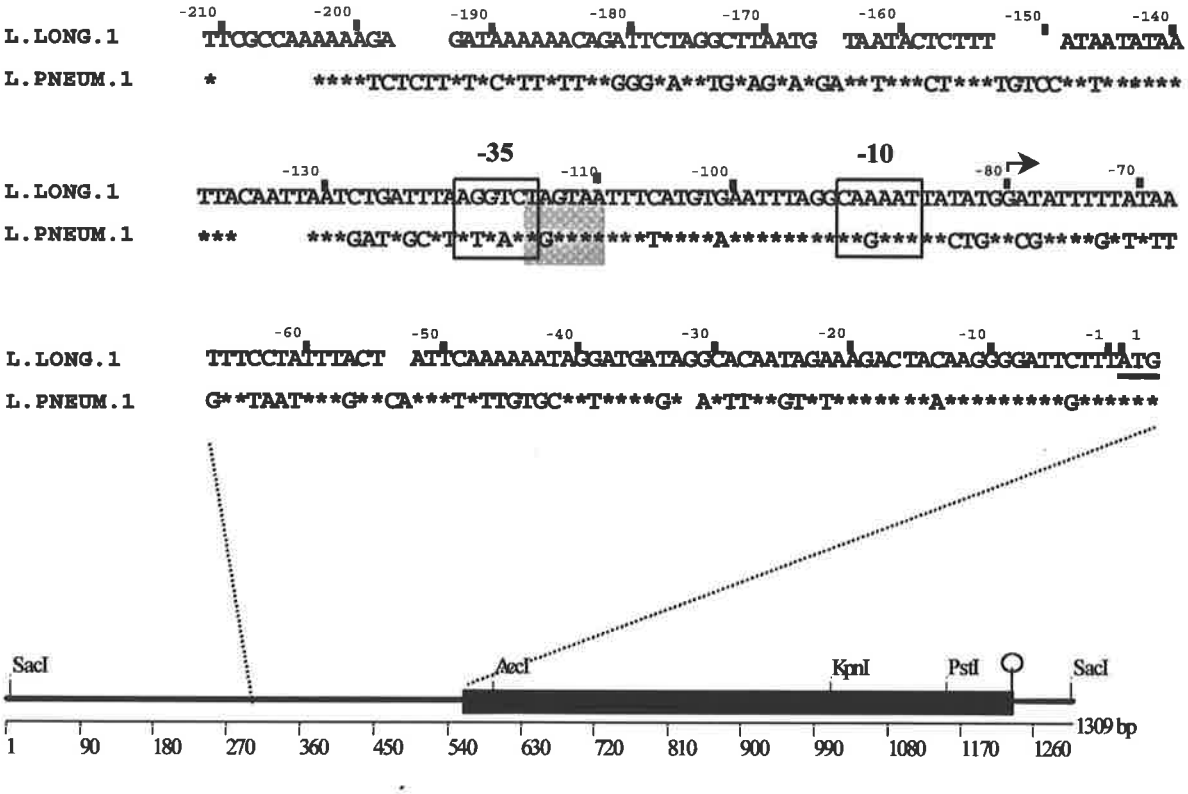
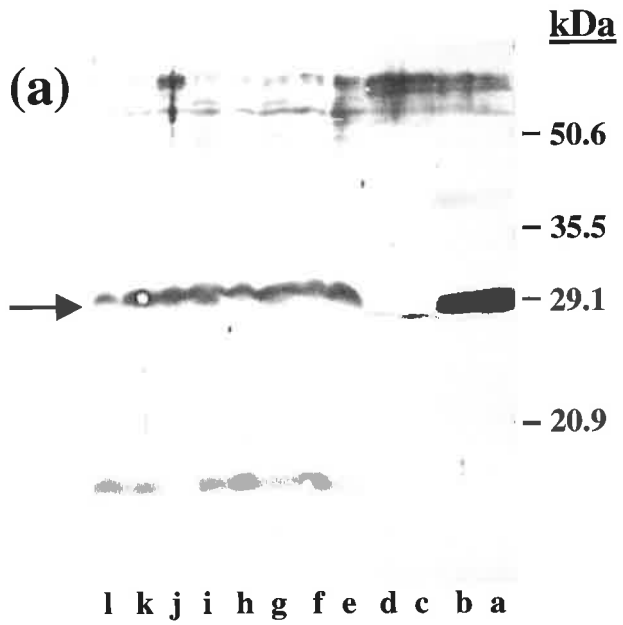

-210
-200
-190
-180
-170
-160
-150
-140
L.LONG.1
TTCGCCAAAAAAGA GATAAAAAACAGATTCTAGGCTTAATG TAATACTCTTT ATAATATAA
L.PNEUM.1
-35
-10
-130
-110
-100
-80
-70
L.LONG.1
TTACAATTAATCTGATTTAAGGTCTAGTAATTTCATGTGAATTTAGGCAAAATTATATGGATATTTTTATAA
L.PNEUM.1
-60
-50
-40
-30
-20
-10
-1
1
L.LONG.1
TTTCCTATTTACT ATTCAAAAAATAGGATGATAGGCACAATAGAAAGACTACAAGGGATTCTTTATG
L.PNEUM.1
SacI
AccI
KpnI
PstI
SacI
1309 bp
1
90
180
270
360
450
540
630
720
810
900
990
1080
1170
1260

Figure 5.6

Western immunoblot analysis of (A) whole membrane protein profiles and (B) outer membrane protein profiles with anti-*L. longbeachae* Mip polyclonal antisera. Protein samples were prepared at 30°C and 37°C, separated on 15% SDS-PAGE and transferred onto nitrocellulose membrane. Lane **a**: *E. coli* DH5α[pIMVS27] (30°C); lane **b**: *E. coli* DH5α[pIMVS27] (37°C); lane **c**: *L. pneumophila* Philadelphia (30°C); lane **d**: *L. pneumophila* Philadelphia (37°C); lane **e**: *L. longbeachae* sg 1 ATCC 33462 (30°C); lane **f**: *L. longbeachae* sg 1 ATCC 33462 (37°C); lane **g**: *L. longbeachae* sg 1 A5H5 (30°C); lane **h**: *L. longbeachae* sg 1 A5H5 (37°C); lane **i**: *L. longbeachae* sg 1 L6C9 (30°C); lane **j**: *L. longbeachae* sg 1 L6C9 (37°C); lane **k**: *L. longbeachae* sg 2 ATCC 33484 (30°C); lane **l**: *L. longbeachae* sg 2 ATCC 33484 (37°C). Arrow shows position of Mip protein band.


kDa
(a)
50.6
35.5
29.1
20.9
l k j i h g f e d c b a

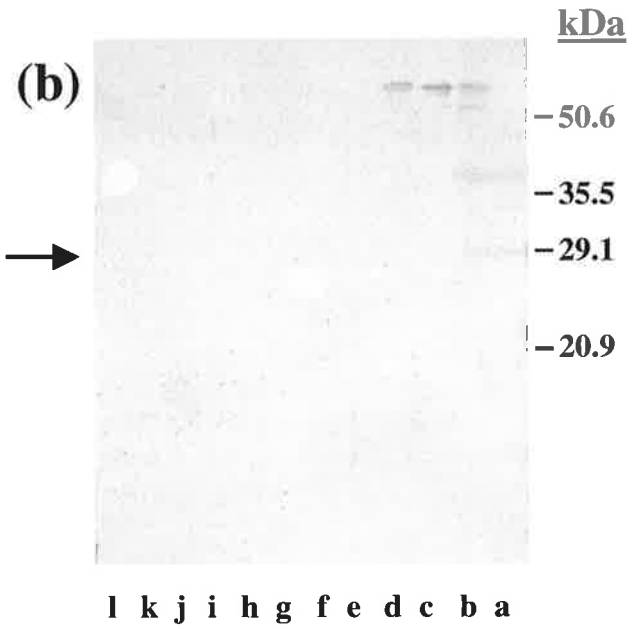

kDa
(b)
50.6
35.5
29.1
20.9
l k j i h g f e d c b a

Table 5-1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
Strains used in study		
E. coli DH5α[pIMVS26]	*E. coli* strain DH5α containing recombinant plasmid construct pIMVS26	This study
E. coli DH5α[pIMVS27]	*E. coli* strain DH5α containing recombinant plasmid construct pIMVS27	This study
Plasmids		
pIMVS26	PGEM-7Zf(-) with ca. 8 kb fragment of *L. longbeachae* sg 1 ATCC genomic DNA	This study
pIMVS27	pGEM-7Zf(-) carrying an approx. 1300 bp *Sac*I fragment of pIMVS26	This study

Table 5-2 Oligonucleotide primers

Primer designation	Oligonucleotide sequence	Experimental application
MIPB1	5' - gag gca gga tcc act gtc tcg ggc gct -3'	PCR amplify *mip* gene sequence from *L. pneumophila*
MIPB2	5' - cac ctt tta tga gga tcc tta gcc ttt -3'	PCR amplify *mip* gene sequence from *L. pneumophila*
807	5' – caa tta atc tga ttt aag gtg -3'	Sequencing *mip* gene of *L. longbeachae* ATCC 33462
806	5' – cca gac gta tta gct aaa gg -3'	Sequencing *mip* gene of *L. longbeachae* ATCC 33462
805	5' - gat ggt act gta ttt gat ag -3'	Sequencing *mip* gene of *L. longbeachae* ATCC 33462
844	5' - gag tat gat gag aaa gaa -3'	Amplification and sequencing of *L. longbeachae mip* genes
845	5' - aca att aat ctg att taa gg -3'	Amplification and sequencing of *L. longbeachae mip* genes
RT-1	5' - ggc tgc aac tga tgc tac atc gct t -3'	Primer extension assay

Chapter 6

Development of a system for genetic exchange in *Legionella longbeachae* serogroup 1: construction of isogenic *mip* mutants

6.1 Introduction

The establishment of reliable molecular genetic exchange techniques in *Legionella longbeachae* sg 1 was important for analysis of factors that play a role in pathogenesis.

The following chapter describes the use of plasmid vector pCACTUS-*mob* (Doyle *et al.*, unpublished observations) for genetic manipulation of *L. longbeachae* sg 1 and the generation of isogenic *mip* mutants. Plasmid pCACTUS-*mob* is a suicide vector that has several features that make it suitable for establishment of a genetic exchange method. Firstly it has a temperature-sensitive replicon, which, at the non-permissive temperature, in the presence of antibiotic selection, selects for integration of the vector into the chromosome by the carriage of the mutated *L. longbeachae* sg 1 gene via homologous recombination. Secondly, the vector encodes the *sacB* gene which allows resolution of the recombination event to be selected for by plating cells on media containing 6% sucrose. The *sacB* gene of *Bacillus subtilis* encodes levan sucrose, a 50 kDa enzyme secreted in culture medium by the organism (Gay, *et al.*, 1983). In the presence of high levels of sucrose, the enzyme causes the accumulation of branched chain sucrose polymers (levans) within the cell cytoplasm resulting in lysis or inhibition of growth of the organism. Thus selection on 6% sucrose results in resolution of co-integrates by selecting for resolution of the vector from the chromosome.

Plasmid pCACTUS-*mob* was used to generate isogenic *mip* mutants in *L. longbeachae* sg 1 ATCC 33462 and a virulent Australian clinical isolate A5H5. The system was adapted for use in *Legionella* and conditions such as vector delivery, resolution frequencies and the number of colonies screened before the desired mutant was obtained were assessed. The *mip* mutants and complemented strains discussed in this chapter were assessed in an animal model of virulence and in an *Acanthamoeba* model of intracellular infection to determine the significance of the Mip protein in *L. longbeachae* sg 1 (chapter 7).

6.2 Methods and materials specific to this chapter

6.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used or constructed are listed in Table 6-1and Table 2-1.

6.2.2 Allelic exchange mutagenisis

Allelic exchange was carried out to generate mutations in the *mip* gene of *L. longbeachae* sg 1 ATCC 33462 and A5H5 using the vector pCACTUS. The vector pCACTUS is a derivative of plasmids containing the *sacB* gene of *Bacillus subtilis* (pIB279) and (pIB307) and contains a temperature-sensitive pSC101 replicon (Blomfield, *et al.*, 1991). The plasmid also contains a chloramphenicol resistance marker for selection. A derivative of this plasmid, pCACTUS-*mob*, contains a *mob* region so that conjugation could be used to transfer the plasmid to host bacterial strains.

For conjugation experiments pCACTUS plasmid constructs containing the mutated *mip* gene were electroporated into the donor strain *E. coli* S-17, prior to conjugation into the *L. longbeachae* sg 1 strain of interest. A filter mating method and a plate mating method were used to transfer plasmid constructs from *E. coli* S1-17 to *L. longbeachae* sg 1 strains (section 2.7). Plates were incubated at 30°C for 2-4 weeks to select for transconjugants.

Electroporation was also used to deliver, approx. 1 μg of CsCl gradient purified pCACTUS plasmid construct to strains of *L. longbeachae* sg 1 (section 2.7). The transformed cells were incubated in BYE broth at 30°C for 5-6 hours and then plated onto CYE plates containing Km. The transformed cells were then incubated at 30°C for approx. 1-2 weeks to select for transformants.

L. longbeachae transformants or transconjugants generated by either construct delivery method were confirmed by microscopic examination for typical *Legionella* colonial morphology, by failure to grow on HBA and by agglutination with a *L. longbeachae* sg 1 specific polyclonal latex serum reagent (Medvet Science Pty. Ltd., Adelaide, S.A., Australia).

For allelic exchange, one randomly chosen Km^r or Cml^r *L. longbeachae* sg 1 colony was chosen from the resultant transformants/transconjugants generated by either method. The colony was incubated in 5 ml of BYE at 30°C with appropriate antibiotic selection for 16 - 20 hours. The culture was then serially diluted in BYE broth and plated on CYE containing Cml or Km at the non-permissive temperature for pCACTUS replication in *Legionella longbeachae* sg 1 (39°C) for 3-5 days. Plating at the non-permissive temperature for replication of pCACTUS in *L. longbeachae* sg 1 selected for the co-integration of the plasmid construct into the chromosome via homologous recombination. One antibiotic resistant colony resulting from this selection process was chosen empirically and incubated in 5 ml of BYE broth with appropriate antibiotic selection for 16 - 20 hours at 30°C. The culture was then serially diluted in BYE broth and plated on CYE media containing 6% sucrose. Plates were incubated at 30°C for 5-7 days to select for resolved co-integrates.

Colonies appearing on the sucrose plates were patched onto CYE plates and screened by PCR (see below) to assess whether successful allelic exchange had occurred. Potential mutant colonies were further characterised by Southern hybridization to confirm chromosomal rearrangement and by western immunoblot to confirm loss of production of Mip protein.

6.2.3 Rapid PCR screening of potential *mip* mutants of *L. longbeachae* serogroup 1

Potential mutant colonies appearing on the 6% sucrose plates were screened by a rapid PCR method to assess whether successful allelic exchange had occurred. Briefly, a small amount of growth from individual colonies to be screened was inoculated into the wells of a 96 well PCR microtitre tray (Perkin Elmer) containing 150 µl of sterile water. The cells were lysed by heating in a microwave oven (650 Watt) on high for 2.5 min to release genomic DNA. A 10 µl aliquot of lysate for each colony was added to the corresponding wells of a fresh 96 well PCR tray containing 15 µl of PCR mix. The PCR mix (section 2.8) contained primers #844 and #845 designed to amplify the entire *mip* gene from *L. longbeachae* strains (Table 5-2). The PCR reaction was performed under standard conditions (section 2.8) at an annealing temperature of 45°C. The amplified product for each colony was analysed on a 2% agarose gel to determine whether deletions had occurred.

6.3 Results

6.3.1 pCACTUS constructs used for optimisation of the genetic exchange method

Several constructs were generated initially to develop the method of genetic exchange in *L. longbeachae* using pCACTUS. Two derivatives of this plasmid were used and are shown in Fig 6.1. Plasmid pCACTUS-*mob* is a derivative of pCACTUS and was constructed in addition to the original vector so that conjugation could be exploited as a means of gene delivery. The *mob* region was originally inserted into the *Bam*HI site of the multiple cloning site of pCACTUS but was later cloned into a non-essential region of this vector (C. Clark *et al* unpublished observations) (Fig 6.1). To generate isogenic *mip* mutants several pCACTUS constructs were made that contained the *mip* gene of ATCC 33462 altered *in vitro* (Table 6-1). Plasmid pIMVS27 contained the wild type *mip* gene from ATCC 33462 on a 1.3 kb *Sac*I fragment and was used to generate mutations in *mip* prior to cloning into pCACTUS (Fig 6.2).

Plasmid construct pCACTUS49 used to construct a deletion mutation in the *mip* gene of *L. longbeachae* sg 1 ATCC 33462 was made in several stages. Plasmid pIMVS27, containing the entire *mip* gene from ATCC 33462, was digested with *Acc*I and *Pst*I to delete a 650-bp fragment from within the coding region of the gene (Fig 6.2). The plasmid digest was gel purified, the restriction sites blunt ended, then re-circularised, resulting in construct pIMVS28. Plasmid pIMVS28 was transformed into DH5α and the resultant transformants screened by Western immunoblot to confirm that the Mip protein was no longer expressed (data not shown). The remaining 850-bp insert of pIMVS28 was then excised and cloned into the *Sac*I site of pCACTUS, yielding pCACTUS49 (Fig 6.2).

Plasmid constructs pCACTUS47 and pCACTUS48 were made in a similar way except that a single digest was performed with *Acc*I or *Pst*I, resulting in a frame shift mutation, confirmed by sequence analysis (data not shown), as opposed to a deletion mutation in *mip* encoded on pIMVS27. This generated intermediate plasmid constructs pIMVS30 and pIMVS31 respectively (Fig 6.2). These plasmid constructs were each transformed into DH5α and resultant colonies screened by western blot to confirm loss of production of Mip protein (data not shown). The *Sac*I fragments from pIMVS30 and pIMVS31 were cloned into the vector pCACTUS-*mob* generating constructs pCACTUS48 (*Acc*I frameshift), and pCACTUS47 (*Pst*I frameshift) (Fig 6.2).

Plasmid construct pCACTUS50, used for electroporation experiments, was constructed by cloning the purified *aphA*-3 non-polar kanamycin resistance cartridge (Ménard, *et al.*, 1993), from plasmid pUC18K, into the *Sma*I site within the multi-cloning siteof pCACTUS. The *aphA*-3 kanamycin resistance cartridge (approx. 850 bp) was purified from vector pUC18K by agarose gel electrophoresis after digestion of the vector with *Sma*I (section 2.6). The construction of this vector allowed the use of Km in addition to Cml to select for transformants. The *Sac*I fragment from pIMVS28 (Fig 6.2) containing the *Acc*I-*Pst*I deleted *mip* gene was then cloned into the polylinker of pCACTUS containing the Km^r cassette yielding pCACTUS50 (not shown).

6.3.2 Transfer of pCACTUS-*mob* into *L. longbeachae* serogroup 1

Two methods of conjugation, plate mating and filter mating, were assessed for delivery of pCACTUS-*mob* into *L. longbeachae* sg 1 strains, and were adapted from methods used previously with *Legionella* (Bradley, *et al.*, 1980, Chen, *et al.*, 1984, Keen, *et al.*, 1985, Tully, *et al.*, 1992a). The conjugation methods developed utilised the natural resistance of *L. longbeachae* sg 1 to aztreonam to select against donor strains and hence negated the need to isolate an antibiotic resistant recipient strains.

The conjugation frequency was determined for each method and expressed as the number of antibiotic transformants divided by the number of recipients. This ranged from 1.9×10^{-3} to 4.8×10^{-6} transconjugants per donor cell (Table 6-2). The conjugation frequencies were comparable to those reported for transfer of various plasmids into other species of *Legionella* including *L. pneumophila, L. micdadei* and *L. bozemanii* (Chen, *et al.*, 1984, Cianciotto, *et al.*, 1988, Engleberg, *et al.*, 1988, Keen, *et al.*, 1985, Mintz and Shuman, 1987, Tully, *et al.*, 1992a). Some variation was seen in the conjugation frequencies for the two methods. The filter mating method generally yielded higher numbers of transformants in comparison to the plate mating method (Table 6-2). This was not unexpected as this conjugation method often works best for unrelated bacterial species. However, irrespective of the mating method used, transconjugants were always obtained. Conjugation was not demonstrated for *L. longbeachae* strain A5H5 and repeated attempts to introduce pCACTUS-*mob* constructs into this strain by this method were unsuccessful. Since conjugation was not a suitable method of vector delivery for all strains of *L. longbeachae* sg 1, electroporation was attempted.

Plasmid construct pCACTUS50, encoding resistance to kanamycin, was used for introduction of mutated *mip* genes into strains of *L. longbeachae* sg 1 by electroporation. It has been shown that chloramphenicol is poorly expressed in *Legionella* (Mintz and Shuman, 1988, Szeto and Shuman, 1990), however, many authors have used kanamycin resistance as it is well expressed in *L. pneumophila* (Cianciotto, *et al.*, 1989b, Hacker, *et al.*, 1993, Keen,

et al., 1985, Mintz and Shuman, 1988, Wintermeyer, *et al.*, 1994). Use of kanamycin to select for transformants may therefore shorten the incubation time required for resistant colonies to appear. Typically, the time frame for detection of transformants by conjugation was 2-4 weeks (Table 6-2).

Electroporation generally resulted in the appearance of transformants within a shorter time frame (8-15 days) than that observed for conjugation methods (Table 6-3). Transformants were detected, after plating on selective media, and the transfer rate was calculated as the number of transformants obtained per microgram of CsCl purified plasmid construct used. The transfer frequency was variable for both the ATCC 33462 and A5H5 strains and ranged from $1 \times 10^2 - 5.7 \times 10^3$ transformants/μg of DNA (Table 6-3). The transformation frequencies obtained for ATCC 33462 and A5H5, although moderate, are consistent with variability reported for other gram-negative bacteria transformed by this method (Wirth, *et al.*, 1989). Electroporation was used as the preferred method of gene delivery of pCACTUS constructs into strains of *L. longbeachae,* to generate isogenic *mip* mutants, due to the relatively short incubation time required for transformants to appear in comparison with conjugation (Table 6-3).

6.3.3 Determination of non-permissive temperature of pCACTUS-*mob* in *L. longbeachae*

Having established that pCACTUS constructs could be transferred to strains of *L. longbeachae* sg 1 and appeared to be stably maintained in this species (data not shown) other factors critical to the successful use of this suicide vector in *Legionella* needed to be determined. The restrictive temperature of pCACTUS-*mob* in the *Enterobacteriaceae* is usually 42°C (C. Clark, personal communication). Studies in our laboratory suggested that 40°C - 41°C would be the maximum temperature that would still allow growth of *L. longbeachae* sg 1 (Dr. T. W. Steele, personal communication). Therefore, the restrictive temperature of pCACTUS-*mob* in *L. longbeachae* was determined to allow for optimal

growth, while at the same time selecting for integration of the suicide vector into the chromosome via homologous recombination. Two strains were chosen for this study an *E. coli* K-12 strain S17-1 containing pCACTUS-*mob* and *L. longbeachae* sg 1 ATCC 33462 [pCACTUS47].

Cultures of both strains were grown in broth at 30°C, serially diluted, plated on solid media containing 5 µg/ml chloramphenicol and incubated at either 39°C or 40-41°C. Control plates that did not contain any antibiotic were plated with the same dilutions of each strain and incubated at the same temperatures. At 39°C growth of the control strain S17-1[pCACTUS-*mob*] was inhibited for growth on selective plates in comparison with the same dilution plated on non-selective Meuller-Hinton plates (MH) (data not shown). A similar growth restriction of S17-1[pCACTUS-*mob*] was also observed at 40°C - 41°C. This indicated that both temperatures were restrictive for replication of pCACTUS-*mob* in *E. coli*. *L. longbeachae* sg 1 ATCC 33462[pCACTUS47] grew well on selective plates incubated at 39°C in comparison with non-selective CYE plates, however, at 40°C - 41°C growth was severely reduced on both (data not shown). This suggested that at 39°C chromosomal integration of pCACTUS had occurred by homologous recombination, due to the presence of *mip* on the construct. Therefore, 39°C was used as the restrictive temperature to select for integration of the vector into the *L. longbeachae* chromosome via homologous recombination, while still allowing growth of the organism.

6.3.4 Spontaneous sucrose resistance

The level of spontaneous sucrose resistance was mesured to ensure that colonies that resulted from the allelic exchange process were due to resolution of co-integrates rather than spontaneous sucrose resistance of *L. longbeachae* sg 1. Spontaneous sucrose resistance can either be the result of loss of the pCACTUS plasmid or spontaneous chromosomal mutation. A culture of *L. longbeachae* ATCC 433462[pCACTUS-*mob*] was grown overnight with chloramphenicol selection, serially diluted, and plated onto media containing Cml or Cml

plus 6% sucrose. The spontaneous rate of resistance was calculated by dividing the number of CFU/ml determined from the Cml-6% sucrose plates by the CFU/ml from the Cml plates. The frequency of resistance was 1. 6 × 10^{-5}, indicating that the level of spontaneous resistance was low and that the plating efficiency of the culture had been reduced by approx. 6 × 10^{4} fold. This level of resistance was in the order expected for this gene product (C. Clark, personal communication) and was consistent with the results reported for use of *sacB* as a selective marker in *L. pneumophila* (Cianciotto, *et al.*, 1988) and *A. hydrophila* (Wong, *et al.*, 1997).

6.3.5 Construction and characterisation of a *mip* mutant in *L. longbeachae* serogroup 1 ATCC 33462

An isogenic *mip* mutant was generated in *L. longbeachae* ATCC 33462 using the vector construct pCACTUS49 (Fig 6.2). Plasmid pCACTUS49 was transferred into *E. coli* strain S17-1 by electroporation. Strain *E. coli* S17-1[pCACTUS49] was then used as a donor in conjugation experiments with ATCC 33462, using the plate mating method, to deliver the mutated construct into *L. longbeachae* sg 1.

The conjugation frequency was 4.8 × 10^{-6} Cml^{r} transconjugants per recipient (Table 6.2). One colony was chosen randomly to perform the allelic exchange process and was plated at the non-permissive temperature for pCACTUS-*mob* replication in *L. longbeachae* (39°C) under selective pressure (chloramphenicol) to select for integration of the construct into the chromosome via homologous recombination. Integration was followed by resolution of pCACTUS-*mob* from the chromosome by plating the co-integrates onto CYE agar containing 6% sucrose. The frequency of resolution, expressed as the number of sucrose tolerant Cml sensitive colonies per Cml^{r} colony, was 1.03 × 10^{-4} (Table 6.2).

A rapid PCR method was used to screen potential isogenic *mip* mutants constructed by this method. Primers # 844 and # 845 (Fig 6.2) amplified an approx. 850 bp product in the case of wild type *L. longbeachae* ATCC 33462 strain and an approx. 650 bp product in

colonies where a deletion in the *mip* gene had occurred due to replacement of the wild gene with the mutated gene (data not shown). Only one colony containing the mutant allele was detected in the 100 colonies screened by PCR giving an approx. 1% mutation rate with no direct selection (Table 6-2).

Southern hybridisation analysis of mutant strain, subsequently designated B10, confirmed that the correct chromosomal rearrangement had occurred in *L. longbeachae* sg 1 ATCC 33462. Genomic DNA from B10 and ATCC 33462 was digested with *Kpn*I, that has a restriction site within the wild type *mip* gene (Fig 6.2), and duplicate Southern transfers were probed with Digoxigenin labelled pIMVS27 and pCACTUS-*mob* (Fig 6.3A). The mutant strain had only one hybridising fragment (Fig 6.3A, lane b), lacking the internal *Kpn*I restriction site, removed by the *Acc*I/*Pst*I deletion, while the wild type parent strain had two hybridising fragments (Fig 6.3A, lane a). No hybridising bands were detected when genomic digests were probed with labelled pCACTUS-*mob* indicating the vector sequence had completely resolved from the chromosome and was lost from the strain (data not shown).

The mutant strain, B10, was additionally examined by western immunoblot to confirm loss of production of the Mip protein. Whole cell protein extracts were prepared from test strains and blots probed with polyclonal anti-*L. longbeachae* sg 1 Mip antisera (section 5.2.4). Mutant strain B10 did not produce a Mip protein (Fig 6.3B, lane f) in comparison to the parent strain ATCC 33462 (Fig 6.3B, lane e).

6.3.6 Construction and characterisation of a *mip* mutant in *L. longbeachae* serogroup 1 strain A5H5

Plasmid construct pCACTUS50 was used to construct a deletion mutation in the *mip* gene of *L. longbeachae* sg 1 strain A5H5. This construct was delivered into A5H5 by electroporation as conjugation was not successful with this strain. The efficiency of transformation by electroporation was 5.7×10^3 transformants per microgram of DNA

(Table 6-3). One colony was chosen randomly to perform the allelic exchange process and was plated at the non-permissive temperature of 39°C. The recombination frequency for this event was 0.375 indicating that virtually all of the colonies subjected to this selective pressure contained integrated pCACTUS construct due to homologous recombination. The frequency of resolution of this construct on 6% sucrose was 8.3×10^{-5} sucrose tolerant colonies per Km^r colony.

Rapid PCR identified potential isogenic *mip* mutant colonies (10%) where a deletion in the gene had occurred. Southern analysis of B8, one of the mutants identified, generated a hybridisation pattern identical to ATCC 33462 *mip* mutant B10 with only one hybridising band as it lacked an internal *Kpn*I site (Fig 6.3A, lane b). The mutant strain also did not produce a Mip protein as evidenced by western immunoblot (Fig 6.3B, lane k).

6.3.7 Construction and characterisation of a *mip* mutant in *L. longbeachae* serogroup 1 ATCC 33462 (R).

Plasmid construct pCACTUS50 was used to construct an isogenic *mip* mutant in a more recently acquired stock of the ATCC 33462 type strain, designated ATCC 33462 (R). This construct was delivered by electroporation with a transformation rate of 1×10^2 transformants per microgram of DNA (Table 6-3).

One colony was chosen for the allelic exchange process and plated at 39°C to select for integration into the chromosome. This occurred at a recombination efficiency of 1.3 (Table 6-3) and was similar to other calculated recombination frequencies indicating that virtually all colonies contained integrated pCACTUS construct. The frequency of resolution of the vector sequence was 2.7×10^{-5} and was also similar to that observed for other allelic exchange experiments (Table 6-2 and Table 6-3).

Rapid PCR identified one mutant strain, designated E1, in 100 colonies screened giving a mutation rate of 1% in the absence of direct selection (Table 6-3). The PCR pattern for this mutant colony was identical to that observed for ATCC 33462 and A5H5 described

previously (data not shown). Southern hybridisation indicated that the correct chromosomal gene rearrangement had occurred in strain E1 and a pattern identical to that observed for B8 and B10 (Fig 6.3A). Additionally, an identical filter probed with pCACTUS-*mob* (data not shown) indicated that the vector sequence had been excised from the chromosome. Western immunoblot of strain E1 (Fig 6.3B, lane i) determined that the mutant strain did not produce a Mip protein in comparison with the parent isolate (Fig 6.3B, lane h).

6.3.8 Complementation of *mip* mutants

To ensure that the mutation process developed using pCACTUS in *L. longbeachae* sg 1 did not affect genes other than *mip*, complemented mutants strains were constructed using vector construct pIMVS29. Plasmid pIMVS29 was generated by cloning the *Sac*I fragment from pIMVS27, which contained the entire *mip* gene from ATCC 33462, into the vector pWKS130 (Wang and Kushner, 1991). Plasmid pWKS130 is a low copy number pSC101 based vector, encoding resistance to kanamycin and is designed for cloning, complementation analysis, and sequencing, in *E. coli*. It was thought that as it is a pSC101 based replicon, as is pCACTUS, it would be a suitable vector for stable replication in *L. longbeachae* sg 1.

The recombinant plasmid construct pIMVS29 was checked to ensure that Mip protein was produced expressed in *E. coli* DH5α, by screening of transformants by western blot using anti-*L. longbeachae* sg 1 Mip polyclonal antiserum (section 5.2.4). All transformants, that contained pIMVS29, produced the expected approx. 27 kDa Mip protein (data not shown).

Mutant strains B10 and B8 was complemented using pIMVS29 by electroporation (section 2.7). Transformants were observed within 5 days and the transformation rate was 2.2×10^4 and 3.2×10^3 respectively (Table 6.3). This result indicated that vector pWKS130 was suitable for use in *L. longbeachae* sg 1 as a complementing vector.

Several transformants, containing pIMVS29, were screened by western immunoblot for each mutant strain to ensure expression of Mip had been restored. A representative result is shown in Fig 6.3B. Whole cell protein profiles transferred by western to nitrocellulose membrane were reacted with anti-*L. longbeachae* sg 1 Mip antiserum. As expected, a protein product of approx. 27 kDa was observed for wild type *L. longbeachae* sg 1 ATCC 33462 (Fig 6.3B, lane e) and A5H5 (Fig 6.3B, lane j). A protein band was absent in ATCC 33462 *mip* mutant B10 (Fig 6.3B, lane f) and A5H5 *mip* mutant B8 (Fig 6.3B, lane k). The complemented A5H5 B8 *mip* mutant strain B8.22 produced a 27 kDa band in the presence (Fig 6.3B, lane m) or absence (Fig 6.3B, lane l) of antibiotic selection for pIMVS29. Similarly, the complemented ATCC 33462 B10 *mip* mutant strain B10.9 produced a 27 kDa band in the absence of antibiotic selection for pIMVS29 (Fig 6.3B, lane g). Complemented *L. longbeachae* sg 1 *mip* mutant strains B10.9 and B8.22 were chosen for subsequent studies.

6.4 Discussion

To identify factors with a role in intracellular infection we sought to develop a system for site specific mutagenesis in *L. longbeachae* sg 1 using the suicide vector pCACTUS-*mob*. The vector has a temperature sensitive replicon to allow integration into the chromosome of *L. longbeachae* via homologous recombination and this was achieved at 39°C. Additionally, the vector contained the *sacB* gene of *B. subtilis*. Previous studies reported that the *sacB* gene was a suitable counter-selectable marker in *L. pneumophila* as it could reduce the plating efficiency of the organism by 10^5-fold on sucrose containing medium (Cianciotto, *et al.*, 1988). Since *Legionella* derive most of their energy from the oxidation of amino acids rather than fermentative processes they do not use sucrose as a sole carbon source (Dowling, *et al.*, 1992). The ability of an organism to utilise sucrose as a sole carbon source can be problematic, although not insurmountable, for use of pCACTUS-*mob* (Wong, *et al.*, 1997).

A reliable method of transfer of genes altered *in vitro* into to *L. longbeachae* sg 1 was first required before allelic exchange could be attempted. Conjugation was used initially to deliver pCACTUS constructs to *L. longbeachae* sg 1 strains as this method had been used successfully with *L. pneumophila* (Chen, *et al.*, 1984, Cianciotto, *et al.*, 1988, Dreyfus and Iglewski, 1985, Engleberg, *et al.*, 1988). A plate mating method and a filter mating method were evaluated and yielded transconjugants at a frequency ranging from $1.9 \times 10^{-3} - 4.8 \times 10^{-6}$ antibiotic resistant *L. longbeachae* sg 1 per recipient (Table 6-2). The efficiency of conjugation was similar to that observed for plasmid transfer into *L. pneumophila* (Chen, *et al.*, 1984, Cianciotto, *et al.*, 1988, Dreyfus and Iglewski, 1985, Engleberg, *et al.*, 1988, Mintz and Shuman, 1988, Wiater, *et al.*, 1994a). This method could not deliver pCACTUS into A5H5 even after many repeated attempts. Widely varying conjugation frequencies have been reported for strains of *L. pneumophila* (Marra and Shuman, 1989, Mintz and Shuman, 1988, Tully, *et al.*, 1992a). Therefore, it is not surprising that within *L. longbeachae,* some strains would be better recipients than others. Additionally, strain A5H5 contains a native plasmid that may prevent conjugation by surface exclusion.

Although conjugation was successful with ATCC 33462, the time frame for growth of transconjugants after plating of the mating mix was long, typically 3-4 weeks, and this may be explained by two factors. Firstly, the temperature (30°C) used for selection of the transconjugants is not optimal for growth of *Legionella*. Secondly, chloramphenicol resistance, encoded on pCACTUS, may be poorly expressed in *L. longbeachae.* Levels of this antibiotic have been shown to be critical for *L. pneumophila* although at low concentrations it has been used successfully as a selective marker in this species (Mintz and Shuman, 1988). Kanamycin resistance is expressed well in *Legionella* and has been used most widely for this species (Mintz and Shuman, 1988).

Plasmid construct pCACTUS50 contained the *aphA*-3 kanamycin resistance cassette from pUC18K and was delivered to strain A5H5 and ATCC 33462 by electroporation. The

rate of transformation varied for each strain and ranged from $1 \times 10^2 - 5.7 \times 10^3$ Km^r colonies/ μg of plasmid DNA. A transformation frequency of 10^5 transformants per microgram of plasmid DNA (pMMB33) has been reported for *L. pneumophila* although the recipient strain used was a restriction defective mutant (Cianciotto, *et al.*, 1989a). Electroporation has been used to deliver plasmid DNA to *L. pneumophila* by other workers although transfer frequencies were not reported (Marra, *et al.*, 1992, Wiater, *et al.*, 1994b). The time frame for transconjugants to come up on plates was considerably less, approx. 8-15 days, using this method as opposed to conjugation (21-28 days). Therefore, electroporation was used as the method of choice for delivery of pCACTUS constructs into *L. longbeachae* sg 1 strains. Additionally, electroporation negates the need for generating antibiotic resistant recipients, however, it can sometimes result in a lower frequency of transfer (Ott, 1994).

Integration of the pCACTUS construct into the chromosome of *L. longbeachae* strains occurred readily with almost all colonies selected at the non-permissive temperature containing integrated vector (Table 6-2 and 6-3). Resolution of the integrated vector and associated allelic exchange also occurred frequently ranging from 1.03×10^{-4} - 8.3×10^{-5} sucrose tolerant antibiotic sensitive colonies per antibiotic resistant colony. This yielded 100-1000's of sucrose resistant colonies per experiment. It is expected that the number of sucrose tolerant colonies as a result of successful resolution is much greater (10-100 times) than those that are spontaneous sucrose resistant colonies. This was observed in all cases of allelic exchange mutagenesis of *L. longbeachae* sg 1 using pCACTUS.

The pCACTUS-*mob* vector and allelic exchange method developed to generate isogenic *mip* mutants in *L. longbeachae* sg 1 strains has several advantages in comparison with other allelic exchange protocols. First, the method of delivery of pCACTUS constructs into *L. longbeachae,* although occurring at moderate frequencies (Table 6-2 and Table 6-3), does not preclude the generation of isogenic mutants since only one transformed colony is required for the mutagenesis process. Therefore this construct is amenable to species and strains of *Legionella* that have poor transformation frequencies (eg *L. pneumophila*

Philadelphia). Restriction systems, known to exist in *L. pneumophila* (Chen, *et al.*, 1986), often prevent successful DNA delivery into this strain, resulting in poor conjugation frequencies (Marra and Shuman, 1989). The pCACTUS-*mob* system negates the need to identify naturally occurring mutants of the recipient strain of interest that lack an endogenous restriction system thereby making them better recipients in subsequent mating experiments (Marra and Shuman, 1989). Additionally, the successful introduction of pCACTUS into *L. longbeachae* sg 1 strains using electoporation negates the need to generate antibiotic resistant recipients required for conjugation. Counter selection against donor strains using natural resistance to aztreonam was exploited with *L. longbeachae* sg 1 in conjugation experiments, since spontaneous or constructed resistant recipients need to be tested for virulence to ensure that attenuation has not occurred.

Only one potential transformed colony is required to generate a mutant strain due to the instability of the recombination event occurring prior to resolution (Fig 6.5). Resolution can lead to the generation of wild type or mutant genotype, and the frequency of each allele is dependent on the type of construct (eg frame shift or deletion) and the size of the altered gene fragment cloned into pCACTUS (Fig 6.5).

Direct selection of mutants is not required and only small numbers of resolved colonies need to be screened. This was achieved with *L. longbeachae* using a rapid PCR method utilising a microwave lysate of the original identified colonies, and thus a result could be achieved in one day. Generally 50-100 colonies were screened with potential mutant colonies identified at a rate of 1-10% per number of colonies screened (Table 6-2 and Table 6-3). It has been shown that multiple passage on artificial media can lead to loss of infectivity of *L. pneumophila* strains (McDade and Shepard, 1979). Therefore any method for site-specific mutagenesis should minimise manipulation of the strain of interest to prevent attenuation of virulence. For this reason, complimented isogenic *mip* mutants were constructed. Results of *in vivo* and *in vitro* biological assays of these strains (discussed in

chapter 7) concluded that the allelic exchange process itself did not attenuate virulence of the original strain.

6.5 Summary

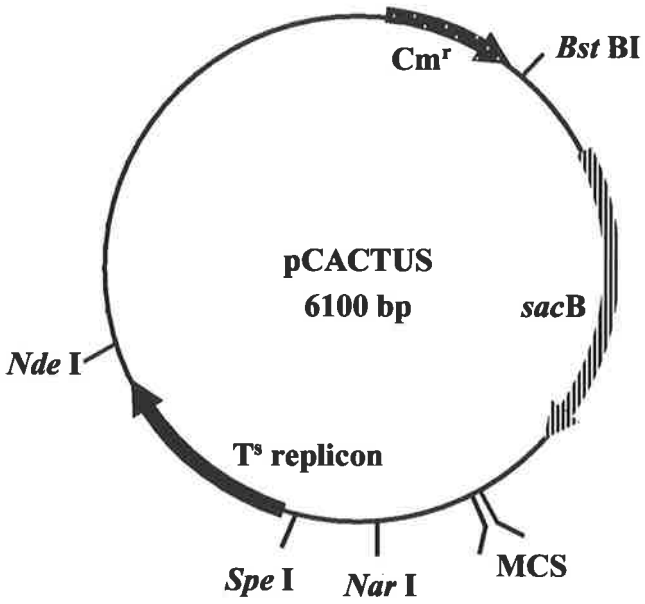
A genetic manipulation system was developed for use in *L. longbeachae* sg 1 using the suicide vector pCACTUS-*mob*. The pCACTUS-*mob* construct containing the *mip* gene, altered *in vitro*, could be delivered to strains of *L. longbeachae* sg 1 by conjugation or electroporation. The frequency of transformants yielded by either method was reasonable although this is not a prerequisite for the use of this system. The allelic exchange process yielded high resolution frequencies thereby allowing identification of isogenic mutants in the *mip* gene in two strains of *L. longbeachae* sg 1.

Figure 6.1

(i): Plasmid map of pCACTUS allelic exchange vector (C. A. Clark, unpublished observations). The vector pCACTUS is a derivative of plasmids containing the *sacB* gene of *Bacillus subtilis* (pIB279) and (pIB307) and contains a temperature-sensitive (T^s) pSC101 replicon. The plasmid also contains a chloramphenicol resistance marker for selection. Multiple cloning site (MCS) with unique restriction enzyme sites *Ase*I, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I and *Sac*I.

(ii): Plasmid map of pCACTUS-*mob* a derivative of pCACTUS, containing a mobilisation region (*mob*) for conjugative transfer.

(i)


Cm^r
Bst BI
pCACTUS
6100 bp
sacB
Nde I
T^s replicon
Spe I
Nar I
MCS

(ii)

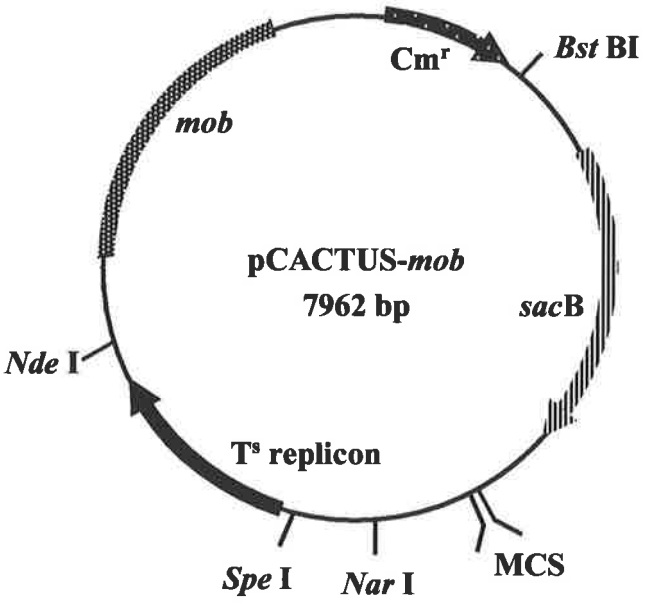

Cm^r
Bst BI
mob
pCACTUS-mob
7962 bp
sacB
Nde I
T^s replicon
Spe I
Nar I
MCS

Figure 6.2

Generation of pCACTUS-*mob* constructs pCACTUS47, 48 and 49 used for development of genetic exchange in *L. longbeachae* sg 1 and the construction of isogenic *mip* mutants. All mutations in the wild type *mip* gene of *L. longbeachae* sg 1 ATCC 33462 were made in pIMVS27, the original plasmid clone derived from the genomic bank constructed in ATCC 33462 (section 5.2.3). Plasmids pIMVS28, 30 and 31 were intermediate plasmids that were transformed into DH5α to screen transformants for expression of Mip. The *Sac*I fragment from these vector constructs was then cloned into pCACTUS-mob to generate the final construct used for the allelic exchange process. Small solid arrows show primers #844 and #845 used to amplify the entire *mip* gene from strains of *L. longbeachae* sg 1 and for initial screening of mutants.

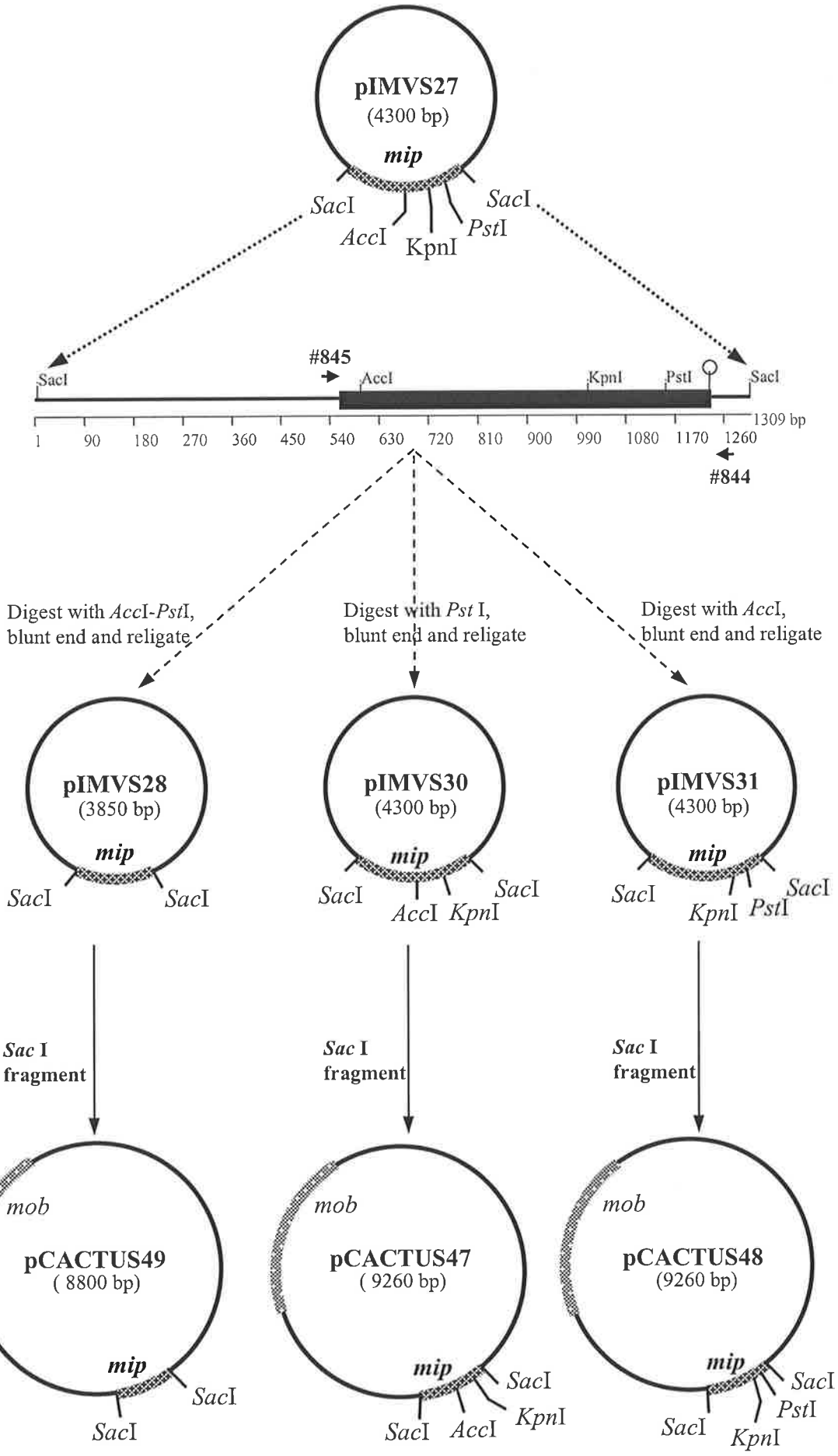

pIMVS27
(4300 bp)
mip
SacI
AccI
KpnI
PstI
SacI
#845
SacI
AccI
KpnI
PstI
SacI
1309 bp
1 90 180 270 360 450 540 630 720 810 900 990 1080 1170 1260
#844
Digest with AccI-PstI, blunt end and religate
Digest with Pst I, blunt end and religate
Digest with AccI, blunt end and religate
pIMVS28
(3850 bp)
mip
SacI
SacI
pIMVS30
(4300 bp)
mip
SacI
SacI
AccI
KpnI
pIMVS31
(4300 bp)
mip
SacI
SacI
KpnI
PstI
Sac I fragment
Sac I fragment
Sac I fragment
mob
pCACTUS49
(8800 bp)
mip
SacI
SacI
mob
pCACTUS47
(9260 bp)
mip
SacI
SacI
AccI
KpnI
mob
pCACTUS48
(9260 bp)
mip
SacI
SacI
PstI
KpnI

Figure 6.3

(i): Southern hybridisation demonstrating mutagenesis by allelic exchange of the *L. longbeachae* sg 1 *mip* gene. Genomic DNA was digested with *Kpn*I and probed with Digoxigenin labelled pIMVS27. Lanes: **a**, *L. longbeachae* sg 1 A5H5; lane **b**, *mip* mutant B8. The solid arrow indicates the 7.3 kb fragment generated in B8 due to the loss of the internal *Kpn*I site which generates 1 kb (dashed arrow) and 7 kb fragments in the parent strain. A similar pattern was observed for *L. longbeachae* sg 1 ATCC 33462 (data not shown).

(ii): Western immunoblot analysis of *mip* mutants and complemented *mip* mutant strains. Whole protein cell profiles were electrophoresed on 15% polyacrylamide, transferred to nitrocellulose by western blot then probed with anti-*L. longbeachae* sg 1 Mip polyclonal antiserum (section 5.2.4). Lane **a**: *E. coli* DH5α[pGEM-7Zf(-)]; lane **b**: *E. coli* DH5α[pIMVS26]; lane **c**: *E. coli* DH5α[pIMVS27]; lane **d**: *L. pneumophila* sg 1 (Philadelphia): lane **e**: *L. longbeachae* sg 1 ATCC 33462; lane **f**: *L. longbeachae* sg 1 ATCC 33462 *mip* mutant B10; lane **g**: *L. longbeachae* sg 1 ATCC 33462 complemented *mip* mutant strain B10.9; lane **h**: *L. longbeachae* sg 1 ATCC 33462 (recent stock); lane **i**: *L. longbeachae* sg 1 ATCC 33462 (recent stock) *mip* mutant strain E1; lane **j**: *L. longbeachae* sg 1 strain A5H5; lane **k**: *L. longbeachae* sg 1 A5H5 *mip* mutant B8; lane **l**: *L. longbeachae* sg 1 A5H5 complemented *mip* mutant strain B8.22; lane **m**: *L. longbeachae* sg 1 A5H5 complemented *mip* mutant strain B8.22 (with kanamycin selection).

(i)

(ii)

Figure 6.4

Genetic interactions between pCACTUS and the *L. longbeachae* sg 1 chromosome. The interrupted line represents the pCACTUS construct containing the mutated *mip* gene of *L. longbeachae* sg 1 ATCC 33462 and the solid line the *L. longbeachae* sg 1 chromosome. The thickened lines represent the allelic regions on the chromosome and pCACTUS which carry the *mip* gene. The illustrated cross-over may represent either insertion or release of pCACTUS.

(i): At the non-permissive temperature of 39°C in *L. longbeachae* sg 1 insertion by a single reciprocal recombination event yields a circular chromosome in which pCACTUS is located between the two *mip* alleles (mip^- and mip^+).

(ii): Release of pCACTUS from the chromosome by recombination results in an exchange of alleles between pCACTUS and the chromosome. Plating of the integrated colony selected in (i) above at 30°C on 6% sucrose selects for resolved co-integrates that do not harbour pCACTUS and hence selects for the recombination event leading to exchange of the *mip* alleles. Two outcomes are possible at the 30°C stage as the integrate exists in two forms. The ratio of colonies showing the correct chromosomal gene rearrangement is dependent on the type of mutation generated in the gene of interest, in this case the *mip* gene of *L. longbeachae* sg 1.

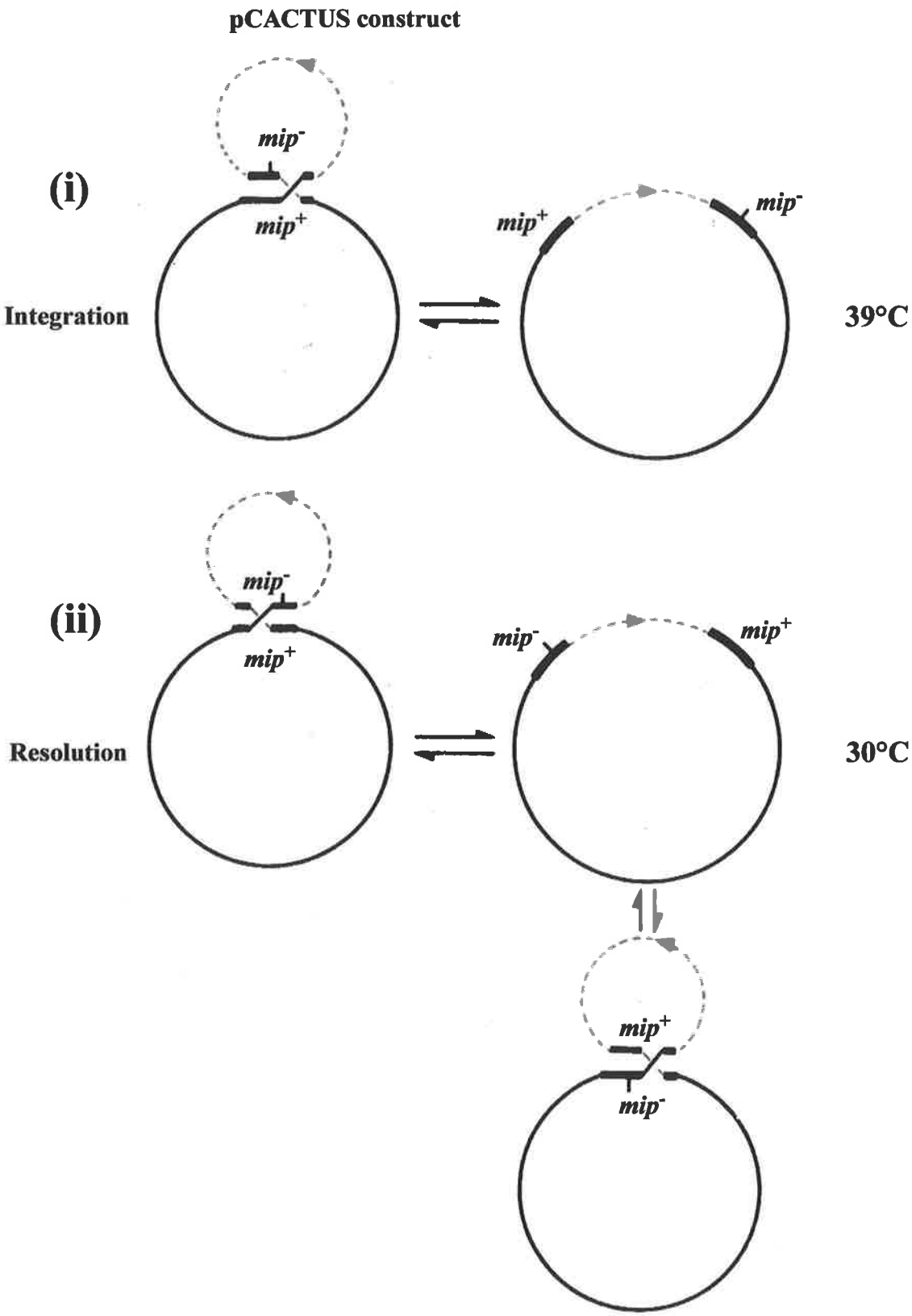

pCACTUS construct
(i)
mip-
mip+
Integration
mip+
mip-
39°C
(ii)
mip-
mip+
Resolution
mip-
mip+
30°C
mip+
mip-

Table 6-1 Bacterial strains and plasmids

Strain or plasmid constructed	Relevant characteristics	Source or reference
Strain		
B10	*L. longbeachae* sg 1 strain ATCC 33462 (original) with a *mip* *Acc*I-*Pst*I deletion	This study
E1	*L. longbeachae* sg 1 strain ATCC 33462 (recent) with a *mip* *Acc*I-*Pst*I deletion	This study
B8	*L. longbeachae* sg 1 strain A5H5 with a *mip* *Acc*I-*Pst*I deletion	This study
B8.22	Strain B8 complemented with plasmid pIMVS29	This study
B10.9	Strain B10 complemented with plasmid pIMVS29	This study
Plasmid		
pIMVS26	PGEM-7Zf(-) with an approx. 8 kb fragment of *L. longbeachae* sg 1 ATCC 33462 genomic DNA	This study
pIMVS27	pGEM-7Zf(-) with an approx. 1.3 kb *Sac*I fragment of pIMVS26 encoding the *mip* gene	This study
pIMVS28	pGEM-7Zf(-) containing an *Acc*I-*Pst*I deleted *mip* gene, generated in pIMVS27	This study
pIMVS30	pGEM-7Zf(-) containing a frame shift mutation in a *Pst*I site of *mip* generated in pIMVS27	This study
pIMVS31	pGEM-7Zf(-) containing a frame shift mutation in an *Acc*I site of *mip* generated in pIMVS27	This study
pCACTUS47	pCACUS-*mob* containing mutated *mip* gene from pIMVS30	This study
pCACTUS48	pCACTUS-*mob* containing mutated *mip* gene from pIMVS31	This study
pCACTUS49	pCACTUS-*mob* containing deleted *mip* gene fragment from pIMVS28	This study
pCACTUS50	pCACTUS containing *aphA*- 3 Km^r cartridge from pUC18K and the deleted *mip* gene from pIMVS28	This study
pIMVS29	pWKS130 containing entire *mip* gene on the approx. 1.3 kb *Sac*I fragment from pIMVS27	This study

Table 6-2 Conjugation of pCACTUS constructs into *L. longbeachae* serogroup 1 ATCC 33462

Donor: ***E. coli*** S17-1 containing	pCACTUS47	PCACTUS48	PCACTUS49	pCACTUS47	PCACTUS-*mob*
conjugation method	Plate mating	Plate mating	Plate mating	Filter mating	Filter mating (O/N)
Incubation time [a]	21 days	28 days	26 days	20 days	15 days
Conjugation frequency [b]	6×10^{-4}	1.08×10^{-4}	4.8×10^{-6}	1.9×10^{-3}	1.16×10^{-4}
Recombination frequency [c]	0.89	0.214	1.8×10^{-3}	nd	nd
Resolution frequency [d]	4.6×10^{-5}	4.3×10^{-4}	1.03×10^{-4}	nd	nd
Mutation rate [e]	3 %	5.8 %	1 %	nd	nd
Mutant designation	-	-	B10	nd	nd

nd: not determined.

[a] time taken for the transconjugants to appear on selective media after plating.

[b] expressed as number of antibiotic resistant *L. longbeachae* per recipient.

[c] expressed as the number of antibiotic resistant colonies (39°C) per antibiotic resistant colony 30°C (1 =100%).

[d] expressed as the number of sucrose tolerant antibiotic sensitive colonies per antibiotic resistant colony.

[e] expressed as the percentage of potential mutants displaying the correct chromosomal rearrangement per number of colonies screened.

Table 6-3 Electroporation of pCACTUS constructs into *L. longbeachae* serogroup 1

***L. longbeachae* recipient** **PCACTUS construct**	A5H5 pCACTUS50	ATCC 33462 PCACTUS50	ATCC 33462-B10 pIMVS29	A5H5-B8 pIMVS29
Incubation time [a]	8 days	15 days	5 days	5 days
Transformation rate [b]	5.7×10^3	1×10^2	2.2×10^4	3.2×10^3
Recombination frequency [c]	0.375	1.3	nd	nd
Resolution frequency [d]	8.3×10^{-5}	2.7×10^{-5}	nd	nd
Mutation rate [e]	10 %	1 %	nd	nd
Mutant designation	B8	E1	B10.9	B8.22

[a] time taken for transformants to appear on selective media after plating.

[b] expressed as number of antibiotic resistant *L. longbeachae* colonies per µg of plasmid DNA.

[c] expressed as the number of antibiotic colonies (39°C) per antibiotic resistant colony 30°C (1=100%).

[d] expressed as the number of sucrose tolerant antibiotic sensitive colonies per antibiotic resistant colony.

[e] expressed as the percentage of potential mutants displaying the correct chromosomal rearrangement per number of colonies screened.

nd: not determined.

Chapter 7

Assessment of *L. longbeachae* serogroup 1 *mip* mutants in an animal model of infection and an *Acanthamoeba* model of infection

7.1 Introduction

The Mip protein of *L. longbeachae* is highly homologous to Mip proteins of other *Legionella* species, with conservation of sites critical for enzymic PPIase activity. Therefore, it is likely to have a similar function and mechanism of action. The cell membrane profile exhibited by *L. longbeachae* strains is distinctive in comparison to *L. pneumophila* sg 1 (Philadelphia), generally having fewer proteins in total and most notably lacking MOMP a very abundant membrane protein in *L. pneumophila*. It is possible that other outer membrane proteins such as Mip, involved in early uptake and intracellular survival as reported for *L. pneumophila,* may have greater importance in species such as *L. longbeachae* which lack an abundant MOMP protein.

To understand the significance of Mip in *L. longbeachae* sg 1 isogenic *mip* mutants were constructed in *L. longbeachae* sg 1 ATCC 33462 and an Australian clinical isolate, strain A5H5. Complemented *mip* mutants were also constructed to determine whether the allelic exchange method affected genes other than *mip,* and to satisfy Koch's molecular postulates. The *mip* mutants, which represent the first reported genetic manipulation of this species, were tested for their ability to infect U937 macrophage like cells and *Acanthamoeba,* and for their ability to establish lethal infection in guinea pigs.

7.2 Methods and materials specific to this chapter

7.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used or constructed in this Chapter are listed in Table 2-1 and Table 6-1

7.2.2 Infection of *Acanthamoeba* with *Legionella* strains

Acanthamoeba group 2 spp. (Strain ACO97), used in co-culture experiments, was originally isolated from commercial potting mix. The identity was confirmed by Mr. Brett Robinson (South Australian Water Corporation, Bolivar, South Australia, Australia). When required, ACO97 was seeded onto amoeba agar plates (Amoebae saline plus 0.01% malt extract (Oxoid), 0.01% yeast extract (Oxoid) and 1% agar no. 1 (Oxoid)), overlaid with a suspension of heat killed *E.coli* ATCC 25922, and incubated at 25°C. The suspension of *E. coli* ATCC 25922 was prepared by scraping the growth from five confluent plates of growth into 5 ml of amoebae saline (2 mM NaCl, 0.016 mM $MgSO_4$, 0.027 mM $CaCl_2$, 1 mM Na_2HPO_4 and 1 mM KH_2PO_4) and then heating at 68°C for 20 minutes to render the suspension non-viable. Seeded plates were checked daily for signs of contamination and migration of amoebae. Amoebae were harvested into amoebae saline when growth was maximal and used immediately in co-culture experiments, or stored at 4°C, as a stock suspension.

7.2.2.1 Liquid co-culture of *Legionella*

Liquid co-culture of *Acanthamoeba* strain ACO97 and *Legionella* species were set up essentially using the method of Cianciotto and Fields, (1992). Co-cultures were prepared in amoebae saline, in duplicate, containing approx. 10^3 *Legionella* bacteria/ml and 10^4 *Acanthamoeba* cysts/ml. *Legionella* suspensions were prepared by emulsifying growth from a 72 hour plate in sterile tap water to yield approx. 10^9 organisms/ml by comparison with a McFarland standard (section 3.2). The suspension prepared for each test strain was diluted in

amoebae saline in 100-fold serial dilutions to yield approx. 10^3 bacteria/ml. Duplicate 4 ml aliquots of this stock suspension were then inoculated with amoebae. The total number of viable *Legionella* bacteria in the co-culture was determined retrospectively by plating the stock suspension onto CYE media. *Acanthamoeba* were enumerated in a KOVA Glasstic slide (Hycor Biomedical Inc, CA), after harvesting from plates and diluting in amoebae saline. Co-cultures were incubated at 30°C, and samples taken at days one, three and seven to determine whether multiplication of *Legionella* had occurred. The samples were diluted in 0.2M HCl and 0.2M KCl buffer solution, pH 2.2, to lyse the amoebae, and the lysates plated onto CYE media.

7.2.2.2 Soil co-culture of *Legionella*

Soil co-culture, to assess intracellular multiplication of *Legionella* strains in *Acanthamoeba* ACO97, was set up essentially as for liquid co-culture, except that *Legionella* and *Acanthamoeba* were added to potting mix (Nu-Earth, Meadows, S.A., Australia). The potting mix was steamed for approx. 1 hour to remove any pre-existing *Legionella* spp., and other heat sensitive organisms. A 20 gram quantity of steamed soil was placed in a sterile container, moistened by the addition of sterile distilled water and seeded with 10^3 *Legionella* bacteria and 10^4 amoebae, prepared as outlined above. Each co-culture was set up in duplicate. The sample was mixed well and incubated at 30°C for 15 days. Samples were taken at various intervals to determine if intracellular replication of *Legionella* had occurred. At each interval a 1 gram aliquot of soil was removed from the container, diluted 1:3 in sterile tap water, mixed thoroughly, and allowed to settle for 15 minutes. An aliquot of the settled suspension was then diluted in HCl-KCl acid buffer (pH 2.2), to reduce unwanted soil micro-organisms, and dilutions were plated onto CYE-VPP selective media.

7.3 Results

7.3.1 Effect of a *mip* mutation on the intracellular infectivity of *L. longbeachae* sg 1

To determine whether Mip promotes infection of amoebae in *L. longbeachae* sg 1, the ability of isogenic *mip* mutants to infect *Acanthamoeba*, a common soil amoeba was assessed. Two systems were used to assess levels of multiplication of *Legionella* strains, with potting mix considered a more natural, non-aquatic, environment for *L. longbeachae* sg 1. The potting mix was steamed for approx. 1 hour to kill any pre-existing *Legionella* spp. However, the steaming process did not sterilise the soil as spore-forming organisms were not killed by this process. The same multiplicity of infection was used for both systems and samples were taken frequently during the experiment to determine the level of multiplication of *Legionella* (Fig 7.1). The mean ± SD CFU was determined for each time point and Student-Newman-Keuls comparison of means ($p \leq 0.05$) was used to determine statistical significance.

L. pneumophila sg 1 (Philadelphia), *L. longbeachae* sg 1 ATCC 33462 (Original) and *L. longbeachae* sg 1 (A5H5) multiplied in liquid co-culture similarly to that determined for other *Legionella* organisms (Fig. 7.1A and B) (Cianciotto and Fields, 1992, O'Connell, *et al.*, 1995). An initial lag period was followed by a steady increase in bacterial numbers during the course of the experiment. The *mip* mutants of both strains of *L. longbeachae* sg 1 had different growth patterns compared with their parent strain and also were distinct from each other (Fig 7.1A and B). Mutant B8 increased in numbers at a lower rate than A5H5 with a statistically significant difference in recovery observed at day seven. Statistically significant differences were not seen at days one and three most likely due to large variation in the counts and the low sample numbers. However, the expected growth trend was observed and the end result was similar to those determined for the *L. pneumophila* sg 1 *mip* mutant (Cianciotto and Fields, 1992). Complimented *mip* mutant B8.22, also grew in amoebae (Fig 7.1A) and was recovered at day seven with numbers that were not statistically different from the wild type strain A5H5. The complemented strain also showed no statistically significant difference in numbers at day one or day three suggesting a growth rate comparable to the parent strain.

The *mip* mutant, B10, constructed in ATCC 33462 (O) was unable to replicate in liquid co-culture and this was observed in several independent repeat experiments (data not shown).

A *mip* mutant, designated E1, constructed in a recently acquired isolate of the ATCC 33462 strain of *L. longbeachae* sg 1 (R), was also tested in the liquid co-culture system (Fig 7.1B). The ATCC 33462 (R) strain replicated in a manner similar to other *Legionella* wild type strains tested in this model showing an initial lag period followed by an increase in bacterial numbers until day seven, with approx. 10^4-10^5 CFU/ml, similar to A5H5 and *L. pneumophila* sg 1 (Philadelphia) (Fig 7.1A). This strain appeared to replicate in *Acanthamoeba* more efficiently that the original isolate with a 1-2 log increase in bacterial numbers recovered at days one and three in comparison with the original isolate (Fig 7.1B). Interestingly, the corresponding *mip* mutant, designated E1, constructed in the recent ATCC 33462 (R) isolate was unable to replicate in this system on two repeat occasions (Fig 7.1B). This result was identical to that shown for *mip* mutant B10 constructed in the original strain of ATCC 33462 (O) (Fig 7.1B).

L. longbeachae sg 1 strain A5H5 and the corresponding *mip* mutant B8 were both able to replicate in potting mix showing similar growth trends as seen with liquid co-culture (Fig. 7.1C). Statistically significant differences in strain recovery were observed at day 7 and day 11. The numbers of organisms observed at day 15, were not statistically different, most likely due to large variation in counts and low sample numbers as stated for liquid co-culture, although the expected growth trend was observed. *L. pneumophila* sg 1 (Philadelphia) replicated in this system but an initial lag phase was observed at day three where numbers dropped to undetectable levels. This may reflect the low number of organisms added initially to the co-culture or may be due to inappropriate levels of sampling for that time point. However, in all experiments *L. pneumophila* sg 1 (Philadelphia) multiplied in this system.

L. longbeachae sg 1 ATCC 33462 (O) and mutant derivative B10 were both unable to replicate in the potting soil system and in several independent repeat experiments. A recently acquired stock of the *L. longbeachae* sg 1 type strain, ATCC 33462 (R) was able to replicate

in soil co-culture (data not shown) but the corresponding *mip* mutant constructed in this strain, E1, was not assessed as it was unable to multiply in liquid co-culture (Fig 7.1B).

7.3.2 Infectivity of *mip* mutants in macrophage-like U937 cells.

The *mip* mutants B8 and B10 were assessed for their ability to infect macrophage-like U937 cells by determining ID50s at 72 hour post infection compared with the parent strain (section 3.2.6). This work was kindly performed by Dr. Nicholas Cianciotto and Dr. Shaila Banvi, Northwestern University, Chicago. Two infections were performed for each test strain and compared with a control strain (*L. pneumophila* strain 130b) and the results are shown in Table 7-1. The results for *L. pneumophila* 130b, A5H5 and ATCC 33462 (O) are identical to those shown in Table 3-6 but are included here again so a direct comparison can be made between parental and isogenic *mip* strains.

The *mip* mutant B10 was greatly impaired in infectivity however, the parent strain ATCC 33462 (O) was also similarly impaired in this cell model. *L. longbeachae* sg 1 ATCC 33462 is also avirulent in an animal model of infection as discussed in chapter 3. The mutant and parent strain both had high ID_{50}s of approx. 10^4 bacteria suggesting that the strains are impaired for intracellular replication within U937 cells although they may be capable of surviving within these cells (O'Connell, *et al.*, 1996b). The results for the *mip* mutant B8 were equivocal as one experiment suggested that infection of the U937 cells by this strain was comparable with the parent strain A5H5 and control strain *L. pneumophila* 130b, while a repeat experiment showed a high ID_{50} (Table 7-1).

7.3.3 Animal model of infection

Guinea pigs exposed to a dose of approx. 10^9 or 10^{10} CFU of aerosolised *L. longbeachae* sg 1 ATCC 33462 (O) and (R) were asymptomatic (chapter 3). For this reason, only the A5H5 *mip* mutant B8 was assessed in the aerosol model of animal infection along with complemented strain B8.22. Infection of guinea pigs was performed as per section 3.2.3.

Two doses of the mutant strain B8 were tested in this model and the percentage weight gain/loss during the course of infection was plotted for each animal and compared with the plot for the parent strain A5H5 and the complemented strain B8.22 (Fig. 7.2).

The parent strain killed three out of five animals (all symptomatic) within five days after exposure (Fig. 7.2A). Guinea pigs exposed to 10^9 CFU dose (approx. 10^5 retained organisms) of *mip* mutant strain B8 showed no evidence of disease (Fig. 7.2C). Evidence of disease, predominantly weight loss, was observed in some of the animals exposed to a 10^{10} CFU dose (approx. 10^6 retained organisms) of the same mutant strain. Despite the 10 × higher dose no deaths occurred (Fig. 7.2D). The *mip* mutant B8 was unable to cause lethal infection with either test dose.

Reintroduction of the intact wild type *mip* gene from *L. longbeachae* sg 1 to generate strain B8.22, was able to fully complement the mutation in strain B8 leading to restored virulence (Fig. 7.2B). All of the animals exposed to a 10^9 dose of strain B8.22 were dead within 5 days of exposure.

7.4 Discussion

The role of the Mip protein of *L. longbeachae* sg 1 was examined by assessing the behaviour of *mip* mutants for their ability to infect guinea pigs and to multiply intracellularly in *Acanthamoeba*. The role of the Mip protein in *L. longbeachae* sg 1 as a potentiator of intracellular infection is suggested by the behaviour of the *mip* mutants in the *Acanthamoeba* co-culture models. The mutant in strain A5H5 showed a similar growth pattern to the *mip* mutants of *L. pneumophila* sg 1 and *L. micdadei* (Cianciotto and Fields, 1992, O'Connell, *et al.*, 1995). The *mip* mutant B10 constructed in *L. longbeachae* sg 1 ATCC 33462 (O) was unable to multiply in either of the amoebae models developed. This may reflect a greater level of attenuation of the ATCC parent strain, originally acquired in 1987.

A recently acquired stock of the ATCC 33462 type strain (R) tested in the *Acanthamoeba* co-culture models multiplied in both the liquid and the soil system. The result

for the soil co-culture system was in contrast to that observed for the original ATCC 33462 (O) isolate suggesting that the original stock may have undergone further attenuation. However, construction of a *mip* mutant, designated E1, in ATCC 33462 (R) showed that it behaved identically to mutant B10 in liquid co-culture in that it was also unable to multiply in this system. This suggests that the result obtained for the *mip* deletion in *L. longbeachae* sg 1 ATCC 33462 is valid and not due to attenuation of the strain.

Differences were observed between *L. longbeachae* sg 1 ATCC 33462 and A5H5 in both *Acanthamoeba* models, with the ATCC 33462 type strain showing a lower level of infectivity in comparison with A5H5. This strain difference is most significant in the animal model where *L. longbeachae* sg 1 ATCC 33462 was unable to establish fatal infection (chapter 3). This result was also confirmed by U937 infectivity studies that showed that the ATCC 33462 (O) isolate was greatly impaired for infection of these macrophage like cells, while A5H5 showed levels of infectivity comparable to the control *L. pneumophila* sg 1 (130b) strain. The result with the ATCC 33462 type strain *L. longbeachae* sg 1 strain are in contrast to that of O'Connell *et al* (1996b) who reported that this strain was infective for these cells. Another group of workers tested ATCC 33462 in Mono Mac 6 and in *A. castellanii* and were unable to demonstrate replication in either host cell type (Neumeister, *et al.*, 1997). A second report by this same group showed moderate replication of ATCC 33462 in Mono Mac 6 cells (Neumeister, *et al.*, 2000). Perhaps the ATCC 33462 type strain *L. longbeachae* sg 1 is highly sensitive to multiple passage or the freeze drying process used to send cultures to overseas laboratories.

The *mip* mutant strains behaved differently in comparison with each other in this model. As expected, mutant strain B10 was unable to multiply in U937 cells while the results for mutant B8 were equivocal. The U937 result for *mip* mutant B8 is of interest as in one assay it was able to infect U937 cells with an ID_{50} of less that 200 bacteria, comparable with the wild type parent strain and *L. pneumophila* sg 1 130b strain. However, in an independent assay, the ID_{50} was approx 10^4 bacteria which is in the order of that observed for the avirulent

ATCC 33462 type strain and corresponding *mip* mutant B10 suggesting that it was unable to establish infection. It is unusual for a strain to be capable of infecting U937 cells and unable to cause disease in an animal model of infection and it is generally assumed that this ability is a hallmark of virulence in *Legionella pneumophila* (Cianciotto, *et al.*, 1990b, O'Connell, *et al.*, 1996b).

There appears to be unknown strain differences between *L. longbeachae* sg 1 strains and *L. pneumophila*. The *L. longbeachae* sg 1 *mip* mutant, strain B8, may lack some other factor required for *in vivo* growth that is influenced by presence of Mip. Overall the results for the U937 studies are consistent in that no strain has been shown to be unable to infect macrophages and retain full virulence in an animal model (Cianciotto, *et al.*, 1990b). In *L. pneumophila* strains that have lost their ability to infect macrophages are uniformly avirulent in animals. Perhaps Mip in *L. longbeachae* sg 1 has functions unique or additional for intracellular survival compared with *L. pneumophila.* This may be in relation to the apparent lack of MOMP and the ecological niche of *L. longbeachae* sg 1.

The results obtained in the animal model for the *mip* mutant in *L. longbeachae* sg 1 strain A5H5 are of interest as no other *mip* mutant, other than *L. pneumophila* sg 1 has been assessed in an animal model. The results are consistent with those seen for the *mip* mutant in *L. pneumophila* sg 1 in that a mutation in this gene leads to attenuation of virulence (Cianciotto, *et al.*, 1990b). In contrast to the results of Cianciotto *et al* (1990b) the *mip* mutant (B8) did not cause death in any guinea pigs under two test dose conditions assessed. The test doses used in this study resulted in lower numbers of bacteria being deposited into the lungs of guinea pigs (approx. 10^5 or 10^6 CFU) than those achieved by intra-tracheal inoculation used in the study by Cianciotto *et al* (1990b). The aerosol model of infection makes it difficult to achieve the higher numbers of deposited bacteria as in the intratracheal model and hence it cannot be concluded if the effect of the *mip* mutation in *L. longbeachae* 1 strain A5H5 would have induced death at doses greater than 10^{10}. It is tempting to speculate that this would be the case as *L. longbeachae* sg 1 differs from *L. pneumophila* sg 1 in that it

does not possess a major outer membrane protein (MOMP) (Ehret and Ruckdeschel, 1983, Hindahl and Iglewski, 1986, Hindahl and Iglewski, 1987) (chapter 4 – unpublished observations). MOMP is believed to play a role in uptake of *L. pneumophila* into macrophages, through its ability to bind complement component C3b (Bellinger-Kawahara and Horwitz, 1990). Therefore, *L. longbeachae* may be more susceptible to changes in outer membrane proteins.

Complemented *mip* mutant strain B8.22 was virulent in a guinea pig model of infection and was also able to multiply in *Acanthamoeba* in a manner similar to the wild type parent strain A5H5. The re-introduction of the intact *mip* gene into the *mip* mutant B8, leading to restored virulence, was important as it not only confirmed the importance of the Mip protein in *L. longbeachae* sg 1 but also showed that the allelic exchange method developed for construction of isogenic mutants in *L. longbeachae* had not affected other genes involved in virulence.

The Mip protein has a significant role in pathogenesis of *L. longbeachae* sg 1 and its survival in the environment. It was proposed originally that the Mip protein in *L. pneumophila* may be linked with inhibition of phagosome-lysosome fusion (Cianciotto, *et al.*, 1989b, Engleberg, *et al.*, 1989). However, Mip promotes infection by *L. micdadei,* an organism that does not block phagosome-lysosome fusion (Rechnitzer and Blom, 1989) and therefore the potential targets of Mip activity cannot be limited to phagosome-lysosome fusion events (O'Connell, *et al.*, 1995). Interestingly, *L. longbeachae* sg 1 strain A5H5 appears similar to *L. micdadei* when examined by electron microscopy (discussed in chapter 4) in that fusion with lysosomes appears to occur and the organism resides in a phagolysosome. It would be interesting to test the *L. micdadei mip* mutant in the aerosol guinea pig model used in this study to determine whether it behaves in a similar manner to the *L. longbeachae* sg 1 A5H5 *mip* mutant. No animal virulence studies were performed with the *L. micdadei mip* mutant and although the results in amoebae and U937 cells were similar to those reported for the *L. pneumophila mip* mutant it does not preclude the possibility that the

mutation may have a more deleterious effect on pathogenesis. One can speculate that this may be the case as this species and *L. longbeachae* sg 1 may share a similar intracellular life style and hence Mip may play a similar, important, as yet unknown function in these species. *C. burnetii,* an intracellular pathogen that survives within a phagolysosome, also has a 25 kDa Mip protein further suggesting that Mip is involved in functions not related to the phenomenon of phagosome lysosome fusion (Mo, *et al.*, 1995).

7.5 Summary

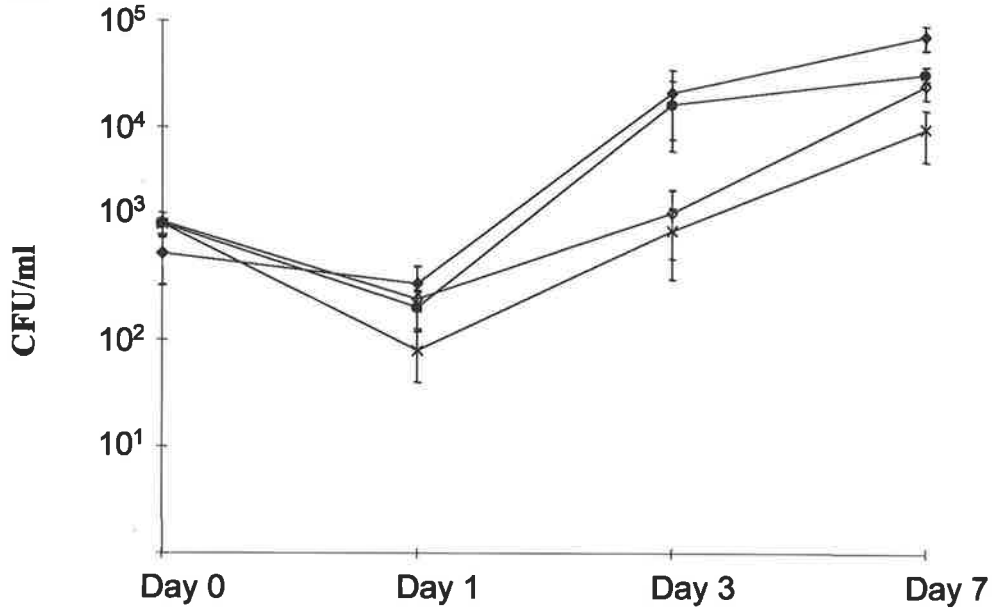
Isogenic *mip* mutants constructed in *L. longbeachae* sg 1 strain ATCC 33462 and A5H5 were tested *in vitro* for their ability to multiply in *Acanthamoeba* and U937 cells and for their ability to establish infection *in vivo* in a guinea pig aerosol model. The ATCC 33462 *mip* mutant was unable to infect a strain of *Acanthamoeba* spp. in both a liquid and a potting mix co-culture system, whereas the A5H5 *mip* mutant behaved in a manner similar to *L. pneumophila* sg 1 and *L. micdadei*, ie. a reduced capacity to infect and multiply within *Acanthamoeba*. In U937 cells the *mip* mutant of ATCC 33462 was unable to establish infection while the result for the *mip* mutant in strain A5H5 was equivocal. The A5H5 *mip* mutant and its parent strain were assessed also for their ability to establish lethal infection after aerosol exposure. Compared to the virulent parent strain, the mutant strain did not kill any guinea pigs under two different dose regimes. A complemented *mip* mutant strain containing the wild type *mip* gene introduced *in trans* regained full virulence in the animal model suggesting that the mutation generated in *mip* had not affected other genes involved in virulence. The data indicate that the Mip protein plays an important role in the intracellular life cycle of *L. longbeachae* sg 1 species and is required for full virulence.

Figure 7.1

Co-culture of *Acanthamoeba* with strains of *Legionella*. The experiments shown are representative of a minimum of two independent experiments.

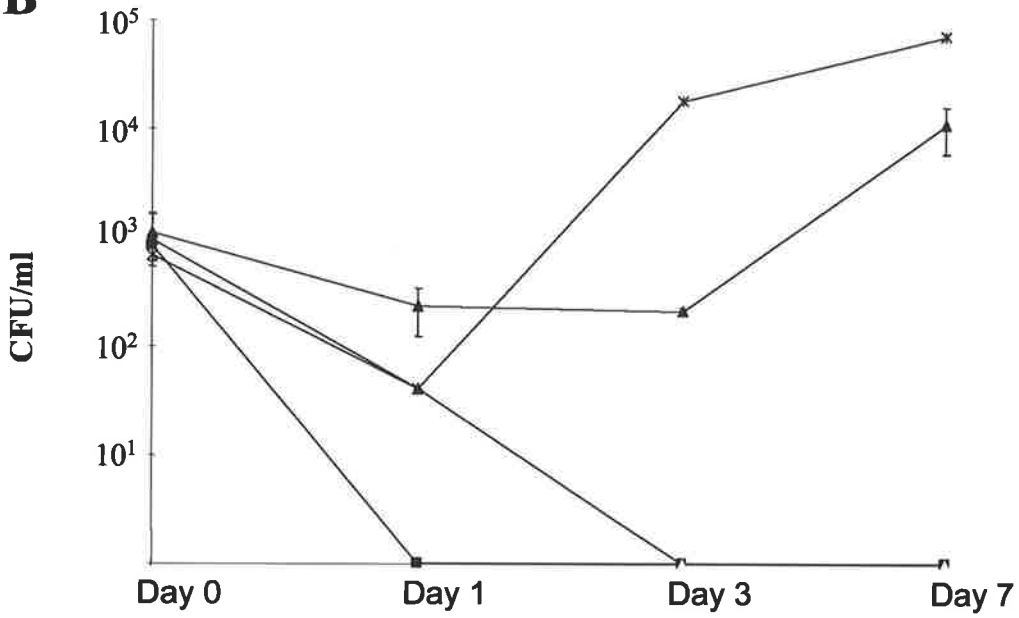
A) and (B): Liquid co-cultures were set up in saline with approximately 10^4 amoebae/ml and 10^3 CFU/ml *L. pneumophila* (Philadelphia) (◆), *L. longbeachae* sg 1 ATCC 33462 original isolate (O)(▲), *L. longbeachae* sg 1 ATCC 33462 recent isolate (R) (✳), *mip* mutant B10 (■), *nip* mutant E1 (△), *L. longbeachae* sg 1 (A5H5) (●), *mip* mutant B8 (×) and complemented *nip* mutant B8.22 (◊). Samples were taken at various time intervals and the number of *Legionella* organisms was determined by plating on selective media. Each time point represents the mean number of CFU recovered and the vertical bars indicate standard deviation.

A



Incubation time (days)

B



Incubation time (days)

Figure 7.1 (continued)

(C): Amoebae were cultured in an artificial potting mix system with approximately 10^4 amoebae/gram and 10^3 CFU/gram of *L. pneumophila* sg 1(Philadelphia) (◆), *L. longbeachae* sg 1 ATCC 33462 original isolate (O)(■), *mip* mutant B10 (△), *L. longbeachae* sg 1 (A5H5) (●) and *mip* mutant B8 (×). Samples were taken at various time intervals and the number of *Legionella* organisms determined by plating onto selective media. The number of viable *Legionella* organisms were determined at various time points by treatment of soil sample with acid buffer and plating on selective media. Each time point represents the mean number of CFU recovered and the vertical bars indicate standard deviation.

C

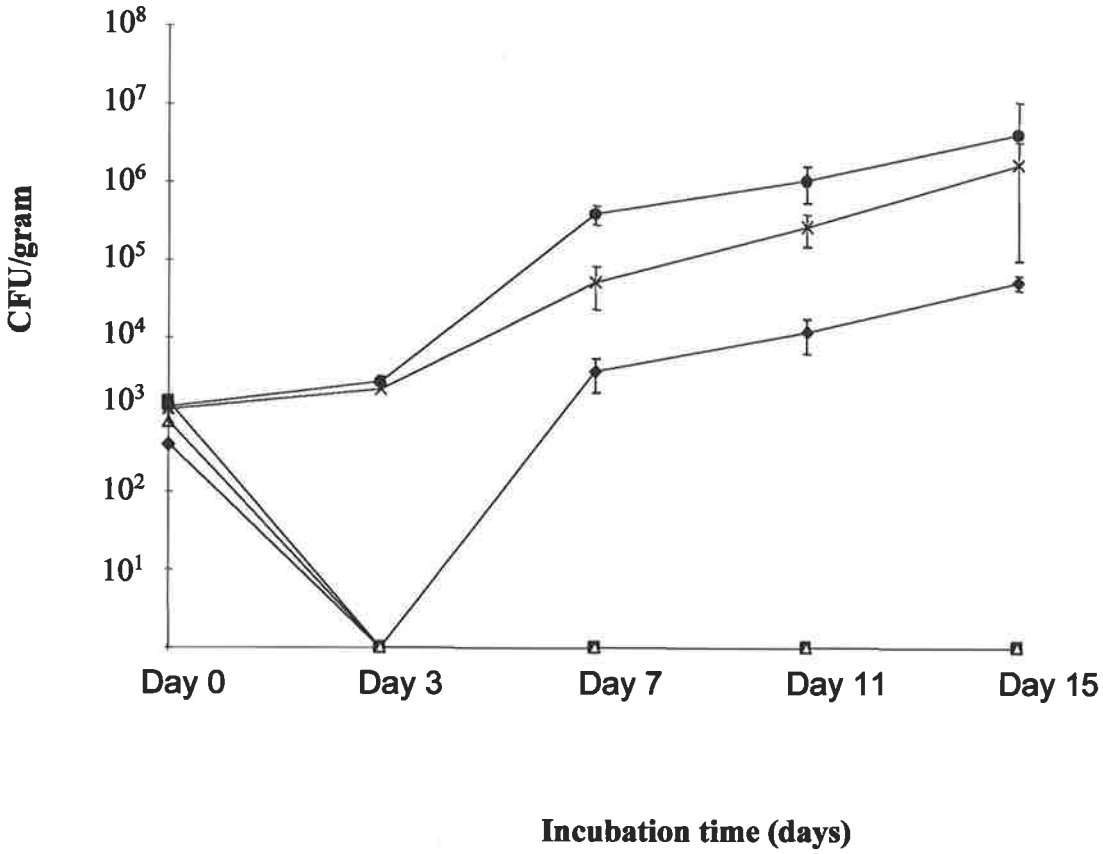

CFU/gram
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Day 0
Day 3
Day 7
Day 11
Day 15
Incubation time (days)

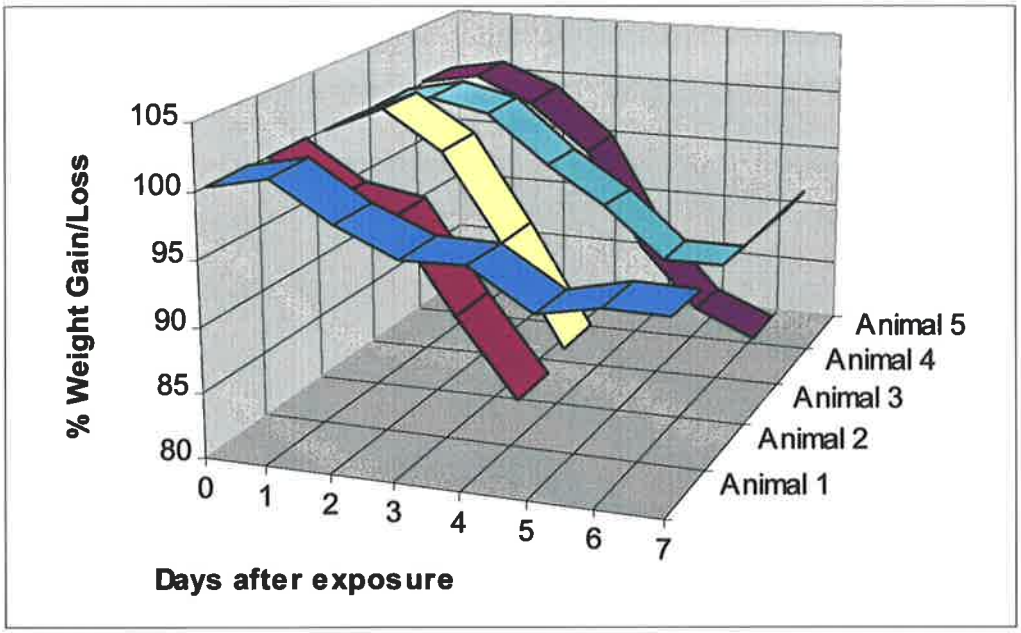
Figure 7.2

Percentage weight gain or loss in guinea pigs exposed to an aerosol of different strains of *Legionella longbeachae* sg 1. Guinea pig death is indicated by termination of the ribbon graph prior to the end of the experiment at day seven.

(A): Animals exposed to a dose of approx. 10^9 *L. longbeachae* sg 1 strain A5H5.

(B): Animals exposed to a dose of approx. 10^9 *L. longbeachae* sg 1 complemeted *mip* mutant strain B8.22.

(A)


% Weight Gain/Loss
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

(B)

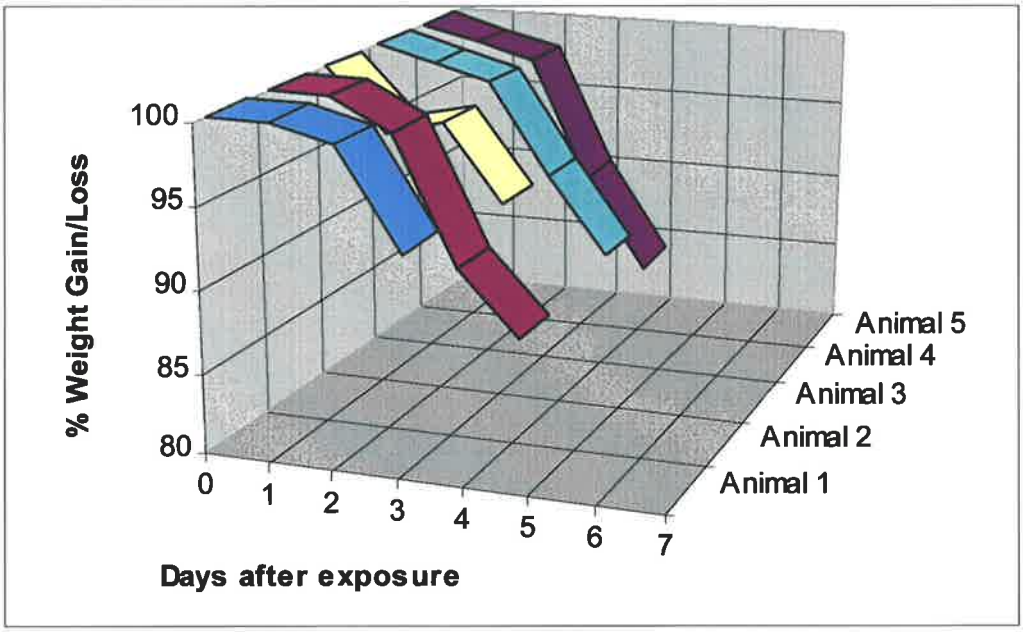

% Weight Gain/Loss
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

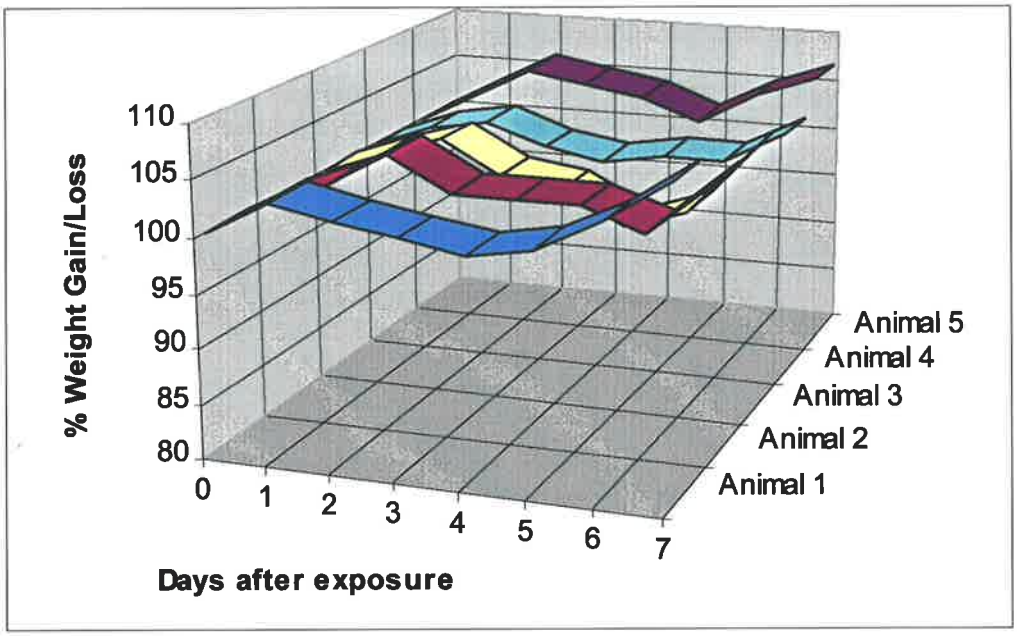
Figure 7.2 (continued)

Percentage weight gain or loss in guinea pigs exposed to an aerosol of different strains of *Legionella longbeachae* sg 1. Guinea pig death is indicated by termination of the ribbon graph prior to the end of the experiment at day seven.

(C): Animals exposed to a dose of approx. 10^9 *L. longbeachae* sg 1 A5H5 *mip* mutant B8.

(D): Animals exposed to a dose of approx. 10^{10} *L. longbeachae* sg 1 A5H5 *mip* mutant B8.

(C)


% Weight Gain/Loss
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

(D)

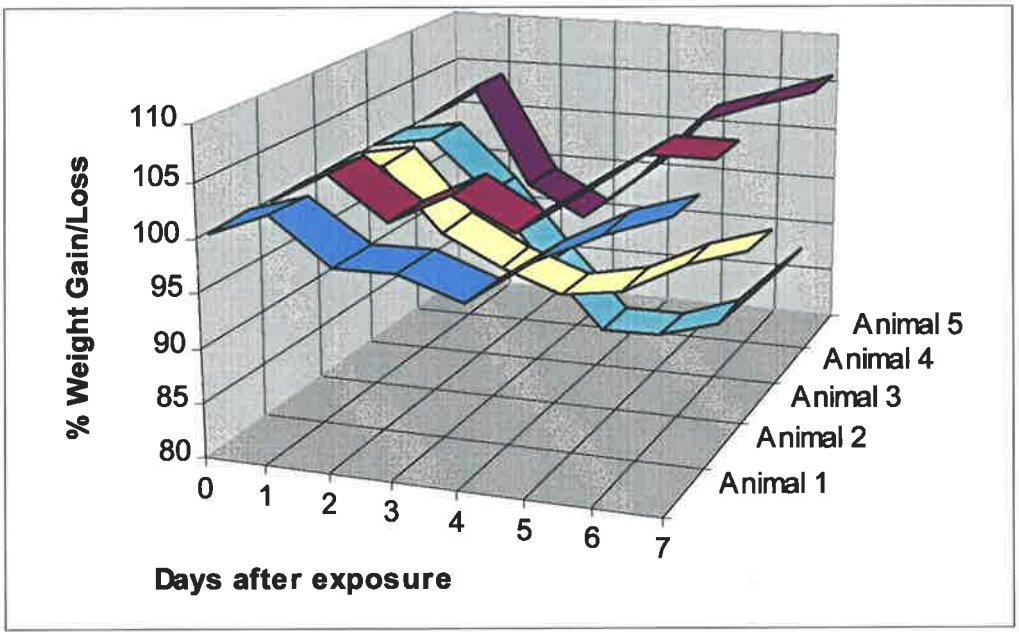

% Weight Gain/Loss
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

Table 7-1 Infectivities of *L. longbeachae* sg 1 *mip* mutants for U937 cells

***Legionella* species**	**Strain**	**$Log_{10}ID_{50}$[a]**	**Infectivity for guinea pigs**
L. pneumophila sg 1	130b	2.73 ± 0.13 3.08 ± 0.28 2.04 ± 0.19 2.38 ± 0.18 3.00 ± 0.28 3.10 ± 0.13	N/A
L. longbeachae sg 1	ATCC 33462 Long Beach 4	1.93 ± 0.13[b]	N/A
L. longbeachae sg 1	ATCC 33462 (O) Long Beach 4	4.55 ± 0.13 -	avirulent
L. longbeachae sg 1 ATCC 33462 *mip* mutant	B10	4.32 ± 0.14 5.87 ± nc[c]	avirulent
L. longbeachae sg 1	A5H5	2.60 ± 0.28 3.14 ± 0.10	virulent- 3/5 deaths
L. longbeachae sg 1 strain A5H5 *mip* mutant	B8	2.25 ± 0.28 4.01 ± 0.15	avirulent

N/A: result not available for this strain.

a: The ID_{50} is expressed as a log value ± the standard deviation (expressed as a log value). Values for different infections are shown for each strain if available.

b: value determined in study by O'Connell *et al* (1996b).

c: not calculated

Chapter 8

Identification of a two-component regulatory system on a *Legionella longbeachae* serogroup 1 plasmid: implications for virulence

8.1 Introduction

L. longbeachae sg 1 is a clonal species of *Legionella* (Lanser, *et al.*, 1990, Ratcliff, R. PhD thesis, 2000, University of Adelaide). Restriction fragment length polymorphism (RFLP) typing and allozyme analysis used to differentiate strains of *L. longbeachae* showed a close relationship between geographically isolated strains (Lanser, *et al.*, 1990). This observation was confirmed by RFLP using a large number of clinical and environmental Australian and overseas isolates in this study. Despite the similarity, an animal virulence model identified three distinct virulence groupings within *L. longbeachae* sg 1 (discussed in chapter 3). An analysis of membrane and LPS profiles of representative isolates of *L. longbeachae* sg 1 belonging to these three virulence groups determined that they were all very similar.

One factor that may determine virulence of a clone of genetically similar strains is the presence of plasmids. Few studies have been undertaken with *Legionella* to determine the role played by plasmid encoded functions. It was originally postulated based on three lines of evidence that these extra-chromosomal elements might be important in *Legionella*. Firstly, multiple passage of *L. pneumophila* on laboratory media leads to loss of virulence (Elliot and Johnson, 1982, McDade and Shepard, 1979). Secondly, some strains of *Legionella* are resistant to penicillin antibiotics and are known to produce beta-lactamase (Thornsberry and

Kirven, 1978). Thirdly, a toxin appears to be important in the disease process (Baine, 1985, Friedman, *et al.*, 1980, Husmann and Johnson, 1994, Kirby, *et al.*, 1998). Plasmids have been identified in both clinical and environmental strains of *Legionella* however their contribution to virulence has not been determined (Aye, *et al.*, 1981, Brown, *et al.*, 1982, Edelstein, *et al.*, 1986, Johnson and Schalla, 1982, Knudson and Mikesell, 1980, Maher, *et al.*, 1983, Mikesell, *et al.*, 1981, Nolte, *et al.*, 1984, Tompkins, *et al.*, 1987). It has been speculated that plasmid encoded functions may be involved in the survival of *Legionella* in the environment rather than in pathogenicity (Ott, 1994) as clinical isolates of *L. pneumophila* tend to lack plasmids while environmental isolates frequently contain plasmids (Brown, *et al.*, 1982, Maher, *et al.*, 1983).

In this chapter, the presence of plasmids in *L. longbeachae* sg 1 was investigated. In particular, a large plasmid (pA5H5) in a virulent Australian clinical isolate of *L. longbeachae* sg 1, strain A5H5 was investigated. Partial sequence analysis revealed a region on the plasmid that had significant inferred homology with the OmpR family of two-component transcriptional regulatory proteins. Downstream of this gene was an open reading frame encoding a potential sensor kinase protein. Proteins of this two-component protein family control a variety of responses to environmental signals including regulation of virulence factors (Bernadini, *et al.*, 1990, Dorman, *et al.*, 1989, Pickard, *et al.*, 1994, Saier, 1994, Stock, *et al.*, 1989).

To determine if the two-component regulatory system had an effect on the virulence of A5H5, an isogenic mutant was constructed in the regulatory gene, designated *lrpR* (*Legionella longbeachae* regulatory protein gene). The mutant strain was assessed for the ability to infect U937 cells, *Acanthamoeba* and guinea pigs.

8.2 Methods and materials specific to this chapter

8.2.1 Bacterial strains and plasmids

Strains of bacteria and plasmids used or constructed in this study are listed in Table 2-1 and Table 8-1.

8.2.2 Construction of *L. longbeachae* serogroup 1 strain A5H5 plasmid bank

CsCl gradient purified plasmid DNA from *L. longbeachae* sg 1 strain A5H5 was separated on a 0.8% Low Melt Point agarose gel. Two bands were observed and the upper band was gel purified as described in section 2.6.4. The purified top band plasmid DNA was digested with *Hin*dIII, and the resulting random size plasmid DNA fragments were cloned into sequencing vector pGEM-7Zf(-), similarly digested with *Hin*dIII. The ligation mix was purified prior to electroporation into *E. coli* DH5α (chapter 2), and transformants were patched onto Amp/IPTG/X-gal plates.

8.2.3 Construction and complementation of the *lrpR* mutant

Allelic exchange was carried out to generate a mutation in the *lrpR* gene using a suicide vector pCACTUS. Electroporation was used to introduce plasmid construct pCACBS2, containing the mutated *lrpR* gene, into *L. longbeachae* sg 1 strain A5H5 and kanamycin (10 μg/ml) was used to select for transformants. Allelic exchange mutagenesis was performed as described previously (chapter 6) except that kanamycin at a concentration of 10μg/ml was used. Colonies from the sucrose plates were patched onto CYE plates and screened by Southern hybridisation to confirm potential mutants. Mutant strains were complemented using plasmid pIMVS35, containing the entire wild type *lrpR* gene, introduced into the mutant strain by electroporation.

8.2.4 Infection of macrophage-like U937 cells with strains of *Legionella longbeachae* serogroup 1

U937 cells are a histiocytic lymphoma cell line that differentiate into non-dividing adherent cells when treated with phorbol esters (Sundstrom and Nilsson, 1976). The U937 monocyte cell line (obtained from Dr. Li Ping, Infectious Diseases Laboratories, IMVS, Adelaide) was maintained in RPMI –1640 media containing foetal bovine serum 10 % (v/v), glutamine 1% (w/v) (RPMI assay media) with penicillin 100 µg/ml and streptomycin 100 µg/ml (Gibco-BRL) (RPMI maintenance media). This medium was used routinely for maintenance of the U937 cells in tissue culture but for experimental assays antibiotics were not added unless otherwise stated. PMA (phorbol myristate acetate) (Sigma), used to differentiate U937 cells, was dissolved in DMSO and stored at -20°C at 10 µg per ml. Cells were incubated in 5% CO_2 at 37°C.

The PMA differentiated cell assay was essentially as described by Baker *et al* (1999). To differentiate the replicative non-adherent U937 cells into adherent macrophage like cells, high density cultures (approx. 10^6 cells /ml) were washed in PBS and seeded into 24 multi-well plates (Corning 25820) at approx. 1×10^6 cells/well in RPMI maintenance media containing 6 ng/ml PMA. Cells were differentiated with PMA for 72 hours and were then washed three times in PBS prior to setting up the infection assay.

Legionella strains were prepared for infection assays by resuspending growth from a 48 hour plate in RPMI medium without foetal calf serum to give approx. 10^9 bacteria/ml by standardising against an OD reading at 550 nm (chapter 3). The *Legionella* suspension was then diluted in RPMI assay media to add to tissue culture cells. *Legionella* were added to the differentiated U937 cells at a ratio of 1:1 or 10:1 and incubated for 2 hours to allow cell invasion to occur. The cells were washed once in PBS and then RPMI assay media containing 200 µg /ml gentamycin was added. The plates were then incubated for 1 hour to kill extracellular bacteria. Cells were washed three times with PBS and all wells, except those designated as the time = 0 hour time point for each strain, were then incubated in RPMI assay

medium. Counts were determined at the time = 0 hour time point by lysing the washed cells with 0.5 ml of 0.1% (v/v) Triton-X100 in PBS. Subsequent time points were taken at 24, 48 and 72 hours. *Legionella* bacterial counts were determined retrospectively by dilution in RPMI assay medium and plating onto CYE medium. Colonies were counted and the CFU/ml determined for each time point.

8.2.5 Sequencing protocols

To sequence the insert of emperically chosen plasmid clones, containing random *Hin*dIII fragments of native plasmid pA5H5, mini-prep plasmid DNA was purified as follows. Briefly, plasmid DNA (20µl) was treated with RNAse A (0.1mg/ml) for 30 min at 37°C, cooled on ice (1 min), spun at 17,000 × g (1 min) and the supernatant precipitated with 20 µl of 7.5 M NH_4Ac and 40 µl of isopropanol. After centrifugation for 15 min at 17,000 × g the DNA pellet was washed once in 75% ethanol - 25% 50 mM NaAc and once with 100% ethanol. The pellet was dried *in vacuo* and resuspended in 10 µl of purified water. Plasmid insert DNA was sequenced using dye primer sequencing chemistry (chapter 2). Sequencing of the insert of CsCl purified plasmid pIMVSX2 was initied using dye primer sequence chemistry (chapter 2). The remaining sequence was determined using dye terminator chemistry with synthetic oligonucleotide primers (Table 8-3) as described in chapter 2.

8.3 Results

8.3.1. Plasmid profiles of *L. longbeachae* strains

Several methods were trialed initially for the extraction of plasmid DNA from *Legionella* isolates (data not shown). Others workers had noted that variation in detecting plasmids in *Legionella* could be caused by the method of extraction and by the media used for growth (Aye, *et al.*, 1981, Mikesell, *et al.*, 1981). In the present study, the three step alkali

lysis method was the most successful, consistent method for the extraction of plasmids from *Legionella* (Fig 8.1) (Sambrook, *et al.*, 1989).

Plasmid DNA extracts of *L. longbeachae* sg 1 strains ATCC 33462, L6C9 and A5H5, *L. longbeachae* sg 2 ATCC 33484 and *L. pneumophila* (Philadelphia) were prepared from broth cultures grown at two different temperatures (30°C and 37°C). Initial examination of the extracts suggested that plasmid DNA was present only in *L. longbeachae* sg 1 strains A5H5 and L6C9 and in *L. longbeachae* sg 2 ATCC 33484 (data not shown). Restriction digestion of the plasmid DNA extracts for each with *Eco*RI, confirmed the presence of plasmid DNA in these strains (Fig 8.1, part i). Discrete fragments were generated for *L. longbeachae* sg 1 A5H5 and L6C9 and *L. longbeachae* sg 2 ATCC 33484 (Fig 8.1) in agreement with initial observations of native plasmid DNA in these strains. A smear would be observed when chromosomal DNA was digested with the restriction enzyme and this was seen in the lanes containing ATCC 33462 (Fig 8.1, part i, lane c and d) and *L. pneumophila* (Fig 8.1, part i, lane a and b), which is consistent with previous results. The total size of the combined fragments indicated that the size of the plasmids in the *L. longbeachae* strains were quite large (approx. 100 kb). There appeared to be a slight increase in the amount of plasmid DNA recovered when the extraction was performed on bacteria harvested at 30°C in comparison to 37°C, for strains A5H5 and L6C9 (Fig 8.1, part i).

Plasmid DNA was detected in nearly all isolates of *L. longbeachae* sg 1 analysed subsequently (Fig 8.1, part ii). Plasmid DNA was also detected in *L. longbeachae* sg 1 strains A5E1, K5H9, K4A1, K8B9 and Atlanta-5 but not in strain LA-24 (data not shown). A plasmid band could not be detected in ATCC 33462, irrespective of the method of extraction used (Fig 8.1, part i, lane e). This result is consistent with that determined by Aye *et al* (1981). However, the detection of plasmid DNA in *L. longbeachae* sg 2 ATCC 33484 is in contrast to that of Aye *et al* (1981), and may be a reflection of the extraction method, media and growth conditions used in that study. A plasmid band was not detected in *L. pneumophila* (Philadelphia), despite several attempts (data not shown), consistent with the work of Aye *et*

al (1981). In some strains, multiple bands were observed (Fig 8.1), however, this is likely to represent multiple forms of the same native plasmid.

Plasmids may therefore play an important role in *L. longbeachae* strains as a high proportion (91 %) appeared to contain extra-chromosomal DNA.

8.3.2 Partial characterisation and analysis of native plasmid pA5H5

Initially a plasmid bank was constructed containing fragments of pA5H5 so that partial sequence analysis could be performed. Plasmid DNA was extracted from empirically chosen *E. coli* DH5α transformants from the pA5H5 plasmid bank and sequenced using dye primer chemistry in both directions. The sequence data generated for each clone was analysed using the BLASTX program. Several clones of interest were identified and the results are summarised in Table 8-2. The data shown is for raw data sequence and represents partial identities.

The inferred protein product of plasmid DNA insert from *E. coli* DH5α clone 4.36 had significant identity to transposon related proteins. An unknown protein in transposon Tn10 and a transposase encoded on plasmid pXT107 in *E. coli* (94% identity) gave the highest scores (Bogosian, *et al.*, 1993, Halling, *et al.*, 1982). Probability scores were also highly significant ($2.3e^{-57}$ – $18e^{-60}$). Sequencing down stream of this region using a custom made synthetic oligonucleotide, p4-36 (Table 8-3), and dye-terminator sequencing chemistry also revealed strong DNA identities (32%-95%) with significant probability scores ($7.4e^{-31}$- $2.3e^{-125}$) to transposase proteins from other bacteria (data not shown).

Plasmid 5.21 insert had identity with pilin and fimbrial protein precursors from *S. typhi* and *E. coli* (Finlay, *et al.*, 1986, Frost, *et al.*, 1984). Pro-pilin is the precursor of the sex pilus sub-unit, a filamentous surface appendage required for cell-to-cell contact during bacterial conjugation. The pro-pilin of *S. typhi* has 42% similarity with the TraA protein of plasmid F (Finlay, *et al.*, 1986). Sequencing downstream of this region with a custom designed synthetic oligonucleotide p5-21 (Table 8-3) also showed weak identity to this family

of proteins (data not shown). The insert DNA of clones 5.21, 5.44, 5.18 and 5.10 (Table 8-2) showed homology with *tra* genes encoded on plasmids from other bacterial species, suggesting that pA5H5 may be conjugative, a phenomenon described for other *Legionella* plasmids (Mintz, *et al.*, 1992a, Tully, 1991).

The DNA insert of clone 5.44 shared weak identity with the plasmid encoded virulence factor IpaB of *S. flexnerii* (Ménard, *et al.*, 1993). Clone 5.27 encoded an insert with strong identity with the UmuC protein of *E. coli,* involved in UV protection and SOS mutagenesis (Kitagawa, *et al.*, 1985). The Umu proteins belong to the ImpB, MucB and SamB family of proteins that also show significant identity with this clone (Table 8-2). The *samAB* operon in *S. enterica* serovar Typhimurium is located on a 60MDa cryptic plasmid and is involved in UV mutagenesis and may play a role in sensitivity of bacterial strains to UV light (Takehiko, *et al.*, 1992). Interestingly, a plasmid has been reported in *L. pneumophila* sg 1 that confers resistance to UV light (Tully, 1991).

Other inferred protein products of cloned insert DNA that had good probability scores and identities with proteins in the data base were clones 5.7, 3.41 and 5.11 (Table 8-2). Clone 5.7 had strong homology to a hypothetical predicted coding region in *Methanococcus jannaschii* (Bult, *et al.*, 1996). This region also has identity with hypothetical proteins in other organisms such as *M. tuberculosis*. Clone 5.11 had inferred identity with a hypothetical protein from the yeast *S. cerevisiae* (Table 8-2). Clone 3.41 had homology to molybdopterin biosynthesis proteins of the HesA/MoeB/ThiF family from organisms including *E. coli* and *H. influenzae* (Fleischmann, *et al.*, 1995, Nohno, *et al.*, 1988). MoeB is involved in the biosynthesis of molybdopterin, necessary for molybdoenzymes.

Clone 5.17 was chosen for further study since the plasmid insert DNA of this clone had significant inferred identity with the OmpR family of transcriptional regulatory proteins (Stock, *et al.*, 1989). Strongest identity was observed with the *rstA* gene product of *E. coli* (Hill, *et al.*, 1989, Roecklein, *et al.*, 1991, Roecklein and Keumpel, 1992). The identity scores with RstA were high (41% identity) with a strong probability of $7.6e^{-39}$. Proteins of the two

component regulatory family control a variety of responses to environmental signals including regulation of virulence factors (Bernadini, *et al.*, 1990, Dorman, *et al.*, 1989, Pickard, *et al.*, 1994, Saier, 1994, Stock, *et al.*, 1989).

Southern hybridisation was performed, using labelled plasmid DNA from clones that had shown identity to potential virulence related proteins in the database (Table 8-2), to determined in similar genes were found on plasmids in other strains of *L. longbeachae* sg 1. When labelled pIMVS5-17 was used to probe a panel of *L. longbeachae* sg 1 strain plasmid DNA hybridising bands were detected in Australian strains K5H9 and A5H3 and overseas isolates D-493, D-1624, D-1738, D-1820 and D-1992 (data not shown). Similarly, labelled pIMVS5-44 cross hybridised with several strains of *L. longbeachae* sg 1 including and Australian isolate, A5H3 and four overseas isolates, D-493, D-1624, D-1820 and D-1959, including some of the strains that hybridised with pIMVS5-17 (data not shown).

The detection of an *ompR*-like transcriptional regulatory gene by partial sequencing of the insert DNA of clone 5.17 and the high degree of identity with the OmpR family of proteins suggested that the plasmid of pA5H5 warranted further analysis. In particular the study was focused on the *ompR*-like gene and whether a sensor kinase was also encoded on pA5H5.

8.3.3 Cloning and sequencing of the *ompR*-like gene on pA5H5

A larger fragment of DNA was cloned from pA5H5, to complete the sequence of the *ompR*-like gene discovered on pIMVS5-17, and to determine if a related sensor kinase gene was encoded downstream, thus constituting a two-component regulatory system. Plasmid pA5H5 was digested with restriction enzymes cutting in the polylinker cloning site of the vector pGEM-7Zf(-) (*Apa*I, *Bam*HI, *Cla*I, *Eco*RI, *Kpn*I, *Sph*I, *Sma*I and *Xba*I). Southern transfer and subsequent hybridisation with labelled pIMVS5-17, determined fragments suitable for cloning (data not shown). An approx. 9 kb *Xba*I fragment was the smallest single restriction fragment that hybridised with the probe and this fragment was subsequently cloned

into pGEM, generating plasmid construct pIMVSX2. Plasmid pIMVSX2 was confirmed to contain the *ompR*-like gene by dye terminator sequencing using oligonucleotides, p5-17 and p5-17R (Table 8-3). The oligonucleotides were designed from the raw sequence data obtained from dye primer sequencing of the insert of pIMVS5-17.

The sequence of the approximately 9 kb *Xba*I fragment cloned in pIMVSX2 was then determined. The sequencing strategy is shown in Figure 8.2 and the primers used listed in Table 8-3. The complete nucleotide sequence of 9367 bp has been submitted to the databases at Genebank/EMBL under the accession number AF288536. A print out showing all ORFs is shown in appendix I.

8.3.4 Analysis of the DNA sequence of the 9.3 kb *Xba*I fragment cloned from pA5H5 in pIMVSX2

Sequencing of the entire 9367 bp *Xba*I fragment of pIMVSX2 revealed eight potential open reading frames (ORFs) (Fig 8.2). A search using BLASTP determined significant sequence identity of two ORFs with members of the response regulator and sensor kinase family of proteins, which form a two-component response regulator system, common to many bacteria (Stock, *et al.*, 1989). The genes encoding the putative two-component regulatory system found on pA5H5 were designated *lrpR* (*L. longbeachae* sg 1 regulatory protein) and *lskS* (*L. longbeachae* sg 1 sensor kinase) (Fig. 8.2).

8.3.4.1 ORF 6 – *lrpR* gene

ORF6 (nt 4725 – 5417) was 692 bp long. A potential ribosome binding site (RBS) (AAGAGG) was found 17 nt upstream from the AUG initiation codon. The spacing of the binding site with respect to the RBS is not ideal, although spacings of this size have been reported for other bacterial genes (Stormo, *et al.*, 1982), and may reflect a control mechanism. Analysis of the DNA sequence downstream of the stop codon of ORF6 did not identify a Rho-independent termination sequence (Rosenberg and Court, 1979). Termination may occur

by a Rho-dependent mechanism involving a C over G rich region upstream of the 3' endpoint of the mRNA (Alifano, *et al.*, 1991).

An inferred protein product of 230 amino acids in size with a predicted molecular mass of 26,326.6 Da was predicted. The inferred protein, designated LrpR, showed significant sequence homology with numerous members of the two-component family of response regulator proteins (Stock, *et al.*, 1989, Stock, *et al.*, 1990). In particular, high identity scores were observed with transcriptional regulatory proteins RstA from *E. coli* (44.7 % identity) (Accession no. U41101) (Hill, *et al.*, 1989, Roecklein, *et al.*, 1991, Roecklein and Keumpel, 1992) and RisA from *Bordetella bronchiseptica* (39.4 % identity) (Accession no. Z97065) (Jungnitz, *et al.*, 1998). The RisA regulatory protein which has been identified in *Bordetella bronchiseptica* has been shown to be involved in the survival of the organism within eukaryotic cells. Significant homology was also observed with the OmpR protein of *E. coli* (37 % identity) (Accession no. J01656) (Fig 8.3), the best known and characterised member of this family (Comeau, *et al.*, 1985, Wurtzel, *et al.*, 1982).

The LrpR protein also shares features characteristic of the response regulator family of proteins, including conserved residues associated with the conserved hydrophobic core (Stock, *et al.*, 1990) (Fig 8.3). Hydrophobic residues in the C-terminal DNA binding domain, conserved among the aligned members of the OmpR family of proteins likely to have a similar structure, are also conserved in LrpR, including three invariant amino acids (Martínez-Hackert and Stock, 1997, Mizuno and Tanaka, 1997) (Fig. 8.3).

Hydrophobicity plots show that the structure of LrpR and OmpR are similar (Fig 8.4). No dominant areas rich in hydrophobic or hydrophillic amino acids were noted and a signal sequence was not predicted for LrpR. It is likely that this protein is cytoplasmic based upon similarities with OmpR (Jo *et al.*, 1986).

8.3.4.2 ORF8- *lskS* gene

Downstream of *lrpR,* but on the complementary strand, was an open reading frame, designated ORF8, that had significant inferred identity with the sensor kinase family of two-

component regulatory systems. ORF8 (nt 7484- 9088) was 1604 bp in length with a potential RBS (5' AGAG 3') located approximately 10 nt upstream from the AUG initiation codon.

ORF4 encoded an inferred protein product of 534 amino acids in size with a predicted molecular weight of 60,716 Da. The inferred protein shares significant identity with the sensor kinase family of proteins, part of the two-component family of response regulators, of which EnvZ is the best known. All sequences producing high scoring segment pairs for the query sequence ORF8 all fell within the sensor kinase family of proteins (data not shown). The inferred protein of ORF8, designated LskS, showed the highest identity scores with RstB, a sensor protein of *E. coli* (24 % identity) (Accession no. P18392) (Hill, *et al.*, 1989, Roecklein and Keumpel, 1992, Roecklein, *et al.*, 1991) and the osmolarity sensor protein EnvZ from *Rickettsia prowazekii* (23 % identity) (Accession no. A71701) (Andersson, *et al.*, 1998). The CpxA protein of *E. coli* (24 % identity) (Accession no. P08336) also exhibited significant identity scores with LskS (Albin, *et al.*, 1986, Weber and Silverman, 1986). This protein is involved in several diverse cellular processes such as biosynthesis of isoleucine and valine, TraJ protein activation activity for *tra* gene expression in F plasmid and synthesis, translocation, or stability of cell envelope proteins. Another protein of interest that had significant identity with LskS is the PcoS protein of *E. coli* that is a sensor kinase related protein encoded on plasmid pRJ1004 (22.4 % identity) (Accession no. X83541) (Brown, *et al.*, 1995). Significant identity was also observed with the EnvZ osmolarity sensor kinase protein of *E. coli* (22 % identity) (Accession no. P02933), the best known and characterised member of the two-component sensor kinase family of proteins (Comeau, *et al.*, 1985, Mizuno, *et al.*, 1985).

When LskS is compared with the EnvZ protein of *E. coli,* strong regions of identity are observed throughout the protein sequence (Fig. 8.5). Although the overall identity is only 22 % there are significant stretches of identity observed within the C-terminii of the proteins (Fig 8.5). The histidine protein kinase family is defined by a region of conserved sequence generally located near the C terminus (Stock, *et al.*, 1989). Within the homologous domains

of the histidine kinase proteins there are three conserved regions. There is good identity within these three regions when LskS is compared with EnvZ, and in particular seven residues are totally conserved, consistent with observations made for this family of proteins (Fig 8.5). The remaining portions of each of the kinase family of proteins tends to be quite variable and therefore the overall low identity scores are consistent with this (Stock, *et al.*, 1989).

Many of the histidine kinases are membrane associated with two hydrophobic trans-membrane sequences bordering a domain that is localised to the outer surface of the cytoplasmic membrane (Stock, *et al.*, 1989). The amino acid sequence of LskS is suggestive of a an inner membrane protein with two hydrophobic membrane spanning regions joined by a hydrophillic stretch of amino acids that reside in the peri-plasmic space, consistent with the model of secondary structure predicted for the sensor kinases (Stock, *et al.*, 1989). Additionally hydropathy plots predicting the secondary structure of LskS also show that it is similar to EnvZ (Figure 8.6). EnvZ is typical of membrane receptor proteins with two domains separated by a hydophobic membrane spanning sequences (Forst, *et al.*, 1988).

An interesting feature was observed for termination of transcription in the case of the LskS protein in that the predicted end point of transcription is within 3 bp of the predicted stop codon of ORF3 running in the opposite direction (Fig 8.2). It is possible that transcription of *lskS* is regulated in part by RNA polymerase occlusion. This is the phenomenon where two polymerases operating in opposite directions collide with each other thereby terminating transcription in one or both genes.

8.3.4.3 ORF7

ORF 7 was 1823 bp long running from nucleotides 5657 to 7480. A potential RBS (5' GAGA 3') was located at nt 7 upstream from the AUG start codon. A stem loop structure of significant free energy was not located downstream of the UAA termination codon suggesting that a rho-independent mechanism is not involved in termination. As suggested above for the *lskS* it is likely that termination of transcription occurs by RNA polymerase occlusion.

The inferred protein product encoded by ORF7 was 607 amino acids in size with a predicted molecular mass of 67,420 Da. The protein had identity (30% identity) (probability $5e^{-06}$) with a putative integrin-linked kinase from *Caenorhabditis elegans* (Accession no. AJ249344) (Lynch, *et al.*, 1999). This kinase may function as an adaptor to phosphorylate the serine residue of protein kinase B, a protein that regulates cellular activities such as glycogen metabolism and apoptosis (Lynch, *et al.*, 1999). Homology was also observed with hypothetical proteins (approx. 30% identity) related to the ankyrin repeat family of proteins. The highest score (32% identity) was observed with ORF FPV031from Fowlpox virus that belongs to this family (Laidlaw, *et al.*, 1998). Ankyrin repeat proteins carry out a wide range of biological activities and have been detected in organisms ranging from viruses to humans (Sedgwick and Smerdon, 1999). The ankyrin repeat motif has been recognised in over 400 proteins including transcriptional regulators, cytoskeletal organisers, developmental regulators and toxins. The many different roles for ankyrin-repeat proteins makes a common function such as an enzymatic activity unlikely, however, the role of ankyrin repeats in mediating protein-protein interactions has been well documented (Sedgwick and Smerdon, 1999).

8.3.4.4 ORF5

Putative ORF5 is encoded on the complementary strand of DNA of pIMVSX2 (Fig 8.2), and was 1334 bp from nt 3199 to 4533. A good RBS binding (5' GGAGA 3') site was found 8 nucleotides upstream of the AUG start site. A significant stem loop structure was not located down stream of the UAA stop site.

The inferred protein product of ORF5 was 444 amino acids in length with a predicted molecular weight of 51,720.8 Da. The inferred protein product had significant identity ($p=2e^{-25}$ - $2e^{-27}$) to the recently described cyanophycin synthetase enzyme of *Anabena variabilis* (28 % identity) (Accession no. AJ005201) (Ziegler, *et al.*, 1998), and a similar enzyme in a *Synechocystis* strain PCC6308 (28 % identity) (Accession no. AF220099) (unpublished data). Cyanophycin synthetase catalyses the *de novo* synthesis of cyanophycin (multi-L-arginyl-poly-L-aspartate), a nitrogen rich reserve material deposited in the cytoplasm in

cyanobacteria. The C-terminal portion of the deduced amino acid sequence of cyanophycin synthetase shows sequence similarity to enzymes of the superfamily of ligases involved in biosynthesis of murein and folyl-poly(γ-glutamate) (Ziegler, *et al.*, 1998). Interestingly, the inferred protein product of ORF5 also produced significant alignments with proteins of the D-alanine-D-alanine ligase (D-alanylalanine synthetase) family of proteins. Homology was observed with the DdlB ligase protein from *E. coli* (32% identity) (Accession no. P07862) (Robinson, *et al.*, 1986), Ddl from *Haemophilus influenzae* (30 % identity) (Accession no. P44405) (Fleischmann, *et al.*, 1995) and DdlB from *Pseudomonas aeurignosa* (28 % identity) (Accession no. U19797) (Liao, *et al.*, 1996). The D-alanine ligase in *E. coli* is a cytoplasmic protein involved in cell wall formation and is part of the peptidoglycan biosynthetic route (Robinson, *et al.*, 1986). By similarity, the D-alanine ligases of the other bacterial species are proposed to function in cell wall formation in the peptidoglycan biosynthetic route. Interestingly, a hypothetical 41.2 kDa protein in the *cps* region (orf7) of *Klebsiella pneumoniae* was also identified in the identity scores (25 % identity)(Accession no. Q48453). Proteins in this region are involved in capsular polysacharide synthesis in a virulent strain of this organism (Arakawa, *et al.*, 1995).

8.3.4.5 ORF4

Putative ORF4 is 908 bp from nt 3119 to 2211. The potential ORF is encoded on the complementary strand of DNA of pIMVSX2 (Fig 8.2). A potential ribosome binding site (5' GGA 3') was found 11 bp upstream from the AUG start site. A significant stem loop structure was not detected down stream from the UAA stop codon. The size of the inferred translated protein product of this ORF was 302 amino acids in size with predicted molecular weight of 33,531 Da.

The inferred protein product from this gene did not produce high scoring segments when analysed by the programme BLASTP. The significance of this hypothetical protein is unclear.

8.3.4.6 ORF3, ORF2 and ORF1

Downstream of ORF4 are three potential ORFs, designated ORF3, ORF2 and ORF1 that may be translationally coupled. ORF3 was 578 bp long, extending from nt 1920 to 1342. A strong RBS was not found upstream of this ORF although at position 17 there was a GG sequence that may represent a potential start site of translation. The predicted protein product of this ORF was 192 aa in size with an estimated molecular weight of 21,713 Da. A BLASTP search showed no significant identity with any protein in the database. The function of this hypothetical protein is unclear.

ORF2 was 437 bp long, extending from nt 918-1355. A potential RBS (5' GGGAG 3') was found 7 nt upstream from the AUG start site for translation, and overlapped the stop site of ORF3 by seven base pairs. The inferred protein product was 145 aa in size with a predicted molecular mass of 16,116.5 Da. A BLASTP search failed to find significant identity with any proteins in the data bases.

ORF1 was 824 bp in size extending from nt 113 - 937. A potential RBS (5' GAG 3') was found 6 nt upstream of the start site of translation of this ORF, overlapping the ORF2 stop codon by 13 nt. The inferred protein product was 274 aa in size with a predicted molecular mass of 31,540.3 Da.

The inferred protein product of ORF1 had significant identity with the streptomycin and spectinomycin adenyltransferase resistance proteins. Significant probability scores ranged from $2e^{-32}$ to $1e^{-5}$ with all sequences producing significant alignments falling into this category (data not shown). High homology scores were observed with the streptomycin 3''-adenylyltransferase of *Staphylococcus aureus* (32% identity) (Accession no. P04827) (Murphy, 1985) and the streptomycin 3''-adenylyltransferase protein (AadA) of *Salmonella choleraesuis* (30 % identity) (Accession no. S25252)(Leung, *et al.*, 1992). The protein product of these genes mediates resistance to the antibiotic streptomycin. Many examples of this type of gene are found in prokaryotes, some of which are encoded on plasmids (Bito and

Susani, 1994, Kazama, *et al.*, 1995, Tait, *et al.*, 1985). Interestingly, plasmid pIMVSX2 confers spectinomycin resistance to *E.coli* (data not shown).

8.3.5 Construction of the *lrpR* mutant

An isogenic *lrpR* mutant was constructed in strain A5H5 by allelic exchange using the suicide vector construct pCACBS2. Plasmid construct pCACBS2 was generated in several stages (Fig 8.7). First, the *lrpR* gene cloned in plasmid pIMVS5-17 was interrupted by insertion of a non-polar Kanamycin resistance cassette (*aphA*-3) (Ménard, *et al.*, 1993) into a unique *Acc*I restriction site in the coding sequence of *lrpR* (Fig 8.2), thus generating intermediate plasmid construct pIMVS5-17K. The insertionally mutated *lrpR* gene in pIMVS5-17K was excised from the plasmid construct by digestion with *Bam*HI and *Sph*I, restriction sites unique to the polylinker region of the cloning vector pGEM-7Zf(-). The *Bam*HI-*Sph*I fragment was gel purified and cloned into pCACTUS. The resultant construct, designated pCACBS2, was introduced into strain A5H5 by electroporation.

The allelic exchange process was essentially as outlined in chapter 6 with the exception that co-integrates were resolved by plating on 6% sucrose ± 10 μg/ml Km. Insertion of the non-polar Km^r casstette into *lrpR* should render resolved strains resistant to this antibiotic in conjunction with the allelic exchange process. Transformation rates, recombination frequencies and resolution frequencies were comparable to allelic exchange results obtained for construction of other mutants in *L. longbeachae* sg 1 using pCACTUS derived constructs (discussed in chapter 6). However, it was noted that no colonies were detected on the sucrose plates containing Km.

Colonies appearing on the 6% sucrose plates were screened by plasmid analysis to identify transformants that had undergone complete allelic exchange resulting in replacement of the wild type *lrpR* gene for the mutated copy. Plasmid DNA extracted from the potential mutant strains and digested with *Acc*I, a restriction site internal to the regulatory gene sequence in A5H5 (Fig 8.2). Triplicate Southern blots of the resulting restriction fragments

were hybridised with labelled pIMVS5-17, purified *aphA*-3 Km^r cassette and pCACTUS-*mob* vector. The wild type strain, A5H5, showed two hybridising fragments due to cleavage of the internal *Acc*I site in *lrpR,* while mutant strains had only one hybridising fragment due to loss of this site (Fig 8.8B). The size of the single fragment generated in the mutant strains was consistent with the addition of the approximately 850 bp Km (*aphA*-3) cassette. When the plasmid DNA digest was probed with the labelled Km cassette a single hybridising band was observed in the case of the mutant strain but no hybridisation was observed for the wild type pA5H5 plasmid DNA, as expected (Fig 8.8C). Plasmid DNA digests probed with pCACTUS showed no hybridising band indicating that the vector sequence had been lost from pA5H5 (Fig 8.8A). One mutant strain was chosen for all further analysis and was designated A3.

8.3.6 Complementation of the *lrpR* mutation

To ensure that the mutation process did not affect genes other than *lrpR*, the mutant strain A3 was complemented with vector construct pIMVS35. Vector construct pIMVS35 containing the entire *lrpR* gene was constructed as follows. Plasmid pIMVSX2 was digested with *Bam*HI and *Eco*RI. An approx. 4 kb band *Bam*HI-*Eco*RI restriction fragment, containing the *lrpR* gene, was excised and purified. The fragment was cloned into pWKS130, a low copy number pSC101 based vector (Wang and Kushner, 1991), similarly digested with both enzymes. The ligation mix was electroporated into *E. coli* DH5α and transformants containing the vector construct were selected on CA containing 25 μg/ml kanamycin. Transformants were screened by colony hybridisation using labelled pIMVS5-17 as a probe, to confirm the presence of the vector construct. Potential positive transformants were additionally screened by plasmid extraction and restriction digestion to confirm the presence of a single copy of the 4 kb insert (data not shown). The plasmid construct from one positive transformant, designated pIMVS35, was used to complement the mutation in strain A3. Plasmid pIMVS35 was introduced into mutant A3 by electroporation and the mix plated onto CYE containing 50 μg/ml kanamycin. Transformants were observed within 6 days and the

transformation rate was comparable to that achieved for use of this vector in complementation studies with the *mip* gene of *L. longbeachae* sg 1 (discussed in chapter 6). Transformants were screened for the presence of pIMVS35 by colony hybridisation using labelled pWKS130 vector as a probe. Potential positive colonies were further analysed by Southern hybridisation. Plasmid DNA extracted from transformants, electrophoresed on agarose gels and transferred onto nylon membranes prior to hybridisation with Digoxigenin labelled pWKS130. Plasmid DNA from all potential positive transformants hybridised with the probe, but not strain A3 or A5H5 as expected (data not shown). Strain D2 was used for complementation experiments.

8.3.7 Effect of *lrpR* on the intracellular replication of *L. longbeachae* sg 1 A5H5 in U937 cells

To determine if the regulatory gene *lrpR* has a role in virulence by promoting intracellular infection of macrophages, the relative ability of the mutant strain A3 to infect U937 cells was examined. U937 cell monolayers were inoculated with comparable amounts of A5H5 and A3 organism and then examined for changes in the number of bacteria recovered over a 72-hour period.

An initial experiment was set up with *L. pneumophila* sg 1 (Philadelphia) as a positive control to determine optimal ratios of U937 cells and *Legionella* bacteria required for intracellular multiplication. Monolayers were inoculated with two infective ratios (1:1 and 10:1) (bacteria:cells) of wild type *L. pneumophila* sg 1 (Philadelphia) and *L. longbeachae* sg 1 A5H5 and the number of bacteria (CFU/ml) was determined over a 48 hour period (Fig 8.9, part i). All strains were able to multiply intracellularly at both multiplicities of infection and increased in numbers by approx. 3 logs over the 48-hour incubation period. The inoculum ratio of 10:1, however, yielded approximately one log-fold higher numbers of bacteria at each time point in comparison to the assay performed with a multiplicity of infection of 1:1. Based on these results, a ratio of inoculation of 10:1 bacteria

to U937 cells was used for all subsequent experiments. This ratio of infection has been used by other workers to assess strains of *Legionella* in U937 cells (Cianciotto, *et al.*, 1989b, O'Connell, *et al.*, 1995).

The mutant strain A3 and wild type parent strain A5H5 were assessed in U937 cells at a ratio of 10:1 (bacteria:cells) and changes in the number of intracellular bacteria were determined over a 72 period compared with each other (Fig 8.9, part ii). The mean number of CFU (± standard deviations) was determined for each point, and the Student-Newman-Keuls comparison of means ($P < 0.05$) was used to determine statistical significance.

L. longbeachae sg 1 A5H5 and the mutant strain A3 both multiplied in U937 cells and were recovered at the end of the experiment at approx. $10^6 - 10^7$ CFU/ml (Fig 8.9, part ii). The mutant strain appeared to multiply as well as the parent A5H5 in U937 cells throughout the experiment and was recovered at 24, 48 and 78 hours at levels that were not were not statistically significant from the parent strain A5H5. However, a statistically significant difference in recovery was noted at t=0 hours for strain A3 in comparison with A5H5. At this time point there was an four-fold difference in recovery of the mutant strain suggesting that it is defective in the earliest stages of intracellular infection.

8.3.8 Effect of *lrpR* on intracellular multiplication of A5H5 in *Acanthamoeba*

To determine whether *lrpR* effects the ability of *L. longbeachae* sg 1 strain A5H5 to infect and multiply in amoebae, we assessed the relative ability of the *lrpR* mutant (A3) to infect and multiply within *Acanthamoeba*. The mutant strain A3 was compared with wild type parent strain A5H5 and the complemented strain D2 in liquid co-culture as described in section 7.2.2.1. The systems were set up with the same numbers of co-infecting *Legionella* and *Acanthamoeba* and samples were taken at regular intervals to determine intracellular multiplication of the *Legionella* strain. The only difference in this experiment was that the mutant strain was grown on CYE plus 10μg/ml of kanamycin to maintain selection for the mutation. The mean number of CFU was determined for each time point, and the Student-

Newmans-Keuls comparison of means ($P< 0.05$) was used to determine statistical significance.

The parent strain *L. longbeachae* sg 1 A5H5, multiplied well in liquid co-culture, similarly to that shown previously (Doyle, *et al.*, 1998). There was an initial lag phase observed, followed by a steady rise in bacterial numbers, due to intracellular multiplication, and the strain was recovered at the end of the experiment having increased 2-3 logs (Fig 8.10). The regulatory gene mutant A3 showed an attenuated growth pattern in comparison with the parent strain (Fig. 8.10). Strain A3 was able to multiply in *Acanthamoeba* after an initial lag period, as observed for the parent strain, however, it appeared to increase in numbers at a lower rate than A5H5, with statistically significant differences in number of bacteria observed at the end of the experiment at day seven. Statistically significant differences were observed for A3 in comparison with A5H5 at day three.

Complemented *lrpR* mutant D2 also grew in *Acanthamoeba*, however, the number of bacteria recovered at the end of the experiment were statistically different from the wild type parent strain A5H5 suggesting that the mutation had not been fully complemented or that the complementing plasmid had been lost (Fig 8.10). The complemented strain did not show statistically significant differences in comparison with A5H5 at day one and three of the experiment however suggesting a growth rate comparable with the parent strain at these points.

8.3.9 Effect of *lrpR* in establishment of disease in a guinea pig animal model of infection

To determine if the mutation generated in the *lrpR* regulatory gene on pA5H5 had an effect on virulence, strain A3 and A5H5 were compared in an aerosol animal model of infection for their ability to establish lethal infection. Additionally, the complemented mutant strain D2, containing a vector construct encoding the wild type *lrpR* gene, was also tested for virulence in the animal model. Guinea pigs were exposed to an aerosol dose of the test strain (approximately 1×10^9 CFU) and retained doses determined for each animal in all

experiments as described in chapter 3. Results for each isolate were plotted as percentage weight gain or loss for each animal against the number of days after exposure (Fig 8.11). Results for all aerosol experiment are summarised in Table 8-4. Statistical analysis of the data was used to determine groupings using the SAS statistical package version 6 (SAS Institute Inc.), and was performed by Ms Nicole Chamberlain from the Department of Public Health, University of Adelaide. Cluster analysis was used to place the test strains into groups according to the chosen variables, namely median time to death and total number of animals that died in each case (Table 8-5.1 and 8-5.2).

The parent strain *L. longbeachae* sg 1 A5H5 was virulent in this model causing death in 3 of 5 animals tested, all symptomatic within 3 days after exposure (Fig 8.11A) (Doyle, *et al.*, 1998). The mutant strain A3 was tested on three separate occasions and it was attenuated in this model of infection compared with the parent strain (Fig 8.11D, E, F). The mutant strain was only able to establish lethal infection in one guinea pig in the test group of five animals in all assays performed (Table 8-4) (Fig 8.11D, E, F). Experimental doses and retained doses determined for each test exposure of strain A3 (Table 8-4) were similar to each other and to that determined for A5H5 indicating that the lower number of deaths observed for A3 was not due to lower numbers of bacteria deposited into the lungs of the guinea pigs.

Re-introduction of the wild type *lrpR* gene from pA5H5 on a vector construct was able to fully complement the mutation in strain A3, leading to restored virulence (Table 8-4). The complemented strain D2 killed three out of five animals, in two independent experiments, consistent with the results obtained for the wild type parent strain A5H5 (Fig 8.11B and C). The retained dose determined for stain D2 for each experiment was comparable to that determined for strain A3 and strain A5H5 indicating that increased deaths observed for this strain were not simply due to increased bacterial numbers deposited into the lungs of test animals (Table 8-4).

Statistical analysis of the raw data for each animal in the test groups defined two statistically significant clusters of strains based on median time to death and number of

animals dead in each test group (Table 8-5.1 and 8-5.2). Median time to death was used in this analysis and not mean time to death as used for analysis of virulence of *L. longbeachae* strains (discussed in chapter 3). The median is a measure of central tendency and was used as the data set was very small and therefore the data was non-normally distributed. Replicate experiment for the same strain were designated as one, two or three (ie. A3-1, A3-2 and A3-3). Clustering based on median time to death grouped all of the mutant strains together while the wild type parent A5H5 clustered together with the two complemented D2 stains (D2-1 and D2-2) (Table 8-5.1 and 8-5.2). Similar results were obtained for the data set when the total number of deaths that occurred for each test strain was compared by cluster analysis (Table 8-5.1 and 8-5.2). All three mutant strains clustered together while the wild type parent strain and the complemented mutant strain formed the second statistically significant cluster.

In the third exposure performed with strain A3, an interesting result was noted. On day seven, technically after the end point of the experiment, one of the animals in the test group began to rapidly decline in health and was subsequently euthanased so that the lungs could be examined to determine if reversion had occurred *in vivo*. Interestingly, there appeared to be two distinct colony morphologies detected, similar to that shown for strain D2 (Fig 8.12). Lungs taken from animals immediately after exposure to strain A3, in all three independent experiments did not exhibit this colony morphology (data not shown). Similarly this colony morphology was not seen on plates of viability counts of A3.

8.3.10 Effect of *lrpR* on cell surface properties

Since *lrpR* appears to have an effect on colony morphology, whole protein, membrane protein, outer membrane and LPS profiles were examined. Protein and LPS extracts from mutant strain A3, parent strain A5H5 and complemented strain D2 were examined by SDS-PAGE to detect any potential differences between isolates.

No obvious differences in protein profiles were observed when whole protein, whole membrane or outer membrane extracts from A5H5, A3 and D2 were compared by SDS-

PAGE (data not shown). Similarly, analysis of lipopolysaccharide profiles showed that all three strains had a similar banding pattern that was distinct from *L. pneumophila* (Philadelphia) and *L. longbeachae* sg 2 ATCC 33484 but not discernible from each other (data not shown).

8.4 Discussion

L. longbeachae is a highly clonal species as judged by RFLP analysis, multi-enzyme electrophoresis (Lanser, *et al.*, 1990) and sequence analysis (Ratcliff, *et al.*, 1998). This is also suggested by the protein and LPS studies discussed in chapter 4. Therefore, factors encoded on plasmids may play a role in the differential ability of some strains to cause disease in an animal model, as described in chapter 3. Plasmids were detected in nearly all strains of *L. longbeachae* analysed (91 %) and appeared to be quite large (≥ 100 kb), suggesting that they may impart some advantage to the organism and influence virulence. Interestingly, the *L. longbeachae* sg 1 ATCC 33462 and LA-24 strains, both avirulent in an animal model of infection, did not possess plasmids. Since large plasmids appeared to be so widespread in this otherwise tightly clonal species a large native plasmid, pA5H5, from a virulent Australian isolate of *L. longbeachae* sg 1 was investigated.

Partial sequencing of pA5H5 was undertaken to determine if there were any inferred gene products encoded on the plasmid with homology to known virulence factors. A plasmid bank was constructed containing random cloned fragments of pA5H5 and empirically chosen clones were sequenced using dye primer chemistry. Several clones in the bank had significant identity with proteins in the data bases. A transposon related element appears to be encoded on pA5H5, suggesting that the native plasmid may contain functions that have been acquired from mobile genetic elements from other bacteria. Plasmid pA5H5 may also be conjugative as identities with *tra* gene products were observed. Additionally, identity was also observed with fimbrial protein precursors that have similarity with the TraA protein of the F plasmid (Finlay, *et al.*, 1986). The pA5H5 native plasmid may also confer resistance to UV light as

significant identity was seen with the Umu proteins of *E. coli*, that are involved in UV protection and SOS mutagenesis (Kitagawa, *et al.*, 1985).

Southern hybridisation was performed using labelled plasmid DNA from clones of interest, to determine if gene products encoded on pA5H5 were unique. Two plasmids containing pA5H5 DNA insert (pIMVS5-17 and pIMVS5.44) were used to probe a panel of *L. longbeachae* sg 1 plasmid extractions. Hybridising bands were detected on plasmids from different isolates of *L. longbeachae* sg 1, some from overseas, suggesting that related gene sequences exist on plasmids in these strains.

Sequence analysis, and Southern hybridisation studies suggest pA5H5 encodes important factors related to virulence, and therefore may represent a mechanism by which strain diversity is achieved in a highly clonal species of *Legionella*. Interestingly, several attempts to cure pA5H5 were all unsuccessful (data not shown). This result supports the concept that important functions encoded on pA5H5 select for its retention. Additionally, pA5H5 appears to be very stable and is maintained after multiple passage (up to 30 times) on non-selective media (Megan Lake, 1998 – unpublished observations).

One significant observation was a pA5H5 derived clone that had significant identity with the OmpR family of transcriptional regulatory proteins, that are part of a two-component regulatory system. The complete gene sequence for this *ompR*-like regulatory gene, designated *lrpR* (*L. longbeachae* sg 1 regulatory protein), was determined. Sequencing downstream from this gene, revealed an ORF with significant identity to the sensor kinase family of proteins and this gene was designated *lskS* (*L. longbeachae* sg 1 sensor kinase). The *lskS* gene in conjunction with *lrpR* make up a putative two-component regulatory system on a native plasmid in *L. longbeachae* sg 1 strain A5H5. A two-component regulatory system has been reported on a plasmid from *Enterococcus faecium* and *Pseudomonas syringae* (Arthur, *et al.*, 1992, Mills, *et al.*, 1993). The inferred LrpR and LskS proteins had strong identity with many members of their respective families of proteins and also shared conserved features (Mizuno and Tanaka, 1997, Stock, *et al.*, 1989, Stock, *et al.*, 1990). Two-component systems,

described for many prokaryotes, regulate a wide variety of responses including: porin gene expression, chemotaxis, nitrogen fixation and also the expression of virulence factors (Stock, *et al.*, 1989).

Other genes of interest were found on pIMVSX2 that had significant identities with other proteins in the databases. The G + C content of the entire insert was 35 mol %. The G + C content ranged from as low as 32 mol % to 36.2 mol %. The G + C content of *Legionella spp.* ranges from 38-46 mol % (Fallon, 1990). This is suggestive of horizontal transfer of some or all of the genes encoded on this region of pA5H5. Additional support for this is highlighted by the fact that stem loop structures indicative of rho-independent transcriptional terminators were not found after any ORFs encoded on pIMVSX2. It has been shown for several genes in *L. pneumophila*, *L. micdadei* and *L. longbeachae* sg 1 that rho-independent transcriptional stop signals are common in *Legionella* (Doyle, *et al.*, 1998, Engleberg, *et al.*, 1989, Heuner, *et al.*, 1995, Hoffman, *et al.*, 1992a, O'Connell, *et al.*, 1995).

Three open reading frames were found on the complementary strand of pIMVSX2 that appear to be translationally coupled as start and stop codons of the ORFs overlap (Rex, *et al.*, 1994). The phenomena of translational coupling has been reported in both chromosomal (Pati, *et al.*, 1990, Rex, *et al.*, 1994) and plasmid DNAs (Athanasopoulos, *et al.*, 1995, Ruiz-Echevarria, *et al.*, 1995). Genes arranged in this way are usually functionally related much like genes in an operon. One of the genes in this cluster had significant inferred identity with the streptomycin and spectinomycin adenyltransferase resistance proteins and may be a new member of the AadA family of proteins. Related genes are found in operons on other plasmids, such as pSa (Tait, *et al.*, 1985). No typical prokaryotic RBS found for the *aadA* gene on pSa and the mechanism and the mechanism for initiation of translation of this gene is unclear (Tait, *et al.*, 1985). Similarly, the RBS for the *aadA*-like gene on pA5H5 was not typical. Often these genes are encoded on transposons (Murphy, 1985) and R-factor R538-1 (Hollingshead and Vapnek, 1985), and hence it is likely that they have been acquired by horizontal gene transfer. The remaining ORFs in this cluster did not have significant

identities with proteins in the data bases and hence their function is unknown. Interestingly, when the pA5H5 derived construct pIMVSX2 is introduced into *E. coli* DH5α it confers resistance to spectinomycin up to 150 μg/ml. This suggests that ORF9 confers resistance to this antibiotic. *L. longbeachae* sg 1 strain A5H5 is naturally resistant to spectinomycin at levels of 500 μg/ml.

The role of the putative two-component regulatory system was investigated by mutation of the *lrpR* gene by insertion of a non-polar *aphA*-3 kanamycin resistance cassette. The mutant strain was assessed for the ability to establish infection in a guinea pig model, to infect U937 cells and to replicate in *Acanthamoeba.*

It was shown that the *lrpR* mutant, strain A3, multiplied as well as the parent strain A5H5 in U937 cells. However, it did appear to be disadvantaged during the initial uptake stages of infection as significantly fewer mutant bacteria were recovered after the initial two hour uptake step in comparison to the parent strain. This statistically significant observation was observed in three independent assays. This difference was not due to lower numbers of A3 bacteria initially added to the assay as viable counts of suspensions used to seed the monolayers were highly comparable. Once inside the U937 cells the mutant replicated as well as the parent strain and was recovered at the end of the experiment in comparable numbers. The t=0 hour result for the *lrpR* mutant in the U937 cells was similar to that observed for the *mip* mutant of *L. pneumophila* and *L. micdadei* when similar numbers of mutant and wild type bacteria are compared (Cianciotto, *et al.*, 1989b, O'Connell, *et al.*, 1995). However, the *lrpR* mutant multiplies similarly to the parent strain after the initial time point which is in contrast to the *mip* mutant results. The result for the *lrpR* mutant is most similar to that observed for the $pilE_L$ mutant of *L. pneumophila* (Stone and Abu Kwaik, 1998). The $pilE_L$ mutant is defective for attachment to U937 cells but exhibits a wild type phenotype for intracellular multiplication in comparison to the parent strain once it obtained access to the intracellular niche. The assay described in this thesis was unable to distinguish between defects in attachment and those of invasion or early intracellular killing. In order to

answer these questions, assays would need to be performed where U937 cells are treated with cytochalsin D to prevent uptake of attached bacteria (King, *et al.*, 1991). Hence the reduced recovery of the *lrpR* mutant shortly after inoculation may be due to a defect in attachment to or uptake into U937 cells or early survival events immediately after uptake. Interestingly, when the mutant was tested in U937 cells at a multiplicity of infection of 1:1 it had a more severe defect in uptake and intracellular growth in comparison with the parent strain (data not shown). This observation suggests that the defect is partially overcome by addition of larger numbers of bacteria to the monolayer.

The *lrpR* mutant was also disadvantaged in an *Acanthamoeba* model of infection, however, which is in contrast to the results of Stone and Abu Kwaik (1998) for the $pilE_L$ mutant which grows similarly to the parent strain. The *lrpR* mutant was not as efficient in intracellular replication or invasion as it was consistently recovered in lower numbers than the parent strain at each time point throughout the experiment. The reduced intracellular multiplication in *Acanthamoeba* of the *lrpR* mutant is similar to that observed for the *mip* mutation (Cianciotto and Fields, 1992, Doyle, *et al.*, 1998, O'Connell, *et al.*, 1995), although the defect in intracellular multiplication was not as severe. No distinction was made in this assay between attachment and intracellular multiplication. It would be interesting to look at numbers of bacteria immediately after attachment to *Acanthamoeba* to determine if a similar effect is seen in this model as that observed in U937 cells for the *lprR* mutant. Strain D2 was also tested in this model and it was recovered at the end of the experiment at levels that were statistically significantly lower than the parent strain. This suggests that the mutation was not fully complemented by introduction of the wild type *lrpR* gene. This may be due to differences in levels of expression of *lrpR* when present on a plasmid. It has been noted before for the defect in the *mip* mutant that the complemented strain was not always identical to the wild type strain (Cianciotto, *et al.*, 1989b).

The *lrpR* mutant phenotype is similar to findings described by Gao *et al* (1997) for a class of mutants (group five) that were defective in intracellular multiplication in protozoan

and U937 cells and hence designated *pmi* mutants (protozoan and macrophage infectivity loci). Group five *pmi* mutants were defective in multiplication in U937 cells and *H. vermiformis*, however, a decrease in intracellular numbers was not seen at t=0 hours in either cell type in contrast to the *lrpR* mutant. Two mutants within the class five group were severely defective in attachment to both cell types. This suggests that the mutation in the *lrpR* gene may not influence attachment but may play a role in events immediately after uptake that effect survival in a similar way to that proposed for *mip* (Cianciotto, *et al.*, 1989b, Doyle, *et al.*, 1998, O'Connell, *et al.*, 1995).

The *mil* (macrophage specific infectivity locus) mutants of *L. pneumophila* are defective in intracellular multiplication in U937 cells but essentially wild type for replication in *A. polyphaga* (Gao, *et al.*, 1998a). In contrast, the *lprR* mutant is essentially wild type for replication in U937 cells but is disadvantaged in replication in *Acanthamoeba*. This is not surprising as mechanisms of attachment and uptake of *L. pneumophila* in mammalian cells and amoebae are different. Attachment to and invasion of *H. vermiformis* is mediated by attachment to a 170-kDa lectin receptor present on the surface of the amoebae (Harb, *et al.*, 1998, Venkataraman, *et al.*, 1997) while attachment to mammalian cells occurs by complement and non-complement receptors (Gibson, *et al.*, 1994, Husmann and Johnson, 1992, Payne and Horwitz, 1987).

The *lrpR* mutant, strain A3, was compared with parent strain A5H5 and complemented strain D2, in an animal model of infection by aerosol exposure to a dose of 10^9 bacteria. It was shown that the mutant strain was attenuated *in vivo* in three independent assays. The mutant strain only killed one out of five animals, in a slower progressing disease, in contrast to A5H5 that rapidly killed three out of five animals. The complemented strain was fully restored for virulence in this model and killed three out of five animals in two independent assays. Statistical cluster analysis showed that the parent strain and complemented strain consistently grouped together, separate from the mutant strain, when both median time to death and number of animals killed were used as variables.

Examination of the lungs of a guinea pig infected with strain A3, euthanased after the endpoint of the experiment due to poor health, showed that organisms recovered from the lungs had two distinct colony morphologies, similar to that observed for the complemented mutant strain D2. Differing colony morphology was noted for the *L. pneumophila pilD* mutant (Liles, *et al.*, 1999). Therefore, the *lrpR* mutant was examined for changes in cell envelope profile in comparison to the parent strain and complemented strain D2. No obvious differences were seen in either strain when total protein, whole membrane or outer-membrane profiles were examined by SDS-PAGE. Similarly, the LPS profiles of the three strains were also compared since it has been reported that a *L. pneumophila* spontaneous LPS mutant has an unstable phenotype (Lüneberg, *et al.*, 1998). All three *L. longbeachae* sg 1 strains had an identical LPS profile. However, it does not rule out the possibility of subtle changes in LPS structure.

Reversion of the *lrpR* mutation could not be demonstrated genetically suggesting that a larger panel of isolates may need to be examined. It is possible that the mutation in *lrpR* may have a pleotropic effect and hence psuedo-reversion may be occurring. Colonial morphology changes suggests some form of phase variation may be associated with the *lrpR* gene. Phase variation has also been associated with the *bvg* two-component regulatory systems in *B. bronchiseptica* (Banemann and Gross, 1997, Carbonetti, *et al.*, 1993). It is also possible that the *lrpR* gene may be acting *in trans* to activate or control genes encoded in the chromosome of *L. longbeachae* sg 1. It is well known that proteins from two component regulatory systems can complement each other *in trans* (Wanner, 1992).

8.5 Summary

A high proportion of *L. longbeachae* sg 1 isolates contained plasmid DNA, (91 %), suggesting that genes encoded by these elements may be important in determining virulence of a particular strain. Partial sequence analysis of a large native plasmid (approx. 120 kb), designated pA5H5, from a highly virulent Australian isolate revealed a two-component

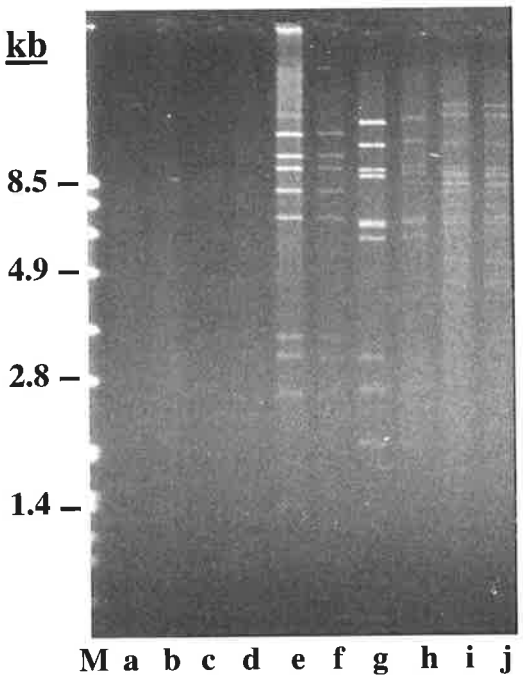
regulatory system with inferred identity to the OmpR family of two-component transcriptional regulatory proteins and EnvZ sensor kinases. An isogenic mutant was constructed in the transcriptional regulatory gene, designated *lrpR* (*L. longbeachae* sg 1 regulatory protein) and this strain was tested in *Acanthamoeba*, U937 cells and in a guinea pig animal model. The mutant was reduced in intracellular multiplication within *Acanthamoeba* but not U937 macrophage like cells. However, the *lrpR* mutant did appear reduced in invasion at the early stages of infection of U937 cells. The *lrpR* mutant was attenuated for virulence in a guinea pig animal model of infection.

Figure 8.1

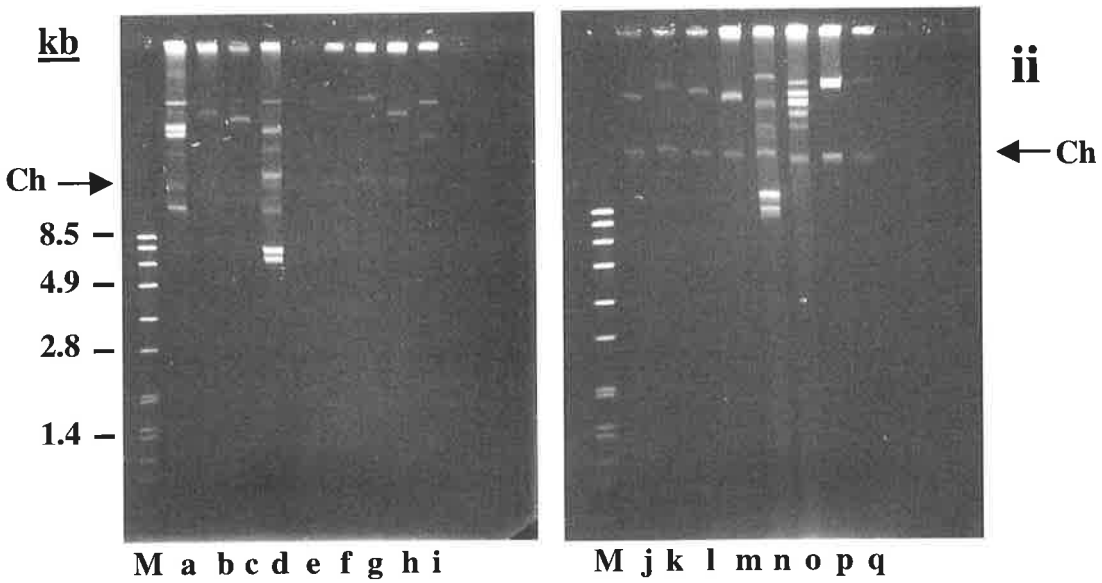
Plasmid profiles of *Legionella* strains.

(i): Plasmid DNA, extracted from some strains of *Legionella* at 30°C and 37°C, were digested with *Eco*RI, electrophoresed on 0.8% agarose gels and stained with ethidium bromide. Lane **a**: *L. pneumophila* strain Philadelphia (30°C); lane **b**: *L. pneumophila* strain Philadelphia (37°C); lane **c**: *L. longbeachae* sg 1 ATCC 33462 (30°C); lane **d**: *L. longbeachae* sg 1 ATCC 33462 (37°C); lane **e**: *L. longbeachae* sg 1 strain A5H5 (30°C); lane **f**: *L. longbeachae* sg 1 strain A5H5 (37°C); lane **g**: *L. longbeachae* sg 1 strain L6C9 (30°C); lane **h**: L6C9 (37°C); lane **i**: *L. longbeachae* sg 2 ATCC 33484 (30°C); lane **j**: *L. longbeachae* sg 2 ATCC 33484 (37°C). Lane **M**: SPP-1 molecular weight marker.

(ii): Plasmid DNA was extracted from strains of *Legionella longbeachae* and run on 0.8% agarose gels. Lane **a**: *L. longbeachae* sg 1 strain A5H5; lane **b**: *L. longbeachae* sg 1 strain L6C9; lane **c**: *L. longbeachae* sg 1 strain A4C5; lane **d**: *L. longbeachae* sg 1 strain A5H3; lane **e**: *L. longbeachae* sg 1 ATCC 33462 (type strain); lane **f**: *L. longbeachae* sg 1 strain D-63; lane **g**: *L. longbeachae* sg 1 strain D-493; lane **h**: *L. longbeachae* sg 1 strain D-880; lane **i**: *L. longbeachae* sg 1 strain D-1028; lane **j**: *L. longbeachae* sg 1 strain D-1056; lane **k**: *L. longbeachae* sg 1 strain D-1738; lane **l**: *L. longbeachae* sg 1 strain D-1750; lane **m**: *L. longbeachae* sg 1 strain D-1751; lane **n**: *L. longbeachae* sg 1 strain D-1624; lane **o**: *L. longbeachae* sg 1 strain D-1820; lane **p**: *L. longbeachae* sg 1 strain D-1959; lane **q**: *L. longbeachae* sg 1 strain D-1992. Lane **M**: SPP-1 molecular weight marker. Arrow indicates likely chromosomal contamination (Ch), observed in all plasmid profile tracks


kb
8.5
4.9
2.8
1.4
M a b c d e f g h i j

i


kb
Ch
8.5
4.9
2.8
1.4
M a b c d e f g h i
M j k l m n o p q
Ch

ii

Figure 8.2

Sequencing strategy, orientation of open reading frames and restriction sites of plasmid DNA insert of pIMVSX2.

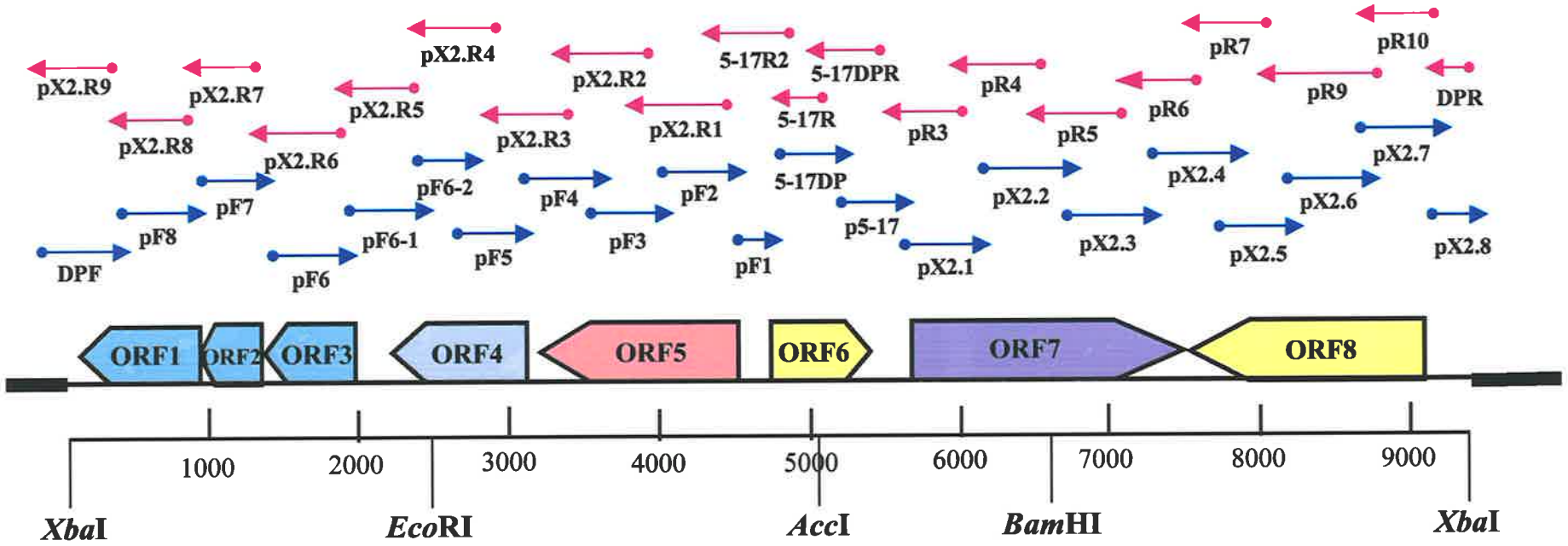
- The sequencing strategy of pIMVSX2 is represented above the scale line with arrow heads indicating the direction of sequencing. The designation of each sequencing primer is labelled above each arrow head. The nucleotide sequence of these primers is listed in Table 8-3.

  Red arrows represent sequencing of the complementary strand of pIMVSX2.

  Blue arrows represent sequencing of the normal strand of pIMVSX2.

  Bold black arrows represent the position and direction of open reading frames and direction of transcription is indicated by the arrow heads.

- Bold lines on the left and right of the inset correspond to the vector pGEM-7Zf(-) DNA.



KEY

ORF6	= *lrpR*	ORF5	= cyanophycin synthetase	ORF4	= unknown	ORF2	= unknown
ORF8	= *lskS*	ORF7	= kinase/ankyrin repeat protein	ORF3	= unknown	ORF1	= *aadA*

Figure 8.3

Amino acid comparison of the transcriptional regulatory proteins OmpR and RisA with LrpR.

* indicate amino acids in LrpR that are identical to RisA and OmpR.

- The grey shaded boxes represent residues associated with the conserved hydrophobic core of response regulators (Stock, *et al.*, 1990).

- The red boxes represent the three highly conserved amino acid residues in response regulators (Stock, *et al.*, 1990).

- The blue boxes represent the three invariant amino acids in the carboxy DNA binding domain of response regulators (Mizuno and Tanaka, 1997).

- Orange boxes represent hydrophobic residues conserved among the aligned members of the response regulator family that are likely to have a similar structure (Mizuno and Tanaka, 1997).

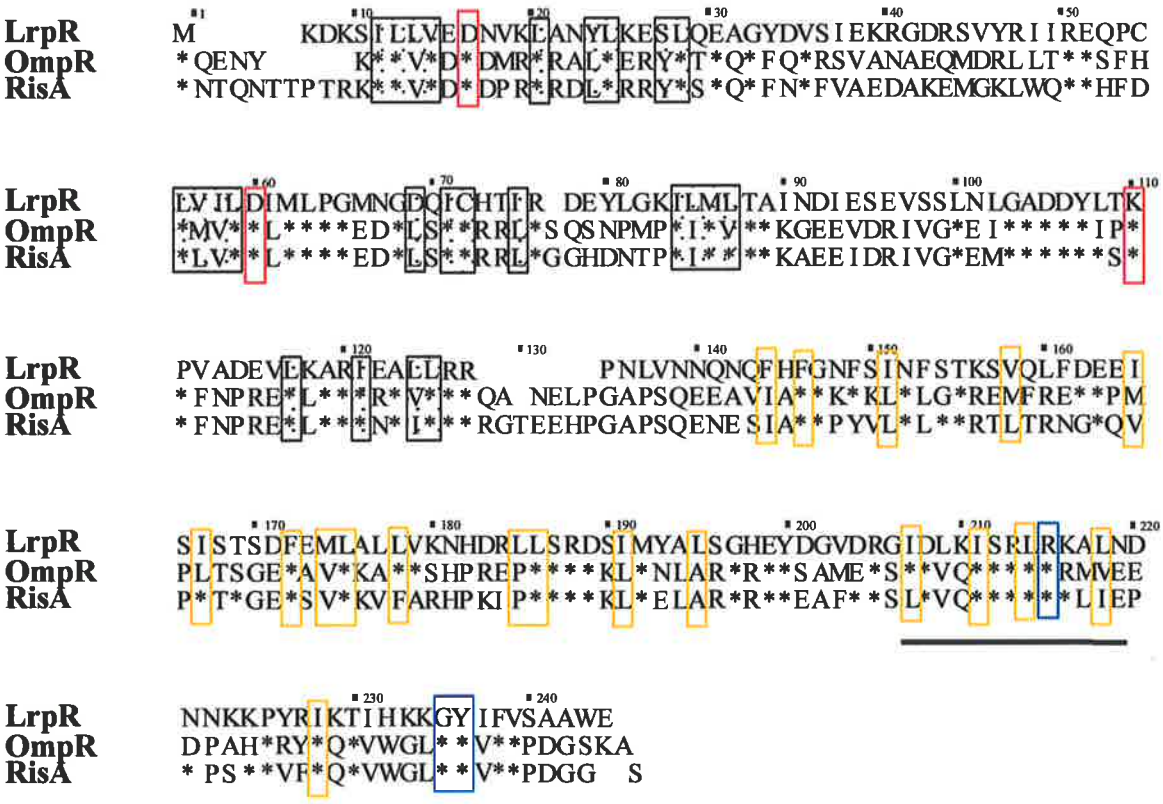
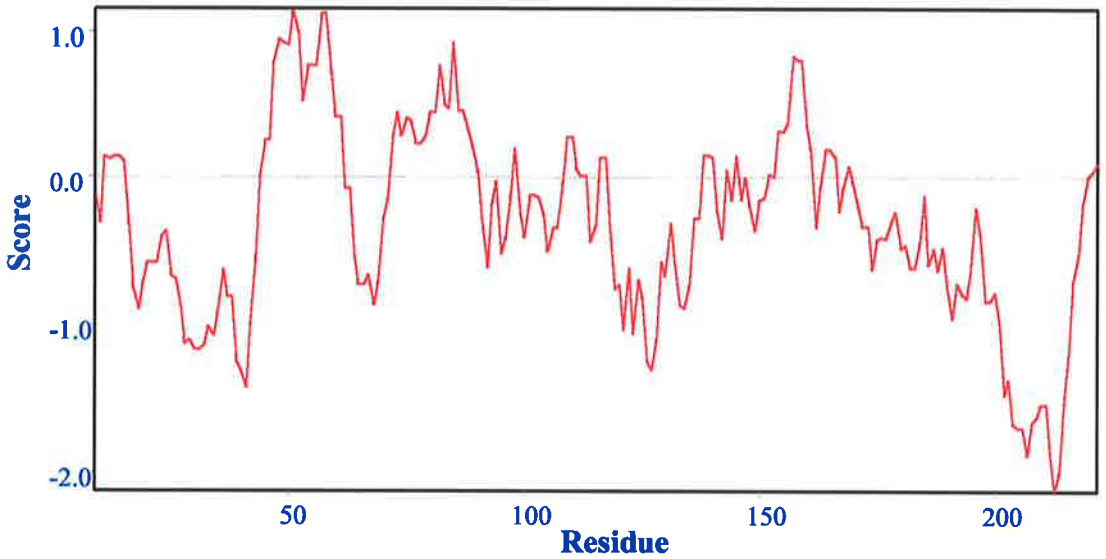
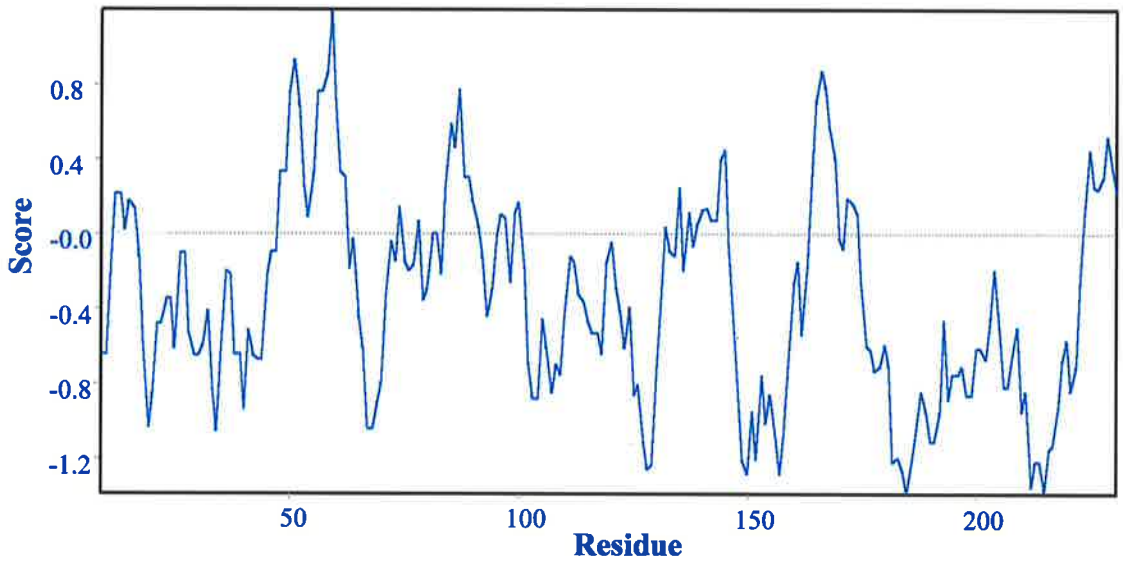

LrpR
OmpR
RisA
1 10 20 30 40 50
M KDKSIILLVEDNVKLANYLKESLQEAGYDVSIEKRGDRSVYRIIREQPC
*QENY K**V*D*DMR*RAL*ERY*T*Q*FQ*RSVANAEQMDRLLT**SFH
*NTQNTTPTRK**V*D*DPR*RDL*RRY*S*Q*FN*FVAEDAKEMGKLWQ**HFD
60 70 80 90 100 110
LVILDIMLPGMNGDQICHTIRDEYLGKILMLTAINDIESEVSSLNLGADDYLTK
*MV**L****ED*LS**RRL*SQSNPMP*I*V**KGEEVDRIVG*EI*****IP*
*LV**L****ED*LS**RRL*GGHDNTP*I**KAEEIDRIVG*EM******S*
120 130 140 150 160
PVADEVLKARIEALLRRPNLVNNQNQFHFGNFSINFSTKSVQLFDEEI
*FNPRE*L**R*V**QANELPGAPSQEEAVIA**K*KL*LG*REMFRE**PM
*FNPRE*L**N*I**RGTEEHPGAPSQENESIA**PYVL*L**RTLTRNG*QV
170 180 190 200 210 220
SISTSDFEMLALLVKNHDRLLSRDSIMYALSGHEYDGVDRGIDLKISRLRKALND
PLTSGE*AV*KA**SHPREP*****KL*NLAR*R**SAME*S**VQ*****RMVEE
P*T*GE*SV*KVFARHPKIP*****KL*ELAR*R**EAF**SL*VQ*****L IEP
230 240
NNKKPYRIKTIHKKGYIFVSAAWE
DPAH*RY*Q*VWGL**V**PDGSKA
*PS**VF*Q*VWGL**V**PDGG S

Figure 8.4

Hydropathy predictions of the LrpR protein from *Legionella longbeachae* sg 1 strain A5H5 and OmpR protein from *E. coli*. Plots were produced by OMIGA 1.1 using the method of Kyte-Doolittle (Kyte and Doolittle, 1982).



Key
Kyte-Doolittle hydropathy: LrpR



Key
Kyte-Doolittle hydropathy: OmpR

Figure 8.5

Amino acid comparison of the sensor kinase (histidine protein kinase) EnvZ of *E. coli* with the proposed sensor kinase LskS from *L. longbeachae* sg 1 strain A5H5, plasmid pA5H5.

* indicate amino acids in LskS that are identical to EnvZ.

- Bold blue lines indicate homologous regions I, II and III determined for the histidine protein kinase protein family (Stock, *et al.*, 1989).
- The red boxes represent invariant residues within the homologous domains.

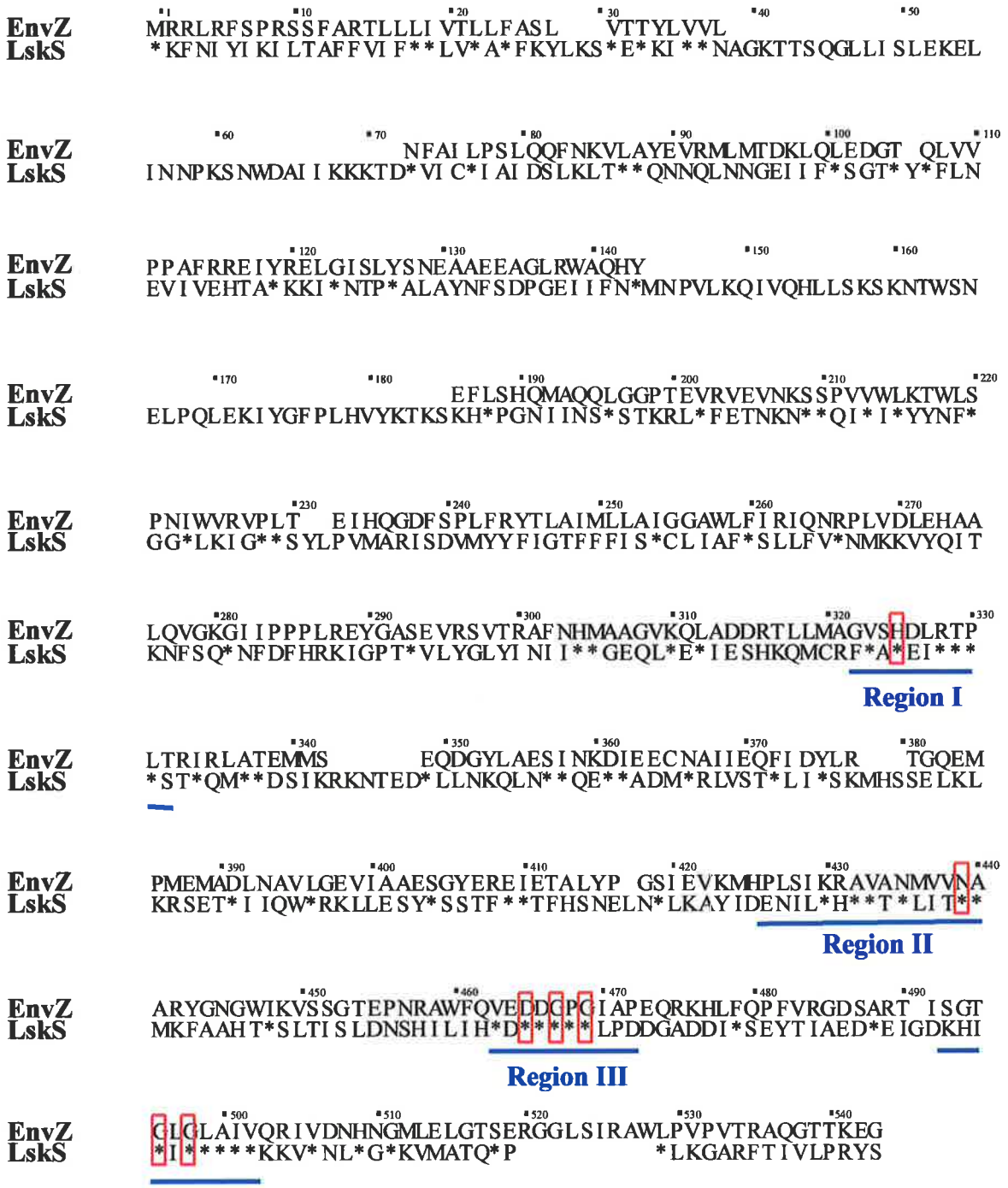
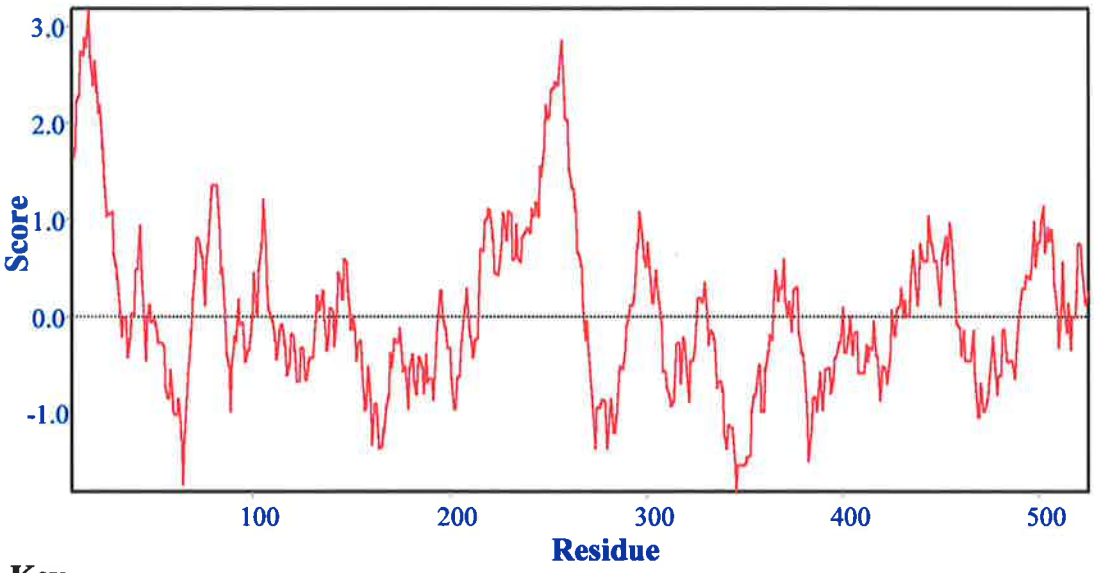

EnvZ
LskS
MRRLRFSPRSSFARTLLLI VTLLFASL VTTYLVVL
*KFNI YI KI LTAFFVI F**LV*A*FKYLKS*E*KI**NAGKTTSQGLLI SLEKEL
NFAI LPSLQQFNKVLAYEVRMLMTDKLQLEDGT QLVV
INNPKSNWDAI I KKKTD*VI C*I AI DSLKLT**QNNQLNNGEI I F*SGT*Y*FLN
PPAFRREIYRELGISLYSNEAAEEAGLRWAQHY
EVI VEHTA*KKI*NTP*ALAYNFSDPGEI I FN*MNPVLKQI VQHLLSKSKNTWSN
EFLSHQMAQQLGGPTEVRVEVNKSSPVVWLKTWLS
ELPQLEKI YGFPLHVYKTKSKH*PGNI INS*STKRL*FETNKN**QI*I*YYNF*
PNIWVRVPLT EIHQGDFSPLFRYTLAIMLLAIGGAWLFIRIQNRPLVDLEHAA
GG*LKI G**SYLPVMARISDVMYYFIGTFFFIS*CLIAF*SLLFV*NMKKVYQIT
LQVGKGI IPPPLREYGASEVRSVTRAFNHMAAGVKQLADDRTLLMAGVSHDLRTP
KNFSQ*NFDFHRKIGPT*VLYGLYI NI I**GEQL*E*IESHKQMCRF*A*EI***
Region I
LTRIRLATEMMS EQDGYLAESINKDIEECNAIIEQFIDYLR TGQEM
*ST*QM**DSIKRKNTED*LLNKQLN**QE**ADM*RLVST*LI*SKMHSSELKL
PMEMADLNAVLGEVIAAESGYEREIETALYP GSIEVKMHPLSIKRAVANMVVNA
KRSET*I IQW*RKLLESY*SSTF**TFHSNELN*LKAYIDENIL*H**T*LIT**
Region II
ARYGNGWIKVSSGTEPNRAWFQVEDDGPGIAPEQRKHLFQPFVRGDSART ISGT
MKFAAHT*SLTISLDNSHILIH*D*****LPDDGADDI*SEYTIAED*EIGDKHI
Region III
GLGLAIVQRIVDNHNGMLELGTSERGGLSIRAWLPVPVTRAQGTTKEG
*I*****KKV*NL*G*KVMATQ*P *LKGARFTIVLPRYS

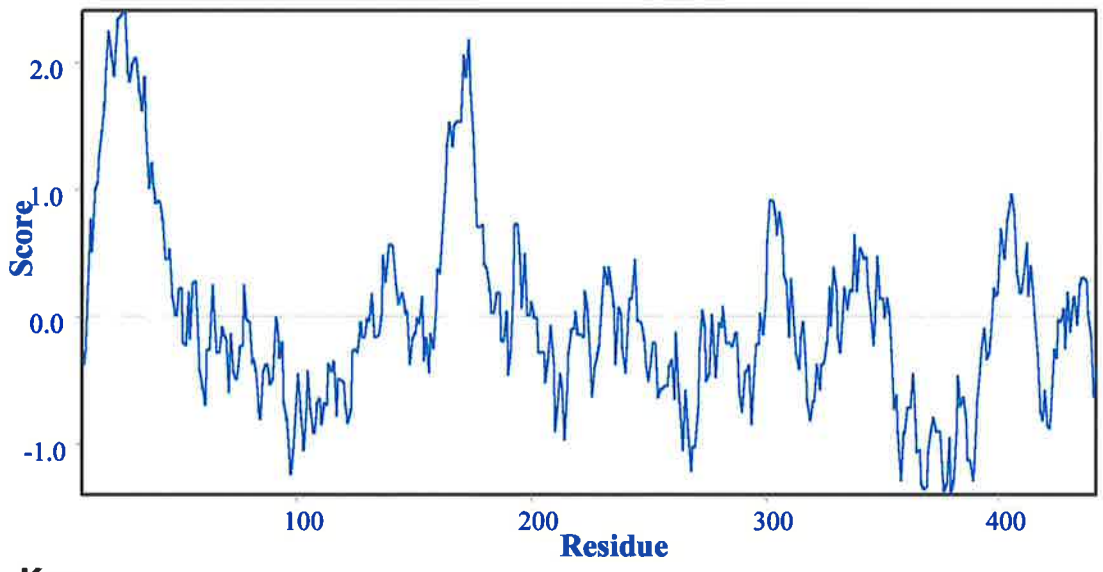
Figure 8.6

Hydropathy predictions of the LskS protein from *Legionella longbeachae* sg 1 strain A5H5 plasmid pA5H5 and the EnvZ protein from *E. coli*. Plots were produced by OMIGA 1.1 using the method of Kyte-Doolittle (Kyte and Doolittle, 1982).



Key

Kyte-Doolittle hydropathy: LskS



Key

Kyte-Doolittle hydropathy: EnvZ

Figure 8.7

Construction of pCACBS2 for generation of an insertion mutation in *lrpR* of pA5H5. Plasmid pIMVS5-17 was derived from an empirically chosen *E. coli* DH5α clone from the plasmid bank constructed from pA5H5. A non-polar *AphA*-3 kanamycin resistance cassette was blunt end cloned into the unique *Acc*I site within the *lrpR* gene generating plasmid pIMVS5-17. Intermediate plasmid pIMVS5-17K was digested with *Sph*I and *Bam*HI to remove the *lrpR* gene fragment containing the insertion mutation. The resultant fragment was cloned into pCACTUS generating pCACBS2 that was used for the allelic exchange process. Plasmid pCACBS2 was introduced into *L. longbeachae* sg 1 strain A5H5 by electroporation.

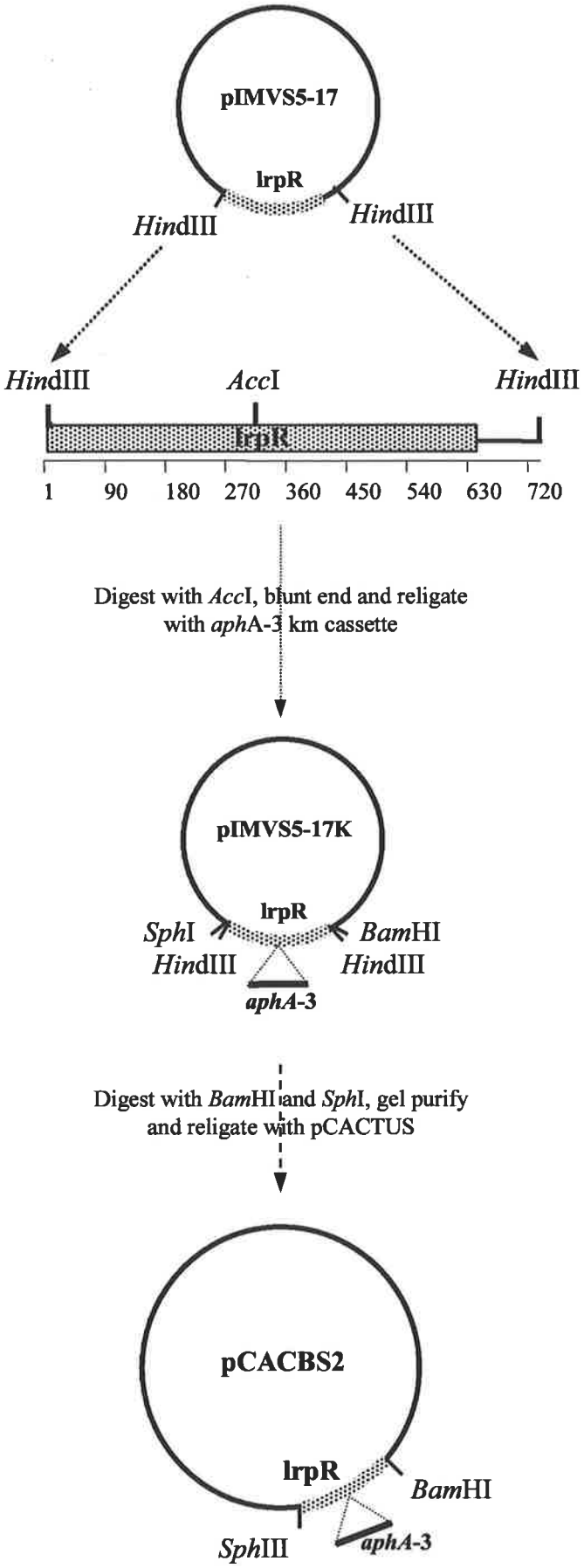

pIMVS5-17
lrpR
HindIII
HindIII
HindIII
AccI
HindIII
lrpR
1
90
180
270
360
450
540
630
720
Digest with AccI, blunt end and religate
with aphA-3 km cassette
pIMVS5-17K
lrpR
SphI
BamHI
HindIII
HindIII
aphA-3
Digest with BamHI and SphI, gel purify
and religate with pCACTUS
pCACBS2
lrpR
BamHI
SphIII
aphA-3

Figure 8.8

Southern analysis of *Legionella longbeachae* sg 1 plasmid DNA. Plasmid DNA was extracted from strains of *L. longbeachae* sg 1, digested with *Acc*I and the fragments transferred onto nylon membrane. Triplicate filters were probed under high stringency conditions with **A**: Digoxigenin-labelled pCACTUS-*mob*; **B**: Digoxigenin-labelled pIMVS5-17 and **C**: Digoxigenin-labelled *Aph3*-A kanamycin resistance cassette. Lanes **a**: pA5H5; lane **b**: pA3. Arrows indicate diagnostic bands.

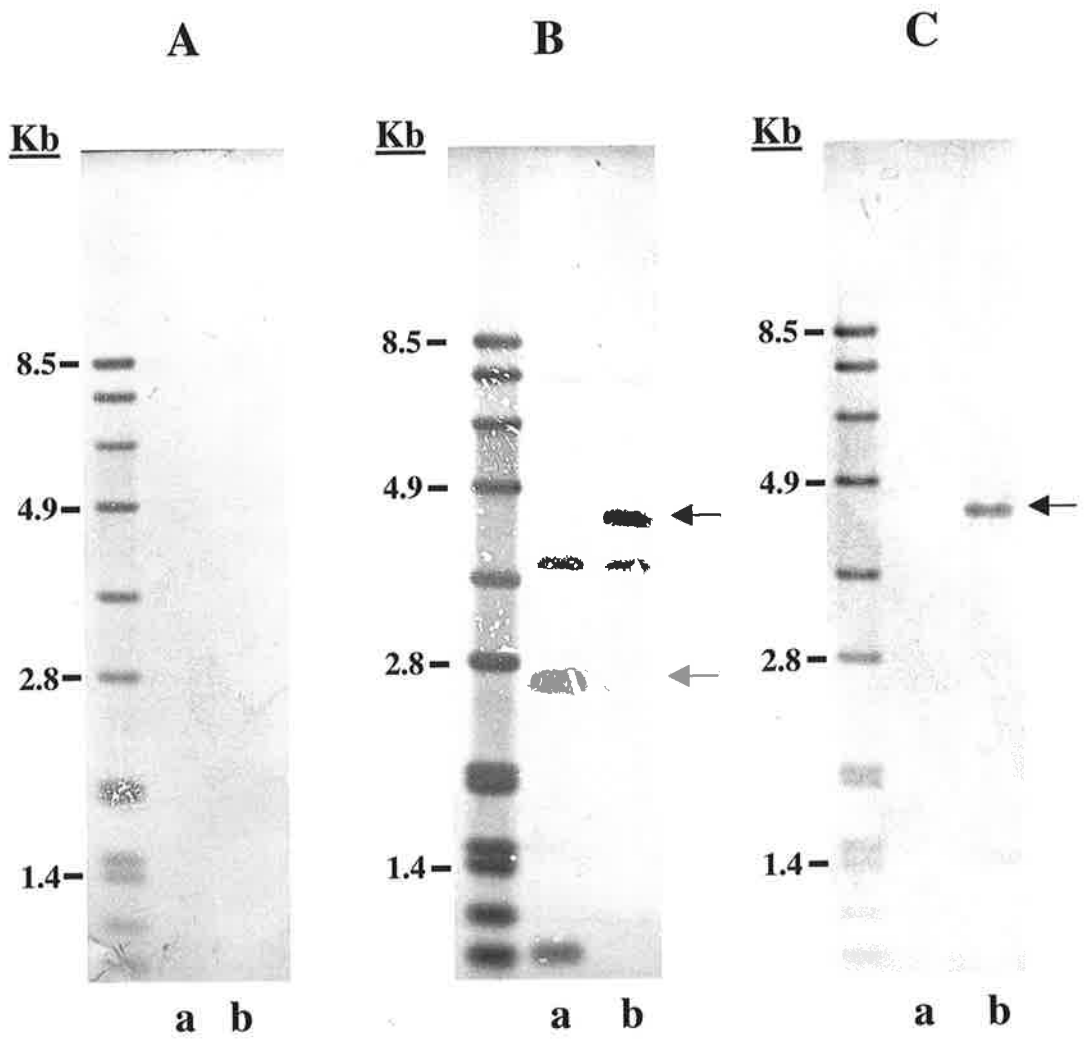

A
B
C
Kb
8.5
4.9
2.8
1.4
a
b

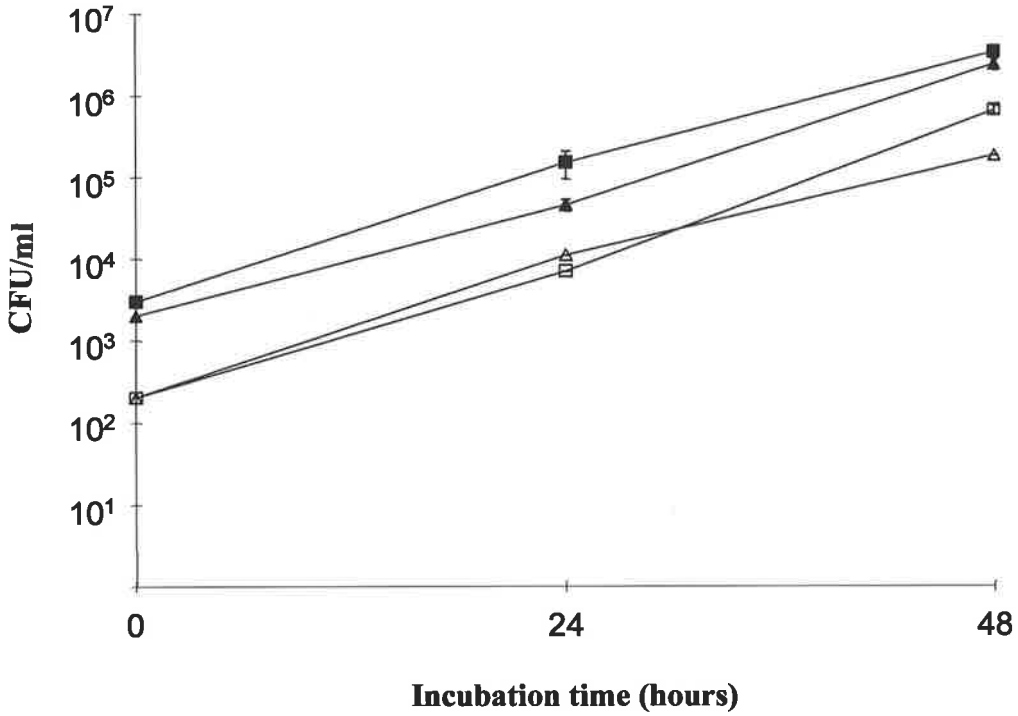
Figure 8.9

Intracellular replication of *Legionella* strains in U937 cells. Monolayers were infected with each strain and samples were taken at 24, 48 and or 72 hours after incubation to determine the number of intracellular bacteria. Note that time zero, indicated on the x axis, corresponds to the first sampling time and represents t=3 hours after initial inoculation of the monolayer. Each data point represents the mean CFU/ml recovered from the well and the vertical bars indicate standard deviation.

(i): Monolayers were inoculated with 8×10^5 CFU *L. pneumophila* sg 1 (Philadelphia) (△), 6×10^6 CFU *L. pneumophila* sg 1 (Philadelphia) (▲), 1.15×10^7 CFU *L. longbeachae* sg 1 A5H5 (■) and 1.1×10^6 CFU *L. longbeachae* sg 1 A5H5 (□).

(ii): Monolayers were inoculated with 1.56×10^7 CFU *L. longbeachae* sg 1 *lrpR* mutant strain A3 (▲) and 1.44×10^7 CFU *L. longbeachae* sg 1 strain A5H5 (□).

(i)


CFU/ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
24
48
Incubation time (hours)

(ii)

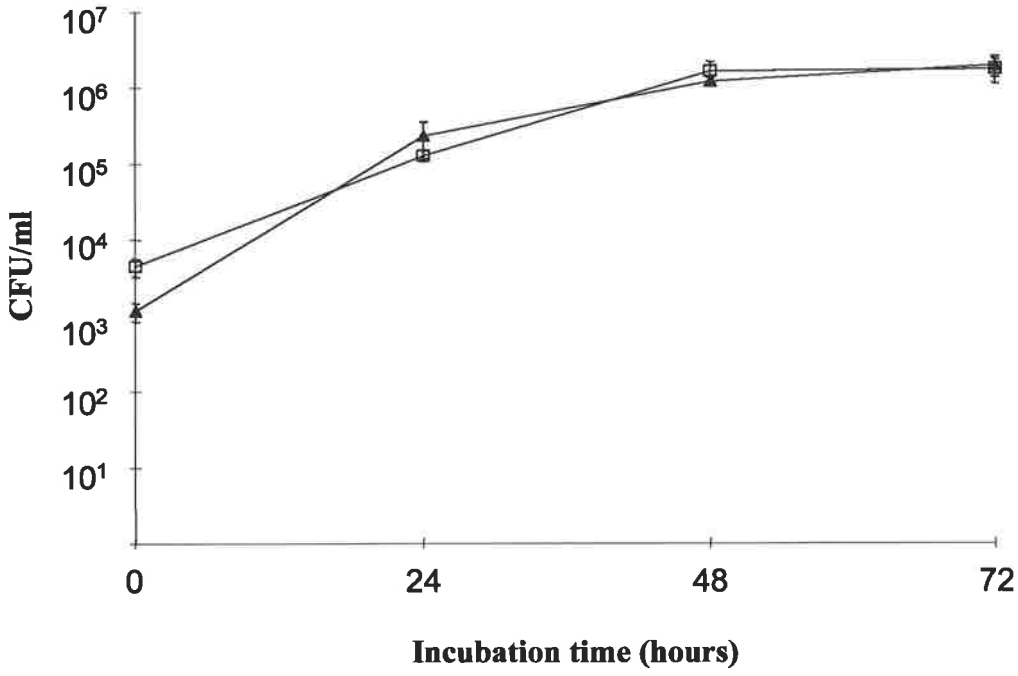

CFU/ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
24
48
72
Incubation time (hours)

Figure 8.10

Co-culture of *Acanthamoeba* with strains of *Legionella*. The experiments shown are representative of a minimum of two independent experiments.

Liquid co-cultures were set up in saline with approximately 10^4 amoebae/ml and 10^3 CFU/ml *L. longbeachae* sg 1 strain A5H5 (◆), *lrpR* mutant strain A3 (△) and complemented *lrpR* mutant, strain D2 (■). Samples were taken at various time intervals and the number of *Legionella* organisms was determined by plating on selective media. Each time point represents the mean number of CFU recovered and the vertical bars indicate standard deviation.

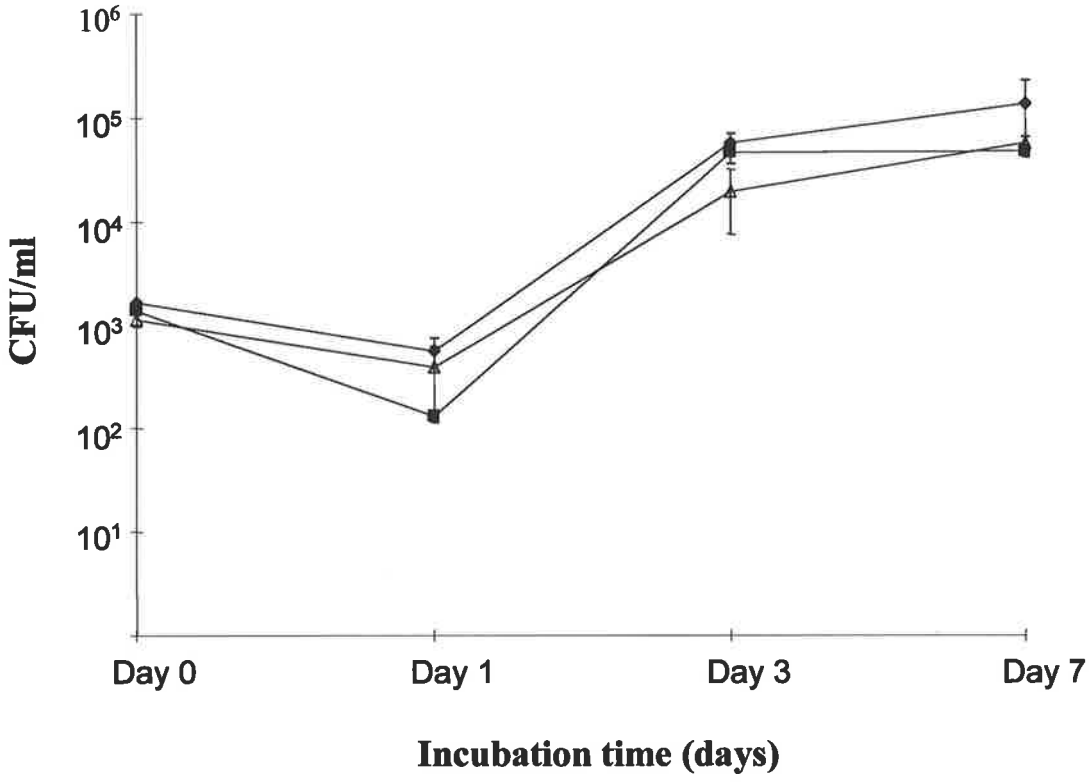

10^6
10^5
10^4
10^3
10^2
10^1
CFU/ml
Day 0
Day 1
Day 3
Day 7
Incubation time (days)

Figure 8.11

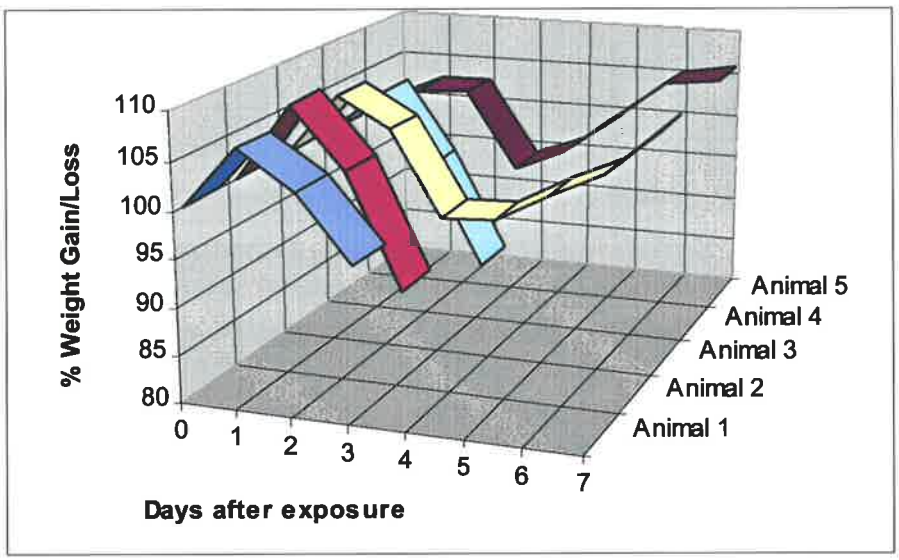
Percentage weight gain or loss in guinea pigs exposed to an aerosol dose of strains of *L. longbeachae* sg 1. Guinea pig death is indicated by the termination of the ribbon graph prior to the end of the experiment on day seven.

(A): Guinea pigs exposed to a dose of approx. 10^9 A5H5 wild type strain.

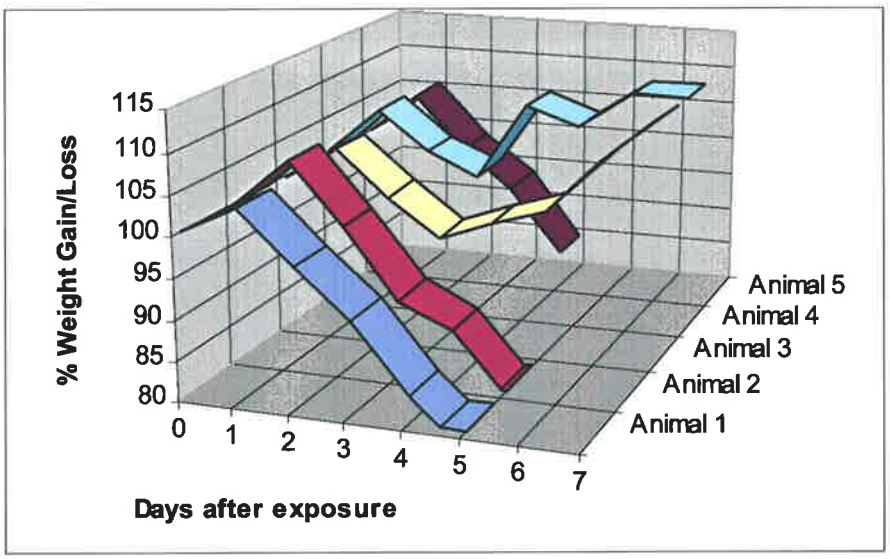
(B): Guinea pigs exposed to a dose of approx. 10^9 D2 complemented mutant strain.

(C): Repeat of B above.

A


% Weight Gain/Loss
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

B


% Weight Gain/Loss
115
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

C

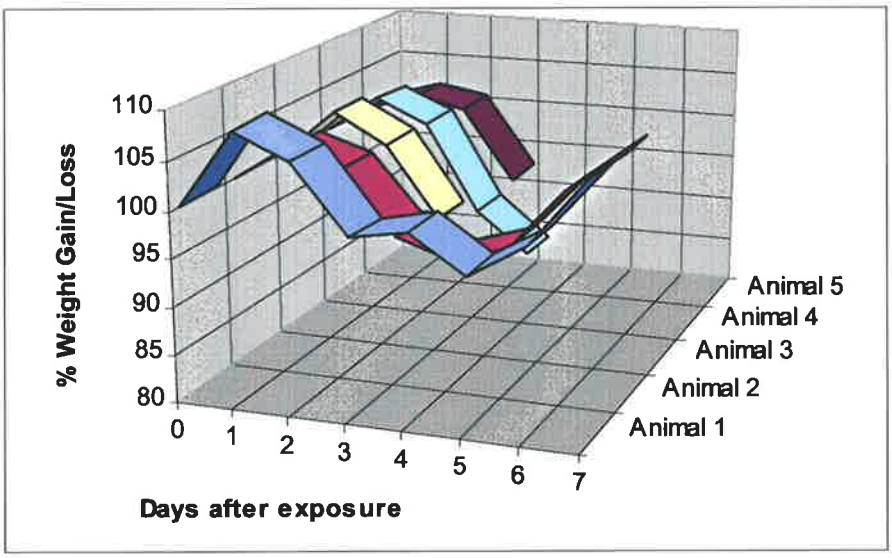

% Weight Gain/Loss
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

Figure 8.11 (continued)

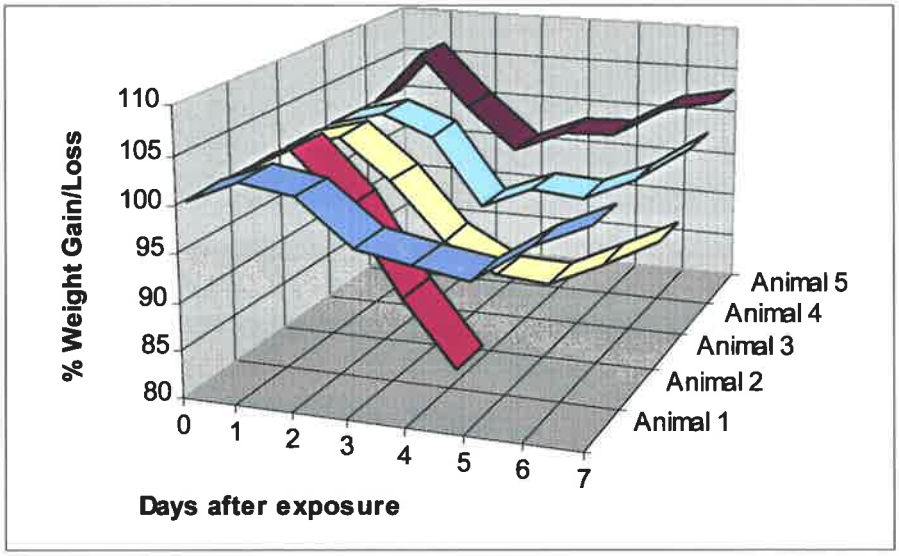
Percentage weight gain or loss in guinea pigs exposed to an aerosol dose (10^9 organisms) of *lrpR* mutant (strain A3), in three independent experiments. Guinea pig death is indicated by the termination of the ribbon graph prior to the end of the experiment on day seven.

(D): Experiment one.

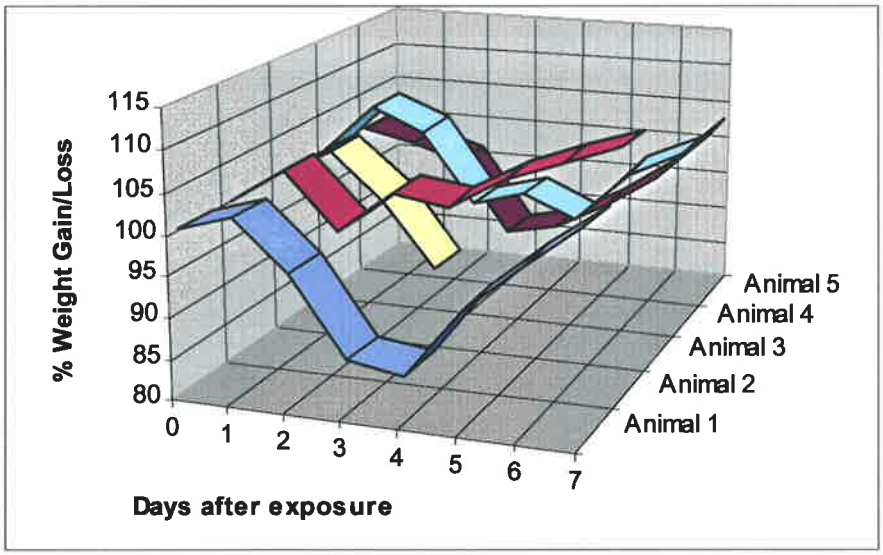
(E): Experiment two.

(F): Experiment three.

D


% Weight Gain/Loss
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

E


% Weight Gain/Loss
115
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

F

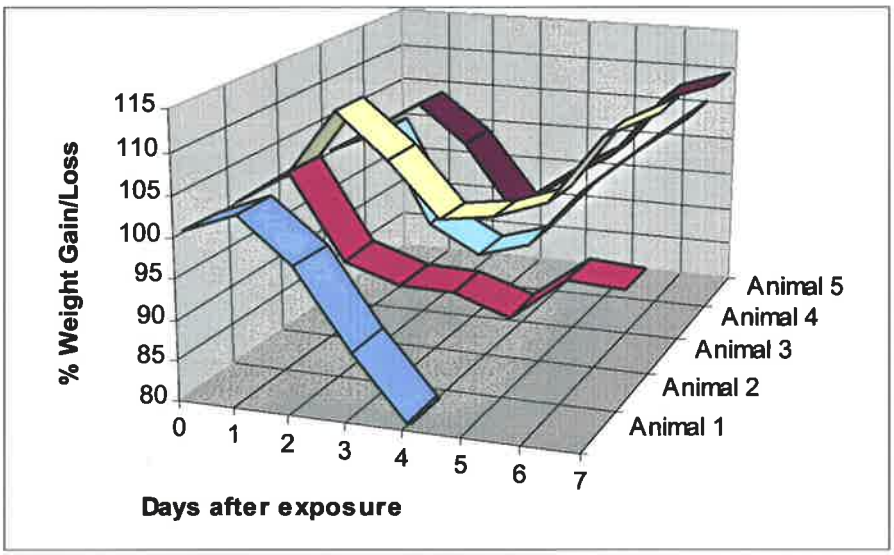

% Weight Gain/Loss
115
110
105
100
95
90
85
80
0
1
2
3
4
5
6
7
Days after exposure
Animal 5
Animal 4
Animal 3
Animal 2
Animal 1

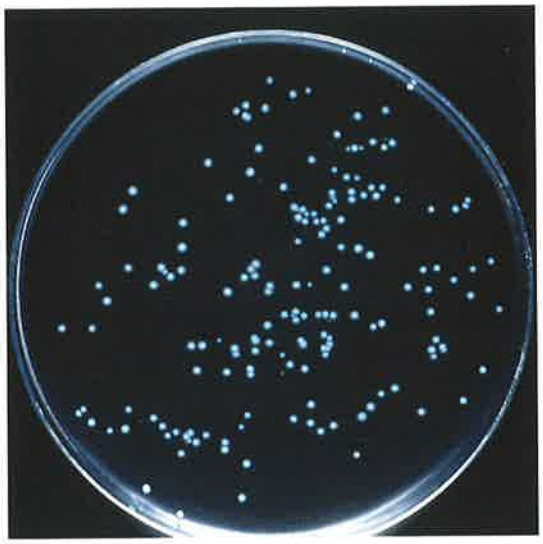
Figure 8.12

Growth of *L. longbeachae* sg 1 strains on CYE agar.

(i): viability count of suspension of strain A5H5 prepared in sterile tap water.

(ii): suspension of lung homogenate of strain A3. Bacteria were recovered from the lungs of a guinea pig that died after the end of an experiment where animals were exposed to a 10^9 dose of *lrpR* mutant, strain A3. The figure shows colonial variation is also representative of viability count plates of suspensions of complemented strain D2, prepared in sterile tap water.

(i)



(ii)

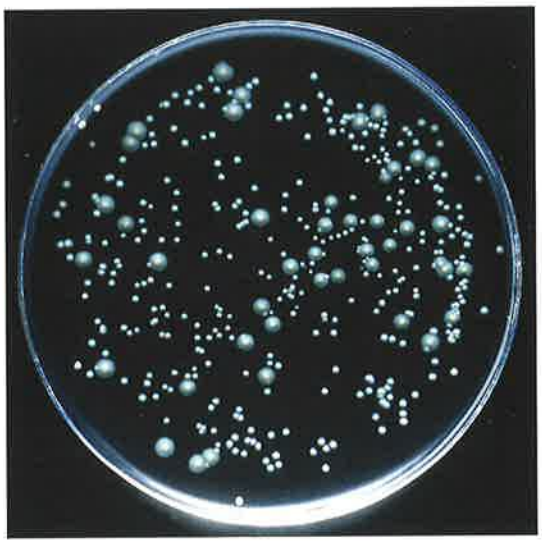


Table 8-1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
Strains		
5-17	*E. coli* DH5α containing plasmid pIMVS5-17.	This study
A3	*L. longbeachae* sg 1 strain A5H5 with a non polar *aphA*-3 Km^r cassette insertion mutation in the *lrp*R gene of the native plasmid pA5H5	This study
D2	Strain A3 complemented with plasmid pIMVS35	This study
Plasmids		
pA5H5	Native plasmid from *L. longbeachae* sg 1 strain A5H5	This study
pIMVS5-17	pGEM-7Zf(-) containing an approx. 850 bp fragment of pA5H5 DNA	This study
pIMVS5-17K	pGEM-7Zf(-) containing mutated *lrp*R gene generated in pIMVS5-17	This study
pIMVSX2	pGEM-7Zf(-) containing an approx. 10 kb *Xba*I fragment of pA5H5 plasmid DNA	This study
pCACBS2	pCACTUS containing mutated *lrp*R gene from pIMVS5-17K	This study
pIMVS35	pWKS130 containing the entire *lrp*R gene encoded on an approx. 4 kb *Bam*HI-*Eco*RI fragment derived from pIMVSX2	This study

Table 8-2 BLASTX sequence homologies of cloned pA5H5 DNA

Plasmid[a]	BLASTX homology Results[b]	Probability score[c]
pIMVS4-36	F - unknown protein (transposon Tn10): 94% identity F- transposase (*E. coli* plasmid pXT107): 94% identity	$1.8e^{-60}$ $2.3e^{-57}$
pIMVS5-21	F- *S. typhi* fimbrial protein precursor from plasmid pED208 with similarity to TraA: 37% identity F- propilin from plasmid ColB4: 28% identity F - *E. coli* fimbrial protein precursor Pil1 from plasmid F : 25% identity	0.00052 0.998 0.999
pIMVS5-7	F- *Methanococcus jannaschii* predicted coding region MJ1123 R- hypothetical 97.5 kDa protein from a 60 kb conjugative bacteriocin-producing plasmid (pMRC01) in *Lactococcus. lactis*	$3.0e^{-16}$ $1.5e^{-6}$
pIMVS5-44	F - *E. coli* TraG protein from F plasmid - 20% identity F - *S. flexneri* IpaB 62kD membrane protein from plasmd pWR100: 40% identity	0.65 0.9990
pIMVS5-17	F - *E .coli* RstA transciptional regulatory protein: 41% identity F - *B. subtilis* PhoP transciptional regulatory protein: 48% identity F- *E. coli* OmpR transciptional regulatory protein: 45% identity	$7.6e^{-39}$ $9.4e^{-26}$ $2.6e^{-18}$
pIMVS5-27	R - *E. coli* UmuC protein: 37 % identity R - *S. typhimurium* UmuC protein : 39% identity R - *S. typhimurium* SamB protein: 33% identity R - *S. typhimurium* ImpB protein from plasmid TP110: 33% identity	$2.3e^{-21}$ $2.2e^{-18}$ $1.0e^{-14}$ $4.6e^{-9}$
pIMVS3-41	F- *E. coli* molybdopterin biosynthesis protein MoeB: 58% identity F- *H. influenzae* molybdopterin biosynthesis protein MoeB: 58% identity	$1.4e^{-16}$ $2.6e^{-15}$
pIMVS5-11	F- homology with YM9718.13c protein from *Saccharomyces cerevisiae*: 37% identity	$1.7e^{-7}$
pIMVS5-18	R - *E. coli* TraI protein (DNA helicase I) from plasmid R100: 37% identity R - *E. coli* TraI protein (DNA helicase I) from plasmid F: 36% identitiy	0.998 0.9998
pIMVS5-10	R - *S. typhimurium* TraI protein encoded on plasmid pKM101: 35% identity R - hypothetical protein 611 from *Coxiella burnetii* with similarity to *E. coli* TraI: 27% identity R - mobilisation protein TraI from *E. coli* conjugative plasmid pCU1: 32% identity R - decorin-binding protein A from *Borrelia garinii* and *B. afzelii*: 31% identity	0.98 0.56 0.98 0.98

a: purified plasmid DNA from empirically chosen and numbered *E. coli* clones from the plasmid bank generated from pA5H5. Plamids were designated with the prefix pIMVS and then the clone number.
b: partial forward and reverse sequence data from individual plasmid clones.
c: probability scores determined by the BLASTX search.
F/R: denotes the direction of the dye primer sequence reaction for which the homology scores were produced

Table 8-3 Oligonucleotide primers

Primer	DNA sequence	Experimental application
pF8	5' –cta tca aga tca tga agc atc gc - 3'	Sequencing of pIMVSX2 (normal)
pF7	5' –tcc ttt aac aat gct tct gct tga gc- 3'	"
pF6-2	5' –tgc ttt cgg act ggg tac ggg c - 3'	"
pF6-1	5' –agc caa cta agc ttc aat cat agc c- 3'	"
pF6	5' –aga tga ctt cca ttg ctc cct gg - 3'	"
pF5	5' – tac gcg atc act atc aac cag c- 3'	"
pF4	5' - ttc atg aga ata ctc cag tgc - 3'	"
pF3	5' - aag agc aac tcc ttg ggc tgc c - 3'	"
pF2	5' - ttt agt atc ctc atc aaa gcg - 3'	"
pF1	5' - ttt atc tcc aca gat tcg ccc - 3'	"
p5-17	5' – cca tga tag gtt gct gag tcg ag - 3'	"
pX2.1	5' - gag atg agc cat gaa aga cgc tgc - 3'	"
pX2.2	5' - tgg aac tgg acc atg gga tgc gc - 3'	"
pX2.3	5' - aac ctg gca atg gta agt tgt gg - 3'	"
pX2.4	5' – gaa ctg gag aag tag ttg atg gcg - 3'	"
pX2.5	5' - gtg tgc gct gca aat ttc atg gc - 3'	"
pX2.6	5' - aca gag gtg gga cct att ttc cg - 3'	"
pX2.7	5' - ttg taa gct aat gca tac ggg g - 3'	"
pX2.8	5' - ttc tgg cat aat tat cct acg - 3'	"
pR10	5' – tcc aga gta ctt tct tgt gaa aag gc- 3'	Sequencing of pIMVSX2 (complementary)
pR9	5' – agg taa ttg tag aac ata ctg cg- 3'	"
pR7	5' - ata aac agc tga aca gca tcc - 3'	"
pR6	5' - agg tta tgg cta cac aat cac cg - 3'	"
pR5	5' - agt tct aag gga gtt ggt ggc g - 3'	"
pR4	5' - aac atc ttc tcc ttc aaa tcg c - 3'	"
pR3	5' - cat ata act gct cac ttg ggc c - 3'	"
p5-17R2	5' - ttc aaa acc tcg tct gca aca gg - 3'	"
p5-17R	5' - gcg ata gac cga tct atc acc acg - 3'	"
pX2.R1	5' - cat tgt gag cat tgc tat ccc g - 3'	"
pX2.R2	5' - gat ttc ctc ttg tgg tta agc c - 3'	"
pX2.R3	5' - gtc agg aca ttt ctc ttc cat ggc - 3'	"
pX2.R4	5' - aca tgg cgc agg tgg tag cgg - 3'	"
pX2.R5	5' - act ttc aag aga acc aag acc g - 3'	"
pX2.R6	5' - tcc tga tga gcg tta tca tgt ccg - 3'	"
pX2.R7	5' - ctc acc cag ata cca taa atc ggc - 3'	"
pX2.R8	5' –atg gtt ctt atc ttg tag gtg g - 3'	"
pX2.R9	5' –tcg atc gaa acc tat agc cgc cg- 3'	"
p4-36	5' – agc tta acg ttg gct tgc cac gc - 3'	Dye terminator sequencing of pIMVS4.36
p5-21	5' – gat gat att cac taa cta tgc g - 3'	Dye terminator sequencing of pIMVS5.21

Table 8-4 Aerosol inoculation of *L. longbeachae* serogroup 1 strains

Strain	Dose (CFU total) [a]	Retained Dose [b]	Deaths [c]	Comments
A5H5	1.49×10^9	3.0×10^5	3/5	-death within 3 days
A3	1.48×10^9	6.8×10^5	1/5	-death at day 3 -animal that died was symptomatic [e]
A3 [d]	1.6×10^9	2.8×10^5	1/5	-death at day 3
A3 [d]	1.47×10^9	2.8×10^5	1/5 [e]	-death at day 4
D2 [d]	1.46×10^9	5.1×10^5	3/5	- all deaths before the end of day 5
D2 [d]	1.6×10^9	4.6×10^5	3/5	- all deaths before the end of day 4

a: determined by viable count

b: number of viable organisms retained in the lungs after exposure to test dose

c: shown as number of deaths per number of guinea pigs tested

d: represents repeat experiments were guinea pigs were exposed to the same test strain

e: symptoms include weight loss, lethargy and laboured breathing. Lungs taken from dead animals showed evidence of consolidation

Table 8-5.1 Cluster analysis groupings based on median time to death

Cluster	Strain	Median[a]	Minimum	Maximum
1	A3-1	8	5	8
	A3-2	8	4	8
	A3-3	8	5	8
2	A5H5	4	4	8
	D2-1	6	5	8
	D2-2	5	4	8

a: Median number of days before death due to a particular test stain.

Table 8-5.2 Cluster analysis groupings based on the number of animals dead in each test group

Cluster	Strain	Dead[a]	STD[b]
1	A3-1	1	0.44721
	A3-2	1	0.44721
	A3-3	1	0.44721
2	A5H5	3	0.54772
	D2-1	3	0.54772
	D2-2	3	0.54772

a: Number of guinea pigs killed out of a total of five exposed to the test strain

b: Standard deviation based on the number of animals dead.

Chapter 9

Concluding discussion

" Why is there an increased incidence of disease due to *L. longbeachae* sg 1 in Australia ?" Are Australian isolates inherently more virulent than overseas isolates ? Does *L. longbeachae* sg 1 share virulence factors with *L. pneumophila* the best studied species from this genus of respiratory pathogens? These questions formed the basis of the studies undertaken in this thesis. Few studies had been focused on *L. longbeachae* probably due to the relatively low reported incidence of disease due to this species of *Legionella* overseas. Most studies of *Legionella* have focused on *L. pneumophila* or *L. micdadei* as these two species are clinically, the most significant in the United States (Reingold, *et al.*, 1984). A general approach to understanding the nature of pathogenesis of *L. longbeachae* was undertaken to try to elucidate the answers to some of these questions.

A guinea pig model of experimental legionellosis was established to assess virulence of isolates of *L. longbeachae* sg 1. A panel of clinical and environmental isolates from different geographical locations within Australia was chosen as a representative cross selection of isolates from this country. These isolates were compared to a panel of 12 isolates obtained from CDC in the United States. Interestingly, the results of the study determined that there were three statistically significant groupings of strains, based on the severity of disease produced in the animals. This result was not surprising given that it has been proposed that, based on U937 infection studies, one isolate of a given species judged as non-infective and therefore by default avirulent in an animal model does not mean that all strains within that species are non-infective (O'Connell, *et al.*, 1996b). All Australian isolates

clustered in the virulent type 1 and type 2 category. Some overseas isolates also belonged to these two groups, however, they were generally fewer in numbers in comparison with Australian isolates. Type 3 strains represented those isolates that were relatively avirulent and this group consisted of only overseas isolates. No Australian isolate tested was avirulent in the aerosol model. The data suggested that Australian strains may be inherently more virulent than overseas isolates, thus partly explaining the increased incidence of disease due to this species here. However, further testing of strains within this species would be required to make any definite conclusions due to the small number of animals tested for each strain and the relatively small number (18) of isolates tested.

Animal model results and complementary U937 ID_{50} results obtained for the ATCC 33462 type strain *L. longbeachae* sg 1 warrant some comment. Our original isolate of this strain was avirulent in an animal model of disease and was not able to replicate in U937 cells. This result was surprising as it had been shown previously that the ATCC 33462 *L. longbeachae* sg 1 type strain was infective for U937 cells suggesting that it would be capable of establishing disease in an animal model (O'Connell, *et al.*, 1996b). To rule out laboratory attenuation a recent stock of this strain was then purchased. The recent isolate of ATCC 33462 was also avirulent in the animal model, consistent with the result for the original stock, although this isolate was not tested in the U937 cell model. Interestingly, the ATCC 33462 type strain has been tested in macrophage-like Mono Mac 6 cells and in *A. castellanii* by other workers who found that it was unable to replicate in either host cell type (Neumeister, *et al.*, 1997). This result suggests that problems may occur when the same isolate is tested in different laboratories. Some strains of *Legionella* may be more sensitive to attenuation resulting from delayed storage or freeze drying processes, factors more likely to occur when cultures are sent to overseas laboratories.

The results of the animal studies were of interest as previous studies with this species of *Legionella* suggested that it is remarkably clonal (Lanser, *et al.*, 1990). Additionally, sequencing of a portion of the *mip* gene from many strains of this species also suggests a

remarkable level of homogeneity, further suggesting the clonal nature of this species (R. Ratcliff, 2000, PhD thesis). Recently a paper was published suggesting that Australian isolates of *L. longbeachae* are not clonal (Montanaro-Punzengruber, *et al.*, 1999). Pulse field gel electrophoresis (PFGE) was used to differentiate strains of *L. longbeachae* into three clonal groupings. The result is interesting but not conclusive for several reasons. First, strains were typed as *L. longbeachae* by antibody methods not by genetic methods such as ribotyping or the method proposed by Ratcliff *et al* (1998), therefore it is not known if all the isolates used in the study are *L. longbeachae* species or indeed serogroup 1 isolates. Second, the analysis does not take into account the presence of extra-chromosomal elements such as plasmids that are present in this species. Third, the number of *Sfi*I fragments generated ranged from four to seven, well below the criteria proposed for interpretation of PFGE data (Tenover, *et al.*, 1995). A study of PFGE patterns of *L. pneumophila* was based on 5-10 bands (Hlady, *et al.*, 1993). Additionally a recent publication using PFGE for typing of *L. pneumophila* suggested that the enzyme *Sfi*I did not give reproducible results and that an alternative enzyme might give better results (DeZoysa and Harrison, 1999). Fourth, the use of a single enzyme for the study of large populations of organisms collected over extended periods of time is not recommended (Tenover, *et al.*, 1995). However, heterogeneity has been shown among strains of *L. micdadei* that are similar phenotypically (Luck, *et al.*, 1995).

A representative isolate was chosen from each of the three virulence groupings, determined in the animal model, to begin analysis of *L. longbeachae* sg 1 pathogenesis. The three isolates were screened for the presence of flagella and MOMP gene sequences, known to exist in *L. pneumophila*, and for differences in membrane protein profiles. Membrane protein profiles determined that *L. longbeachae* sg 1 strains lacked a distinct abundant MOMP-like protein and had fewer proteins in general in comparison to *L. pneumophila*. No differences were detected when the three isolates were compared to each other. At the molecular level a *flaA*-like gene and an *ompS*-like gene sequence (encoding MOMP) did not appear to be present in *L. longbeachae* sg 1 using Southern hybridisation under high

stringency conditions, however, a band was detected at low stringency hybridisation (approx. 30 % bp mismatch). This may represent a MOMP-related gene sequence which was also detected in *L. micdadei* at this stringency. Cloning and sequencing of this gene would determine if it encodes an outer membrane protein related to MOMP of *L. pneumophila* or an unrelated unique outer membrane protein. MOMP protein of *L. pneumophila* can fix complement component C3 (Bellinger-Kawahara and Horwitz, 1990) thus mediating phagocytosis of the organism via complement receptors CR1 and CR2 (Payne and Horwitz, 1987). Therefore the lack of a MOMP-like protein in *L. longbeachae* would suggest that perhaps other outer membrane components of this species may have a more significant role in pathogenesis.

Electron microscopic studies using the *L. longbeachae* sg 1 avirulent ATCC 33462 type strain and virulent strain A5H5 suggest that this species of *Legionella* is taken up by classical phagocytosis. A heavy layer of electron dense material suggestive of "ribosome studding" was not observed for either strain and it is likely that *L. longbeachae* species have an intracellular life cycle distinct from *L. pneumophila.* The results suggest that *L. longbeachae* bacteria may reside in a phagosome that fuses with host cell lysosomes in a manner similar to that described for *L. micdadei.* If this is the case then factors that enable the bacteria to cope with the stress induced by fusion with lysosomal bodies may be more important in these species than in *L. pneumophila*, a species of *Legionella* that survives in a specialised phagosome that does not fuse with host cell lysosomes.

The *mip* gene of *L. longbeachae* sg 1 was examined in detail in this study as it was the first virulence factor described for *Legionella* at the time this study was initiated and was also known to be present in this species (Cianciotto, *et al.*, 1990a, Cianciotto, *et al.*, 1990b, Engleberg, *et al.*, 1989). Additionally, the lack of an obvious MOMP-like protein in *L. longbeachae* sg 1 suggested that this species may be more susceptible to changes in outer membrane proteins. Cloning and sequencing of the *mip* gene of *L. longbeachae* sg 1 ATCC 33462, *L. longbeachae* sg 2 ATCC 33484 and an Australian clinical isolate A5H5 determined

that the DNA sequences were virtually identical with the exception of a 2 bp variation in the gene sequence of the sg 2 type strain. The inferred Mip proteins were identical, and highly similar to *L. pneumophila* (Engleberg, *et al.*, 1989, Ludwig, *et al.*, 1994) and *L. micdadei* (Bangsborg, *et al.*, 1991). Amino acids critical for PPIase activity were conserved in the *L. longbeachae* Mip protein and although this function was not determined experimentally this suggests a similar function and mechanism of action. Primer extension experiments also determined that the putative -10 and -35 transcriptional promoter sequences of *L. longbeachae* sg 1 *mip* differed slightly from that determined for *L. pneumophila.* It is possible that the proteins may be regulated differently as a large area of compression upstream of the *L. longbeachae* sg 1 *mip* gene indicates secondary structures may be present.

Isogenic *mip* mutants were constructed by site-specific mutagenesis using the suicide vector pCACTUS-*mob.* The pCACTUS-*mob* vector and allelic exchange method has several advantages in comparison with other allelic exchange protocols used to construct mutants in *Legionella.* Firstly, in theory, only one transformant is required for the generation of a mutant therefore it is suitable for use in strains that have naturally low frequencies of transformation. Secondly, conjugation or electroporation can be used to deliver the plasmid construct into strains of *Legionella* without the need of helper plasmids. Thirdly, direct selection of mutants is not required and relatively small numbers of resolved colonies need to be screened. The addition of an *aphA*-3 kanamycin resistance cassette into the polylinker of pCACTUS markedly improved the times for transformants to appear. This is probably due to the relatively better expression of kanamycin resistance in comparison with chloramphenicol resistance which is also encoded on pCACTUS (Mintz and Shuman, 1988).

Isogenic *mip* mutants in *L. longbeachae* sg 1 A5H5 and the type strain ATCC 33462 were tested in biological models and the results indicated that there were distinct differences between the *L. longbeachae* strains in comparison to *L. pneumophila.* The loss of Mip in the ATCC 33462 strain rendered it unable to infect and multiply in *Acanthamoeba* while the A5H5 *mip* mutant was attenuated for intracellular growth in a manner similar to that observed

for the *mip* mutants of *L. pneumophila* and *L. micdadei*. The A5H5 *mip* mutant was avirulent in an animal model of infection in contrast to the *L. pneumophila mip* mutant. The animal model used in this study was different to that used to test the *L. pneumophila mip* mutant and hence the two systems cannot be compared directly. However, it is possible that the loss of Mip may have a more significant effect in this species of *Legionella* due to overall differences observed between these two species in this study. A *mip* mutant was constructed in *L. micdadei* (O'Connell, *et al.*, 1995) the second most common *Legionella* species associated with disease in the United States (Reingold, *et al.*, 1984). However, it was not tested in an animal model. Since electron microscopy studies suggest that these two species may share a similar intracellular life cycle, distinct from *L. pneumophila,* it would be of value to test the *L. micdadei mip* mutant in an animal model of infection. The Mip protein may have a more significant role in pathogenesis of *Legionella* species that do not share all of the proposed virulence factors determined for *L. pneumophila* or the same intracellular lifecycle. Currently, only the *L. longbeachae* sg 1 *mip* mutant has been compared to the *L. pneumophila mip* mutant in an animal model of infection. Interestingly, *C. burnetii,* an intracellular pathogen that survives within a phagolysosome, also has a 25 kDa Mip protein (Mo, *et al.*, 1995). A *mip* mutant has not been constructed in this organism and therefore it is not known what effect it would have in an animal model of infection. Mip may therefore be important for cellular functions unique or additional for intracellular survival to those required for *L. pneumophila.*

The role of plasmids was investigated in *L. longbeachae* sg 1. A high degree of similarity was observed when virulent and avirulent isolate protein profiles were examined (discussed in chapter 8). It was thought that functions encoded on extra-chromosomal elements might account for the differences in virulence observed in the animal model. Interestingly, large plasmids were detected in nearly all strains of *L. longbeachae* sg 1 examined (91 %), with the exception of two avirulent isolates, suggesting that plasmids may be important in *L. longbeachae* sg 1.

A large plasmid from a highly virulent strain (A5H5), designated pA5H5, was chosen for analysis. This plasmid appeared to be large, approx. 120 kb, suggesting that it could potentially code for many proteins, some of which may play an important role in survival and or virulence of the strain. The plasmid appeared to be highly stable and all attempts to cure A5H5 were unsuccessful. Additionally, the plasmid was present in the cell after multiple passage of the strain suggesting that retention of the plasmid is favourable.

Partial sequencing of cloned portions of this large plasmid determined that it encodes a number of interesting functions. Identity was observed with the inferred proteins of several *tra* genes indicating that it is likely to be conjugative. Strong inferred identity was also observed with UV mutagenesis and SOS repair proteins, known to exist on other bacterial plasmids. Interestingly, a plasmid has been identified in *L. pneumophila* that confers resistance to UV light (Tully, 1991). The most significant finding, however, was the discovery of a sequence with identity to the OmpR family of transcriptional regulatory proteins. Cloning and sequencing of the region around this gene determined that a potential two-component regulatory system was encoded on pA5H5. The existence of this type of system on a plasmid has been reported previously (Arthur, *et al.*, 1992, Mills, *et al.*, 1993). The putative gene for the transcriptional regulatory gene was designated *lrpR* (*Legionella longbeachae* regulatory protein) and the putative gene for the potential sensor kinase *lskS* (*Legionella longbeachae* sensor kinase). The inferred protein sequences of each of these proteins has strong identity with other proteins in their respective families and conserved features determined for each (Mizuno and Mizushima, 1990, Mizuno and Tanaka, 1997, Stock, *et al.*, 1989, Stock, *et al.*, 1990).

Construction of an isogenic *lrpR* mutant determined that this gene appears to be required for full virulence of *L. longbeachae* sg 1 strain A5H5 in an animal model of infection. The mutant strain was also attenuated for replication in *Acanthamoeba* and invasion of U937 cells. What effect this potential two component system is having in *L. longbeachae* sg 1 strain A5H5 is unknown. Bacteria possess systems for sensing external

environments and responding by coordinately controlling the expression of genes whose products are employed to assist survival of the cell under different conditions. Two component regulatory systems are important in this sensory response (Albright, *et al.*, 1989, Parkinson and Kofoid, 1992). It can be speculated that *L. longbeachae* sg 1 uses the *lprR-lskS* locus to sense its environment and then react with appropriate gene expression. However, the factors that may be regulated by this locus remain to be determined.

Utilisation of a two-component regulatory system to regulate intracellular virulence gene expression is well documented. The *ompR-envZ* system is important for *S. flexnerii* virulence gene expression and the *phoPQ* system of *S. typhimurium* is involved in macrophage survival and mouse virulence (Bernardini, *et al.*, 1990). Two component regulatory systems also control synthesis of factors such as curli expression involved in inert surface colonization and bio-film formation in *E. coli* (Vidal, *et al.*, 1998), and formation of tubular structures known as Sifs (*Salmonella*-induced filaments) in *S. typhimurium* (Mills, *et al.*, 1998). An *ompR-envZ* system is also important for regulation of expression of Vi polysaccharide, a capsular antigen thought to be involved in the virulence of *S. typhi* (Pickard, *et al.*, 1994). There are several reports describing genes encoding bacterial exopolysaccharide antigens that are regulated by two-component sensory systems. For example, in *P. aeruginosa, algR* regulates the transcription of *algD*, necessary for alginate production (Deretic, *et al.*, 1989). The *ompR* gene has been reported to regulate the *S. typhimurium tppB* locus that codes for a tripeptide permease (Gibson, *et al.*, 1987) and the *E. coli* genes coding for microcin B17 (Hernandez-Chico, *et al.*, 1982).

The results for the *lrpR* mutation are not dissimilar to that observed for the *risA* mutation of *Bordetella bronchiseptica* (Jungnitz, *et al.*, 1998). The *risA* gene is part of a second two component regulatory system (*risA-risS*) discovered in this bacterium and is required for bacterial resistance to oxidative stress, production of acid phosphatase and *in vivo* persistence in mice. LrpR may regulate expression of an unknown oxidoreductase that mediates resistance to intracellular killing mechanisms which may be critical in strains of *L.*

longbeachae species that may not avoid phagosome-lysosome fusion. The ability of the *risA* mutant to survive in macrophages was not assessed and hence a direct comparison with our U937 results could not be made. However, the RisA protein was one of two regulatory proteins that had the highest homology scores with LrpR. Similarly, an invasion deficient mutant of *Burkholderia pseudomallei* was mapped to a two component regulatory locus on the chromosome (Jones, *et al.*, 1997). However, in RAW macrophages replication of the mutant was not effected as it was recovered at the end of the incubation period in similar numbers to the parent. The defect in the mutant appears to be specific to invasion of non-professional phagocytes. This mutant has not been tested in an animal model of infection. The intracellular pathogen *Mycobacterium tuberculosis* contains a putative two component regulatory system (*mtrA-mtrB*) similar to the *ompR-envZ* system reported for other bacteria (Via, *et al.*, 1996). Upon macrophage infection the *mtrA* gene is induced although it remains to be determined whether a mutation in this gene will yield a similar phenotype to existing *ompR*-like mutations or the *lrpR* phenotype. It is possible that *lrpR* may control invasion related loci.

Many questions remain unanswered regarding the exact role of the putative *lrpR* and *lskS* two-component system in virulence of *L. longbeacha* sg 1, strain A5H5. Two component systems are generally involved in the coordinate regulation of several gene products and their inactivation usually results in a highly pleiotropic effect (Mizuno and Mizushima, 1990). Two dimension gel electrophoresis of whole protein extracts, comparing wild type strain A5H5 and its *lrpR* derivative would enable the detection of proteins that are down regulated in the mutant strain and proteins that appear to be repressed by *lrpR*. Additionally, primer extension analysis would help to validate putative open reading frames detected on the insert of pIMVSX2, and hence which products are likely to be real.

The discovery of an ORF on the insert of pIMVSX2 with homology to spectinomycin resistance proteins and the demonstration of resistance of *E. coli* strains harbouring this construct to this antibiotic is an interesting discovery. It is not surprising to find an antibiotic

determinant on this plasmid as antibiotics are secondary metabolites produced largely by fungi and soil bacteria, an ecological niche for *L. longbeachae* species. Natural tagging of the plasmid with an antibiotic resistance marker may allow selective transfer of pA5H5 to other strains of *Legionella* and *E. coli.* Previously this could not be achieved. It will be important to introduce pA5H5 into the plasmid-less avirulent *L. longbeachae* sg 1 type strain (ATCC 33462) and to assess this strain in an animal model. The conversion of the type strain to a virulent phenotype in the animal model would be conclusive evidence that factors encoded on pA5H5 play a role in virulence.

The potential played by plasmids in *L. longbeachae* sg 1 may be substantial. Auto-transmissible bacterial plasmids may play a lead role in horizontal gene transfer (Amábile-Cuevas and Chicurel, 1992). Transposons can insert into and excise out of plasmids and the presence of these elements increases the probability of recombinational events involving neighbouring DNA sequences (Amábile-Cuevas and Chicurel, 1992). It will be important to determine if pA5H5 is conjugative. The findings of the study of pA5H5 are interesting in light of the recent discovery of the *dot/icm* genes, capable of conjugative transfer, in *L. pneumophila* (Segal, *et al.*, 1998, Segal and Shuman, 1997, Vogel, *et al.*, 1998, Winans, *et al.*, 1996). These genes are believed to have evolved from a plasmid gene transfer system. Interestingly, the LrpR protein has homology with the VirG regulatory protein from the pTi pasmids of *Agrobacterium tumefaciens* (Powell, *et al.*, 1987). VirG is required for the regulation of at least two *vir* loci. Therefore it is likely that plasmids may have an important role in other species of *Legionella*.

Concluding remarks

The highly clonal soil borne pathogen *L. longbeachae* sg 1 exhibits a wide degree of variability in its ability to cause disease in an animal model of infection. This variability may be due, in part, to the presence of large plasmids carried by this species. This thesis underlines the necessity to further study non-*L. pneumophila Legionella* species as it is

becoming apparant that other species may have different intracellular life styles from that proposed for *L. pneumophila*. This is particularly relevant in light of the recent publication by Joshi and Swanson (1999), highlighting that *L. micdadei* and *L. pneumophila* appear to employ different strategies to replicate in amoebae and macrophages.

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APPENDIX I

Sequence of cloned pA5H5 fragment from pIMVSX2

```
   1  TCTAGATGGT TTGAATGATT TGATGATCGT GTAGCAATGT AGCAGAATAC
  51  CTGGAAATGA CTGATCTTAC TGAAATAATA CTAATGGCAA ATATAGTCTC
 101  CTGCCCTGCT ATCTAGGCTA ACTTTATTAA GGCGCAAGGA TCTTTTAGAT
 151  TTATTGATAA CTTTTGATCG ATAATTCTAC TTAATATAAA ATCAGCACAG
 201  GGTTTTACAA GCACATTAAT ATCATCCCAA TATTCATCTT CCAAACCAAT
 251  GCATATGTGT TTCGCTCTGT TCATCACGGG CTGATACATT TTAGGTAGAC
 301  GATCAATCAC CCAATCGGCG GCTATAGGTT TCGATCGAAT CTCATTAGTT
 351  TCTAATGTAC TCCAAATCCG TGCATAGGTT AGTAATACAT TTCGAGTGTC
 401  CTGTTCAAGT TCCGCTGAAA GTCTATCAAG ATCATGAAGC ATCGCTTTTA
 451  TAAAGTCGCT ATATGGGACC GGATCTAATA GTTGCTTAGG GCTTTCCCCC
 501  ATCAATGTAT GACTCTTCAA TAAGACCTGC GTGACAATCA GCGCAAGATC
 551  CGGCATTGCA CGATCAGACC ATGGTTCAAA ATTTCCCATT TCAAATGATG
 601  AGCGAAGCCA TTCGCCATAT TGAAAATCAA AAAGGGGTGG ATAAATCCAA
 651  GGATTAATTG CGACCCTTTC AACAAGGGTC ATTTCTATAG GTGGCTTCGT
 701  ACTTTTCATG TAAACGCCTG ATATTTGGAG TAGATGGTTA GTCAACTCCA
 751  TCTTTTCTTT TGACGTTGTG GTACGATTTG TTACAACAAA AAAATCAATG
 801  TCACTGTATT TTTGCAAACC ACCTACAAGA TAAGAACCAT AAAGATAAAC
 851  TCCCAAAAGA TCTGCGCCTA AAATCTTTGT TAGTACCTCA AGACATTCAT
                                      <- ORF 1
 901  TGAGTTGTTG CTTTACATCA CTGTTGTTTT CCATCATTAA CACTCAGCCA
 951  TTTTTTAAAA TCCTTTAACA ATGCTTCTGC TTGAGCAATA CAAGTTATTA
1001  CTGCACTCTC AGGAATAATA TCGCCAGAAT AATCTGCAAT ATTGCGTTGT
1051  TTACGCATAG CATCCAATAT AATCACTGTT TTTTTATCAA TACCTAAAGT
1101  CTGAGGCAAT AACTGAATCA TCGTTTGATG ATGTCCAGGT TTAGAAGTCA
1151  AAGTTCGATA TCCATGAGAT TGAAGTGCTG CATTTGCTAT TTGCATAATT
1201  GCTTTATAGG CAGCATCAAA GCGATTCTCC GCACTAACCA AGTCAATTTT
1251  TGAATCCACA ATATTACGTT CAGCAGCATG CAAAAGCCGA TTTATGGTAT
1301  CTGGGTGAGA TGGTATTTTT TCCAGGCTGA TGCCAATTAA ATTATCCAAG
      <- ORF 2
1351  GTCATGTTCA GCTCCCATAA TAAATAGCTT GGGATTATTT AAAACCTCCT
1401  GAATGAAAAG ATCTTGTTTT TGTAAAGATG ACTTCCATTG CTCCCTGGAA
1451  TATATTTTGG GGTTAATTTC TCTCCCTAAA CTCGCTTGGG CAGCATAAAG
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1501 AGCTGCAACT GCTTCTGTAA AATCAACCTC ACCAATAATA AGCACATCAA

1551 TATCACTTCC AAGGTTTTCG GTTCCTTTGG CCACCGAACC AAAAACAAAA

1601 GCTACTTCAA TTTTTTCAGC TAATGAAGTT AAAAAATCAA ACAACACATC

1651 CACCAAACCA GACGTTTTCT TTAAAATGCT TGAAAGCTCC GTATAAATAG

1701 GAAAATTTCG ATTTGCTTGG TAATAAACTT GATTACCGCT CTGCTCGCGC

1751 AGAAGTACTT TGGATTTAGC AAGTTTGGAT AGTTCTCGAT GAAGTGTACC

1801 CGCTGTGGTA TTCGTTAACC GCGCAATCTC TCGGACATGA TAACGCTCAT

1851 CAGGATGCAG CAATAAAAGT GCCAGCACAC GCCGCCGATA TTCAGAAAAC

ORF 3

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1951 ATCATAGCCT AAAAAGCTAC ACCATTCAAT CAGATAAATA AGAAAACCAT

2001 AAAAAGAAAT ATCCGTTATA AATCAACTAT CCACTGGATC ACTTCAACAC

2051 ACAACCAATG ACCTCAATGA TCTTGTAGAT ATCGAAACTT AAGGGCTCAG

2101 TCTTGAGAAA GCATGAAATG GTATTTCCCA ATTACGTAAT TGTCTTATTG

2151 GAAATAAAAT GGGGCCTTGA ATTAAAGTTC TTTTCAAAAA TCAATAAACC

2201 CCTACTTCTA TTACCATGGC TTCATATTAA AAGGAGATGG GTTAAAAATT

2251 TCTTGTTCCC CATCCTCAGA TTTATTATGT ATCTCCTCAT TAGCTTCTTG

2301 TTTGGAGAGT TGATTTTTAT TAGACGGTCT TGGTTCTCTT GAAAGTGGAC

2351 TTGCAGTAAG GCCTTGCTCT TTCGTTACCT CATCTCTTTC TTGTGCTTTC

2401 GGACTGGGTA CGGGCACTAA ATTATTTGCT AGCTGTTGAA TAAATGCATC

2451 GTATAAAGCT AATGTGGGCA TGGTAATGCG AAGAGAACAT ATGCTTCCCT

2501 CCTCATCTTG AAGGATTTGT ATGCAATCAT CAGAAAGATG ATTTTCTTCT

2551 TTAAATTCAT TGAATTCTTT TATAATCGCT TCCATGAATT TTTTTAATTC

2601 TTCTCTTTGT TCTTCAGTTA ATTTACTAGG TTCACATTGT AATTTAATTG

2651 AAAGAGTCAT TGATACGCGA TCACTATCAA CCAGCAATAA TCCTTTGGCG

2701 ATCAATTCCG CAATTATTTC TGGGTCAAAA CCAGACTCTA AATCAAGTTC

2751 TTCTGAACCA AACACGCCTA CCAATTCATC TTCAGACCCA ACTACTTTGC

2801 CAGGTTTAGT TAGGAATTTA ACTTCACCAG GACTCGCTTT TTCCTCAGAT

2851 GTCTTCTTAT CAGAGGTGGC ACTATCACCT CCGCTACCAC CTGCGCCATG

2901 TCCGGAACAA AGGCGCTTAG GTGCGATTTC TTTAAAACAA GTCCCGCAGC

2951 GTACTTTTGC ACCTTCCTCA GCAGCCTCTT TTTTTCTGC TTCCTTTCTA

3001 AATTGTTGTA AAGCTAATGC TATAGAGGAG GTTTTGGGTT CATCTGCCGT

3051 TATGGATTCA AGGACAGGCT GATGTTGTTC TTTGTCTTTT TTCATGAGAA

ORF 4

3101 TACTCCAGTG CAAGTTCATA ATGGTTTCAT CCGAATAAAG ATTTACTTAT

3151 CTGTTATTAT ACCGAGTTAA AAAAACCCAA TTGTATATAT AATGTACATT

3201 AATTGGGTTG CTCGTTTCTT CGACTTAAAA CATCGAGTAC ATAATCCCAA

3251 ATTTTACCTG CTACATTAAT TCCTTTCCCA TCAGATGGAT TGAGATGTAA

3301 TTCAATGTAA GGGAAGCTAT TATTTTCAAT AATTCCAAAT TGTTGCTTAT

3351 GCCATGGAAG AGAAATGTCC TGACAAATAA AATCAAGACC TGTAAGCGGT

3401 ACATTGAGTA TTTTATGTAC TTTTTCCAAT AACAGTTTAT TCTCAGGATG

3451 TATCAGGTCG ATAACATTAC TGATTTTTGC ACCACTACTC ATAGTAACTT

3501 TTTTGGCAAT AAAGATTTGT TGTCCTTTGT TTATTACCGT ATTAAGAGCA

3551 ACTCCTTGGG CTGCCAAAAT TTTCATTAAG TTGCTATTTC GAGTAATTTT

3601 GTAGGGATAT TCACCATGTC TTTCTTTCCA CGTAACTTTA TTTTTTTTAT

3651 CAATTAACTC TTGAAGTGTA GTTACACCAT CGCCAACAAT GCCCTCAGGT

3701 TGTCGTCTCA CACAAGCGAT TAATGAATCA TTAATAATTA ATGACCGATA

3751 AACATCACCA GGAATGTGTT TTTCAACCAG CACGCGAAAA TCAACCTGCT

3801 TTACTATTTT TATCGCTTCT TTTAATTCAT TTGGAGTTTG AATAGTACAT

3851 GAAGTATGAA TACTTAGAGA TTCAGATAGT GGCTTAACCA CAAGAGGAAA

3901 TCCCAATGAG ATACCATAGT TGTAGGCGTT TTTGCTGGAA ATAAAAACCC

3951 TCCCTAAGGG GCAGGGAATT TGGCTATCTA ATAATTTTTT CTTGAGGCTC

4001 CATTTATTAT CATATTTAGT ATCCTCATCA AAGCGTGTAT TTTTTTGAGA

4051 AATAAGAGAG ATTGGAGTCT GTATGAAATA ATATTTTTTC CTATTGTAGA

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4151 TCTAGCCCTC TTTTTTGTGC TTCATCCCAA AATGCAAGGA CACTGTTATT

4201 GAATTGTTTC CTATCAATGG TCTGAGTCTT GGTATATAGT TTTATTTTTT

4251 GGAAAATATT AATGACCAAC TGATTTAATA GGTTATTGAA TCGTAAAAAA

4301 TCCTGCTCTG ATAGGAGAAA GAGTTTAGAT AAAAGATAGA TAAATGGATT

4351 GAATATAAAA TAATGGAATA AATTAAAAAA TTTTCTTCGA AAATGAGAAC

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ORF 6

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5101 ATCAAAATCA ATTTCACTTT GGCAATTTTT CTATCAATTT TAGTACTAAG

5151 AGTGTTCAGC TCTTTGATGA AGAAATTTCA ATAAGTACCA GTGATTTTGA

5201 AATGCTTGCT TTGTTGGTTA AGAACCATGA TAGGTTGCTG AGTCGAGATA

5251 GCATTATGTA TGCTCTTTCT GGGCATGAAT ATGATGGGGT TGATAGAGGT

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5351 AAAGCCCTAT CGTATAAAAA CAATTCATAA AAAAGGATAT ATATTTGTAT

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5451 TTATCAAAAA TAGTAAAAAA TTCTTTTGCC TCGACCTTGG GATTTTTCGT

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5551 CTCGACAAAA GCATCATTTT AGAACGCTTG TACAATATAT ATACATATTA

5601 GCCGAGTGTT TTTGATAAAA TCAACCTATT ATTTTAAGTA ATAGTCGAGA

ORF 7 ➡

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5701 AGATCATCCA CTGCCTGAAA ATCAGAGAGG TGTGGTGATA AATCCTCACT

5751 TAAGACAACG GTTCAAGCAA TTTGTTCCAA TTGGCGTCTA TTTAGAAGGC

5801 TCTGATTTGA AGTATTATTT TTTCGATCAG TGTGATAAAT CCCCAAAAAT

5851 CTATAGTAGT TTGGATGAAC TCAAACGAGA CTTACCGGAA GAACTAGACA

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7051 TTCGCCACCA ACTCCCTTAG AACTCAAACA ATCTGTTAGA CAAATATTTC

7101 AGATGAGTCA ACACAAGGCT GATGAACTAC TTTCATTACC TATTGCGAAA

7151 CGTGAATATA GCAAATGTAG TGAAGGTACT CTTTATATTC CTGGAGATGT

7201 AGTGGAAAAA TTGCATGGAA TCTTGCAATC TGCAAAGGGG AATAATGAAC

7251 TATTATCGGC GTTTGAATCA GAGCTTAGTA TGGTTATCAG TGCTCTTGAA

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8851 TTGCTATAAG ACAAATAACA TTATCTGTTT TTTTCTTTAT TATTGCATCC

8901 CAGTTTGATT TCGGATTATT AATTAACTCT TTTTCTAAAC TAATTAAAAG

8951 TCCTTGTGAC GTCGTTTTTC CTGCATTAAC AACTATCTTT GTCTCAACTG

9001 ACTTTAAATA TTTAAAAAAT GCCAAAACAA GAATTAAAAA AATCACGAAG

ORF 8

9051 AACGCAGTCA AAATTTTAAT ATAAATATTA AATTTCATTA CTATGCACTC

9101 TAAATAAGAA TAATAGATGC CTTTTCACAA GAAAGTACTC TGGATTATCT

9151 TTGATAATTT CAATATCTAA TTCTGGCATA ATTATCCTAC GTTTTGTCAC

9201 TCTTTATAAC TCTTGTAATT AGCCTATTTA AGGCATTCAA ATTATCGATA

9251 TTCGTTATGG CCTCATCAAA TAATGATAAA GTCTCTGGAT CGTGTGTTTC

9301 TAAATACTTG AATGCTTTTT TTGGACCTTG ATAACGCAAT TCTTTGAATA

9351 CAAAATAATC CTCTAGA

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Sequence Analysis of the *mip* Gene of the Soilborne Pathogen *Legionella longbeachae*

ROBYN M. DOYLE,[1]* TREVOR W. STEELE,[1] ALAN M. McLENNAN,[1] IAN H. PARKINSON,[2] PAUL A. MANNING,[3] AND MICHAEL W. HEUZENROEDER[1]

Infectious Diseases Laboratories,[1] Division of Tissue Pathology,[2] Institute of Medical and Veterinary Science, Adelaide, South Australia 5000, and Microbial Pathogenesis Unit, The University of Adelaide, Adelaide, South Australia 5005,[3] Australia

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To understand the basis of pathogenesis by *Legionella longbeachae* serogroup 1, the importance of the Mip protein in this species was examined. Amino-terminal analysis of the purified, cloned *L. longbeachae* serogroup 1 ATCC 33462 Mip protein confirmed that the cloned gene protein was expressed and processed in an *Escherichia coli* background. DNA sequence analysis of plasmid pIMVS27, containing the entire *L. longbeachae* serogroup 1 *mip* gene, revealed a high degree of homology to the *mip* gene of *Legionella pneumophila* serogroup 1, 76% homology at the DNA level and 87% identity at the amino acid level. Primer extension analysis determined that the start site of transcription was the same for both species, with some differences observed for the −10 and −35 promoter regions. Primers designed from the *mip* gene sequence obtained for *L. longbeachae* serogroup 1 ATCC 33462 were used to amplify the *mip* genes from *L. longbeachae* serogroup 2 ATCC 33484 and an Australian clinical isolate of *L. longbeachae* serogroup 1 A5H5. The *mip* gene from A5H5 was 100% identical to the type strain sequence. The serogroup 2 strain of *L. longbeachae* differed by 2 base pairs in third-codon positions. Allelic exchange mutagenesis was used to generate an isogenic *mip* mutant in ATCC 33462 and strain A5H5. The ATCC *mip* mutant was unable to infect a strain of *Acanthamoebae* sp. both in liquid and in a potting mix coculture system, while the A5H5 *mip* mutant behaved in a manner siilar to that of *L. pneumophila* serogroup 1, i.e., it displayed a reduced capacity to infect and multiply within *Acanthamoebae*. To determine if this mutation resulted in reduced virulence in the guinea pig animal model, the A5H5 *mip* mutant and its parent strain were assessed for their abilities to establish an infection after aerosol exposure. Unlike the virulent parent strain, the mutant strain did not kill any animals under two different dose regimes. The data indicate that the Mip protein plays an important role in the intracellular life cycle of *L. longbeachae* serogroup 1 species and is required for full virulence.

Legionella longbeachae serogroup 1 was first recognized as a cause of pneumonia in 1981 (30). In May 1987, *L. longbeachae* serogroup 1 was isolated for the first time from a patient in Australia (25). Since then, numerous cases of infection caused by this species have been reported (8, 24), and presently approximately 50% of all pneumonia cases in South Australia are attributable to this species (8, 39a, 45), a statistic which reflects the national trend. Subsequent studies showed that *L. longbeachae* serogroup 1 was present in commercial potting mix and in the soil of potted plants of patients and that it survived for long periods in these environments, indicating that soil, rather than water, may be the natural habitat of this species and a possible source of infection in the community (40). Restriction fragment-length polymorphism and allozyme studies performed to compare *L. longbeachae* serogroup 1 isolates from clinical and environmental origins demonstrated that they were all closely related and similar to isolates from *L. longbeachae* serogroup 1 ATCC 33462, indicating a close relationship between organisms isolated from countries as far apart as Australia and the United States (24).

No virulence studies of *L. longbeachae* serogroup 1 have been done, although *L. longbeachae* serogroup 2 has been examined by intraperitoneal injection into guinea pigs and for the ability to infect and multiply in a protozoan model of infection with *Tetrahymena pyriformis* and *Hartmannella verformis* (17, 44). Recent publications detailing in vitro models for intracellular growth of *L. longbeachae* serogroup 1 have shown that it can replicate in U937 cells (35) but is unable to replicate in Mac 6 cells or in *Acanthamoebae castellanii* (33). Little is known about the intracellular life cycle of this species, the factors which may contribute to pathogenesis, and whether these factors are shared with *Legionella pneumophila* serogroup 1.

L. longbeachae serogroup 1 pathogenesis studies have focused on the Mip protein and have examined the significance of this protein in pathogenesis by the organism. The Mip protein of *L. pneumophila* serogroup 1 has been established as a virulence factor of the organism, playing an important role in the intracellular life cycle, as mutant strains which lack the protein are significantly impaired in their ability to infect alveolar macrophages and protozoa (9, 12). They are also attenuated in their ability to cause disease in experimentally infected guinea pigs (11). The *L. pneumophila* serogroup 1 Mip protein displays homology to the FK506 binding protein (FKBP) class of immunophilins and shows characteristic peptidyl prolyl *cis-trans* isomerase (PPIase) activity (18). A homolog of the Mip protein also occurs in *Legionella micdadei* (2), a species of *Legionella* associated with disease in humans, and a *mip* mutant in this species also shows reduced intracellular infection (34). Mip analogs have been detected in all species of *Legionella* examined so far, including *L. longbeachae* serogroup 1 (10, 37, 38). Mip-like analogs which also display homology to the FKBP class of proteins have been reported in

* Corresponding author. Mailing address: P.O. Box 14, Rundle Mall, Adelaide, South Australia 5000, Australia. Phone: 618 8222 3274. Fax: 618 8222 3543. E-mail: Robyn.Doyle@imvs.sa.gov.au.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
L. pneumophila serogroup 1 (Philadelphia)	ATCC 33152; type strain	CDC[a]
L. longbeachae serogroup 1	ATCC 33462; type strain	CDC
L. longbeachae serogroup 2	ATCC 33484; type strain	CDC
A5H5 (*L. longbeachae* serogroup 1)	Australian clinical isolate	This study
B10	ATCC 33462 *L. longbeachae* serogroup 1 strain with a *mip* deletion mutation	This study
B8	*L. longbeachae* serogroup 1 A5H5 with a *mip* deletion mutation	This study
B8.22	Strain B8 complemented with plasmid SKW27	This study
E. coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_k^- m_k^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*	20
E. coli S17-1	*recA* derivative of *E. coli* 294 (*hsdR* Pro) with RP4-2Tc::Mu (Ap Km Nm Tc::Mu) Km::Tn*7* in the chromosome	39
Plasmids		
pGEM-7Zf(−)	Ap^r cloning vector	Promega
pUC18K	pUC18 with the *aphA-3* Km^r resistance cassette	P. Sansonetti, reference 31
pCACTUS	Cm^r cloning vector containing *sacB* and a temperature-sensitive replicon	C. A. Clark
pIMVS26	pGEM with ca. 8-kb fragment of *L. longbeachae* serogroup 1 ATCC 33462 genomic DNA	This study
pIMVS27	pGEM carrying a *Sac*I fragment of pIMVS26	This study
pIMVS28	pGEM containing deleted *mip* gene generated in pIMVS27	This study
pCACTUS49	pCACTUS containing deleted *mip* gene fragment from pIMVS28	This study
pCACTUS50	Derivative of pCACTUS49 containing Km^r from pUC18K and *mob* deletion	This study
pWKS130	Km^r cloning, sequencing vector	46
pIMVS29	pWKS130 containing entire *mip* gene on *Sac*I fragment from pIMVS27	This study

[a] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.

other intracellular pathogens such as *Chlamydia trachomatis* (27) and *Coxiella burnetti* (32), with PPIase activity having been demonstrated for both organisms (28, 32). Hence, Mip-like proteins with homology to the FKBP class of immunophilins may play a critical role in the life cycles of these organisms (19).

In this report, we document the cloning and sequence analysis of the *mip* gene from *L. longbeachae* serogroup 1 ATCC 33462 and compare the results with those from *L. pneumophila* serogroup 1 (16), *L. longbeachae* serogroup 2 ATCC 33484, *L. micdadei* (2), and an Australian clinical isolate of *L. longbeachae* serogroup 1, strain A5H5. To understand the significance of Mip in *L. longbeachae* serogroup 1, we constructed and characterized isogenic *mip* mutants in *L. longbeachae* serogroup 1 ATCC 33462 and the Australian clinical isolate of this species, strain A5H5. The mutants, which represent the first reported genetic manipulation of this species, were tested for their abilities to infect a strain of *Acanthamoebae* and to establish infection in guinea pigs. There were apparent differences between the two isolates of *L. longbeachae* serogroup 1 in both of these models.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial isolates of *Legionella*, *E. coli* strains, and plasmids used or constructed in this study are listed in Table 1. *Legionella* strains were routinely cultured on charcoal yeast extract α-ketoglutarate (CYE) plates (24) at 35°C. Legionella broth was used as a liquid growth medium (41). When required, selective agents were used at the following concentrations: chloramphenicol (CM), 5 μg/ml; kanamycin (KM), 25 μg/ml; and aztreonam, 4 μg/ml. For the amoeba coculture experiments, *Legionella* organisms were plated onto CYE plates containing pimafucin (250 mg/liter), polymixin B (80,000 IU/liter), and vancomycin (2 mg/liter) (CYE-VPP). *E. coli* strains were grown in Luria broth or on Columbia agar, and where appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; CM, 25 μg/ml, and KM, 25 μg/ml.

Antisera and antibodies. *L. pneumophila* serogroup 1 polyclonal monospecific anti-Mip antisera, used initially to screen the *L. longbeachae* serogroup 1 plasmid bank, were a kind gift from N. P. Cianciotto (Department of Microbiology and Immunology, Northwestern University, Chicago, Ill.). Polyclonal antiserum was prepared specifically against *L. longbeachae* serogroup 1 Mip, excised from a 15% polyacrylamide gel, and emulsified in phosphate-buffered saline (PBS), pH 7.2. The acrylamide mix was injected subcutaneously into two New Zealand White rabbits. The injection was repeated after 2 weeks, and the serum was harvested at 6 weeks. The antiserum was extensively absorbed with *E. coli* DH5α(pGEM-7Zf[−]) prior to use.

Western immunoblot. Total cell protein extractions were prepared by the method of Pearlman et al. (36). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Lugtenberg et al. (26) with a 15% running gel. Western immunoblot was performed per the procedure of Towbin et al. (43), using the staining procedure of Hawkes et al. (21), with 4-chloro-1-napthol.

Construction and screening of the plasmid bank. Whole chromosomal DNA was extracted from *L. longbeachae* serogroup 1 ATCC 33462, by the method of Manning et al. (29), and digested with *Bam*HI-*Eco*RI. The fragments were cloned into pGEM-7Zf(−) and transformed into DH5α. A clone carrying an 8-kb fragment was identified by colony immunoblot with *L. pneumophila* serogroup 1 anti-Mip serum. This clone, designated DH5α(pIMVS26), expressed a protein of approximately 27 kDa, as demonstrated by Western immunoblot. A subclone expressing the protein was generated by *Sac*I digestion of pIMVS26 and recloning into pGEM-7Zf(−). A clone containing a 1.3-kb *Sac*I fragment was identified, and the plasmid was designated pIMVS27.

DNA sequencing. Sequencing was performed with the Applied Biosystems model 373A DNA sequencer. Plasmid pIMVS27 was sequenced in the forward direction with Dye Primer kits (ABI, Foster City, Calif.). The protocol was applied to templates generated by nested deletion of pIMVS27 with the Erase-a-Base kit (Promega, Madison, Wis.), according to the manufacturer's instructions. The complementary strand of the clone was determined by using the Dye Terminator kit (ABI), with primers designed from the forward-sequence data, with double-stranded pIMVS27 as the template. The entire *mip* gene sequence was analyzed by DNASIS and PROSIS (Hitachi Software). Two primers, 844 (5′-GAGTATGATGAGAAAGAA-3′) and 845 (5′-ACAATTAATCTGATTT AAGG-3′), were designed from the completed sequence to amplify the entire

mip gene from ATCC 33484 and strain A5H5. The expected 850-bp PCR product was purified with the QIAquick PCR purification kit (Qiagen) and sequenced with the Dye Terminator kit (ABI).

Primer extension from total bacterial RNA. Primer extension analysis was used to map the 5′ end of the *mip* mRNA with a synthetic oligonucleotide primer (5′-GGCTGCAACTGATGCTACATCGCTT-3′). Total bacterial RNA was extracted from *L. longbeachae* serogroup 1 ATCC 33462, DH5α(pIMVS27), and DH5α(pGEM-7Zf[−]) by the hot-phenol method of Aiba et al. (1) and treated with RNase-free DNase I (Boehringer Mannheim). The oligonucleotide primer was radioactively labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Boehringer Mannheim). The primer was hybridized to 20 μg of total RNA, and the mix was extended per the method of Williams et al. (47), with Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim). The reaction was loaded onto a 6% acrylamide-urea sequencing gel and visualized by autoradiography. Plasmid pIMVS27 was sequenced with the DNA sequencing kit version 2 (Amersham, Buckinghamshire, United Kingdom).

Allelic exchange mutagenesis, construction, and complementation of *mip* mutants. Allelic exchange was carried out to generate mutations in the *mip* gene with the suicide vector pCACTUS. Vector pCACTUS is a derivative of plasmids containing the *sacB* gene of *Bacillus subtilis* (pIB279) and pIB307, containing a temperature-sensitive pSC101 replicon (6). pCACTUS also contained a *mob* region and a chloramphenicol resistance gene. Plasmid pCACTUS49 was introduced into *L. longbeachae* serogroup 1 ATCC 33462 by conjugation with the modified method from Bradley et al. (7) from a 48-h plate subculture of *Legionella* growth. The mating was incubated for 6 h at 30°C on CYE plates, serially diluted in PBS, and plated onto CYE plates containing 5 μg of CM per ml and 4 μg of aztreonam per ml. The natural resistance of *L. longbeachae* to aztreonam was used to select against the donor. All plates were incubated at 30°C. Electroporation was used to introduce pCACTUS50 into *L. longbeachae* serogroup 1 A5H5. Electrocompetent A5H5 cells were prepared according to the method of Dower et al. (13), except that PBS was used in the initial washes. Glycerol-treated A5H5 cells and plasmid DNA (approximately 1 μg) were subjected to an electric pulse of 2.3 kV in a 0.2-cm cuvette (Bio-Rad) with a Bio-Rad gene pulser at 100 Ω. The cells were incubated in broth at 30°C for 5 to 6 h and plated onto CYE plates containing KM.

The resulting Km^r or Cm^r *L. longbeachae* colony was cultured in broth at 30°C with the appropriate antibiotic. Subsequent culture on CYE plates containing CM or KM at the nonpermissive temperature for pCACTUS replication in *L. longbeachae* (39°C) resulted in the cointegration of the plasmid into the chromosome via homologous recombination. One resultant antibiotic-resistant colony was incubated in broth with antibiotic selection at 30°C and plated onto CYE containing 6% sucrose to select for resolved cointegrates. Colonies from the sucrose plates were patched onto CYE plates and screened by PCR to assess allelic exchange, and potential mutants were confirmed by Southern blot hybridization and immunoblot. Mutant strains were complemented with plasmid pIMVS29, which was introduced by electroporation.

Southern blot hybridization. DNA was transferred to nylon membranes (Hybond-N+; Amersham) by the method of Southern (42) and hybridized with digoxigenin (DIG)-labeled probe at 42°C overnight. Probes were labeled with DIG and hybridized with the filter under conditions described previously (24). The filters were developed according to the manufacturer's protocol (Boehringer Mannheim).

Infection of *Acanthamoebae* with *Legionella* strains. *Acanthamoebae* group 2 spp. used in coculture experiments were originally isolated from potting mix. Their identities were confirmed by Brett Robinson, South Australian Water Corporation, Bolivar, South Australia, Australia, and one strain, designated ACO97, was chosen for all experimental work.

Liquid cocultures of *Acanthamoebae* ACO97 and *Legionella* species were set up essentially as described by other workers (12). Duplicate cocultures containing approximately 10^3 *Legionella* organisms per ml and 10^4 *Acanthamoebae* cysts per ml were set up in 4 ml of amoeba saline (2 mM NaCl, 0.016 mM $MgSO_4$, 0.027 mM $CaCl_2$, 1 mM Na_2HPO_4, 1 mM KH_2PO_4). *Legionella* organisms were prepared by suspending growth from a 72-h CYE plate in sterile tap water to give approximately 10^9 organisms/ml by comparison with a turbidity standard (McFarland standard number 4); this was confirmed spectrophotometrically by using an optical density of 1.0 at 550 nm. These organisms were serially diluted and plated onto CYE agar to determine numbers of viable bacteria. Cocultures were incubated at 30°C. Samples were taken at days 1, 3, and 7, diluted in 0.2 M HCl-KCl buffer (pH 2.2), and plated onto CYE plates. Potting mix coculture samples were set up essentially as for liquid coculture, except that *Legionella* and *Acanthamoebae* were added to presteamed potting mix (Nu-Erth, Meadows, South Australia, Australia). Twenty grams of steamed soil seeded with *Legionella* and amoebae was incubated at 30°C, and samples were taken at days 3, 7, 11, and 15. At each interval, a 1-g aliquot of soil was removed, diluted in sterile tap water, mixed thoroughly, allowed to settle for 15 min, and then diluted in 0.2 M HCl-KCl acid buffer to reduce the number of unwanted soil microorganisms. Aliquots were plated onto CYE-VPP.

Animal studies. (i) Intraperitoneal inoculation. Outbred guinea pigs (IMVS colored stock; Institute of Medical and Veterinary Science-Veterinary Services, Gilles Plains, South Australia, Australia), weighing between 300 and 600 grams, were inoculated intraperitoneally with a suspension of *Legionella* prepared in sterile tap water, as outlined for coculture experiments, and enumerated initially in a counting chamber (Hausser Scientific Partnership, Horsham, Pa.). The actual dose administered was accurately determined retrospectively by serial dilution and plating on CYE plates.

(ii) Aerosol inoculation. Guinea pigs were infected by exposure to an aerosolized dose of *Legionella* within a closed chamber. The test strain dose was prepared as outlined for the amoeba coculture, except that strain B8.22 was grown on CYE plates containing KM and was enumerated retrospectively. The chamber was constructed of Perspex (Lucite) and measured 220 by 220 by 240 mm, with a removable top. A nebulizer pump therapy kit (Ventalair Forte II; Allersearch, Granville, Australia) was used to generate the aerosol, which had an average particle size of 3.9 microns, as specified by the manufacturer. An inlet was constructed on one side of the chamber, through which the nebulizer hose was inserted; this hose was sealed in place. The hose connected the nebulizer bowl on the inside of the chamber to the nebulizer pump unit on the outside. The nebulizer pump generated positive pressure in the chamber which was vented through a small outlet valve on the opposite side of the box. The chamber was placed in a laminar flow hood during aerosolization as a safety measure. Guinea pigs were placed in the chamber, and a 3-ml test suspension (containing approximately 10^9 or 10^{10} *Legionella* organisms total) was aerosolized into the chamber over a 15-min interval. The guinea pigs were held in the chamber for a further 5 min. One animal in each test group was killed immediately after exposure to enumerate the *Legionella* organisms introduced into the lungs. Lungs were homogenized in 100 ml of sterile tap water by using a Waring commercial blender (Waring Products, New Hartford, Conn.), and viable counts were determined by plating the homogenate, in duplicate, onto CYE and CYE-VPP plates.

Animals were checked three times daily for signs of illness, and their weights were recorded. Lungs were taken from animals that died to confirm experimental pneumonia.

Nucleotide sequence accession numbers. The *mip* gene sequence data obtained for *L. longbeachae* serogroup 1 ATCC 33462 and *L. longbeachae* serogroup 2 ATCC 33484 in this study are available under GenBank and EMBL accession numbers X83036 and AF000958, respectively.

RESULTS

Analysis of *L. longbeachae* serogroup 1 Mip. Amino-terminal analysis of clone DH5α(pIMVS26) was performed to ensure the identity of the Mip protein from *L. longbeachae* serogroup 1 and that it was processed in *E. coli*. N-terminal sequencing of the purified Mip protein from clone DH5α(pIMVS26) was performed at Macquarie University Centre for Analytical Biotechnology (Macquarie University, School of Biological Sciences, New South Wales, Australia) on a 470A Applied Biosystems protein sequencer. The protein was homologous to the first 16 amino acids in the processed form of the Mip outer membrane protein from *L. pneumophila* serogroup 1 (16), except for a threonine residue at position 8 in place of an alanine residue (Ala-Thr-Asp-Ala-Thr-Ser-Leu-**Thr**-Thr-Asp-Lys-Asp-Lys-Leu-Ser-Tyr). Subsequent sequencing of plasmid pIMVS27 showed one potential open reading frame (ORF) of 699 bp. A strong ribosome binding site was also found in close proximity to the putative ATG start site for translation. Downstream of the ORF, a stop codon was seen in conjunction with a region of dyad symmetry, corresponding to a putative transcriptional terminator, similar to that seen for the *L. pneumophila* serogroup 1 *mip* gene (16). This most likely represents a factor-independent transcription termination signal and has also been proposed for the *L. micdadei mip* gene (2).

The inferred translated *mip* gene product was a polypeptide of 233 amino acids with a predicted molecular mass of 24,661 Da. The inferred amino acid sequences of the Mip proteins from *L. longbeachae* serogroup 1 A5H5 and the ATCC 33484 serogroup 2 isolate were identical with that of the ATCC 33462 *L. longbeachae* serogroup 1 Mip protein (Fig. 1) and were very similar to that of the *L. pneumophila* serogroup 1 Mip protein (16), displaying approximately 87% identity, and also to that of the *L. micdadei* Mip protein (2) (Fig. 1). The first 20 amino acids suggested a signal sequence and in conjunction with the N-terminal analysis suggest that Mip is processed in *E. coli* in a manner similar to the way it is processed in *L. pneumophila* serogroup 1. The key sites proposed to be involved in the PPIase activity (19) determined for the *L. pneumophila* sero-

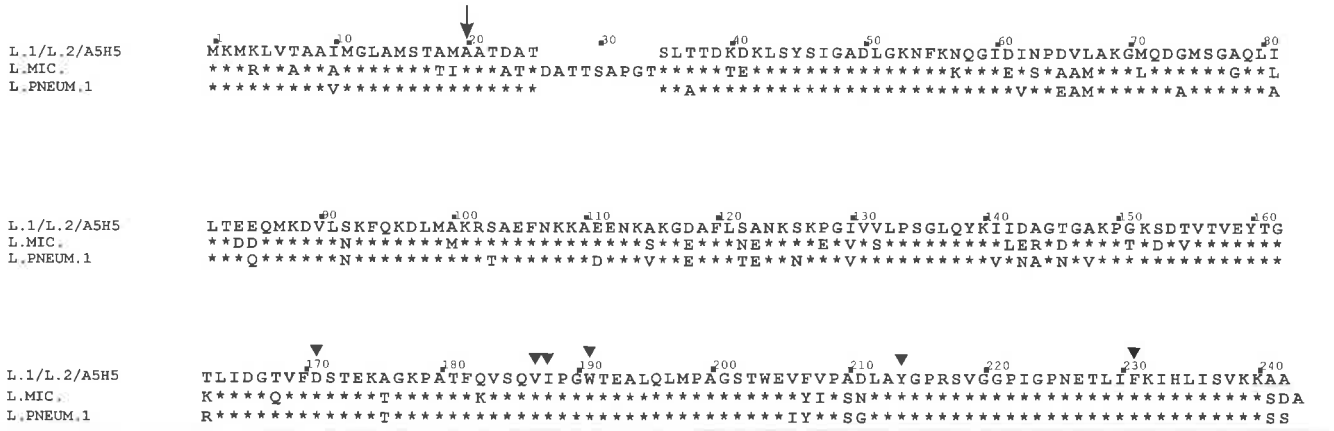

FIG. 1. Amino acid comparison of the Mip proteins of *L. longbeachae* serogroup 1 ATCC 33462 (L.1), *L. longbeachae* serogroup 1 A5H5 (A5H5), *L. longbeachae* serogroup 2 ATCC 33484 (L.2), *L. pneumophila* serogroup 1 (L. PNEUM. 1), and *L. micdadei* (L. MIC.). Asterisks indicate amino acids identical to those of *L. longbeachae*; triangles indicate amino acids predicted to form part of the active site for PPIase activity of Mip. The arrow indicates the site of signal peptidase cleavage.

group 1 (Philadelphia) Mip protein (18) are conserved in *L. longbeachae* serogroup 1 Mip (Fig. 1), suggesting a similar function and role in pathogenesis.

Analysis of *L. longbeachae* serogroup 1 *mip* transcriptional signals. To confirm the start site for transcription of *mip*, and to compare this with the case for *L. pneumophila* serogroup 1, primer extension analysis was performed. Identification of the 5′ ends of the *mip* mRNA isolated from *L. longbeachae* serogroup 1 and the *E. coli* clones was determined by synthesis of cDNA with an oligonucleotide primer that was complementary to a region of DNA 54 bp downstream from the putative ATG start site on the *mip* mRNA. Identical cDNA bands were synthesized from RNA from *L. longbeachae* serogroup 1 ATCC 33462 and DH5α(pIMVS27), with no band detected in the control track where DH5α(pGEM7Zf[−]) was used as a template (data not shown). By comparing these bands with the sequencing reaction of pIMVS27, primed with the same oligonucleotide, the 5′ end of the *mip* mRNA was mapped to the G residue at nucleotide position 473 of the *L. longbeachae* serogroup 1 *mip* gene sequence. This result confirmed that the start sites for transcription in *L. longbeachae* serogroup 1 and *L. pneumophila* serogroup 1 (16) were identical in both species (Fig. 2A). The probable −10 and −35 promoter consensus sequences were identified and compared with those for *L. pneumophila* serogroup 1 (Fig. 2A). The −10 region was the same for *L. longbeachae* serogroup 1 and *L. pneumophila* serogroup 1; however, a −35 region was identified (Fig. 2A) in *L. longbeachae* serogroup 1 that is a more likely part of the promoter sequence than that suggested for *L. pneumophila* serogroup 1 (16). The spacing of the −10 and −35 regions for *L. longbeachae* serogroup 1 is a closer match with the consensus sequences determined for *E. coli*, and the spacing is optimal (17 ± 1 nucleotide).

Construction and complementation of *L. longbeachae* serogroup 1 *mip* mutants. Isogenic *mip* mutants were generated in *L. longbeachae* serogroup 1 ATCC 33462 and strain A5H5 by

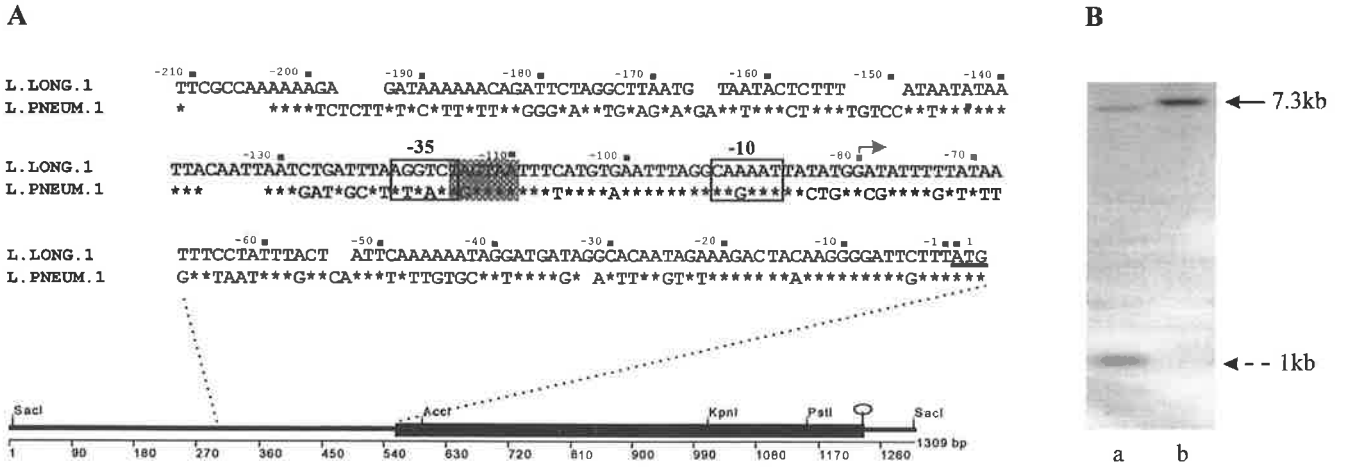

FIG. 2. (A) Line diagram depicts the DNA sequence determined from sequencing pIMVS27. The solid box shows the *mip* gene from *L. longbeachae* serogroup 1 ATCC 33462, selected restriction sites in the *mip* gene, and the stem loop structure at the end of the ORF. The inset sequence is the DNA sequence upstream of the ATG start site for translation in *L. longbeachae* serogroup 1 and *L. pneumophila* serogroup 1, showing the −10 and −35 promoter regions determined by primer extension analysis. The shaded box indicates the −35 promoter region proposed for *L. longbeachae*. The nonshaded box is the −35 region proposed in reference 16. The start site for transcription is shown with a solid arrow. (B) Southern hybridization demonstrating mutagenesis by allelic exchange of the *L. longbeachae* serogroup 1 *mip* gene. DNA was digested with *Kpn*I and probed with DIG-labeled pIMVS27. Lanes: a, *L. longbeachae* serogroup 1 A5H5; b, B8. The solid arrow indicates the 7.3-kb fragment generated in B8 due to the loss of an internal *Kpn*I site which generates 1- and 7-kb fragments in the parent strain. A similar pattern was observed for *L. longbeachae* serogroup 1 ATCC 33462 (data not shown).

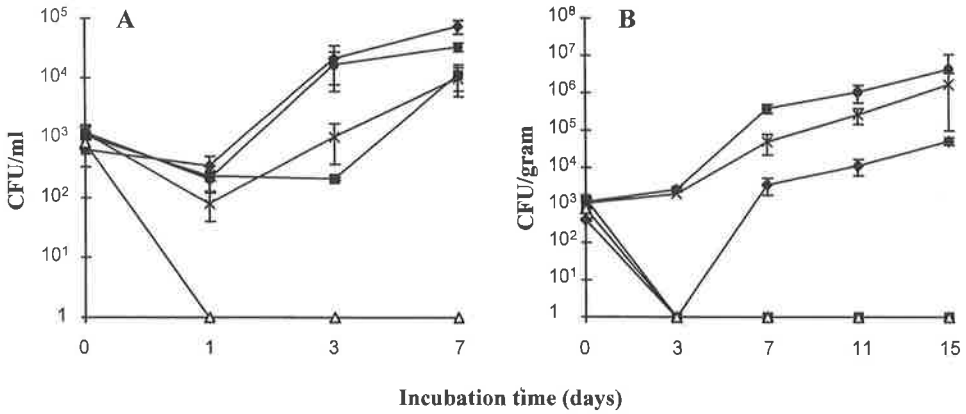

FIG. 3. Coculture of *Acanthomoebae* with strains of *Legionella*. (A) Amoeba liquid cocultures were set up in saline with approximately 10^4 amoebae/ml and 10^3 CFU (each) of *L. pneumophila* serogroup 1 (Philadelphia) (◆), *L. longbeachae* serogroup 1 ATCC 33462 (■), B10 (△), A5H5 (●), and B8 (×) per ml. Samples were taken at various time intervals, and the number of *Legionella* organisms was determined by plating on selective media. Each time point represents the mean number of CFU recovered, and the vertical bars indicate standard deviations. (B) Amoebae were cocultured in an artificial potting mix system with strains of *Legionella* as indicated above. Numbers of viable *Legionella* organisms were determined at various time points by treatment of the soil sample with acid and plating on selective media. The experiments shown are representative of two independent experiments.

allelic exchange with a plasmid construct, pCACTUS49, constructed in several stages. First, the *mip* gene in pIMVS27 was mutated by digestion with *Acc*I and *Pst*I to delete a 650-bp fragment within the coding region (Fig. 2A). The resulting construct, designated pIMVS28, was transformed into DH5α, and transformants were screened by Western blot to confirm the loss of production of Mip (data not shown). The residual 850-bp *Sac*I fragment of pIMVS28 was cloned into pCACTUS to yield pCACTUS49. Plasmid pCACTUS49 was introduced into S17-1, which then served as a donor strain in subsequent conjugation experiments.

Due to difficulties in conjugation with strain A5H5, construct pCACTUS50 was made and delivered by electroporation. Plasmid pCACTUS50 was constructed from pCACTUS49 by removing the *mob* site by restriction digestion and inserting the Km^r marker from pUC18K into the *Sma*I site of the polylinker in order to use KM in addition to CM to select for transformants.

To identify colonies that had undergone complete allelic exchange, PCR analysis was performed with primers 844 and 845. A fragment of approximately 650 bp amplified in the case of the mutant strain was in contrast to an 850-bp fragment for the wild type (data not shown). Chromosomal DNA from the putative *mip* mutants and the parent was then digested with *Kpn*I, a restriction site internal to the *mip* gene sequence (Fig. 2A). Duplicate Southern blots were probed with DIG-labeled pIMVS27 and pCACTUS. The mutant strains had only one hybridizing fragment (Fig. 2B, lane b) lacking the internal *Kpn*I site, which had been removed by restriction deletion, while the parent strain had two hybridizing fragments (Fig. 2B, lane a). No bands were detected with the pCACTUS probe, indicating the vector sequence had been completely resolved from the chromosome (data not shown). In addition, Western immunoblot showed loss of Mip production in the mutant strains (data not shown).

To ensure that the mutation process had not affected genes other than *mip*, complemented mutant strains were constructed with vector pIMVS29. This construct was derived by cloning the *Sac*I fragment from pIMVS27, containing the entire *L. longbeachae* serogroup 1 *mip* gene, into vector pWKS130. Only strain B8 was complemented, as B10 and the parent strain were both avirulent. The complemented *mip* mutant in A5H5 was screened by Western immunoblot to confirm the production of Mip (data not shown).

Effect of *mip* on intracellular infection. To determine whether Mip promotes infection of amoebae in *L. longbeachae* serogroup 1, we assessed the abilities of both *mip* mutants to infect *Acanthamoebae*, a common soil amoeba. Two systems were used to assess the levels of multiplication of *Legionella* strains, with potting mix considered a more natural, nonaquatic environment for *L. longbeachae* serogroup 1. The potting mix was steamed for approximately 1 h to kill any preexisting *Legionella* spp.; however, the steaming process did not sterilize the mix, as spore-forming organisms were not killed. The same multiplicity of infection was used for both systems, and samples were taken frequently during the experiment to determine the level of multiplication of *Legionella* (Fig. 3). The mean number of CFU (± standard deviations) was determined for each time point, and the Student-Newman-Keuls comparison of means ($P < 0.05$) was used to determine statistical significance.

L. pneumophila serogroup 1 (Philadelphia), *L. longbeachae* serogroup 1 ATCC 33462, and strain A5H5 multiplied in liquid coculture similarly to other *Legionella* organisms (12, 34) (Fig. 3A), showing an initial lag period with a steady increase in bacterial numbers during the experiment. The *mip* mutants of the two strains of *L. longbeachae* serogroup 1 showed growth patterns different from that of their parent strain and also from those of each other. Mutant B8 increased in numbers at a lower rate than A5H5, with a statistically significant difference in recovery observed at day 7. Statistically significant differences were not seen at days 1 and 3, most likely due to large variations in the counts and the low sample numbers. However, the expected growth trend was observed, and the end result was similar to that determined for the *L. pneumophila* serogroup 1 *mip* mutant (12). Complemented *mip* mutant B8.22 also grew in amoebae (data not shown) and was recovered at day 7 in numbers that were not statistically different from those of the wild-type strain A5H5. Strain B10 was unable to replicate in this system and in several repeat experiments.

L. longbeachae serogroup 1 A5H5 and B8 were both able to replicate in potting mix, showing similar growth trends, as seen with the liquid coculture (Fig. 3B). Statistically significant differences in strain recovery were observed at days 7 and 11. The numbers of organisms observed at day 15 were not statistically different, most likely due to reasons stated above; however, the expected growth trend was observed. *L. pneumophila* serogroup 1 (Philadelphia) replicated in this system, with an initial

TABLE 2. Intraperitoneal inoculation of *Legionella* strains

Strain	Dose (total CFU)	No. of guinea pigs killed/no. tested	Comments
L. pneumophila serogroup 1 (Philadelphia)	5×10^8	3/3	Spleens contained 10^7 to 10^8 organisms
L. longbeachae serogroup 1 ATCC 33462	2×10^9	0/3	
L. longbeachae serogroup 2 ATCC 33484	5×10^8	0/3	
L. longbeachae serogroup 1 A5H5	1×10^9	1/3	Death at 4 days

lag phase observed at day 3, where numbers dropped to undetectable levels; this may reflect the low number of organisms added initially to the coculture or the inappropriate levels of sampling for that time point. In all experiments, however, *L. pneumophila* serogroup 1 (Philadelphia) multiplied. *L. longbeachae* serogroup 1 ATCC 33462 and mutant B10 were unable to replicate in this system and in several repeat experiments.

Animal model of infection. Two models were established in the laboratory and assessed for their ability to allow a comparison of the virulences of experimental *Legionella* strains. The intraperitoneal model allowed accurate doses to be administered and test strains to be compared (Table 2). *L. pneumophila* serogroup 1 (Philadelphia) was virulent in this model, with death occurring in all animals within 30 h. The *L. longbeachae* serogroup 1 strains, however, rarely caused death by this mode of transmission. The *L. longbeachae* serogroup 1 and serogroup 2 ATCC type strains were completely avirulent in this model, while *L. longbeachae* serogroup 1 A5H5 did produce symptoms and death in 1 of 3 animals after 4 days. The data indicated that this was not a suitable model to assess the mutant strains, given the relative avirulence of *L. longbeachae* in this model.

The aerosol model allowed doses of *Legionella* to be administered by the respiratory route of entry. The symptoms produced and the time course of the disease were similar to those predicted by other workers (11). *L. pneumophila* serogroup 1 and strain A5H5 were both virulent by this model and caused death in 3 of 5 animals tested. Examination of the lungs taken from a guinea pig that died due to exposure to A5H5 revealed that the air spaces of the lung parenchyma were filled with a dense cellular infiltrate consisting of neutrophils and monocyte cells, and the histological appearance was consistent with a severe acute pneumonia similar to that seen with *L. pneumophila* serogroup 1 (3, 4, 15). Animals exposed to aerosols of *L. longbeachae* serogroup 1 ATCC 33462 and *L. longbeachae* serogroup 2 ATCC 33484 developed no symptoms and were avirulent, although the numbers of *Legionella* organisms retained in the lung with each test strain were comparable (Table 3). For this reason, only the A5H5 *mip* mutant (B8) was assessed in the aerosol model, along with the complemented strain B8.22. Guinea pigs were tested with two doses of the mutant strain, and the percentage weight gain or loss was plotted for each animal and compared with that for animals inoculated with the isogenic parent strain as well as with that for animals inoculated with the complemented strain (Fig. 4). The parent strain killed 3 of 5 animals (all symptomatic) within 5 days after exposure (Fig. 4A). Guinea pigs exposed to 10^9 CFU (approximately 10^5 retained organisms) of B8 showed no evidence of the disease (Fig. 4B). Some evidence of disease, predominantly weight loss, was observed in some of the animals exposed to a 10^{10}-CFU dose (approximately 10^6 retained organisms) of the same mutant strain (Fig. 4C). The *mip* mutant in strain A5H5 did not cause death with either dose. Reintroduction of the intact wild-type *mip* gene from *L. longbeachae* serogroup 1 was able to fully complement the mutation in strain B8, leading to restored virulence (Fig. 4D).

DISCUSSION

In this study, the *mip* gene from *L. longbeachae* serogroup 1 was sequenced, and the role played by this protein in facilitating infection of guinea pigs and *Acanthamoebae* was examined. The *mip* gene sequences for *L. longbeachae* ATCC 33462 and A5H5 were identical, while the sequence for *L. longbeachae* ATCC 33484 differed from the former by two bases (positions 517 and 523). The translated protein sequences were identical and highly conserved in comparison to those from *L. pneumophila* serogroup 1 and *L. micdadei*. The start sites of transcription for *L. longbeachae* serogroup 1 and *L. pneumophila* were identical, and this confirms the high degree of conservation of *mip* genes and hence the probability that the proteins have similar functions. PPIase activity was not determined for *L. longbeachae* serogroup 1 Mip, but conserved amino acids critical to this enzymatic activity suggest the protein has a similar mechanism of action.

The role of the Mip protein as a potentiator of intracellular infection in *L. longbeachae* is further suggested by the behavior of the *mip* mutants in the *Acanthamoebae* coculture models. The mutant in strain A5H5 showed a growth pattern similar to those of the *mip* mutants of *L. pneumophila* serogroup 1 and *L. micdadei* (12, 34). The *mip* mutant in *L. longbeachae* serogroup 1 ATCC 33462 was unable to multiply in the amoeba models and warrants further analysis, but these results may simply reflect a greater level of attenuation of the ATCC parent strain. Differences were observed between the two parent

TABLE 3. Aerosol inoculation of *Legionella* strains

Strain	Dose (total CFU)	No. of guinea pigs killed/no. tested	Comments	Retained dose
L. pneumophila serogroup 1 (Philadelphia)	1×10^9	2/3	Death within 5 days	$\sim 2 \times 10^5$
L. longbeachae serogroup 1 ATCC 33462	1×10^9	0/3	No symptoms	2×10^5
L. longbeachae serogroup 2 ATCC 33484	1×10^9	0/3	No symptoms	$\sim 2 \times 10^5$
L. longbeachae serogroup 1 A5H5	1×10^9	3/5	Death within 5 days (days 2 and 4)	3.5×10^5
B8	1×10^9	0/5	No symptoms	1×10^5
B8	1×10^{10}	0/5	Slight symptoms in most animals	1.6×10^6

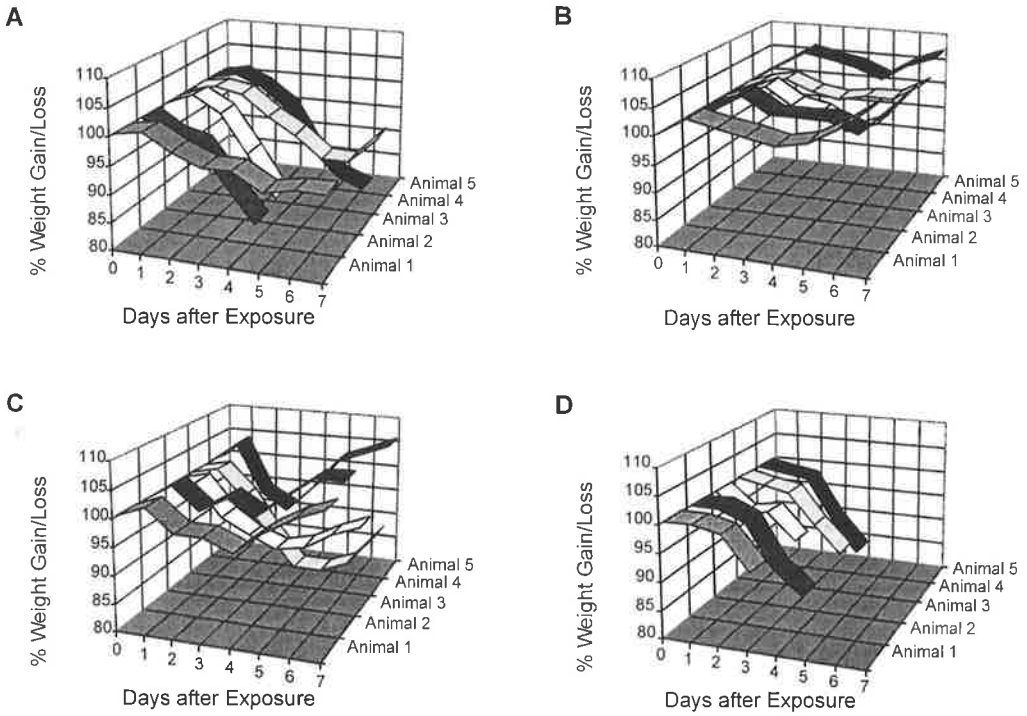

FIG. 4. Percentage weight gain or loss in guinea pigs exposed to an aerosol of different strains of *Legionella longbeachae* serogroup 1. (A) Animals exposed to a dose of 10^9 *L. longbeachae* serogroup 1 A5H5 organisms; (B) animals exposed to a dose of 10^9 B8 organisms; (C) animals exposed to a dose of 10^{10} B8 organisms; (D) animals exposed to a dose of 10^9 B8.22 organisms. Guinea pig death is indicated by the termination of the ribbon graph prior to the end of the experiment on day 7.

strains, in both models, with the type strain ATCC *L. longbeachae* showing a lower level of infectivity in comparison to strain A5H5. This strain difference was most significant in the animal model, where *L. longbeachae* serogroup 1 ATCC 33462 was unable to establish infection in either model, while strain A5H5 was virulent.

The results obtained in the animal model for the *mip* mutant in *L. longbeachae* serogroup 1 A5H5 are of interest, as no other *mip* mutant, other than those of *L. pneumophila* serogroup 1, has been assessed in an aerosol animal model. The results are consistent with those seen for the *mip* mutant of *L. pneumophila* serogroup 1 (11). The mutant was unable to cause death in guinea pigs under two test dose conditions. However, the test doses trialed in this study resulted in lower numbers of bacteria being deposited into the lungs than those achieved by intratracheal inoculation in the study by Cianciotto et al. (11). The aerosol model of infection makes it difficult to achieve higher numbers of deposited bacteria, and hence we cannot say whether the *mip* mutation in *L. longbeachae* serogroup 1 would have yielded different results at higher doses. It is tempting to speculate that this would be the case, as *L. longbeachae* serogroup 1 differs from *L. pneumophila* serogroup 1 in that it does not possess the major outer membrane protein (references 14, 22, and 23 and unpublished observations). The major outer membrane protein is believed to play a role in uptake of *L. pneumophila* serogroup 1 into macrophages through its ability to bind complement component C3b (5). Therefore, *L. longbeachae* serogroup 1 may be more susceptible to changes in outer membrane proteins. The difference between the wild-type parent and the *mip* mutant in *L. longbeachae* serogroup 1 on the severity of the symptoms shown indicates a significant effect on the organism. The Mip protein is likely to have a significant role in pathogenesis of the organism or in survival in protozoa and the environment.

Given the close clonal nature of *L. longbeachae* serogroup 1 (24), why is the American ATCC *L. longbeachae* serogroup 1 isolate less virulent than the Australian clinical isolate? Are there fundamental differences between the two strains that may account for these discrepancies? Work is currently under way to investigate these questions.

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