The Influence of Phenotype on the Stress Resistance of *Legionella pneumophila.*

By

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A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy

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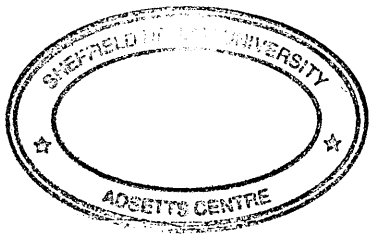
Collaborating Organisation : Aston University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ABBREVIATIONS.

ORGANISMS

A. castellanii	*Acanthamoeba castellanii*
A. polyphaga	*Acanthamoeba polyphaga*
A. palestinensis	*Acanthamoeba palestinensis*
C. psittaci	*Chlamydia psittaci.*
E. coli.	*Escherichia coli*
H. vermiformis	*Hartmannella vermiformis*
L. micdadei	*Legionella micdadei*
L. longbeachae	*Legionella longbeachae.*
L. pneumophila	*Legionella pneumophila*
L. monocytogenes	*Listeria monocytogenes.*
M. tuberculosis	*Mycobacterium tuberculosis*
M. avium	*Mycobacterium avium*
S. enteritidis	*Salmonella enteritidis*
S. typhimurium	*Salmonella typhimurium*
V. cholerae	*Vibrio cholerae.*
V. vulnificus	*Vibrio vulnificus.*
sp.	species.

CHEMICALS.

ABTS	2,2-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid.
BCA	bicinchoninic acid
BIT	benzisothiazolone
BSA	bovine serum albumin.
$CaCl_2.7H_2O.$	calcium chloride (hydrous).
CMIT	5-chloro-N-methylisothiazolone.

$CuSO_4$	copper sulphate.
ddH_2O	double distilled water.
DAPI	4'6-diamidino-2-phenylindole
EDTA	ethylene diamine-tetra-acetic acid (di-sodium salt).
$Fe_4(P_2O_7)_3.SO_4$	ferric pyrophosphate
FG	Feeley-Gorman
HCl	hydrochloric acid.
KH_2PO_4	potassium orthophosphate
K_2HPO_4	di-potassium hydrogen phosphate.
$MgCl_2$	magnesium chloride
$MgSO_4.7H_2O.$	magnesium sulphate (hydrous)
NaCl	sodium chloride.
Na_2CO_3	sodium carbonate.
$NaHCO_3$	sodium bi-carbonate.
NaOH	sodium hydroxide.
$(NH_4)_2SO_4$	ammonium sulphate.
PBS	phosphate buffered saline.
PMA	phorbal myristate acid.
PHMB	polyhexamethylene biguanide
PYG	peptone yeast extract glucose.
RPMI	Roswell Park Memorial Institute.
Tris	tris(hydroxymethyl)- aminomethane.
YE	yeast extract.

UNITS

cfu	colony forming units per ml.
d.	day
g	gram
x g	force due to gravity
h	hour.
In	natural log.
kDa	kilo Dalton.
kHz	kilohertz.
M	molar
mM	millimolar.
mA	milliamperes.
mg	milligram
min.	minute.
ml	millilitre.
mm^2	milimetre-squared.
µm	micrometre.
nm	nanometre.
OD	optical density
ppm	parts per million
rpm	revolutions per minute.
sec.	second.
µl	microlitre.
µg	microgram
V	volt
v/v	volume per volume.
wk.	week
w/v	weight per volume.
yr.	years.
>	greater than
<	less than.

MISCELLANEOUS

1°	primary
2°	secondary.
DNA	deoxyribonucleic acid
dotA	defective for organelle trafficking.
ELISA	enzyme-linked immunosorbant assay.
GAl	galactosamine.
GAl/NAc	*N*-acetyl-D-galactosamine.
gsp	global stress protein.
HSP	heat shock protein.
icm	intracellular multiplication.
IgG	immunoglogulin G.
LPS	lipopolysaccharide.
MBC	minimum bactericidal concentration.
MIC	minimum inhibitory concentration.
mip	macrophage initiator protein.
MOMP	major outer membrane protein.
OMP	outer membrane protein.
oxyR	oxidation response gene.
RNA	ribonucleic acid.
rpoS	RNA polymerase encoding gene.
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
soxRS	superoxide response gene.

LIST OF DEFINITIONS

Aged : Bacteria which were held in coculture and harvested 72 h. after release from the host cell. These bacteria underwent a morphological change from small and rounded to enlarged rod shapes. This change in morphology was concommitant with a loss in motility.

Freshly Harvested : The removal of the legionellae from the coculture immmediately upon release of the bacteria from the host cell.

Microcosm : Small scale representation of an aquatic environment.

Phenotype : Total appearance of an organism, determined by interaction during development between its genetic constitution (genotype) and the environment.

Sigma Factor (σ Factor) : Protein component of prokaryotic RNA polymerase.

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ABSTRACT

The influence of phenotype on the stress resistance of *Legionella pneumophila*.
By Helena Rachel Scaife.

Legionella pneumophila, the causative agent of Legionnaires' disease, is an aquatic intracellular organism capable of replicating within both amoebae and human phagocytic cells. A comprehensive study has been undertaken to examine the hypothesis that intracellular replication of *L. pneumophila* induces enhanced resistance to external stress stimuli relative to that of *in vitro* grown legionellae.

Microscopical studies have shown that *L. pneumophila* grown in YE broth consistently develop a rod-shaped morphology and are non-motile. In contrast, *L. pneumophila* grown within *Acanthamoeba polyphaga* or U937 monocytes exhibit a smaller, rounded morphology and are highly motile. After *ca.* 72 h post lysis, the intracellular bacteria adopted a morphology similar to that of broth grown legionellae in stationary phase. These bacteria were termed 'aged'.

Time-kill assays have shown that *L. pneumophila* grown within *A. polyphaga* or U937 monocytes are more resistant than broth grown legionellae to a number of different stress conditions including exposure to the antibiotics traditionally used in the treatment of Legionnaires' disease, elevated temperatures associated with water treatment processes, mechanical stress and starvation. 'Ageing' of intracellular *L. pneumophila* before exposure to the stress stimuli, resulted in a marked loss of stress resistance.

To study the physiological basis of the increased stress resistance of intracellular grown *L. pneumophila*, a preliminary study of the surface properties of the variously grown legionellae was undertaken. The outer membrane protein (OMP) profiles of the *L. pneumophila* were prepared using sarkosyl and analysed by SDS-PAGE. Proteinase K digestion of the outer membrane (OM) was employed to prepare the LPS layer. The preliminary comparative study of the *L. pneumophila* OM has established that the mode of growth influences both the OMP profile and the LPS layer. In particular, intra-amoebic grown *L. pneumophila* possesses a novel protein of 15 kDa and intra-monocytic grown legionellae one of 24 kDa, both of which are lost upon 'ageing'.

The results of the project have shown a link between the presence of novel sarkosyl insoluble OMPs, bacterial morphology and enhanced resistance of the intracellular grown *L. pneumophila* to external stress stimuli. It could, therefore, be suggested that *L. pneumophila* undergo a prior adaptation to stress conditions during intracellular replication. Upon 'ageing' of intracellular grown legionellae the novel insoluble OMPs were lost with a concomitant change in morphology and loss in resistance to external stress stimuli.

The findings of this work have practical implications with respect to the clinical treatment of Legionnaires' disease and the eradication of the causative agent from water systems.

CHAPTER 1 INTRODUCTION.

In July 1976, approximately 4,400 people attended the annual convention of the Pennsylvannia American Legion which was held at the Bellevue-Stratford Hotel in Philadelphia, USA. In the days following the convention, 182 cases of pneumonia were diagnosed, 147 patients were hospitalised, and 29 people died. The 'mystery' disease was quickly dubbed 'Legionnaires' disease'. The causative agent proved to be a member of a previously unrecognised family of Gram negative bacteria. The specific organism was later named *Legionella pneumophila* (Brenner *et al.*, 1979). Following the Philadelphia outbreak, considerable money and research effort was spent, and is still being spent, in an endeavour to determine how this bacterium whose natural environment is water was capable of evolving into a pathogen of man. Much of this work has been reported in reviews by Winn (1988); Dowling (1992); Rechnitzer (1994); and Barker and Brown (1994; 1995). Inspite of this intense scientific effort, every year more gaps in our knowledge of this bacterium are identified. For example, recent evidence has shown that intracellular replication in both amoebae and human macrophages induces a phenotype that is dramatically different physiologically to that obtained by *in vitro* replication (Hoffman *et al.*, 1990; Abu Kwaik *et al.*, 1993 and Barker *et al.*, 1993). These differences may account for earlier reports that intra-amoebic replication enhances the resistance of *L. pneumophila* to chlorine (Navratil *et al.*, 1990) and biocides (Barker *et al.*, 1992) and may also account for the lack of correlation between *in vitro* testing of antibiotics and clinical efficacy (Fitzgeorge *et al.*, 1985 and Barker and Brown, 1995). However, it is difficult to draw any firm conclusions from this work, owing to the widely differing experimental conditions used by these various groups.

This review of previous work will be very brief, other than in areas identified as pertinent to this project; namely, *in vitro* growth and intracellular replication, the

influence of the replication pathway on the surface properties of the bacterium and the response of the bacterium to external stress stimuli.

1.1 BACKGROUND WORK.

It is clear that *L. pneumophila* have been present in the aquatic environment for many years (Fliermans *et al.*, 1983) and could possibly be described as old or ancient organisms. However, the potential of *L. pneumophila* as a pathogen appears not to have been realised until the advent of man-made thermal habitats. The first major outbreak of infectious disease which was attributed directly to the *L. pneumophila* occurred in Philadelphia, USA in 1976. The agent causing this outbreak of respiratory disease was not isolated until early 1977. McDade *et al.* (1977) isolated the agent from the lung tissue of fatal cases by culture in guinea pigs and yolk sacs of embryonated eggs. The causative organism appeared to be a small, Gram negative rod, morphologically identical to the bacteria found in infected lung tissue from autopsy material (Chandler *et al.*, 1977). The bacteria proved to be the first recognised member of an extremely large family, the Legionellaceae and was called *Legionella pneumophila.* Following identification of the causative agent of the Philadelphia outbreak, many previously unexplained epidemics of respiratory disease were attributed to *L. pneumophila.* For example, the outbreak at St. Elisabeth's Hospital, Washington DC., in 1965, with 81 cases and 12 deaths, and the Pontiac outbreak of acute febrile illness without pneumonia in 1968, with 144 cases but no deaths. The earliest identified outbreak of a respiratory disease, which was retrospectively identified as Legionnaires' disease, occurred in 1957 (Tobin *et al.*, 1980 and Osterholm *et al.*, 1983). The first sporadic cases of Legionnaires' disease were identified soon after the Philadelphia outbreak (McDade *et al.*, 1977), with earlier cases being diagnosed retrospectively (1943- Hebert *et al.*, 1980 and 1947-McDade *et al.*, 1979). It may thus be concluded that Legionnaires' disease is not a 'new' disease.

Legionella pneumophila is a member of the family Legionellaceae. These bacteria are Gram negative, aerobic, non-sporeforming, non-acid-fast, unencapsulated bacilli

(Brenner *et al.*, 1986). Their major source of energy for growth appears to be amino acids which are catabolised by the Kreb's cycle (George *et al.*, 1980). Since the original isolation of the bacterium by McDade, over 39 species of *Legionella* and 61 serologically distinct groups have been described in the literature. Approximately half (18) of the species have been associated with respiratory tract infections in man (Fields, 1996). *Legionella* sp. are second only to *Streptococcus pneumoniae* as a cause of severe community acquired pneumoniae, accounting for 14-37 % of reported cases (Bates *et al.*, 1992). The majority of these (80 %) are attributed to *L. pneumophila,* of which 50 % are due to serogroup 1 (Reingold *et al.*, 1984 and Rechnitzer, 1994). The remaining 20 % of cases are reported as being caused mainly by other serogroups of *L. pneumophila*, *L. micdadei*, and more commonly in Australia by *L. longbeachae* (England *et al.*, 1981; Reingold *et al.*, 1984 and Nimmo and Bull, 1995). The severity of the disease arising from the different *Legionella* sp. ranges from a mild form, called Pontiac fever, which is a self-limiting, non-pneumonic, febrile infection with high attack rate (Glick *et al.*, 1978; Winn, 1988; Rechnitzer, 1994 and Barker and Brown, 1995) to severe Legionnaires' disease. The typical clinical picture of Legionnaires' disease is that of a severe and acute fulminating pneumonia together with hepatic, renal and cerebral involvement (Fraser *et al.*, 1977; Beaty *et al.*, 1978; Kirby *et al.*, 1980 and Rechnitzer, 1994). Most organs have been implicated in the manifestation of this disease (Rechnitzer, 1994). Clinical reports of multiple sites of infection associated with the *Legionella* bacteraemia (Edelstein *et al.*, 1979) strongly suggests a haemotogenous spread of the bacteria following the initial infection and lesion of the lungs. It is not surprising, therefore, that Legionnaires' disease is reported as having a relatively high fatality rate. Lind (1983) reported that fatality due to Legionnaires' disease may be up to 14 % in isolated cases, whilst O'Mahony *et al.* (1990) suggested that it may be as high as 27 % in a major outbreak.

One of the difficulties encountered by the early investigators of Legionnaires' disease was the wide range of symptoms presented by patients, many of which were mis-interpreted as arising from other diseases, such as influenza. The spectrum of illness

induced by this bacterium is thought to be a manifestation of the host's response, although it could also be due to variation in the virulence of the *L. pneumophila* strain, or the size of the infective dose received. Severe Legionnaires' disease symptoms are commonly associated with people who are already immunocompromised, and the elderly. It is not surprising, therefore, that outbreaks most commonly occur amongst groups of people in large communal establishments such as old peoples' homes and hospitals. The occurrence of Legionnaires' disease at Stafford District General Hospital, England, in 1985, is typical of such an outbreak (Badenoch, 1986). Outbreaks of Legionnaires'disease in the community at large are often associated with hotels and holiday apartments, many of which remain unused for periods of time. An interesting recent development has been the occurrence of two outbreaks of Legionnaires' disease on the cruise ship, "Edinburgh Castle," (Daily Telegraph, June, 1998).

Inspite of extensive epidemiological studies, the source and mode of infection of the 1976 outbreak of Legionnaires' disease in Philadelphia was never found. The time spent in the lobby was the only link established between the hotel and the infection suggesting that an airbourne organism may be involved (Winn, 1988). Subsequent studies have shown that *Legionella* sp. are waterborne organisms, and that legionellosis is aquired exclusively by the inhalation of aerosol-borne bacteria originating from environmental sources; no person-to-person transmission has been documented (Brieman, 1993 and Onody *et al.*, 1997). Although *L. pneumophila* is an aquatic organism, specific conditions are required for the development of Legionnaires' disease. The "chain of causation" of Legionnaires' disease consists of six links : a natural reservoir, amplifying factor(s), dissemination of the bacteria, a virulent strain, inoculation of an appropriate site, and lastly the susceptibility of the host (Fraser, 1984). The processes leading to Legionnaires' disease are summarised in Figure 1.1. *Legionella* sp. are common in the natural environment and have been isolated from the majority of natural water sources including lakes, rivers and marine waters (Fliermans *et al.*, 1981 and Ortiz-Roque and Hazen, 1987). Fliermans (1983) suggested that *Legionella* has probably been present in water for a long period of time in a form

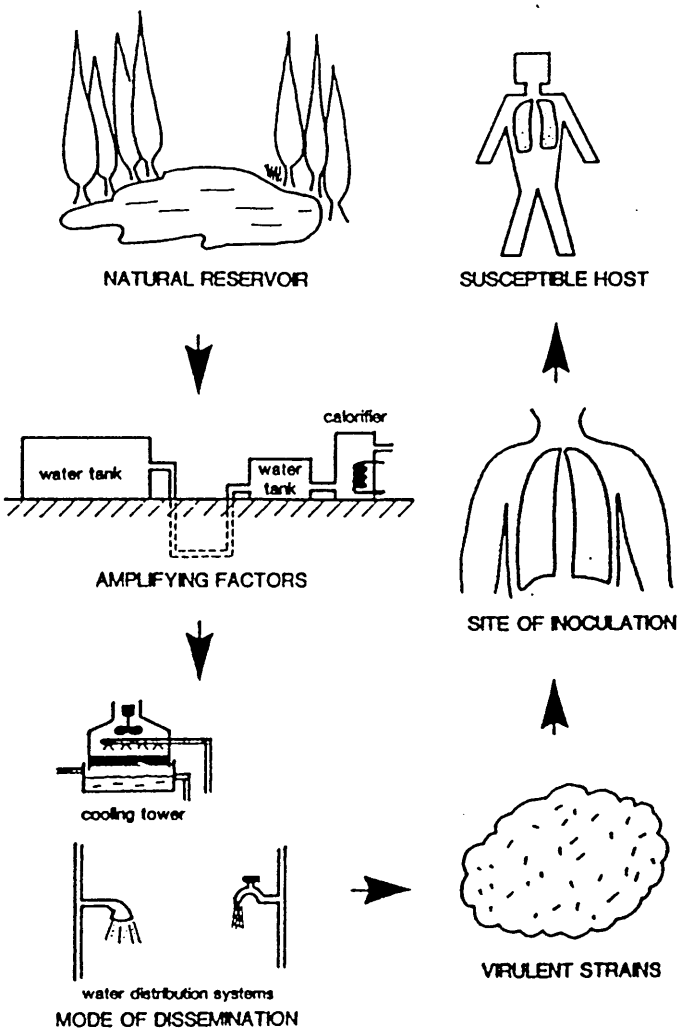


Figure 1.1. The "chain of causation" of Legionnaires' disease. (After Fraser, 1984).

harmless to man, and that it has been the development of man-made thermal habitats that has allowed its emergence as a pathogen.

Legionella sp. are reported as being widely distributed in potable and non-potable water systems. Desplaces *et al.* (1984) isolated *Legionella* sp. from 44 % of tap water samples in Paris. A similar survey by Bartlett *et al.* (1983) in the UK, found 53 % of hotels and 70 % of hospitals contaminated by the bacteria. Vickers *et al.* (1987) also reported that 60 % of hospitals in Pennsylvannia, USA, were similarly contaminated. Since the discovery of intra-amoebic replication by certain bacteria, there has been much speculation as to the relevance of this to human infection. It is generally accepted that amoebae play an important role in the persistence of *L. pneumophila* in aquatic systems, since growth of the bacterium in the absence of these organisms has not been reported (Rowbotham, 1986; Wadowsky *et al.*, 1988 and Fields *et al.*, 1993). Amoebae have been shown to be present in many water systems implicated in outbreaks of Legionnaires' disease (Barbaree *et al.*, 1986; Fields *et al.*, 1989;1990 and Brieman *et al.*, 1990). The role of amoebae in an outbreak is not clear since contamination of potable water systems by these organisms is very common (Rowbotham, 1980; Tyndall and Dominique, 1982 and Henke and Siedel, 1986). It is possible that amoebae provide *L. pneumophila* with a protective niche throughout the process of water treatment in addition to serving as a replication reservoir. The mechanism of intracellular replication of *L. pneumophila* is examined in depth later in this review, and consequently, it will not be considered further at this juncture.

The mode of transmission from contaminated water systems to the human respiratory tract is currently subject to debate. The early detection of *L. pneumophila* in aerosols led to the suggestion that the inhalation of such aerosols is the mode of acquisition of the disease (Muder *et al.*, 1986; Dennis and Lee, 1988 and O'Brien and Bhopal, 1993). A bacterial aerosol has been defined as being "a mixture in air of small droplets of water and dried 'droplet nuclei' that contain bacteria and are small enough to remain suspended for considerable periods" (Dennis and Lee, 1988). The smaller the droplets,

the greater the penetration of the lungs by the infective aerosol. For infection to occur the droplets of the aerosol must be < 5 μm in diameter (Fraser, 1980; Gibson *et al.*, 1983 and Fitzgeorge *et al.*, 1985). In the domestic environment, it is unlikely that aerosols containing legionellae are produced continually from any one source. More probably, they are produced as a bolus when sources, such as showers, are first turned on (Colbourne *et al.*, 1984) or after water systems have been disturbed (Shands *et al.*, 1985). Under such conditions of release, the ability of legionellae to survive within an aerosol droplet becomes a major factor in determining its virulence (Dennis and Lee, 1988). The longer bacteria remain viable within an aerosol droplet, the greater the accumulation of legionellae in the lungs of susceptible individuals, and the more likely such people are to succumb to infection.

Epidemiological evidence suggests that aerosols are capable of travelling distances of 900m (Bhopal *et al.*, 1991), or possibly 'miles' (Berk *et al.*, 1998) and that aerosol-borne legionellae can survive for up to 2 h at 65 % relative humidity (Hambleton, 1983). There is increasing evidence, however, that the inhalation of aerosols contaminated with planktonic *L. pneumophila* is not the mode of transmission of the bacterium from its aquatic reservoir to its potential human host. The infective dose required for the development of Legionnaires' disease in man is not known (Fraser, 1980; O'Brien and Bhopal, 1993 and Barker and Brown, 1995). However, Tyndall *et al.* (1985) suggested that it may be as high as 1.4×10^7 bacteria, based on evidence from animal studies. Using this figure, and knowledge of contamination levels, the authors calculated that a subject would need to inhale aerosols from the average contaminated cooling tower for a period of nine years in order to accumulate the proposed infective dose of the bacterium. Similarly, Dennis *et al.* (1984) suggested a subject would have to breathe aerosolised air around a contaminated shower for 227 min. in order to inhale one bacterium.

The close relationship between amoebae and *L. pneumophila* is now considered to play a role in the transmission of Legionnaires' disease. The detection of airborne amoebae

in water aerosols (Rodriguez-Zaragoza *et al.*, 1993) has led to the suggestion that *L. pneumophila* infected amoebae may be the primary vector for Legionnaires' disease. Infected amoebae may contain up to 10^4 bacteria per cell (Rowbotham, 1986). It is suggested that a few deeply inhaled legionellae-laden amoebae could, on lysis, introduce large numbers of *L. pneumophila* at locally high concentrations into the lower respiratory tract of humans. This presents *L. pneumophila* with the optimum conditions for uptake and replication in nearby monocytes and macrophage. Recent evidence presented by Berk *et al.* (1998) suggested a possible variation of this mode of infection. It is reported that *A. polyphaga* and *A. castellanii* eject vesicles containing viable *L. pneumophila* just prior to encystment of the population as a means of survival during biocidal treatment of water systems. The vesicles have been shown to be resistant to biocide exposure for up to 24 h, and to be very resistant to dessication. It was suggested that contaminated vesicles can transport viable *L. pneumophila* for great distances from the reservoir in aerosols and, possibly, in dusts. In addition, as the vesicles are much smaller than the parent amoebae, they can be expected to penetrate deeper into the alveoli and small bronchioles before entrapment. It is possible, however, that all three mechanisms of transmission are operative at the same site of contamination.

O'Brien and Bhopal (1993) suggested that non-clinical legionellosis may follow exposure to low numbers of bacteria alone, with the clinical, more severe form, occurring as a result of exposure to either a large dose of bacteria, or to legionellae within an amoebae. This hypothesis is reinforced, to some extent, by the observation of a high prevalence of elevated antibody titres in the background population of sites of major outbreaks, such as the hotel employees in Philadelphia and hospital staff in Stafford. Cirillo *et al.* (1994) on the other hand, reported that intra-amoebic grown *L. pneumophila* are most virulent at the moment of release from the host cell. Thus a subject inhaling a few infected amoebae, or vesicles, may develop a more severe form of Legionnaires' disease, than one inhaling a greater number of less virulent planktonic bacteria via an aerosol.

The severity of the symptoms of Legionnaires' disease presented by a patient depend, to some extent, on the virulence of the inhaled organisms (Rechnitzer, 1994). Virulence has been directly correlated with the ability of the bacteria to invade host cells, multiply intracellularly and cause cellular damage and disease (Rechnitzer, 1994). The histopathological feature of Legionnaires' disease is a severe confluent lobular or lobar pneumonia, with lesions of alveolites and bronchiolites (Blackmon *et al.*, 1978 and Winn and Myerowitz, 1981). However, before reaching the distal part of the lungs, where the conditions are such that they can induce an inflammatory response and cause tissue damage, legionellae have to overcome the lung defences, including mechanical barriers and the cellular and humoral immune systems. Figure 1.2 summarises the lung defence system. The role of mechanical barriers in the protection against *Legionella* infection has not been extensively investigated. However, inhalation of a contaminated aerosol is known to be rapidly followed by bacterial replication; which suggests that mechanical defences such as secretory immunoglobulins, mucus secretion and ciliary clearance, are ineffective against *Legionella* infection. Specific antibodies are known to markedly enhance the uptake of *L. pneumophila* by macrophage and monocytes, but intracellular replication is not prevented. It must be concluded, therefore, that humoral immunity has only a limited role in the defence against *Legionella* infection (Rechnitzer, 1994). In contrast, cell-mediated immunity has been shown to play a major role in mans' defence against Legionnaires' disease. Cytokines, including interferon-gamma and tumour necrosis factor, are produced following exposure of the macrophage to *Legionella* antigens. Cytokine-activated monocytes are able to inhibit intracellular growth of *L. pneumophila*, in contrast to non-activated monocytes which support the growth of the organisms (Horwitz and Silverstein, 1981 and Bhardwaj *et al.*, 1986). *Legionella* antigens also induce enhanced natural killer cell activity and production of interferon-gamma (Blanchard *et al.*, 1985a; 1985b). The cytokine activated killer cells have also been reported as being able to kill legionellae-infected macrophages (Blanchard *et al.*, 1987 and Resnick *et al.*, 1988).

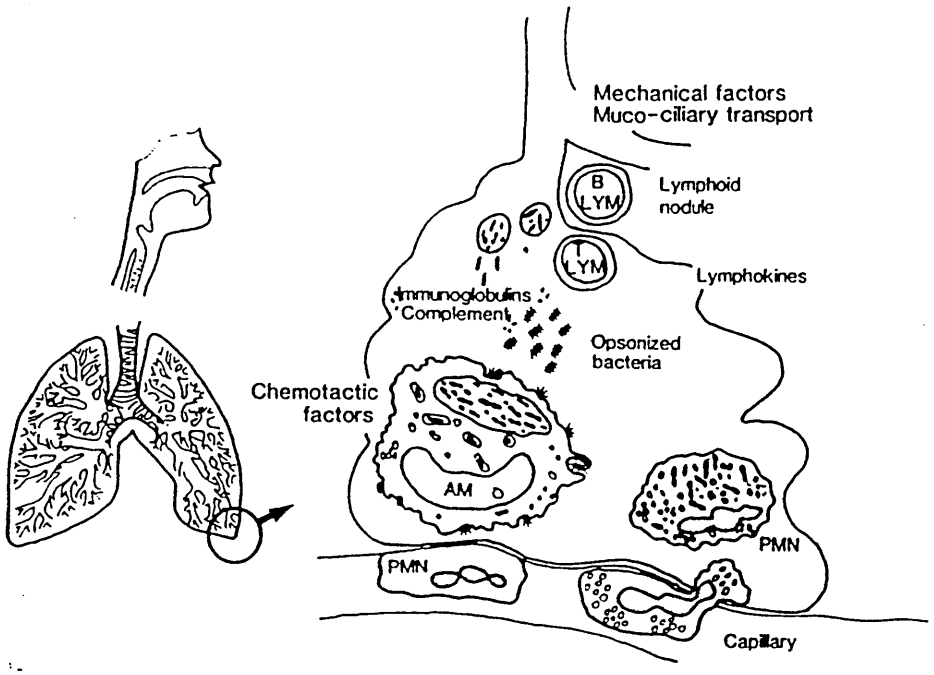


Figure 1.2. The lung defences. AM, alveolar macrophages; PMN, polymorphonuclear neutrophils; B and TLYM; B and T lymphocytes. (After Rechnitzer, 1994).

Recent evidence has been presented which suggests that the most severe form of Legionnaires' disease is induced by the co-inhalation of amoebae and legionellae. Brieland *et al.* (1996; 1997) have shown that the presence of amoebae inhibits cytokine activation of monocytes, there-by reducing the effectiveness of the immune system. The mode of action of the amoebae in inhibiting recruitment of activated monocytes into the lungs is, as yet, unknown.

1.2 ULTRASTRUCTURE OF GRAM NEGATIVE BACTERIA.

Bacteria can be divided into two broad classes, Gram positive and Gram negative, depending on their response to a staining technique developed in 1884 by Christian Gram. Gram positive and Gram negative bacteria differ considerably in the structure of their cell walls, and in the role this structure plays in coping with environmental changes. Consequently, this review of bacterial ultrastructure will be limited to the outer cell surface, with particular reference to Gram negative bacteria and more specifically *L. pneumophila*. Neidhardt *et al.* (1990) described the cell wall of a Gram positive bacteria as resembling a thick blanket when compared to the thin sheets of Gram negative bacteria. Typical cell wall structures are shown schematically in Figures 1.3 (a) and (b) (After, Lambert, 1988a).

The Gram positive bacterium cell wall consists of a thick multi-layered peptidoglycan layer interdispersed with small amounts of other polymers, notably teichoic acids (Figure 1.3 a). This polymeric structure consists of many layers wrapped around the length and width of the cell, thereby forming a sack that determines the size and shape of the organism. The main function of this layer, however, is to protect the cytoplasmic membrane from extracellular chemical agents.

The Gram negative bacteria have evolved a radically different solution to the problem of protecting their cytoplasmic membrane. These bacteria have a second membrane, the outer membrane (OM), with a much thinner peptidoglycan layer attached to its inner surface (Figure 1.3 b). The OM is chemically distinct from most biological membranes

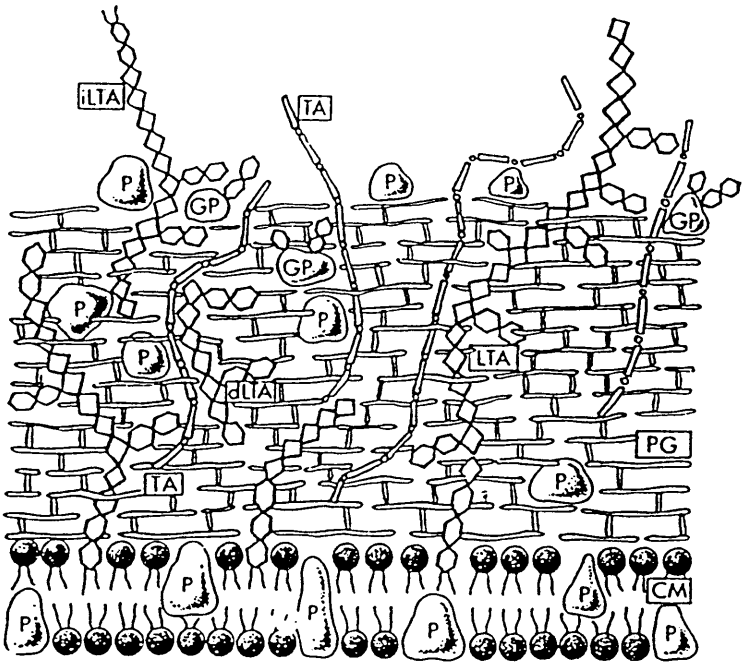


Figure 1.3 (a). Schematic representation of a cross section of the Gram positive bacterial cell envelope. CM, cytoplasmic membrane; PG, peptidoglycan; P, protein; GP, glycoprotein; TA, teichoic acid or techuronic acid; LTA, lipoteichoic acid; dLTA, deacylated lipoteichoic acid, iLTA, inverted lipoteichoic acid (glycolipid protuding away from the cell surface.) (After Lambert, 1988a).

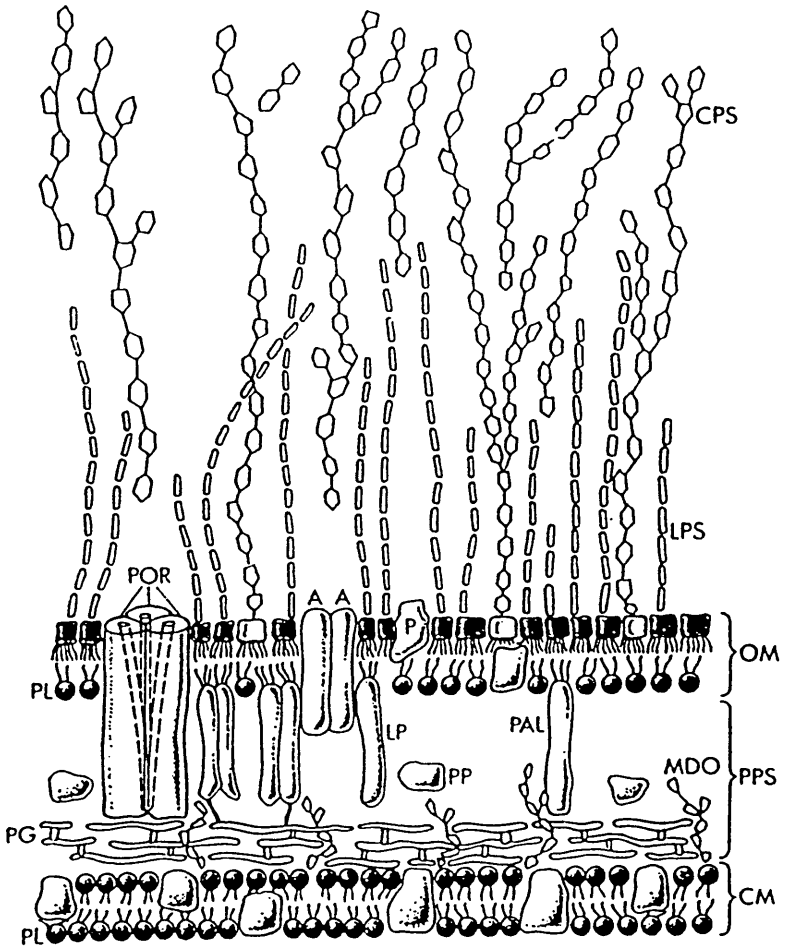


Figure 1.3 (b). Schematic representation of a cross section of the Gram negative bacterial cell envelope. CM, cytoplasmic membrane; PPS, periplasmic space; OM, outer membrane; PG, peptidoglycan; P, protein, PP, periplasmic protein; LP, lipoprotein; PAL, peptidoglycan-associated lipoprotein; POR, porin; CPS, capsular polysaccharide; LPS lipopolysaccharide; MDO, membrane-derived oligosaccharide; A, other outer membrane protein; PL, phospholipid. (After Lambert, 1988a).

and has the ability to resist damaging chemicals. It is a bilayered structure, the inner layer of which is similar in composition to that of the cytoplasmic membrane. The outer layer, on the other hand has a unique constituent in the place of phospholipids found in the membranes of Gram positive bacteria. This is the bacterial lipopolysaccharide (LPS) layer, a complex molecule not found elsewhere in nature. As a result, the layers of this membrane are extremely asymetrical, and the properties of this unusual bilayer differ considerably from those of a typical biological membrane.

The LPS layer consists of three parts; Lipid A, which anchors the LPS in the outer layer of the OM; a core of short chain sugars, and an outer of long chain sugars, the O-antigen. The core structure of LPS is relatively constant amongst Gram negative bacteria and characteristically includes the sugars keto-deoxyoctonic acid (KDO) and heptose. The O-antigen consists of long chain carbohydrates, up to forty sugars in length, which are hydrophilic in nature and cover the bacterial surface. Although this is a loose structure, at least compared with the peptidoglycan layer of Gram positive bacteria, it is highly effective in excluding hydrophobic compounds. Mutants that don't possess the O antigen become sensitive to compounds like bile salts and certain antibiotics, to which the wild type are resistant.

The OM barrier constitutes both an advantage and a hazard to Gram negative bacteria. For example, some bacteriophage use proteins in the OM as attachment sites on their host bacteria; conversely, the OM confers resistance to many antibiotics, especially penicillins. The presence of an OM in Gram negative bacteria also has unexpected biological consequences. The LPS of the OM is highly reactive when it is introduced into animals. The lipid A component has a large number of biological activities depending on its concentration. In small amounts it elicits fever and activates a series of immunological and biochemical events that lead to mobilisation of host defence mechanisms. In large concentrations, this compound, also known as endotoxin, can cause shock and even death (Neidhardt *et al.*, 1990). The O antigen portion, as part of

its name denotes, is antigenic. The O antigens come in many forms and are used to define species and sub-species of Gram negative bacteria (Neidhardt *et al.*, 1990).

L. pneumophila display an ultrastructure typical of Gram negative bacteria and as such have a cell wall consisting of an outer membrane, a peptidoglycan layer and a cytoplasmic membrane (Flesher *et al.*, 1979). A number of cellular components have been purified and proposed as virulence factors. The virulence factors are located in the outer membrane, bound to the peptidoglycan layer, and possibly expressed on the cell surface, or are part of the cytoplasmic membrane. The *Legionella* cell envelope *per se* has unusual characteristics and a unique LPS structure. A band pattern atypical of Gram negative bacteria is obtained when the LPS of *L. pneumophila* is subjected to sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Ciesielski *et al.*, 1986; Conlan and Ashworth, 1986 and Nolte *et al.*, 1986). Chemical analysis has showed it to possess a unique fatty acid composition characterised by a large number of branched-chain fatty acids and by the absence of hydroxy-fatty acids generally associated with lipid A (Otten *et al.*, 1986 and Sonensson *et al.*, 1989). Several serological studies have revealed that LPS is the serogroup-specific antigen and is responsible for the diversity of serogroups 1 to 14 of *L. pneumophila* (Nolte *et al.*, 1986 and Otten *et al.*, 1986). Gabay *et al.* (1985) described LPS as the major antigen recognised by sera from patients with Legionnaires' disease.

Extensive investigation of whole bacteria, or individual membranes, has isolated many proteins unique to *L. pneumophila*. Of particular importance are three proteins with a molecular weight of 24 kDa, 28 kDa and 60 kDa. The 28 kDa protein, in particular, has been shown to be present in virtually all *Legionella* sp. (Butler *et al.*, 1985 and Gabay *et al.*, 1985). This protein, termed the major outer membrane protein (MOMP), has been characterised as a porin and is considered to be tightly bound to the LPS (Hindahl and Iglewski, 1986). Analysis suggested (Hoffman *et al.*, 1992) that it is composed of a 28 kDa and a 31 kDa sub-unit cross-linked by interchain disulphide bonds and anchored in the peptidoglycan layer. The 31 kDa sub-unit is thought to be

the anchor-unit as this has not been isolated from OM preparations (Butler *et al.*, 1985; Butler and Hoffman, 1990 and Hoffman *et al.*, 1992). Gabay *et al.* (1985) suggested that the MOMP was not an important antigen as it is infrequently recognised by patient sera. Payne and Horwitz (1987) reported that the MOMP plays an important role in the uptake of *L. pneumophila* by human macrophages.

All fourteen serogroups of *L. pneumophila* have been shown to express a *mip* gene and have a 24 kDa Mip protein on their surface (Cianciotto *et al.*, 1989; 1990a, 1990b).The latter has been shown to be essential for full infection of both amoebae and human phagocytic cells. Twenty-nine other *Legionella* sp. have been shown to express a similar Mip-like protein. These are reported as possessing molecular weights of between 24 and 31 kDa and react with specific Mip antisera. If the Mip related proteins of other *Legionella* sp. are functionally dissimilar to the Mip protein of *L. pneumophila* itself, it may explain the apparent greater virulence and wide range of symptoms presented by patients with Legionnaires' disease.

Heat shock proteins (HSP), or stress proteins, are highly conserved proteins with a molecular weight of 60 kDa, which have important biological functions. HSP60 acts as a chaperone and is probably involved in the folding and export of proteins. It is, therefore, essential for bacterial growth (Hindersson *et al.*, 1991 and Zeilstra-Ryalls *et al.*, 1991). Its role in pathogenicity is unknown. Under normal conditions, most HSPs are constitutively expressed at a low level by all eukaryotic and prokaryotic cells (Miller *et al.*, 1989). A variety of stress stimuli, including heat shock, nutrient deprivation, oxygen radicals, and intracellular replication, induce a marked increase in HSP synthesis (Gibson *et al.*, 1994 and Boulanger *et al.*, 1995). The selective synthesis of stress proteins during intracellular growth is a common feature of all intracellular bacteria such as *Salmonella typhimurium* (Buchmeier and Heffron, 1990), *Mycobacterium tuberculosis* (Lathigra *et al.*, 1991) and *Brucella abortus* (Lin and Ficht, 1995). All *Legionella* sp. and serogroups express a 58 to 60 kDa protein during intracellular replication (Hoffman *et al.*, 1989 and Sampson *et al.*, 1990). The heat

shock protein (HSP) is mainly found in the cytoplasmic fraction, but may be expressed on the surface of intracellular bacteria (Plikaytis *et al.,* 1987; Hoffman *et al.,* 1989; 1990 and Sampson *et al.,* 1990). HSP is recognised by the majority of patient sera and cross reacts with other heat shock proteins, such as the common antigen of *Pseudomonas aeruginosa*, the GroEL of *E. coli*, the *M. tuberculosis* 65 kDa antigen and *Coxiella burnetti* HSP (Shinnick, 1991). *L. pneumophila* possess a genus specific epitope which can be recognised on all species (Steinmetz *et al.,* 1991).

Barker *et al.* (1993) reported that surface properties of intra-amoebic grown *L. pneumophila* were different in several respects to those of legionellae grown *in vitro*. The presence of a 15 kDa protein, additional LPS bands and altered fatty acid content were all noted for intra-amoebic grown *L. pneumophila*. Miyamoto *et al.* (1993) reported a 24 kDa protein which was unique to *L. pneumophila* grown in macrophages. Experimentation showed that it was not a Mip protein. Unlike Barker *et al.* (1993), these workers examined whole cell proteins which makes it difficult to isolate the origin of this protein.

Ludwig *et al.* (1991) isolated a 19 kDa OM lipoprotein which has been analysed and cloned. This protein is immunogenic and tightly bound to the peptidoglycan. The protein has been named the peptidoglycan-associated (Ppl) protein. The legiolysin (Lly) protein, a 39 kDa protein, causes haemolysis and browning of culture media through pigment production, but has no proteolytic activity. The Lly protein has been isolated from the whole cell extract, but its exact location is as yet unknown. The expression of legiolysin seems unique to *L. pneumophila,* whether avirulent or virulent (Bender *et al.,* 1991; Rdest *et al.,* 1991 and Wintermeyer *et al.,* 1991).

There have been several reports suggesting that the more acute Legionnaires' disease may be caused by the inhalation of legionellae-laden amoebic cysts or vesicles which burst open within the lungs (Rowbotham, 1986; 1992; O'Brien and Bhopal, 1993 and Berk *et al.*, 1998). Other workers suggested that intra-amoebic growth of legionellae

may prime the bacteria for infection of man (Barker and Brown, 1995 and Brieland *et al.*, 1996). Gabay *et al.* (1985) examined the response of sera from patients infected with *L. pneumophila* serogroup 1 towards surface antigens of broth grown *L. pneumophila*. It was reported that patient sera recognised the LPS antigens of *L. pneumophila*, but not the MOMP antigens. However, it is unlikely that the *L. pneumophila* found at the commencement of human infection would possess the broth grown phenotype. It would be of interest to examine the response of rabbit raised anti-*L. pneumophila* antibodies against the surface antigens of both broth grown and intracellular grown *L. pneumophila* to determine the effect of bacterial phenotype and growth phase on the host immune response.

1.3 BACTERIAL GROWTH.

1.3.1 *IN VITRO* GROWTH.

The development of media for the *in vitro* culture of *L. pneumophila* received considerable attention in the late 1970s following the original outbreak of Legionnaires' disease in Philadelphia, USA. In the following years a range of artificial media, both complex and chemically defined, were developed for *in vitro* culture of *L. pneumophila*. *Legionella* sp. are unable to grow on many standard bacteriological media, such as blood agar and unenriched chocolate agar. The bacterium was first successfully grown on Muller-Hinton agar supplemented with haemoglobin and IsoVitale X enrichment (Feeley *et al.*, 1978). It was noticed that repeated passage on this medium resulted in variants of *L. pneumophila* which had lost the ability to cause disease in animals. In 1978, Feeley *et al.* reported the successful *in vitro* culture of *L. pneumophila* using a complex medium, the Feeley-Gorman (FG) medium. This later became the basis of YE broth (Feeley *et al.*, 1979). The addition of ACES buffer (Pasculle *et al.*, 1980) and α- ketoglutarate (Edelstein, 1982) further improved the growth of *L. pneumophila*. In 1979, Warren and Miller and Pine *et al.* independently reported chemically defined media which would support the growth of *L. pneumophila*. The yields from these media were, however, less than that from an equivalent complex

medium culture. Warren and Miller (1979) and Pine *et al.* (1979) reported that the optimum growth conditions for *L. pneumophila* were incubation at 37°C with shaking, in a medium buffered to a pH 6.5-6.9. The morphology of *in vitro* grown *L. pneumophila* has been reported as varying from rod-like when grown in complex medium (Warren and Miller, 1979; Brenner *et al.*, 1986 and Barker *et al.*, 1992) to filamentous when cultured in chemically defined media (Warren and Miller, 1979). Pine *et al.* (1979) also reported that the morphology was dependent on the growth conditions and the phase of growth.

Growth limitation induced by nutrient depletion has been shown to give rise to bacteria with reduced growth rates and radically altered cell envelopes (Brown and Melling, 1969; Ellwood and Tempest, 1972; Holme, 1972; Brown, 1975 and Lambert 1988a). This has been reported to influence greatly the susceptibility to antimicrobial agents (Brown, 1975 and Gilbert and Wright, 1986) and to antibiotics (De La Rosa *et al.*, 1982; Brown and Williams, 1985a; 1985b and Tuomanen *et al.*, 1986) for a wide range of organisms (Tempest *et al.*, 1968; Meers and Tempest, 1970; Minnickin *et al.*, 1971; Dean, 1972; Holme, 1972; Dean *et al.*, 1976 and Gilbert and Brown, 1980). The problem of growth phase and the use of *in vitro* grown bacteria for assessing their response to external stress stimuli has been reviewed by Brown *et al.* (1990). These authors highlighted the influence that growth phase may exert on the outcome of any investigation using *in vitro* grown bacteria. It was suggested that if exponential phase bacteria were required they should be harvested several generations after the inoculation of the culture and before the onset of stationary phase. Failure to do so could lead to mixed populations of bacteria whose properties do not reflect a single growth phase.

1.3.2 INTRACELLULAR REPLICATION.

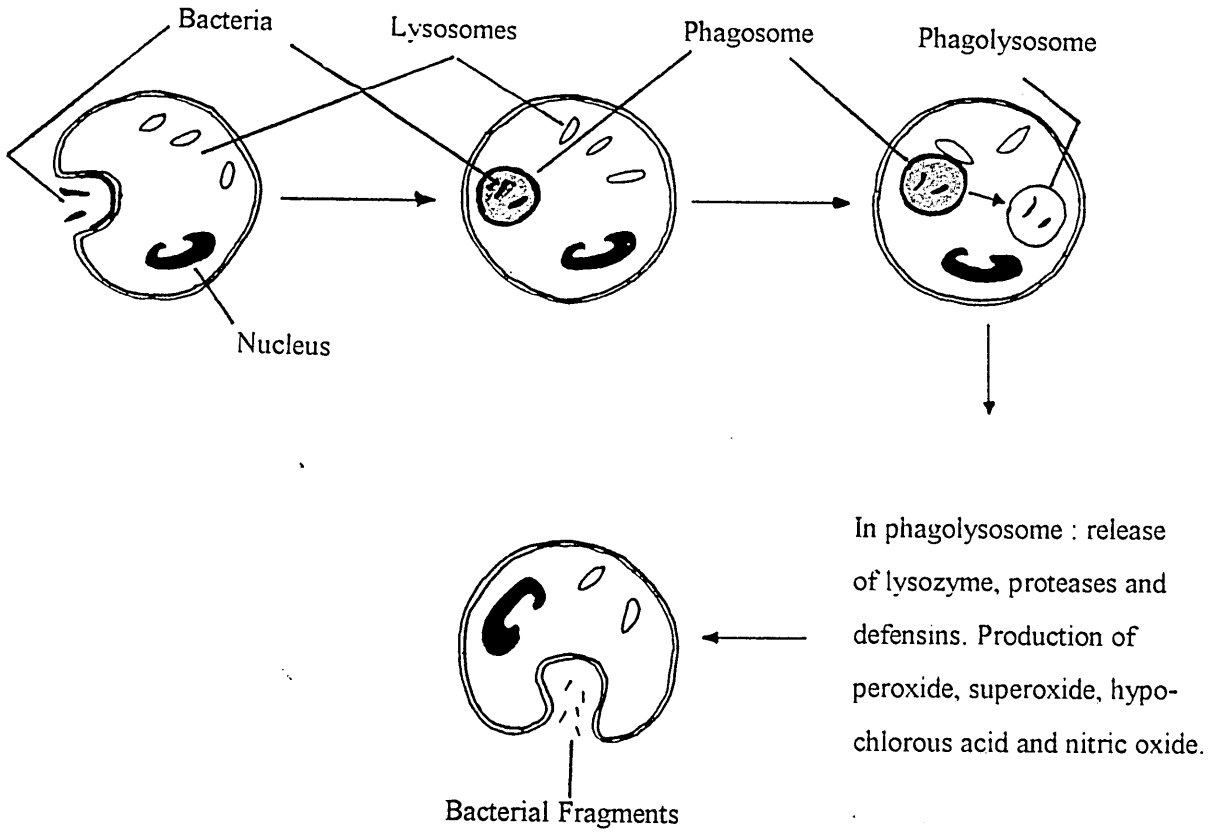
Intracellular bacteria are a group of pathogens which have the capacity to enter host cells and replicate inside them. Whilst some of these microorganisms reside in the intracellular milieu only transiently, others, such as *Chlamydia* sp., spend most or all of

their life inside host cells. These obligate intracellular bacteria have become so accustomed to their intracellular habitat that they can no longer survive in the extracellular environment (Schachter, 1988). Upon infection of humans, the preferred habitat of most intracellular bacteria are the professional phagocytic cells but many are also capable of entering non-professional phagocytic cells (Goebel, 1988). In order to enter the latter cells successfully, bacteria have evolved a specific invasion mechanism. These bacteria express surface proteins called 'invasins', that provoke endocytic uptake by non-professional phagocytic cells. These proteins have been identified on the surfaces of bacteria such as *Yersinia* sp. and *Salmonella* sp. (Goebel, 1988 and Miller *et al.*, 1988).

L. pneumophila is a ubiquitous organism not possessing a mammalian reservoir, but which has evolved the capacity to cause human disease (Barker and Brown, 1995). The *Legionella* sp. are unusual in that they are one of only a small number of bacterial species which can infect and replicate within free living amoebae as well as invading and replicating in human phagocytic cells. Other bacteria reported as exhibiting similar dual replicative capacity include *Mycobacterium* sp. (Harf, 1993 and Steinert *et al.*, 1998) and *Listeria monocytogenes* (Ly and Muller, 1990a; 1990b and Harf, 1993). *L. pneumophila* is also unusual in its lack of specificity of host cell for replication. Rowbothom (1986) first reported that *L. pneumophila* can replicate in species of *Hartmannella, Acanthamoeba* and *Naegleria.* Fields (1996) extended this list to include 13 species of amoebae and two species of ciliated protozoa. Amoebae are mainly aquatic organisms but they have also been isolated from a variety of sources which at some time or another have been in contact with water, including soil, sewage, compost and dust in the air. Any of these sources may contain amoebae infected with legionellae. Other bacteria have been reported as surviving as parasites or endosymbionts of free living amoebae, a number of which are mammalian pathogens. Among these *Vibrio cholerae* (Thom *et al.*,. 1992), *L. monocytogenes* (Ly and Muller, 1990a and 1990b) and the more recently recognised nosocomial pathogens *Pseudomonas* sp., *Xanthamonas maltophilia* and *Flavobacterium* sp. (Harf and

Monteuil, 1989 and Monteuil *et al.*, 1992). *Edwardsiella tarda* and *Aeromonas salmonicida* are reported as being capable of replicating within the ciliated protozoa *Tetrahymena pyriformis* (King and Schotts, 1988).

With a few possible exceptions, bacteria are taken up into human phagocytic cells and amoebae by the process of endocytosis. Three major mechanisms of endocytosis have been identified; classical phagocytosis, coiling phagocytosis, and receptor-mediated endocytosis. The process of classical phagocytosis is presented schematically in Figure 1.4. Briefly, bacteria are enveloped by the host cell and are taken up into a vacuole or phagosome. In most instances, this step is followed by fusion of the lysosomes with the phagosome to form a phagolysosome. This initiates the release of acidic toxic products that kill most bacteria and degrade them into fragments (Salyers and Whitt, 1994). A small number of bacteria enter the human phagocytic cell by a novel process of endocytosis called coiling phagocytosis. These bacteria include *L. pneumophila* (Horwitz, 1984) *Chlamydia psittaci* (Wyrick and Brownridge, 1978), *Trypanosoma brucei* (Stevens and Moulton, 1978) and *Borellia burgdorferi* (Szczespanski and Fliet, 1978). This process is summarised in Figure 1.5. Very briefly, the presence of a bacterium in the vicinity of a phagocyte stimulates the potential host cell to develop a pseudopod. Following attachment of the bacterium to the pseusdopod, the pseudopod coils around the bacterium drawing it into the host cell. Eventually, the multi-layered coil becomes a single enclosed phagosome within the host cell. Once this stage has been reached, the pathway for the fragmentation of the bacteria is similar to that of classical phagocytosis. The nature of the stimulus for pseudopod production by a potential host cell and entry of the host cell by coiling phagocytosis is not fully understood, but is possibly related to bacterial surface antigens. Horwitz (1984) showed that coating *L. pneumophila* with antibody against the organism (opsonisation) neutralises the coiling phagocytosis phenomenon. However, the organisms still enter the host cell but via classical phagocytosis.



Bacteria are first engulfed by classical phagocytosis into a phagosome. Fusion of phagosomes and lysosomes releases toxic products that kill most bacteria and degrade them into fragments. Debris from dead bacteria is then released by exocytosis.

Figure 1.4 Process of Classical Phagocytosis.

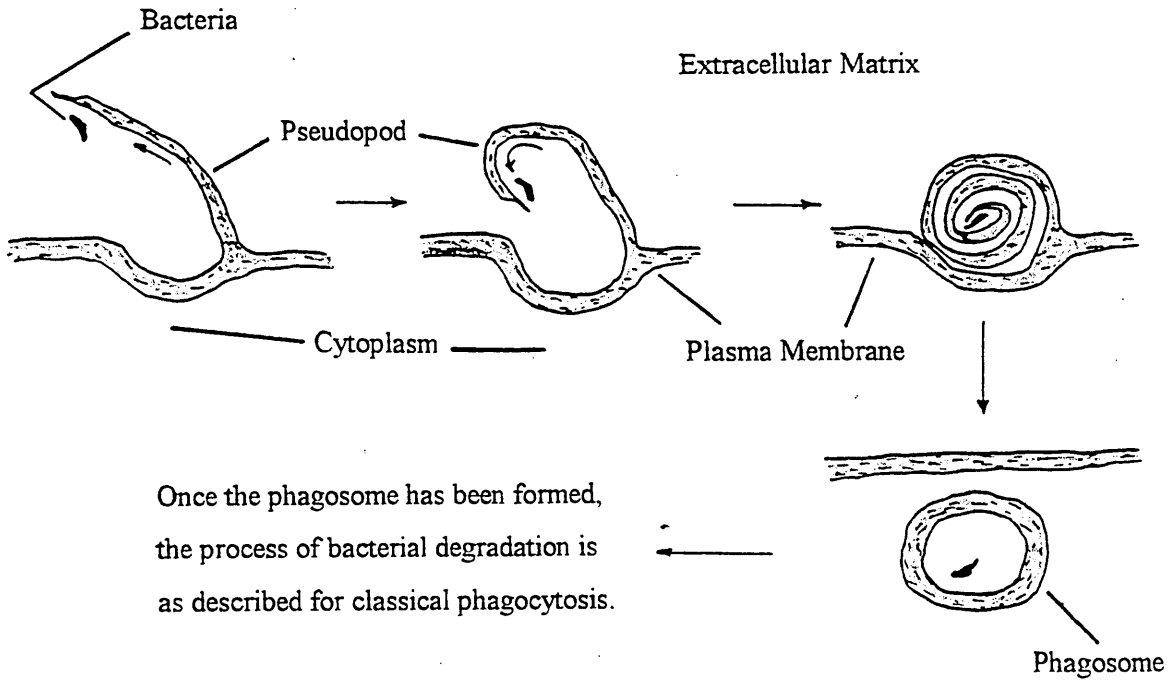


Figure 1.5 Process of Coiling Phagocytosis

The third mechanism of endocytosis, receptor-mediated endocytosis, has been associated with the uptake of a small number of bacteria by amoebae. These include *L. pneumophila* (Fields, 1996), *C. psittaci* (Hodinka and Wyrick, 1986) and *L. micdadei* (Abu Kwaik *et al.*, 1998). This process is outlined in Figure 1.6. The surface membrane of amoebae contains areas of protein-coated pits, in which are located surface receptors. The pits continually pinch off to form coated vesicles. Any bacteria attached to the receptors would thus be internalised as the phagosome is formed. Once formed, the phagosome fuses with a lysosome which triggers the release of acidic toxic products that kill and degrade most bacteria.

Intracellular bacteria are a group of organisms that have evolved mechanisms for avoiding degradation within the host cell. The majority of bacteria use one of two avoidance mechanisms; escape from the phagosome or inhibiting the fusion of the phagosome with lysosomes. The former mechanism is utilised, for example, by Gram negative bacteria such as *Shigella flexneri* (Sansonetti *et al.*, 1986) and *Rickettsia prowazekii* (Winkler and Turco, 1988) and the Gram positive bacterium *L. monocytogenes* (Frehel *et al.*, 1986) which escape from the phagosome into the host cell cytoplasm by degrading the phagosomal membrane. The bacteria then replicate using the host cell nutrients in the less hostile cytoplasmic environment. A greater number of intracellular bacteria have, however, evolved mechanisms to prevent digestion by inhibiting the fusion of the phagosome with the endosomal and lysosomal compartments, thus preventing acidification and fragmentation (Frehel *et al.*, 1986 and De Chastellier *et al.*, 1993, 1995). This bacterium defence mechanism appears to be triggered by the phagosomal environment. Bacteria utilising this mechanism of intracellular survival include *Mycobacterium avium, Sarcobium lyticum* and *L. pneumophila. Chlamydia* sp. and *Coxiella burnetti* are exceptions to this generalisation (Hackstadt and Williams, 1981). *Chlamydia* sp. remain within a phagosome throughout the entire life cycle after entering the host cell with the entire complement of proteins required to inhibit phagolysosomal fusion. In contrast,

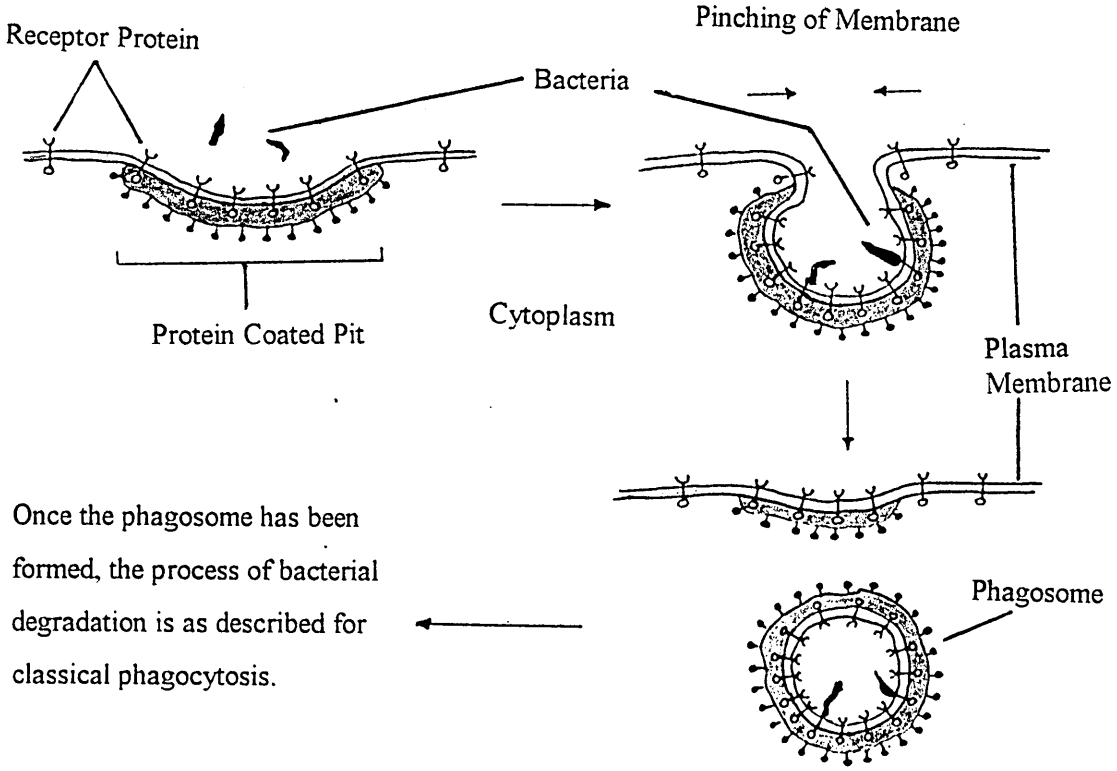


Figure 1.6 Process of Receptor-Mediated Endocytosis

C. burnetti use the acidification process brought about by phagolysosomal fusion as a trigger for intracellular replication. A very small group of bacteria have evolved the capability of replication in more than one host cell type.

Legionella sp., *Mycobacterium* sp., and *L. monocytogenes* are all reported as replicating within both amoebae and human phagocytic cells (Pearlman *et al.*, 1988; Ly and Muller, 1990 and Steinert *et al.*, 1998). Fields (1996) suggested that the only known differences between the infection of amoebae and mammalian cells are the processes associated with the early stages of these infections. The intracellular life cycle of *L. pneumophila* in both amoebae and human phagocytic cells is summarised in Table 1.1 (After Fields, 1996).

	Amoebae	Human Phagocytes
Entry	170 kDa Gal or Gal/NAc lectin receptor	CR1 / CR3 complement receptors.
	Microfilament-independent uptake (receptor mediated endocytosis)	Microfilament-dependent uptake (coiling phagocytosis)
	Requires host cell protein synthesis	Does not require host cell protein synthesis
Multiplication	No phagosome / lysosome fusion	
	Phagosome containing bacteria fuse with host cell endoplasmic reticulum	
	Bacteria become motile inside host cell	
Release	Bacteria lose motility within 24 h release from host cell.	

Table 1.1. Features of the infective process in amoebae and human phagocytic cells.

Recent publications suggest that the attachment and invasion of amoebae by *L. pneumophila* is mediated by the presence of a 170 kDa Gal or GalNAc lectin receptor on the surface of the host cell (Venkataraman *et al.*, 1997). In addition, the authors claim that attachment and invasion are associated with tyrosine dephosphorylation of multiple amoebic proteins, including the 170 kDa Gal/GalNAc

lectin receptor. Attachment of *L. pneumophila* to human phagocytic cells has been shown to be mediated by a three component system, consisting of monocyte complement receptors CR1 and CR3, fragments of complement component C3 and the major outer membrane protein (MOMP) on the surface of *L. pneumophila*. C3 fixes selectively to MOMP by the alternative pathway of complement activation (Payne and Horwitz, 1987; Bellinger-Kawahara and Horwitz, 1990 and Marra *et al.*, 1990).

Differences in the mechanism of bacterial uptake by amoebae and macrophage were first reported by Elliot and Winn (1986). These authors suggested that *L. pneumophila* enters macrophage by classical phagocytosis and amoebae by a form of receptor-mediated endocytosis. Horwitz (1984); Oldham and Rodgers (1985); Halablab *et al.* (1990) and Swanson and Isberg, (1995), on the other hand, argue that *L. pneumophila* enter macrophage by the process of coiling phagocytosis. The mode of entry of the bacterium into amoebae is also subject to argument. Elliot and Winn (1986); Fields *et al.* (1993) and Abu Kwaik (1996) all argue that *L. pneumophila* enters amoebae by some mechanism other than coiling phagocytosis, possibly by a variant of receptor-mediated endocytosis. Bozue and Johnson (1996), on the other hand, claim to have observed the uptake of *L. pneumophila* by *A. castellanii* by the process of coiling phagocytosis.

As indicated previously, once a *L. pneumophila* bacterium has entered the phagosome of a human macrophage or amoeba, the process leading to replication is essentially the same. *L. pneumophila* has the ability to subvert the host cell phagosome processes at a very early stage of phagocytosis. To avoid acidification and fusion with a lysosome, the *L. pneumophila* in the phagosome recruit mitochondria, small vesicles and ribosomes to the outer surfaces of phagosome membrane shortly after uptake. The phagosome associates with the rough endoplasmic reticulum (RER) in the host cell after a lag period of 3-6 h (Horwitz, 1993 and Swanson and Isberg, 1995). This trafficking pathway results in a ribosome-studded vesicle known as the replicative phagosome. Formation of this compartment within either amoebic or human host cells appears to be

required for intracellular replication. Abu Kwaik (1996) suggested that it may contribute a rich source of nutrients for the replicating bacteria. The molecular mechanisms used by the bacteria to establish this unique structure within the host cells are as yet unknown. Once enclosed within the phagosome, the bacteria undergo active replication after an intial lag phase of 6-8 h post infection, but remain rod-shaped and non-motile (Rowbotham, 1986; Pruckler *et al.*, 1995, and Shuman *et al.*, 1998). This stage of replication has been observed to be followed by expansion of the infected phagosome to fill the host cell, the acquisition of directional motility by the bacteria, and a reduction in their size (Rowbotham, 1986 and Pruckler *et al.*,1995). The replication process has been shown to culminate in the rupture of the host cell and liberation of bacteria into the surrounding environment. On release from the lysed host cell, the bacteria are highly motile, small and rounded in shape. In the absence of further potential host cells, the bacteria undergo a phenotypic change such that approximately 24 h after release they have become non-motile, larger and rod-shaped. This phenotype is typical of *in vitro* grown legionellae (Rowbotham, 1986; Barker *et al.*, 1992 and Fields, 1996).

Genetic studies have identified a cluster of genes (encoding proteins) required for host cell infection and intracellular replication by *L. pneumophila* (Berger and Isberg, 1993 and Brand *et al.*, 1994). Studies have shown that *L. pneumophila* has a 24 kDa surface protein (Mip) which is required for full infection of both amoebae and human phagocytes (Cianciotto *et al.*, 1989; 1990a and Engleberg, 1989). Cianciotto *et al.* (1990a) demonstrated that mutants defective in *mip* exhibit reduced infectivity towards U937 monocytes and explanted human macrophages. The *mip* defective mutant has lower virulence than its isogenic parent as measured in guinea pigs after intracheal inoculation. Mip has been shown to be specific to *L. pneumophila*, but *mip*-like genes are found throughout the Family Legionellaceae (Cianciotto *et al.*, 1990b and Bangsborg *et al.*, 1991). Barker and Brown (1995) suggested that infection of amoebae and macrophage may have a common molecular basis and the ability of *L. pneumophila* to cause human diseases may be a consequence of its prior adaptation to intracellular

growth within environmental protozoa. Other genetic loci that confer and/or regulate the virulence of *L. pneumophila* have been reported. The intracellular multiplication (*icm*) locus is required for replication and for virulence in guinea pigs (Marra *et al.*, 1992). Adjacent to the *icm* locus is the *dotA* (defective for organelle trafficking) gene (Berger and Isberg, 1993 and Berger, 1994). This gene has been reported as encoding a protein which is an integral part of the *L. pneumophila* cytoplasmic membrane (Roy and Isberg, 1997). Roy and Isberg (1997) suggested that DotA works in association with other proteins to transmit a signal to the host cell to direct the trafficking of the cellular organelles in the phagosome. The proximity of the *icm* locus to the *dotA* gene on the *L. pneumophila* chromosome, and the similarity of intracellular growth defects resulting from mutations in either region, led Roy and Isberg (1997) to suggest that these genes may all encode a multi-protein system that is required for the establishment of a replication niche once the legionellae are ingested by human phagocytic cells.

It is possible that the expression of the *mip* gene, *dotA* gene and / or the *icm* locus may be affected by temperature. Coordinated regulation of virulence genes has been reported in a number of pathogens including *Shigella* and *Yersinia* sp., *V. cholerae*, *Bordetella pertussis* and *Staphylococcus aureus*. These bacteria respond to specific environmental stimuli such as temperature, pH, concentration of specific ions, osmolarity, or a combination of these factors.The isolation of *Legionella* strains from water systems operating between 6°C and 63°C indicates, however, that bacteria are capable of survival over a surprisingly wide temperature range (Fliermans *et al.*, 1981). Some environmental isolates have been shown to grow optimally at 30°C (Hoffman, 1984) and their temperature range for replication has varied from 20 to 43°C (Yee and Wadowsky, 1982; Wadowsky *et al.*, 1985 and Schulze-Robbecke *et al.*, 1987). Anand *et al.* (1983) showed acanthamoebae can kill and digest *Legionella* at 20°C, whereas at 35°C the same *Legionella* strains infect and kill the host cell. Mauchline *et al.* (1993) showed *L. pneumophila* to be significantly less virulent in animal pathogenicity tests after growth in chemostat cultures when the temperature was decreased from 37°C to 24°C. The loss of virulence was reversed when the temperature was returned to 37°C.

This phenomenon would also explain the emergence of *Legionella* sp. as pathogens only after the invention of man-made thermal habitats. Mauchline *et al.* (1994) suggested that, as the temperature of many water systems lies between 35°C and 45°C, these systems not only act as places for the growth and dissemination of legionellae but also may prime the organism for causing disease in humans.

The availability of nutrients can affect the growth of all bacteria, whether *in vitro* or within a host cell. The acquisition of iron has been reported as being essential for the growth of *L. pneumophila* as it serves as a cofactor for aconitase, superoxide dismutase and other enzymes (Steinman, 1992; Mengaud and Horwitz, 1993 and Hickey and Cianciotto, 1994). *L. pneumophila* have, therefore, evolved mechanisms to scavenge free iron or iron bound to the cellular chelators from the immediate environment. However, at present the scavenging mechanisms of *L. pneumophila* are unknown. Legionellae do not appear to have adopted widely recognised scavenging mechanisms used by other bacteria, such as the binding to, or utilisation of, transferrin or lactoferrin within the host cells. If insufficient iron is gained during intracellular replication then an iron-depleted phenotype is formed (Griffith *et al.*, 1983; Brown *et al.*, 1984 and Shand *et al.*, 1985). Such phenotypic changes have been reported to alter virulence characteristics, sensitivity to antibiotics and host cell defences (Anwar *et al.*, 1984; 1985; Brown and Williams 1985ab and Cochrane *et al.*, 1987). Barker *et al.* (1992) showed that *L. pneumophila* grown in an iron depleted chemically defined medium were markedly more resistant to biocide BIT but more susceptible to biocides PHMB and CMIT and than those grown in a standard chemically defined medium. The iron depleted bacteria were more susceptible to each biocide than the intra-amoebic grown legionellae. This would suggest that iron depletion alone is not the cause of the enhanced resistance to biocides of intra-amoebic grown *L. pneumophila*.

1.4 RESPONSE OF BACTERIA TO STRESS.

All pathogenic bacteria are subject to stress stimuli whether in their natural environment or within host cells. The specific physiological state of the bacteria greatly

influences their response to the stress stimuli (Dawes, 1984). Many environmental changes exert their effect via the proton motive force which influences the phenotypic response of the bacteria, including the growth rate (Konings and Veldkamp, 1980 and Kjelleberg *et al.*, 1987). It is well known that when organisms which are replicating exponentially encounter an adverse environment, a reduction in growth rate occurs and the bacteria enter stationary phase. The imposition of nutrient deprivation, whether *in vitro* or intracellularly, causes the physiology of the bacteria to adapt in a number of ways : (i) Usage of the nutrient is rationed within the bacterium by the use of alternative substrates, modification of the bacterial composition, and /or reduction in amounts of bacterial macromolecules containing such nutrients. (ii) Alteration of the bacterial surface occurs to increase the efficiency of uptake of the growth limiting substrate. (iii) The bacterial growth rate alters to the maximum possible within that environment (Brown *et al.*, 1990).

The intracellular environment is probably one of the most hostile any bacteria is likely to encounter (Rowbotham, 1986). Infection of a host cell by a bacterial parasite is a complex and dynamic interaction involving a series of recognition events and phenotypic alterations during which cells of both the host and microbe undergo a process of mutual recognition and adaptation. Upon phagocytosis, bacteria are simultaneously exposed to a number of stimuli. They respond to these by a dramatic alteration in gene expression (Buchmeier and Heffron, 1990; Sorger, 1991; Mekalanos, 1992; Abshire and Neidhardt, 1993 and Abu Kwaik *et al.*, 1993). To avoid, or to counteract, the adverse conditions encountered within the host cell, intracellular bacterial pathogens have developed a variety of strategies. Mechanisms utilised include escape from the phagosome into the cytoplasm, blockage of maturation of the phagosome, modification of the phagosome along the endosomal- lysosomal pathway, or the development of resistance to microbiocidal compounds. (Horwitz, 1983; Horwitz and Maxfield, 1984; Xu *et al.*, 1994; Clemens and Horwitz, 1995; Clemens, 1996 and Rathman *et al.*, 1996). An example of the first avoidance mechanism is found in the facultative intracellular pathogen *L. monocytogenes.* During replication within

the cytoplasm, no *L. monocytogenes* stress proteins are induced which suggests that the cytoplasmic micro-environment does not contain conditions adverse to these microorganisms. In contrast, other facultative intracellular pathogens such as *S. typhimurium, M. tuberculosis, Yersinia entercolitica, B. abortus* and *L. pneumophila* continue to reside within a phagosome. This is achieved by the synthesis of numerous proteins in response to the phagosomal micro-environment. It has been reported that at least 30 % of intracellular growth induced proteins expressed by *L. pneumophila* are also induced by one or more *in vitro* stress stimuli (Abu Kwaik *et al.*, 1993). However, none of the *in vitro* stress stimuli examined by these workers could mimic the intracellular environment in its entirety. It was suggested that the phenotypic alteration by intracellular pathogens is controlled by multiple regulons and is most probably a simultaneous response to a combination of stresses and other stimuli yet to be identified (Abu Kwaik *et al.*, 1997).

The literature contains a number of references to the response of *L. pneumophila* to external stress stimuli (Antibiotics- Chen *et al.*, 1993; Baltch *et al.*, 1995 and Onody *et al.*, 1997; Temperature - Stout *et al.*, 1986; Groothuis *et al.*, 1985 and Starvation - Pasko-Kolva *et al.*, 1991; 1992; 1993).

1.4.1. EXPOSURE TO ANTIBIOTICS.

Bacteria are capable of resisting, or avoiding, the action of antibiotics in a number of ways, the most important of which are :

(i) The formation of enzymes which inactivate the antibiotics.

(ii) The modification of the target site so that it is insensitive to the antibiotic.

(iii) The prevention of antibiotic entry to the target site. (Lambert, 1984).

Since early use of antibiotics, mechanism (i) has provided the greatest obstacle to the effective chemotherapy of infectious diseases. The most striking example is the production of β-lactamases. These are enzymes which inactivate penicillins and cephalosporins by hydrolysing their β-lactam ring (Salyers and Whitt, 1994). Other

enzymes are produced which also inactivate antibiotics by the addition of groups which destroy their antimicrobial activity. These include adenylating, phosphorylating and acetylating enzymes which are responsible for resistance to aminoglycosides (Salyers and Whitt, 1994).

Resistance mechanism (ii) has been recognised for many years, but has not posed a major therapeutic problem. A classic example of this mechanism is provided by pneumococci. These bacteria are resistant to sulphonamides, due to a decreased affinity of the target enzyme, tetrahydropteric acid synthetase, for these antibiotics (Salyers and Whitt, 1994). Mechanism (iii) is also quite common. The ability of Gram negative bacteria to resist antibiotics, which are effective against Gram positive bacteria, generally results from an inability of the antibiotics to cross the Gram negative outer membrane (Nikaido, 1976). With the exception of the β-lactams, the target sites of action of the major groups of antibiotics are intracellular. Any changes in cell wall composition which alter the rate of antibiotic penetration are likely to affect the sensitivity to antibiotics which act at intracellular sites. This is due to the fact that the antibiotic must first cross the cell wall before reaching the permease systems in the cytoplasmic membrane. The capability of all bacteria to alter the chemical composition of their cells in direct response to changes in nutrient availability and growth rate is well reported (Ellwood and Tempest, 1972 and Brown *et al.*, 1990).

The antibiotic treatment of diseases induced by intracellular bacteria is made more problematical by the very nature of the location of the bacteria. In most instances, an antibiotic must pass, unaltered, into the host cell phagosome and overcome any defences offered by the phagosome before it reaches the bacterium outer surface. The ideal antibiotic, therefore, is one which readily enters the phagocytic cell and is capable of accumulating rapidly to sufficient levels so as to exert an effective antimicrobial action on the target bacteria.The difficulty in achieving this objective probably accounts for the poor correlation between the results of *in vitro* testing and observed clinical efficacy (Barker and Brown, 1995). The difficulty of treating disease induced by many

of the intracellular pathogens is illustrated by Legionnaires' disease. Antibiotics such as lipid insoluble penicillins, cephalosporins and aminoglycosides (Stokes *et al.*, 1989) and tazobactam (Edelstein and Edelstein, 1994) are all effective against extracellular but not intracellular *L. pneumophila*. It has been suggested, that only drugs which can accumulate in phagocytes, such as the macrolides, quinolones and rifampicin can be considered effective against intracellular *L. pneumophila* (Barker and Brown, 1995). Recent observations by Barker *et al.* (1992), have suggested, however, that the problem of effective antibiotic treatment of Legionnaires' disease may be more complex than originally thought. These authors report that intra-amoebic growth enhances the resistance of the bacteria to biocides and suggests that this may be related to an observed change in phenotype. If *L. pneumophila* undergo a similar modification after replication in phagocytic cells, then bacteria within the cell may respond to antibiotics in a different manner to those grown *in vitro*.

Clinical experience since the outbreak of Legionnaires' disease in 1976 supports the choice of erythromycin, a macrolide, for the initial treatment process (Fraser *et al.*, 1977; Kirby *et al.*, 1980 and Bartlett *et al.*, 1986). A combination of rifampicin and erythromycin has been reported to be more effective in patients who are critically ill or severely immunocompromised (Macfarlane, 1987). Unfortunately , each antibiotic has undesirable side effects. New macrolides, such as azithromycin and clarithromycin, have been developed and have been shown to be possible alternatives to erythromycin if the patient is able to take oral therapy (Edelstein, 1993). The literature also contains a number of reports of the successful treatment of Legionnaires' disease by the use of fluroquinolones (Winter *et al.*, 1988; Unertl *et al.*, 1989 and Chidiac and Mouton, 1991). These antibiotics are bactericidal against *L. pneumophila*, unlike erythromycin (Barker and Farrell, 1990) and clarithromycin (Edelstein, 1993), which are only inhibitory in their action. It is not surprising, therefore, that Horwitz and Silverstein (1983) observed the regrowth of *L. pneumophila* in infected phagocytes after the removal of erythromycin from the extracellular matrix. Winter *et al.* (1988) suggested that ciprofloxacin, a quinolone, in combination with erythromycin may favourably

affect the outcome of severe Legionnaires' disease. There have also been encouraging results with the use of perfloxacin and levofloxacin. Dournon *et al.* (1990) found that treatment with perfloxacin alone may be as effective as combined therapy with erythromycin and rifampicin for patients with severe legionellosis. Levofloxacin is a new fluoroquinolone with enhanced antibacterial activity against *L. pneumophila* and excellent penetration of human phagocytic cell (Baltch *et al.*, 1995 and Smith *et al.*., 1997). The inhibitory activity of levofloxacin is greater than that of ciprofloxacin, except against members of the family Enterobacteriaceae and *P. aeruginosa*. Ciprofloxacin is still the more active against these bacteria (Takasuna *et al.*, 1992).

Due to the serious nature of Legionnaires' disease and the requirement for the prompt initiation of antimicrobial therapy it is important that more accurate assessments of antibiotic efficacy against *L. pneumophila* are made. The onset of replication within human phagocytic cells occurs very soon after the initial inhalation of a contaminated aerosol. It is probable that by the time the drug therapy commences the bacteria will possess the intracellular phenotype. A search of the literature suggests that all the information on antibiotic efficacy has been gained by examining the ability of antibiotics to kill *L. pneumophila* remaining within phagocytes or those grown *in vitro*. It is not clear from these reports whether failure of the antibiotics to kill the intracellular bacteria arises from an inability of the antibiotic to penetrate the phagocyte, a failure to accumulate sufficiently to kill the bacteria, or a possible enhanced resistance of the legionellae. As far as it is known, there have been no published studies on the antibiotic susceptibility of intracellular grown legionellae freed from the host cell without prior subculture.

1.4.2. BACTERIAL SURVIVAL AT ELEVATED TEMPERATURES.

Legionella sp. are frequent contaminants of potable and non-potable waters and are particularly associated with domestic hot water systems (Fliermans *et al.*, 1981; Dennis *et al.*, 1984 and Ciesielski *et al.*, 1984). Water held in pipes between a circulating supply and an outlet (dead-legs) can provide a suitable environment for the

proliferation of legionellae which, when disseminated from taps and showers as aerosols, have been linked with Legionnaires' disease (Dufour and Jakubowski, 1982; Stout *et al.*, 1982; Wadowsky *et al.*, 1982; Bartlett and Bibby, 1983; Meenhorst *et al.*, 1983; Bartlett *et al.*, 1984 and Neill *et al.*, 1985).

The identification of hot / warm water systems as the amplifying stage and the dissemination of *L. pneumophila* led to considerable effort in quantifying the thermal resistance of the bacteria and developing methods for its eradication. *Legionella* have been isolated from waters with temperatures between 6 and 63°C (Fliermans *et al.*, 1981 and Habich and Muller, 1988). The growth of legionellae has been shown to be temperature dependent, replication is optimal between 30 and 45°C (Plouffe *et al.*, 1983; Vickers *et al.*, 1987; Lee *et al.*, 1988 and Zacheus and Martikainen, 1996). All legionellae die rapidly on exposure to high water temperatures (Dennis *et al.*, 1984; Groothuis *et al.*, 1985 and Stout *et al.*, 1986). It is widely known that water systems contain other bacteria, in addition to legionellae. The literature appears to contain only one systematic comparative study of the temperature resistance of *L. pneumophila* relative to that of other waterborne bacteria. Dennis *et al.* (1984) compared the survival of *Legionella* to that of coliforms, *Micrococcus* sp., *Pseudomonas* sp. and *Sarcinia* sp. at temperatures between 46 and 58°C. Marked differences in heat susceptibility of the various bacteria were only observed in the upper region of the temperature range. At 54°C *Legionella* was more resistant than the coliforms, *Micrococcus* sp., and *Pseudomonas* sp. but was markedly more susceptible than the *Sarcinia* sp. At 58°C, the *Micrococcus* sp. was twice as resistant as *L. pneumophila*. In a general review, Lee and West (1991) comment that the majority of the Gram negative heterotrophic bacteria found in water systems are much more heat sensitive than *Legionella* strains.

As far as it is known, the literature contains only one direct reference to the effect of heat stress on the ultrastructure of *L. pneumophila*. Harley *et al.* (1997) reported that prolonged exposure of *L. pneumophila* to 50°C for 30 min resulted in a distortion of

shape and thickening of the cell walls of the bacteria, probably due to shrinkage of the cytoplasm. The vacuoles were reported as being intact and unchanged in size and shape.

Several groups of workers have approached the question of survival of *L. pneumophila* in water at high temperatures in a more practical manner by performing field studies or using "model" hot water systems (Groothuis *et al.*, 1985; Stout *et al.*, 1986; Farrell *et al.*, 1990; Makin and Hart, 1991; Haldane *et al.*, 1993 and Zacheus and Martikainen, 1996). Groothuis and Veenendaal (1983) reported that 25 % of *L. pneumophila* survived after 1 h exposure at 50°C. No bacteria survived when the temperature was raised to 55°C. Fliermans *et al.* (1981) also reported that *L. pneumophila* rapidly loses viability on exposure to temperatures above 50°C, but surprisingly reported that the bacterial electron transport system is still active at 60°C. Many workers reported that *L. pneumophila* cannot be completely eradicated by flushing the system with hot water at 70°C because of 'dead ends' in the pipe network which are not reached by the hot water (Groothuis *et al.*, 1985; Farrell *et al.*, 1990; Makin and Hart, 1991 and Zacheus and Martikainen, 1996). The HSE Approved Code of Practice for the operation of large scale hot water systems recommends that hot water should be stored at a minimum of 60°C, and the returning water system maintained at above 50°C to prevent colonisation by legionellae. There have been several reports of systems where the re-emergence of legionellae has occurred when the water temperature has been allowed to return below 50°C (Groothuis *et al.*, 1985 and Farrell *et al.*, 1990). There is evidence, however, that failure to maintain the water at the recommended temperatures is not the sole cause of the re-emergence of *L. pneumophila* in a previously decontaminated system. Many genera of free living protozoa are capable of forming cysts which are resistant to extremes of temperature, dessication and disinfection (Biddick *et al.*, 1984). For example, *A. polyphaga* cysts are capable of surviving exposure to temperatures above 60°C (Biddick *et al.*, 1984). *L. pneumophila* within encysted amoebae may be capable of recovery and replication when the amoebae come out of encystment under more favourable conditions. Similarly, Kilvington and Price (1990) have shown that

L. pneumophila within cysts of *Acanthamoeba polyphaga* can survive exposure to 50 mg/l of chlorine. Navratil *et al.* (1990) also reported that *L. pneumophila* released after growth in *Hartmannella vermiformis* are more resistant to subsequent exposure to chlorine. This is in general agreement with findings of Barker *et al.* (1992) who showed that intra-amoebic grown *L. pneumophila* are more resistant to biocides than *in vitro* grown bacteria. There is strong evidence, therefore, that the continued re-emergence of *L. pneumophila* in water systems is a consequence of the failure to eradicate infected amoebae from the system, and not a failure to kill planktonic bacteria. A second possible source of "re-infection" of water systems arises from the presence of biofilms. Biofilms are a complex matrix in which a large variety of bacteria and other microbiota co-exist. They are found in many water systems and their development is related to the intrinsic biological activity of aerobic and anaerobic bacteria, as well as water quality. States *et al.* (1993) suggested that *L. pneumophila* may survive extreme temperatures by association with biofilms. This was confirmed by Rogers *et al.* (1994a and 1994b) who found that hot water systems operating at 50°C may contain a reservoir of viable *L. pneumophila* in biofilms along with a supporting population of micro-organisms which appeared to enhance the survival of the legionellae.

1.4.3 EFFECT OF MECHANICAL STRESS ON SURVIVAL

The cell wall of *Legionella* sp. display an ultrastructure typical of Gram negative rods (Flesher *et al.*, 1979). Mechanical strength and cell shape are determined by the peptidoglycan layer (Lambert, 1988a). In most Gram negative bacteria the peptidoglycan is anchored to the OM by covalently linked low molecular weight lipoproteins (Brown, 1975). Amano and Williams (1983) reported that the peptidoglycan layer associated proteins are more complex in *L. pneumophila* than in other bacteria, and fall into two categories. The first group consists of non-covalently (loosely) associated proteins. The second group are more tightly bound. The authors suggested that some of the latter group of proteins may be linked by disulphide bonds. The role of these linking proteins in maintaining the structural rigidity of

L. pneumophila is unclear. However, Hatch *et al.* (1984) and Newhall and Jones (1983) reported that cysteine-rich proteins linked by disulphide bonds to each other, and other proteins, maintain the structural rigidity of *Chlamydia* sp.. Major changes occur when the chlamydiae transform from elementary (round) bodies to reticulate (rod-shaped) bodies which are thought to represent a reduction in the number of disulphide links within the outer membrane, and by implication a reduction in rigidity. Weichart and Kjelleberg (1996) have taken this hypothesis further by utilising disintegration by ultrasonic vibration to examine the effect of growth conditions on the mechanical strength and rigidity of *Vibrio vulnificus.* The authors reported that starved bacteria with a small, rounded morphology were more resistant to mechanical stress imposed by ultrasonic vibration than those which were actively growing.

1.4.4 EFFECT OF NUTRIENT DEPRIVATION ON SURVIVAL.

The environments of most bacteria in nature are generally very different from those of the laboratory, and oligotrophic conditions are more the norm than the exception. Fluctuations in nutrient availability can induce many bacteria to undergo a transition from balanced growth to unbalanced, or complete cessation of growth, and *vice versa.* The response of bacteria to nutrient flux has been addressed during the last decade, but is still a controversial issue. Early studies simulating the nutrient availability in 'natural' environments showed many bacteria to adopt a morphology somewhat different to that arising from standard laboratory cultures. These bacteria were very small and rounded relative to their counterparts grown in nutrient rich culture media, and were regarded as being 'nutrient stressed' or 'starved'. Many workers reported them as developing a 'normal' morphology on the provision of adequate nutrients (Conn, 1948; Casida, 1965; Ensign, 1970 and Hurst, 1977). Dawson *et al.* (1981) termed them 'dwarfs' and considered them as merely another form of 'normal' bacteria. MacDonnell and Hood (1982) urged caution pointing out that many bacteria in the natural environment were 'dwarfs' and that this may be the common form of many bacteria. *Legionella* sp. are typical of such bacteria. Ubiquitous in water, legionellae are found primarily with a

small, rounded morphology and only adopts the so-called 'normal' form of non-motile rods when cultured in complex media under highly specific conditions in the laboratory.

The precise morphological and physiological state of bacteria in the natural environment is subject to debate. Sieburth (1979) suggested that the small, rounded forms found in freshwater environments are bacteria in the process of adaptation from a nutrient rich environment arising from agricultural run-off, sewage and similar sources, to an oligotrophic environment. The author suggested that the adaptation arises from an inability of the bacteria to sustain 'normal' rates of metabolism in the oligotrophic environment. Novitsky and Morita (1978) on the other hand, suggested that a stress response resulting in ultramicrobacteria is an important feature of the survival strategy of many truly native bacteria. It is quite possible that both arguments are correct. Nutrient availability in the majority of natural water environments would be expected to be in a state of dynamic flux. It would not be surprising, therefore, if the bacterial morphology and physiology follows a similar pattern of flux to that of nutrient availability.

Inspite of the obvious difference between the morphological and physiological characteristics of many bacteria in their natural environment and laboratory cultures, the subject of starvation response has been studied only in recent years. In a major review in 1983, Kjelleberg *et al.* suggested that the process of ultramicrobacteria formation or 'dwarfing' contains two steps :

(i) 'Dwarfing' *per se*, which occurs over the first four to five hours during which there is obvious metabolic activity and no loss of viability. This process was reported as containing two distinct phases, fragmentation of the bacteria over the first one to two hours, giving rise to an increase in cell numbers, and continuous size reduction of fragmented cells, but no further increase in bacterial numbers.

The intervals suggested by Kjelleberg *et al.* (1993) appear to have been derived entirely from marine bacterial studies. Whilst it is highly probable that the freshwater bacteria undergo a similar process, the suggested time periods may be different.

(ii) Starvation, which occurs after the dwarfing phases, is reported as being a period of continuing size reduction with little or no metabolic activity and a slow loss in viability. The latter aspect has received considerable attention in recent years and has given rise to considerable debate concerning the concepts of dormancy and viable but non-culturable (VBNC) bacteria.

The problem of ultra-low metabolic activity in bacteria subject to long term starvation has also been considered at length by Kapreylants *et al.* (1993). These workers proposed the existence of 'dormant' cells, and defined them as being "cells which have a reversible state of low metabolic activity such that the cells can persist for extended periods without division". The basic argument for the widespread existence of vegetative , non-sporulating bacteria in the dormant form is that "in most natural microbial environments only a very small fraction of the bacteria present can be enumerated using agar plate technique" (Mason *et al.*, 1986). Discrepancies between the total and viable count have been widely reported for bacteria in soil (Bakken and Olsen, 1987 and Morita, 1988) and water (Roszak and Colwell, 1987; Morita, 1988 and Barer *et al.*, 1993). This phenomenon has also been observed in Gram negative bacteria during laboratory investigations of their progressive starvation. It has been suggested that ultramicrobacteria are dormant forms of non-sporulating bacteria and as such are not able to produce colonies on the surface of agar (Stevenson, 1978 and Morita, 1988).

A further example of 'vegetative dormancy' is the formation of 'viable but non-culturable' (VBNC) forms of some Gram negative bacteria. Bacteria in this state cannot be recovered by routine isolation methods. Barer *et al.* (1993) describe organisms in the VBNC state as non-culturable bacteria which can yield culturable cells

under certain conditions. These authors distinguished between VBNC bacteria and cells which are non-culturable but retain certain assayable activity. These were termed active but non-culturable (ANC).

A variety of techniques have been used to demonstrate the VBNC state in Gram negative bacteria of the genera *Vibrio* (Xu *et al.*, 1982 and Colwell *et al.*, 1985), *Escherichia* (Xu *et al.*, 1982) *Salmonella* (Roszak *et al.*, 1984), *Aeromonas* (Allen-Austin *et al.*, 1984), *Legionella* (Hussong *et al.*, 1987), *Campylobacter* (Rollins and Colwell, 1986) and *Shigella* (Colwell *et al.*, 1985). Advances in microscopical counting techniques are such that the direct counting technique of Kogure *et al.* (1979) is no longer used. More recently, bacteria in the VBNC state have been enumerated by DNA hybridisation (Hay *et al.*, 1995) and a variety of monoclonal-linked and fluorescent stains. Due to the limitations of the above techniques, the phenomenon of VBNC has not been investigated in Gram positive bacteria. The evidence suggesting that ultramicrobacteria and VBNC cells are dormant is rather ambiguous, and a constant topic for debate. This is especially true as the dormant state is a 'grey area' between what is normally considered 'alive' and what is 'dead'.

By definition, to describe a bacterium as being in a dormant state, one must prove the bacteria of interest cannot grow under the current incubation conditions, but is capable of replication under more appropriate conditions. A number of studies have indicated that the VBNC cells can start to multiply on solid media after the application of a resuscitation procedure. A variety of mechanisms have been used to resuscitate bacteria, many of which are controversial. The main problem is establishing that all the bacteria are truly non-culturable, and that it is not a few culturable cells which are responsible for regrowth once conditions have improved. Colwell *et al.* (1985) used the passage of VBNC Gram negative bacteria through an animal ileal loop for resuscitation. Roszak *et al.* (1984) described the resuscitation of *S. enteritidis* starved for 4 d by incubation in liquid nutrient medium. However, data on the total counts was not reported and resuscitation after 21 d of starvation was not possible. It is therefore

possible that the resuscitated bacteria were not all truly VBNC. MacDonnell and Hood (1982) have also described the resuscitation in dilute nutrient broth but showed that the concentration range which allowed regrowth was rather narrow. Other methods have involved temperature shifts (Colbourne and Dennis, 1989 and Nillson *et al.*, 1991). Colbourne and Dennis (1989) have described the successful resuscitation of *L. pneumophila* from the VBNC state by raising the temperature from 37°C to 45°C for 10 min. The results are disputed by West *et al.* (1993) who reported the failure of this technique to resuscitate *L. pneumophila* in their studies. The existence of bacteria in the VBNC state and the resuscitation of these bacteria is of great importance in the monitoring of pathogens in the environment and has been debated extensively; but, as yet, no firm conclusions have yet been drawn.

1.5 AIMS.

Following the original outbreak of Legionnaires' disease in 1976, considerable effort has been directed towards understanding all aspects of the causative agent, the bacterium *L. pneumophila*. The importance of growth and morphology in determining the physiological characteristics of the bacterium has only recently been recognised. The aims of this thesis were to gain more knowledge of the influence of phenotype on the response of *L. pneumophila* to external stress stimuli. The aims of this work were :

1. To investigate the growth of *L. pneumophila in vitro* and intracellularly within amoebae and monocytes.

2. To investigate the influence of phenotype on the resistance of *L. pneumophila* to various stress stimuli.

3. To carry out an initial investigation of the surface properties of variously grown *L. pneumophila* in order to study the physiological basis of *Legionella* stress resistance.

CHAPTER 2. MATERIALS.

2.1 ORGANISMS.

2.1.1 BACTERIUM.

A virulent strain of *Legionella pneumophila* serogroup 1 (subgroup Knoxville) was used throughout this project. This strain was the causative organism of an outbreak of Legionnaires' disease in Stafford, England (Badenoch, 1986). It was obtained from the *Legionella* Reference Laboratory, Central Public Health Laboratory, Colindale, London, UK and was stored on polystyrene beads at -70°C (Barker *et al.*, 1986). The bacteria were cultured by aseptically transferring a bead into 50 ml of yeast extract (YE) broth and incubating with shaking (250 rpm) at 37°C for 48 h. to yield a suspension containing *ca.* 10^8 colony forming units per millilitre (cfu/ml). The strain was subsequently maintained by passaging in YE broth or by growing on Buffered Charcoal Yeast Extract (BCYE) agar at 37°C.

2.1.2 AMOEBAE.

A strain of *Acanthamoeba polyphaga* was kindly donated by Dr. T. Rowbotham, Public Health Laboratories, Leeds, UK. This strain of amoebae was associated with an outbreak of Legionnaires' disease in the UK, the causative agent was *L. pneumophila* serogroup1. The amoebae were stored in flat bottomed tissue culture flasks with vented caps (Becton and Dickinson (UK) Ltd, Oxford, UK) containing peptone-yeast extract glucose (PYG) broth (Section 2.2.3). The flasks were stored in the dark at room temperature and the amoebae allowed to encyst.

When amoebic trophozoites were required the encysted amoebae were used to seed a new flask containing PYG broth. The encysted amoebae were incubated at 37°C in the dark and a monolayer of amoebic trophozoites allowed to form. The growth of the cells was monitored daily using phase contrast microscopy. When the monolayer of amoebae was confluent on the surface of the flask, the medium was decanted and fresh

broth added aseptically. The amoebae were further incubated at 37°C in the dark. This process was repeated every 2 to 3 d. to maintain the cells in a viable state. Incubation in the dark was necessary as experience showed the monolayers failed to form when the amoebae were exposed to light.

2.1.3 MONOCYTIC CELLS.

Undifferentiated U937 monocytic cells, which are derived from a human histiocytic lymphoma cell line, have been reported to support intracellular growth of *L. pneumophila* (Pearlman *et al.*, 1988 and Steinert *et al.*, 1994). U937 cells were obtained from the European Tissue Culture Centre, Centre of Applied Microbiology and Research, Porton Down, UK. The cells were grown in vented flat bottomed tissue culture flasks (Becton and Dickinson) in a non-adherent and replicative form. The medium used was HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) Roswell Park Memorial Institute 1640 (RPMI) medium (Sigma, Poole, UK), supplemented as described in Section 2.2.4. After incubation at a temperature of 37°C in an atmosphere of 5 % carbon dioxide for a period of 3 to 4 d. the cell number was reduced by pouring away the suspended cells and adding fresh medium to the few cells remaining in the flask. These were subsequently allowed to grow and replicate for a further 3 to 4 d., or until the cells were confluent.

Long-term storage of the cells was achieved by centrifugation of 50 ml of cell suspension (*ca.* 10^6cells/ml) at 800 x g for 6 min.. The resulting pellet was resuspended in 6 ml of cold storage medium which consisted of 90 % (v/v) of foetal bovine serum and 10 % (v/v) dimethyl sulphoxide (DMSO). The resulting cell suspension was allowed to cool on ice for 1 h in a sterile cryovial (Bibby, Nemours, France) prior to storage in liquid nitrogen for a maximum of 3 yr.

2.2 PREPARATION OF UNDEFINED MEDIA.

2.2.1 BUFFERED CHARCOAL YEAST EXTRACT (BCYE) AGAR.

Charcoal containing CYE agar has been widely reported as an effective medium for the growth of *L. pneumophila* (Feeley *et al.*, 1979; Pasculle *et al.*, 1980; and Edelstein, 1981; 1982). The composition of BCYE agar used was as described by Edelstein (1981) and is given in Table 2.1

Component	g/litre
ACES*	10.0
KOH	2.2
α-ketoglutarate monopotassium salt	1.0
Yeast Extract	10.0
Fermtech Agar	10.0
Activated Charcoal	0.5
ddH_2O	to 1 litre

* ACES= *N* (2-acetoamido)-2-aminoethane sulphonic acid

Table 2.1 Composition of BCYE agar

The pH of the medium was adjusted to 6.9 +/- 0.05 using either 5 M HCl or 5 M KOH and autoclaved at 121°C for 20 min.. The addition of charcoal permitted the autoclaving of the agar as it removes free fatty acids and oxygen radicals which form during this sterilisation process (Hoffman *et al.*, 1983). Feeley *et al.* (1979); Hoffman *et al.* (1983); and Armon and Payment (1990), have reported that supplements of ferric pyrophosphate and L-cysteine are necessary for the effective growth of *Legionella* on this medium. Consequently, after cooling to 50°C, filter sterilised solutions of ferric pyrophosphate and L-cysteine (final concentrations of 0.0025 g/ml and 0.004 g/ml, respectively) were added just prior to the agar being poured into sterile triple-vented petri dishes.

To ensure that the quality of the agar was consistent, one plate from each batch was inoculated with ten-fold dilutions of a *L. pneumophila* suspension (*ca.* 10^5-10^6 cfu/ml). A BCYE agar plate from a previously tested batch was also inoculated by the same method and acted as a control. To confirm the purity of the prepared agar plates, a freshly poured plate was incubated at 37°C for 2 d. The prepared agar plates were stored at 4°C and were used within one month of production.

2.2.2 YEAST EXTRACT (YE) BROTH.

The composition of YE broth is similar to that of the BCYE agar described in Section 2.2.1 with the important exception that neither agar nor charcoal were added to the medium. The absence of charcoal enables the growth of *L. pneumophila* to be observed. Unfortunately, sterilisation by autoclaving in the absence of charcoal results in the release of toxic compounds which inhibit the growth of legionellae; possibly fatty acids (Hoffman *et al.*, 1983) or oxygen radicals (Pine *et al.*, 1979). The problem was overcome by filter sterilising the medium by passage through a 0.22 µm syringe filter (Schleicher and Schuell, Dassel, Germany) after the addition of the supplements, ferric pyrophosphate and L-cysteine (Johnson *et al.*, 1982). The medium was filtered into 50 ml sterile disposable tubes (Sarstedt, Leicester, UK) which were stored at 4°C until required. The medium was used within one month of production.

2.2.3 PEPTONE YEAST EXTRACT GLUCOSE BROTH (PYG).

A. polyphaga trophozoites were grown and maintained in PYG broth, the composition of which is given in Table 2.2

Component	g/l
Protease peptone	12.0
Yeast Extract	5.0
Glucose	10.0
Ferric Sulphate	0.003
Amoebic saline (details in section 2.4.1)	to 1 litre

Table 2.2 Composition of PYG broth

The pH was adjusted (if required) to 6.9 +/- 0.05 by means of 5 M HCl or 5 M KOH prior to sterilisation by autoclaving at 121°C for 20 min.. Once cooled the medium was stored at 4°C and used within one month of preparation.

2.2.4 ROSWELL PARK MEMORIAL INSTITUTE 1640 (RPMI) MEDIUM.

Undifferentiated U937 monocytic cells were maintained in HEPES RPMI 1640 medium (Sigma). The medium selected did not contain sodium bicarbonate as sodium ions are known to inhibit the growth of *L. pneumophila* and would, therefore, prevent intra-monocytic replication (Feeley *et al.*, 1979; Dennis *et al.*, 1981; Tesh and Miller, 1982; Weiss and Westphal, 1984; Barker *et al.*, 1986 and Lee and West, 1991). The medium was supplemented with 10 % (v/v) of heat inactivated foetal bovine serum and 1 % (v/v) 100 mM glutamine. The foetal bovine serum is an essential nutrient for the support of lymphoma cell growth. Heat inactivation was necessary to inactivate complement and remove destructive immunoglobulins. These cause the monocytic cells to excrete toxins which affect *L. pneumophila* survival. This was achieved by incubation at 56°C for 1 h. Glutamine acts as a high energy source (Sigma Technical Services, Poole, UK).

2.3. PREPARATION OF CHEMICALLY DEFINED MEDIUM.

The composition of the chemically defined medium (CDM) used during growth studies was that as described by Warren and Miller (1979). Details of the medium composition are given in Table 2.3.

Component	Final Concn. (µg / ml)
L-Alanine	500
L-Arginine.HCl	900
L-Asparagine	150
L-Aspartic Acid	1,000
L-Cysteine.HCl.H_2O	400
L-Cystine	75
L-Glutamic Acid	1,650
L-Glutamine	250
Glycine	1,350
L-Histidine.HCl	300
L-Isoleucine	555
L-Leucine	555
L-Lysine.HCl	750
L-Methionine	300
L-Phenylalanine	450
L-Proline	250
L-Serine	650
L-Threonine	450
L-Tryptophan	400
L-Tyrosine	75
L-Valine	600
KH_2PO_4	1,000
K_2HPO_4	4,000
NaCl	5,000
$MgSO_4.7H_2O$	10
$CaCl_2.7H_2O$	10
$(NH_4)_2SO_4$	2,000
$Fe_4(P_2O_7)_3$ (soluble)	250
$NaHCO_3$	420

Table 2.3 Composition of Chemically-defined Medium

The pH of the medium was adjusted to 6.9 +/- 0.05, using either 5 M HCl or 5 M KOH. The medium was filter sterilised through a 0.22 µm membrane (Schleicher and Schuell) into 50 ml sterile disposable sample tubes (Sarstedt) which were stored at 4°C for a maximum duration of one month.

2.4 SUSPENDING MEDIA

2.4.1 AMOEBIC SALINE.

Amoebic saline consisted of 1 % (v/v) stock A solution and 1 % (v/v) stock B solution in double distilled water (ddH_2O). The composition of stock A and stock B solutions are given in Table 2.4.

Stock A	g/litre
NaCl	12.0
$MgSO_4.7H_2O$	0.4
Na_2HPO_4	14.2
KH_2PO_4	13.9
Stock B	
$CaCl_2$	0.4

Table 2.4. Composition of Amoebic Saline

The amoebic saline was sterilised in sealed pyrex bottles by autoclaving at 121°C for 20 min. and was stored at room temperature.

2.4.2 PHOSPHATE BUFFERED SALINE (PBS).

PBS consisted of 137 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM potassium di-hydrogen phosphate and 8 mM di-potassium orthophosphate. Magnesium chloride (1 mM) and calcium chloride (1.8 mM) were added to the stock PBS to make tissue-culture PBS. This solution was used to resuspend *L. pneumophila* or host cells prior to injection of the rabbits used in the immunological studies.

2.4.3 DOUBLE DISTILLED WATER (ddH_2O).

Deionised, double distilled water was prepared using the Milli-Q RG system, (Millipore, Walford, UK). The metal ion composition of the water was kindly determined by H. Birtwistle using Inductively Coupled Plasma (ICP) analysis.

2.4.4 TAP WATER.

Tap water used during long-term survival studies was taken from the laboratory tap situated in the Owen Building at Sheffield Hallam University, Sheffield, UK. The metal ion content of tap water was again kindly determined by H. Birtwistle using Inductively-Coupled Plasma (ICP) analysis.

2.5 CHEMICALS.

Chemicals used throughout this project were obtained from BDH Chemicals Ltd. (Poole, UK), Sigma-Aldrich (Poole, UK), Fisons (Loughborough, UK), Merck (Lutterworth, UK) and Life Technologies, (Paisley, UK). AnalaR and Aristar grades, or equivalent, were used. Ciprofloxacin was obtained from Bayer Plc. (Slough, UK).

2.6 GLASSWARE.

All glassware, with the exception of gel plates, was machine washed using a mild detergent and thoroughly rinsed in ddH_2O prior to use. The glass bottles used during long-term survival studies were made ultra-clean by washing in 10 M HCl to remove any remaining residues followed by five washes in single distilled water and five washes in ddH_2O.

Gel electrophoresis plates were washed in ddH_2O before and after use. If the gel plates were used for the separation of lipopolysaccharide, they were immersed overnight in a tank containing 1 M NaOH. The soak in NaOH was necessary to destroy any proteinase K remaining on the glass plates from the previous separation. Residual proteinase K would degrade subsequent protein samples loaded on to a gel formed using the plates. The glass bowl used during the silver staining of lipopolysaccharide was washed in 10 M NaOH followed by thorough washing in ddH_2O to remove grease that would harm the gel. During the silver staining process the glass measuring cylinders were not machine washed but were cleaned using 10 M NaOH and copious amounts of ddH_2O.

CHAPTER 3. METHODS.

3.1 GROWTH STUDIES.

3.1.1 SPECTROPHOTOMETRIC MEASUREMENTS OF BACTERIAL GROWTH.

Optical density measurements are commonly used as a means of rapidly determining the concentration of a solution or a suspension. In essence, a beam of light can be shone through a known 'thickness' of liquid and the amount of light transmitted determined. Optical density (OD) is given by

$$OD = \ln x \frac{\text{(incident light intensity)}}{\text{(transmitted light intensity)}}$$

It can be seen from this relationship that the value of OD increases as the amount of light transmitted decreases. From the Beer-Lambert Law, it can be shown that OD is proportional to the concentration (C) of the solution or suspension. In practice, however, this relationship holds true only for low values of C (< 0.01 M) (Skoog and Leary, 1992). Skoog and Leary (1992) also reported that the value of C determined varies with the wavelength of the light used. Most workers in the field of *L. pneumophila* research report optical density measurements at a light wavelength of 660 nm (OD_{660}). A review of the literature, however, has failed to find a definitive reason for using this wavelength. In addition, there is increasing evidence that OD values >1 are unreliable due to instrument induced errors (Skoog and Leary, 1992). An investigation was, therefore, undertaken to determine whether light with a wavelength of 660 nm was the best choice for OD measurements of suspensions containing *L. pneumophila* . The concentration of *L. pneumophila* above which the Beer-Lambert relationship no longer holds true was also determined.

3.1.1.1 SCANNING SPECTROSCOPY.

A scanning spectrophotometer (Unicam UV2, uv/vis scanning spectrometer, Unicam, Cambridge, UK) was used to determine the optical density of various *L. pneumophila* cultures at all wavelengths between 400 and 750 nm. The samples were *L. pneumophila* cultures after 0, 8, 24, 48 and 96 h. of growth in YE broth at 37°C with shaking. The problem of background colouration was overcome by separating the bacteria from the supernatant by centrifugation at 4,400 x g for 10 min.. The resulting supernatant was then used to 'zero' the instrument prior to OD measurements being made on the corresponding culture. Thus any OD measurement made should be due to the bacteria alone. Typical results are shown in Figures 3.1 (a) and 3.1 (b).

It will be noted that the majority of OD values determined are greater than 1 and must, therefore, be considered unreliable. The above work was repeated after a 1 in 10 dilution of the bacteria in the respective supernatant. Typical results are shown in Figure 3.1 (c) and Figure 3.1 (d).

It was apparent from this work that acceptable OD values (<1) are obtained after a 1 in 10 dilution, provided that the wavelength of the light used was greater than *ca.* 600 nm. In view of this observation, and to conform to the choice of other workers in the field, it was decided that all OD measurements would be made at a wavelength of 660 nm.

3.1.1.2 DEVIATION FROM BEER-LAMBERT LAW.

As indicated previously, the Beer-Lambert Law ($OD \propto C$) relationship breaks down as the concentration of the solution or suspension increases above *ca.* 0.01 M. In addition, the relatively large physical size of bacteria can be expected to result in shadowing within the test solution. That is, some bacteria will effectively obscure others from the incident light. Thus giving rise to false OD readings. It was, therefore, necessary to determine the OD_{660} value above which the Beer-Lambert Law was no longer valid for *L. pneumophila* suspensions. A culture of *L. pneumophila* was serially diluted 1 in 2 using its supernatant as the diluent and the corresponding OD_{660} values measured. The

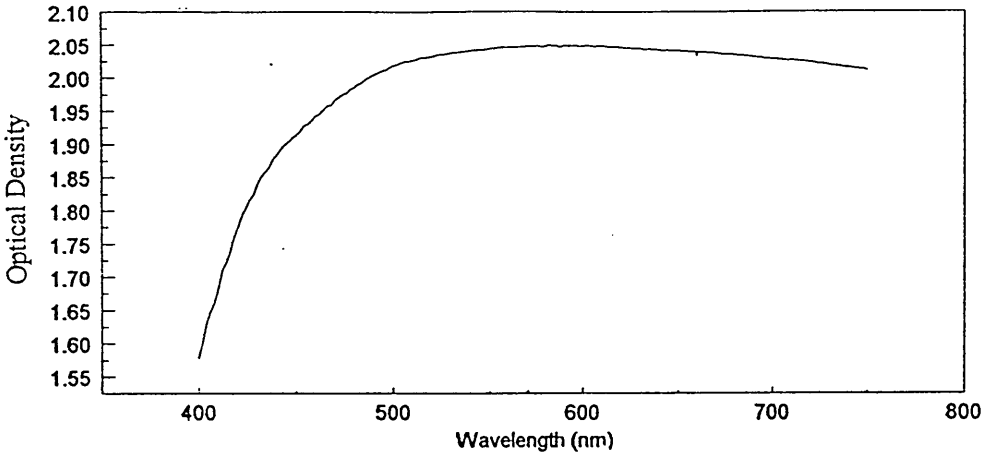


Figure 3.1 (a) Variation of optical density of YE broth grown *L. pneumophila* with wavelength of transmitted light. 24 h growth culture. Plot produced by scanning spectrometer working between 400 and 750 nm wavelengths.

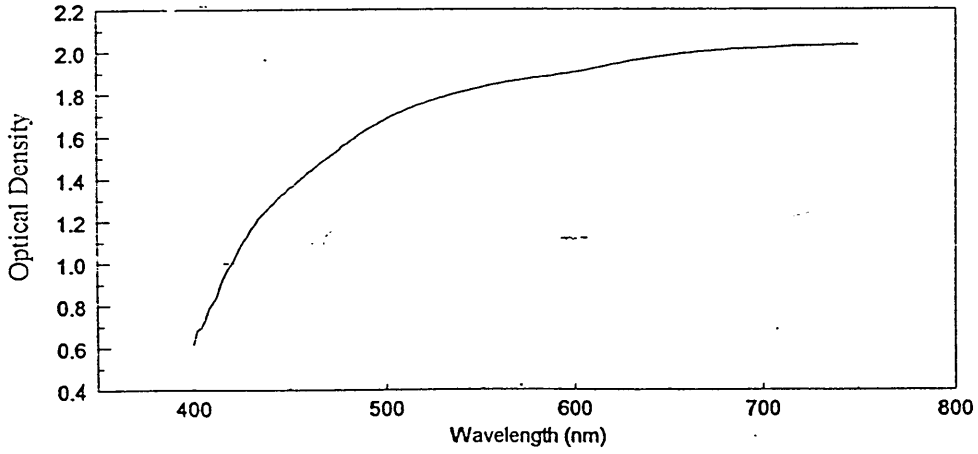


Figure 3.1 (b) Variation of optical density of YE broth grown *L. pneumophila* with wavelength of transmitted light. 48 h growth culture. Plot produced by scanning spectrometer working between 400 and 750 nm wavelengths.

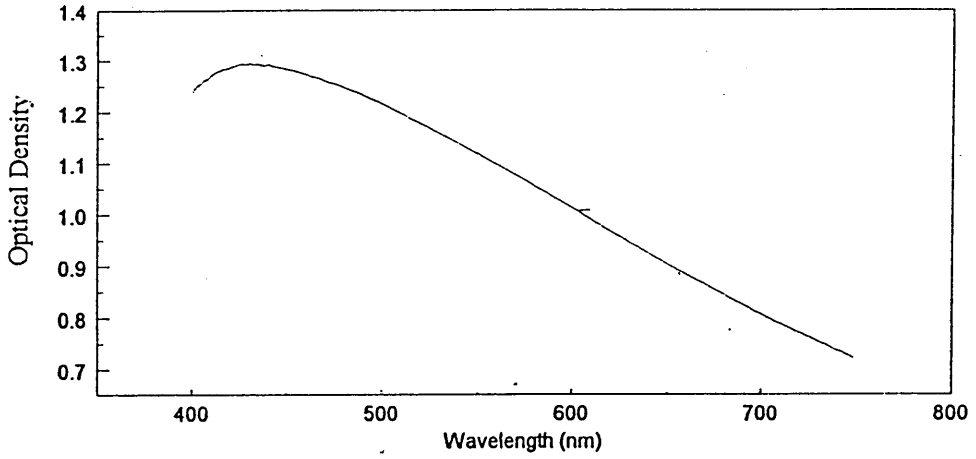


Figure 3.1 (c) Variation of optical density of YE broth grown *L. pneumophila* with wavelength of transmitted light. 24 h growth culture diluted 1 in 10 in supernatant. Plot produced by scanning spectrometer working between 400 and 750 nm wavelengths.

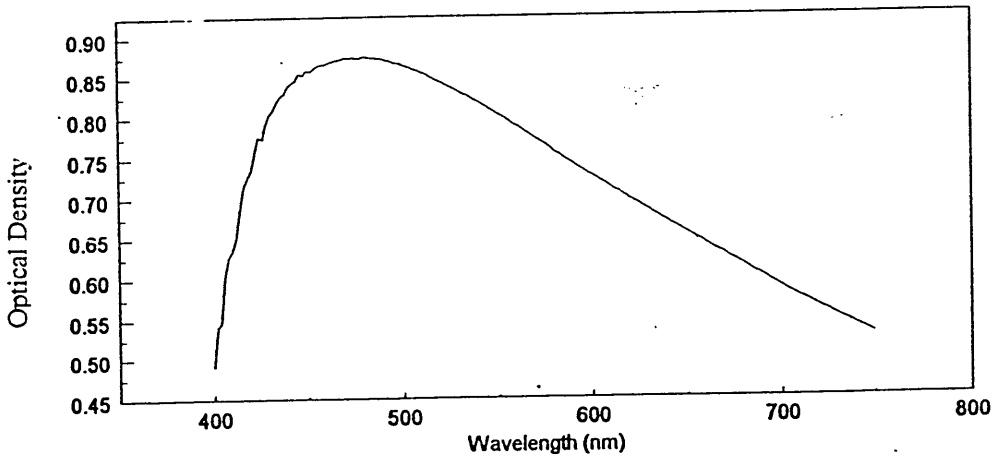


Figure 3.1 (d) Variation of optical density of YE broth grown *L. pneumophila* with wavelength of transmitted light.48 h growth culture diluted 1 in 10 in supernatant. Plot produced by scanning spectrometer working between 400 and 750 nm wavelengths

maximum dilution used was 1 in 2,048. The 'true' OD_{660} values of the samples were calculated by assuming that the value obtained for the most dilute solution is correct. The relationship between measured and calculated OD values is shown in Figure 3.2. The information presented in Figure 3.2 shows that a significant deviation from the Beer-Lambert Law only occurs when the observed OD_{660} value is > *ca.* 0.5. It was therefore, decided that all suspensions with OD_{660} values > 0.4 would be diluted 1 in 10 in the respective supernatant and re-measured to enable a more accurate bacterial optical density to be calculated.

3.1.2 GROWTH OF *L. PNEUMOPHILA* IN YE BROTH AND CDM.

An initial culture of *L. pneumophila* was prepared by inoculating 50 ml of YE broth contained in a sterile 250 ml conical flask with a loop full of BCYE agar grown *Legionella* and incubating overnight (12-16 h.) at 37°C with shaking (250 rpm) (New Brunswick Scientific, Edison, New Jersey, USA). This initial culture was used to inoculate 50 ml volumes of either prewarmed and aerated YE broth or CDM contained in sterile 250 ml conical flasks. Inoculated media were incubated at 37°C with shaking. The growth of the *L. pneumophila* was monitored at hourly intervals by optical density measurements (OD_{660}) for up to 72 h. Details of the optical density measurement technique are given in Section 3.1.1.

To ensure comparability, each growth medium was inoculated to the same OD_{660} value. The YE broth culture was prepared by measuring the OD_{660} value of the initial culture and inoculating 50 ml of the fresh medium to give an initial OD_{660} value of 0.05. To avoid cross contamination of the CDM by YE broth, a volume (10 ml) of the initial culture was centrifuged at 5,800 x g for 5 min. (MSE Scientific Instruments, Crawly, UK) to pellet the bacteria. The pellet was resuspended in 10 ml of CDM and re-centrifuged to ensure complete removal of the YE broth. The resulting pellet was resuspended in a 10 ml volume of CDM and the optical density of the suspension determined. This suspension was used to inoculate a sterile flask containing 50 ml of CDM to an initial OD_{660} value of 0.05.

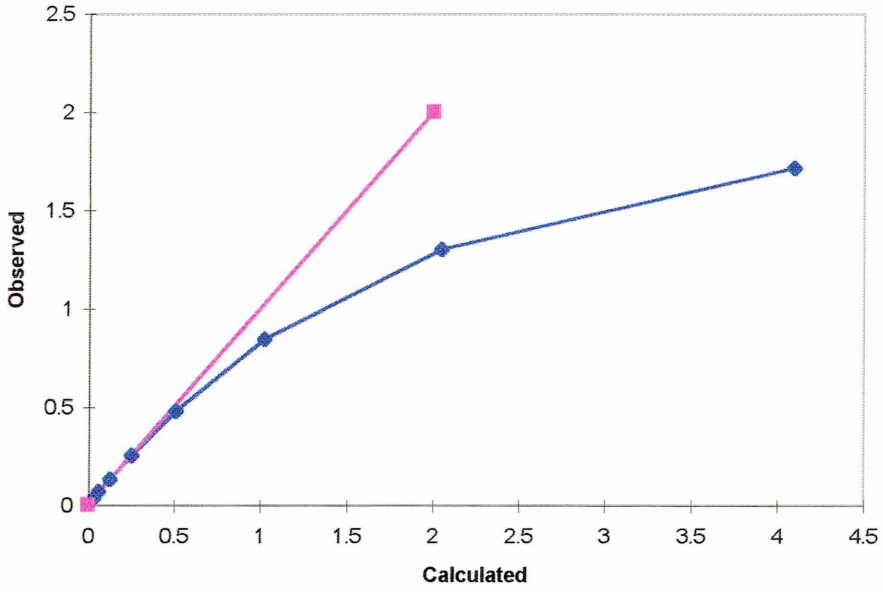


Figure 3.2. Deviation from the Beer-Lambert Law.

(■) - the theoretical relationship between observed (measured) OD_{660} values and values calculated from 1 : 2 dilutions of YE broth grown *L. pneumophila*.
(◆) - the practical or real relationship between observed OD_{660} values and calculated values. A marked deviation of measured values of OD_{660} from the theoretical occurs at values > 0.5.

A preliminary analysis of the growth curves showed that YE broth produced greater yields of *L. pneumophila* with shorter generation times than the CDM under the same conditions. YE broth was, therefore, adopted as the artificial growth medium of *L. pneumophila* throughout the project. A detailed analysis of the growth kinetics of *L. pneumophila* are presented in Chapter 4. All subsequent growth curves were performed in YE broth in the manner described above. Analysis of the growth curves suggested that an OD_{660} of between 0.4 and 0.5 corresponded to the exponential phase of growth of *L. pneumophila*. This was usually achieved after 4 to 6 h. post inoculation. *L. pneumophila* was considered to be in the stationary phase of growth when the OD_{660} value rose to between 2.4 and 2.6, usually 24 h. post inoculation.

3.1.3 ENUMERATION OF *L. PNEUMOPHILA* BY VIABLE PLATE COUNTING.

Determination of the viability of *L. pneumophila* was performed by plating serial ten-fold dilutions on to duplicate pre-dried BCYE agar plates. These were incubated at 37°C for a period of 5 to 7 d. Investigation of long-term survival of *L. pneumophila* in water microcosms, infectivity assays and the effects of antibiotics and heat on survival were monitored by this method of viable counting.

As the project progressed it became apparent that the above technique was too insensitive at low bacterial concentrations. The viability of *L. pneumophila* at low concentrations was determined by plating 100 µl of the undiluted suspension on to BCYE agar. The limit of detection by this method has been estimated to be *ca.* 10 cfu/ml.

3.1.3 1 STATISTICAL TESTS.

The reproducibility of the *L. pneumophila* serial dilution recovery method on BCYE agar was determined by calculating both the accuracy and reproducibility of serial dilutions. Duplicate agar plates were inoculated with 25 µl volumes of bacterial suspension using five replicate dilution series. In addition, one series of dilutions was

inoculated (25 µl volumes) on to five pairs of agar plates in order to assess the reproducibility of the pipetting procedure. Data from each experiment was examined and subjected to an analysis of variance. The experimental data and statistical analysis are shown in Tables A1 to A4 of Appendix 1 (a).

In order to test the reproducibility of the viable spread plate counting method, 100 µl of a culture of *L. pneumophila* grown to stationary phase in YE broth was serially diluted 1 in 100 in amoebic saline. The number of bacteria were counted using an improved Neubaur haemocytometer prior to the diluting of the suspension to give a concentration of approximately 2000 bacteria per ml in the final suspension. This can be expected to produce *ca.* 200 colony forming units per 100 µl after incubation at 37°C for 5 to 7 d. Five replicate samples of the final dilution were made, and from each, 100 µl was plated on to five BCYE agar plates, giving 25 plates in all. The plates were incubated at 37°C for 5 to 7 d. and the resulting colonies counted. The results are shown in Table A5 and Table A6 of Appendix 1(b).

3.1.4 A COMPARISON OF SPECTROPHOTOMETRIC MEASUREMENTS WITH VIABLE COUNTS.

Growth curves were determined as described in Section 3.1.2. The optical densities of the growing cultures were monitored at hourly intervals. Simultaneously, the viable count of the culture was measured by inoculating duplicate BCYE agar plates with serial ten-fold dilutions of the culture, as described in Section 3.1.3. After 5 d. incubation at 37°C the resulting colonies were counted and the cfu/ml values compared to the respective OD_{660} values. This comparison is presented in Figure 3.3

3.1.5 COCULTURE TECHNIQUES.

3.1.5.1 ASSESSING VIABLE AND TOTAL COUNT OF MONOCYTES AND AMOEBAE.

The viability of the *Acanthamoeba* and the monocytic cell line was assessed by the trypan blue stain exclusion assay (Sigma Technical Services, Poole, UK, and Holden *et*

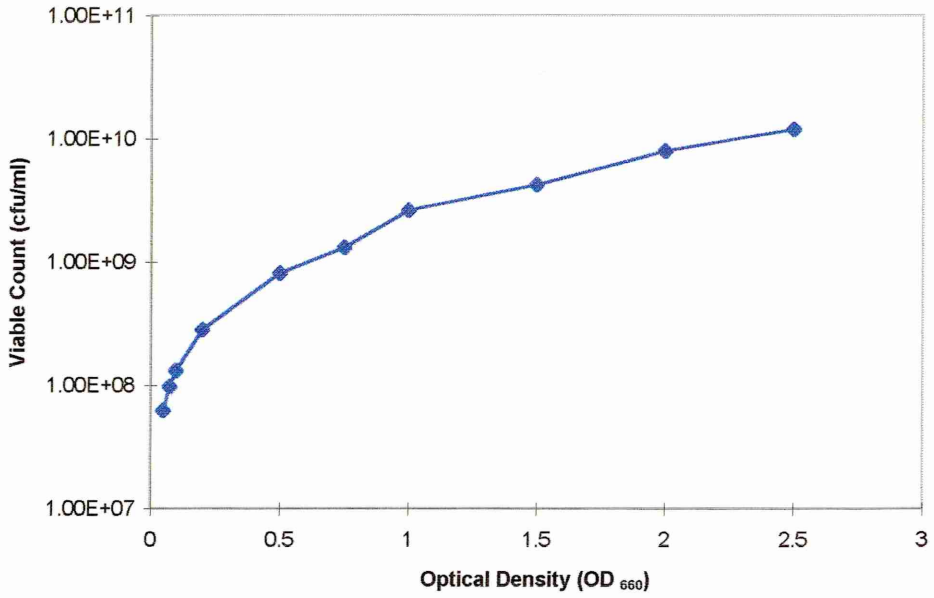


Figure 3.3 Relationship between the concentration of *L. pneumophila* as determined by the viable count method and measured OD_{660} values of the corresponding culture.

al., 1984). This involved the addition of an equal volume of 0.4 % (w/v) trypan blue (Sigma) to a sample of host cell suspension (40 µl : 40 µl). After mixing, 10 µl of the solution was placed in the counting chamber of an improved Neubaur haemocytometer. The number of viable cells (unstained cells) and the total number (stained and unstained cells) were counted in the central square ($1mm^2$). To test the reproducibility of this counting method ten replicates of amoebae and monocytes were counted and a statistical test of confidence applied to the results, as shown in Table A7 and Table A8 of Appendix 1 (c).

3.1.5.2 GROWTH OF *L. PNEUMOPHILA* WITHIN *ACANTHAMOEBA POLYPHAGA*.

It has been reported previously that the uptake of *L. pneumophila* by acanthamoebae is poor if the amoebae are allowed to continue to grow in a nutrient rich environment (Moffat and Tompkins, 1992). To overcome the problem of removing the amoebae from their growth medium, the amoebae were cold shocked off the surface of the tissue culture flasks by placing the flasks at -20°C until the medium had just frozen. The medium containing the suspended amoebae was subsequently thawed at 37°C before centrifugation at 400 x g for 6 min.. The amoebae were washed in amoebic saline, a nutrient free solution, prior to infection with YE broth grown *L. pneumophila*. The concentration of the amoebae was determined by counting in a haemocytometer. The bacteria used to infect the amoebae had been previously grown in YE broth to exponential phase. The culture was centrifuged at 2,080 x g for 30 min. and the pellet produced resuspended in amoebic saline. This suspension was further centrifuged to ensure complete removal of the YE broth. The resulting pellet was resuspended in amoebic saline and the bacterial concentration determined by optical density measurement. The amoebae were inoculated with legionellae to give an approximate ratio of 1 bacterium to 1 amoeba. The cocultures containing amoebae were incubated at 37°C for 2 to 3 d. in the dark. The infection of the amoebae was monitored by phase contrast microscopy and the bacteria harvested once infected amoebae had lysed and small highly motile *L. pneumophila* were seen in the medium.

3.1.5.3 GROWTH OF *L. PNEUMOPHILA* WITHIN U937 MONOCYTES.

Intra-monocytic cocultivation of *L. pneumophila* was carried out in a similar manner to that of intra-amoebic grown bacteria except that the coculture was performed in a nutrient rich environment. This was necessary as U937 cells do not survive under nutrient limited conditions. Cold shock was not required as the cells are non-adherent. Three to four day old cells were washed into fresh RPMI medium by centrifugation at 400 x g for 6 min. The resulting pellet was resuspended in fresh RPMI and the centrifugation process repeated. The concentration of the U937 cells and their viability was ascertained by trypan blue exclusion staining and haemocytometer counting (Section 3.1.5.1). Broth grown *L. pneumophila* for coculture with the U937 monocytes was prepared by centrifuging at 2,080 x g for 30 min. prior to resuspension of the resulting pellet in RPMI medium. The suspension was again centrifuged at 2,080 x g for 30 min. to ensure complete removal of YE broth. The resulting pellet was resuspended in RPMI medium and the concentration of the bacteria determined by OD measurements. The suspension was then used to inoculate the coculture to a ratio of one bacterium-to-one host cell. The cocultures were incubated at 37°C in an atmosphere of 5 % carbon dioxide. Monitoring of the cocultures by phase contrast microscopy revealed the presence of small, highly motile intra-monocytic grown *Legionella* after 4 to 5 d.

3.1.5.4 HARVESTING OF INTRACELLULAR-GROWN *L. PNEUMOPHILA*.

To harvest the bacteria, the cocultures were vortexed for 1 min. before centrifugation at at 400 x g to deposit any remaining cell debris. The supernatant was subsequently centrifuged at 2,080 x g for 30 min.. The resulting pellet was washed and resuspended in 1 ml of either amoebic saline (intra-amoebic *Legionella*) or RPMI medium (intra-monocytic *Legionella*) prior to streaking out on to BCYE agar for single colonies to confirm purity. The intracellular grown *Legionella* was used to either inoculate subsequent cocultures of amoebae or monocytes to gain further quantities of intracellular grown bacteria or for experimental use.

3.1.6 MORPHOLOGICAL STUDIES.

Samples for microscopic examination were prepared by the hanging drop technique. The prepared samples were examined by means of phase contrast microscopy at magnifications of x 400 and x 1000, the latter using oil immersion.

3.1.7 'AGEING' OF INTRACELLULAR BACTERIA.

Intracellular grown *L. pneumophila* were held in coculture for a further 72 h. after host cell lysis. Phase contrast microscopy was employed to confirm that all the legionellae had undergone a change from small, highly motile bacteria to that of rod-shaped and non-motile; similar to that of broth grown *L. pneumophila*. The bacteria which had undergone this process were harvested (Section 3.1.5) and termed 'aged' for the purpose of this thesis.

3.1.8 REQUIREMENT OF A WHOLE HOST CELL FOR REPLICATION.

Samples of broth grown *L. pneumophila* were harvested and prepared for coculture with amoebae and monocytes as described in Sections 3.1.5.2 and 3.1.5.3, respectively. The prepared *L. pneumophila* were added to give a final concentration of *ca.* $1x10^6$ cfu/ml to each of the following :

(a) Coculture Medium alone (amoebic saline or RPMI).

(b) Conditioned Medium. This was coculture medium in which host cells have been resuspended and lysed by sonication. The lysate was filtered through a 0.22 μm membrane to remove the debris.

(c) *A. polyphaga* or U937 monocytes resuspended in the respective coculture medium to a concentration of *ca.* 10^5 cells/ml (Section 3.1.5.2. and 3.1.5.3.)

(d) Lysed host cells. Amoebae and monocytes (*ca.* 10^5 cells/ml) were prepared for coculture as described in Section 3.1.5.2 and 3.1.5.3, respectively. After resuspension in the respective coculture medium, the host cell suspensions were vortexed for 3 min.. Complete disintegration of the host cells was confirmed by microscopic examination.

The suspensions were monitored by phase contrast microscopy and the concentration of legionellae monitored daily by the viable count method for a duration of 5 d. (Section 3.1.3).

3.1.9 COMPARISON OF THE UPTAKE AND INFECTION OF VARIOUSLY GROWN *L. PNEUMOPHILA* BY ACANTHAMOEBAE AND MONOCYTIC CELLS.

Invasion assays were performed to investigate the effect of *L. pneumophila* concentration upon uptake by, and subsequent replication within, amoebae and U937 monocytes. A series of cocultures was prepared as described in Sections 3.1.5.2 and 3.1.5.3. The concentration of host cells was standardised by haemocytometer counting and the suspensions inoculated with *L. pneumophila* to give bacteria-to-host cell ratios of 1 : 10,000, 1 : 1, 100 : 1 and 1000 : 1. The latter ratio was confined to the amoebic cocultures. Assays were performed using broth grown *Legionella* in exponential phase or stationary phase, and freshly harvested intra-amoebic or intra-monocytic grown legionellae. As a control, amoebic saline or RPMI was inoculated with *L. pneumophila* to a concentration *ca.* 10^5 cfu/ml. It was noted during earlier work (Section 3.1.5.4) that the morphology of intracellular grown legionellae changed to that of broth grown if they were not immediately harvested and used. The above assay was repeated to determine whether this change in morphology was reflected in the infectivity of the bacteria.

The infection process was monitored by phase contrast microscopy and the concentration of legionellae was measured daily by the viable count method for a duration of 7 d. (Section 3.1.3). The viability of the amoebae and monocytic cells was also assessed on a daily basis by trypan blue staining (Section 3.1.5.1) with an uninoculated suspension of the respective cells acting as a control.

3.1.10 THE EFFECT OF THE *LEGIONELLA* SOURCE UPON UPTAKE AND REPLICATION WITHIN AMOEBAE.

Amoebae were prepared for coculture as described in Section 3.1.5.2. to a final bacteria-to-host cell ratio of 10 : 1. The sources of *L. pneumophila* were as follows :

(a) Intra-amoebic grown *L. pneumophila* prepared as in Section 3.1.5.2.and 3.1.5.4.
(b) Intra-amoebic grown *L. pneumophila* 'aged' in the coculture menstruum for 72 h. after host cell lysis.
(c) Broth grown legionellae in stationary phase, prepared for coculture as in Section 3.1.5.2.

The uptake and replication of the legionellae was monitored by phase contrast microscopy and the concentration of *L. pneumophila* measured daily by the viable count method for a duration of 5 d. (Section 3.1.3).

3.2 THE EFFECT OF STRESS CONDITIONS ON THE SURVIVAL OF VARIOUSLY GROWN *L. PNEUMOPHILA*

The susceptibility of variously grown *L. pneumophila* isolates to a range of stress parameters has been determined. The isolates used were either freshly harvested intracellular, 'aged' intracellular or *in vitro* grown in origin. The intracellular grown bacteria were cultured in either *A. polyphaga* or undifferentiated U937 monocytic cells (Sections 3.1.5.2 and 3.1.5.3). The *in vitro* grown legionellae were cultured in YE broth to exponential and stationary phase (Section 3.1.2).

3.2.1 SUSCEPTIBILITY TO ANTIBIOTICS

3.2.1.1 PREPARATION OF STOCK SOLUTIONS OF ANTIBIOTICS.

Stock solutions of ciprofloxacin (Bayer, Slough, UK; 84.4 % purity), rifampicin (Sigma, 95 % purity) and erythromycin (Sigma, 98 % purity) were prepared to stock concentrations of 1000 μg/ml (w/v). Prior to dilution in ddH_2O, the rifampicin was

dissolved in di-methyl sulphoxide (DMSO) (5 % of final volume) and erythromycin was dissolved in methanol (5 % of final volume). The solutions were sterilised through a 0.22 μm filter (Schleicher and Schuell) into 30 ml sterile sample tubes (Bibby) and stored at 4°C for a maximum duration of 2 wk.

3.2.1.2 DETERMINATION OF MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATION.

The minimum inhibitory concentration (MIC) of the antibiotics was determined by the broth dilution technique (Schlegal, 1986). Serial two-fold dilutions of each antibiotic were prepared in 7 sterile test-tubes, each containing a final volume of 5 ml of YE broth. The initial starting concentrations of the antibiotics were: ciprofloxacin-7.68 μg/ml , rifampicin-0.512 μg/ml , and erythromycin-16.0 μg/ml. Each series of test-tubes were inoculated with either exponential phase or stationary phase broth grown or freshly harvested intra-monocytic grown *L. pneumophila* to an OD_{660} of 0.02 (*ca.* 2 x 10^7 cfu/ml), and giving a total of 9 series of 7 test-tubes. After incubation at 37°C for 48 h. with shaking, the minimum concentration of antibiotic that completely inhibited the growth of the bacteria was taken as being the MIC. The minimum bactericidal concentration (MBC) was determined by plating 100 μl of each dilution which showed no visible growth during the MIC assay, on to pre-dried BCYE agar. The minimum concentration of antibiotic which prevented growth on agar was taken as the MBC.

3.2.1.3 TIME-KILL ASSAYS

The susceptibility to antimicrobial agents of variously grown *L. pneumophila* and 'aged' intracellular grown bacteria was determined by time-kill assays. The assays were performed in 250 ml conical flasks containing 50 ml of YE broth. *L. pneumophila* at a concentration equivalent to an OD_{660} value of 0.02 (*ca.* 2 x 10^7 cfu/ml) were exposed to ciprofloxacin (1 μg/ml), rifampicin (5 μg/ml) and erythromycin (8 μg/ml) acting independently and a combination of erythromycin (8 μg/ml) and rifampicin (5 μg/ml). Control samples consisted of bacterial suspensions (*ca.* 2 x 10^7cfu/ml) without added

antibiotics. The assays were incubated at 37°C, with shaking, throughout the time of exposure to the antibiotic. Viable counts were taken at time zero and after 6 and 24 h., as described in Section 3.1.3 .

The efficacies of antibiotics are usually determined by their ability to kill growing bacteria. To examine the effects of antibiotics on non-growing *L. pneumophila,* bacterial suspensions were centrifuged at 2,080 x g for 30 min. The resulting pellets were resuspended in amoebic saline and the suspensions centrifuged for a further 30 min. at 2,080 x g. The washed pellets were resuspended in amoebic saline and their OD_{660} values determined. The bacteria were inoculated into 50 ml volumes of amoebic saline, a nutrient free solution, to give OD_{660} values of 0.02 (*ca.* 2 x 10^7cfu/ml). This stops further growth of the bacteria without inducing lysis. The time kill assays were repeated as described above.

3.2.2 SUSCEPTIBILITY TO ELEVATED TEMPERATURE.

3.2.2.1 SUSCEPTIBILITY OF VARIOUSLY GROWN *L. PNEUMOPHILA* TO HEAT.

The effect of different temperatures (37°C, 50°C, 55°C, 60°C, 65°C) on the survival of broth grown *L. pneumophila* in exponential and stationary phase and *Legionella* grown within amoebae or monocytes was investigated. The legionellae were centrifuged at 2,080 x g for 30 min. and the resulting pellet resuspended in 5 ml of amoebic saline. The centrifugation process was repeated. The resulting pellet was again resuspended in 5 ml of amoebic saline. The concentrations of the bacterial suspensions were established by haemocytometer counting and the suspension diluted 1 in 10,000 in amoebic saline. This medium was used as the menstruum, rather than YE broth, to prevent bacterial replication. This suspension was used to inoculate 10 ml volumes of amoebic saline, pre-heated to the test temperature, to give a final concentration of *ca.* 2,000 bacteria per ml. The sample of *L. pneumophila* incubated at 37°C served as a control for the purposes of this work. Volumes (100 µl) of each suspension were

spread on to pre-dried BCYE agar at time zero and after 2, 4, 6, 8 and 10 min. of heat exposure to determine the concentration of viable bacteria (Section 3.1.3).

3.2.2.2 TIME-KILL ASSAYS

The survival of broth grown (exponential phase and stationary phase), intra-amoebic and intra-monocytic grown *L. pneumophila* was determined by time kill assays. The test temperature chosen for this experiment was 50°C. This was found by experimentation to be the highest temperature which did not induce a state of non-recoverability in any of the *Legionella* growth forms during the 10 min. test period (Section 3.2.2.1). As *L. pneumophila* cells are normally incubated at 37°C for optimum growth, samples held at this temperature acted as the controls. The variously grown bacteria were prepared for the test by centrifuging suspensions at 2,080 x g for 30 min. and resuspending the resulting pellet in amoebic saline. Volumes (50 ml) of amoebic saline in 250 ml conical flasks were held in pre-heated water baths and inoculated to an OD_{660} value of 0.05 (*ca.* 5×10^7 cfu/ml) with one of the prepared suspensions. Viable counts were performed at time zero and after periods of 40, 80, 120 and 240 min., as described in Section 3.1.3.

3.2.3 SUSCEPTIBILITY TO MECHANICAL STRESS (ULTRASONIC VIBRATION).

3.2.3.1 ASSESSMENT OF SURVIVAL USING SPECTROPHOTOMETRIC MEASUREMENTS.

An investigation into the susceptibility of broth grown *L. pneumophila* in exponential and stationary phase to sonic disintegration was performed. Volumes (50 ml) of bacterial suspension were centrifuged at 4,400 x g for 10 min.. The resulting pellet was resuspended in amoebic saline and the OD_{660} value determined. Amoebic saline (15 ml) was pre-heated to 37°C prior to inoculation to an OD_{660} value of 0.05 (*ca.* 5×10^7cfu/ml). A 1 ml volume of this suspension was placed into a pre-warmed eppendorf tube held in water maintained at 37°C. The suspension was sonicated for various periods of time using 1 sec pulses with intervals of 10 sec. between pulses for cooling. The apparatus used was an MSE Soniprep disintegrator (MSE Scientific

Instruments) working at maximum power at an amplitude of 22 μm and a frequency of 25 kHz. The OD_{660} value of the suspension was measured after 0, 3, 6, 9, 12, 15, 18, 20, 30, 40, 50 sec. of sonication. The stock suspension served as a control. Its OD_{660} value was measured at the commencement of the experiment and at 10 min. intervals throughout the investigation to monitor the survival of the legionellae. The ratio of OD_{660} value of the sample after sonication to that of the control, expressed as a percentage, was used as a measure of the susceptibility of the bacteria to sonication. Initial results of this investigation showed that OD_{660} measurements were incapable of differentiating the mechanical stress resistance of the different growth phases of broth grown *L. pneumophila* (Figure 3.4). An alternative form of evaluation based on the viable count method was, therefore, investigated.

3.2.3.2 VIABLE COUNT METHOD.

Broth grown *L. pneumophila* and freshly harvested and 'aged' intra-amoebic grown legionellae were diluted in cold amoebic saline (15 ml) to give a concentration of 2000 bacteria per ml, as described in Section 3.2.2.1. The stock suspension was held on ice prior to sonication. A 1 ml volume of suspension was placed into a pre-cooled eppendorf tube held in a beaker of ice. This method reduced the loss of sample volume due to evaporation caused by heat arising from the sonication process. The suspension was sonicated for various periods of time using 1 sec pulses with intervals of 10 sec. between pulses for cooling. After sonication a 100 μl aliquot was plated on to BCYE agar (Section 3.1.3). Viable counts were assessed after 0, 5, 10, 20, 30, 60, 90, 120, 150, 180, 210, 240, 300 sec.. The time points for the exponential phase bacteria were modified to 0, 3, 6, 9, 12, 15, 20 sec. after it was found that these organisms were highly susceptible to long periods of sonication. The stock suspension served as a control sample. Its viable count was measured at time zero and at 10 min. intervals throughout the experiment to assess whether storage on ice affected the viability of the legionellae.

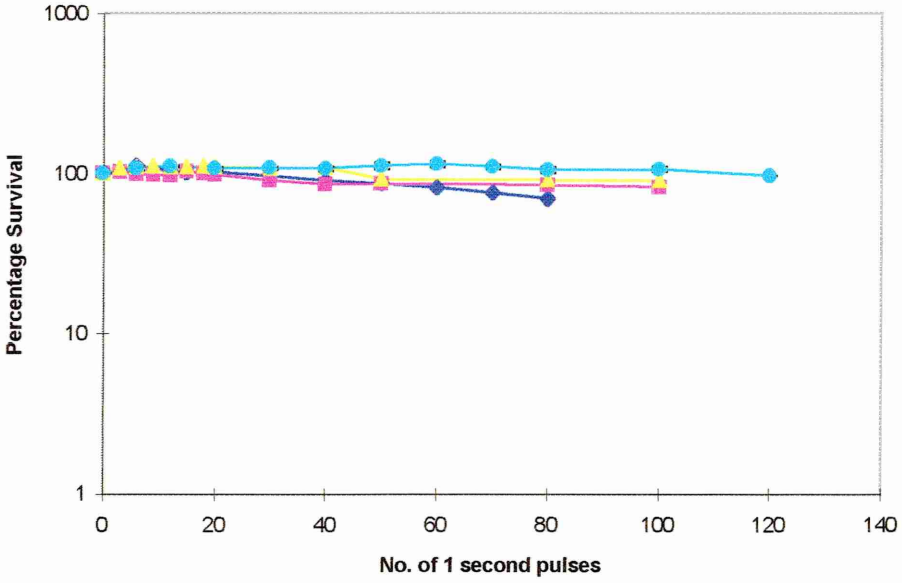


Figure 3.4. Survival of YE broth grown *L. pneumophila* exposed to mechanical stress (1 sec pulses of sonication). Test cultures grown to early exponential phase (◆), mid exponential phase (■), early stationary phase, (▲) and stationary phase (●).

3.2.4 LONG-TERM STARVATION OF VARIOUSLY GROWN *L. PNEUMOPHILA* IN DEIONISED AND TAP WATER.

3.2.4.1 PREPARATION OF MICROCOSMS.

The microcosms consisted of ultra-clean bottles (Section 2.6) of prepared deionised water or tap water, inoculated with variously grown *L. pneumophila.* The inocula used were either broth grown (in exponential or stationary phase) or freshly harvested intracellular-grown (intra-amoebic or intra-monocytic). The inocula were prepared by centrifuging 10 ml volumes of bacterial suspensions at 2,080 x g for 30 min. The resulting pellets were resuspended in water and re-centrifuged again at 2,080 x g for a further 30 min. to ensure complete removal of growth medium. The washed pellets were resuspended in 10 ml of water prior to aseptic inoculation of the microcosms to an OD_{660} value of 0.01 (*ca.* 1 x 10^7cfu/ml). In each case the water used was the same as that of the microcosm to be inoculated.

3.2.4.2. STORAGE AND SAMPLING OF THE MICROCOSMS.

The microcosms were incubated at 37°C in the dark (wrapped in aluminium foil). The survival of the *L. pneumophila* was assessed at time zero and on a fortnightly basis until the bacteria could not be detected by viable counts on BCYE agar, i.e < 10 cfu/ml. At each sampling point the viable plate count, the total count, and the ability of the legionellae to infect monocytes or amoebae and replicate, was determined. Difficulties were encountered during this work in that the broth grown *L. pneumophila* in deionised water were not recoverable at the first fortnightly sampling point. The investigation was, therefore, repeated with the sampling intervals adjusted as the experiment progressed. For example, a short sampling interval of 3 to 4 d. was used for the broth grown *L. pneumophila* in deionised water. Longer intervals were used for all the other microcosms until the point of non-recoverability of the bacteria was approached. The sampling interval was then reduced to 3 to 4 d. in order to recover the few remaining culturable bacteria. In addition, the concentration of viable *L. pneumophila* was measured using a commercial vital staining kit, the BacLight Live / Dead staining kit (Molecular Probes, Leiden, Holland) which had recently become available.

3.2.4.3 SURVIVAL ASSESSMENTS.

(i) Viable plate count.

The method of preparation of the sample for viable plate counting was modified as the investigation progressed. During the early stages, i.e relatively high bacterial concentrations, the methods outlined in Section 3.1.3. were employed. Once the bacteria appeared to be non-recoverable using the undiluted suspension, attempts were made to recover viable legionellae by the modification of the centrifugation and membrane filtration techniques reported by Boulanger and Edelstein (1995). Volumes (1.5 ml) of water from each microcosm were centrifuged at 11,600 x g for 10 min. to pellet the bacteria. The pellets were subsequently resuspended in 100 µl of sterile ddH_2O and plated on to BCYE agar (Section 3.1.3). A total of six samples taken at weekly intervals were examined for each microcosm. At the end of this period, a 20 ml volume of microcosm water was also concentrated by centrifugation at 2,080 x g for 30 min. The resulting pellet was resuspended in 50 µl of sterile ddH_2O and spread onto a BCYE agar plate. The agar plates were incubated at 37°C for a minimum of 9 d. before examination.

After six attempts at recovering *L. pneumophila* by the above method a 20 ml volume from each microcosm was concentrated on to a sterile 0.22 µm nitrocellulose membrane by vacuum filtration. The membrane was then placed on to BCYE agar and the plate incubated as described above.

(ii) Total count.

Two bacterial stains which are commonly used for the staining of bacteria for the examination by fluorescence microscopy are acridine orange and 4'6-diamidino-2-phenylindole (DAPI) stain (Sigma, Poole, UK). The performance of both stains with *L. pneumophila* was evaluated. The microscope used was an Olympus BX60 with a BX-FLA fluorescence attachment.(Olympus, London, UK). The observations were made at light wavelengths of 465 nm (acridine orange) and 365 nm (DAPI). Both techniques required the fixing of the stained bacteria on to a black 0.22 µm

polycarbonate membrane (Sartorius, Lutterworth, UK) by vacuum filtration. The apparatus used is shown in Figure 3.5.

The acridine orange fluorescence staining method employed was that of Hobbie *et al.* (1977), as modified by Bitton *et al.* (1993). Considerable difficulties were encountered using this technique in that the fluorescence faded rapidly during examination at x 400 magnification, making the counting of small intracellular bacteria extremely difficult.

Samples from the same microcosms were also examined after staining with DAPI. The problem of fading of the stain was not encountered using this method. DAPI staining was, therefore, adopted for the purpose of this work. The initial examination of samples stained by this technique showed bacterial concentrations were far too high for accurate counting. A dilution of 1 in 100 in filtered ddH_2O was found to reduce the bacterial numbers to a countable level. The technique used for staining samples by the DAPI stain was as follows: 100 µl of a 0.1 % (w/v) solution of DAPI was added to a 1 ml volume of diluted microcosm water. The samples were incubated for 15 min. at room temperature. A polycarbonate membrane (Sartorius) was placed in the filtration apparatus (Figure 3.5) and 2 ml of filtered ddH_2O added. After incubation in the presence of the stain, the sample was added to the water and mixed thoroughly by pumping up and down with a pipette prior to vacuum filtration. After filtration, the membrane was removed from the apparatus and allowed to dry for *ca.* 10 min. on a microscope slide. A drop of epifluorescence oil was added to the membrane followed by a cover-slip. The sample was examined at x 400 magnification using ultraviolet light at a wavelength *ca.* 365 nm. The number of bacteria in each of 15 fields was counted and the number of bacteria per ml calculated as follows:

$$\text{No. of bacteria/ml} = \text{Av count/field} \times \text{Dilution factor} \times \frac{\text{Effective filtration area}}{\text{Area of field of view}}$$

The accuracy and reproducibility of the counting technique was assessed by counting bacteria in 15 fields of view on each of 5 slides prepared from the same sample of

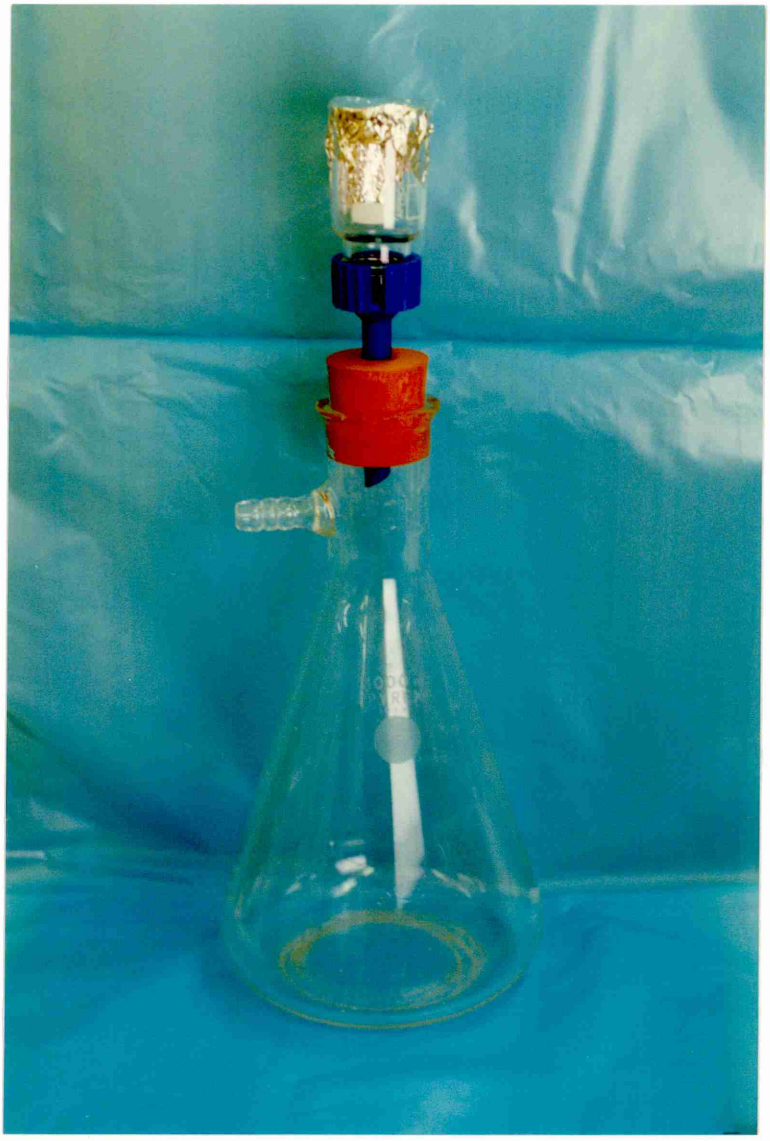


Figure 3.5 Vacuum Filtration Apparatus. Employed for mounting stained bacteria on polycarbonate membranes for epifluoresence microscopy.

microcosm water. Experimental data and statistical analysis are given in Table A9 and Table A10 of Appendix 1, Section (d).

(iii) Infectivity towards host cells.

The ability of *L. pneumophila* to retain their infectivity towards host cells, after storage in the water microcosms, was investigated by studying the uptake of the surviving *L. pneumophila* by amoebae or monocytes. Host cells were prepared for coculture as described in Sections 3.1.5.2 and 3.1.5.3, respectively. The suspensions were diluted to give a host cell concentration of between 1 x 10^5 cells/ml and 2 x 10^5 cells/ml. Volumes (1.5 ml) of suspended cells were placed in sterile bijouxs and 500 µl of microcosm water added. The cocultures were incubated at 37°C and a viable count determined as described previously (Section 3.1.3) at time zero and after 4 and 7 d.

(iv) Vital staining

The manufacturers of the vital staining kit have shown that it is capable of distinguishing between live and dead Gram negative bacteria. The theory behind this is given in Appendix 2. To assess its performance the stain was initially used to examine a sample of broth grown *L. pneumophila* in exponential phase and one which had been heat-killed by holding at 80°C for 30 min.. When examined by ultraviolet light (*ca.* 365 nm) using a blue filter (wavelength *ca.* 465 nm), the live bacteria were observed to fluoresce green. When a green filter was used (wavelength *ca.* 530 nm), the dead bacteria fluoresced red.

The vital staining kit was used to examine the microcosms at time zero and at 4 weekly intervals throughout the long-term survival investigation. The kit consisted of a mounting medium and two solutions, A and B, which were mixed in equal proportions immediately prior to use. A 3 µl volume of mixed stain was added to 1 ml of diluted microcosm water. The sample was incubated at room temperature in the dark for 15 min. prior to vacuum filtration on to a black polycarbonate membrane, as described in Section 3.2.4.3 part (ii). Following drying on a microscope slide, a drop of mounting

media was placed on the membrane and a cover-slip added. The number of live bacteria was assessed at x 400 magnification by counting 10 fields and calculating the mean concentration per ml.

3.2.4.4 RECOVERY OF POTENTIALLY VIABLE BUT NONCUTURABLE CELLS.

Once the viable count of *Legionella* is *ca.* < 10 cfu/ml the bacteria are no longer detectable on BCYE agar. Microscopical investigations using the vital staining technique have suggested, however, that failure to infect host cells, or be detectable on BCYE agar, does not necessarily mean the complete absence of viable bacteria. It was, therefore, considered necessary to investigate methods of recovering small numbers of potentially viable, but non-culturable bacteria, from the water microcosms. Sampling of the microcosms was continued at 7 d. intervals for a period of 6 wk. after the bacteria became non-recoverable on BCYE agar. Several different methods were used in an attempt to detect the presence of viable bacteria in each of the samples. Details of the techniques are given below.

(i) Resuscitation using a nutrient rich environment.

Volumes (1.5 ml) of water were concentrated by centrifugation at 11,600 x g for 10 min. and the resulting pellet used to inoculate 5 ml of YE broth. The sample was incubated at 37°C with shaking and examined after 5 d. for visible signs of bacterial growth.

(ii). Resuscitation using host cells.

The method used was a modification of that employed during the microcosm infectivity studies (Section 3.2.4.3. (iii)). In this instance, the host cell suspension was inoculated with a pellet prepared by concentrating a 1.5 ml volume of microcosm water. The viable count was monitored as described in Section 3.1.3.

(iii). Resuscitation using amoebae.

The technique used was that described by Steinert *et al.* (1997). After 48 h. growth in PYG broth, the amoebae were removed from the surface of the flask by cold shock

(Section 3.1.5.2). The concentration of the amoebae was determined via haemocytometer counting and the trophozoites diluted in PYG broth to a concentration of *ca.* 10^5 cells/ml. Volumes (1 ml) of this amoebic suspension were pipetted into the wells of a 24-well infection plate (Costar, High Wycombe,UK). The plate was incubated overnight (12-16 h.) in the dark at 37°C to encourage the amoebae to adhere to the surface of the wells. After incubation the PYG broth was removed and the cells washed with 1 ml of amoebic saline to remove any remaining nutrients. The amoebic saline was then replaced with a 1 ml volume of microcosm water per well. Following inoculation, the well plates were centrifuged at 400 x g for 3 min. and incubated in the dark at 37°C. At time zero and after 7 d. the protozoa were removed from the surface of the well plate by agitation and a viable count of the *Legionella* performed as described in Section 3.1.3.

(iv) Resuscitation via heat shock.

Colbourne and Dennis (1989) suggested that very small numbers of legionellae can be resuscitated by a short exposure to heat. Volumes (1 ml) of microcosm water were incubated at 45°C for 10 min.. This was then plated on to duplicate BCYE agar plates. The plates were incubated for 7 d. at 37°C prior to examination.

3.3 ANALYTICAL TECHNIQUES.

3.3.1 STANDARDISATION OF BACTERIAL NUMBERS.

It is important that, within experimental error, each investigation employs the same number of bacteria. Previous workers have standardised the number of bacteria by harvesting into a fixed volume of suspending medium until a pre-determined optical density was achieved (Butler *et al.*, 1985 and Gabay *et al.*, 1985). In many cases, the sample volumes were large (*ca.* 10 ml) and the bacterial concentration low ($OD_{660} < 1$). In contrast, the nature of the current work has necessitated the use of small volumes of highly concentrated bacteria. In an endeavour to overcome the problem of standardisation of bacterial numbers, the following method was adopted (Personal communication, T. Buhler). The OD_{660} value of the *Legionella* culture was determined, as described in Section 3.1.1. This value was used to calculate the volume of bacterial

suspension to be concentrated which would contain bacteria equal in number to that expected in 50 ml suspension with an OD_{660} of 1 or 1 ml suspension with an OD_{660} of 50. The volume of suspension to be concentrated was calculated using the following formula :

$$\text{Volume of suspension (ml)} = \frac{50}{OD_{660} \text{ of suspension}}.$$

The calculated volume was concentrated by centrifugation at 4,400 x g (J2-21 Beckman Instruments) for 10 min. at 4°C. The resulting pellet was resuspended in 1 ml of cold amoebic saline and stored as 0.5 ml volumes in eppendorf tubes (Sarstedt) at -20°C prior to use.

3.3.2 ANALYSIS OF *LEGIONELLA* PROTEIN PROFILES.

3.3.2.1 PREPARATION OF *LEGIONELLA* MEMBRANE PROTEINS

Legionella membrane proteins were prepared by the method of Ames (1974). A sample of *L. pneumophila* was removed from storage at -20°C and thawed at room temperature for 30 min. prior to sonication. A MSE Soniprep sonic disintegrator was used at maximum power with an amplitude of 22 μm and a frequency of 25 kHz (MSE Scientific Instruments). The sample was held in an ice bath to minimise the effect of heat and sonicated with a thin long tip probe for 10 cycles of 10 sec. pulses, with 20 sec. pauses for cooling. After sonication the sample was stored on ice prior to centrifugation (MSE Scientific Instruments) at 5,800 x g for 10 min. to pellet unbroken cells and debris. The pellet was removed from the supernatant which was centrifuged again at 5,800 x g for 5 min.. This process was repeated 4 times or until the supernatant appeared clear. The resulting supernatant was placed in a polyallomer microtube (Beckman Instruments) and centrifuged in an Ultracentrifuge T100 (Beckman Instruments) at 100,000 x g for 90 min. at 4°C. The supernatant and the corresponding pellet, containing cytoplasmic and outer membrane proteins, were stored at 4°C prior to analysis by sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.3.2.2 PREPARATION OF *LEGIONELLA* PROTEIN SAMPLES FOR GEL SEPARATION.

Sample buffer was added to the pellets of protein prepared as above. The volume added was just sufficient for resuspension of the pellet, (5-40 µl). Sample buffer was also added to the corresponding supernatant sample in the proportion 1 : 3, sample buffer : supernatant. Both sets of samples were incubated at 80°C for 10 min. to denature the proteins prior to separation by SDS-PAGE. The constituents of sample buffer were 10 mM Tris/HCl pH 8.6, 1 mM EDTA, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 30 % (w/v) glycerin and 0.1 % (w/v) bromophenol blue in ddH_2O.

3.3.2.3 PREPARATION OF WHOLE HOST CELL PROTEINS.

A sample of either *A polyphaga* trophozoites or U937 monocytes was washed in amoebic saline by centrifugation at 400 x g for 6 min. The resulting pellet was resuspended in 50 ml of amoebic saline and OD_{660} value of the suspension determined. The volume of the suspension was adjusted to an OD_{660} value of 1.0 by means of amoebic saline. A 50 ml volume of the suspension was centrifuged at 400 x g for 6 min. to pellet the cells. The resulting pellet was resuspended in 40 µl of sample buffer and incubated at 80°C for 10 min. to denature the proteins prior to separation by SDS-PAGE.

3.3.2.4 SEPARATION BY SDS-PAGE

Separation of the whole cell protein pellets and supernatants was performed using a Mini-Protean gel apparatus (Biometra, Maidstone, UK). Two glass gel plates were used, one of which was slightly shorter than the other and notched at the top. The gel plates were prepared by initially washing in ddH_2O and drying in a flow of air to remove grease spots which could cause air bubbles to form in the gel. The larger plate was laid on the bench and a 1 mm thick rubber sealer was placed around the sides and bottom of the plate. The shorter notched plate was placed on top of the sealer so the plates were symmetrical (Figure 3.6). The plates were held together in an upright

position using clips. A gel *per se* consisted of a 6 % stacking and a 12 % separating layer. The constituents of the gel are given in Table 3.1

Stock solution	Separating layer	Stacking layer
30:08 (w/v) Acrylamide : bis-acrylamide	8.26 ml	1.00 ml
Tris/HCl pH 8.8 (1.5 M)	5.00 ml	
Tris/HCl pH 6.8 (1.0 M)		1.40 ml
SDS 10 % (w/v)	200 µl	60 µl
ddH_2O	7.06 ml	3.46 ml
TEMED*	20 µl	20 µl
Ammonium persulphate 0.1 % (w/v)	120 µl	60 µl

* = N,N,N'N'-tetramethylene diamine

Table 3.1. Constituents of the SDS-PAGE gel.

The separating gel was poured between the glass plates leaving a gap of *ca.* 1.5 cm at the top. This was filled with sterile water to cause the gel to set with a straight gel front. The separating gel was allowed to set for 20 min. whilst the stacking gel was prepared. Once the separating gel was set, the water was replaced by the stacking gel and a clean teflon comb inserted between the gel plates to give the required number of wells (Figure 3.6). The stacking gel was allowed to set for *ca.*10 min. before the comb was removed, thus creating the sample wells. The wells were flushed out using electrode buffer (50 mM Tris, 344 mM Glycine, 0.1 % (w/v) SDS) immediately after the comb was taken out to remove any un-polymerised acrylamide solution. If allowed to polymerise the surplus acrylamide produces irregular shapes in the wells leading to distortion of the protein bands. The rubber spacer was removed prior to the attachment of the gel to the electrophoresis tank using clips on the sides of the gel. The shorter notched plate faced inwards towards the central electrophoresis tank. The central tank and troughs were filled with electrode buffer. A bent needle and syringe were used to remove air bubbles trapped beneath the bottom of the gel prior to loading of the samples. Samples of resuspended pellet (2-5 µl) or supernatant (5-10 µl) in sample

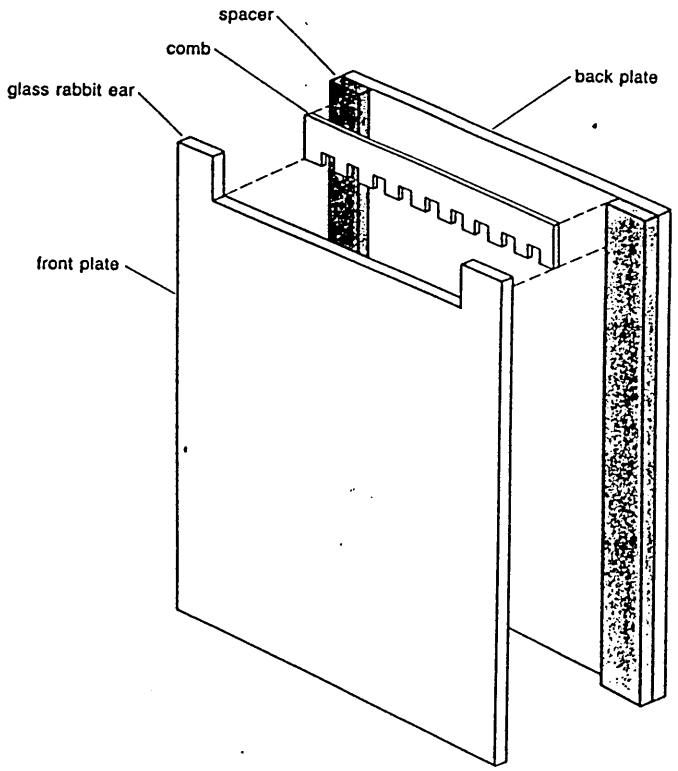


Figure 3.6. SDS-PAGE gel apparatus, exploded view. In use, the front and back gel plates are separated by spacers. The gap formed is filled with the gel and the comb inserted to form wells.

buffer were loaded into the wells of the gel using a 50 µl Hamilton syringe (Fisons). Bio-rad low range molecular weight markers were diluted 1 : 3 in sample buffer and loaded into the outer wells to enable the molecular weight of the membrane proteins to be determined. Denaturation was not necessary as the markers used were individual proteins with specific molecular weights. These are given in Table 3.2.

Marker	Approx. molecular weight (kDa)
Phosphorylase b	97.4
Serum Albumin	66.2
Ovalbumin	45.0
Carbonic anhydrase	31.0
Trypsin inhibitor	21.5
Lysozyme	14.4

Table 3.2 Bio-rad Low Range Molecular Weight standard markers

Unused wells were loaded with sample buffer (5 µl). This ensures greater efficiency of separation of the proteins in the loaded wells.

The gels were run at a fixed voltage of 40 V (current *ca* 0.01 mA) for an initial 45 min. and then at 120 V (current *ca*. 0.08mA) for 75 min. or until the dye front had migrated to the bottom of the gel. The initial low voltage was applied to maximise the resolution of the bands by 'compacting' the samples prior to migration through the separating gel.

Once the dye front had migrated to the bottom of the gel, the gel was removed from the electrophoresis chamber and the gel plates parted using a thin spatula. The gel was lifted from the larger plate and stained and then destained, following the method of Ames (1974) outlined below.

3.3.2.5 STAINING AND DESTAINING

Protein gels were stained for 1 h, with gentle agitation, in Coomassie Blue stain containing 50 % (v/v) methanol, 10 % (v/v) glacial acetic acid and 0.1 % (w/v) Brilliant

blue R-250 (Sigma) in ddH_2O. This solution caused both the protein bands and the background to be stained blue. The background stain was removed from the gel by repeated washing in a destain solution consisting of 50 % (v/v) methanol and 10 % (v/v) acetic acid in ddH_2O. The protein bands were not destained by this process.

Once destaining was complete, the gel was washed in ddH_2O and placed on a piece of 3MM absorbant paper (Whatman, Maidstone, UK). The paper was placed on to a slab dryer (Bio-Rad) and a vacuum applied. The gel was dried on to the piece of paper at 80°C for 3 h.

3.3.2.6 DETERMINATION OF THE MOLECULAR WEIGHTS OF *LEGIONELLA* PROTEINS.

The molecular weights of the separated *Legionella* proteins were determined from a standardisation curve prepared for each gel. The standardisation curve was produced by plotting the calculated R_m value of each standard protein marker against the $\log_{10}$ value of the corresponding molecular weight. The molecular weights of the separated *Legionella* proteins can be determined from this curve after calculation of their respective R_m values. The relative mobility (R_m) of each standard protein marker and unknown protein was calculated by means of the formulae :

$$\text{Relative mobility (Rm)} = \frac{\text{Distance migrated by the protein (mm)}}{\text{Length of the separating gel}}$$

3.3.3 PREPARATION OF OUTER MEMBRANE PROTEINS (OMP).

3.3.3.1 STANDARD METHOD

The technique used was the same as that described in Section 3.3.2.1 for the preparation of cell membrane proteins. To destabilise the cytoplasmic membrane, 125 µl of 10 % (w/v) lauryl sarcosinate (sarkosyl) was added after the sonication of the sample and prior to incubation at room temperature for 1 h. This technique failed to produce clear distinct bands when the resulting OMP pellet was separated on an SDS-PAGE gel and stained with Coomassie blue. Various modifications of the standard

method were examined, details of which are given in Appendix 2. Method 7 was found to give clear distinct bands.

3.3.3.2 MODIFIED METHOD (No. 7).

The method described by Barthe *et al.* (1988) was investigated. Briefly,125 µl of 10 % (w/v) sarkosyl was added to a sample of cell suspension prior to incubation at room temperature for 1 h. The sample was then sonicated in an ice bucket for 20 cycles of 10 sec pulses with 20 sec pauses for cooling. After sonication a 500 µl volume of 50 mM Tris (pH 7.2) containing lysozyme (1 mg/ml) and sarkosyl 2 % (w/v), was added to the suspension prior to centrifugation at 5,800 x g for 10 min.. The pellet formed was removed and the supernatant centrifuged for a further 5 min.. The resulting supernatant was placed in a polyallomer tube (Beckman) and centrifuged at 100,000 x g for 30 min. at 4°C. After centrifugation, the pellet formed was separated from the supernatant.The latter was stored at 4°C prior to analysis by SDS-PAGE. The pellet was resuspended in 500 µl of 50 mM Tris, pH 7.2, containing sarkosyl 2 % (w/v) and the centrifugation was repeated. The resulting supernatant was removed and the pellet stored at 4°C.

3.3.4 PROTEIN ASSAY.

A bicinchoninic acid microquantity protein assay (Smith *et al.*, 1985) was performed to establish the quantity of protein in the outer membrane pellets and the corresponding supernatants prepared by the above method (Section 3.3.3.2). This assay was performed in an attempt to ensure that equal quantities of protein were applied to all the SDS-PAGE gels. Lysozyme is added to the *L pneumophila* pellet and supernatant during their preparation. Lysozyme itself is a protein and its presence could give rise to a high protein content in the pellet or supernatant. To enable the true protein content of the pellet and supernatant to be calculated the quantity of protein arising from the added lysozyme was determined. This was achieved by repeating the OMP preparation process omitting the *L. pneumophila*. The resulting supernatant and pellet were both stored at 4°C prior to the assay.

A series of dilutions (0-500 μg/ml) of bovine serum albumin (BSA) in ddH_2O were made. Volumes (100 μl) of each dilution were pipetted, in duplicate, into the wells of a polystyrene microtitre plate (Immulon II, flat bottomed 96 well plate, Dynatech, Billingshurst, UK). The *Legionella* outer membrane pellets were resuspended in 200 μl of ddH_2O. Volumes (10 μl and 20 μl) were pipetted into the wells of the 96 well plate. In addition, each of the background pellets produced from the bacterial free suspensions, were resuspended in 200 μl of ddH_2O and 10 μl and 20 μl volumes pipetted into wells of the 96 well plate. Volumes (10 and 20 μl) of the supernatants from the *Legionella* preparations and 20 μl of the background supernatant were also placed in the wells. A volume (100μl) of micro-working reagent (M-WR) was added to each well. The M-WR was prepared according to the formulation of Smith *et al.* (1985). The reagent is made from three different micro-reagents, MA, MB, MC, whose compositions are given in Table 3.3.

Micro-reagent	Constiuents
MA	8.0 % (w/v) $Na_2CO_3.xH_2O$
	1.6 % NaOH
	1.6 % Tartaric acid (Disodium salt)
	pH was adjusted to 11.25 using $NaHCO_3$
MB	4.0 % bicinchoninic acid
MC	4 vol. of 4.0 % (w/v) $CuSO_4.5H_2O$ + 100 vol (MB)
M-WR	1 vol.MA + 1 vol. MC

Table 3.3. Composition of micro-working reagent (M-WR) and its constituents

The prepared well plate was incubated at 60°C for 1 h. After incubation the optical density of the suspensions was measured at a light wavelength of 550 nm using a plate reader (Anthos reader 2001, Anthos Labtec Instruments, Austria). The protein content of each supernatant or pellet suspension attributable to *L. pneumophila* alone was determined as follows: the OD_{550} value due to M-WR (blank, no BSA) was deducted

from the values obtained for each of the BSA samples. The resulting values were plotted against the respective predetermined protein concentrations to form a standard curve (Figure 3.7). The concentration of *Legionella* protein in the pellet and supernatant preparations were determined from this curve from a knowledge of their true OD_{550} values. These were obtained by measuring the well plate OD_{550} value and deducting from it the value attributable to the background of lysozyme and M-WR.

The protein content of the exponential phase and stationary phase broth grown *L. pneumophila* supernatants were determined as 517.5 and 586.5 μg/ml, respectively. Volumes (10 μl) of supernatant containing *ca.* 5 μg of protein were subsequently loaded into each well of the gel prior to analysis. The protein content of the pellet preparation of broth grown *L. pneumophila* in exponential phase and stationary phase were determined as 1.35 mg/ml and 2.69 mg/ml, respectively. The pellet preparations from legionellae in exponential phase were subsequently resuspended in 200 μl of ddH_2O and the pellet preparations from the stationary phase bacteria were resuspended in 400 μl of ddH_2O. Volumes (1 μl) of each suspension were loaded into wells of an SDS-PAGE gel prior to analysis. This again allowed for the separation of *ca.* 5 μg of protein per well.

3.3.5 PREPARATION OF LIPOPOLYSACCHARIDE LAYER

3.3.5.1 PROTEINASE K DIGEST

The lipopolysaccharide (LPS) layer was extracted from outer membrane preparations (Section 3.3.2) by boiling at 100°C in lysing buffer (200 μl) for 10 min. prior to the addition of 10 μl of a solution of proteinase K in lysing buffer (1 mg/ml). The lysing buffer consisted of 62.5 mM Tris-HCl, pH 6.8, containing 10 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 3 % (w/v) SDS and 0.01 % (w/v) bromophenol blue. The sample was incubated at 60°C for 1 h. before centrifugation at 11,600 x g for 5 min.. The supernatant was retained and stored at 4°C prior to analysis by SDS-PAGE, using the method described in Section 3.3.2.3.

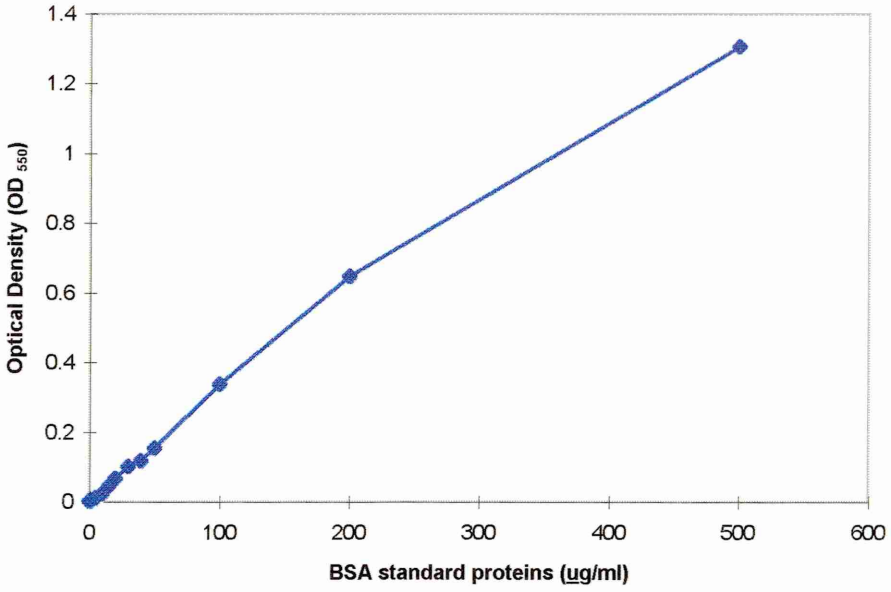


Figure 3.7 Standard curve showing relationship between optical density (OD_{550}) measurements and predetermined protein concentrations of BSA. The OD_{550} values of the protein content of BSA solutions, arising from the bicinchoninic acid protein assay, were determined. The curve was used to determine the protein content of YE broth grown *L. pneumophila* sarkosyl insoluble OMP preparations and the corresponding supernatants.

Volumes (2-5 µl) of the LPS samples were loaded into the wells of an SDS-PAGE gel. Purified lipopolysaccharide from *E. coli* 0111 : B4 was loaded on to the gel as a reference (Tsai and Frasch, 1982). The lyphophilised powder (Sigma) was hydrated to a concentration of 1 mg/ml with sterile ddH_2O and subsequently stored at -20°C. The *E.coli* LPS was mixed with an equal volume of sample buffer (Section 3.3.2.2) and incubated at 80°C for 10 min. prior to loading 5 µl into the outer wells of the gel. The LPS samples were separated in the same manner as the protein samples, as described in Section 3.3.2.3.

3.3.5.2 SILVER STAINING

Following separation of the LPS samples by SDS-PAGE, the gel was stained using the method described by Tsai and Frasch (1982). The gel was immersed overnight (12-16 h.) in 200 ml of fixing solution (40 % (v/v) ethanol, 5 % (v/v) acetic acid in ddH_2O) contained in a clean glass dish (Section 2.6). The fixing solution was replaced with 100 ml of oxidising solution (40 % v/v ethanol, 5 % v/v acetic acid, 0.7 % (w/v) periodic acid in ddH_2O) and gently agitated at room temperature for 20 min.. The gel was subsequently given 4 x 15 min. washes in 200 ml volumes of ddH_2O prior to staining by immersion in 150 ml of silver nitrate solution for 15 min., (1 g silver nitrate, 246 ml ddH_2O, 2.8 ml 1 M sodium hydroxide, 3 ml 25 % (v/v) ammonia). Following staining, the gels were given 4 x 15 min. washes in ddH_2O prior to development. The gels were gently agitated in 200 ml developing solution (25 mg citric acid, 250 µl formaldehyde, 250 ml ddH_2O) until the LPS ladders appeared to the required intensity. The gels were immediately washed in ddH_2O to stop the reaction and then washed 4 x 15 min. in ddH_2O to ensure complete removal of the developing solution. If the gel became overstained, the gel was washed 4 x 15 min. in ddH_2O prior to gentle agitation in a 25 % solution of Max-Fix photographic fixing solution (Kodak Ltd, Liverpool, UK) until the gel faded to the required intensity. The gel was finally washed exhaustively in ddH_2O to remove the fixing solution prior to photographing and drying on a slab dryer under vacuum (Section 3.3.2.4).

3.3.5.3 INTERPRETATION OF LPS LADDER PATTERNS.

During SDS-PAGE analysis, negatively charged LPS chains migrate towards the anode due to association with lipid A. The distance migrated is dependent on the length of the sugar chain and not on their molecular mass. The shorter the LPS chain the further it migrates down the gel. Protein molecular weight markers, therefore, cannot be used to calculate the molecular mass of LPS chains (Personal communication, H. Chart).

3.4 IMMUNOLOGICAL STUDIES

3.4.1 IMMUNOLOGICAL DETECTION

Two different techniques of immunological detection were used during this project, Enzyme-linked immunosorbant assays (ELISA) and immunoblotting. Enzyme-linked immunosorbant assays were performed to determine the titre of the antibodies raised in rabbits and in sera from patients previously infected by *L. pneumophila* serogroup 1 (Kindly donated by T. Boswell, Public Health Laboratories, Heartlands Hospital, Birmingham, UK). This technique was also used to examine the capabilities of the various antibodies to bind to OMP antigens from the pellet preparation, the corresponding supernatant, and the proteinase K extracted LPS antigens. Once the titre of the antibodies was determined, immunoblotting was used to examine the binding of antibodies to specific OMP or LPS antigens after their electrophoretic transfer to a nitrocellulose membrane.

3.4.1.1 BASIC IMMUNOLOGICAL DETECTION TECHNIQUES

The ELISA and immunoblotting techniques each consisted of 5 basic stages. The two techniques were very similar, once the initial immobilisation of the antigens had been achieved. In the case of the ELISA technique, antigens were immobilised to well surfaces of a microtitre plate. The immunoblotting technique employs electrophoretic transfer of antigens from a gel on to a membrane, after separation by SDS-PAGE. The basic stages of the two techniques are as follows:

1. Immobilisation of proteins or LPS antigens in the wells of a microtitre plate, or on to a membrane by electrophoretic transfer.

2. Unoccupied sites are saturated (blocked) to prevent nonspecific binding of antibodies, which are protein themselves, to the well surface, or the membrane.

3. The coated well plate surface, or membrane, is probed for antigens of interest with specific, primary antibodies (e.g. antibodies raised in rabbits against *Legionella* or those from patients infected with *L. pneumophila* serogroup 1).

4 Secondary antibodies, specific for the primary antibody type and conjugated to enzyme markers are used to label the antigen-antibody complexes (e.g. anti-rabbit IgG peroxidase or anti-human IgG peroxidase).

5. The peroxidase-labelled antigens are made visible by incubation with appropriate substrates which form insoluble coloured products.
The practical procedure is summarised in Figure 3.8.

3.4.2 ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA) TECHNIQUE.

3.4.2.1 PRELIMINARY WORK

To establish the optimum concentration of antigen that would bind antibodies, a 'chequer-board' titration was performed. A polystyrene microtitre plate (Immulon 2, flat-bottomed 96 well plate) was coated with serial dilutions of the antigen across the plate followed by serial dilutions of commercially available anti-*L. pneumophila* serogroup 1 antibody, (PHLS Laboratories, London, UK), down the well plate as outlined in Figure 3.9. The outer-most rows and columns were used for controls of no antigen, no 1° antibody and no 2° antibody. These controls were standard throughout the ELISA assays.

3.4.2.2 IMMOBILISATION - COATING OF MICROTITRE PLATE (STEP 1).

An OMP pellet, prepared as described in Section 3.3.3.2, was initially diluted 1 in 400 in 50 mM Na_2CO_3 buffer prior to serial two-fold dilution from 1 in 400 to 1 in 204,800 in the same buffer. Volumes (100 µl) of each dilution were pipetted into each set of

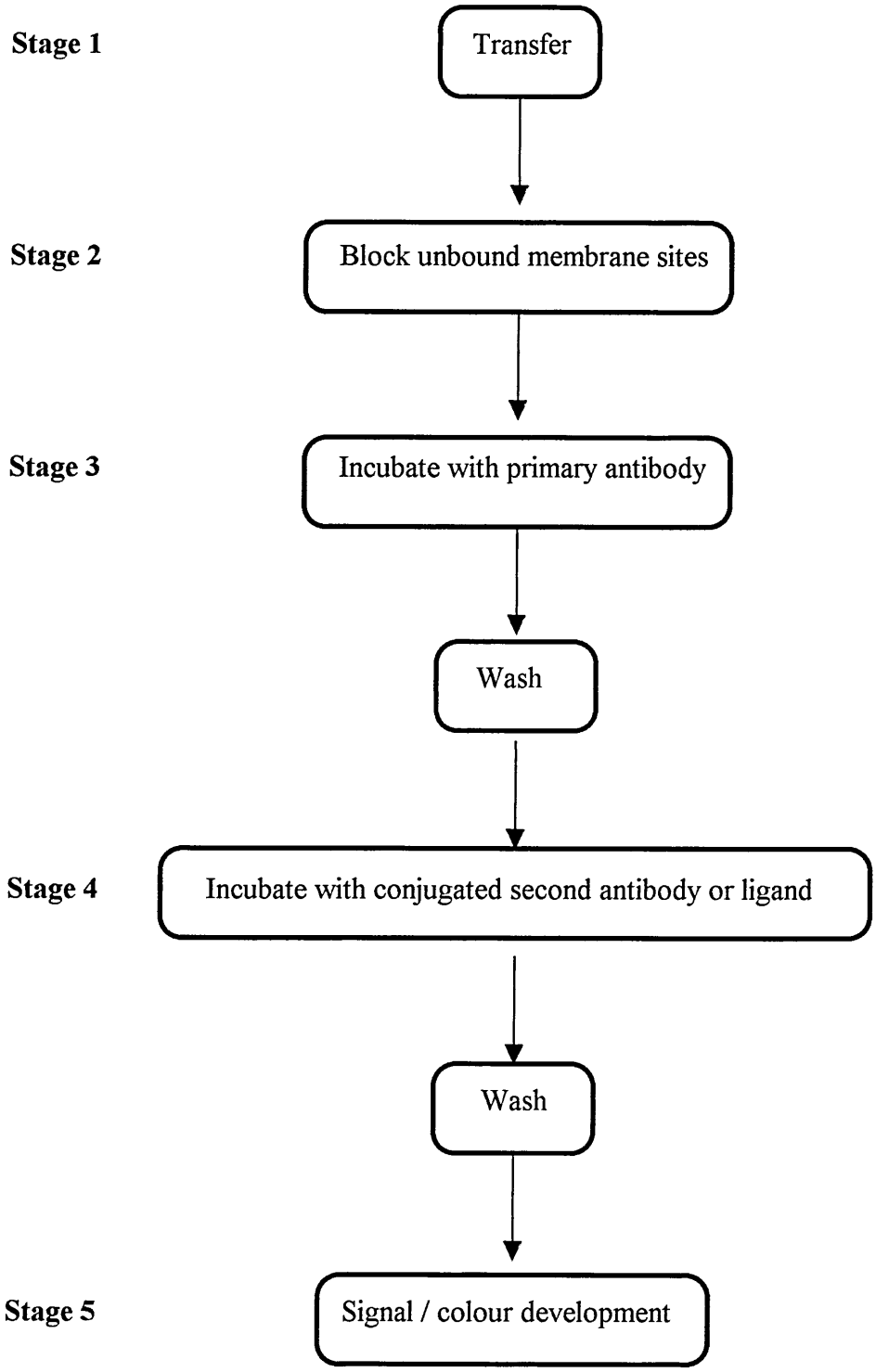


Figure 3.8 Outline of ELISA / Immunoblotting procedure.

wells (Figure 3.9). The plate was placed in a moist chamber and incubated overnight at 37°C.

3.4.2.3 BLOCKING (STEP 2).

After immobilisation, the unbound antigen was removed from the wells and 200 µl of blocking solution was pipetted into each well, including those acting as controls. The plates were incubated at 37°C for 2 h. Various blocking solutions namely, skimmed milk (1 % and 5 % w/v) in PBS, Tween, and bovine serum albumin (BSA, 1 % w/v) in PBS were examined during the preliminary ELISA assays. A blocking solution consisting of BSA (1 % w/v) in PBS was found to give the greatest differentiation between the diiferent antigen / antibody concentrations.

3.4.2.4 BINDING OF PRIMARY (1^{O}) ANTIBODY (STEP 3).

After incubation, the blocking solution was removed and the wells washed once with PBS. A microtitre plate (round bottom, 96 well plate, Fisons, Loughborough, UK) was used to prepare serial two-fold dilutions of the test sera in blocking solution. Volumes (100 µl) of the diluted sera were transferred to the corresponding wells of the coated ELISA plate, as outlined in Figure 3.9. The ELISA plate was then covered and incubated at 37°C for 1 h. It was then washed three times in PBS.

3.4.2.5 BINDING OF SECONDARY (2^{O}) ANTIBODY (STEP 4).

A 100 µl volume of peroxidase-labelled monoclonal anti-rabbit IgG conjugate (Sigma A-2074) diluted 1 in 10,000 in blocking solution was added to each well of the plate. The plate was covered and incubated at 37°C for 2 h. after which it was washed five times in PBS.

3.4.2.6 BINDING OF SUBSTRATE / COLOUR DEVELOPMENT (STEP 5).

The substrate used was 2,2-azino-bis 3-ethyl benzthiazoline-6-sulphonic acid (ABTS). One 10 mg tablet was initially dissolved in 100 ml of 0.05 M phosphate-citrate buffer, pH 5.0. Phosphate-citrate buffer was prepared by adding 25.7 ml of 0.2 M dibasic

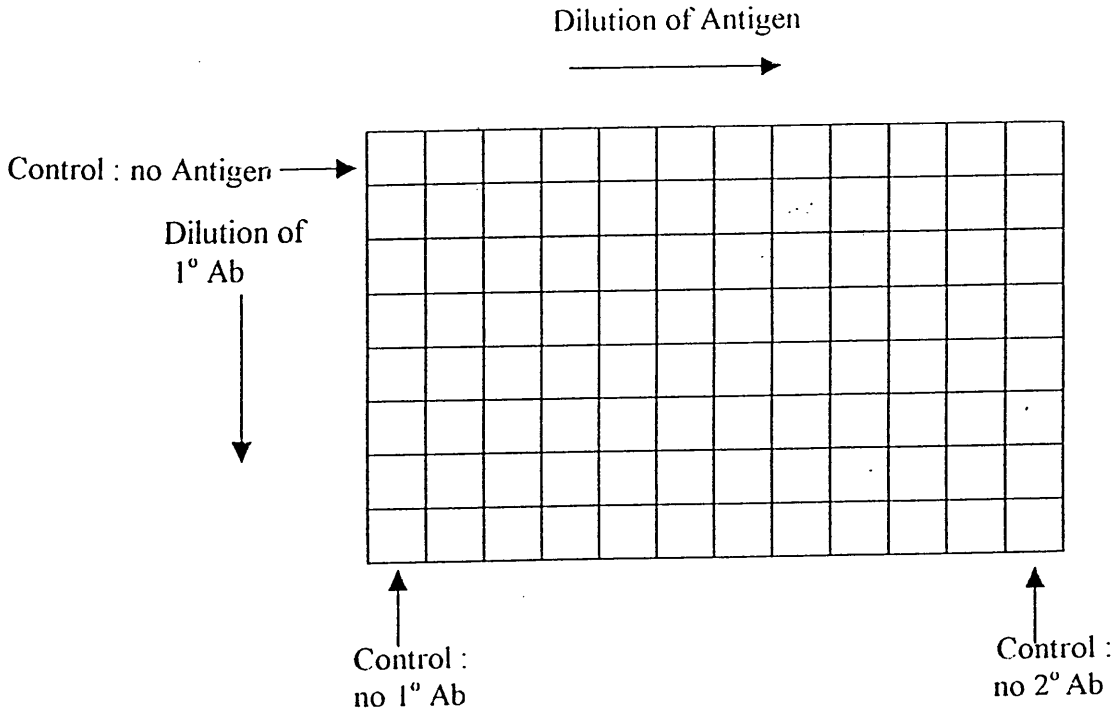


Figure 3.9 Preliminary ELISA plate array for chequer-board titration to determine optimum antigen and antibody dilutions for further ELISA.

sodium phosphate and 24.3 ml of 0.1 M citric acid to 50 ml of ddH_2O. A 125 µl volume of 6 % hydrogen peroxide was added to the substrate solution immediately prior to its use. A 100 µl volume of substrate solution was placed in each well and the plate incubated at 37°C for 1 h. The optical density of the green colour formed during incubation was read at 405 nm using a plate reader (Anthos reader 2001, Anthos Labtec Instruments, Austria)

The above method was found to give weak colour development and correspondingly poor differentiation. Experimentation has shown that colour development can be enhanced by modification of the substrate composition (Personal communication; T. Pepper). The modified substrate was prepared by dissolving one 10 mg tablet of ABTS in 16.6 ml of phosphate-citrate buffer, the pH of which was adjusted to 4.4 using 10 M HCl. Immediately prior to use 5 µl of 6 % hydrogen peroxide was added to each 10 ml of buffer. The optimum antigen (pellet suspension) concentration required to coat the plates for titration of the antibodies raised in rabbits was determined by plotting 'true' OD_{405} value (measured OD_{405} value minus the mean OD_{405} value produced by the controls) against the antigen dilution factor.

3.4.3 RAISING OF ANTIBODIES AGAINST INTRA-AMOEBIC, INTRA-MONOCYTIC AND BROTH GROWN *L. PNEUMOPHILA* AND HOST CELLS.

3.4.3.1 PREPARATION OF SOLUTION TO IMMUNISE RABBIT.

A total of five rabbits were immunised with one each of the following:

Rabbit 1- Whole *A. polyphaga* trophozioites.

Rabbit 2- Whole U937 monocytic cells.

Rabbit 3- Intra-amoebic grown *L. pneumophila.*

Rabbit 4- Intra-monocytic grown *L. pneumophila.*

Rabbit 5- Broth grown *L. pneumophila* (stationary phase).

L. pneumophila were concentrated by centrifugation at 2,080 x g for 30 min.. The amoebae and monocytes were centrifuged at 400 x g for 6 min.. Each resulting pellet was resuspended in 5 ml of tissue culture PBS (Section 2.4.2). The bacterial suspensions were centrifuged again at 2,080 x g for 30 min. and the host cells at 400 x g for 6 min.. Each pellet was resuspended in 5 ml of tissue culture PBS. This process was repeated four times to ensure complete removal of the *Legionella* growth medium for each sample. Prior to the fourth centrifugation, the concentration of each suspension was determined by haemocytometer counting. The volume of tissue culture PBS required to resuspend each pellet to a concentration of *ca.* 10^9 cells/ml was determined. A 3 ml volume of each suspension was centrifuged, as described above, and the resulting pellets resuspended in 3 ml of PBS containing 0.5 % (v/v) formaldehyde. The cells were incubated at 37°C for 2 h, with shaking. The suspensions were then washed three times by centrifugation at 8,000 x g for 20 min. with 10 ml volumes of PBS resulting in a dilution of the formaldehyde of at least 1000-fold. This was necessary as the original concentration of formaldehyde would have harmed the rabbit upon immunisation. The bacteria were resuspended in PBS to give a final concentration of *ca.* 10^9 cells/ml. The quantity of cells was checked by haemocytometer counting and the suspensions were streaked out on to BCYE agar to confirm purity.

3.4.3.2 IMMUNISATION OF THE RABBITS

The raising of antibodies in rabbits was performed at Aston University by a designated holder of an animal licence. The rabbits used were 3 to 4 month old Belgian Giant rabbits. The rabbits were injected with 200 µl of bacterial suspension into the ear vein every 7 d. After 4 wk., a sample of blood was taken from each rabbit and the antibody titre determined by ELISA.

3.4.3.3 PREPARATION OF THE SERUM

A sample of blood from each rabbit was incubated in a clean 50 ml centrifuge tube (Beckman) at 37°C for 1 h. Calcium chloride was then added to give a final

concentration of 5 mM prior to overnight incubation at 4°C. This caused the blood to coagulate and the resulting blood clot was removed by centrifugation at 250 x g for 10 min. Each supernatant (serum) was dispensed into a sterile cryovial and held at 4°C prior to the ELISA assay.

3.4.4 TITRATION OF ANTIBODIES

3.4.4.1 TITRATION OF ANTIBODIES RAISED IN RABBITS

The ELISA analysis outlined in Section (3.4.1.2) was repeated, but with the important exception that a 1 in 10,000 dilution of antigen (OMP pellet) was immobilised on to each test well. Each sera raised in a rabbit (Section 3.4.1.2) was serially diluted two-fold in blocking solution from a 1 in 30 dilution to 1 in 1,920 in a microtitre plate, in duplicate. Volumes (100 µl) were transferred to the corresponding wells of the coated ELISA plate, as outlined in Figure 3.10. After completion of the assay, the 'true' OD_{405} of each test well was plotted against the dilution factor for each type of rabbit antibody. This enabled the titre of each rabbit raised antibody to be determined and the titres compared to one another.

When the titre of each antibody was found to be sufficiently high for the immunoblotting technique, each rabbit was bled through cardiac puncture and completely drained. The serum was prepared as in Section 3.4.3 and dispensed into 1 ml volumes held in sterile eppendorf tubes. Until required the serum was stored at -20°C but once defrosted it was maintained at 4°C.

The work of Knirel *et al.* (1996) suggested that as *Legionella* LPS is highly immunogenic, antibodies would bind to the LPS and not the OMPs. To investigate the binding capabilities of each rabbit raised antibody the ELISA assays were repeated with minor modifications to the immobilisation process. Plates were coated in either a 1 in 10,000 dilution of OMP antigen (pellet preparation), as described previously, or a 1 in 25 dilution of the corresponding antigen (supernatant) in 50 mM Na_2CO_3 buffer. A 100 µl volume of the supernatant antigen was immobilised on to the surface of each

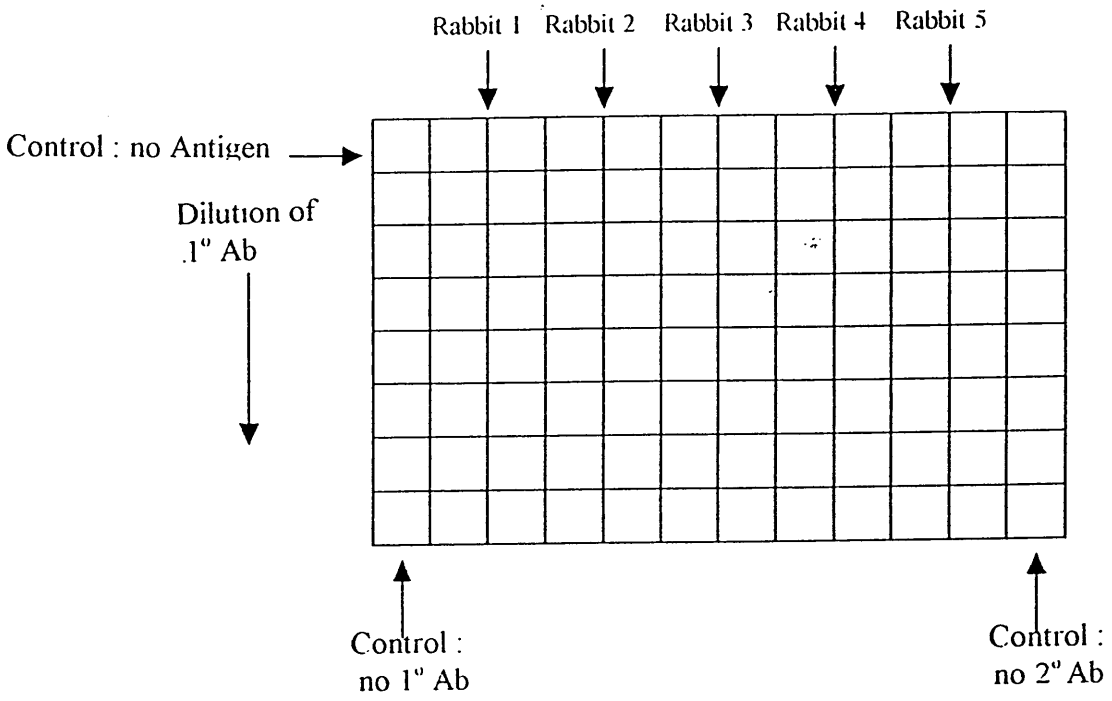


Figure 3.10. ELISA plate array for determination of relative response of rabbit antibodies raised against variously grown *L. pneumophila* or host cells. A fixed concentration of antigen was applied to each well as determinded in Figure 3.9

well by overnight incubation (12-16 h.) at 37°C in a moist chamber. Extracted LPS antigen was diluted 1 in 50 in a diluent consisting of 1 ml of chloroform and 10 ml of absolute ethanol. A 100 µl volume of LPS solution was pipetted into each test well and allowed to evaporate during overnight incubation at 37°C (Bantroch *et al.*, 1994). After completion of the assay as described in Section 3.4.2, the 'true' OD_{405} was plotted against antibody dilution factor for each assay.

3.4.4.2 TITRATION OF PATIENT SERA

The titre of the patient sera was determined using the OMP antigen (pellet preparation), the corresponding supernatant antigen, and the LPS antigen of broth grown *L. pneumophila* in stationary phase, using the methods outlined above. Due to the fact that the primary antibodies were from patients, it was necessary to use peroxidase-labelled goat anti-human IgG peroxidase as the secondary antibody. A 100 µl volume of anti-human IgG diluted 1 in 4,000 in blocking solution was pipetted into each test well and the plates covered. The plates were incubated at 37°C for 2 h. The ELISA was developed and analysed as in Section 3.4.2.6.

3.4.5 IMMUNOBLOTTING

The immunoblotting process employed involved the electrophoretic transfer of either proteins or LPS from an SDS-PAGE gel to a nitrocellulose membrane (0.45 µm pore size, BioRad). Immobilisation of the proteins or LPS on to the membrane can be achieved by either complete immersion of a gel-membrane sandwich in a buffer (wet transfer) or by placing the gel-membrane sandwich between absorbent paper soaked in buffer (semi-dry transfer). Both were evaluated using protein and LPS samples. Semi-dry transfer can be performed using a single buffer or a triple buffer system; both of which were tested. The triple buffer semi-dry system was found to produce the best resolution of protein bands and LPS regions after antibody probing and colour development. The wet transfer and single buffer semi-dry transfer methods are outlined in Appendix 4. The triple buffer semi-dry system employed throughout the immunoblotting experiments is outlined below.

3.4.5.1 IMMOBILISATION BY TRIPLE BUFFER SEMI-DRY ELECTROPHORETIC TRANSFER.

The apparatus employed for the semi-dry electrophoretic transfer was a "Transblot Semi-dry" (BioRad) as shown in Figure 3.11. The gel and membrane were sandwiched horizontally between two layers of buffer wetted absorbent paper in direct contact with two closely spaced solid plate electrodes. The upper plate was the cathode and the lower plate the anode. This system employed three buffers :

anode buffer I (300 mM Tris/HCl, 10 % (v/v) methanol pH 9.9)

anode buffer II (25 mM Tris/HCl, 10 % (v/v) methanol pH 9.4)

cathode buffer (25 mM Tris/HCl, 10 % (v/v) methanol, 40 mM ε-aminocaproic acid, pH 8.4).

After separation of the proteins or LPS by SDS-PAGE, the resulting gel was immersed in anode buffer II for 10 min.. Four pieces of 3MM absorbent paper (Whatman) and one piece of nitrocelullose membrane were cut to the size of the gel. The gel-membrane sandwich was set up, as outlined in Figure 3.11. One piece of paper saturated in anode buffer I was placed on to the anode plate followed by the one piece of anode buffer II soaked paper. The nitrocellulose membrane soaked in anode buffer II, then the gel were placed on to the wetted paper followed by two pieces of paper soaked in cathode buffer. A glass rod was gently rolled over the sandwich to remove any air bubbles. The cathode plate and lid were placed on top of the sandwich and transfer allowed to take place at a constant current of 150 mA; 2-3 V, for 1 h.

3.4.5.2 BLOCKING / STAINING

After transfer was complete, the gel was removed and stained for either proteins or LPS accordingly, (Section 3.3.3.2 or Section 3.3.5.1., respectively). The orientation of the membrane to the gel was marked on the membrane using a pencil. The membrane was removed and immersed in 25 ml of blocking solution (Section 3.4.3.3) followed by incubation overnight (12-16 h.) at 4°C. The membrane was then washed once in 25 ml of PBS (Section 2.4.2).

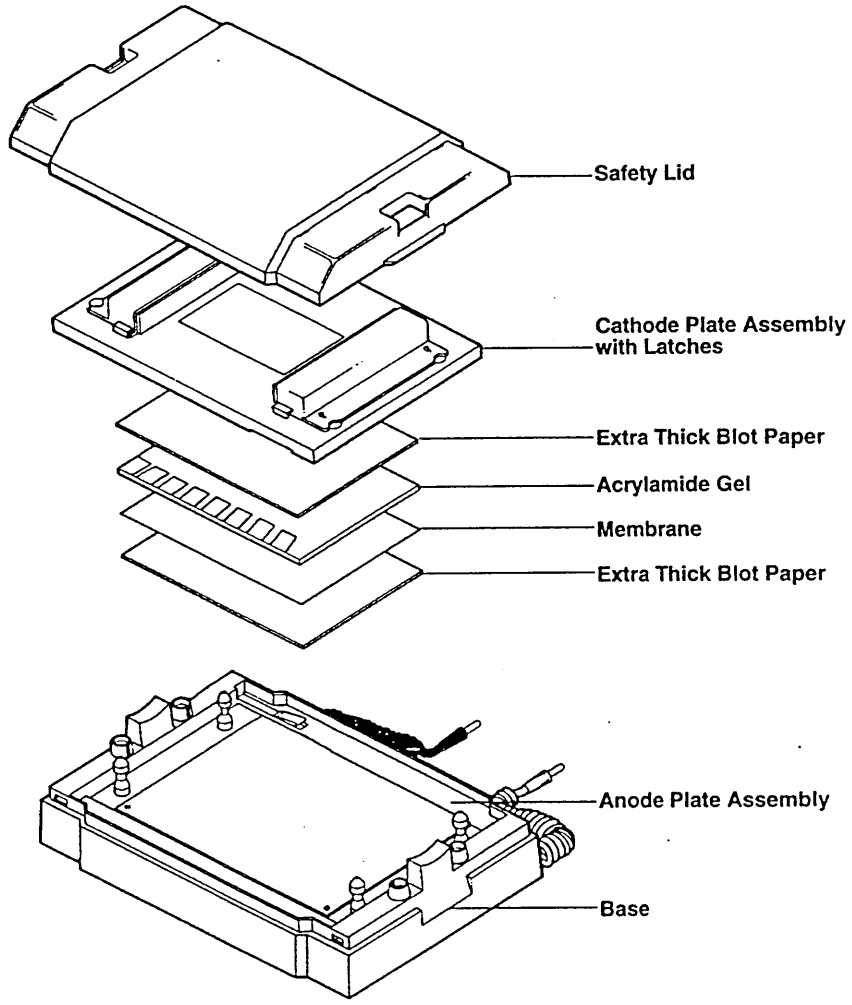


Figure 3.11 Semi-dry "Transblot" apparatus- exploded view. Used for the transfer of LPS or protein from the SDS-PAGE gel to a nitrocellulose membrane. The gel-membrane sandwich is formed between absorbent paper soaked in anode buffer or cathode buffer.

3.4.5.3 BINDING OF PRIMARY ANTIBODY

Rabbit raised anti-*L. pneumophila* antibodies were diluted in 25 ml of blocking solution to give the optimum concentration of antibody which was found to bind the protein or LPS antigens (Section 3.4.3.). The rabbit antibodies raised against whole amoebae or monocytes were diluted 1 in 10 in 25 ml of blocking solution prior to probing the membrane. Due to the small quantities of patient sera available, these sera were diluted 1 in 50 in 5 ml of blocking solution and placed in a 30 ml sample tube (Bibby). The membranes, to be probed with patient sera, were cut into two or three strips which were rolled and placed in the tubes. The surface to which the LPS or proteins had been transferred faced inwards. The tubes were rolled for 3 h. at 37°C. Membranes probed with rabbit sera were incubated in trays at 37°C for 2 h. without agitation. Each membrane was then washed with 25 ml of PBS three times. The membrane strips were removed from the tubes prior to washing. The diluted patient sera were stored at 4°C for re-use, after the addition of 2-3 crystals of sodium azide. The latter acts as a preservative.

3.4.5.4 BINDING OF SECONDARY ANTIBODY

The membranes probed with rabbit sera were immersed in 25 ml of peroxidase-labelled anti-rabbit IgG conjugate diluted 1 in 30,000 in blocking solution. The membrane strips probed with patient sera were immersed in 25 ml of peroxidase-labelled anti-human goat IgG conjugate diluted 1 in 2000 in blocking solution. All membranes were incubated at 37°C for 2 h. and then washed five times with 25 ml volumes of PBS.

3.4.5.5 SUBSTRATE / COLOUR DEVELOPMENT

In an attempt to optimise the colour development and resolution of the protein or LPS bands, two substrate solutions were evaluated both of which contained 4-chloronaphthol as the substrate.

Method 1

A stock solution of 4-chloronaphthol was prepared by dissolving 300 mg of chloronaphthol in 10 ml of absolute ethanol. Just prior to developing the membrane,

250 µl of chloronaphthol stock solution was added to 25 ml of 50 mM Tris (pH 7.6). A copius white precipitate formed which was removed by filtering through Whatman No.1 filter paper. Hydrogen peroxide (125 µl of a 6 % solution) was added to the filtrate and the membrane immersed in 25 ml of substrate solution. The membrane was incubated at room temperature, with agitation, for 10 to 15 min.. The reaction was stopped by washing the membrane in copious amounts of ddH_2O. The colouration of the bound regions developed rapidly in this solution but the results were very smeared and diffuse.

Method 2

Chloronaphthol (50 mg) was dissolved in 15 ml of cold absolute ethanol to which 35 ml of 10 mM Tris/HCl pH 7.5 containing 150 mM NaCl and 100 µl of 6 % hydrogen peroxide was added. The membrane was immersed in 25 ml of substrate solution and allowed to develop at 37°C for 20 to 30 min.. This method was found to produce a better resolution of protein or LPS bands and was adopted for this project, despite the longer period of development. An added advantage was that this substrate solution was easier to prepare. An image of the blot was taken using a gel scanner (Ultra-violet Products, Cambridge, UK) and the membranes stored in the dark at room temperature.

CHAPTER 4 GROWTH STUDIES.

The overall objective of this research project was to compare the phenotypic characteristics of *in vitro* grown and intracellular grown *L. pneumophila* with respect to their resistance to external stress stimuli. In order to achieve this aim it was necessary to establish the conditions which would produce consistent yields of both *in vitro* and intracellular grown legionellae with a predictable morphology.

4.1 *IN VITRO* GROWTH.

A comparative study was made of the growth characteristics and yield of *L. pneumophila* from cultures in YE broth (Edelstein, 1982) and CDM (Warren and Miller, 1979).

The growth kinetics of *L. pneumophila* in YE broth and CDM are presented in Figure 4.1. Comparison of the two growth curves shows legionellae in CDM to have a longer lag phase (*ca.*5 h.) than the bacteria in YE broth (*ca.* <1 h.). Likewise, the generation time of legionellae in CDM during exponential phase was greater than that in YE broth (*ca.* 9 h. *cf. ca.* 2 h.). The YE broth grown culture entered stationary phase at an OD_{660} value of 2.46 and produced a maximum yield of *ca.* 10^{10} cfu/ml. This was higher than the maximum OD_{660} value of 0.288 (*ca.* 4×10^8 cfu/ml) achieved during growth in CDM. It was, therefore, decided to adopt YE broth as the medium for *in vitro* growth of *L. pneumophila* for the purposes of this project.

A brown pigment was observed to form in both the YE broth and CDM cultures. Figure 4.2 illustrates a typical change in supernatant colouration of YE broth cultures due to formation of this pigment. The progressive formation of the pigment was followed throughout the growth period by measuring the OD_{660} value of the culture supernatants. Comparison of this data with the corresponding growth curve in YE broth (Figure 4.3) shows that pigment production commences at the late exponential phase of *L. pneumophila* growth and is maximal in early stationary phase. The

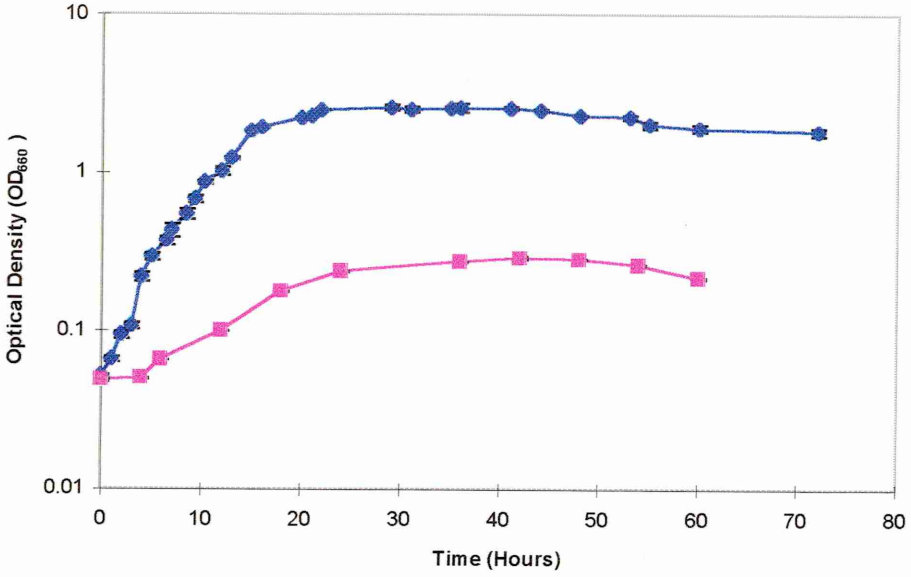


Figure 4.1 Growth kinetics, as measured by OD_{660}, of *L. pneumophila* in YE broth (◆) and CDM (■). Both cultures were inoculated to an OD_{660} value of 0.05 and incubated at 37°C with shaking at 250 rpm. Bars represent the standard errors of the means for three replicate samples.

Figure 4.2. Supernatant colouration of YE broth cultures due to the progressive formation of pigment. Times post inoculation were 0 h, Flask 1; 4 h, Flask 2; 16 h, Flask 3; 24 h, Flask 4; and 48 h, Flask 5.

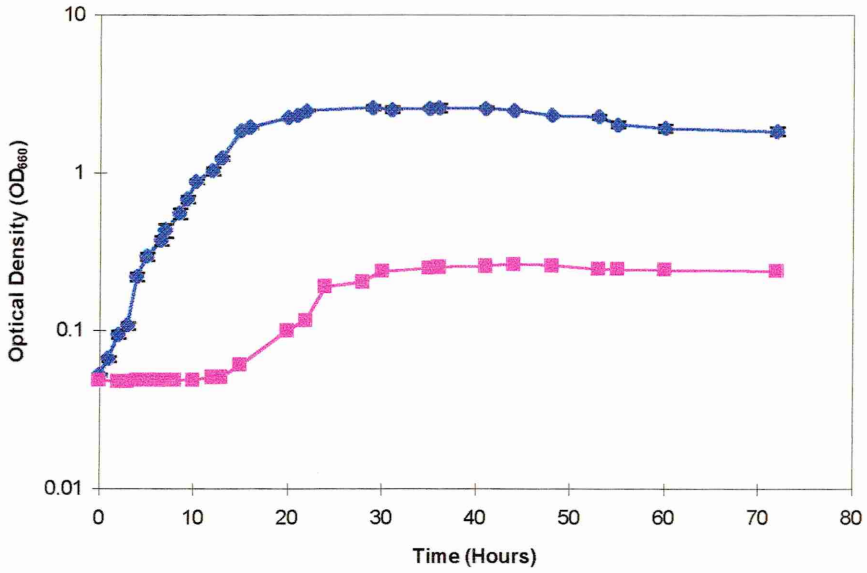


Figure 4.3. Pigmentation of YE broth cultures. Comparison of the pigmentation of the culture supernatant (■) with the corresponding growth curve of *L. pneumophila* in YE broth (◆), as determined by OD_{660} measurements. Bars represent the standard errors of the means for three replicate samples.

formation of pigment was not observed upon incubation without shaking, or on incubation with shaking when inoculated with 1000 fold less bacteria, i.e 10^4 cfu/ml.

A review of the literature suggests that *L. pneumophila* grown in artificial media adopt either a rod-like or a filamentous morphology. A microscopical investigation was, therefore, undertaken to determine the morphology of *L. pneumophila* grown in YE broth in this study and to identify any changes in morphology that may have occurred. Changes in bacterial morphology during growth were monitored by oil immersion phase contrast microscopy at x 1000 magnification. The initial inoculum consisted mainly of single rod-shaped bacteria which were non-motile (Figure 4.4). After *ca.* 5 h., exponential phase commenced and the number of rod-shaped legionellae increased. In addition, many were observed to be undergoing binary division and appeared as pairs, end to end (Figure 4.5). The number of pairs decreased towards the end of the exponential growth phase with the majority of the bacteria adopting a single rod-like morphology. A few bacteria (*ca.* 1-2 per field) adopted a filamentous form (Figure 4.6). At the stationary phase of growth, the bacteria were observed to be single rod-shaped (*ca.* 0.5-0.75 μm x 2-3 μm) and to be non-motile. A small number of atypical bacteria (*ca.* 1-2 per field) which were small, rounded and motile were also present at this stage of growth (Figure 4.7).

4.2 GROWTH WITHIN AMOEBAE AND MONOCYTES.

4.2.1. INTRODUCTION.

A. polyphaga was chosen as the amoebic host cell for the purpose of the current study as it is known to have been the host species associated with an outbreak of Legionnaires' disease in the UK (Barker *et al.*, 1993). Pearlman *et al.* (1988) demonstrated that U937 cells transformed by phorbol myristate acid (PMA) support the intracellular replication of *L. pneumophila*. When treated with PMA, U937 cells differentiate to become adherent, non-replicative cells with characteristics of tissue macrophages. If undifferentiated, the cells remain in suspension, are able to replicate and have the characteristics of monocytic cells. The current study was confined to

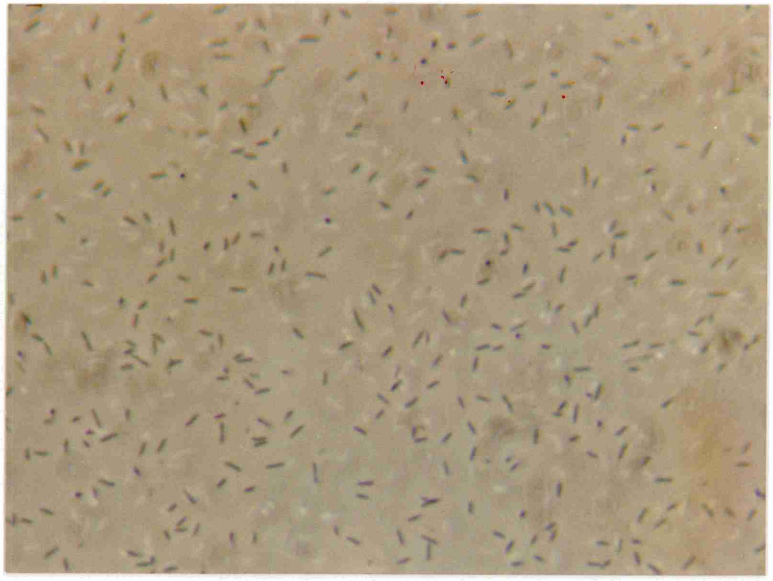


Figure 4.4. Preculture used to inoculate the YE broth in *L. pneumophila* growth studies. The majority of the legionellae are single, small and rod-shaped. Magnification x1000 Oil immersion.

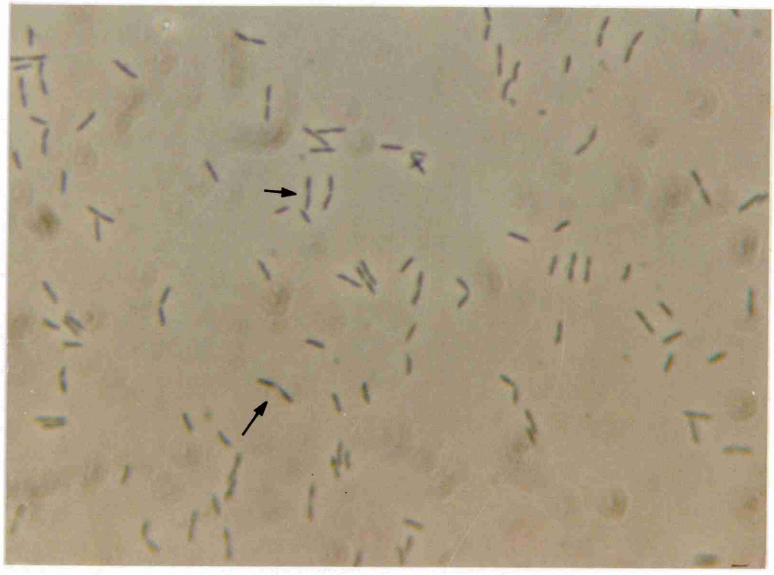


Figure 4.5. *L. pneumophila* in exponential phase. The bacteria are small and rod shaped. Many appear as pairs (arrowed). Magnification x 1000. Oil immersion.

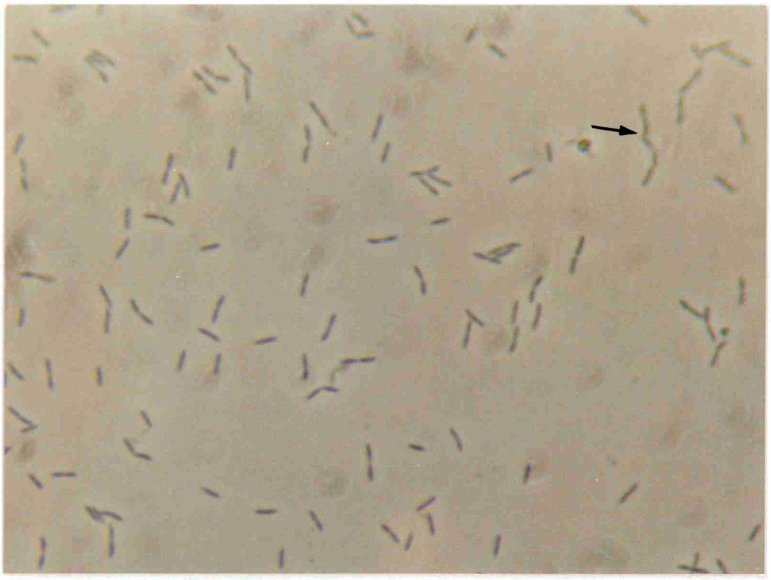


Figure 4.6. *L. pneumophila* towards end of exponential phase of growth. A few filamentous *Legionella* have formed (arrowed). Magnification x 1000. Oil immersion.

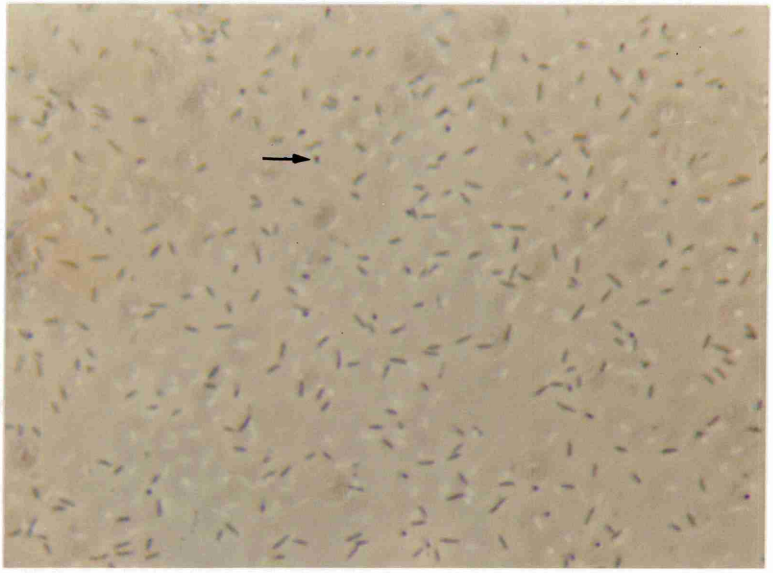


Figure 4.7. *L. pneumophila* in the stationary phase of growth. The majority of the bacteria are single, rod-shaped and smaller in size than those in exponential phase (*cf.* Figure 4.5). A few bacteria have adopted a small, rounded form, *ca.* 1μm in diameter (arrowed). Magnification x 1000 Oil immersion.

undifferentiated monocytic-like cells as preliminary experimentation showed that the differentiation process was time consuming and was unable to reproduce consistently confluent monolayers in sufficient quantities for this project. Intracellular grown *L. pneumophila* have been described by various authors (Rowbotham, 1986, and Barker *et al.*, 1992) as being small, rounded and highly motile in comparison to the morphology of those grown in YE broth. Microscopical studies were undertaken to confirm that the cocultures employed in the current study led to motile bacteria with an altered morphology.

4.2.2 REQUIREMENT FOR A VIABLE HOST CELL.

The ability of *L. pneumophila* to replicate within amoebic and monocytic host cells is well documented (Horwitz and Silverstein, 1980; Anand *et al.*, 1983; Holden *et al.*, 1984 and Rowbotham, 1986). Contamination problems during the coculture studies have shown that some bacterial species, such as *Micrococcus* sp. and *Staphylococcus aureus,* are able to replicate in RPMI medium and survive in amoebic saline. If *L. pneumophila* is capable of replication in a similar manner, or in the presence of lysed host cells, then it is possible that the yield of bacteria from a coculture would contain a mixture of intracellular and *in vitro* grown bacteria. It was, therefore, considered necessary to determine whether or not intracellular grown *L. pneumophila* could continue to replicate after host cell lysis by utilising host cell debris and / or metabolic products as a source of nutrient. A short study was, therefore, undertaken to examine this hypothesis.

Figures 4.8 and 4.9 indicate that the number of viable *L. pneumophila* increases only in the presence of viable host cells. Over a 5 d. period bacterial viable counts in amoebic coculture increased by 2 orders of magnitude and in monocytic cocultures by 1.3 orders of magnitude. In the absence of whole host cells there was a progressive loss of viable *L. pneumophila*. The loss in viability of intra-amoebic grown *L. pneumophila* was 1 order of magnitude, irrespective of suspending medium. The corresponding value for intra-monocytic grown legionellae was 3 orders of magnitude. Close examination of both sets of data suggests, however, that during the first 1 to 2 d. of the test the

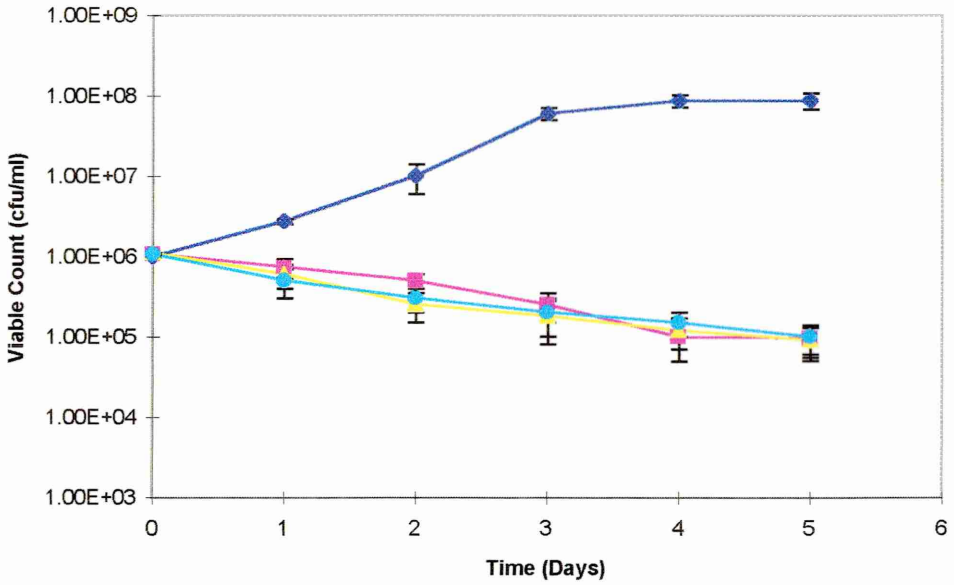


Figure 4.8 Growth of *L. pneumophila* - requirement for viable *A. polyphaga*. Survival of broth grown *L. pneumophila* with lysed (■) or whole amoebic trophozoites (◆) or suspension in conditioned amoebic saline (▲) and amoebic saline alone (●). Bars represent the standard errors of the means for three replicate samples.

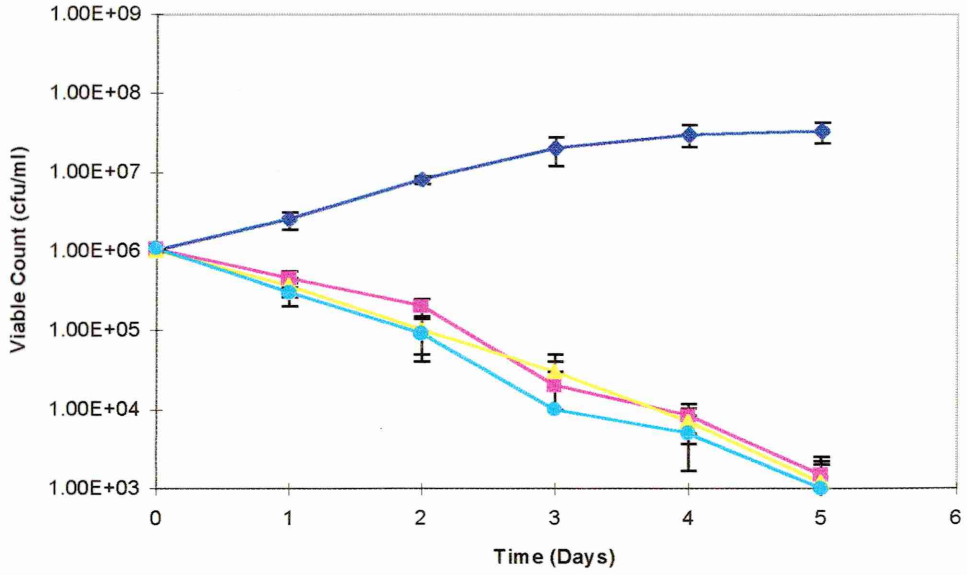


Figure 4.9 Growth of *L. pneumophila* - requirement for viable U937 monocytes. Survival of broth grown *L. pneumophila* with lysed (■), or whole monocytes (◆) or suspension in conditioned RPMI medium (▲) and RPMI medium alone (●).Bars represent the standard errors of the means for three replicate samples.

number of viable bacteria is slightly greater in the medium containing lysed host cell debris than in either that containing metabolic products (conditioned medium) or the suspending medium alone. After this period the loss in bacterial viability is the same, irrespective of the test conditions. From this short investigation, it can be concluded that viable host cells are necessary for effective coculture replication of *L. pneumophila.*

4.2.3 MORPHOLOGICAL STUDIES.

The morphology of *L. pneumophila* was monitored throughout coculture with both amoebic trophozoites and monocytic host cells. Wet preparations were examined by oil immersion phase contrast microscopy at x 1000 magnification. Prior to inoculation, the morphology of the bacteria was confirmed as being typical of broth grown *L. pneumophila, viz.* rod-shaped and non-motile.

After *ca.* 3-4 h. post inoculation, numerous bacteria were observed clustering around a single trophozoite, predominantly in the region of the contractile vacuole (Figure 4.10). The photomicrograph also shows bacteria on the point of entry into the amoebae (arrowed). This stage was followed by the development of a phagosome containing the bacteria. With time, the infected phagosome became enlarged due to the increasing number of replicating legionellae. As the number increased, the replicating legionellae became more motile and rotated as a 'single mass' in one direction. Eventually the infected phagosome was seen to fill the whole of the amoebic trophozoite (Figure 4.11). The phagosome eventually burst releasing small, highly motile bacteria into the extracellular matrix. The intra-amoebic grown legionellae were more rounded than the broth grown bacteria (rod-like) and smaller in size (*ca.* <1 μm x 1 μm *cf* 0.5-0.75 μm x 2-3 μm). These small, highly motile bacteria were observed to be the primary organism of second stage infection of the remaining amoebic trophozoites. Examination of the cocultures showed that the amoebic trophozoites started to encyst *ca.* 3 d. after inoculation. A few cysts were seen to contain replicating *L. pneumophila.* The small highly motile bacteria which failed to infect a host cell were observed to change to the morphology of the original broth grown legionellae inoculum. All legionellae

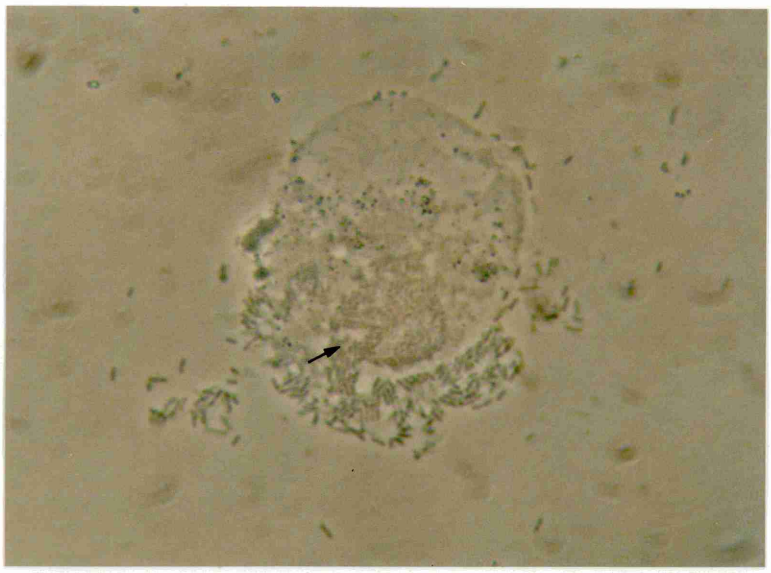


Figure 4.10. Early stages of intra-amoebic replication. *L. pneumophila* clustered around a single *A. polyphaga* trophozoite, predominantly in the region of the contractile vacuole. Legionellae can also be observed within the phagosome and at the point of entry into the amoebae (arrowed). Magnification x 1000 oil immersion.

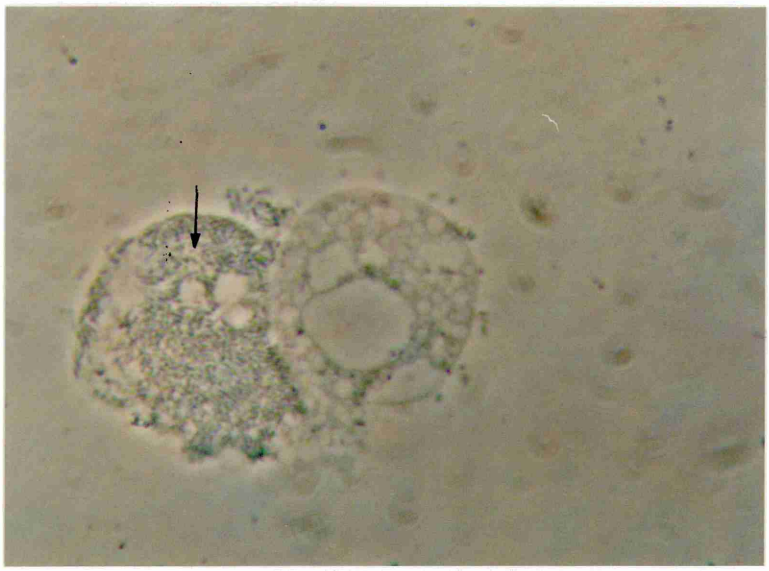


Figure 4.11. *L. pnemophila* infected (arrowed) and uninfected *A. polyphaga* trophozoites. *Legionella* can be seen to be filling the phagosome of the infected amoebae. These *L. pneumophila* were observed to be highly motile. Magnification x 1000 oil immersion.

underwent this transformation within 72 h. of release from the host cell (Figure 4.12). This process was termed 'ageing'.

A microscopical study was also made of intra-monocytic replication of *L. pneumophila*. Considerable difficulties were encountered in obtaining photomicrographs due to the very small size of the monocytes. Figure 4.13 shows a monocytic cell shortly after infection. It should be noted that legionellae within the phagosome still appear to possess the rod like morphology of the inoculated broth grown *L. pneumophila*. These bacteria were observed to be non-motile. Figure 4.14 shows a later stage of replication within the monocyte, *ca.* 24 h. after inoculation. The host cell phagosome is enlarged and the bacteria have become much smaller and were observed to be motile. This stage was followed by rupture of the host cell after *ca.* 16-18 h. post infection. The intra-monocytic grown bacteria which failed to infect further host cells underwent a change in the morphology to one similar to that of the broth grown legionellae. This phenomenon was identical to that observed for intra-amoebic *L. pneumophila*.

It may be concluded from the observations reported above that the uptake and replication of *L. pneumophila* within amoebae and monocytic cells follows a number of distinct stages.

(i) Clustering / adherence of legionellae on the host cell surface (Figure 4.10).

(ii) Entry into the host cell (Figure 4.10).

(iii) Replication of rod shaped non-motile bacteria within the host cell (Figure 4.13).

(iv) Enlargement of the phagosome accompanied by a change in bacterial morphology and the induction of motility (Figures 4.11 and 4.14).

(v) Rupture of the host cell and release of the intracellular grown legionellae.

4.2.4 UPTAKE AND REPLICATION IN HOST CELLS.

The ability of intracellular grown legionellae to infect amoebae and monocytes has not been widely investigated. The majority of investigations into uptake and replication of

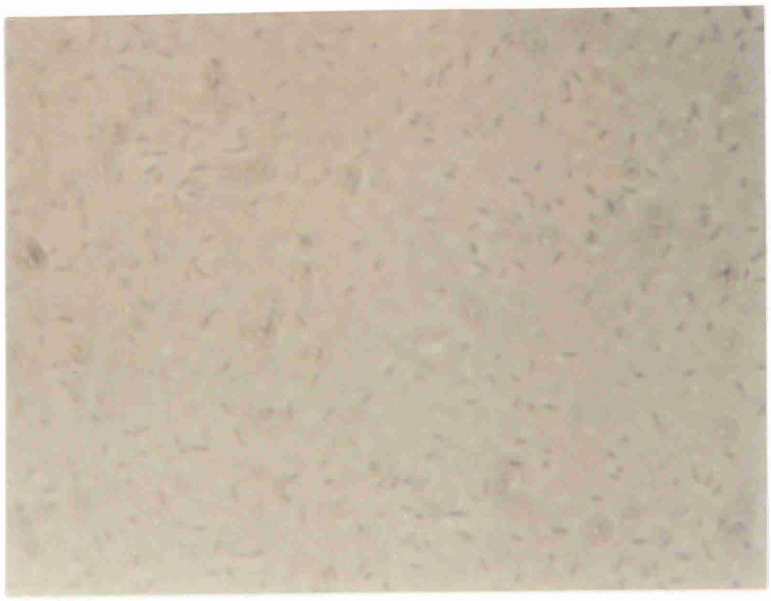


Figure 4.12 'Aged' intra-amoebic grown *L. pneumophila*. Revealing broth grown morphology after 'ageing' for 72 h in coculture. Magnification x 1000 oil immersion.

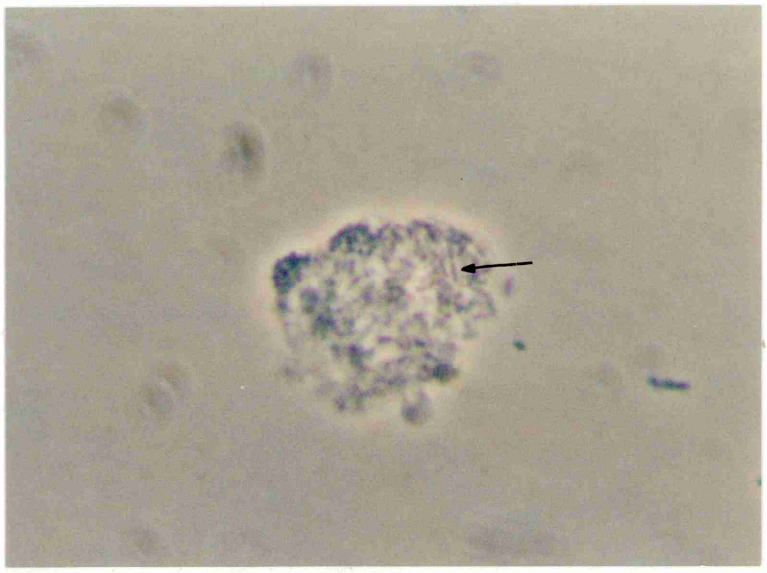


Figure 4.13 Early stage of *Legionella* infection of a U937 monocytic cell. The legionellae within the phagosome, (arrowed), possess the rod-like morphology of the broth grown *L. pneumophila* inoculant. Magnification x 1000 oil immersion, photographically enlarged x 7.

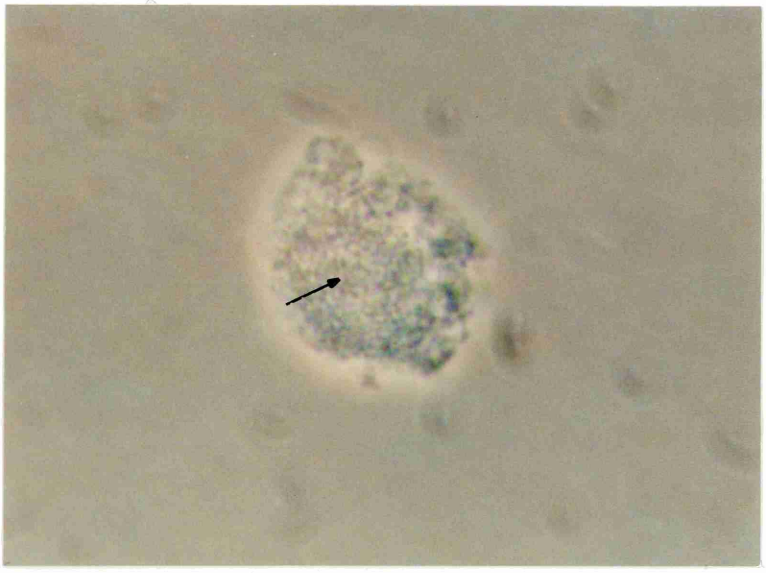


Figure 4.14 *Legionella* infected U937 monocytic cell. The phagosome is enlarged and the legionellae have become smaller and more rounded, (arrowed).
Magnification x 1000 oil immersion, photographically enlarged x 7.

L. pneumophila within either amoebae or human phagocytic cells described in the literature have used broth or agar grown legionellae for the inoculum (Horwitz and Silverstein, 1980; Anand *et al.*, 1983; Pearlman *et al.*, 1988; and Moffat and Tompkins, 1992). In the light of this apparent lack of information, an investigation was conducted to examine the effect of the environmental growth conditions of the bacterial inoculum (bacterial origin) and that of bacteria-to-host cell ratio on the kinetics of host cell infection and intracellular replication. Amoebic trophozoites and U937 monocytic cells were inoculated with broth grown (exponential and stationary phase) or intra-amoebic or intra-monocytic grown *L. pneumophila* to various bacteria-to-host cell ratio (Section 3.1.9). As a control, amoebic saline and RPMI were inoculated with the variously grown *L. pneumophila*. The viability of the legionellae was monitored daily by plate count, and the viability of the host cells by the trypan blue exclusion assay. The effect of the origin of the *L. pneumophila* and the bacteria-to-host cell ratio on the uptake and replication in amoebae is summarised in Figures 4.15 (a-e). The results of a parallel investigation using monocytic cocultures is summarised in Figures 4.16 (a-d).

Reference to the data presented in Figure 4.15 (a-d) shows that for a given bacteria-to-host cell ratio the origin of the legionellae inoculum has only a marginal effect on the time to reach the maximum bacterial yield from an amoebic coculture, and only a small effect on the maximum yield itself.

Figure 4.15 (a) shows that at low bacteria-to-host cell ratio (1 : 10,000) there is an initial period after inoculation during which none of the inoculum bacteria could be detected by growth on BCYE agar. This was anticipated as the inoculum concentration (*ca* 10 cfu/ml) was at the limit of detection using the sampling method employed. After 1-2 d. the number of bacteria increases sufficiently for recovery by the viable plate count method. Replication continues until a maximum viable count of *ca.* 10^8-10^9 cfu/ml was achieved. The origin of the inoculum bacteria appears to have little effect on the time to achieve maximum yield, which in this instance was reached in *ca.* 4 d. The yield from amoebae inoculated with intracellular bacteria was found to be 2.5 times greater than that achieved when the inoculum was broth grown *L. pneumophila*.

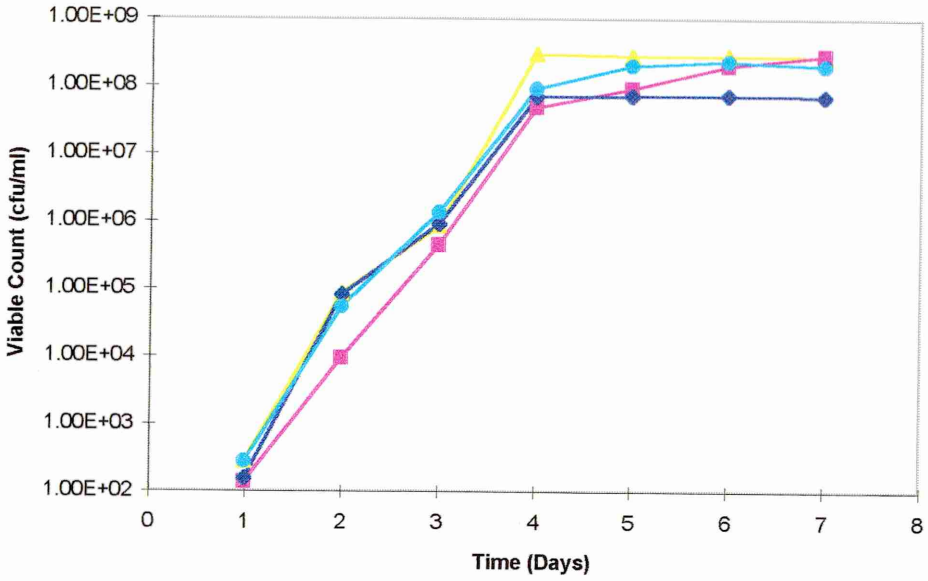


Figure 4.15 (a) Effect of bacterial origin on infection of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1 : 10,000. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●).

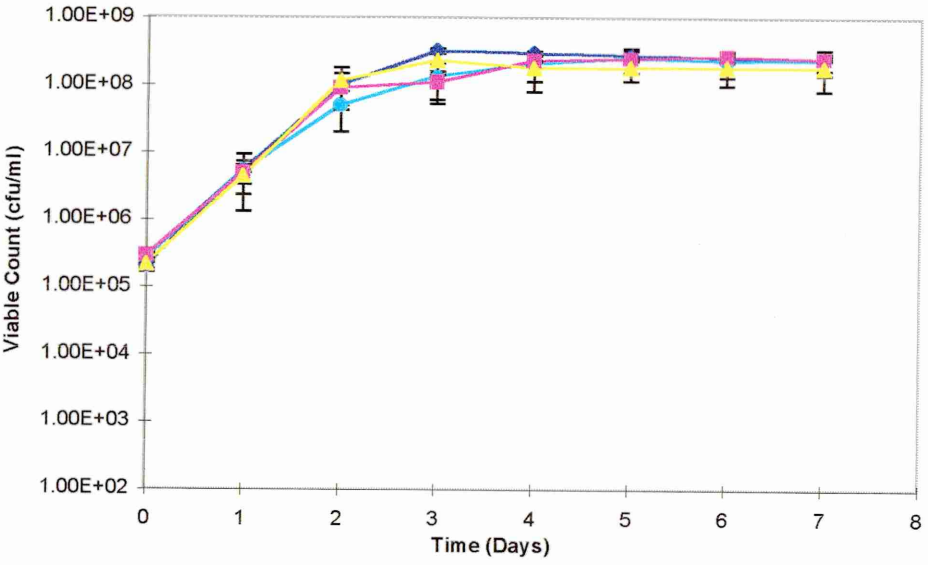


Figure 4.15 (b). Effect of bacterial origin on infection of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means of 3 replicate samples.

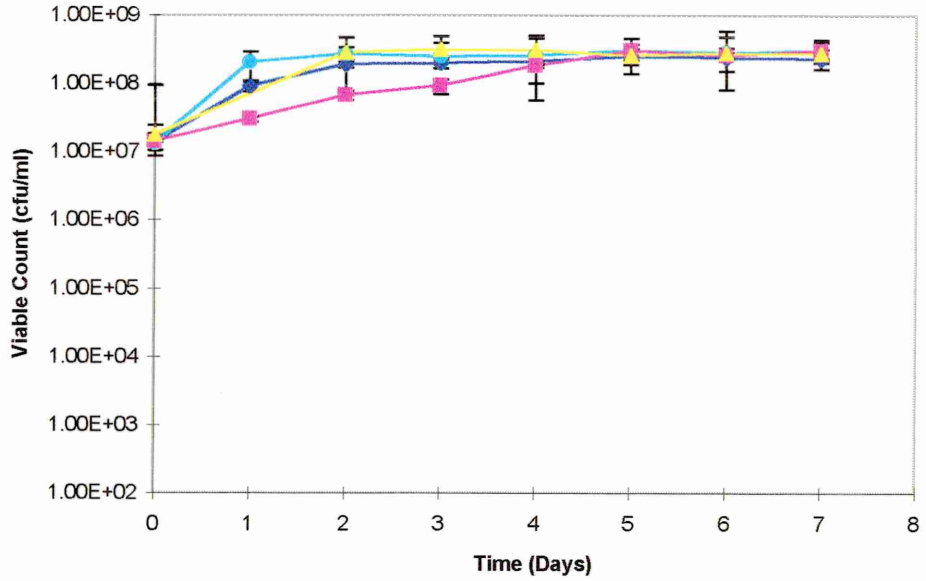


Figure 4.15 (c). Effect of bacterial origin on infection of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 100 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means of 3 replicate samples.

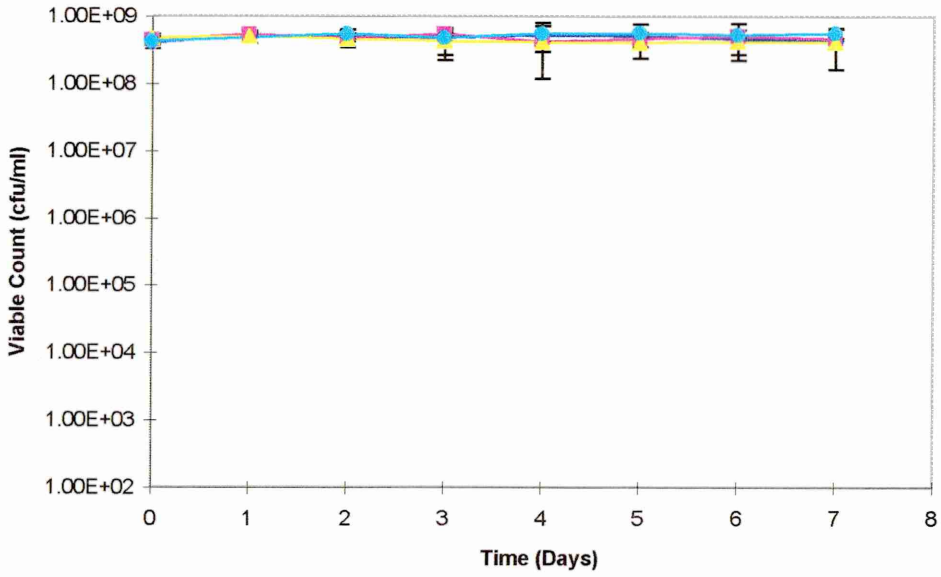


Figure 4.15 (d). Effect of bacterial origin on infection of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1000 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means of 3 replicate samples.

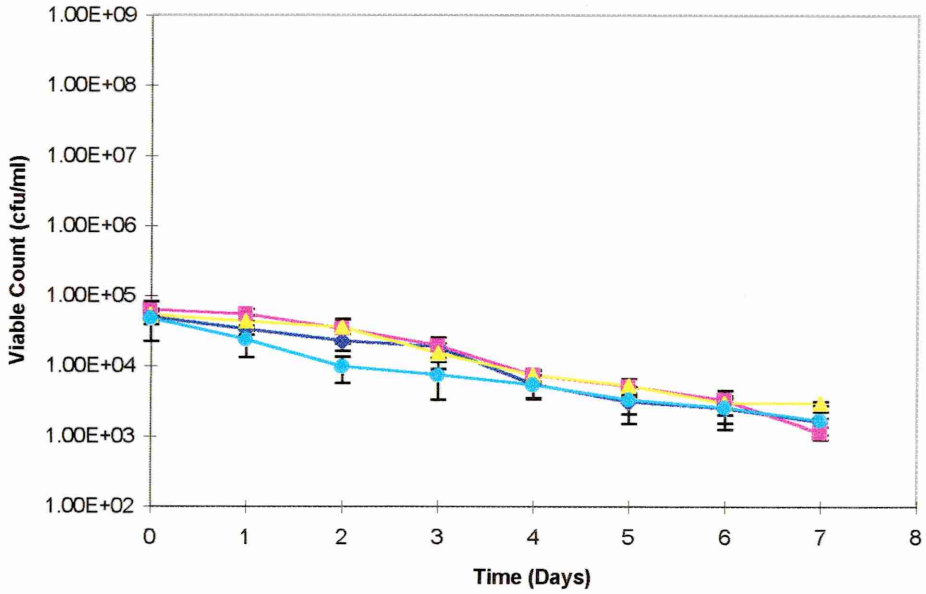


Figure 4.15 (e). Control. Survival of *L. pneumophila* in amoebic saline alone. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means of 3 replicate samples.

Increase of the bacteria-to-host cell ratio to 1 : 1 appears to have only a marginal effect on the general trends observed at the lower bacteria-to-host cell ratio (1 : 10,000), reported above. Figure 4.15 (b) shows that replication of *L. pneumophila* occurs within a few hours of inoculation and the maximum yield (*ca.* 10^8-10^9 cfu/ml) is achieved in *ca.* 3 d. Again, the origin of the inoculum bacteria has very little effect on the time to reach the maximum bacterial yield. The data suggests that a slightly higher yield is obtained when the inoculum *L. pneumophila* is intracellular in origin. However, in this instance, the difference between the yield from intracellular and broth grown *L. pneumophila* is not as great as that observed for the lower bacteria-to-host cell ratio. At a bacteria-to-host cell ratio of 100 : 1 (Figure 4.15 (c)) replication appears to commence within a few hours of inoculation and the maximum yield of bacteria was again *ca.* 10^8-10^9 cfu/ml. In this instance, however, origin of the inoculum does appear to have an affect on the time to reach maximum yield, as well as the size of the maximum yield itself. The maximum yield of bacteria from the coculture was achieved within *ca.* 1 d. when inoculum bacteria were intra-monocytic in origin, within *ca.* 2 d. when it was intra-amoebic or broth grown in exponential phase, and *ca.* 5 d. when it was broth grown in stationary phase. The data obtained from very high bacteria-to-host cell ratios (1000 : 1) (Figure 4.15 (d)) indicates that there was no change in the viable count of *L. pneumophila* throughout the duration of the investigation. Origin of the inoculum legionellae appears to have no effect on this phenomenon. Data from control samples presented in Figure 4.15 (e) indicate that in the absence of host cells, the origin of the *L. pneumophila* has a small effect on the loss of bacterial viability. The viability of all the controls fell by *ca.* 1 order of magnitude during the course of the investigation.

The effect of the origin of the *L. pneumophila* and the bacteria-to-host cell ratio on the uptake and replication in monocytes are summarised in Figure 4.16 (a-d). Figure 4.16 (a) shows that at a low bacteria-to-host cell ratio (1 : 10,000) there is an interval of *ca.* 2 d. after inoculation during which the bacteria are not detectable on BCYE agar. After this period, the bacteria have multiplied sufficiently to be detected on agar and replication continues until a viable count of *ca.* 5×10^7-5×10^8 cfu/ml is achieved after

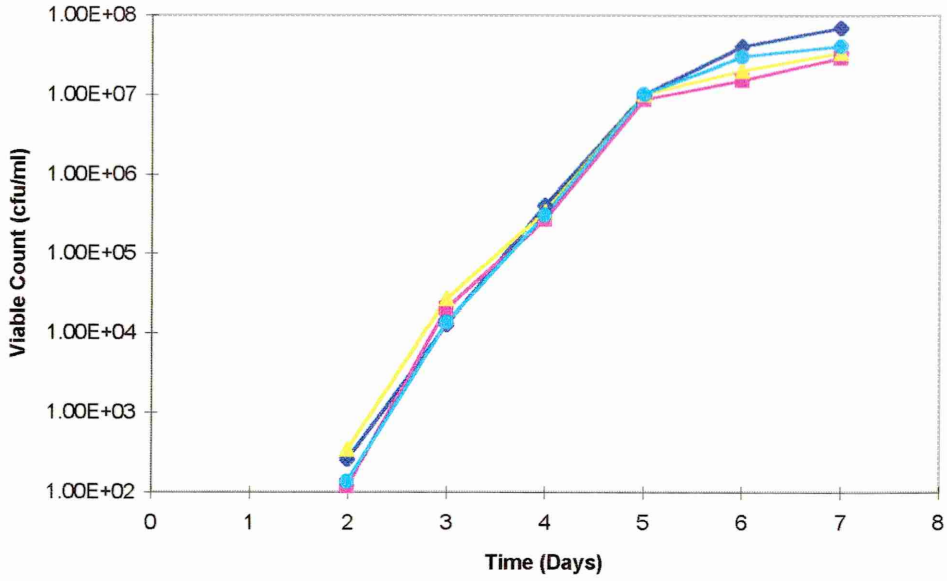


Figure 4.16 (a). Effect of bacterial origin ratio on infection of U937 monocytes by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1 : 10,000. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●).

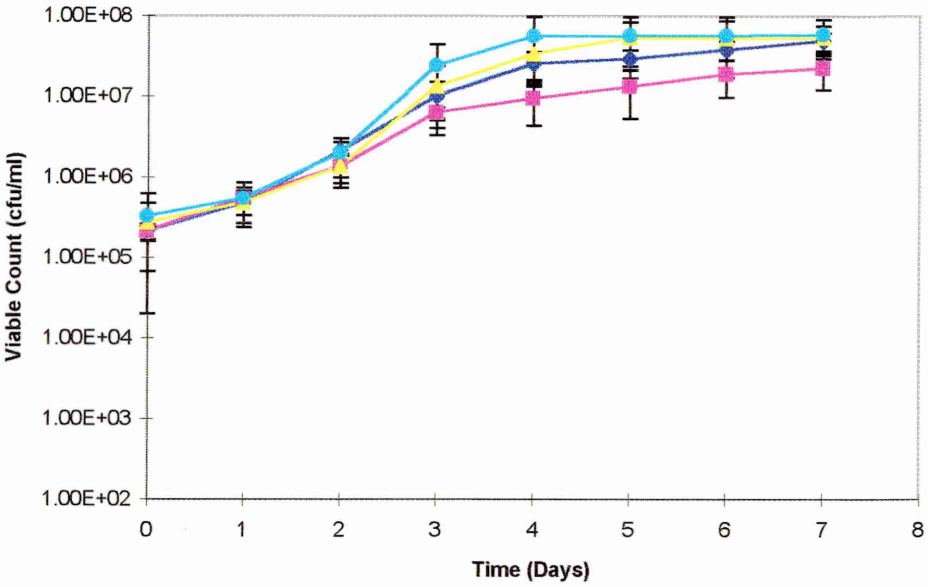


Figure 4.16 (b). Effect of bacterial origin on infection of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means of 3 replicate samples.

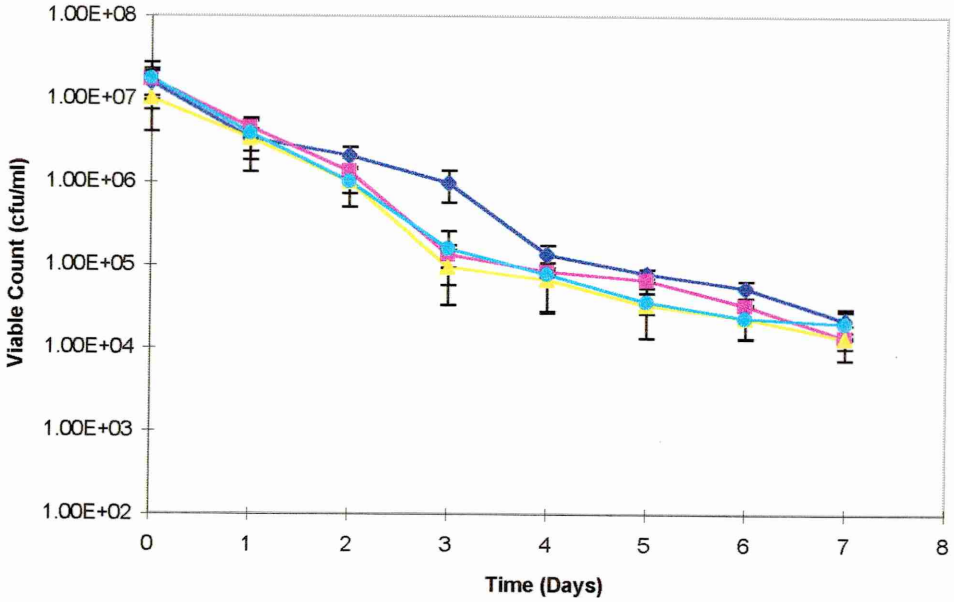


Figure 4.16 (c). Effect of bacterial origin on infection of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 100 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means of 3 replicate samples.

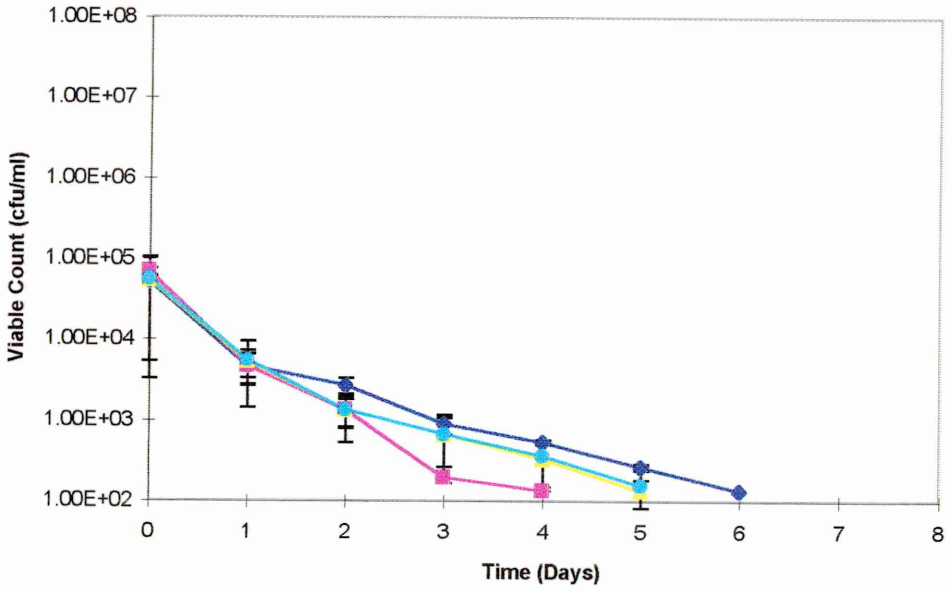


Figure 4.16 (d). Control. The survival of *L. pneumophila* in RPMI medium alone. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means of 3 replicate samples.

ca. 7 d. The origin of the inoculum bacteria has little effect on the initial non-detectable period, the time to achieve maximum bacterial yield, or the size of the yield itself. Figure 4.16 (b) shows that replication of the *L. pneumophila* at a bacteria-to-host cell ratio of 1 : 1 occurs within a few hours of inoculation. The maximum yield (*ca.* 5×10^7-5×10^8 cfu/ml) of bacteria is achieved after *ca.* 4 d. The origin of the inoculum *L. pneumophila* again has little effect on the time to achieve the maximum yield.

When the bacteria-to-host cell ratio was raised to 100 : 1 (Figure 4.16 (c)) a steady decrease in the viable count was observed throughout the 7 d. period of the investigation. The origin of the inoculum *L. pneumophila* appeared to have little effect on the rate of loss of viability, which was *ca.* 2.5-3 orders of magnitude in each case. The control experiments (Figure 4.16 (d)) show a loss in viability of *ca.* 3 orders of magnitude over *ca.* 5-6 d. This is very similar to the rate of decline in viability observed in the high bacteria-to-host cell ratio (100 : 1) study.

Figures 4.17 (a-d) and 4.18 (a-d) show the above data re-plotted to highlight the effect of bacteria-to-host cell ratio on the growth kinetics of *L. pneumophila* in amoebic and monocytic cocultures, respectively. As bacterial replication did not occur at bacteria-to-host cell ratios of 1000 : 1 and 100 : 1 in amoebae and monocytes, respectively, this data was not included in the figures. Comparison of the data suggests two trends common to all cocultures. Firstly, the final yield of bacteria is *ca.* 5×10^7-5×10^8cfu/ml, irrespective of the inoculum origin or the host cell. Secondly, the time to achieve the maximum yield within a given coculture falls as the bacterial concentration of the inoculum rises.

Observations made by Rowbotham, (1986), and during this present study, suggest that the morphology of intra-amoebic grown *L. pneumophila* changes to one similar to that of broth grown, if it is retained for *ca.* 72 h. in the coculture medium after host cell lysis, ('aged'). A short investigation was undertaken to determine whether this morphological change affects the virulence of the bacteria.

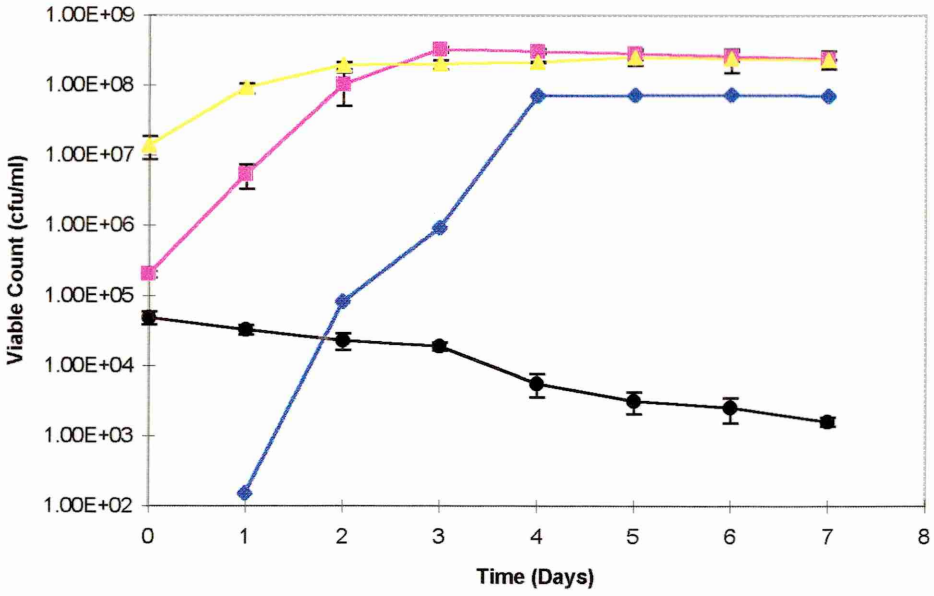


Figure 4.17 (a). Effect of bacteria-to-host cell ratio on the infection of *A. polyphaga* by YE broth grown *L. pneumophila* in exponential phase. Bacteria-to-host cell ratios of 1 : 10,000 (◆), 1 : 1 (■) and 100 : 1 (▲). *L. pneumophila* in amoebic saline alone acted as a control (●).Bars represent the standard errors of the means for three replicate samples.

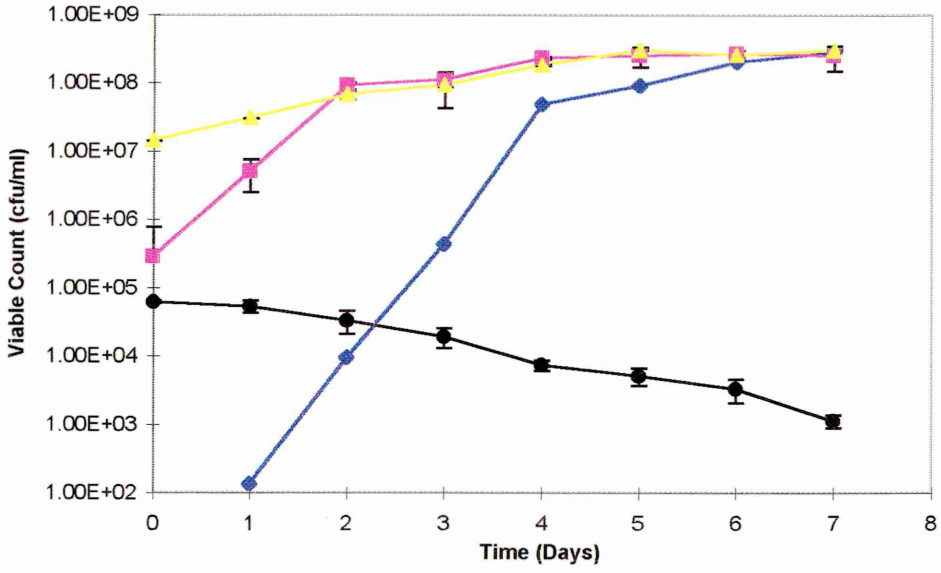


Figure 4.17 (b). Effect of bacteria-to-host cell ratio on the infection of *A. polyphaga* by YE broth grown *L. pneumophila* in stationary phase. Bacteria-to-host cell ratios of 1 : 10,000 (◆), 1 : 1 (■) and 100 : 1 (▲). *L. pneumophila* in amoebic saline alone acted as a control (●). Bars represent the standard errors of the means for three replicate samples.

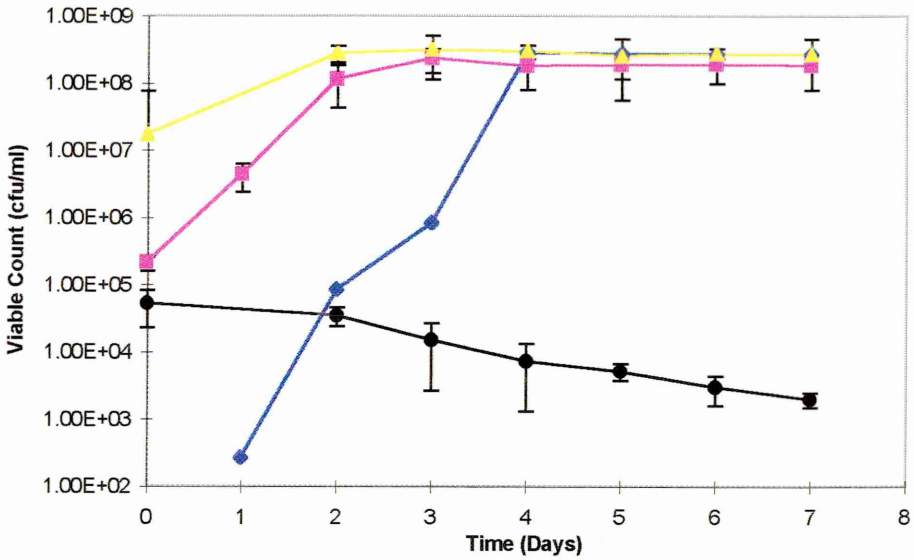


Figure 4.17 (c). Effect of bacteria-to-host cell ratio on the infection of *A. polyphaga* by intra-amoebic grown *L. pneumophila*. Bacteria-to-host cell ratios of 1 : 10,000 (◆), 1 : 1 (■) and 100 : 1 (▲). *L. pneumophila* in amoebic saline alone acted as a control (●).Bars represent the standard errors of the means for three replicate samples.

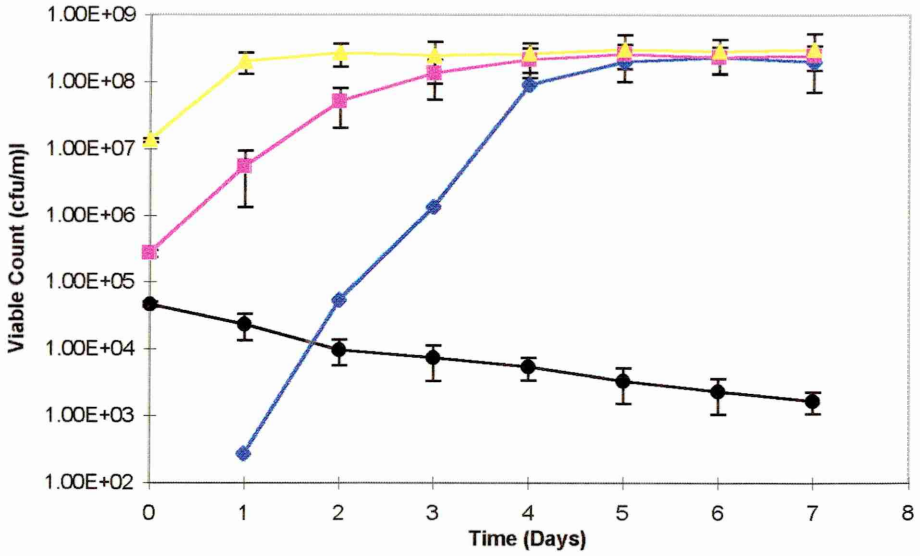


Figure 4.17 (d). Effect of bacteria-to-host cell ratio on the infection of *A. polyphaga* by intra-monocytic grown *L. pneumophila*. Bacteria-to-host cell ratios of 1 : 10,000 (◆), 1 : 1 (■) and 100 : 1 (▲). *L. pneumophila* in amoebic saline alone acted as a control (●).Bars represent the standard errors of the means for three replicate samples.

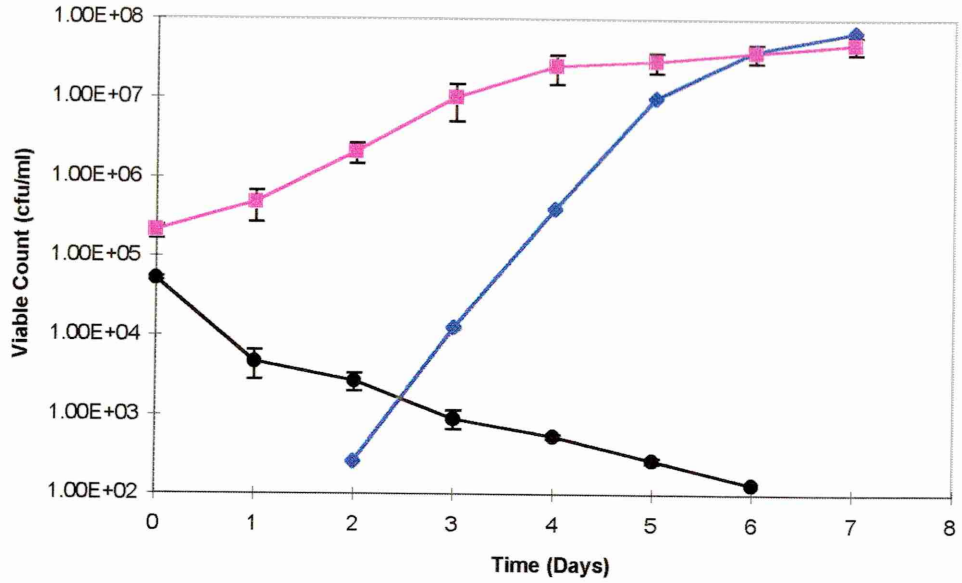


Figure 4.18 (a). Effect of bacteria-to-host cell ratio on the infection of U937 monocytes by YE broth grown *L. pneumophila* in exponential phase. Bacteria-to-host cell ratios of 1 : 10,000 (◆) and 1 : 1 (■). *L. pneumophila* in RPMI medium alone acted as a control (●). Bars represent the standard errors of the means for 3 replicate samples.

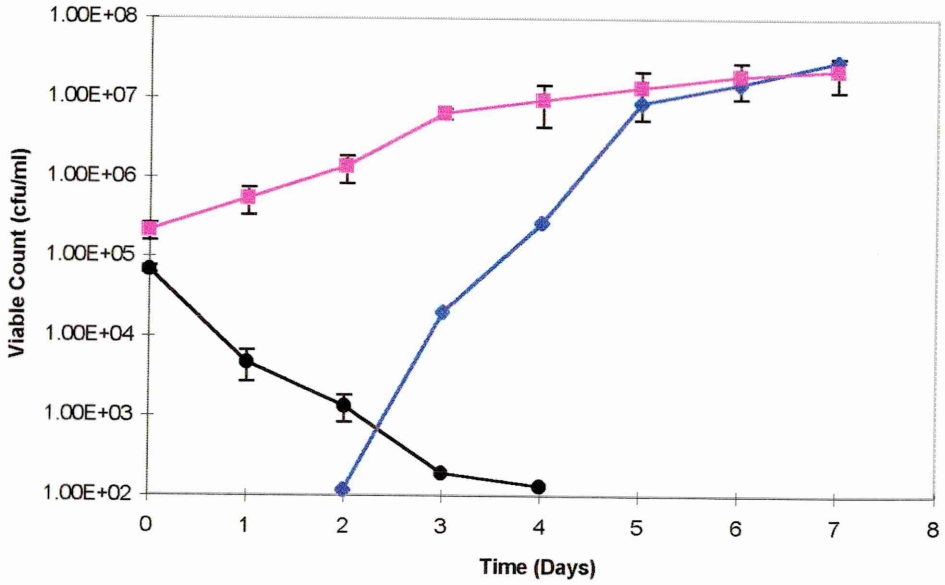


Figure 4.18 (b). Effect of bacteria-to-host cell ratio on the infection of U937 monocytes by YE broth grown *L. pneumophila* in stationary phase. Bacteria-to-host cell ratios of 1 : 10,000 (◆) and 1 : 1 (■). *L. pneumophila* in RPMI medium alone acted as a control (●). Bars represent the standard errors of the means for 3 replicate samples.

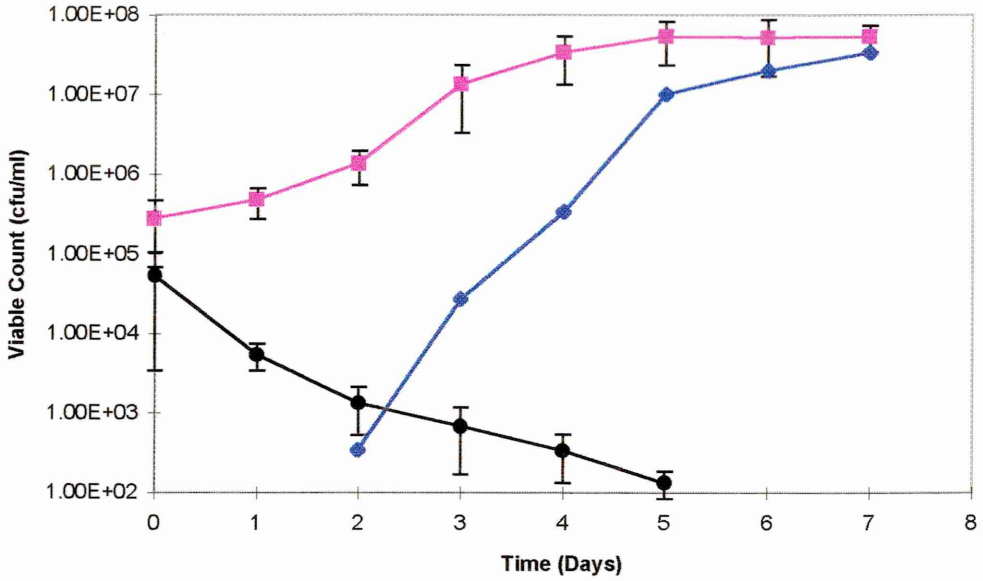


Figure 4.18 (c). Effect of bacteria-to-host cell ratio on the infection of U937 monocytes by intra-amoebic grown *L. pneumophila*. Bacteria-to-host cell ratios of 1 : 10,000 (◆), 1 : 1 (■) and 100 : 1. *L. pneumophila* in RPMI medium alone acted as a control (●). Bars represent the standard errors of the means for 3 replicate samples.

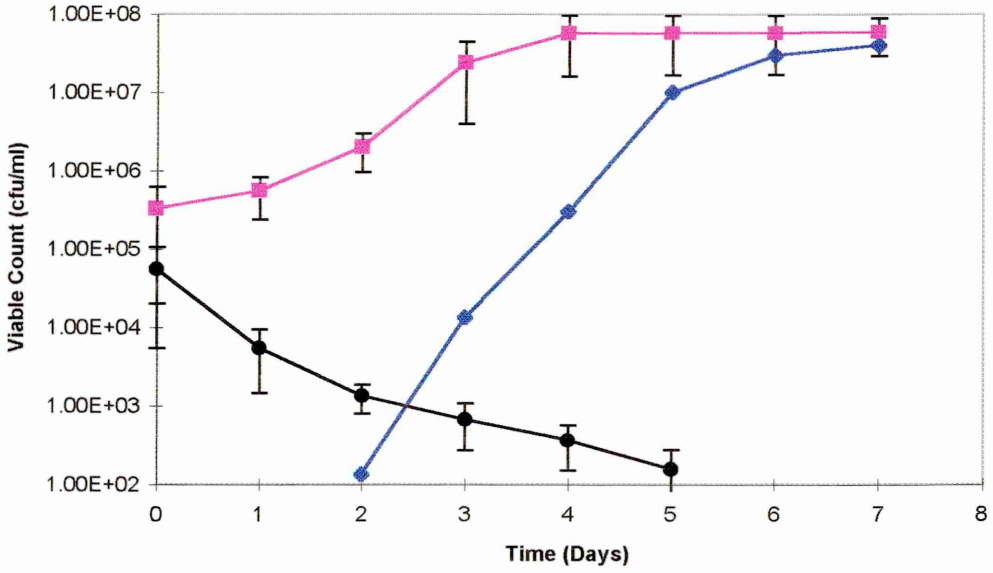


Figure 4.18 (d). Effect of bacteria-to-host cell ratio on the infection of U937 monocytes by intra-monocytic grown *L. pneumophila.* Bacteria-to-host cell ratios of 1 : 10,000 (◆) and 1 : 1 (■). *L. pneumophila* in RPMI medium alone acted as a control (●). Bars represent the standard errors of the means for 3 replicate samples.

The results of this investigation are shown in Figure 4.19. It is apparent from this data that 'aged' intra-amoebic grown *L. pneumophila* behave in a similar manner to broth grown legionellae in stationary phase. Invasion of the host cell and replication appears to occur at the same rate and yields from the cocultures are the same, within experimental error. The coculture inoculated with freshly harvested intra-amoebic grown *L. pneumophila* produced a bacterial yield *ca.* 2.5 times greater than that observed in cocultures inoculated with broth grown or 'aged' intra-amoebic grown legionellae.

4.2.5 HOST CELL SURVIVAL.

As far as it is known no detailed study has been undertaken of the rate of host cell lysis due to the intracellular replication of *L. pneumophila.* One of the factors that could determine the rate of host cell lysis within a coculture is the initial bacteria-to-host cell ratio. The effect of bacteria-to-host cell ratio on the viability of host cells was, therefore, assessed throughout the growth studies work outlined above. The data obtained from this study is summarised in Figures 4.20 (a-e) and 4.21 (a-d) for amoebic and monocytic cocultures, respectively. Both sets of data indicate that within a given coculture the origin of the inoculum *L. pneumophila* has little effect on host cell lysis.

Figures 4.20 (a-c) show that within amoebic cocultures bacteria-to-host cell ratios have only a negligible effect on host cell viability for *ca.* 3 d. post inoculation. Beyond this period, the bacteria-to-host cell ratio appears to have a small effect on host cell viability, the size of the effect increasing with time. After 7 d., the greatest loss of viability was observed in samples inoculated at a ratio of 100 : 1, and least in those at a ratio of 1 : 10,000. The difference between the number of surviving cells was, however, fairly small, *ca.* 2×10^4 *cf.* 8×10^3 cells/ml. The viability of the uninoculated control cells (Figure 4.20 (e)) fell to *ca.* 3×10^4 cells/ml during the same period. Microscopic observations suggested that the increased loss of cell viability was due to a combination of host cell lysis and amoebic encystment. Inoculation at a very high bacteria-to-host cell ratio (1000 : 1) resulted in an immediate and steady fall in cell viability,

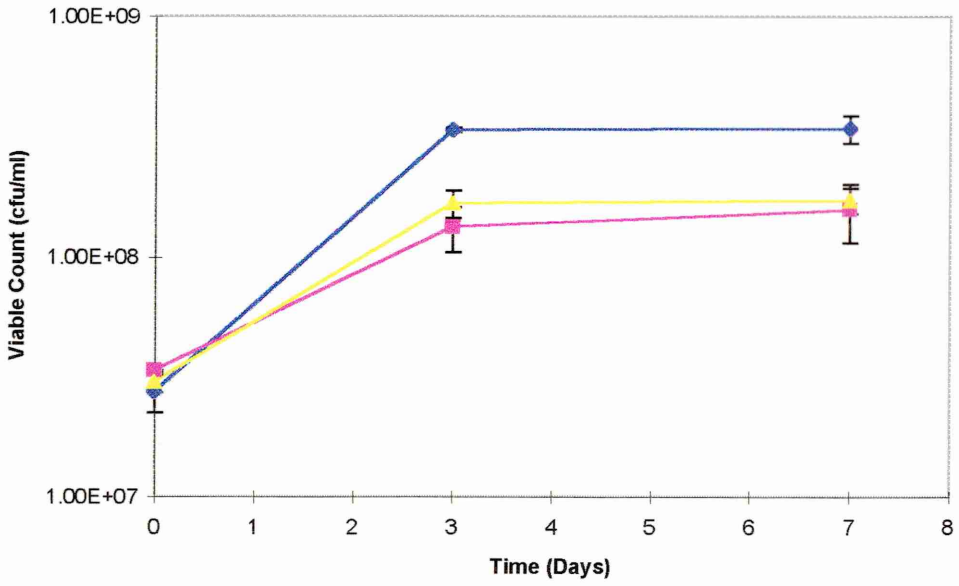


Figure 4.19. Growth of *L. pneumophila* in *A. polyphaga* trophozoites. Effect of inoculum origin at a bacteria-to-host cell ratio of 100 : 1. The inoculum were freshly harvested intra-amoebic grown *L. pneumophila*, (◆), broth grown legionellae in stationary phase (■) and 'aged' intra-amoebic grown bacteria (▲).

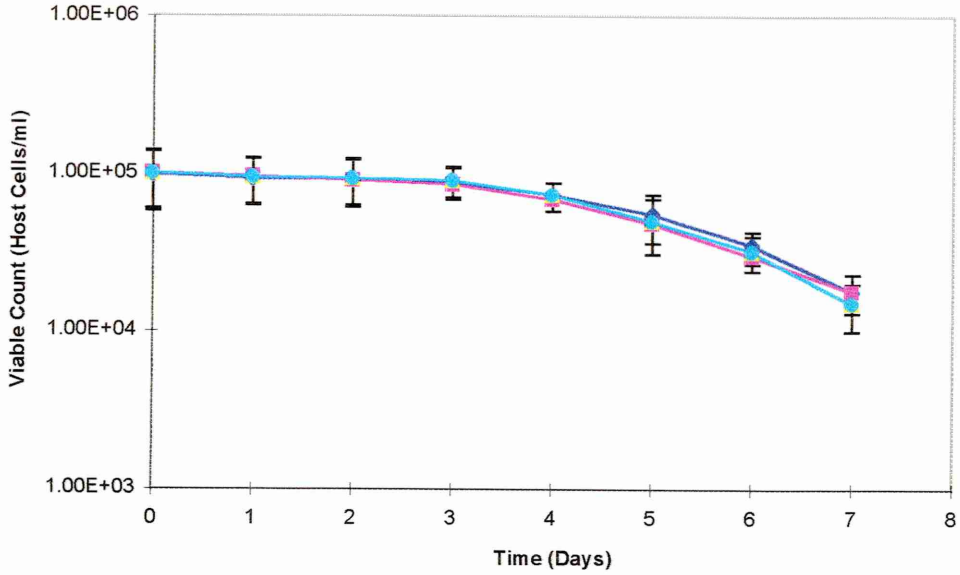


Figure 4.20 (a). Effect of bacterial origin on the lysis of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1 : 10,000. Inoculum *L. pneumophila* grown in: YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●).

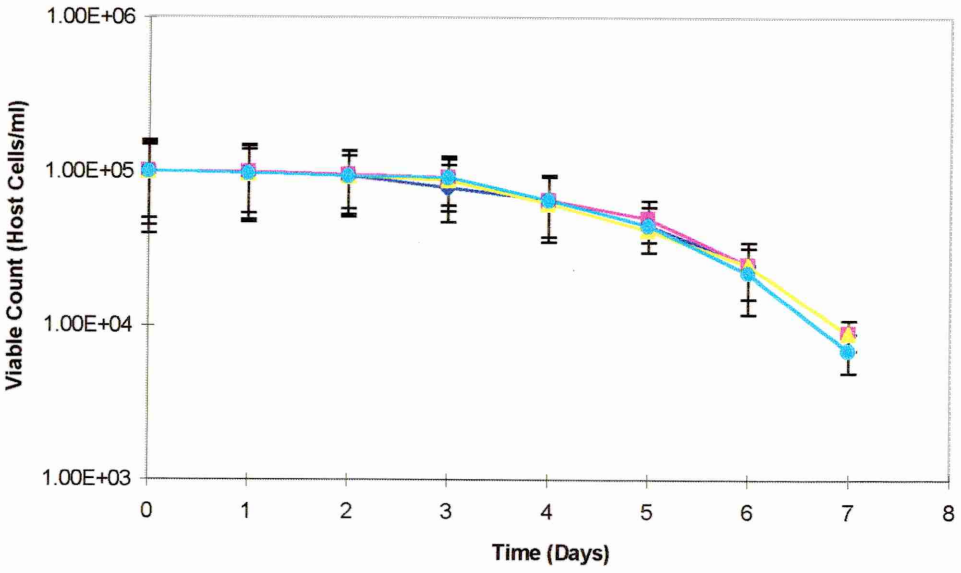


Figure 4.20 (b). Effect of bacterial origin on the lysis of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria to host cell ratio of 1 : 1. Inoculum *L. pneumophila* grown in: YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●).

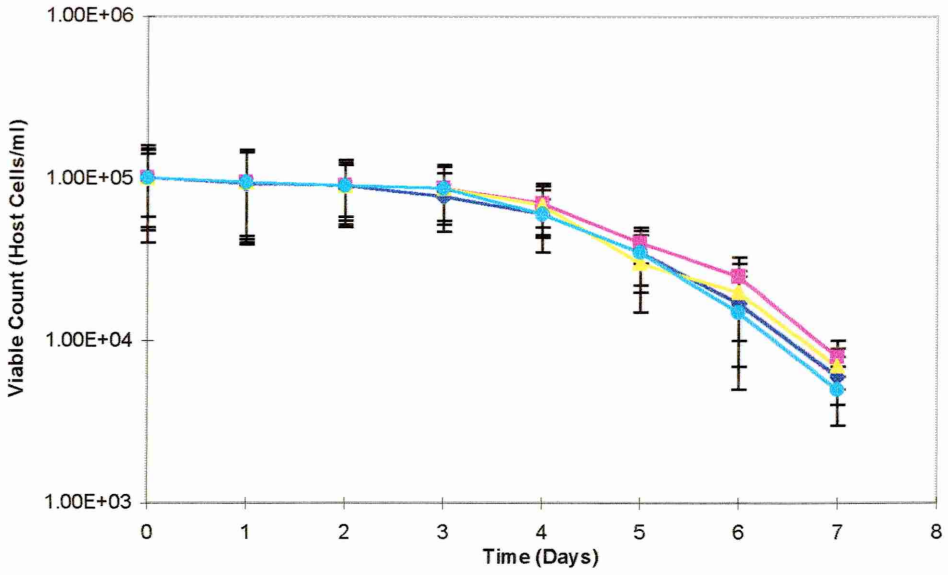


Figure 4.20 (c). Effect of bacterial origin on the lysis of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria to host cell ratio of 100 : 1. Inoculum *L. pneumophila* grown in: YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲)and within monocytes (●).

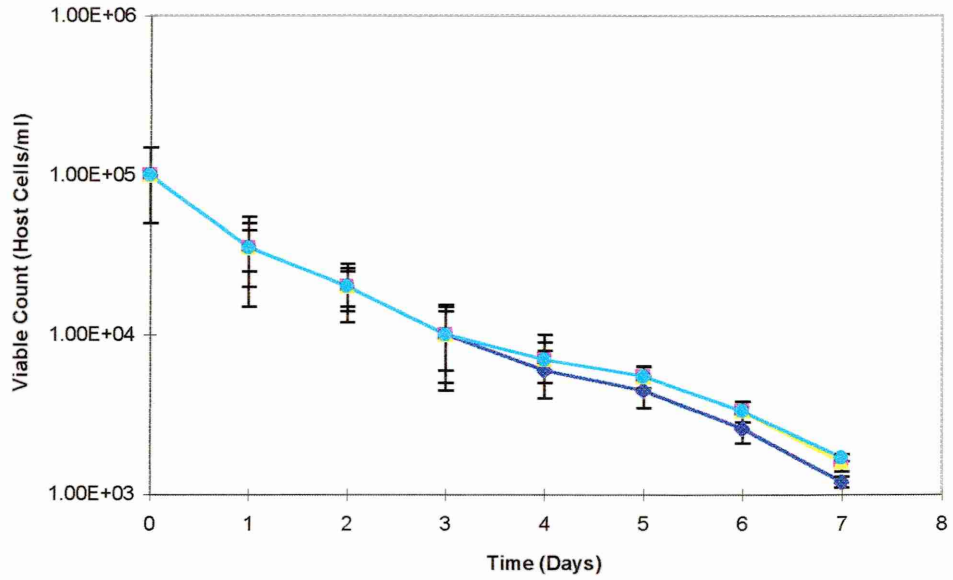


Figure 4.20 (d). Effect of bacterial origin on the lysis of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria to host cell ratio of 1000 : 1. Inoculum *L. pneumophila* grown in: YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲)and within monocytes (●).

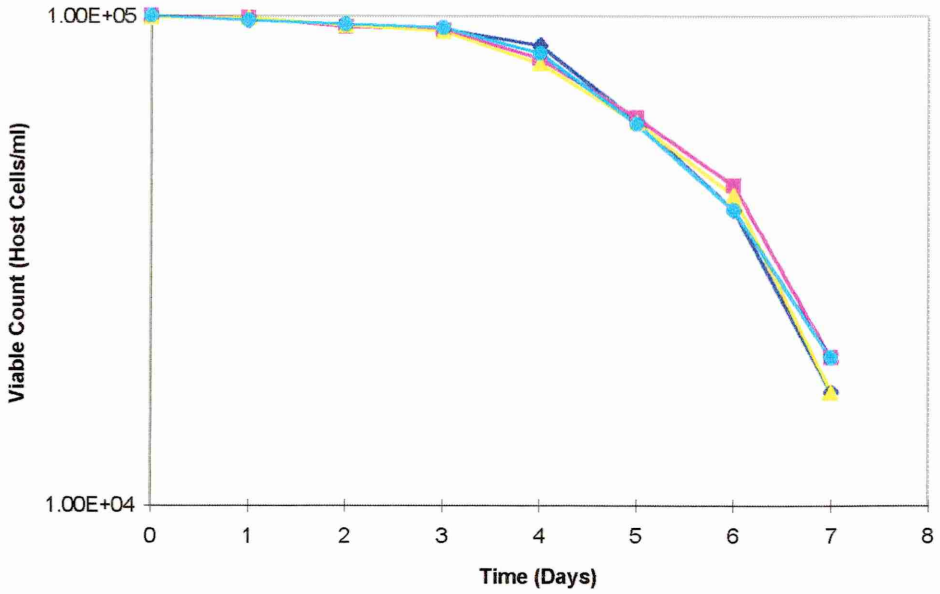


Figure 4.20 (e). Control. Lysis of *A. polyphaga* trophozoites in amoebic saline alone. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●).

ca. 2 x 10^3 cells/ml surviving after 7 d. (Figure 4.20 (d)). Trypan blue exclusion staining showed *ca.* 65 % of the acanthamoebae to have lysed within 24 h. of inoculation.

An important difference between the monocytic coculture and that of the amoebae is the fact that the monocytes are suspended in a nutrient rich medium and are, therefore, able to replicate. Figures 4.21 (a-b) show that at bacteria-to-host cell ratios of 1 : 10,000 and 1 : 1 the change of cell viability with time was similar to that of the uninoculated control (Figure 4.21(d)). A rise in cell viability of *ca.* 0.5-0.75 orders of magnitude was observed during the first 3 d. after inoculation for these two ratios. After this period of growth, cell viability fell in both cocultures and the control. After 7 d. post inoculation the number of viable cells in the coculture had fallen to *ca.* 8 x 10^4 cells/ml. The corresponding values for the 1 : 1 coculture and control were *ca.* 3 x 10^4 and *ca.* 10^5 cells/ml, respectively. Inoculation at high bacteria-to-host cell ratios (100 : 1), Figure 4.21 (c) resulted in a rapid loss of cell viability (*ca.* 2 x 10^2 cells/ml surviving after 7 d.). A 70 % loss of cell viability was observed within 24 h. of inoculation.

As previously indicated, the maximum bacterial yield from both amoebic and monocytic cocultures is *ca.* 5 x 10^7-5 x 10^8 cfu/ml. This concentration has been shown to induce rapid host cell lysis when used as the inoculum. Comparison of the *L. pneumophila* growth data (Figures 4.17 and 4.18) with the corresponding host cell lysis curves (Figures 4.20 and 4.21) suggest a possible link between the two observations. Within any given coculture, rapid cell lysis appears to commence within a short time of the maximum yield of the bacteria being achieved. For example, in the amoebic coculture inoculated with intra-amoebic grown *L. pneumophila* to a bacteria-to-host cell ratio of 1 : 10,000, maximum yield was achieved after *ca.* 4 d. of replication; rapid cell lysis commenced after 3 d. The corresponding values for a ratio of 100 : 1 are *ca.* 2 d. and *ca.* 2 d, respectively. A typical example of this data comparison is presented in Figure 4.22 (a-b). It may be suggested, therefore, that the maximum yield from the cocultures studies is limited to *ca.* 5 x 10^7 -5 x 10^8 cfu/ml by the onset of rapid cell lysis.

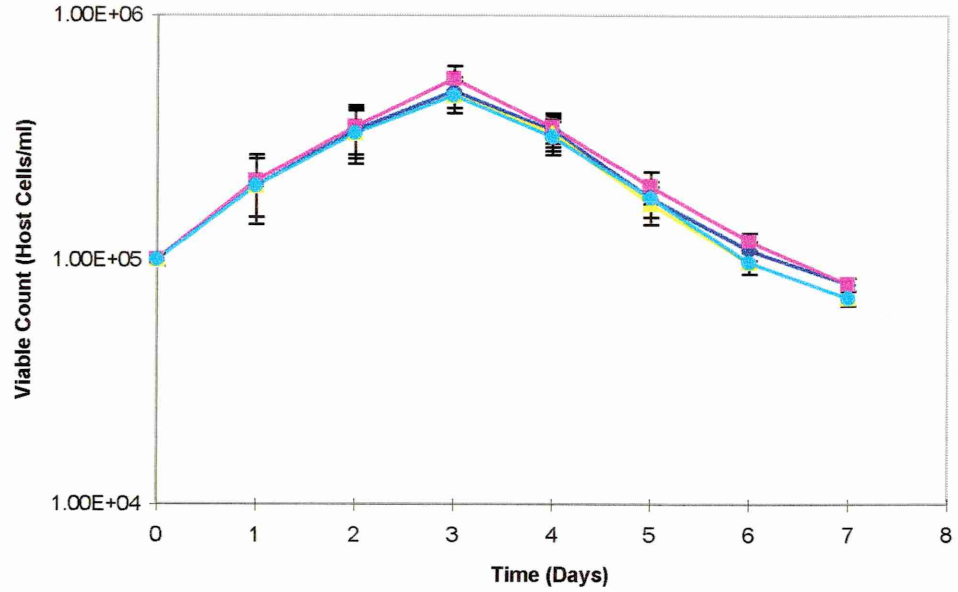


Figure 4.21 (a). Effect of bacterial origin on the lysis of U937 monocytes by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1 : 10,000. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means for 3 replicate samples.

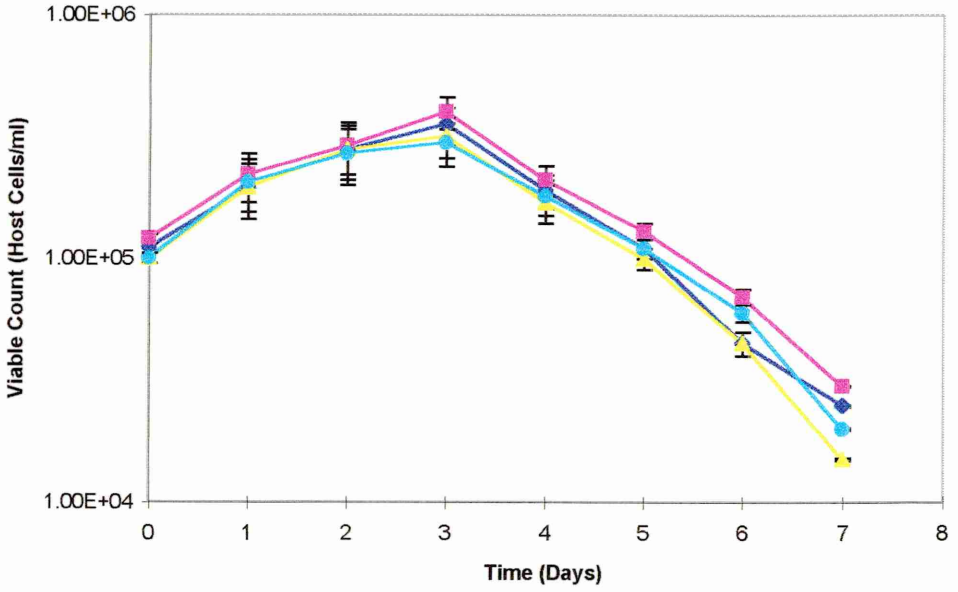


Figure 4.21 (b). Effect of bacterial origin on the lysis of U937 monocytes by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means for 3 replicate samples.

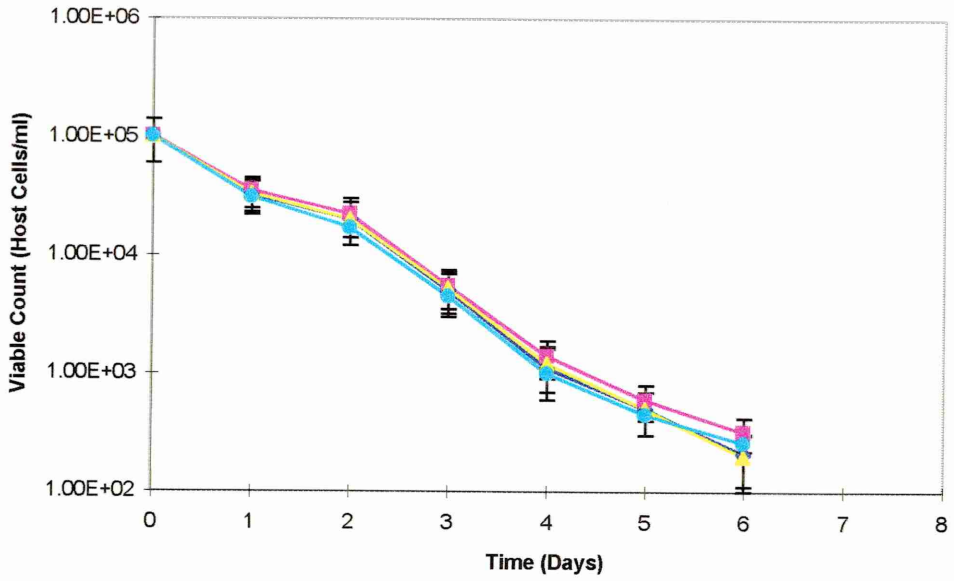


Figure 4.21 (c). Effect of bacterial origin on the lysis of U937 monocytes by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 100 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means for 3 replicate samples.

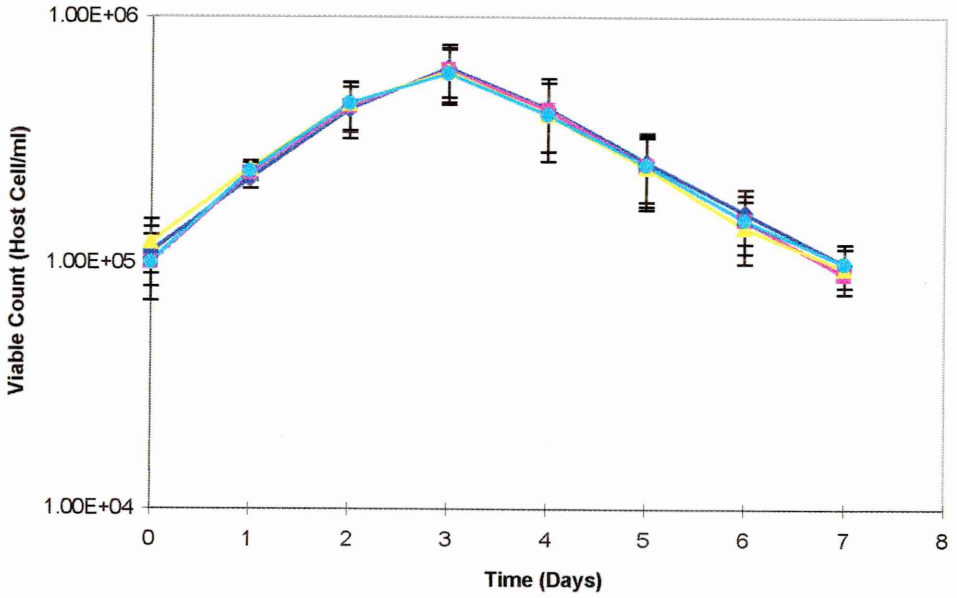
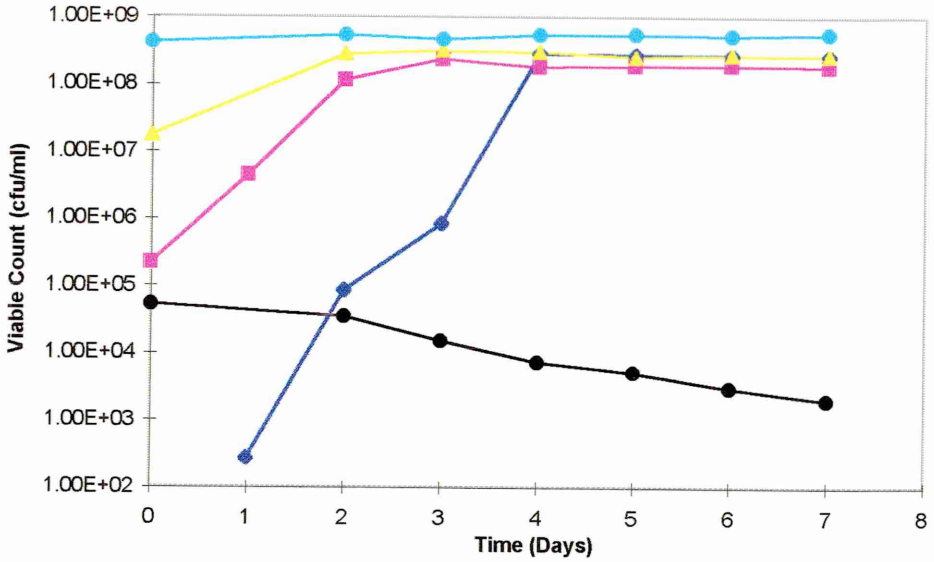
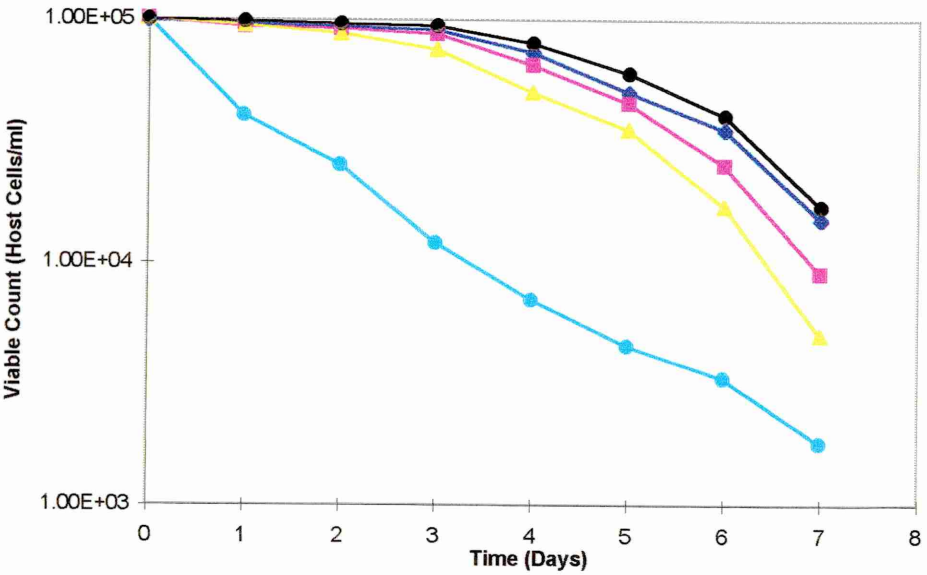


Figure 4.21 (d). Effect of bacterial origin on the lysis of *A. polyphaga* by *L. pneumophila*. Infection at a bacteria-to-host cell ratio of 1000 : 1. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), within amoebae (▲) and within monocytes (●). Bars represent the standard errors of the means for 3 replicate samples.



(a) Infection of *A. polyphaga* by *L. pneumophila* at various bacteria-to-host cell ratios; 1 : 10,000 (◆)., 1 :1 (■), 100 : 1 (▲) and 1000 : 1 (●). *L. pneumophila* in amoebic saline acted as a control (●).



(b). Lysis of *A. polyphaga* by *L. pneumophila* at various bacteria-to-host cell ratios; 1 : 10,000 (◆)., 1 :1 (■), 100 : 1 (▲) and 1000 : 1 (●). *A. polyphaga* in amoebic saline acted as a control (●).

Figure 4.22. Comparison of the two figures shows that the achievement of maximum yield of bacteria from a given coculture is concommittant with the on set of rapid host cell lysis.

There have been a number of reports describing possible causes of host cell lysis (Szeto and Shuman, 1990; Husmann and Johnson, 1994; Muller *et al.*, 1996 and Shuman *et al.*, 1998). These reports are concerned primarily with lysis of the host cell after intracellular bacterial replication. As indicated above, when amoebic or monocytic cells are inoculated at bacteria-to-host cell ratios of 1000 : 1 and 100 : 1, respectively, there is, almost immediately, a rapid decrease in host cell viability. The loss of host cell viability was concomitant with no replication of the legionellae. The phenomenon was also observed by Husmann and Johnson (1994) whilst investigating the viability of J774 monocytic host cells inoculated to a bacteria-to-host cell ratio of 1000 : 1 with *L. pneumophila*. These workers speculated that very high concentrations of *L. pneumophila* produce an extracellular cytotoxic agent in sufficient quantities to cause rapid host cell lysis. Husmann and Johnson (1994) failed to identify a cytotoxic agent within the supernatant. Inspite of the failure of these workers, the supernatants of the coculture from the above growth studies were examined to determine whether an extracellular cytotoxic agent was responsible for the rapid host cell lysis observed in this study.

Samples of the supernatants from the amoebae (1000 : 1) and monocytic (100 : 1) cocultures were centrifuged at 2,080 x g for 30 min. to pellet the cell debris and legionellae. The resulting supernatants were passed through a 0.22 μm filter to remove any remaining bacteria. It has been suggested that the cytotoxic agent could be zinc metalloprotease (Szeto and Shuman, 1990) or low molecular weight proteins (Friedman *et al*, 1980 and Hedlund, 1981). Both of these potential cytotoxins are destroyed by heat. Samples of the supernatants were, therefore, denatured by heating at 80°C for 30 min. Fresh amoebae and monocyte suspensions were prepared in their respective coculture media to a concentration of *ca.* 10^5 cells/ml. A 1 ml sample of the supernatant and denatured supernatant were added to 10 ml of the respective host cell suspension. The host cell suspensions alone served as controls. The viability of the host cells was monitored daily by the trypan blue exclusion assay (Section 3.1.5.1.). The protein profile of each coculture medium was determined by SDS-PAGE using the method described in Sections 3.3.2.2.-3.3.2.4.

The results of this short investigation are presented in Figures 4.23 (a-b) and 4.24. Figures 4.23 (a-b) clearly show that neither the supernatant nor the denatured supernatant had any effect on the viability of either amoebae or monocytic cells. Failure of the supernatants to cause immediate host cell lysis clearly indicates that the causative agent is not present in the supernatant *per se*. The SDS-PAGE gel presented in Figure 4.24 shows the absence of proteins in either the amoebic supernatant (Lanes 1 and 2) or amoebic saline (Lane 3). The monocytic supernatants were found to contain no proteins in addition to those normally found in RPMI medium (Lanes 4 and 5 *cf.* Lane 6)

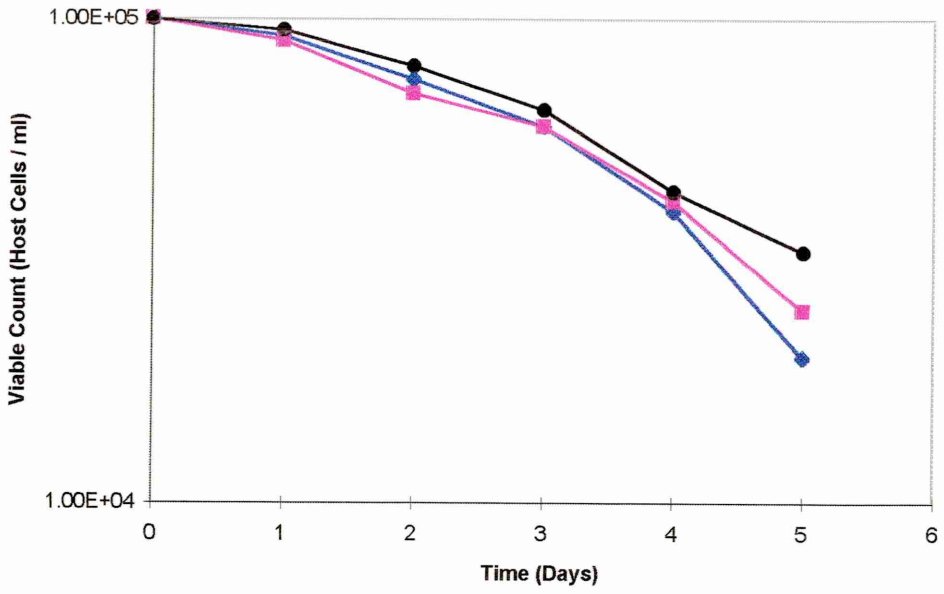


Figure 4.23 (a). Effect of coculture supernatant on lysis of *A. polyphaga* trophozoites. Lysis of *A. polyphaga* on coincubation with amoebic coculture supernatant (◆) and denatured amoebic coculture supernatant (■) and amoebic saline alone (control) (●).

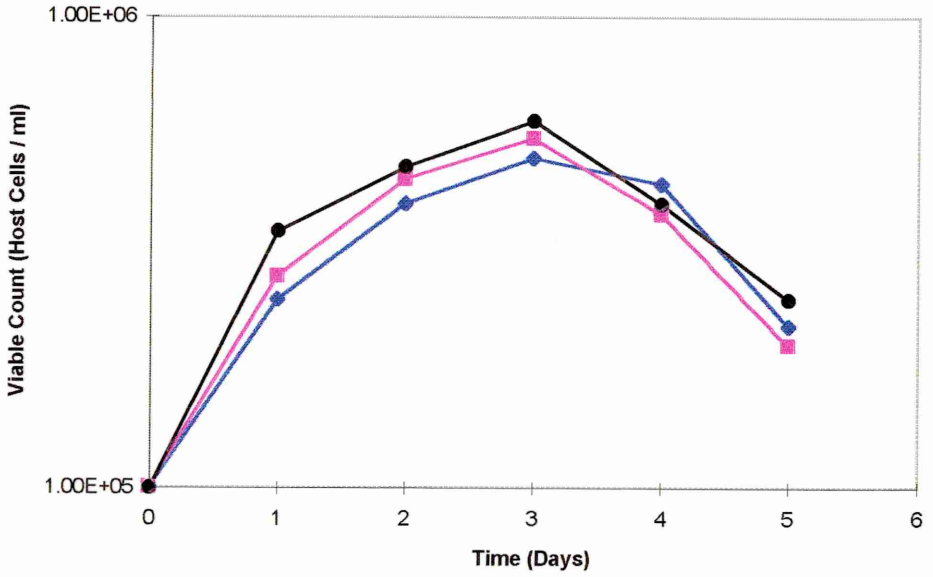


Figure 4.23 (b) Effect of coculture supernatant on lysis of U937 monocytes. Lysis of monocytes on coincubation with monocytic coculture supernatant (◆) and denatured monocytic coculture supernatant (■) and RPMI medium alone (control) (●).

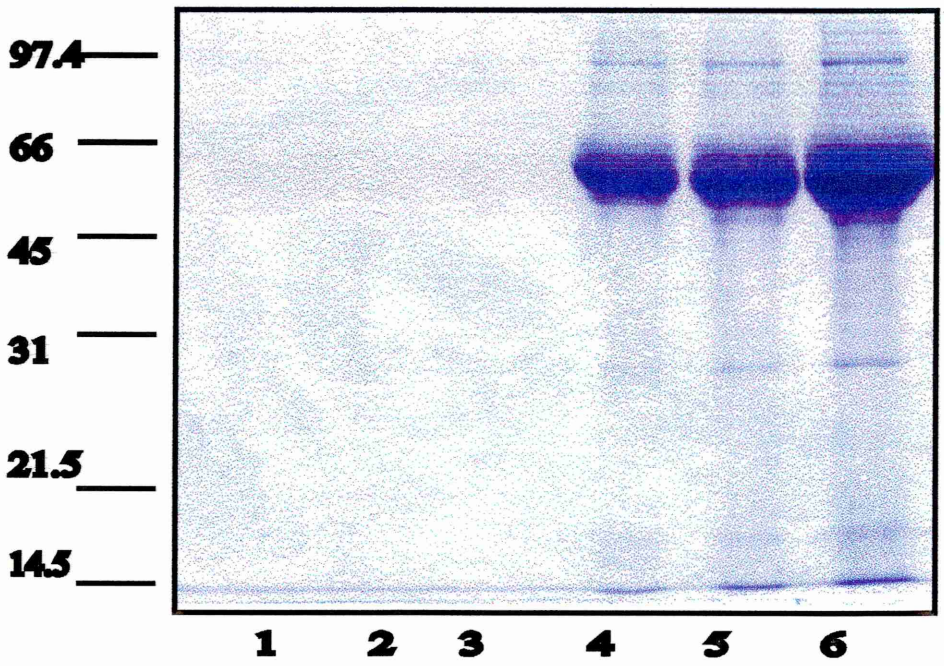


Figure 4.24 SDS-PAGE analysis of coculture supernatants and the corresponding growth media. Lanes 1 and 2, amoebic coculture supernatant, Lane 3, amoebic saline only, Lanes 4 and 5, monocytic coculture supernatant, Lane 6, RPMI medium only.

4.3 DISCUSSION.

The primary objective of this project was to study the phenotypic characteristics of *in vitro* and intracellular grown *L. pneumophila* with respect to their response to external stimuli. For such a comparison to be meaningful, it was necessary to establish growth conditions which would produce consistent bacterial yields with a predictable morphology.

4.3.1 *IN VITRO* GROWTH.

In the current study, growth in the CDM of Warren and Miller (1979) was compared with that in the modified YE broth of Edelstein (1982). The results confirm that the YE broth culture produces the greatest yield of *L. pneumophila*. Pine *et al.* (1979) and Warren and Miller (1979) each found that addition of yeast extract to their media stimulated growth of *L. pneumophila* leading to increased yields. Pine *et al.* (1986) reported that the active principal in yeast extract appears to be purine and pyrimidine derivatives, of which guanine is the most efficacious.

The current investigation showed the lag phase in YE broth to be shorter (*ca.* <1 h.) than that in CDM (*ca.* 5 h.). Warren and Miller (1979) observed similar differences in lag phase when comparing growth kinetics in CDM and F-G medium. No lag phase was observed in F-G medium, whereas a lag phase of *ca.* 3 h. was observed in CDM. Warren and Miller (1979) prepared their inoculum from F-G agar slopes. In the current study the initial culture was grown in YE broth. Often, when bacteria are transferred from complex medium to chemically defined medium, growth is characterised by an extended lag phase followed by depressed growth rates (Pine *et al.*, 1979). The extended lag phase observed in CDM cultures was probably due to the time required for *L. pneumophila* to adapt to the new environment before replication can begin. Pine *et al.* (1979) reported the lag phase in their CDM was reduced upon a second transfer within the medium and maximum rates of growth were achieved, again suggesting adaptation of the bacteria to the medium.

In the current investigation, the generation time of legionellae during exponential phase in YE broth was *ca.* 2 h. compared to *ca.* 9 h. in CDM. Warren and Miller (1979) however, found no difference between the generation time of *L. pneumophila* grown in F-G medium and CDM. This being *ca.* 6 h. in each case. Pine *et al.* (1979), suggested that yeast extract may contain growth promoters which could lead to a shorter lag period and a faster rate of growth. A possible alternative explanation is the action of α-ketoglutaric acid in YE broth. Virtually all organisms generate toxic respiratory by-products during aerobic growth which can inhibit further replication. These include hydroxyl radicals, hydrogen peroxide and superoxide radicals. Alpha-ketoglutaric acid acts as a scavenger for these oxidative radicals, thus removing their inhibitory effect (Hoffman *et al.*, 1983). It may be possible that the longer generation time of the *L. pneumophila* in CDM observed in the current study, compared to that reported by Warren and Miller (1979), is a reflection of the composition of inoculum growth medium (YE broth *cf.* F-G medium). The nutrient availability of the growth medium may influence the physiological state of the inoculum bacteria and thus the subsequent growth rates. Alternatively, bacteria transferred from YE broth to CDM would be subjected to a far greater change in 'nutrient richness' of their environment than those going from F-G medium to CDM. The stress of this greater change could influence subsequent growth rates of the bacteria.

The formation of a soluble brown pigment by *L. pneumophila* was observed in both the YE broth and CDM cultures. This phenomenon is not unique to *L. pneumophila.* Several species of *Bacillus, Serratia, Pseudomonas, Streptomyces* and *Vibrio* have been reported as producing a brown melanin-like pigment (Steinert *et al.*, 1995). Warren and Miller (1979) reported that pigment formation is less in CDM than in F-G medium cultures. The addition of L-tyrosine to their CDM culture stimulated pigment formation. Mintz *et al.* (1988) reported the formation of a soluble brown pigment in a chemically defined culture medium (CAA) devised for the isolation of auxotrophic *L. pneumophila* mutants.

Pigment formation has been shown by the current study to commence during late exponential phase, and to be maximal in the stationary phase of *L. pneumophila* growth in YE broth. Similar observations were reported by Warren and Miller (1979) in CDM and Mintz *et al.* (1988) in CAA medium. Wintermeyer *et al.* (1994) observed the browning of F-G medium during the late stationary phase of *L. pneumophila* growth and suggested that the pigment was conferred by the Lly protein. The Lly protein has been shown to have a significant homology with the MelA protein of *Shewanella colwelliana* (Fuqua *et al.*, 1991). Homogenistic acid has been reported as a product of MelA (Coon *et al.*,1994) and Steinert *et al.* (1995) suggested this is the cause of the browning effect. Wintermeyer *et al.* (1994) also reported that pigment formation was oxygen dependent, which explains the lack of pigment in static cultures. This is in agreement with the findings of Pine *et al.* (1979), and those of the current study, in that no pigmentation occurred in cultures which were not aerated by shaking. The current study has also shown that pigmentation did not form in cultures inoculated with low concentrations of bacteria (*ca.* 10^4 cfu/ml). Pine *et al.* (1979) suggested that an inoculation concentration of at least 10^7 cfu/ml is necessary before brown pigmentation is observed.

The reasons for the formation of the brown pigment during the growth of *L. pneumophila* is not clear. However, pigmentation is known to be a common protector against the lethal effects of light. Hoffman *et al.* (1983) suggested that light accelerates the production of lethal oxidative radicals during respiration by *L. pneumophila*. This is countered by the legionellae by the production of a pigment to reduce light intensity. Steinert *et al.* (1995) investigated the possible role of the pigment in protecting the bacteria from light by mutating the *lly* gene. Wild type and *lly* negative mutants were exposed to light (halogen lamp). The ability of the mutants to replicate in *H. vermiformis*, an amoeba, was also investigated. Steinert *et al.* (1995) concluded that pigmentation was important for the survival of *L pneumophila* stressed by light but does not have any influence on the virulence of *L. pneumophila* or intracellular replication.

Microscopical studies have shown that YE broth grown *L. pneumophila* to be rod shaped and non-motile. The bacteria were observed to be paired, end to end, and occasionally filamentous during exponential growth. Stationary phase was characterised by single rod-shaped bacteria with occasional small, rounded and motile bacteria. The rod-shaped non-motile form of *L. pneumophila* is now widely accepted as being typical of growth in a complex medium. The appearance of paired bacteria during the exponential phase of growth in F-G medium has been reported by Keel *et al.* (1979) who described the legionellae as replicating by pinching binary fission. Pine *et al.* (1979) described the breaking up of chains of bacteria upon entering stationary phase to form single and double 'cigar' shaped bacteria. They also reported that prolonged incubation of a limited substrate causes the rod-shaped bacteria to become coccoid shaped. There are two possible explanations for the appearance of small motile bacteria at the stationary phase of growth in YE broth, reported by Pine *et al.* (1979) and observed in the current work. Prolonged exposure to limiting nutrients would induce a stringent response; which is characterised by the formation of motile coccoidal bacteria (Neidhardt *et al.*, 1990). Under the conditions used in the current work, and those of Pine *et al.* (1979), the rate of nutrient depletion within the medium would be progressive and slow. The formation of coccoidal bacteria would be expected to follow a similar pattern. Coccoidal bacteria may also arise from a process involving autoinduction. The achievement of a critical bacterial cell density, once stationary phase has been entered, could lead to the autoinduction of certain stress regulatory networks, possibly including the stringent response. Autoinduction is a phenomenon which, once triggered, induces a very rapid transformation throughout a bacterial population. Thus, it can be anticipated that the majority of bacteria would change to the coccoidal form once they enter the stationary phase of growth. The observations made in this work, and those of Pine *et al.* (1979) suggest that the first explanation of coccoidal cell formation within the stationary phase of growth is the more likely in this instance.

Warren and Miller (1979) inoculated both F-G medium and their CDM with legionellae which were primarily single rod-shaped bacteria. The legionellae retained their single cell morphology during growth in F-G medium but adopted a filamentous form

throughout growth in CDM. Kjelleberg *et al.* (1993) have reported that *Vibrio* sp. adopted a filamentous form upon nitrogen starvation. Legionellae may respond in a similar manner to a restriction in the availability of nitrogen.

4.3.2 INTRACELLULAR REPLICATION.

Microscopical examination of wet preparations have shown that, within a short period of time after inoculation, bacteria begin to cluster around one end of a few of the amoebae. Similar observations were reported by Rowbotham (1986) who suggested that the initial stage of intra-amoebic replication was a clustering of legionellae at the end of the trophozoite, close to the point at which the contractile vacuole empties. This stage is followed by non-classical phagocytosis of the bacteria. Fields (1996) has suggested that the process of infection of amoebae by *L. pneumophila* is a form of receptor mediated endocytosis. Once enclosed within the phagosome, and adapted to the new environment, the bacteria undergo active replication but remain rod-like and non-motile (Rowbotham, 1986 and Pruckler *et al.*, 1995). Shuman *et al.* (1998), reported a lag phase of *ca.* 6-8 h. post infection before any increase in bacterial numbers is detected. It is suggested that the bacteria are adapting to the nutritional conditions which prevail within the host cell phagosome. Once the bacteria begin to replicate intracellularly, their generation time is *ca.* 2 h. (Shuman *et al.*, 1998). This is the same as the generation time in YE broth observed in the current study. This would imply that the nutritional requirements of the bacteria are met in the phagosome. However, it is highly unlikely that the available nutrients within amoebae or monocytes will be identical to those in YE broth. This stage of replication has been observed to be followed by expansion of the infected phagosome to fill the amoebae and the acquisition of directional motility by the bacteria. Rowbotham (1986) and Pruckler *et al.* (1995) have described the replicating rod-shaped, non-motile *L. pneumophila* as undergoing a rapid transformation to a small, rounded, and highly motile form at a well defined point during intracellular replication. This point appears to be the attainment of of critical bacterial population density, suggesting a form of autoinduction response, leading to both a morphological change and the onset of motility. This phenomenon is discussed further in Chapter 7.

Observations made during this study indicate that the replication process culminates in the rupture of the amoebae and liberation of the bacteria into the surrounding medium. Rowbotham (1986) suggested that the phagosome containing the *Legionella* and the amoebae cell wall did not always rupture at the same time. Rowbotham (1986) also reported that intracellular grown *L. pneumophila* change to the broth grown phenotype (rod-shaped, non-motile) if they do not infect further amoebae within 24 h. after release from the host cell. Similar observations were made during the current work.

Encystment of amoebae was noted during the second stage of *Legionella* infection and replication. Acanthamoebae have been described as forming thick walled resistant cysts compared to other protozoa (Rowbotham, 1986). A few amoebae cysts were observed during the current work to have thick walls and be infected with highly motile legionellae. Rowbotham (1986) also reported mature thick-walled cysts of *A. polyphaga* containing motile *L. pneumophila* serogroup 1. The process leading to infected cysts was described by Rowbotham (1986) as a race between the legionellae replicating and bursting the amoebae, and the amoebic trophozoite forming a mature cyst. Once a mature cyst is formed, the amoebae are refractory to infection. Encystment was considered by Rowbotham (1986) to be the main means by which acanthamoebae escape infection by legionellae.

As far as it is known, no detailed systematic study of the morphological changes of *L. pneumophila* during intra-phagocytic replication has been published. Observations have shown that infection and replication of *L. pneumophila* in U937 monocytic host cells is very similar to that observed within amoebae after phagocytosis. Horwitz (1984) reported that the phagocytosis of *L. pneumophila* by phagocytic cells occurs by an unusual mechanism, coiling phagocytosis, in which a single pseudopod wraps around the bacterium. Shuman *et al.* (1998) reported that following coiling phagocytosis the many layers of the coil resolve to form a single layered membrane-bound compartment, the phagosome or vesicle. Microscopical observation has shown that broth grown legionellae retain their morphology within the phagosome and actively

replicate. This stage is again followed by enlargement of the phagosome, change of bacterial morphology and the onset of motility in the bacterial mass. Intra-monocytic replication culminates in the bursting of the phagocyte and the release of small, highly motile legionellae into the extracellular matrix. These bacteria were also observed to change to the broth grown morphology if they failed to infect a host cell.

The present study has shown that *L. pneumophila* are unable to replicate in the presence of host cell debris, host cell metabolic products (conditioned medium) or in the coculture medium alone. Similar studies by Horwitz and Silverstein (1980) and Pearlman *et al.* (1988) demonstrated that *L. pneumophila* are incapable of replication in cocultures containing only lysed monocytic cells. Pearlman *et al.* (1988) reported a decrease in bacterial viability of 2 orders of magnitude in 48 h. This is similar to the loss in legionellae viability observed during the current investigation (*ca.* 2.5-3 orders of magnitude). Anand *et al.* (1983) reported the failure of *L. pneumophila* to replicate in a nutrient rich (Neffs) medium in the presence of lysed *A. palestinensis*. These workers reported a decrease in *L. pneumophila* viability of *ca.* 4 orders of magnitude within 3 d. This value is somewhat higher than that found in this study (*ca.* 1 order of magnitude loss in bacterial viability) and considerably higher than that reported by Barker *et al.* (1992) (*ca.* 0.3 orders of magnitude in 4 d). Examination of the data presented by Barker *et al.* (1992) shows that this decrease is markedly less than that observed in amoebic saline alone (0.9 orders of magnitude). Similar observations have been made in the current work, but the differences are smaller and confined to *ca.* 1 to 2 d. after inoculation. It is tentatively suggested that during this period the bacteria could scavenge sufficient nutrients from the host cell debris to survive, but not grow. Once this nutrient source is depleted the bacteria would enter a state of starvation.

Failure of *L. pneumophila* to replicate in a medium containing host cell metabolic products alone has also been demonstrated. Similar observations were made by Rodgers and Gibson (1993) using RPMI medium conditioned by U937 monocytes and by Anand *et al.* (1983) in medium containing the metabolic products of *A. palestinensis*. A loss of *L. pneumophila* viability of *ca.* 4 orders of magnitude in

48 h was again reported by Anand *et al.* (1983). The corresponding loss in the current study was again *ca.* 1 order of magnitude. Horwitz and Silverstein (1980) and Steinert *et al.* (1998) adopted a different approach to the study. Horwitz and Silverstein (1980) held *L. pneumophila* and viable monocytic cells within the same medium but separated the two by a fine membrane. A loss of *L. pneumophila* viability of *ca.* 2 orders of magnitude was observed over a 3 d. period. This was a similar reduction to that found in the current investigation. Steinert *et al.* (1998) performed a similar experiment, separating *L. pneumophila* and *A. polyphaga* trophozoites by a 0.1 μm polycarbonate membrane. A slight decrease in the number of viable *L. pneumophila* was observed. The work was repeated using *M. avium* and *E. coli*. These bacteria continued to grow at the same rate as those in direct coculture. Steinert *et al.* (1998) suggested that unlike *L. pneumophila*, *M. avium* and *E. coli* are able to grow saprozoically on products secreted by amoebae.

The observed losses in viability of *L. pneumophila* reported above could be due to the coculture medium itself. The current study has shown the viability of *L. pneumophila* suspended in RPMI alone falls by *ca.* 3 orders of magnitude and that in amoebic saline by *ca.* 1 order of magnitude in 6 d. This suggests the possible presence of an inhibitory factor. It is widely reported that *L. pneumophila* will not grow in RPMI alone but no explanation for this has been offered (Horwitz and Silverstein, 1980; Holden *et al.*, 1984; Abu Kwaik *et al.*, 1993; Rodgers and Gibson, 1993 and Steinert *et al.*, 1994). Barker *et al.* (1986) and Caternich and Johnson (1988; 1989) have reported the inhibitory effects of sodium ions towards *L. pneumophila*. RPMI supplied without sodium bicarbonate buffer is known to contain sodium ions at a concentration of 6.8 mg/ml (Sigma Technical Services). If the medium is purchased with added sodium bicarbonate, rapid loss of viability of the *L. pneumophila* inoculum takes place before infection of the host cell can occur (Personal observation). The concentration of sodium ions required to inhibit *L. pneumophila* growth is *ca.* 100 mM, much lower than might be expected for osmotic stress (Caternich and Johnson, 1989). Sodium ions are known to inhibit certain bacterial enzymes but the reason why legionellae are

sensitive to these ions is not currently understood (Shuman *et al.*, 1998). Amoebic saline also contains sodium ions but not in such high quantities as in RPMI medium (0.26 *cf.* 6.8 mg/ml). This could explain the observed differences in loss of viability between the two media. *L. pneumophila* have also been shown to be sensitive to the presence of superoxides (Pine *et al.*, 1979 and Sadowsky *et al.*, 1993). These may also be present in the coculture media as a product of respiration.

The effect of the growth conditions of the inoculum bacteria upon replication and final yield in coculture was examined. The final yield of *L. pneumophila* in amoebic coculture was found to be *ca.* 10^8 cfu/ml and in monocytic coculture was *ca.* 5×10^7 cfu/ml, irrespective of the bacteria-to-host cell ratio. Horwitz and Silverstein (1980) also found that the final yield of intra-monocytic grown *L. pneumophila* was independent of the inoculum concentration. As in the current investigation, Horwitz and Silverstein (1980) fixed the number of monocytes and varied the number of bacteria in each coculture. The authors reported final *L. pneumophila* yields of 10^6-10^7 cfu/ml were achieved after 2-3 d. of coculture. Steinert *et al.* (1994) reported final yields of 10^6-10^7 cfu/ml in 24 h. after infection of both U937 cells and *A. castellanii* with *L. pneumophila* at a bacteria-to-host cell concentration of 10 : 1. Moffat and Tompkins (1992) reported a higher yield of *ca.* 10^8 cfu/ml when examining amoebic cocultures. These findings are consistent with the yields achieved in the current studies. Anand *et al.* (1983) achieved greater yields of *L. pneumophila* when coincubating with amoebae at high bacteria-to-host cell ratios (1000 : 1). During the current study, no replication of *L. pneumophila* in amoebae was observed at this ratio. However, unlike the current investigation, Anand *et al.* (1983) used a nutrient rich medium in which replication of amoebae could occur during the experiment. This would have the effect of changing the bacteria-to-host cell ratio. Horwitz and Silverstein (1980) also cocultured fixed numbers of bacteria with varying numbers of monocytic cells. The yield of intracellular grown bacteria increased with the number of host cells, but never exceeded *ca.* 5×10^7-5×10^8 cfu/ml. The time to achieve the maximum yield was reported as being virtually unaffected by the number of host cells. It may be suggested, therefore, from the evidence available in the literature, and that

reported in this thesis, that the potential maximum yield of intracellular grown bacteria from any coculture is *ca.* 10^7-10^8 cfu/ml. Bacteria-to-host cell ratio determines the time to achieve that yield. Evidence presented in this study has also shown that *L. pneumophila* at concentrations too low to be detected by standard plating techniques (< 10 cfu/ml) can, nevertheless, infect host cells and replicate to *ca.* 5×10^7-5×10^8 cfu/ml. This has considerable practical implications for the routine monitoring of environments for the presence of pathogens, such as *L. pneumophila*. The failure to detect the presence of bacteria by standard plating techniques does not necessarily mean that they are absent from that environment. Bacteria concentration techniques should be routinely employed. From a clinical view-point, the evidence would imply that very few infectious bacteria (< 10) need to be inhaled to initiate the onset of disease.

Evidence gained in the current study suggests that phenotype has only a small effect on the invasiveness of *L. pneumophila* and the final yield of harvested bacteria in coculture. This is contrary to the findings of Cirillo *et al.* (1994) who reported a ten-fold difference between the invasivenes of intra-amoebic grown *L. pneumophila* and that of BCYE agar grown legionellae for both macrophages and *A. castellanii* trophozoites. Bacteria of the intracellular grown phenotype have been shown by the present investigation to be only *ca.* 2.5 times more invasive than those of the broth grown phenotype. Cirillo *et al.* (1994) suggested that growth in amoebae leads to changes in membrane protein expression by *L. pneumophila* that correlate with an increased capacity to invade both human phagocytic cells and amoebae.

Microscopical observations during this study have suggested a link between motility and the invasiveness of *L. pneumophila*. The small, highly motile intracellular grown *L. pneumophila* were observed to enter amoebae within a shorter period than the non-motile rod-shaped bacteria grown in broth. This could be, in part, a reflection of the time taken by the respective bacteria to attain a position favourable for attachment to, and subsequent infection of, the host cell. Pruckler *et al.* (1995) examined wildtype

L. pneumophila and flagella negative mutants for their ability to infect amoebae and U937 cells. These workers reported that although the virulence of *L. pneumophila* is not dependent upon motility *per se*, the more motile a bacterium is, the greater its ability to invade a host cell. Morphology may also affect the rate of phagocytosis. It is possible that phagocytosis of the small intracellular grown bacteria may require a shorter time period than that needed for the larger rod-shaped *L. pneumophila*. A factor which may affect the time to reach the final yield is the morphological change that the broth grown *L. pneumophila* undergo after replication within the phagosome, prior to induction of motility and the rupture of the host cell. The intra-amoebic grown legionellae may not be required to undergo this change in morphology as they could be pre-adapted to the intracellular environment. Microscopical examination also showed that, after intracellular replication, all the bacteria had the same morphology and motility, and were equally invasive of host cells, irrespective of the inoculum origin. Similar observations were reported by Rowbotham (1986). This author suggested that infection of any remaining amoebae was due to the small highly motile legionellae released from previously infected amoebic trophozoites, and not the remaining original broth grown inoculum.

The literature contains many reports of studies on the coculture of *L. pneumophila* with different host cells. The vast majority of these have concentrated on the factors affecting the legionellae and have assumed that viable host cells would be available for infection after the first round of intracellular replication. As far as it is known, no systematic study has been made of host cell lysis due to successive cycles of intracellular replication by *L. pneumophila*. Observations made in the current study suggest that a critical bacteria-to-host cell ratio exists for both amoebic and monocytic cocultures below which loss of host cell viability in coculture follows a pattern very similar to that of natural cell death. In both instances, the greater the initial bacteria-host cell ratio, the bigger the deviation between the two patterns. The origin of the inoculum *L. pneumophila* used appeared to have no effect on this pattern of behaviour.

At bacteria-to-host cell ratios of 100 : 1 or less, the monocytic cells replicated during the first 72 h. after inoculation. After this period, there was a decrease in the viability of the monocytic cells both in the cocultures and in the uninoculated control. This could be due to the formation of an inhibitory agent within the RPMI medium. Alternatively, the cells may have grown to confluency and are dying off due to lack of space. The slight differences between loss in viability of uninoculated cells and those in coculture is due to lysis of the host cells, post-intracellular replication. In amoebic coculture, no loss in host cell viability was apparent during the initial 72 h. when the bacteria-to-host cell ratios were below 1000 : 1. After this period, encystment commenced in addition to lysis of the amoebae. The former is most probably due to the lack of nutrients in amoebic saline. As before, the differences between the decrease in amoebic viability of the uninoculated cells and those in coculture is probably due to the lysis of the host cells, post-intracellular replication.

Observations made during this study and reports in the literature (Rowbotham, 1986; Pearlman *et al.*, 1988 and Muller *et al.*, 1996) indicate that replicating bacteria induce a dramatic cytopathic effect (lysis) upon the host cell *ca.* 16-18 h. post infection. It has been suggested that *L. pneumophila* secrete cytotoxins, including a zinc metalloprotease and it is these which are responsible for host cell lysis (Keen and Hoffman, 1989; Quinn and Tompkins, 1989 and Szeto and Shuman, 1990). Subsequent work by Blander *et al.* (1990) suggested that this was incorrect. Bacteria with null mutations in the structural gene for the protease were found to replicate within cells and to produce the same cytotoxic effect as wild-type *L. pneumophila*. It has also been suggested that the intracellular replicating bacteria induce apoptosis, or programmed cell death, in the host. This phenomenon has been described for *Shigella flexneri* and *Bordetella pertussis* (Shuman *et al.*, 1998). Muller *et al.* (1996) have described apoptosis in PMA-treated HL-60 cells infected with *L. pneumophila*. These workers demonstrated that HL-60 cells infected with *L. pneumophila* exhibit a ladder pattern of DNA fragmentation and chromatin condensation and segmentation of the nucleus. Both of these are classical characteristics of apoptosis. Other groups have described the induction and attenuation of apoptosis in HL-60 cells by PMA alone (Zhu and Loh,

1996). Abu Kwaik *et al.* (1998) argued that apoptosis is unlikely to play a role in lysis of amoebae. Apoptosis is a mechanism by which multicellular organisms eliminate unwanted cells to avoid injury to the rest of the organism. Since amoebae are unicellular organisms, apoptosis would constitute cell suicide and, therefore, is unlikely to occur. A third suggestion (Shuman *et al.,* 1998) is that the burden of bacterial replication and production of NH_3 from amino acid metabolism, as well as other metabolic products, eventually cause the host cell to die. It is evident, therefore, that the precise mechanism of lysis of amoebae post intracellular replication is yet unknown.

L. pneumophila did not replicate when in coculture with amoebic trophozoites at a bacteria-to-host cell ratio of 1000 : 1 or with monocytes at a ratio of 100 : 1. At this inoculum level, the viability of the *L. pneumophila* in amoebic coculture remained unaltered during the period of the test; whilst that of legionellae in the coculture medium alone fell by *ca.* one order of magnitude. This raises the possibility of scavenging of nutrients from the lysed amoebae by the *L. pneumophila* at a level sufficient to ensure greater survival than in coculture medium alone, but not growth. Anand *et al.* (1983) and Barker *et al.* (1992) have also shown a higher survival rate of *L. pneumophila* in association with lysed amoebae than in coculture alone. Neither groups of workers offer an explanation for this phenomenon. Interestingly, the decrease in viability of the *L. pneumophila* in monocytic coculture at a bacteria-to-host cell ratio of 100 : 1 is similar to that in RPMI medium alone. It is possible that scavenging is still taking place but the beneficial effects are outweighed by the known inhibitory effects of RPMI medium.

The failure of *L. pneumophila* to replicate in either amoebae or monocytic cocultures at high bacteria-to-host cell ratios (> 1000 : 1 and > 100 : 1, respectively) has been shown during this project to be concomitant with rapid lysis of the host cells. A similar phenomenon of high numbers of bacteria destroying host cells without replication has been reported for *Rickettsia prowazekii* (Winkler and Turco, 1988). These authors suggested that the host cell lysis was due to the production of phospholipase by the bacteria. Husmann and Johnson (1994) studying the rapid lysis of J774 monocytic cells

concluded that the coculture supernatant was not cytotoxic towards fresh host cells. This is in agreement with the current work which has shown that the coculture supernatants were not cytotoxic towards U937 monocytic cells or amoebae trophozoites. Conversely, both Friedman *et al.* (1980) and Hedlund (1981) reported the isolation of low molecular weight toxins (*ca.* <1 kDa) from coculture supernatant that were active against Chinese hamster ovary cells and mouse macrophages, respectively. As the current coculture supernatants have been shown to be non-cytotoxic towards host cells, it is highly unlikely they are the causative agents in the present study.

An alternative explanation for rapid host lysis has been proposed by Kirby *et al.* (1998). These workers described the ability of *L. pneumophila* to insert a pore into eukaryotic cell membranes upon intial contact. The authors suggested that this mechanism may alter endocytic trafficking events within the host cell and aid the establishment of a replicative phagosome. It is further suggested that in a high bacteria-to-host cell infection, the simultaneous insertion of a large number of pores by many bacteria may result in rapid host cell death. This hypothesis may explain the different bacteria-to-host cell ratios that induce spontaneous lysis in the two host cell types used in this investigation (1000 : 1 in amoebic coculture, 100 : 1 in monocytic coculture). Assuming cell lysis occurs when a given proportion of the host cell membrane is pierced, it follows that the smaller the cell size, the lower the bacteria-to-host cell ratio necessary to achieve this state. Thus, monocytes would be expected to lyse at lower bacteria-to-host cell ratios than the much larger amoebae. The proposals of Kirby *et al.* (1998) could also offer an explanation for the maximum yield effect observed in cocultures during the present studies. It may be suggested that the maximum yield of *ca.* 5×10^7 -5×10^8 cfu/ml from each coculture is limited by the onset of rapid cell lysis.

4.4 CONCLUSIONS

Work has been undertaken to determine the conditions which would produce consistent yields of bacteria with predictable morphology from both *in vitro* and intracellular cultures. The following conditions have been adopted for the purposes of this thesis :

- YE broth will be adopted for the purpose of *in vitro* growth. Consistent bacterial numbers and morphology can be obtained by careful control of the culture conditions.

- A ratio of 1 : 1 should be adopted for the production of intracellular grown bacteria at a host cell concentration of *ca.* 10^5 cells/ml. At this ratio, maximum yield can be achieved within an acceptable period (2-3 d. post inoculation) whilst utilising a minimal quantity of stock bacterial inoculum. Maximum yield is consistently 5×10^7-5×10^8 cfu/ml.

CHAPTER 5 THE RESPONSE OF *L. PNEUMOPHILA* TO STRESS CONDITIONS.

A review of the literature (Section 1.4) has suggested that little is known of the effect of intracellular growth of *L. pneumophila* in response to stress. The majority of reported studies have utilised either *in vitro* grown *L. pneumophila* or bacteria still enclosed within the host cell. The problem with the latter approach is that it is difficult to distinguish between the response of the bacteria to the imposed stress from that of the host cell. The current work has attempted to overcome this problem by separating the *L. pneumophila* from the host cell prior to imposition of the stress. The stress conditions employed were exposure to three of the antibiotics traditionally used for the treatment of Legionnaires' disease, elevated temperatures, long-term starvation and mechanical stress. These apparently unrelated stress stimuli were chosen as in each case any variation in the response of *L. pneumophila* according to phenotype could have practical implications in, for example, the clinical treatment of Legionnaires' disease and eradication of *L. pneumophila* from water systems.

5.1 EXPOSURE TO ANTIBIOTICS.

Previous studies of the efficacy of antibiotics in killing *L. pneumophila* have tended to utilise either *in vitro* testing or pre-infected phagocytes. However, both techniques suffer from deficiencies. Horwitz and Silverstein (1983) described *in vitro* testing as directly exposing bacteria to antibiotics under standardised physiological conditions which are unrepresentative of the intra-phagocytic environment. Thus, information gained from such tests must be treated with caution, especially when using it as a basis for the therapeutic treatment of Legionnaires' disease. The use of pre-infected phagocytes is one approach aimed at overcoming this problem (Vilde *et al.*, 1986; van den Broek, 1991; Ramirez *et al.*, 1992; Smith, G. M. *et al.*, 1995; Segreti, 1996 and Smith, R. P., 1997). In such tests the absence of re-growth or very slow re-growth of

the intra-phagocytic bacteria on removal of the antibiotic from the extracellular environment is used as the criterium for describing the agent as being bactericidal towards *L. pneumophila*. However, whilst such studies demonstrate the ability of an antibiotic to penetrate and concentrate within phagocytic cells, they cannot give a clear indication of the level of resistance of the intracellular bacteria to the antibiotic under test. By separating the legionellae from the host cell prior to exposure to the antibiotic agent it may be possible to determine the influence of the intracellular environmental conditions on the physiological properties of the *L. pneumophila* bacteria. Freshly harvested intra-amoebic and intra-monocytic grown legionellae were exposed to erythromycin, ciprofloxacin and rifampicin separately and to erythromycin and rifampicin in combination. The sensitivity of these isolated intracellular grown *L. pneumophila* to the antibiotics was compared to that of YE broth grown legionellae in both exponential and stationary phase.

The MIC values of erythromycin, ciprofloxacin and rifampicin were determined for both the broth and intracellular grown bacteria. Growth conditions of the *L. pneumophila* appeared to have no effect on the value determined for a particular antibiotic. The MIC values of erythromycin, ciprofloxacin and rifampicin determined in this study were 0.25, 0.06 and 0.008 μg/ml, respectively. The MBC values of the antibiotics were determined by spread plating each dilution in which growth had not occurred after 48 h incubation. The MBC was taken as the minimum concentration of antibiotics which killed all the bacteria. The MBC value for each antibiotic was found to be independent of the prior growth conditions of the *L. pneumophila*. The MBC values of erythromycin, ciprofloxacin and rifampicin were 1.0, 0.24 and 0.036 μg/ml, respectively.

Time-kill assays were conducted to determine the effect of exposure of broth grown and intracellular grown *L. pneumophila* to therapeutic levels of the various antibiotics, including a combination of erythromycin and rifampicin. Bacterial survival was determined by viable plate count on BCYE agar. The susceptibilities of variously

grown *L. pneumophila* to rifampicin (5 μg/ml) are shown in Figure 5.1. The results clearly indicate that the growth conditions of a bacterium has a marked effect on its sensitivity to the antibiotic. Broth grown legionellae were far more susceptible to rifampicin exposure than intracellular grown bacteria. The greatest susceptibility was exhibited by the broth grown *L. pneumophila* in the exponential phase of growth. These bacteria underwent a loss of 4 orders of magnitude within 6 h. of exposure and *ca.* 6 orders of magnitude within 24 h. This suggests a greater than 99.99 % kill within 6 h. of exposure to rifampicin. The broth grown bacteria in the stationary phase exhibited a somewhat greater resistance to the antibiotic than those in the exponential phase. The loss in viability was 1 order of magnitude and 3.5 orders of magnitude after 6 and 24 h. exposure, respectively. In this instance a killing of more than 99.9 % was only achieved after 25 h. of exposure to rifampicin. The corresponding values after 24 h. exposure to rifampicin for the intra-amoebic and intra-monocytic grown bacteria are 50 % and 25 %, respectively. The continued replication of the various *Legionella* inocula in the control suspensions throughout the test period confirmed that the losses in viability were due to the antibiotic alone and not to natural cell death.

Broadly similar results were obtained on exposure of the bacteria to ciprofloxacin (1 μg/ml), (Figure 5.2). Exposure of broth grown *L. pneumophila* in exponential phase resulted in a loss of 3 orders of magnitude in viability after 6 h. and *ca.* 6 orders of magnitude within 24 h. This represents a killing of 99.9 % within 6 h. of exposure of the bacteria to ciprofloxacin. The losses in viability of the stationary phase bacteria were 1.5 orders of magnitude and 3.0 orders of magnitude within 6 h. and 24 h. exposure, respectively. A bacterial kill of 99.9 % was only achieved after 24 h. of exposure to ciprofloxacin. The corresponding values for intra-amoebic and intra-monocytic grown *L. pneumophila* after 24 h. exposure to ciprofloxacin were 93.5 % and 88 %, respectively.

The data relating to exposure to erythromycin (8 μg/ml) suggests that *L. pneumophila* grown under each condition exhibit a greater resistance to this antibiotic than to either

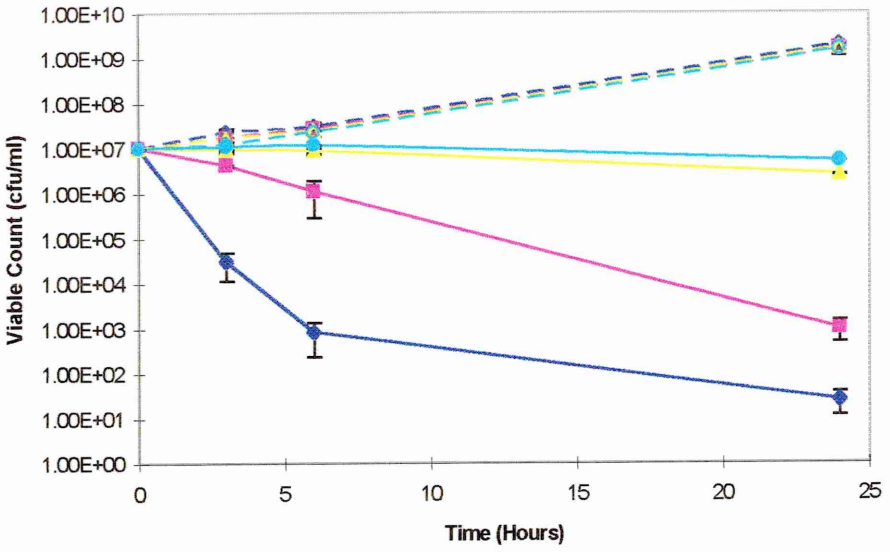


Figure 5.1 Survival of *L. pneumophila* after exposure to rifampicin (5 µg/ml) at 37°C in YE broth. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), or intracellularly in U937 monocytes (▲) or amoebae (●). Results for controls in which antibiotic was not added are shown (◇, □, △, ○), respectively. Bars represent the standard errors of the means for 3 replicate experiments.

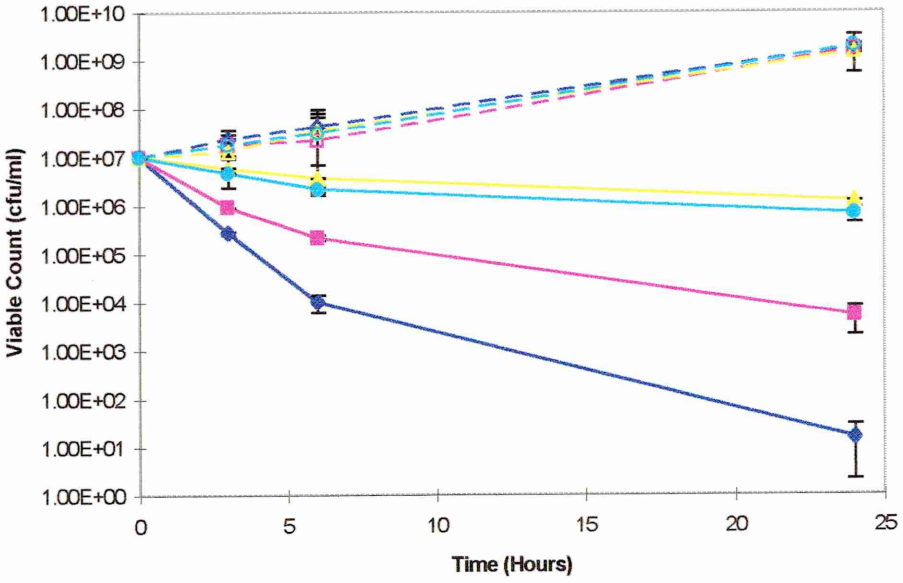


Figure 5.2 Survival of *L. pneumophila* after exposure to ciprofloxacin (1 µg/ml) at 37°C in YE broth. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), or intracellularly in U937 monocytes (▲) or amoebae (●). Results for controls in which antibiotic was not added are shown (◇, □, △, ○), respectively. Bars represent the standard errors of the means for 3 replicate experiments.

rifampicin or ciprofloxacin (Figure 5.3). A 99.9 % killing of the broth grown legionellae in exponential phase was not achieved after 24 h. exposure to erythromycin (*cf* 6 h for rifampicin and ciprofloxacin). The corresponding values of the stationary phase broth grown, intra-amoebic and intra-monocytic grown *L. pneumophila* after 24 h. exposure to erythromycin were 88 %, 20 % and 30 %, respectively.

Clinical experience (Gump *et al.*, 1979; Macfarlane, 1987 and Barker and Brown, 1995) has indicated that a combination of erythromycin and rifampicin (8 μg/ml and 5 μg/ml, respectively) increases prognosis in patients suffering from severe Legionnaires' disease. The prescribed combination was, therefore, examined as part of this study. The results of this investigation (Figure 5.4) reinforced the clinical observations. The activity of the two antibiotics in combination resulted in a 90 % killing of broth grown *L. pneumophila* in exponential phase in 3 h. and reduced the bacteria to a point of being non-recoverable after 6 h. exposure. A similar rapid fall in viability was noted for broth grown *L. pneumophila* in stationary phase during the initial 3 h. of exposure but only 5 orders of magnitude reduction in viability occurred within 24 h. This represents a killing of more than 99.99 % within 24 h. of exposure to a combination of rifampicin and erythromycin. In contrast, 10 % of intra-monocytic grown and 30 % of intra-amoebic grown *L. pneumophila* survived after 24 h. of exposure.

The above work, and that reported in the literature (Chen *et al.*, 1993; Baltch *et al.*, 1995; Nimmo and Bull, 1995 and Onody *et al.*, 1997), has studied the response of legionellae to antibiotic exposure whilst the bacteria are in a nutrient rich medium. That is, the bacteria had the capacity for continuing active replication. No information appears to have been published regarding the response of *L. pneumophila* to antibiotics when in a nutrient free environment. A short investigation was, therefore, undertaken to examine this aspect. The above study of the antimicrobial properties of ciprofloxacin was repeated, but amoebic saline was substituted for the YE broth. The results of this work are presented in Figure 5.5. As anticipated, no growth of the bacteria occurred

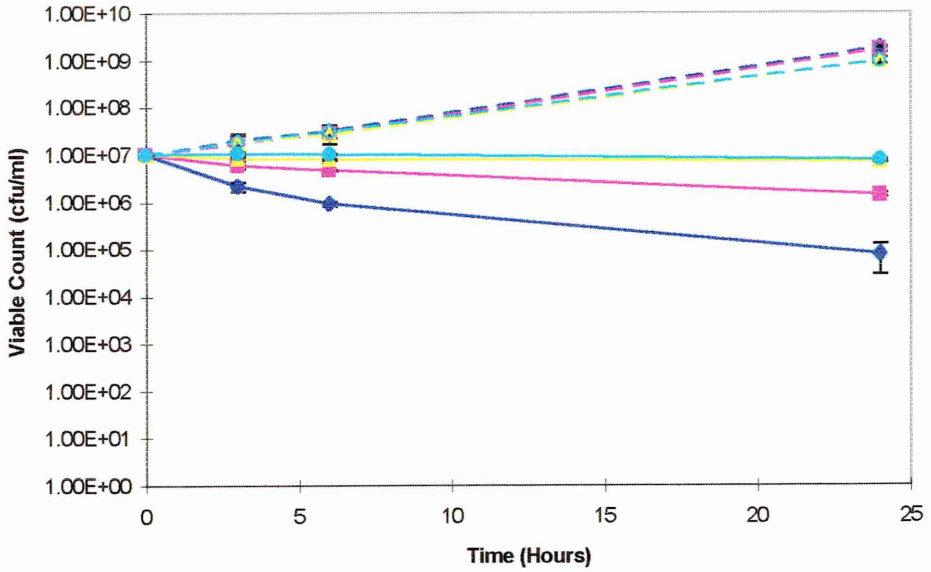


Figure 5.3 Survival of *L. pneumophila* after exposure to erythromycin (8 µg/ml) at 37°C in YE broth. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), or intracellularly in U937 monocytes (▲) or amoebae (●). Results for controls in which antibiotic was not added are shown (◈, □, △, ○), respectively. Bars represent the standard errors of the means for 3 replicate experiments.

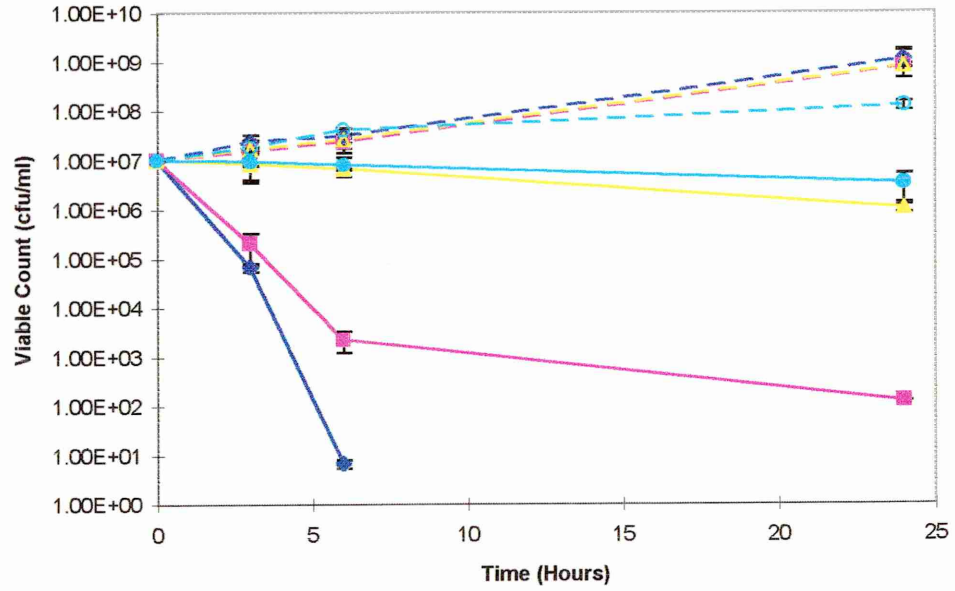


Figure 5.4 Survival of *L. pneumophila* after exposure to rifampicin (5 µg/ml) and erythromycin (8 µg/ml) at 37°C in YE broth. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), or intracellularly in U937 monocytes (▲) or amoebae (●). Results for controls in which antibiotic was not added are shown (◈, □, △, ○), respectively. Bars represent the standard errors of the means for 3 replicate experiments

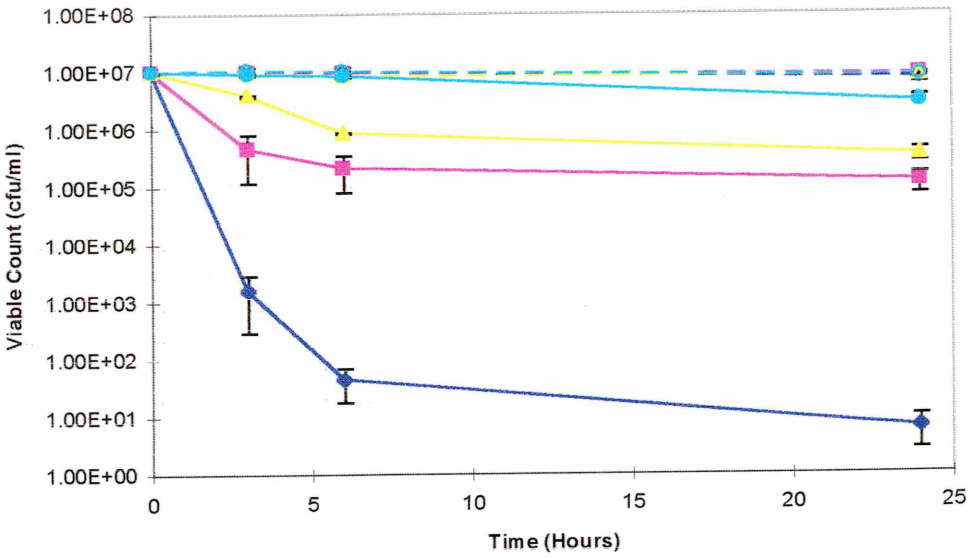


Figure 5.5 Survival of non-growing *L. pneumophila* after exposure to ciprofloxacin (1 μg/ml) at 37°C in amoebic saline. Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), intracellularly in U937 monocytes (▲) or amoebae (●). Results for controls in which antibiotic was not added are shown (◇, □, △, ○), respectively. Bars represent the standard errors of the means for 3 replicate experiments.

and negligble loss in viable count was observed in the control culture over the 24 h. test period. Thus any observed changes in the test culture should be due to the antibiotic alone. Exponential phase broth grown *L. pneumophila* was found to be particularly susceptible to ciprofloxacin. A killing of 99.99 % was observed in 3 h. of exposure. The broth grown bacteria in stationary phase showed increased resistance to ciprofloxacin compared to those in exponential phase. A killing of 99 % was not achieved within the 24 h. test period. As in the previous investigations, the intracellular grown bacteria exhibited a far greater resistance to ciprofloxacin than the broth grown bacteria. After 24 h. exposure to the antibiotic the corresponding values for the intra-amoebic and intra-monocytic grown *Legionella* were 72 % and 95 %, respectively.

Growth and microscopical studies have shown that the morphology of intracellular grown *L. pneumophila* changes to one similar to that of broth grown bacteria in stationary phase if they are left suspended in the coculture medium for more than 24 h. after release from the host cell. This process has been termed 'ageing'. A short investigation was undertaken to determine whether this process influenced the antibiotic resistance of intracellular grown *L. pneumophila*. The response of freshly harvested intra-amoebic grown and intra-monocytic grown *L. pneumophila* to ciprofloxacin (1 μg/ml) was compared to that of the corresponding 'aged' bacteria. The results are presented in Figures 5.6 and 5.7. Figure 5.6 shows that after 'ageing' the resistance of intra-amoebic grown *L. pneumophila* falls to a level similar to that of broth grown bacteria in stationary phase. Approximately 10 % of the freshly harvested intra-amoebic grown legionellae survived after 24 h. exposure to the antibiotic. The corresponding value for the 'aged' bacteria was below 1 %. A similar pattern of response was observed upon 'ageing' intra-monocytic grown *L. pneumophila* (Figure 5.7).

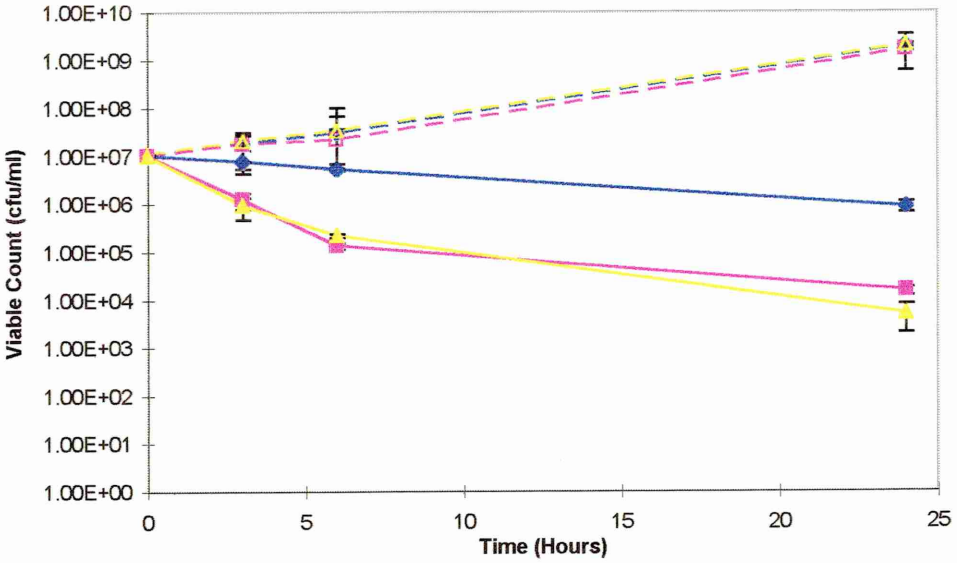


Figure 5.6. Survival of 'aged' intra-amoebic grown *L. pneumophila* after exposure to ciprofloxacin (1 µg/ml) at 37°C in YE broth. The origin of the *L. pneumophila* was freshly harvested intra-amoebic grown, (◆), intra-amoebic grown 'aged' in coculture for 72 h post host cell lysis, (■), and YE broth grown in stationary phase (▲). Results for controls in which antibiotic was not added are shown (◇, □, △), respectively. Bars represent the standard errors of the means for 3 replicate experiments.

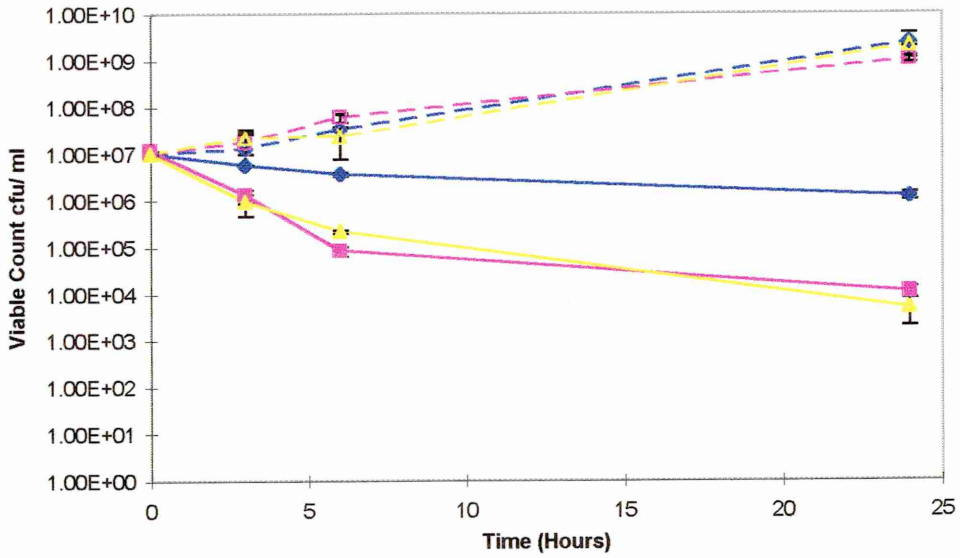


Figure 5.7. Survival of 'aged' intra-monocytic grown *L. pneumophila* after exposure to ciprofloxacin (1 µg/ml) at 37°C in YE broth. The origin of the *L. pneumophila* was freshly harvested intra-monocytic grown, (◆), intra-monocytic grown 'aged'in coculture for 72 h post host cell lysis, (■), and YE broth grown in stationary phase (▲). Results for controls in which antibiotic was not added are shown (◇, □, △), respectively. Bars represent the standard errors of the means for 3 replicate experiments.

5.2 EXPOSURE TO ELEVATED TEMPERATURES.

There are a number of reports of the effect of elevated water temperature on the viability of *L. pneumophila* (Groothuis *et al.*, 1985; Stout *et al.*, 1986; Farrell *et al.*, 1990; Makin and Hart, 1991; Haldane *et al.*, 1993 and Zacheus and Martikainen, 1996). These workers conducted field studies and consequently the data generated refers to *L. pneumophila* with the morphology and phenotypic characteristics associated with the bacteria found within a particular system. As far as it is known there has been no systematic comparative study of the effect of growth conditions on the resistance of *L. pneumophila* to heat published in the literature. A short study was therefore, undertaken to rectify the situation. Time-kill assays were conducted over 10 min. periods at temperatures of 50°C, 55°C, 60°C and 65°C.

The results of this investigation are presented in Figures 5.8 to 5.11. Exposure to temperatures below 55°C had no effect on the viability of any of the *L. pneumophila* examined. At 55°C, the exponential phase broth grown *L. pneumophila* exhibited a 30 % killing within the 10 min. test period (Figure 5.8); the other forms of *L. pneumophila* were unaffected. Exposure to higher temperatures showed the intracellular grown bacteria to possess a far greater resistance to heat stress than broth grown legionellae. At 60°C, the exponential phase broth grown *L. pneumophila* became non-recoverable within 2 min. and the stationary phase bacteria within 4 min. (Figures 5.8 and 5.9). Both forms of intracellular grown *L. pneumophila* remained recoverable after 10 min. exposure at 60°C (Figures 5.10 and 5.11). Approximately 30 % of the intra-amoebic grown bacteria remained viable after this exposure whilst the corresponding value for intra-monocytic grown *L. pneumophila* was 20 %. Raising the temperature to 65°C induced a state of non-recoverability in the broth grown *L. pneumophila* in less than 1 min. At the latter temperatue, the corresponding survival times for intra-amoebic grown and intra-monocytic grown bacteria were *ca.* 2 min. and 1 min, respectively.

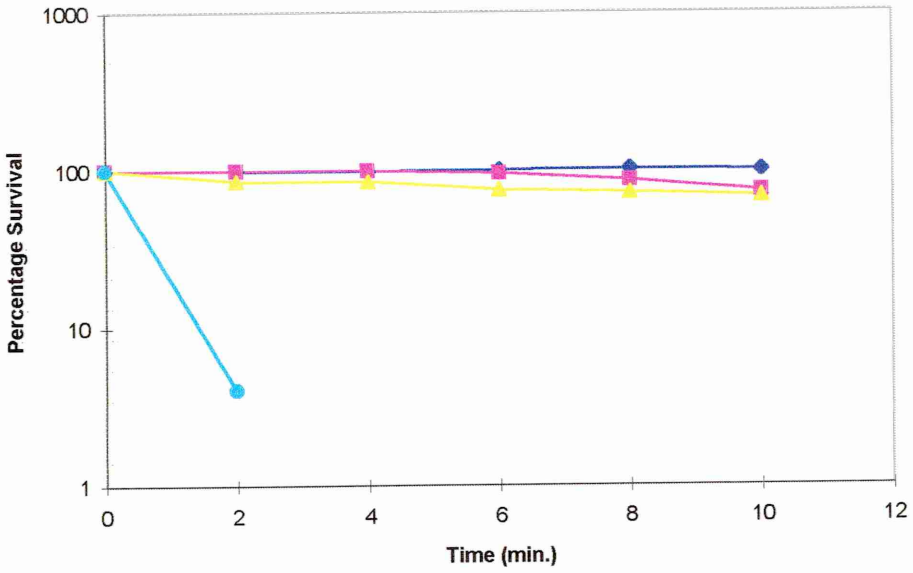


Figure 5.8 Effect of temperature on survival of broth grown *L. pneumophila* in exponential phase. The test suspensions were held in amoebic saline at temperatures of 37°C (◆), 50°C (■), 55°C (▲) and 60°C (●). At 60°C there was no recovery after 2min.

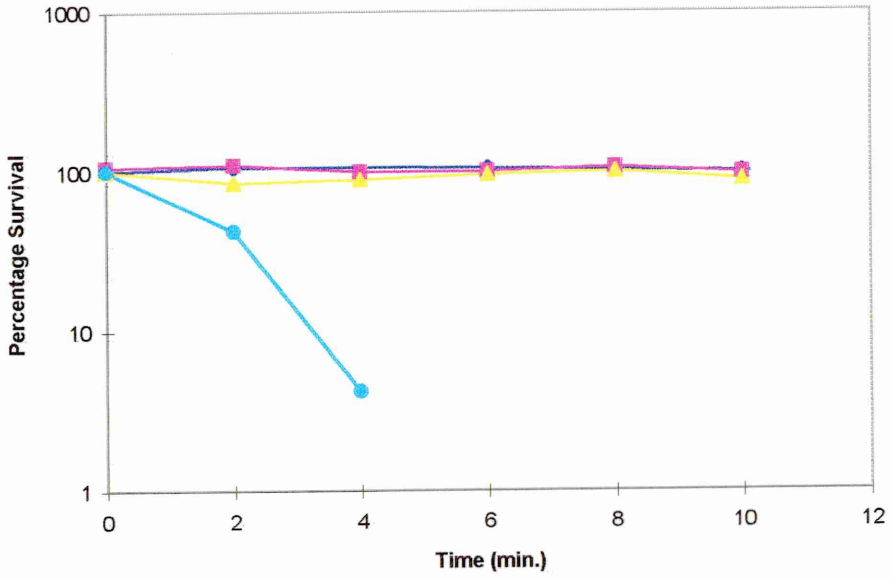


Figure 5.9. Effect of temperature on survival of broth grown *L. pneumophila* in stationary phase. The test suspensions were held in amoebic saline at temperatures of 37°C (◆), 50°C (■), 55°C (▲) and 60°C (●).At 60°C there was no recovery after 4 min.

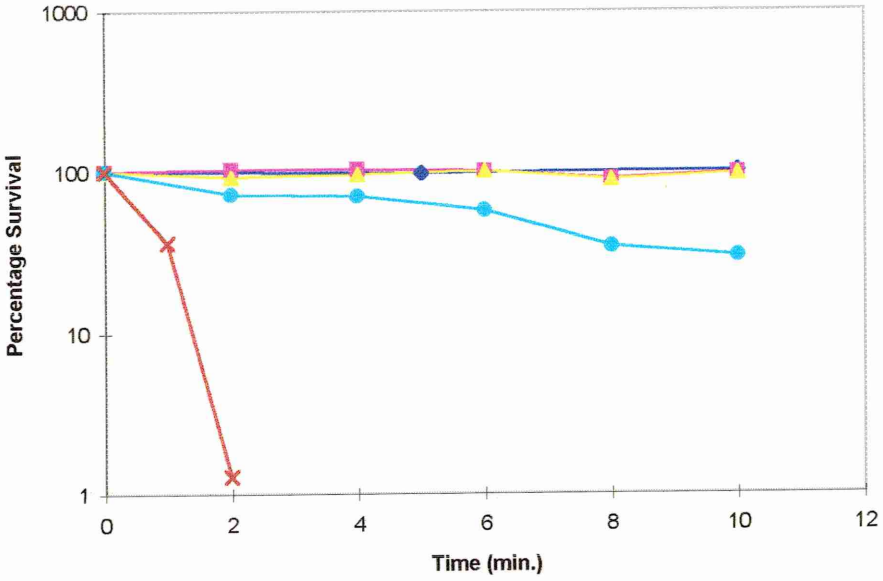


Figure 5.10. Effect of temperature on survival of intra-amoebic grown *L. pneumophila*. The test suspensions were held in amoebic saline at temperatures of 37°C (◆), 50°C (■), 55°C (▲), 60°C (●) and 65°C (✖). At 65°C there was no recovery after 2 min.

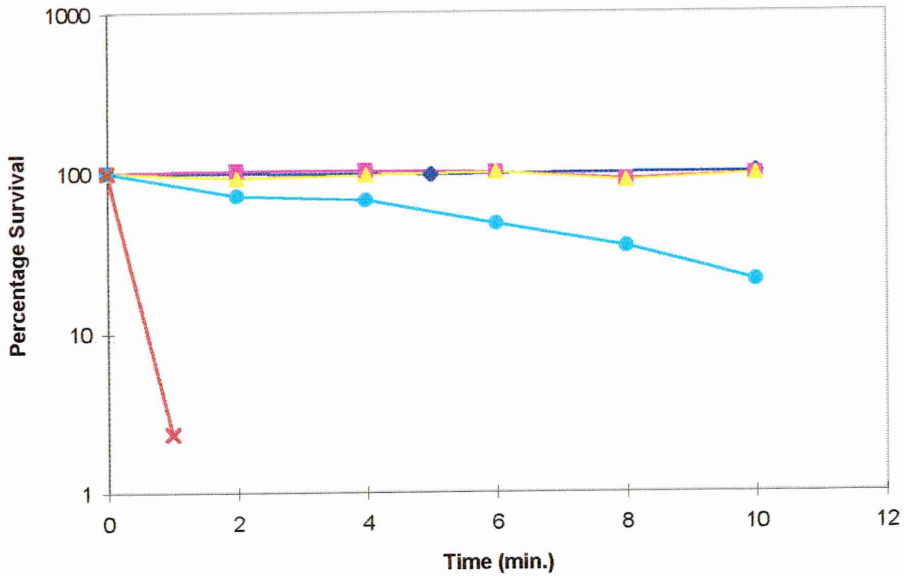


Figure 5.11. Effect of temperature on survival of intra-monocytic grown *L. pneumophila*. The test suspensions were held in amoebic saline at temperatures of 37°C (◆), 50°C (■), 55°C (▲), 60°C (●) and 65°C (✖). At 65°C there was no recovery after 1 min.

The results from the initial study showed that the variously grown *L. pneumophila* survived at least 10 min. exposure to a temperature of 50°C. This temperature corresponds to that employed in many large scale hot water systems, including those found in hospitals, hotels and holiday complexes. It was, therefore, decided to investigate further the survival potential of the variously grown legionellae at 50°C over a 240 min. period. The results are presented in Figure 5.12. The data again shows a marked difference between the heat resistance of intracellular and broth grown *L. pneumophila*. After 240 min. exposure the viability of the broth grown legionellae in exponential phase was reduced by *ca.* 2.4 orders of magnitude (0.4 % survival) and that in stationary phase by *ca.* 1.5 orders of magnitude (3 % survival). The corresponding survival values for the intra-amoebic and intra-monocytic grown bacteria were 50 % and 30 %, respectively. No loss in viability was observed in the control sample.

5.3 EXPOSURE TO LONG-TERM STARVATION.

The occurence of *L. pneumophila* in the natural and man-made aquatic environments has been well documented (Fliermans *et al.*, 1979;1981; Tobin *et al.*, 1981; Dennis *et al.*, 1982; Stout *et al.*, 1982; Wadowsky *et al.*, 1982 and Farrell *et al.*, 1990). The majority of these environments can be expected to be nutrient depleted with respect to bacterial growth. The current work has shown that broth grown *L. pneumophila* has a different phenotype to intracellular grown bacteria and is less resistant to stress. It is possible, therefore, that intracellular growth will also enhance the survival and maintenance of virulence of legionellae under the starvation conditions found in aquatic environments. To investigate these hypotheses, samples of the variously grown *L. pneumophila* were held in microcosms of sterile deionised and sterile tap water for a period of up to 400 d. Total count and bacterial viability were assessed by vital staining / epifluorescence microscopy. The viability of the bacteria was also assessed by viable plate counting on BCYE agar. Once the bacteria were no longer recoverable on agar by normal sampling methods, alternative techniques were used in an endeavour to recover

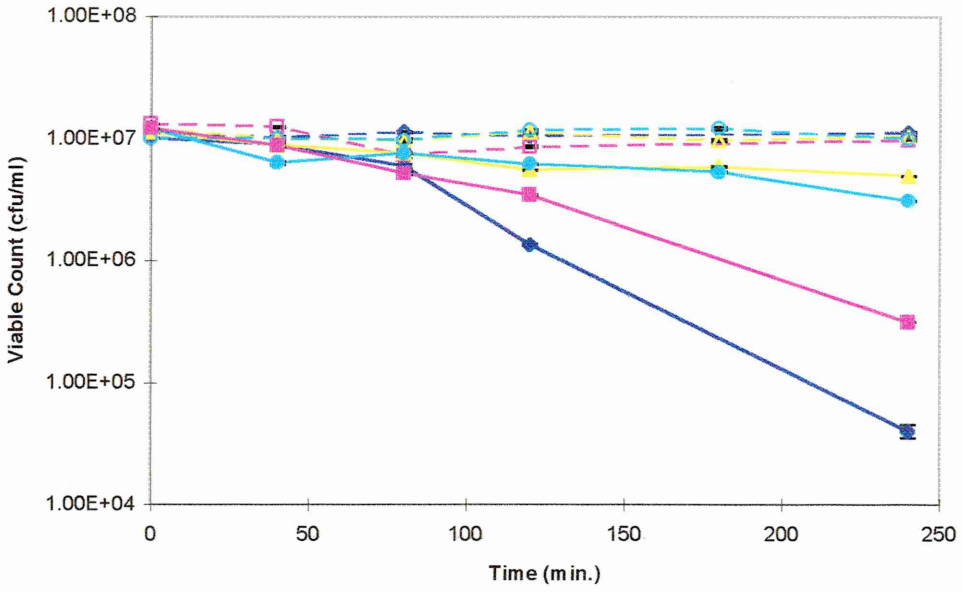


Figure 5.12. Effect of *L. pneumophila* origin on survival after exposure to heat at 50°C in amoebic saline. *L. pneumophila* grown in YE broth to exponential phase (◆), or stationary phase (■), or intracellularly in amoebae (▲) or in U937 monocytes (●). Results for controls which were held at 37°C are also shown (◇, □, △, ○), respectively. Bars represent the standard errors of the means for three replicate samples.

the few remaining culturable bacteria. In addition, infectivity studies were performed by coculturing the starved legionellae with amoebae or monocytes.

5.3.1 RECOVERY ON BCYE AGAR.

The data relating to the survival of the variously grown *L. pneumophila* in the two aquatic environments, as determined by recovery on BCYE agar, are presented in Figures 5.13 and 5.14. These figures clearly show that bacterial origin has a marked effect on survival in both tap water and deionised water. In addition, it is apparent that all the legionellae tested survived longer in tap water than in deionised water. The data shown in Figure 5.13 suggests that broth grown legionellae in exponential phase are the least able to survive starvation in deionised water. A point of non-recoverability (*ca.* 10 cfu/ml) was reached within *ca.* 5 wk. The survival of stationary phase broth grown *L. pneumophila* was somewhat longer, with the bacteria remaining recoverable for a period of *ca.* 8 wk. Intracellular grown *L. pneumophila* survived starvation for much longer periods than the broth grown bacteria. The point of non-recoverability of intra-amoebic grown bacteria was reached after *ca.* 17 wk. and that of intra-monocytic grown bacteria in 19 wk. A similar pattern of survival was observed in the tap water microcosms, but the survival times were longer. The exponential phase broth grown legionellae reached a point of non-recoverability in *ca.* 24 wk. and the stationary phase bacteria in *ca.*32 wk. The intra-amoebic grown bacteria survived *ca.* 50 wk. before becoming non-recoverable. The tap water study also included a sample of 'aged' intra-amoebic grown *Legionella*. It is interesting to note that the survival of these bacteria is worse than that of freshly harvested intra-amoebic grown legionellae but is marginally better than that of the broth grown *L. pneumophila* in stationary phase (*ca.* 35 wk. *cf.* 32 wk.). Attempts at recovery of any viable bacteria remaining in the microcosms by centrifugation or membrane filtration proved unsuccessful.

5.3.2 METAL ION CONTENT.

The concentration of metal ions in the tap water and deionised water before and after the test period are presented in Table 5.1. As anticipated, the tap water contained

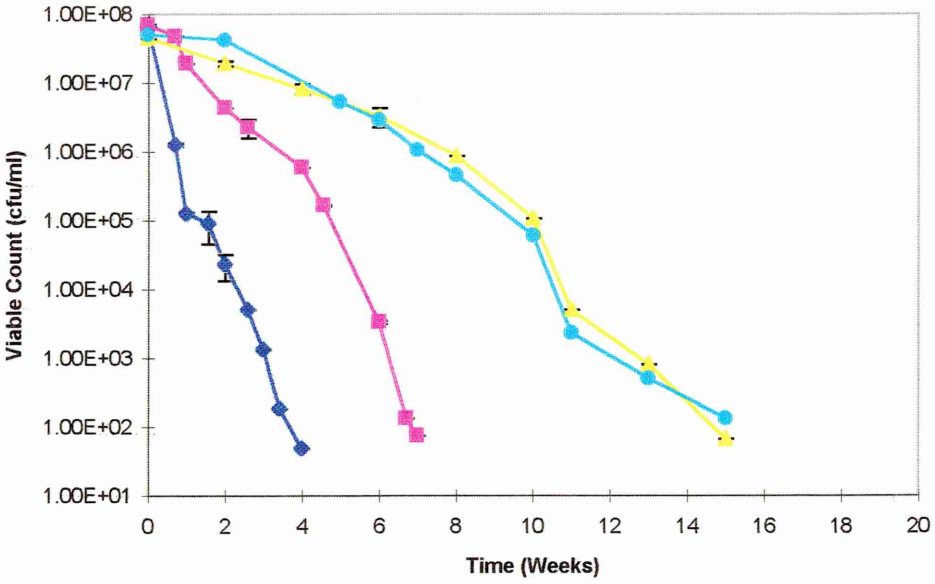


Figure 5.13. Starvation of *L. pneumophila* in deionised water as measured by viable plate count (cfu/ml). Inoculum *L. pneumophila* grown in YE broth to exponential phase (◆), or stationary phase (■), or intracellularly in amoebae (▲) or monocytes (●). Bars represent the standard errors of the means for 3 replicate experiments.

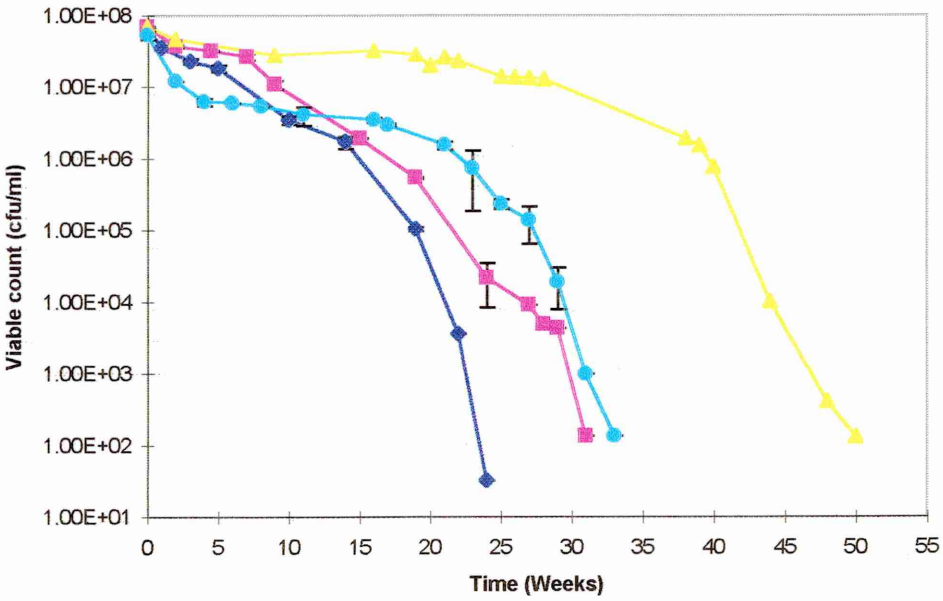


Figure 5.14. Starvation of *L. pneumophila* in tap water as measured by viable plate count (cfu/ml). Inoculum *L. pneumophila* grown in YE broth in exponential phase (◆), stationary phase (■), freshly harvested from amoebae (▲) or after 'ageing' in coculture for 72 h. (●). Bars represent the standard errors of the means for 3 replicate experiments.

Time (yrs)		Na	K	Cu	Ca	Al	Mg	Cr	Fe	Mn	Co	Cd	Pb	Zn
Deionised Water	**0**	< .005	0.0215	<. 0005	< .003	< .008	< .005	< .002	<.0008	<.0038	.0.105	< .004	<.082	0.007
	1	< .005	0.0363	<. 0005	0.149	< .008	0.028	0.074	0.0010	0.004	0.308	0.004	<.082	<.003
Tap Water	**0**	13.00	1.031	0.0243	16.86	< .008	3.58	< .002	< .0008	<.0038	0.135	< .004	<.082	0.0093
	1	11.02	1.177	0.056	18.18	0.029	3.83	0.097	0.0031	0.004	0.419	0.010	<.082	<.003

Table 5.1 Metal ion content (ppm) of deionised and tap water. The waters were examined prior to the addition of bacteria and after one year of incubation by ICP analysis. The bacteria were YE broth grown *L. pneumophila* in stationary phase and were removed by filtration prior to re-analysis.

higher concentrations of Group I and II metal ions than deionised water. Very little difference was noted between the concentration of aluminium and transition metal ions in the two waters. Re-analysis of the waters after the test period showed marked changes in the concentrations of Group I and II metal and aluminium ions. Smaller changes being noted in the concentration of the transition metal ions. The concentration of K^+, Ca^{2+} and Mg^{2+} ions all increased during the test period with the greatest change occurring in tap water. The Na^+ ion concentration in tap water decreased during the test period, but remained unchanged in the deionised water. The pH of the two waters changed little during the test period. That of tap water changed from pH 7.75 to pH 6.93. The corresponding values for deionised water were pH 6.3 and pH 5.97.

5.3.3 INFECTIVITY STUDIES.

Samples of *L. pneumophila* were taken from the microcosms at 1 to 2 wk. intervals and tested for infectivity of *A. polyphaga* and U937 monocytes. The results of the infectivity studies are presented in Figures 5.15 and 5.16 together with data relating to recovery on BCYE agar. The data shows that *L. pneumophila* remains recoverable on BCYE agar and are capable of infecting host cells for longer periods of starvation in tap water than in deionised water. A close correlation between recovery on agar and infectivity of host cells is also apparent. The data suggests, in the first instance, that choice of host may influence recovery. However, the effect is small, and inconsistent, and may arise from the random nature of sampling waters with very low bacterial concentrations and a possible non-uniform distribution of the bacteria within the microcosm. Both sets of data show similar trends in that intracellular grown bacteria remain recoverable and infective for longer periods than bacteria grown in broth. In addition, exponential phase broth grown bacteria remain recoverable and infective for shorter periods than legionellae in the stationary phase of growth. Figure 5.16 also shows that 'ageing' of intra-amoebic grown *L. pneumophila* prior to suspension in tap water modifies its response to starvation. The recovery and infectivity of 'aged' bacteria were observed to be very similar to those of broth grown bacteria in stationary phase.

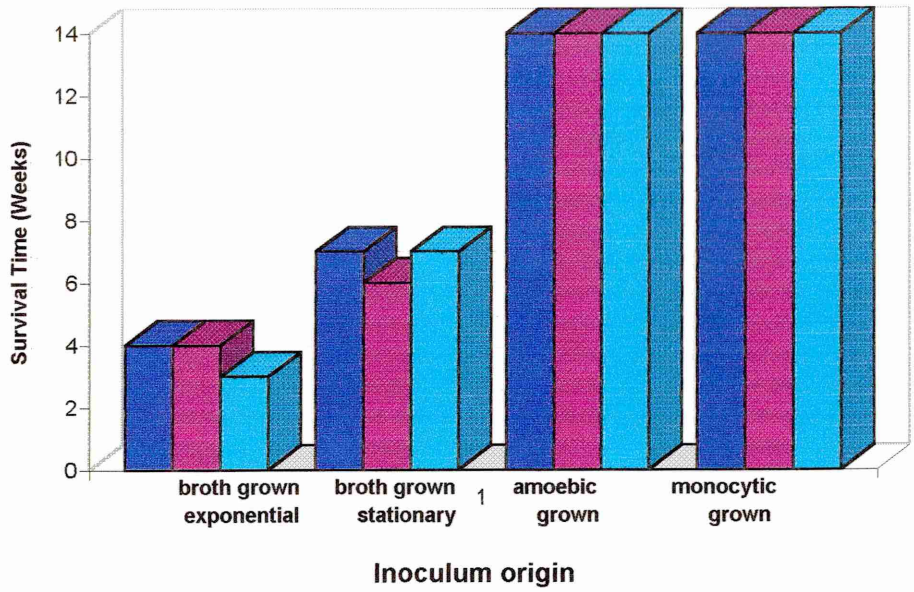


Figure 5.15 Maintenance of virulence of *L. pneumophila* in deionised water. Effect of inoculum *L. pneumophila* origin on the period of infectivity towards *A. polyphaga* trophozoites (■) and U937 monocytes (■), and recovery on BCYE agar (■).

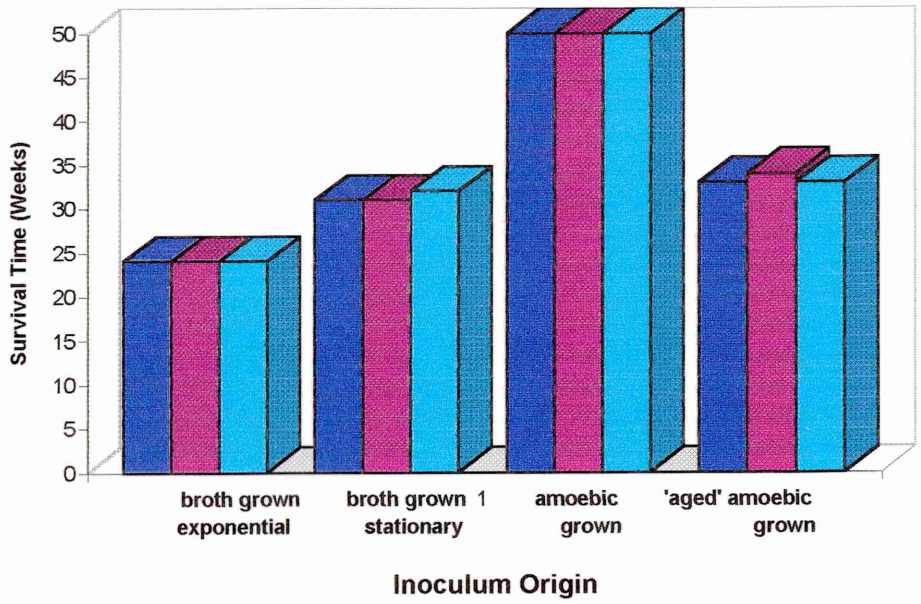


Figure 5.16 Maintenance of virulence of *L. pneumophila* in tap water. Effect of *L. pneumophila* inoculum origin on the period of infectivity towards *A. polyphaga* trophozoites (■) and U937 monocytes (■), and recovery on BCYE agar (■).

5.3.4 RESUSCITATION

A comparison of bacterial total count, recovery on BCYE agar and the number of membrane intact bacteria as determined by vital staining / epifluorescence microscopy, was made for each microcosm. Figure 5.17 presents comparative data typical of that obtained for each microcosm. The full data are presented in Appendix 5. The bacterial total count remained unchanged in all the microcosms throughout the test period, indicating that no replication had taken place. Membrane intact and potentially viable / infective *L. pneumophila* were observed by the vital staining technique for a far greater period of time than the bacteria were culturable on BCYE agar. This would suggest that viable bacteria remain within a microcosm at concentrations far below that required for detection on BCYE agar. A number of techniques for the resuscitation of such bacteria have been proposed (Section 3.2.4). The method of Colbourne and Dennis (1989) (heat shock), Steinert *et al.* (1997) (coculture with amoebae), subculture in YE broth, and prolonged infectivity assays failed to resuscitate non-culturable bacteria from the microcosms.

5.3.5 MORPHOLOGICAL STUDIES.

Microscopical observations show that the broth grown *L. pneumophila* and 'aged' intra-amoebic grown legionellae became smaller and more rounded (*ca.* 1 μm x 1-2 μm) during starvation. After 16-20 wk., approximately 99 % of these bacteria had undergone this change. Examination of the bacteria after the 400 d. test period revealed the broth grown legionellae had decreased in size (*ca.* < 1 μm x <1 μm) to smaller than that of intracellular grown bacteria and became cocci in shape. The intracellular grown bacteria did not undergo the morphological change observed previously upon 'ageing' in coculture. These bacteria remained small and rounded (*ca.* 1 μm x 1 μm).

5.4 EXPOSURE TO MECHANICAL STRESS.

There is some evidence in the literature which suggests that a change of morphology can alter the rigidity and, by implication, the mechanical strength of a bacterium

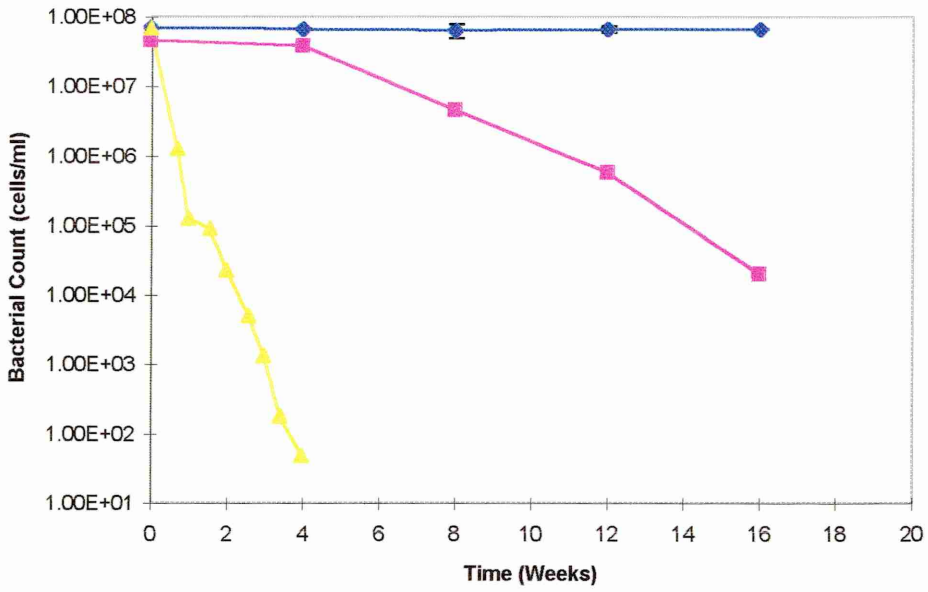


Figure 5.17. Comparison of vital staining and plate count methods to determine bacterial survival. Survival of YE broth grown *L. pneumophila* in exponential phase in deionised water : monitored by total count (◆), and viable count as determined by vital staining (■) and plate count (▲).

(Newhall and Jones, 1983; Hatch *et al.*, 1984 and Lambert, 1988a). In addition, Weichart and Kjelleberg (1996) have demonstrated that starved *Vibrio vulnificus* bacteria possess a greater resistance to damage by ultrasonic waves than the actively growing form. This, they attribute to a change in membrane composition of the bacteria. They also commented on a change in morphology of the bacteria from rod-shaped to coccoidal. Broadly similar morphological changes have been observed in the current work during intracellular replication.

Small samples of variously grown *L. pneumophila,* including 'aged' intra-amoebic grown legionellae, were subjected to mechanical stress by means of ultrasonic vibrations (sonication). The survival of the bacteria was determined by recovery on BCYE agar. The results of this work are presented in Figure 5.18. The data shows that intra-amoebic grown *L. pneumophila* exhibits a significantly greater resistance to mechanical stress than broth grown legionellae. Approximately 270 pulses of ultrasound were required to achieve *ca.* 95 % killing of the intra-amoebic grown *L. pneumophila*, whilst 20 to 40 pulses were necessary to achieve the same level of killing of the broth grown bacteria. Broth grown *L. pneumophila* in exponential phase were observed to be less stress resistant than those in stationary phase (20 *cf* 40 pulses). 'Ageing' of the intra-amoebic grown *L. pneumophila* markedly reduced the ability of the bacteria to withstand mechanical stress. A 95 % killing of 'aged' intra-amoebic grown legionellae was achieved after only 75 pulses of ultrasound.

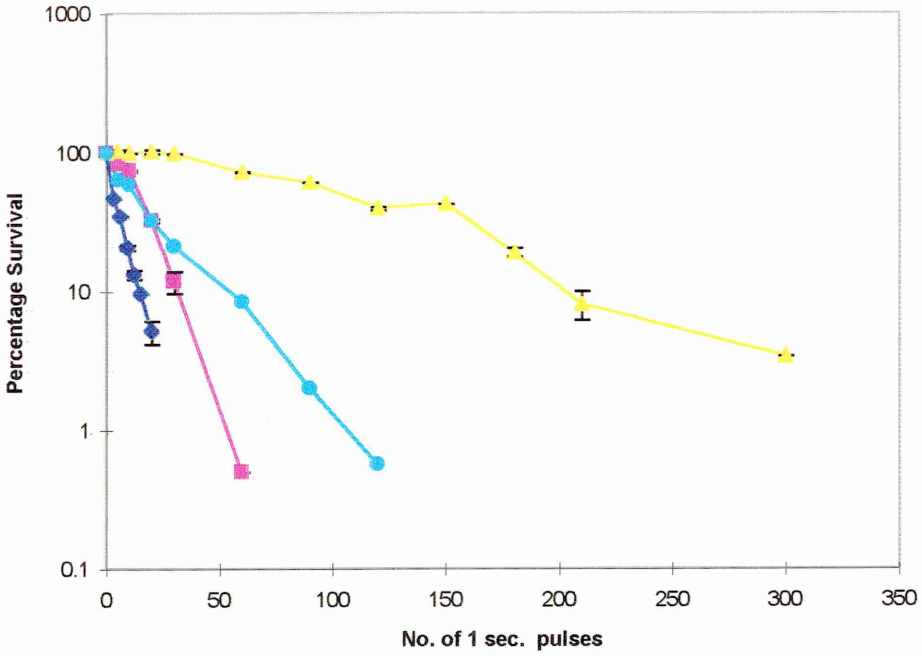


Figure 5.18. Susceptibility of *L. pneumophila* to mechanical stress imposed by ultrasonic vibration (pulses of 1 sec. duration at 25 kHz and 22 μm of amplitude). *L. pneumophila* grown in YE broth to exponential phase (◆), to stationary phase (■), freshly harvested from amoebae (▲), or after 'ageing' in coculture for 72 h. (●). Bars represent the standard errors of the means for 3 replicate experiments.

5.5 DISCUSSION.

5.5.1. EXPOSURE TO ANTIBIOTICS.

Data obtained in the current study has shown that intracellular grown *L. pneumophila* are far more resistant than broth grown legionellae to the antibiotics ciprofloxacin, rifampicin and erythromycin. The pattern of antibiotic resistance shown by the variously grown *L. pneumophila* was identical for each antibiotic tested. In order of increasing resistance this was broth grown legionellae in exponential phase, broth grown legionellae in stationary phase and intracellular grown *L. pneumophila*. This clearly indicates a direct relationship between growth conditions and the sensitivity of *L. pneumophila* to antibiotic exposure. Further evidence for this hypothesis is provided by the observations that 'ageing' of intracellular grown bacteria and the growth phase of broth grown legionellae also exert an effect on the susceptibility to antibiotics. It has been shown that the resistance of intracellular grown *L. pneumophila* to the three antibiotics tested falls to that of broth grown legionellae in stationary phase after 'ageing'. Microscopical studies have shown that the increased sensitivity of the 'aged' intracellular *L. pneumophila* to antibiotics is accompanied by a marked change in morphology. 'Ageing' induces a morphological change from the small, rounded and highly motile form of *L. pneumophila*, characteristic of intracellular growth, to the larger, rod-shaped and non-motile form typical of growth in YE broth. Marked changes in morphology, however, are not a pre-requisite for altered sensitivity of *L. pneumophila* to antibiotics. It has been shown that broth grown legionellae in exponential phase are more readily killed by exposure to antibiotics than those in stationary phase. The morphologies of these two growth phases are very similar. This would suggest that growth phase alone may be sufficient to affect the phenotypic characteristics of broth grown legionellae. This observation is in general agreement with the conclusions drawn by a number of other workers, namely that slow growing bacteria such as those entering or in stationary phase are particularly recalcitrant to antimicrobial agents (Horwitz and Silverstein, 1983; Brown and Williams,1985a;1985b; Williams, 1988 and Brown *et al.*, 1990). It has also been shown that growth rate can alter the composition of the cell envelope of a bacterium (Brown and Melling 1969;

Ellwood and Tempest, 1972; Holme, 1972; Brown, 1975; Lambert, 1988b and Anwar *et al.*, 1990). Anwar *et al.* (1990) and Brown *et al.* (1990) suggested that changes in the bacterial surface composition may cause alterations in the ability of an antimicrobial agent to cross the cell envelope.

Variations in the bacterial cell envelope are thought to alter the action of antibiotics upon the bacteria in a number of ways:

(i) If the primary target for the drug is within the cell envelope itself, a reduction in the relative number of target sites may reduce the overall susceptiblity of the bacteria (Roantree *et al.*, 1977 and Nikaido and Nakae, 1979).

(ii) Alterations to the bacterial surface, particularly those affecting the acidic phospholipid content, LPS or surface charge can affect the initial binding of the antimicrobial agent (Marshak and Tocanne, 1980).

(iii) If an antibiotic is hydrophilic, it must traverse the OM of the bacterium via its porin proteins. Thus a variation in porin protein content would be reflected in the susceptibility of the bacterium,

and conversely,

(iv) if the antimicrobial was hydrophobic, the antibiotic would have to pass through both the hydrophilic and lipophilic compartments of the cell envelope. In this instance, the passage of the drug is influenced, not only by the relative lipophilicity of the antibiotic, itself, but also the lipophilicity of each cell envelope compartment. A variation in the latter would induce a change in the antibiotic susceptibility of the bacterial cells.

Erythromycin alone, or in combination with rifampicin, are the antibiotics recommended for severe cases of Legionnaires' disease (Macfarlane, 1987; Chen *et al.*, 1993 and Edelstein, 1993) and ciprofloxacin has also been shown to be as effective against *L. pneumophila* (Winter *et al.*, 1988). The responses of *L. pneumophila* grown

under various conditions to these three antibiotics acting alone, and a combination of erythromycin and rifampicin were examined during the present investigation.

The data obtained in the current investigation indicates that erythromycin is the least effective against the variously grown *L. pneumophila* examined. This finding is in broad agreement with that of many other workers who have shown that erythromycin only inhibits the growth of intracellular and *in vitro* grown *L. pneumophila* and is not bactericidal (Bartlett *et al.*,1983; Vilde *et al.*, 1986; Liebers *et al.*, 1989; Barker and Farrell, 1990; Rajagoplanan-Levasseur *et al.*, 1990; Kitsukawa *et al.*, 1991; Johnson *et al.*, 1992 and Higa *et al.*, 1993). Fitzgeorge *et al.* (1986) showed that therapy with erythromycin alone was less effective than either rifampicin or ciprofloxacin. The current investigation has also shown that rifampicin is marginally more effective than ciprofloxacin against all the *L. pneumophila* examined, irrespective of growth conditions, and that both are superior to erythromycin. This observation is in agreement with the work of Chen *et al.* (1993), Reda *et al.* (1994) and Nimmo and Bull (1995), who reported rifampicin to be, by far, the most active agent against *in vitro* *L. pneumophila* during time-kill assays and MIC tests.

Rifampicin has been shown in this study and elsewhere (Chen *et al.*, 1993; Reda *et al.*, 1994 and Nimmo and Bull, 1995) to inhibit and kill *L. pneumophila* rapidly during *in vitro* studies. Whilst no *in vivo* bacterial resistance to rifampicin has been reported, it has been shown during *in vitro* studies that a resistance to rifampicin rapidly develops (Moffie and Mouton, 1988 and Barker and Farrell, 1990). Barker and Farrell (1990) reported that rifampicin resistant mutants were found in cultures prior to exposure to the drug and that these bacteria became the predominant population 75 h. after exposure to rifampicin. It was suggested, therefore, that rifampicin alone may not be suitable for treating cases of Legionnaires' disease due to the possibility of the development of *in vivo* resistance to the antibiotic. Moffie and Mouton (1988) argued against single drug therapy with rifampicin on the basis of observed mutation rates as high as 1-4 x 10^{-8} in strains of *L. pneumophila*. In contrast, Onody *et al.* (1997) who

examined 98 clinical isolates of *L. pneumophila*, failed to detect resistance to rifampicin, erythromycin or ciprofloxacin. These authors suggested that therapeutic failures in the patients examined were not related to an increased resistance of *L. pneumophila* to the commonly used antibiotics. Edelstein (1991) found no increase in resistance to rifampicin during treatment of the experimental disease in guinea pigs. Onody *et al.* (1997) argued that as there is no evidence of person to person spread of Legionnaires' disease, therefore, the potential for prior exposure of the bacteria to antibiotics before infection is low.

The current study has shown that ciprofloxacin has bactericidal properties only marginally inferior to those of rifampicin. Meyer (1989) also reported ciprofloxacin to be bactericidal for *Legionella* sp. More importantly, Meyer (1989) also showed that ciprofloxacin only inhibited the intracellular growth of *Legionella* sp. at the concentrations achievable in serum. The enhanced resistance to ciprofloxacin of the intracellular grown *L. pneumophila* observed in the current study and findings of Meyer (1989) suggest that ciprofloxacin is not necessarily bactericidal during the clinical treatment of Legionnaires' disease, only inhibitory. On the other hand, Chen *et al.* (1993) suggested that ciprofloxacin may be an alternative treatment of *Legionella* infections in transplant recipient patients receiving the antibiotic cyclosporin A. Unlike erythromycin and rifampicin, this drug does not alter cyclosporin A levels (Hooper *et al.*, 1988). Unfortunately, as in the case of rifampicin mutational resistance of *L. pneumophila* has also been reported for ciprofloxacin (Havlichek *et al.*, 1987, Moffie and Mouton, 1988 and Barker and Farrell, 1990). However, these mutants have been shown to develop less rapidly.

The studies of Barker and Farrell (1990) revealed that rifampicin resistant mutants were killed more rapidly when a combination of erythromycin and rifampicin were used. The two drugs seem to act in concert. It was suggested that erythromycin inhibits growth of legionellae by preventing bacterial protein synthesis, and hence the rate of development of resistant mutants, whilst the rifampicin kills the bacteria. The results of the current

study confirm that a combination of erythromycin and rifampicin is more effective against all the various growth types of *L. pneumophila* examined. Similar results were reported by Baltch *et al.* (1995). These workers performed time kill assays over a 72 h. period on *in vitro* grown *L. pneumophila* with 0.5 times the MIC of erythromycin and 0.5 times the MIC of rifampicin in combination. It was shown that in combination erythromycin and rifampicin were more effective at killing *L. pneumophila* than the same concentration of either antibiotic acting independently.

The success of the erythromycin and rifampicin combination in *in vitro* studies illustrates the problem of translating laboratory studies into therapy. Horwitz and Silverstein (1983) demonstrated that *L. pneumophila* replicating in phagocytes were resistant to a combination of erythromycin and rifampicin even at concentrations comparable to, or higher than, peak blood levels in humans. In addition they reported that the bacteria retain their capacity to multiply within the phagocyte after the extracellular antibiotics are removed. This suggests that both erythromycin and rifampicin allow bacteria to remain viable whilst within the host cell. The clinical implication of these findings is that early withdrawal of the antibiotic regime would allow re-emergence of the disease.

The MIC values found in the current study for the broth grown *L. pneumophila* in exponential and stationary phase and intracellular grown legionellae were identical with respect of each antibiotic tested. Similarly, the MBC values were also identical for each antibiotic. These observations are possibly due to the method of testing. Intracellular grown legionellae cultured in YE broth for the stipulated 48 h. period of the MIC test would have undergone replication and adopted the phenotype of broth grown legionellae. Consequently, the standard broth grown dilution technique employed to determine the MIC and MBC values is unable to differentiate between the resistance to antibiotics of intracellular and broth grown *L. pneumophila*.

The problems of MIC test conditions influencing the outcome of comparative studies of antibiotic resistance of *L. pneumophila* is clearly illustrated by the contradictory nature of a number of reports in the literature. Vilde *et al.* (1986) reported that the minimum concentration of erythromycin and rifampicin required to inhibit *L. pneumophila* within macrophages was 10-fold higher than the minimum inhibitory concentration (MIC) for broth grown legionellae. In contrast, Higa *et al.* (1998) reported that the minimum inhibiting extracellular concentration (MIEC) of macrolides (erythromycin, clarithromycin and azithromycin), fluoroquinolones (ciprofloxacin, sparfloxacin and levofloxacin) and rifampicin were similar to the respective MIC values for the broth grown *L. pneumophila*. A possible explanation for the observations of Higa *et al.* (1998) can be found in the work of Edelstein and Edelstein (1989). The latter workers reported that legionellae exposed to antibiotics 2 h. post host cell infection were more susceptible than those allowed to infect phagocytes and replicate for 1 d. prior to antibiotic exposure. It may be argued that Higa *et al.* (1998) allowed insufficient time (*ca.* 12 h.) for legionellae to replicate and undergo a phenotypic change prior to antibiotic exposure for a true comparison of intracellular and broth grown bacteria to be made.

The observation that intracellular grown *L. pneumophila* are more resistant to antibiotics than broth grown or 'aged' intracellular grown legionellae has a number of clinical implications. Information about the efficacy of antibiotics is gained primarily from time-kill assays and MIC / MBC tests using *in vitro* grown *L. pneumophila*. In the light of the evidence presented in this thesis, information gained from such studies must be treated with caution. A rank order of antibiotic efficacy may be obtained, but not a true measure of their efficacy against intracellular grown *L. pneumophila*. It has been shown that reliable evidence of antibiotic efficacy can only be obtained by utilising freshly harvested intracellular grown legionellae. Failure to utilise the bacteria immediately upon harvesting can also lead to erroneous results owing to the phenomenon of 'ageing'. The results of MIC / MBC tests conducted in YE broth over the standard 48 h. test period must also be treated with caution owing to the replication

of the intracellular grown *L. pneumophila* and the acquisition of an *in vitro* grown phenotype.

From a therapeutic aspect, the project has suggested that the failure of clinical antibiotic regimes is due only in part to a lack of accumulation of an antibiotic within the phagosome. Based on the evidence presented in this thesis, it may be argued that part of the failure is due to a greater resistance of intracellular grown *L. pneumophila* to antibiotics than suggested by the standard assessment techniques. An added complication is the observation that some antibiotics which appear to be bactericidal during *in vitro* testing are only bacteriostatic with respect to *L. pneumophila* within infected phagocytes (Horwitz and Silverstein, 1983 and Meyer, 1989).

The clinical implication of these findings is that early withdrawal of the antibiotic regime would allow re-emergence of the disease. Complete eradication of the *L. pneumophila* would appear to necessitate the combined action of bacterial growth inhibiting antibiotics and the human phagocytic cell defences. Horwitz and Silverstein (1981) and Horwitz (1982), have both reported that cell-mediated immunity plays a critical role in limiting the multiplication of *L. pneumophila*. The latter requirement is probably the main reason that Legionnaires' disease patients with impaired cell-mediated immunity are often more prone to protracted illnesses and show higher mortality rates, even when treated with antibiotics.

The evidence presented in this thesis and that in the literature suggest that future developments in the antibiotic treatment of Legionnaires' disease must take a multi-pronged approach. The development of a more effective antibiotic *per se*, and, ideally, one which possesses increased penetrative capabilities with respect to the phagocytic cell. In addition, microscopical evidence obtained during this work suggests that legionellae reside within the phagosome of the host cell throughout all, or most, of the period of replication. Ideally, any new drug should be capable of specifically targetting this region of the phagocytic cell. A possible alternative approach could be to

specifically target the *L. pneumophila* bacteria on release from infected phagocytes before they can invade new host cells. The β-lactams and aminoglycosides may be suitable for this purpose as they have been reported to be highly bactericidal towards *L. pneumophila* during *in vitro* testing (Hand, 1984 and Stokes *et al.*, 1989), but are unable to penetrate the phagocytic cell. A combination of such an antibiotic with one displaying good intra-phagocytic bactericidal properties may offer a better prognosis to patients with severe Legionnaires' disease.

5.5.2 EXPOSURE TO HIGH TEMPERATURES.

The occurrence of legionellae in man-made aquatic environments has been shown to be temperature dependent (Plouffe *et al.*, 1983; Vickers *et al.*, 1987; Lee *et al.*, 1988 and Zacheus and Martikainen, 1996). *Legionella* have been isolated from waters with temperatures between 6 and 63°C (Fliermans *et al.*, 1981 and Habich and Muller, 1988). It has been shown that all legionellae die rapidly on exposure to high water temperatures (Dennis *et al.*, 1984; Groothuis *et al.*, 1985 and Stout *et al.*, 1986) but only one study has been undertaken to determine whether growth conditions has an effect on temperature susceptibility (Abu Kwaik *et al.*, 1997). Abu Kwaik *et al.* (1997) compared the survival of exponential phase broth grown *L. pneumophila* exposed to a temperature of 53°C for 1 h. with that of bacteria grown in U937 monocytes and *H. vermiformis* trophozoites. These authors reported a four-fold increase in the resistance of intracellular grown *L. pneumophila* compared to broth grown bacteria. The results of the current study have confirmed these findings and have shown that intra-monocytic grown bacteria are also capable of survival at higher temperatures than broth grown *L. pneumophila*. It has also been shown that intracellular grown *L. pneumophila* are capable of surviving for 1 to 2 min. at 65°C and for more than 10 min. at 60°C. Broth grown bacteria rapidly died at both temperatures. Extended time-kill assays at 50°C confirmed the marked differences between the susceptibilities of intracellular grown *L. pneumophila* and broth grown legionellae to heat exposure. An effect of growth phase on the susceptibility of broth grown legionellae to heat was also observed. The number of viable bacteria in stationary phase was approximately

seven-fold greater than that in exponential phase after 240 min. exposure at 50°C. The results of the time-kill assays and information in the literature clearly indicates a relationship between phenotype and the ability of *L. pneumophila* to survive for extended periods at water temperatures of 50°C or less, and for a few minutes to higher temperatures. Dennis *et al.* (1984) and Stout *et al.* (1986) also performed time kill assays on *in vitro* grown *Legionella* strains. Both sets of authors have described their results in terms of decimal reduction times, that is a time for a 90 % killing at a particular temperature. Unfortunately, marked differences in the initial starting concentrations of *L. pneumophila* and the temperature range employed by the different workers, and in the current work, prevents a direct comparison of the results. Stout *et al.* (1986) used an initial bacterial concentration of *ca.* 10^8 cfu/ml, Dennis *et al.* (1984) *ca.* 10^6 cfu/ml and the current work 2000 bacteria/ml. Dennis *et al.* (1984) reported that bacterial survival times ranged from an infinite period to 6 min as the water temperature was raised from 46 to 58°C. Stout *et al.* (1986) examined higher temperatures in the range of 60 to 80°C and reported survival times of *ca.* 3.6 to 0.8 min. respectively. The work of Stout *et al.* (1986) is, however, considered to be of little practical importance as the workers used unrealistically high concentrations of bacteria and the survival time at 80°C was obtained by extrapolation of the results.

The observation that intra-amoebic grown *L. pneumophila* are capable of surviving higher temperatures than the broth grown bacteria frequently used for laboratory studies is of practical importance in the control of legionellae and their eradication from water systems. The HSE Code of Practice recommends that large scale warm water systems should store water at 60°C or more, returning water should not fall below 50°C, and that the temperature of water leaving a tap should reach 50°C within 1 min. of opening. Under ideal circumstances this practice should maintain a system free of *Legionella*. Adoption of this regime in total is not, however, always practicable. Water temperatures fall along extended pipe runs or in sections where water is drawn off infrequently. All the systems cannot reach, or maintain, the desired temperatures, or temperatures may be deliberately lowered for economic or end-user safety purposes. A

survey by Mauchline *et al.* (1994) found that many parts of large water systems are, in reality, maintained at temperatures of 35-40°C.

Unfortunately, there is widespread evidence that adoption of 'good practice' is no guarantee against the appearance of *Legionella* sp. in a warm water system. Legionellae and amoebae are found in natural waters, and hence both may enter a warm water system via the cold water feed. Thus a potential Legionnaires' disease problem exists from the moment a system is filled. It may be argued that raising the temperature of the water in the calorifier (boiler) to 60°C, or more, should kill both legionellae and amoebae. Evidence has been gained, however, which suggests that legionellae, and possibly amoebae (Biddick *et al.*, 1984), can survive at temperatures up to 65°C. This may be sufficient to enable both the legionellae and the amoebae to survive the heating process, and to move through the calorifier into the storage and supply circuits. In addition, it is well known that stratification occurs within infrequently used hot water storage tanks, with the hotter water rising to the top. The lower temperatures at the bottom of such storage tanks could offer suitable conditions for intra-amoebic replication of *L. pneumophila* and its survival before it enters the supply circuit. The potential problems of the design and use of the supply circuit have been discussed previously (Chapter 1). It may be suggested, therefore, that the ability of *L. pneumophila* to survive for short periods at relatively high temperatures makes it extremely difficult to ensure that a particular warm water supply does not become a source of Legionnaires' disease. A possible approach to this complex problem could be to take steps to ensure that the cold water feed to the system is itself free of legionellae and amoebae. The end use of the water would, probably, prevent the use of chemical-based biocides but such achievment may be possible with the use of UV radiation.

5.5.3 SURVIVAL UNDER STARVATION CONDITIONS.

In the aquatic environment, microorganisms including *L. pneumophila* are subjected to a dynamic flux of nutrient availability, giving rise to a life cycle of unbalanced growth. This is characterised by periods of growth at various rates interspersed with intervals of

non-growth, starvation, recovery and re-growth. The ability of the bacteria to survive a period of starvation is often dependent upon adaptations made during an earlier part of the life cycle (Dawes, 1984). The present investigation has shown that intracellular grown legionellae survive and remain virulent for longer periods of starvation than either broth grown or 'aged' intracellular grown legionellae in both tap and deionised water. Intracellular replication has also been shown to induce modification of the cell envelope composition, (Barker *et al.*, 1993) the switching on / off of various proteins, (Abu Kwaik *et al.*, 1993) and an increased production of poly-β-hydroxybutyric acid, (PHB) (Rowbotham, 1986) which is subsequently used as a food reserve during starvation (Poindexter, 1981 and Kapreylants *et al.*, 1993).

Comparison of survival times on the basis of water type shows that all *L. pneumophila* survive longer in tap water than in deionised water. An ICP analysis has shown that the only major difference between the concentration of metal ions in tap water and deionised water lies in the ions of Group I (Na^+, K^+) and Group II (Ca^{2+}, Mg^{2+}) metals. Analysis before and after the starvation test period showed an increase in the concentration of Ca^{2+} and Mg^{2+} ions in both waters. In the absence of evidence to the contrary, it is suggested that this rise is due to the release of these ions by the starved bacteria. Similarly, the decrease in the Na^+ ions in the tap water could be due to absorption by the *L. pneumophila* bacteria. It is not possible to comment on changes in the Na^+ ion content of the deionised water as the concentrations are below the limit of accuracy of the measuring equipment. Studies have shown the structural layers of Gram negative bacteria such as the *E. coli* envelope have the capacity to bind metals from solutions (Beveridge and Koval, 1981 and Hoyle and Beveridge, 1983; 1984). It has been suggested that divalent metal ions play an important part in maintaining the integrity of the OM, probably by binding LPS molecules together on the outer surface (Lambert, 1984). Ferris and Beveridge (1986), have shown that the metal content of the outer membrane consists of 16 % calcium and 6 % magnesium ions. Both of these metallic ions are important for stabilising the organic charge density within membranes, particularly the phosphoryl groups of the LPS (Ferris and Beveridge, 1984), and have

been described as being an 'inorganic cement' in regard to the membrane structure. These findings are at odds with the observations made in the current study, namely that upon starvation bacteria appear to release Ca^{2+} and Mg^{2+} ions and absorb Na^{+} ions. In this respect it is interesting to note the work of Heller *et al.* (1998), who reported that low concentrations of Na^{+} ions enhance the survival of *L. pneumophila* under starvation conditions. This observation could also explain the greater period of survival of *L. pneumophila* in tap water relative to deionsed water. The Na^{+} ion concentration is markedly higher in tap water than in deionised water. In addition, Beveridge (1988) reported that the loss of Ca^{2+} and Mg^{2+} ions from the outer membrane of bacteria induces them to develop a more curved surface.

Microscopical observations made during the current study have indicated that intracellular grown *L. pneumophila* retain their small, rounded form (*ca.* 1 μm x 1 μm) throughout the period of starvation. In contrast, broth grown legionellae, which are normally large and rod-shaped (*ca.* 1-2 μm x 2-3 μm), were observed to become smaller and more rounded (*ca.* < 1 μm x < 1 μm) upon starvation, acquiring a morphology similar to that of intracellular grown *L. pneumophila.* The mechanism by which this occurs could be that suggested by Beveridge (1988). The formation of small ultramicrobacteria has been reported as being a response of many bacteria to starvation conditions (Torella and Morita, 1981; Roszak and Colwell, 1987 and Barer, 1993), in particular to carbon depletion (Kjelleberg *et al.*, 1993). Torella and Morita (1981) defined ultramicrobacteria as being cells which were undergoing a period of slow or no-growth, whose diameter was less than 0.3 μm and which did not significantly increase in size when inoculated onto a nutrient rich agar medium. The presence or absence of ultramicrobacteria in the microcosms of the present study could not be determined as their size is beyond the resolution of light microscopy. Electron microscopy is necessary for the study of ultramicrobacteria (Roszak and Colwell, 1987).

No variation in the apparent bacterial total count was observed in any microcosm investigated in the current study. The lack of measurable bacterial growth or lysis suggests that cryptic growth has not occurred. The latter phenomenon is that in which the surviving members of a starving population multiply by utilising the nutrients released by lysis of dead bacteria. Comparison of the number of live bacteria, as determined by vital staining, with that measured on BCYE agar, suggested that the latter technique persistently under estimates the true number of viable legionellae within a sample. Possible explanations for this observation have been offered by several groups of workers. West *et al.* (1993) suggested that 'starved' bacteria may die of nutrient shock when plated onto nutrient rich agar. A similar explanation was suggested by Sahney *et al.* (1993), Salyers and Whitt (1994) and St. John and Steinman (1996) who proposed that bacteria are killed by a respiratory burst caused by a rapid increase in nutrient availability. More recently, Bloomfield *et al.* (1998) proposed that the failure of starved bacteria to survive on transfer into a nutrient rich environment was due to the accumulation of toxic superoxides and free radicals.

The lack of correlation between different viable count techniques has also been reported by Hussong *et al.* (1987) and Pasko-Kolva *et al.* (1992) who showed that *Legionella* viability as measured by plate count declined to a greater extent than bacterial lysis as assessed by [^{3}H] thymidine labeling, direct fluorescence microscopy or direct total count. Colwell (1993) observed a similar phenomenon in *Vibrio* sp., *E. coli* and other organisms. This author also suggested that some Gram negative bacteria do not form morphologically identifiable spores or cysts but nevertheless are able to differentiate. Colwell termed the bacteria "viable but non-culturable bacteria" (VBNC). As vital staining in the current study indicated the presence of viable legionellae after recovery on agar was no longer possible, it could be suggested that the legionellae have entered the VBNC state.

The present study has shown that all starved *L. pneumophila* were infective for both amoebae and monocytes whilst recoverable on agar. Once the legionellae were no

longer recoverable on agar, the bacteria were incapable of infecting further host cells. In contrast, Steinert *et al.* (1997) suggested that VBNC *L. pneumophila* can be resuscitated by a host cell infection technique very similar to that used in the current study. Small, but important, differences in experimental detail may offer explanations for the apparent discrepancies between the different sets of results. The work of Steinert *et al.* (1997) involved the starvation of bacteria at 20°C prior to resuscitation in amoebae at 37°C. Bacteria used in the current work were maintained at 37°C throughout the starvation period and during the resuscitation attempt. It could be argued that the rise in temperature from 20°C to 37°C, which did not occur in the current study, triggered the resuscitation of the bacteria by the process of heat shock. Studies discussed in Chapter 4 showed that legionellae which were initially too few to culture on agar were capable of replication in host cells to 10^7-10^8 cfu/ml. These results are in contrast to those found when examining starved legionellae. However, an explanation for this discrepancy can be offered. The intracellular growth studies described in Chapter 4 utilised freshly grown *Legionella* inocula which were diluted in such a manner to ensure uniformity of bacterial distribution. Thus, it can be anticipated that each sample would contain sufficient bacteria to ensure infection of a host cell and initiation of the replication process. In the starvation studies, the microcosms were sampled by removing 500 µl volumes of water from a total volume of *ca.* 200 ml. At very low viable bacterial concentrations the chance of such a small sample containing sufficient viable *L. pneumophila* for intracellular replication becomes random. A further difference could lie in the nature of the bacterium. The growth studies used freshly harvested intracellular grown bacteria. The starvation studies, on the other hand, attempted to recover bacteria whose membrane structure had probably been modified by long-term nutrient depletion.

Colbourne and Dennis (1989) reported the resuscitation of VBNC *in vitro* grown *L. pneumophila* by means of heat shock. These workers applied a temperature increase from 37°C to 42°C of the bacteria for 10 min. prior to attempting recovery on agar. Attempts to resuscitate non-culturable legionellae from the microcosms in the present

invesigation by the method of these workers also failed. These findings are in agreement with those of West *et al.* (1993) who described the failure of heat shock to revive non-culturable *in vitro* grown *Legionella.*

Subculture of the starved non-culturable *L. pneumophila* into YE broth also failed to stimulate bacterial growth. It has been suggested that this is due to cell death arising from nutrient shock (West *et al.*, 1993) or a respiratory burst (Sahney et al, 1993; Salyers and Whitt, 1994 and St. John and Steinman, 1996) brought about by the transfer of a bacteria conditioned to a nutrient-free environment into one which is nutrient rich. The latter explanation has been elaborated by Bloomfield *et al.* (1998) who suggested that transfer of starved bacteria to a nutrient rich medium poses a severe challenge to the adaptive capacity of the organisms. Survival in such a medium involves an adaptation involving protein expression and which is time dependent. The authors proposed that the transfer of bacteria to a nutrient rich environment at temperatures optimal for enzyme activity initiates an imbalance in metabolism, producing a near instantaneous production of superoxides and free radicals. In the absence of phenotypic adaptation the bacteria are unable to detoxify the superoxide, and, therefore, a proportion or all of the bacteria die. Torrella and Morita (1981) also attributed their failure to revive starved bacteria to the use of inappropriately rich media. Tabor *et al.* (1981) reported that the use of minimal nutrient concentrations and prolonged incubation periods are necessary for the successful revival of starved bacteria. Resuscitation of non-culturable *Vibrio* sp. by the use of dilute nutrient broth has been demonstrated by MacDonnell and Hood (1982). However, these workers also reported that the concentration range of nutrients which successfully resuscitated the bacteria was very narrow. It may be possible, therefore, to resuscitate non-culturable *L. pneumophila* if a suitable nutrient composition and concentration range could be identified. Heinmets *et al.* (1953), on the other hand, observed that *E. coli,* considered to be non-viable because of a failure to grow in complete medium, would nevertheless, grow in the same medium after a short inoculation period in the presence of suitable metabolites, such as pyruvate, acetate and oxaloacetic acid. Sources of these

metabolites are incorporated into most modern culture media. It is unlikely, therefore, that this is a problem in the current study.

The concept that bacteria could be viable but non-culturable (VBNC) has been debated for more than 15 years (Kogure *et al.*, 1979; Xu *et al.*, 1982; Colwell *et al.*, 1985; Hussong *et al.*, 1987; Barer *et al.*, 1993; Kjelleberg *et al.*, 1993; Barer, 1997 and Steinert *et al.*, 1997). The phenomenon has been difficult to accept by many microbiologists as the established view is that the capacity of a bacterium to replicate to detectable levels (a colony or broth turbidity) reflects its viability. The idea that bacterium can remain viable but fail to grow on conventional media strikes at the core of the maxim: viability = culturability, and has given rise to the oxymoron 'viable but non-culturable' (VBNC). The main scientific argument for the existence of bacterium in a VBNC state is that it is a genetically determined differentiation process, similar to sporulation, that ensures continued survival in harsh environments until the re-establishment of conditions suitable for growth. As yet, there is a lack of specific evidence to support this theory. The stimuli required to initiate the recovery process and allow replication are unknown. An alternative perspective of this phenomenon is that bacteria in the VBNC state are 'injured' in that they are unable to use certain nutrients. Injured cells may be recognised by failure to grow under selective isolation conditions and that recovery and repair leads to the restoration of growth. The recovery medium through which the bacteria return to culturability is thus critical and highly dependent on the nature of the injury.

Evidence in favour of the existence of bacteria in a VBNC state falls into two categories :

(i) Enumeration of viable bacteria by vital staining and epifluorescence microscopy always produces a result several orders of magnitude greater than that obtained by conventional culture techniques.

(ii) Microbiologists have been unable to isolate causal agents by conventional means when there is clinical evidence of the presence of infectious diseases within the immediate environment.

The latter evidence presents a serious challenge to public health microbiologists. The discrepancy could be attributed to a failure to isolate and identify VBNC bacteria. These concerns have been highlighted in a recent editorial by Barer (1997) who commented on the isolation of VBNC forms of *Vibrio cholerae* and *Campylobacter jejuni* from the natural environment in areas where the diseases are endemic. Conventional microbiological techniques were reported as failing to isolate the bacteria from the local environment.

The ability of *L. pneumophila* to survive and remain virulent towards amoebae and monocytic cells under starvation conditions in tap water has important implications in the management of large warm water systems. A recent survey by Mauchline *et al.* (1994) showed that many large warm water systems are not maintained above the recommended minimum temperature of 50°C; but, are in fact between 35- 40°C, the optimal temperature range for *Legionella* replication. It has been shown in this study that intra-amoebic grown *L. pneumophila* can survive starvation in tap water at 37°C and retain the ability to infect amoebic and monocytic host cells for periods of up to 50 wk.

Biocides are frequently used as a chemical means to eradicate *L. pneumophila* from contaminated systems. It has been shown that intra-amoebic grown *L. pneumophila* are more resistant to antimicrobial agents than originally thought (Navratil *et al.*, 1990 and Barker *et al.*, 1992). It has also been shown that *L. pneumophila* can survive a biocidal treatment by residence inside an amoebic cyst or by association with a biofilm (Kilvington and Price, 1990 and States *et al.*, 1993). Recent evidence suggested that *L. pneumophila* may survive for at least 24 h in biocidal solutions inside a vesicle ejected by an amoebae prior to encystment (Berk *et al.*, 1998). Any *Legionella* bacteria

which survive the decontamination process would be expected to repopulate the water system should the conditions be favourable. For replication of *L. pneumophila* to re-occur in decontaminated warm water systems, viable biofilms or amoebic host cells, or both, must be present. In the absence of these, replication of *L. pneumophila* is not possible, but evidence has been gained which suggests that such *Legionella* can survive starvation in warm tap water for periods of up to 50 wk. and remain virulent during this period. Growth can be expected to resume in the presence of newly formed biofilms or suitable host cells, be they amoebae introduced by fresh cold feed water, or human monocytic cells, after inhalation via a contaminated aerosol.

5.5.4 EXPOSURE TO MECHANICAL STRESS

It has been demonstrated in the current study that intra-amoebic grown *L. pneumophila* are profoundly more resistant to ultrasonic vibration than broth grown *L. pneumophila*. These observations suggest a link between phenotype and resistance to mechanical stress. This hypothesis is reinforced by the observation that the resistance of intracellular grown *L. pneumophila* falls to that of broth grown *L. pneumophila* in stationary phase upon 'ageing'. It has been shown that 'ageing' induces a change in morphology and phenotype from that of intracellular grown to one similar to that of broth grown legionellae.

Microscopical studies have shown that growth conditions markedly effect the morphology of *L. pneumophila*. Intracellular growth induces a small rounded form of legionellae, whilst growth in broth and 'ageing' of intracellular grown *L. pneumophila* results in a larger rod-shaped bacteria. It could be argued that the morphology of the bacteria alone is determining its resistance to mechanical stress. It is well known that long rod-shaped structures are far less resistant than spherical structures to shear stresses, such as those imposed on the bacteria by ultrasonic vibration (Selchon *et al.*, 1984). It is highly unlikely however, that morphology alone is the cause of the current observations as it cannot account for the effect of growth phase on the resistance to stress of broth grown *L. pneumophila*.

Changes to the cell envelope composistion are thought to be responsible for alterations in the overall stability of Gram negative bacteria (van der Linden, 1992). These changes in composition are reported as being dependent on growth conditions (Buchanen and Sowell, 1982; Leduc *et al.*, 1989; Brown *et al.*, 1990; Barker *et al.*, 1993 and Weichart and Kjelleberg, 1996). The latter workers demonstrated that starved *V. vulnificus* possess a greater resistance to damage by ultrasonic waves than those that were actively growing. They noted a difference in the morphologies of the two forms of the bacteria, but suggested that the enhanced resistance of the starved *V. vulnificus* may be related to changes in the fatty acid composition of the cell envelope. Barker *et al.* (1993) reported that intra-amoebic replication of *L. pneumophila* induces a modification of the OMP profile and the LPS, in addition to alteration of the fatty acid composition of the cell envelope. These changes could account for the enhanced resistance to mechanical stress of intra-amoebic grown *L. pneumophila* compared to that of broth grown legionellae. The mechanism by which these compositional changes induce enhanced mechanical strength into the cell envelope of intra-amoebic grown is, however, unclear.

The literature contains several reports which suggest that a change of morphology alters the extent of cross-linking within the cell envelope of Gram negative bacteria (Amano and Williams, 1983; Newhall and Jones, 1983 and Hatch *et al.*, 1984). It has been suggested that the strength of the cell envelope of Gram negative bacteria may be dependent on the form and quantity of cross-linking between the outer membrane and the peptidoglycan layer (Amano and Williams, 1983). These authors demonstrated that the peptidoglycan protein composition of *L. pneumophila* differs from that of other Gram negative bacteria in that (i) the peptide sub-units of the peptidoglycan layer are 80-90 % cross-linked through meso-diaminopimelic acid and alanine, and (ii) the peptidoglycan associated proteins are resistant to proteolysis. The general conclusion from this work was that the cell envelope of *L. pneumophila* is markedly more robust than that of other Gram negative bacteria. Kondo *et al.* (1994) have shown that starved *V. cholerae* display a thicker and more electron dense peptidoglycan layer than actively

growing bacteria. This would imply that the availability of growth nutrients determines the thickness and properties of the peptidoglycan layer. This in turn could influence the structural stability of the cell envelope and thereby its resistance to mechanical stress. In the current study nutrient availability is determined by growth conditions. Intra-amoebic growth is considered to represent the most nutrient depleted environment used in this study. Culture in broth during the exponential phase of growth is thought to be the richest. It is tentatively suggested that the order of resistance to mechanical stress found in this study is largely a reflection of the influence of growth conditions on the thickness of the peptidoglycan layer and the degree of cross-linking with the outer membrane. The morphological shape of the *L. pneumophila* may influence structural stability to some extent, but its effect is considered to be far outweighed by that of cell envelope modification.

It has been established in this thesis that intracellular replication enhances the robustness of the cell envelope of *L. pneumophila*. Reports in the literature suggest that this may be due to a thickening of the peptidoglycan layer and an increase in the amount of cross-linking with the outer membrane. This increase in robustness could have practical implications. For example, an increased toughness of the cell envelope may enable intracellular grown legionellae to withstand more readily any internal mechanical stresses which may develop within the membrane when the bacteria are exposed to raised temperatures. Thickening of the peptidoglycan layer and an increase in the density of cross-linking between it and the outer membrane could also have the effect of impairing the ability of antibiotics to cross the cell wall. Finally, such structural modifications could be reflected in changes in the outer membrane itself, thus altering the availability and / or nature of antibiotic target sites.

5.6 CONCLUSIONS.

The influence of phenotype on the resistance of *L. pneumophila* to various stress conditions has been determined. The stimuli used were exposure to antibiotics, heat, long-term starvation and mechanical stress.

The following conclusions can be drawn from observations made during the above work:

- Intracellular grown *L. pneumophila* are more resistant than broth grown legionellae to all the stress stimuli examined.

- *In vitro* grown *L. pneumophila* in the stationary phase of growth are more resistant to stress than those in the exponential phase.

- A change of phenotype induced by 'ageing' intracellular grown bacteria induces a loss in resistance to stress.

CHAPTER 6 *L. PNEUMOPHILA* SURFACE PROPERTIES

6.1 INTRODUCTION.

A successful pathogen must be capable of (a) entering a host cell, (b) replicating within the host, (c) resisting host cell defences and (d) damaging the host. In most instances the ability of a bacterium to meet these requirements is influenced by the nature of its outer surface. There is evidence to suggest that the composition and related biological properties of the bacterial surface are largely determined by the growth environment, especially specific nutrient deprivation, (Ellwood and Tempest, 1972; Holme, 1972; Brown and Williams, 1985b and Dalhoff, 1985), the rate of growth (Gilbert and Brown, 1978; 1980 and Dalhoff, 1985) and whether the bacteria are replicating in artificial media or within host cells (Barker *et al.*, 1993). Entry of bacteria into a host cell leads to a rapid environmental change for the organism, including nutrient availability. Adaptation to the intracellular environment has been shown to require coordinated gene expression (Susa *et al.*, 1996). Barker *et al.* (1993) reported that surface properties of intra-amoebic grown *L. pneumophila* were different in several respects to those of legionellae grown *in vitro*. The presence of a 15 kDa protein, additional LPS bands and altered fatty acid content were all noted for intra-amoebic grown *L. pneumophila*.

In the light of these findings it was decided to examine the sarkosyl insoluble outer membrane protein (OMP) profile and the LPS of intra-amoebic grown and broth grown *L. pneumophila*. The investigation was extended to include the effects of intra-monocytic growth and 'ageing' of intracellular grown *L. pneumophila*. The latter was included to determine whether the influence of environmental conditions and variations in stress resistance was associated with a change of surface properties.

6.2 ANALYSIS OF MEMBRANE PROTEINS.

The results of the SDS-PAGE analysis of the cytoplasmic and outer cell membranes of broth grown *L. pneumophila* are shown in Figure 6.1, Lanes 1 and 2, together with standard molecular weight markers. Analysis of the gel shows the presence of 12 proteins, the majority of which have molecular weights between 31 and 66 kDa. Comparison of Lanes 1 and 2 indicates that phase of growth has no effect on the size and number of proteins present in the cell membranes of broth grown *L. pneumophila*. The faint banding suggests that observed proteins are only present in small quantities. Analysis of the corresponding supernatants showed the presence of medium to low molecular weight proteins, 30 to 12 kDa (Figure 6.1, Lanes 3 and 4).

The interface between a Gram negative bacterium and its environment is the OM surface. Modifications of this membrane can be anticipated to exert a major effect on the ability of the bacterium to withstand any stress imposed upon it by its environment. It was, therefore, decided to confine further cell membrane protein analysis, during this preliminary work, to the OM alone. A comparative study of the OMPs of the variously grown *L. pneumophila* was undertaken by SDS-PAGE analysis.

The SDS-PAGE analysis of the sarkosyl insoluble OMPs of variously grown and 'aged' intracellular grown *L. pneumophila* are shown in Figure 6.2, together with standard molecular weight markers. The results show that a 28 kDa protein is expressed by all of the *L. pneumophila* investigated. Growth conditions did, however, influence the presence of other proteins. A 15 kDa protein was found exclusively in freshly harvested intra-amoebic grown *L. pneumophila* (Figure 6.2, Lane 3). This protein was not observed in intra-amoebic grown *L. pneumophila* which had remained in the coculture menstruum for 72 h. post host cell lysis (Figure 6.2, Lane 5). Similarly, a 24 kDa protein was found exclusively in freshly harvested monocytic grown *L. pneumophila* (Figure 6.2, Lane 4). This protein was absent after 'ageing' of the bacteria (Figure 6.2, Lane 6). High molecular weight sarkosyl insoluble OMPs (106, 98, 78 and 42 kDa)

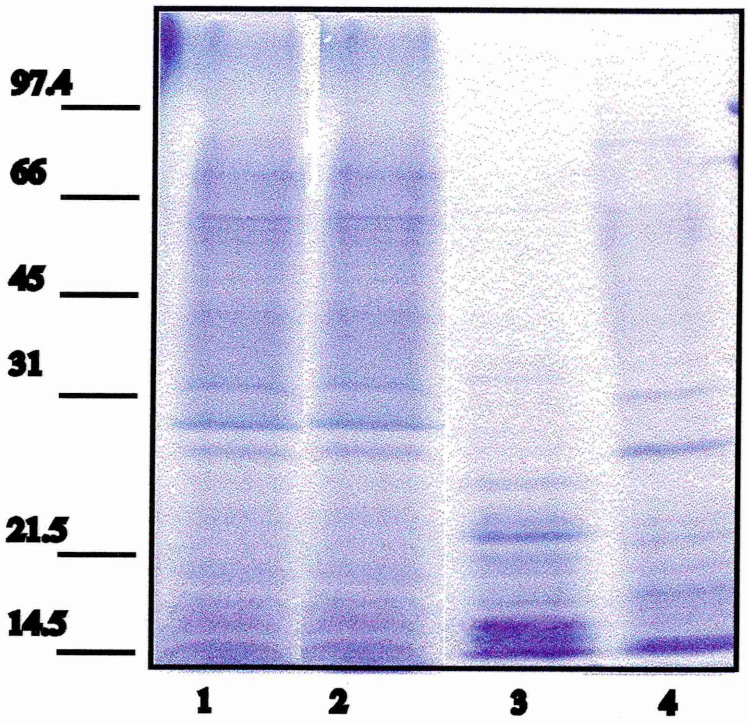


Figure 6.1. SDS-PAGE profiles of *L. pneumophila* total (cytoplasmic and outer) membrane proteins. Growth conditions : Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase); Lane 3, supernatant corresponding to YE broth grown (exponential phase); Lane 4, supernatant corresponding to YE broth grown (stationary phase).

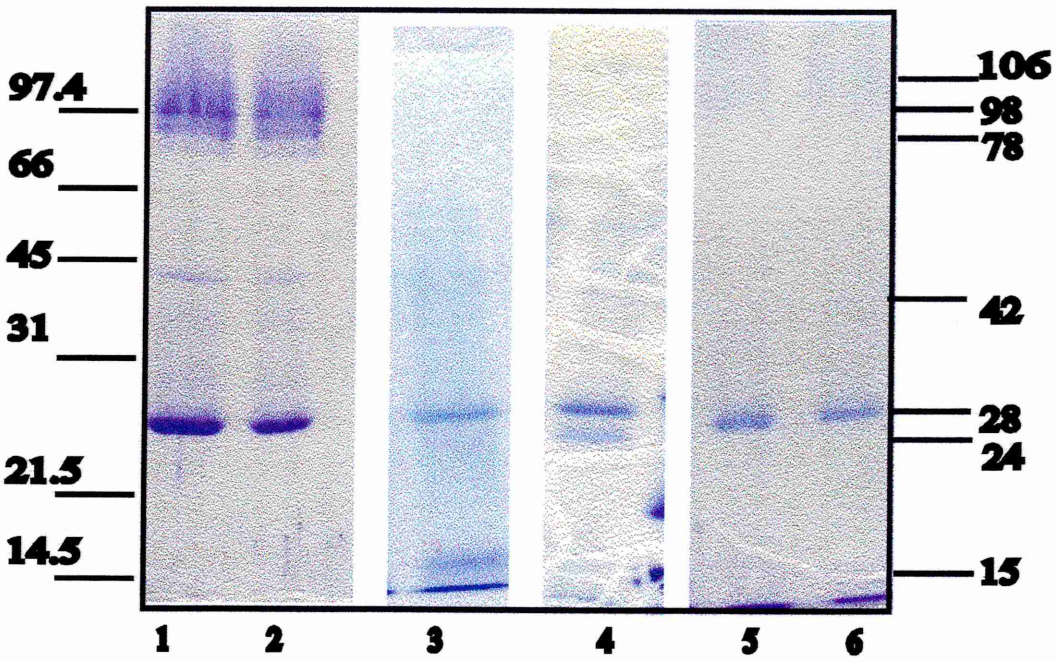


Figure 6.2. SDS-PAGE profiles of *L. pneumophila* sarkosyl-extracted OMPs. Growth conditions : Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase); Lane 3, freshly harvested intra-amoebic grown; Lane 4, freshly harvested intra-monocytic grown; Lane 5, 'aged' intra-amoebic grown; Lane 6, 'aged' intra-monocytic grown.

were found in the broth grown legionellae (Figure 6.2, Lanes 1 and 2) but do not appear to be present in either the freshly harvested or the 'aged' intracellular grown *L. pneumophila*.

Examination of whole cell proteins of U937 monocytes and *A. polyphaga* trophozoites shows that each contains many proteins (Figure 6.3, Lanes 1 and 2, respectively). Analysis of the sarkosyl insoluble plasma membrane fractions revealed that each host cell possesses one sarkosyl insoluble membrane protein. That of the monocytes had a molecular weight of *ca.* 24 kDa and that of the amoebae was *ca.* 15 kDa (Figure 6.3, Lanes 3 and 4, respectively).

Changes in microbial surfaces have been shown to lead to changes in surface antigens (Murphy, 1994). Antigenic variation of surface antigens is one mechanism that is used by bacteria to facilitate persistence and avoid mammalian defence mechanisms. It can be anticipated, therefore, that *L. pneumophila* replicating under different growth conditions would possess different surface antigens. This in turn may lead to the formation of antibodies with varying responses within infected hosts. A preliminary investigation was, therefore, undertaken to determine whether antibodies raised in rabbits against the variously grown legionellae recognise the OM antigens (LPS and OMP) of both intracellular and broth grown *L. pneumophila* serogroup 1 (sub-group Knoxville). The antibodies used were raised against whole cells, and not specific components of the *L. pneumophila* outer membrane.

For the purpose of immunoblotting, the rabbit raised antibodies were titrated by ELISA (Section 3.4.2). The OD_{405} value of the titrated antibody was used as a measure of its binding capacity. The higher the OD_{405} value, the greater the binding capacity of the antibody. Each antibody was titrated against sarkosyl insoluble OMP antigens of broth grown *L. pneumophila* in stationary phase. Figure 6.4 shows the relative amounts of antibody raised against *L. pneumophila* OM antigens in each rabbit. An OD_{405} value of *ca.* 1.0 for the antigen-antibody titration is considered acceptable for immunoblotting

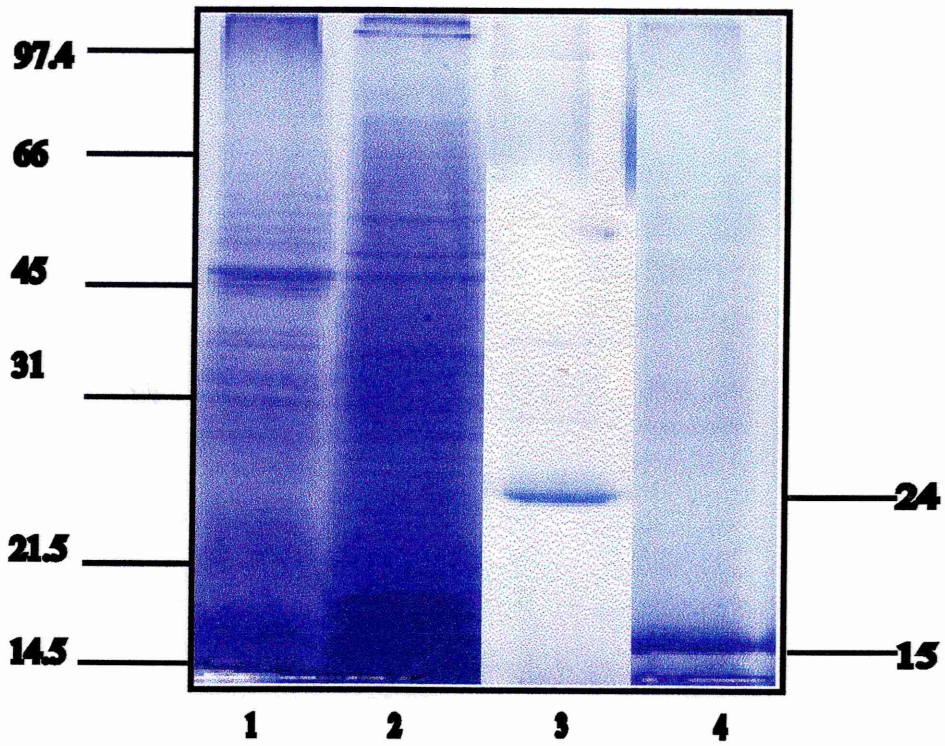


Figure 6.3. SDS-PAGE protein analysis of U937 monocytes and *A. polyphaga* trophozoites. Lane 1, whole U937 monocytes; Lane 2, whole *A. polyphaga*; Lane 3, sarkosyl insoluble membrane preparation of U937 monocytes; and Lane 4, sarkosyl insoluble membrane preparation of *A. polyphaga*.

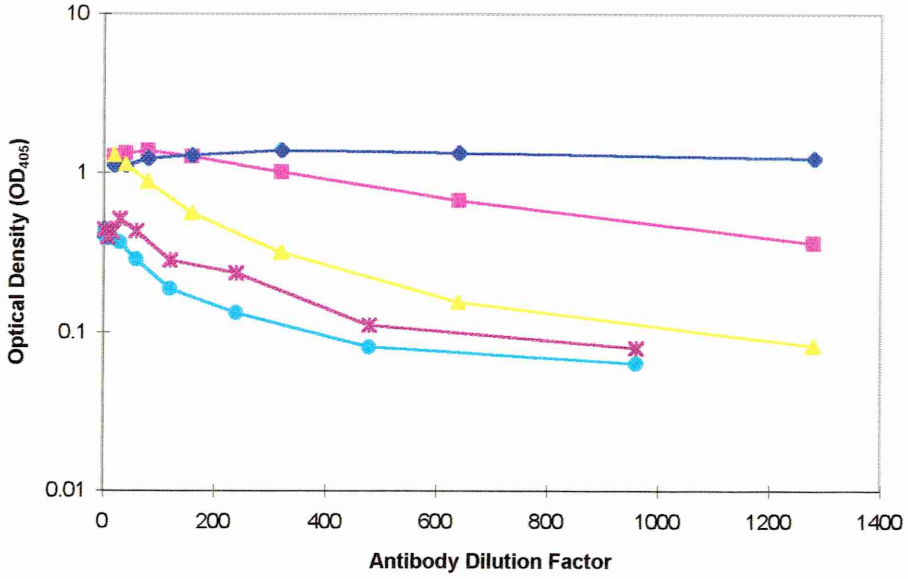


Figure 6.4 ELISA assay : The relative response of rabbit raised antibodies towards sarkosyl insoluble antigens of YE broth grown *L. pneumophila.* Antisera raised against amoebic trophozoites (✖), U937 monocytes (●), intra-amoebic grown (◆), intra-monocytic grown (▲) and YE broth grown (■) *L. pneumophila.*

purposes. Analysis of the ELISA results indicated that this value can be achieved during titrations of antibodies raised against *L. pneumophila*, but not in titrations against the immunised host cells. The overall response of the antibodies raised against *A. polyphaga* and U937 monocytes was very weak. The greatest response of both anti-*A. polyphaga* and anti-U937 monocyte antibodies was observed at a dilution of *ca.*1 : 5. The dilution at which the greatest response to each antibody occurred was adopted for immunoblotting purposes. These are as shown below in Table 6.1 :

Rabbit raised Antibody	Dilution	OD_{405}
anti-intra-amoebic grown *L. pneumophila*	1 : 100	1.18
anti-broth grown *L. pneumophila*	1 : 100	1.18
anti-intra-monocytic grown *L. pneumophila*	1 : 50	1.09
anti-acanthamoebae trophozoites	1 : 5	0. 43
anti-U937 monocytic cells	1 : 5	0.43

Table 6.1 Rabbit raised antibody dilutions used during immunoblotting.

Immunoblotting was performed to examine the response of the rabbit raised antibodies towards the sarkosyl insoluble OMP profiles of the variously grown *L. pneumophila*. The results of the immunoblotting revealed that the anti-*A.polyphaga* and anti-U937 monocytic cell antibodies did not bind to the OM antigens. Figure 6.5 (a-c) shows the response of each rabbit raised anti-*L. pneumophila* antibody towards sarkosyl-insoluble OM antigens of variously grown legionellae. Little difference was noted in the response of antibodies against broth grown, intra-amoebic grown and intra-monocytic grown *L. pneumophila* towards the OM antigens. The various anti-*Legionella* antibodies bound to the OMP profiles of the variously grown legionellae, with the exception of one band in each profile. Comparison of Figure 6.5 (a-c) with a typical Coomassie blue stained OMP profile (Figure 6.2, Lanes 1-4) showed that the unbound bands correspond to the MOMP of *L. pneumophila*. Where binding had taken place, the pattern of antibody binding appeared smeared with little definition of the bands. The

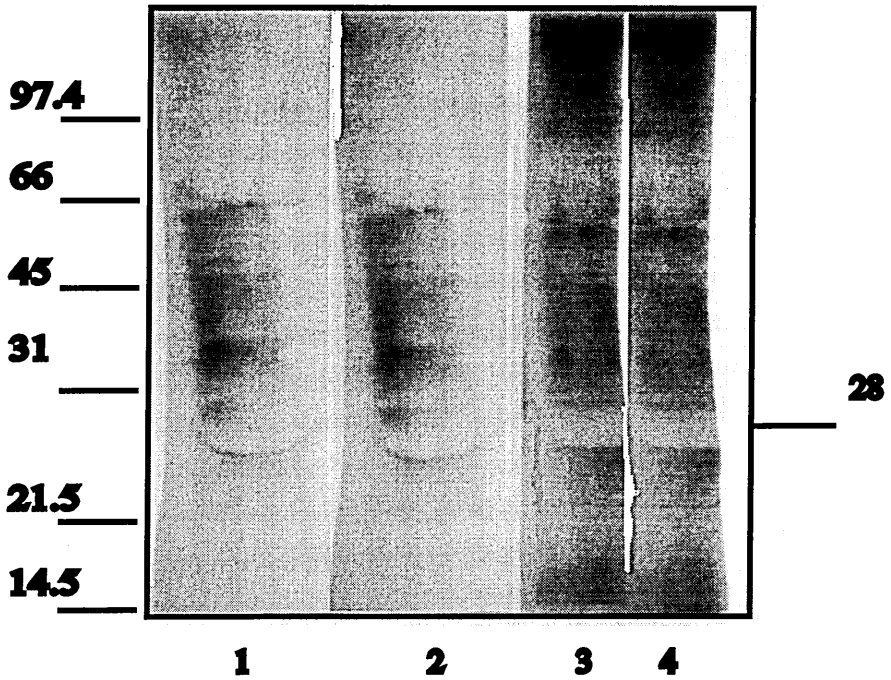


Figure 6.5 (a) Immunoblots of sarkosyl insoluble OM antigens of variously grown *L. pneumophila* reacted with anti-intra-amoebic grown *L. pneumophila* antibodies. Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase), Lane 3, intra-amoebic grown; Lane 4, intra-monocytic grown.

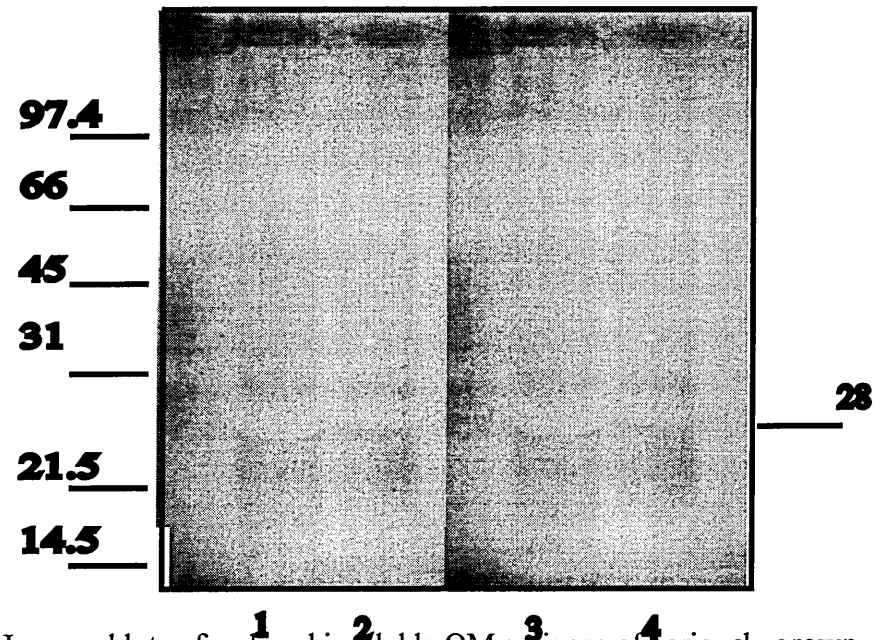


Figure 6.5 (b) Immunoblots of sarkosyl insoluble OM antigens of variously grown *L. pneumophila* reacted with anti-broth grown *L. pneumophila* antibodies. Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase), Lane 3, intra-amoebic grown; Lane 4, intra-monocytic grown

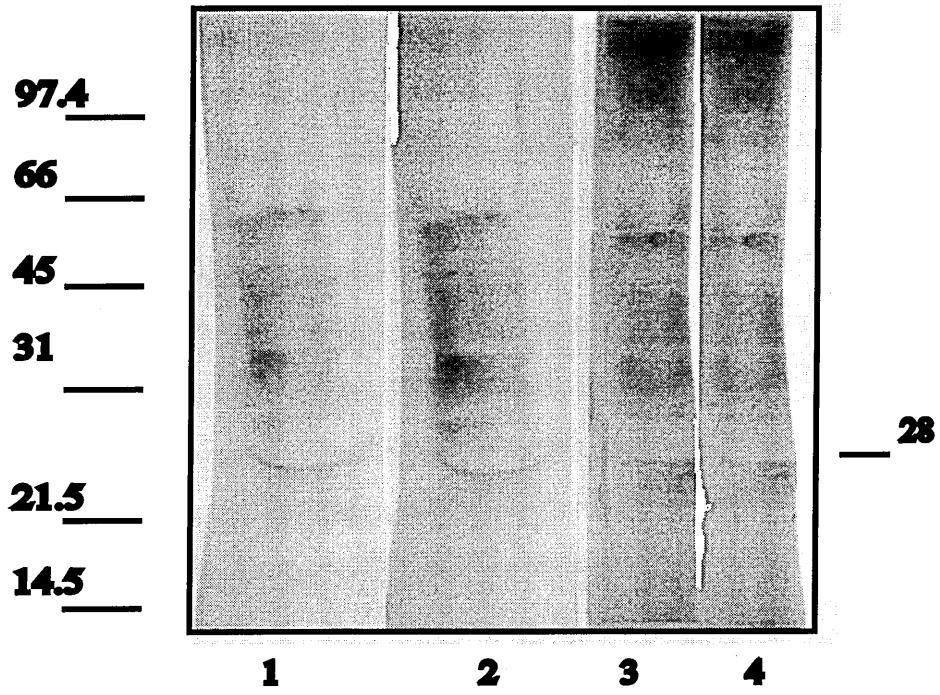


Figure 6.5 (c) Immunoblots of sarkosyl insoluble OM antigens of variously grown *L. pneumophila* reacted with anti-intra-monocytic grown *L. pneumophila* antibodies. Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase), Lane 3, intra-amoebic grown; Lane 4, intra-monocytic grown.

lack of definition of the bands suggests that the antibody responses towards the OM antigens are weak (Figure 6.5 (a-c), Lanes 1-4). Antibodies against intra-monocytic grown *L. pneumophila* appeared to be bound less strongly to the OM antigens than those against intra-amoebic grown *L. pneumophila*, as indicated by a reduced definition. It is possible, however, that this observation is due to a lower antibody concentration being employed.

6.3 ANALYSIS OF OUTER MEMBRANE SUPERNATANT.

SDS-PAGE analysis of the supernatants corresponding to the sarkosyl-insoluble OM preparations of the variously grown *L. pneumophila* was carried out to determine whether any proteins were removed during the preparation process. Each profile showed a smeared pattern with no distinct bands after staining with Coomassie blue, (Figure 6.6, Lanes 1-6). This suggested the possible presence of LPS within the supernatant. It was decided, therefore, to investigate whether the rabbit raised antibodies would recognise antigens within the supernatant. The ELISA assay was repeated using a 1 : 25 dilution of the supernatant corresponding to the broth grown *L. pneumophila* sarkosyl-insoluble OM preparation used as the antigen in the previous ELISA. The results show that the supernatant contains antigens which are recognised by each of the anti-legionellae antibodies (Figure 6.7). Comparison of the data presented in Figure 6.7 with that in Figure 6.4 shows that the response of the various antibodies to the antigens present in the supernatant was similar to that towards antigens in the sarkosyl insoluble OM preparation. The same dilution values were, therefore, adopted for the purpose of the immunoblotting.

Immunoblotting showed that the supernatant antigens were not recognised by antibodies raised against *A. polyphaga* trophozoites and U937 monocytes. Supernatant antigens were recognised by antibodies raised against the variously grown *L. pneumophila* (Figure 6.8 (a-c), Lanes 1-4). The pattern obtained was smeared and diffuse, lacking in definition, and similar in appearance to that achieved by SDS-PAGE analysis and Coomassie blue staining (Figure 6.6). The response of anti-legionellae

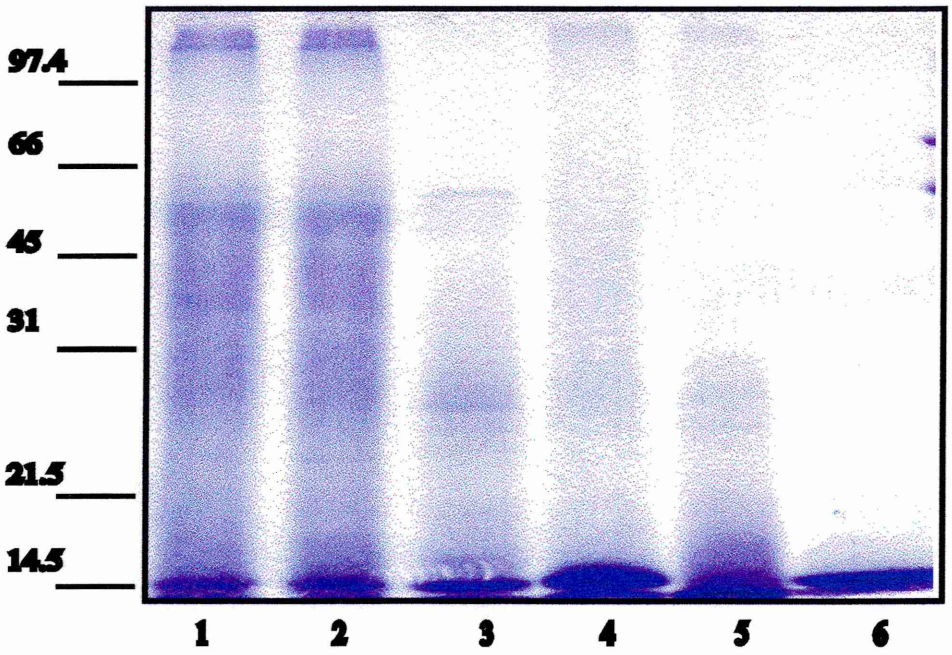


Figure 6.6. SDS-PAGE analysis of the OM supernatants corresponding to the sarkosyl insoluble OMP preparations of variously grown *L. pneumophila*. Growth conditions : Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase); Lane 3, freshly harvested intra-amoebic grown; Lane 4, freshly harvested intra-monocytic grown; Lane 5, 'aged' intra-amoebic grown; Lane 6, 'aged' intra-monocytic grown.

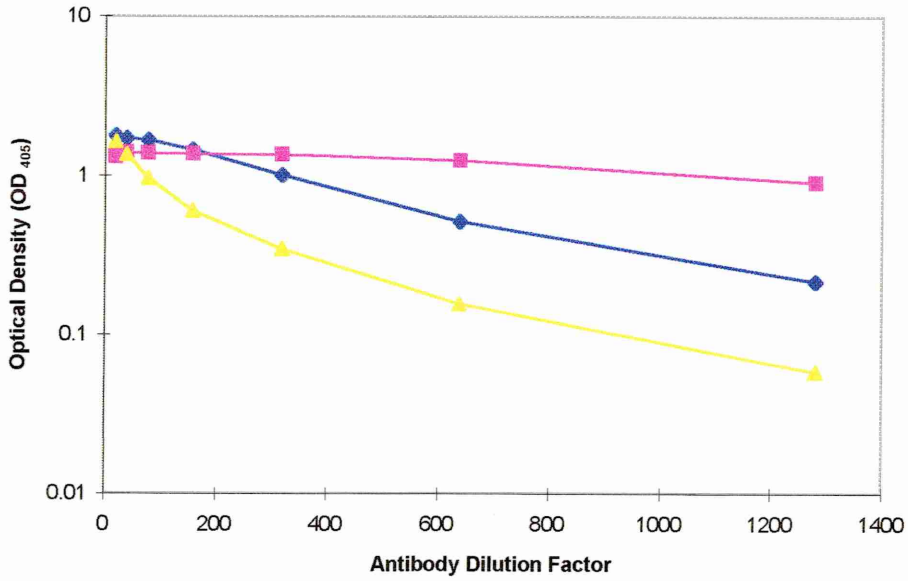


Figure 6.7. ELISA assay : The relative response of rabbit raised antibodies towards YE broth grown OM supernatant antigens : Antisera raised against intra-amoebic grown (◆), YE broth grown (■) and intra-monocytic grown (▲) *L. pneumophila*.

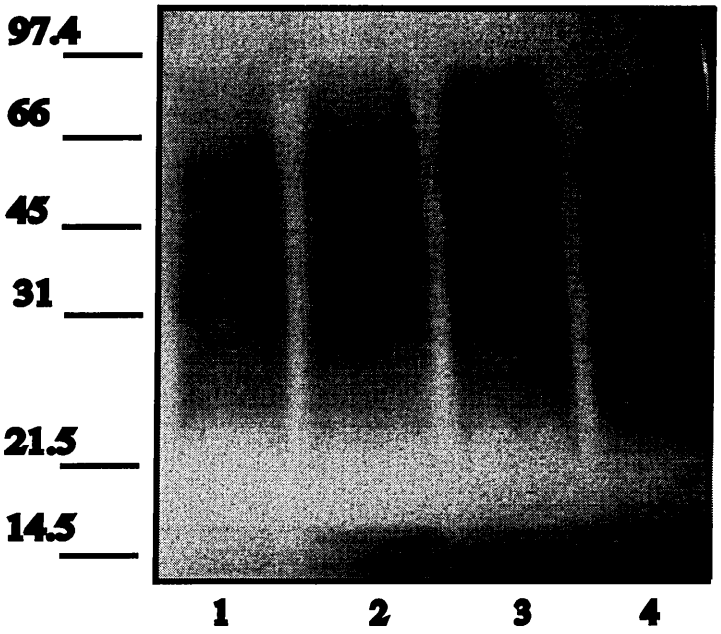


Figure 6.8 (a) Immunoblots of OM supernatant from sarkosyl insoluble preparation of variously grown *L. pneumophila* reacted with anti-intra-amoebic grown *L. pneumophila* antibodies. Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase); Lane 3, intra-amoebic grown; Lane 4, intra-monocytic grown.

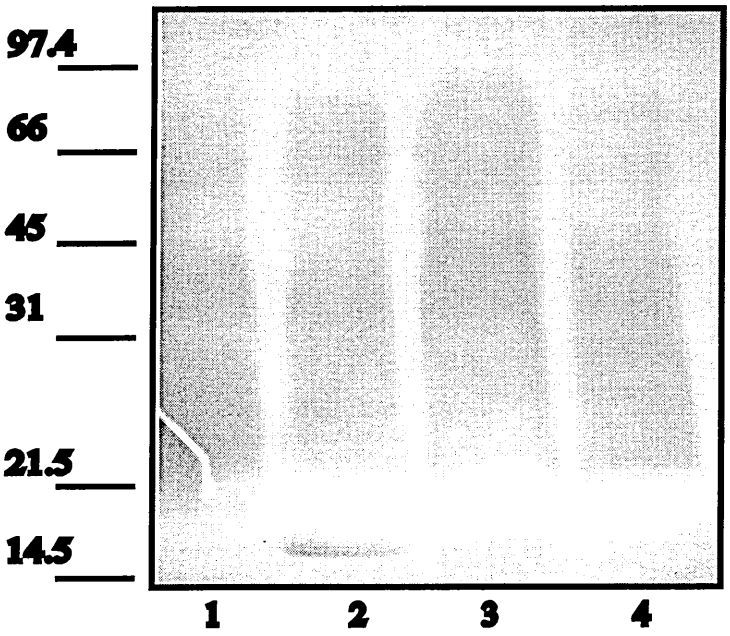


Figure 6.8 (b). Immunoblots of OM supernatant from sarkosyl insoluble preparation of variously grown *L. pneumophila* reacted with anti-broth grown *L. pneumophila* antibodies. Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase); Lane 3, intra-amoebic grown; Lane 4, intra-monocytic grown.

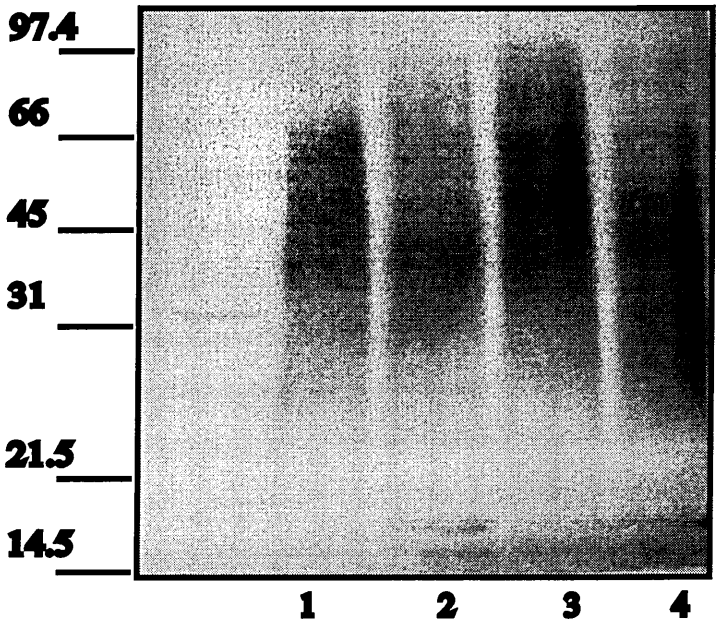


Figure 6.8 (c) Immunoblots of OM supernatant from sarkosyl insoluble preparation of variously grown *L. pneumophila* reacted with anti-intra-monocytic grown *L. pneumophila* antibodies. Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase); Lane 3, intra-amoebic grown; Lane 4, intra-monocytic grown.

antibodies towards the smeared profiles of supernatant antigens suggests the rabbit raised antibodies recognise LPS antigens. LPS is known to produce a smeared profile upon SDS-PAGE analysis (Chart, 1994). LPS is also a major component of the outer membrane and has been described as the major antigen recognised by sera from patients infected with *L. pneumophila* serogroup 1 (Gabay and Horwitz, 1985).

6.4 ANALYSIS OF THE LIPOPOLYSACCHARIDE LAYER.

The SDS-PAGE analysis (Figure 6.9, Lanes 1-4) of the extracted LPS from the variously grown *L. pneumophila* revealed profiles which are atypical of Gram negative bacteria. Gram negative bacteria such as *E. coli* (Figure 6.9, Lane 5) and *Salmonella* sp., exhibit a regular ladder-like banding pattern. The LPS profiles observed in the current study were effectively divided into two regions, the upper and lower regions, by a lightly stained (intra-monocytic grown *Legionella*) (Figure 6.9, Lane 1) or unstained (broth grown- and intra-amoebic grown legionellae) band (Figure 6.9, Lanes 2, 3 and 4 respectively). The ratio of the length of the upper region of each *Legionella* LPS profile to that of the lower regions was *ca.* 3 : 1. No differences were observed between the lower regions of the LPS profiles of the variously grown *L. pneumophila*. The profiles each had a regular ladder-like pattern of 8 narrow bands of *ca.* 0.75-1.0 mm wide with 0.5 mm spacing between them.

The profile in the upper region, varied with the source of the *L. pneumophila*. Broth grown legionellae (both exponential and stationary phase) exhibited 3 narrow diffuse, equally spaced bands at the top of the profile. Below these bands, two widely spaced, broad diffuse bands were observed. The profiles of the intracellular grown *L. pneumophila* were different to that of broth grown legionellae and between intra-amoebic and intra-monocytic grown bacteria. The profile of intra-amoebic grown *L. pneumophila* LPS showed two narrow bands at the top which corresponded approximately in position, size and spacing to the bands at the top of the LPS profile of broth grown legionellae. Below these four diffuse bands or groups of bands were

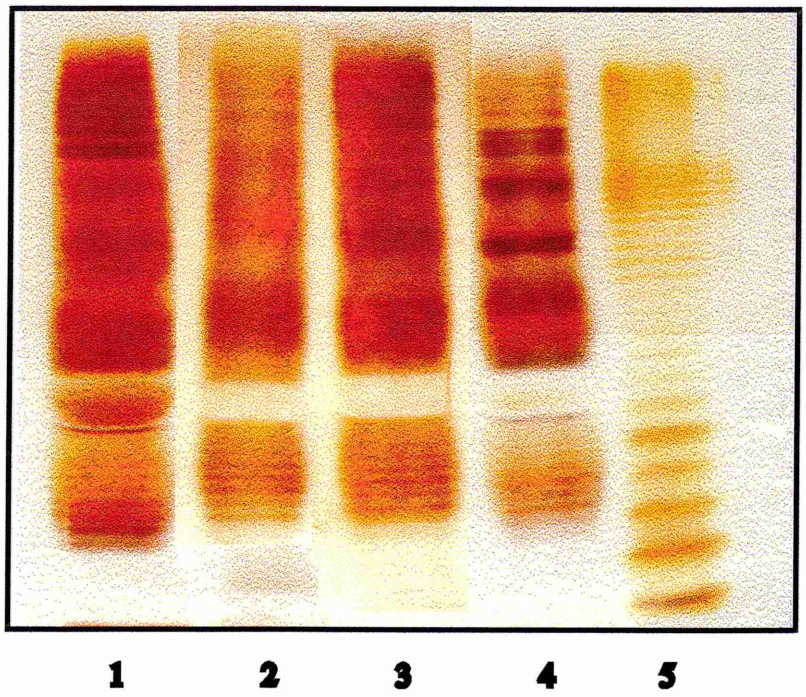


Figure 6.9. SDS-PAGE analysis of LPS extracted from *L. pneumophila* after silver staining. Growth conditions : Lane 1, intra-monocytic grown; Lane 2, YE broth grown (exponential phase); Lane 3, YE broth grown (stationary phase); Lane 4, intra-amoebic grown. Reference : Lane 5, *E.coli* purified LPS.

observed. The first group consisted of three narrow bands, the spacing between them was so small as to make them appear as one band on initial examination. Below this a slightly narrower diffuse band was observed. The third consisted of two very narrow bands with negligible spacing between them. The fourth group was very diffuse and appeared to be a single band. The LPS profile of intra-monocytic grown *L. pneumophila* was very similar to that of the intra-amoebic grown *L. pneumophila* except in that it was difficult to differentiate individual bands. The LPS profile of intra-monocytic grown legionellae was also atypical in that weak staining occurred in the region observed to remain unstained in the LPS profiles of the broth grown and intra-amoebic grown bacteria.

As a continuation of the antibody studies, each of the rabbit raised anti-legionellae antibodies was titrated against broth grown *L. pneumophila* (stationary phase) LPS antigens (5 μg/ml) by ELISA. Figure 6.10 shows the relative responses of the different anti-legionellae antibodies against LPS antigens. The responses of the various antibodies to the antigens present in the LPS was similar to that demonstrated towards antigens from sarkosyl insoluble preparations. Comparison of the data in Figures 6.11 (a-c) shows that the response of each anti-legionellae antibody towards LPS antigens is similar to that towards sarkosyl insoluble OM antigens and the corresponding supernatant antigens. This would suggest that the anti-legionellae antibodies are recognising the LPS antigens within the sarkosyl insoluble OM and supernatant preparations. The dilutions of antibodies used for the immunoblotting of the LPS antigens were the same as those used in the sarkosyl insoluble OM and corresponding supernatant (Table 6.1).

The results of this immunoblotting work are shown in Figure 6.12 (a-c). Examination of the blots shows ladder-like patterns in the lower region similar to those resulting from SDS-PAGE analysis of extracted LPS and silver staining (Figure 6.9). The similarity of the silver stained profiles and those observed on the blots would suggest that binding of the antibodies has occurred in a specific and targetted manner in the

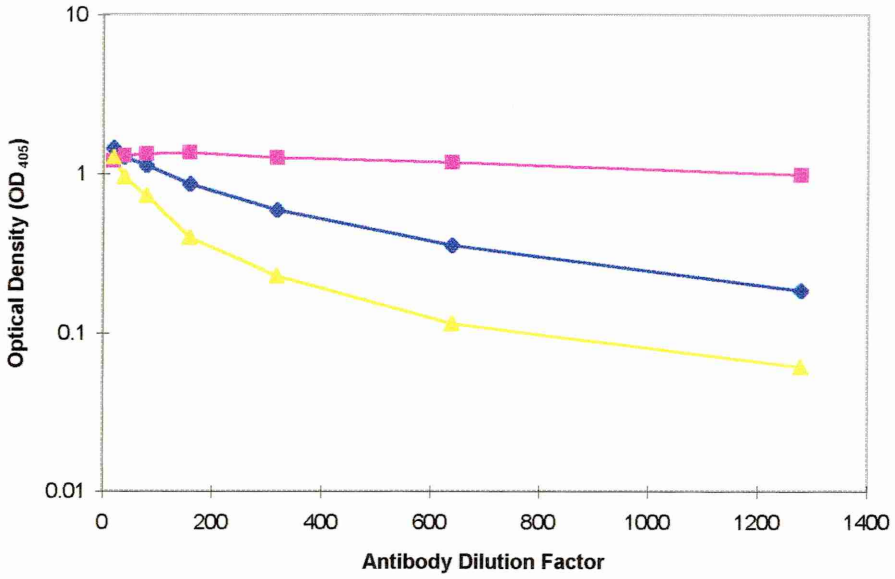


Figure 6.10. ELISA assay : The relative response of rabbit raised antibodies towards YE broth grown LPS antigens : Antisera raised against intra-amoebic grown (◆), YE broth grown (■) and intra-monocytic grown (▲) *L. pneumophila*.

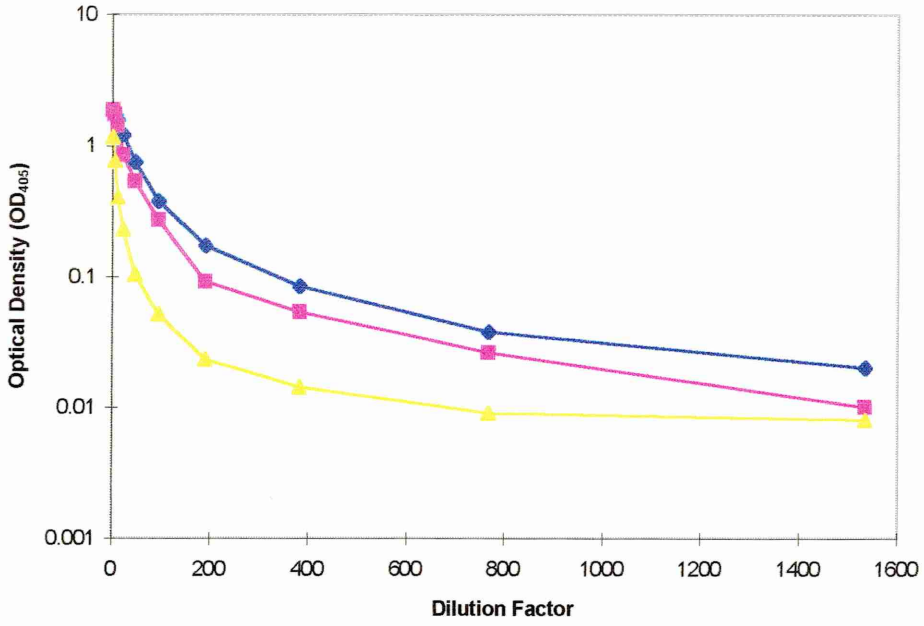


Figure 6.11 (a) ELISA assay : The relative response of rabbbit antibodies raised against intra-amoebic grown *L. pneumophila* towards the sarkosyl insoluble OMP antigens (◆), the corresponding supernatant antigns (■) and the LPS antigens (▲) of YE broth grown *L. pneumophila*.

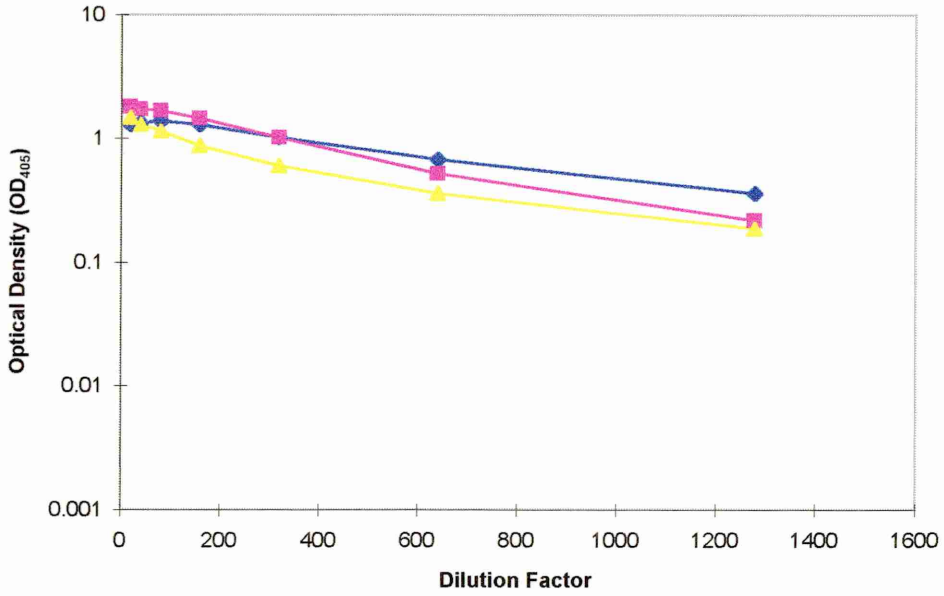


Figure 6.11 (b) ELISA assay : The relative response of rabbit antibodies raised against YE broth grown *L. pneumophila* towards the sarkosyl insoluble OMP antigens (◆), the corresponding supernatant antigns (■) and the LPS antigens (▲) of YE broth grown *L. pneumophila*.

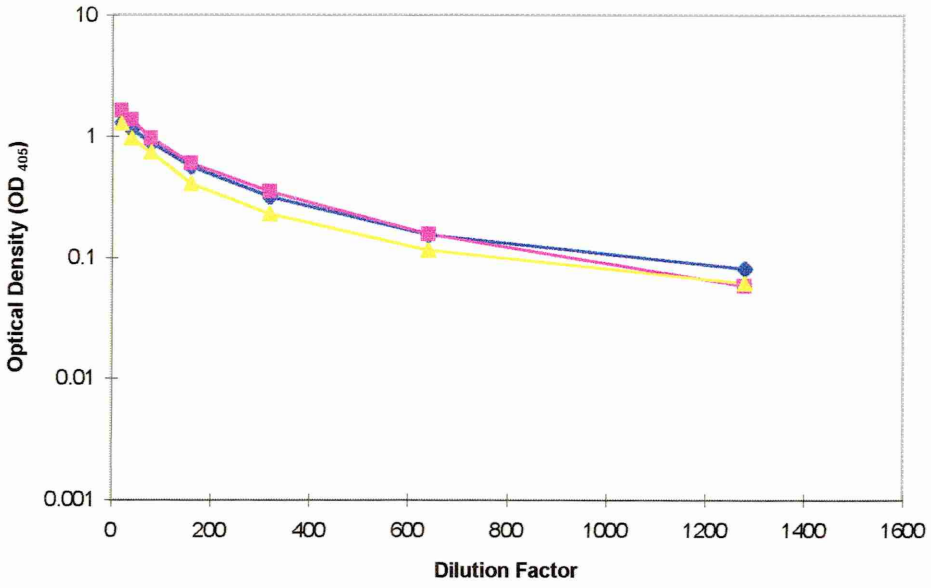


Figure 6.11 (c) ELISA assay : The relative response of rabbit antibodies raised against intra-monocytic grown *L. pneumophila* towards the sarkosyl insoluble OMP antigens (◆), the corresponding supernatant antigns (■) and the LPS antigens (▲) of YE broth grown *L. pneumophila*.

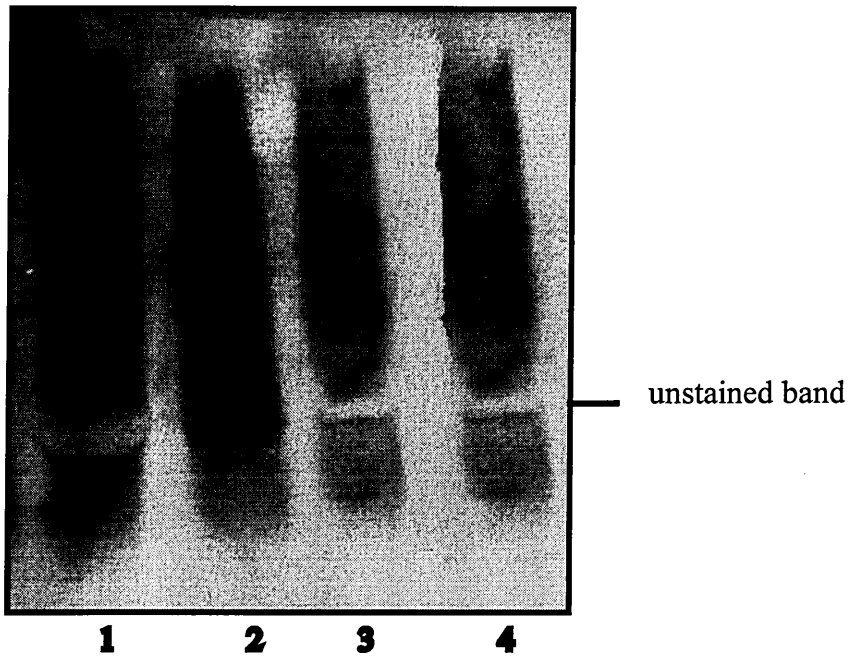


Figure 6.12 (a). Immunoblot analysis of proteinase K extracted LPS of variously grown *L. pneumophila* reacted with anti-intra-amoebic grown *L. pneumophila* antibodies. Lane 1, intra-monocytic grown; Lane 2, intra-amoebic grown, Lane 3, YE broth grown (exponential phase); YE broth grown (stationary phase); Lane 4.

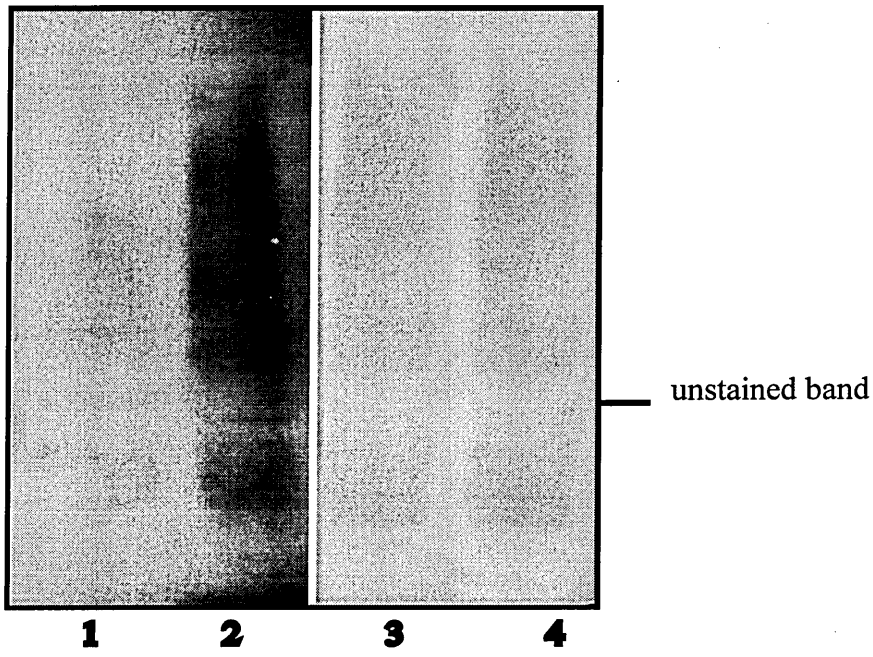


Figure 6.12 (b) Immunoblot analysis of proteinase K extracted LPS of variously grown *L. pneumophila* reacted with anti-monocytic grown *L. pneumophila* antibodies. Lane 1, intra-monocytic grown; Lane 2, YE broth grown (exponential phase); Lane 3, YE broth grown (stationary phase); Lane 4, intra-amoebic grown.

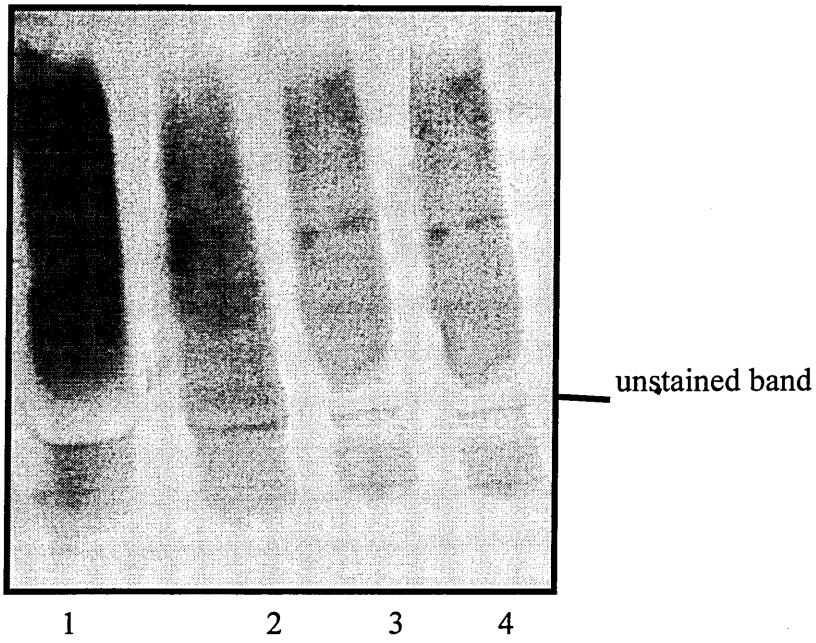


Figure 6.12 (c) Immunoblot analysis of proteinase K extracted LPS of variously grown *L. pneumophila* reacted with anti-intra-broth grown *L. pneumophila* antibodies. Lane 1, intra-amoebic grown; Lane 2, intra-monocytic grown; Lane 3, YE broth grown (exponential phase); Lane 4, YE broth grown (stationary phase).

lower region of the LPS profiles. Conversely, the blots corresponding to the upper region of the LPS profiles were smeared with a general loss of band definition.This would suggest a more general response of the antibodies to the LPS antigens in this region or a considerable amount of binding leading to diffuse bands.

No antibody binding occurred in the unstained region of the LPS profiles of the broth grown and intra-amoebic grown *L. pneumophila* (Figure 6.12 (a-c), Lanes 1, 3 and 4). Binding did occur in the corresponding band of intra-monocytic grown *L. pneumophila* (Figure 6.12 (a-c), Lane 2). This band was observed to stain slightly on analysis by SDS-PAGE. The density of colouration of the blots may be taken as an indication of the strength of binding of the antibody. The blots suggest that binding of anti-intra-amoebic grown and anti-broth grown legionellae antibodies occurs more strongly to the LPS of intra-amoebic grown *L. pneumophila* (Figures 6.12 (a) and (c), Lane 1) than to that of either broth grown or intra-monocytic grown legionellae. Conversely, antibodies against intra-monocytic grown *Legionella* bind most readily to the LPS antigens of intra-monocytic grown *L. pneumophila* (Figure 6.12 (b) Lane 2). Careful examination of the blots also shows that the overall binding strength of the anti-intra-monocytic grown *L. pneumophila* antibodies towards LPS antigens of the variously grown *L. pneumophila* is weaker than that of either anti-broth grown or anti-intra-amoebic grown *Legionella* antibodies. These differences in binding strength could be due to small differences in the concentration of antibodies employed or differences in recognition by the antibodies of the LPS antigens of the variously grown *L. pneumophila*.

Small quantities of sera from patients infected with *L. pneumophila* serogroup 1 were obtained towards the end of the preliminary investigation. The sera were designated patient serum 1, 2 and 3. Each serum was titrated against sarkosyl insolube OM antigens, OM supernatant antigens and LPS antigens of broth grown *L. pneumophila* in stationary phase by ELISA using the technique described previously (Figure 6.13 (a-c)). Little difference was observed between the binding of the patient raised antibodies

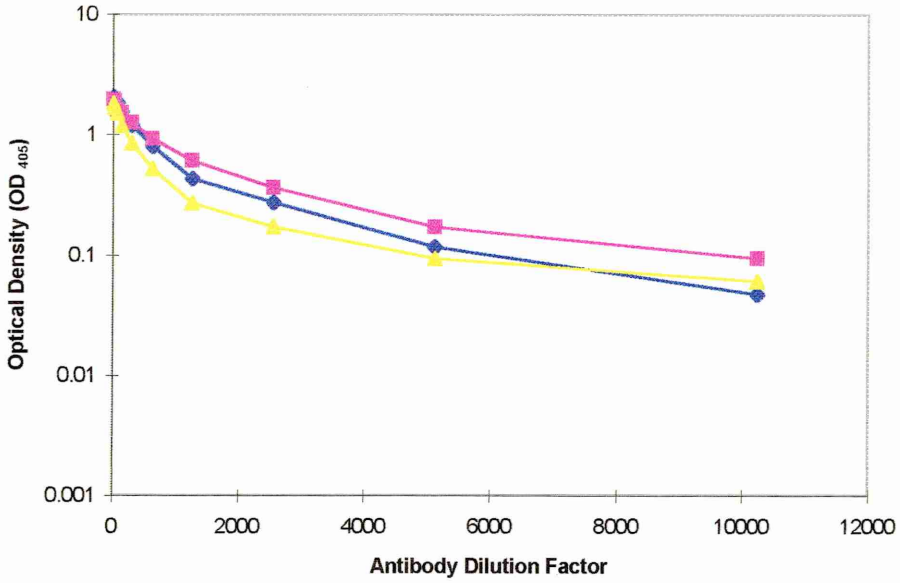


Figure 6.13 (a) ELISA assay : The relative response of Patient Serum 1 towards the sarkosyl insoluble OMP antigens (◆), the corresponding supernatant antigens (■) and LPS antigens (▲) of YE broth grown *L. pneumophila*.

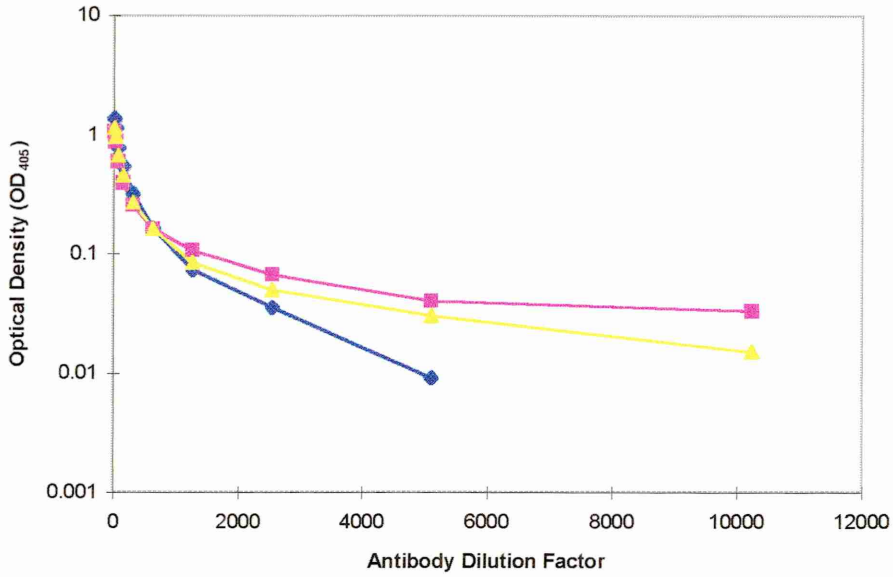


Figure 6.13 (b) ELISA assay : The relative response of Patient Serum 2 towards the sarkosyl insoluble OMP antigens (◆), the corresponding supernatant antigens (■) and LPS antigens (▲) of YE broth grown *L. pneumophila*.

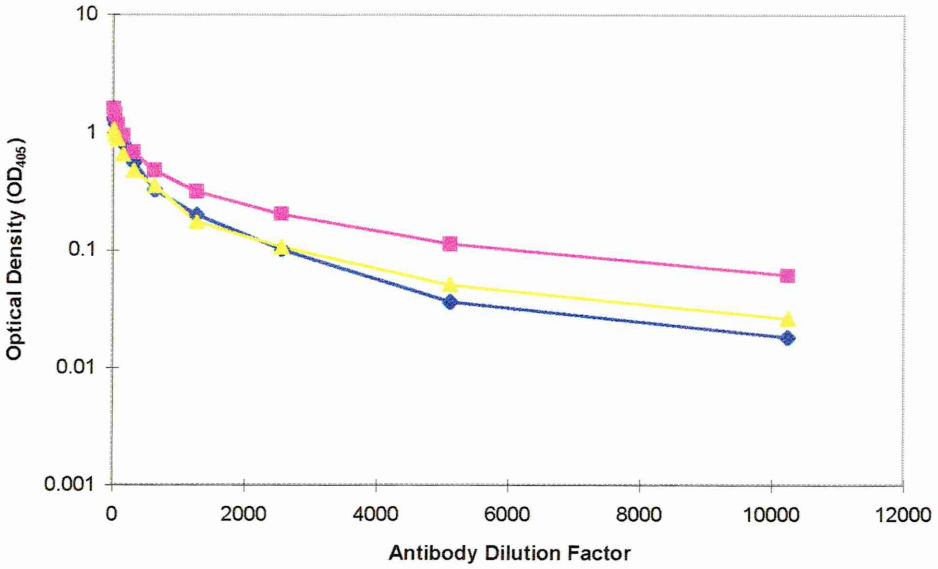


Figure 6.13 (c). ELISA assay : The relative response of Patient Serum 3 towards the sarkosyl insoluble OMP antigens (◆), the corresponding supernatant antigens (■) and LPS antigens (▲) of YE broth grown *L. pneumophila*.

to the LPS antigens, the supernatant antigens and those of the OM preparation. As LPS is present in both the sarkosyl insoluble OM preparation and the corresponding supernatant, it is probable that the patient sera are recognising LPS antigens. As only small quantities of patient sera were available, the immunoblotting work was confined to the LPS antigen profiles of the variously grown *L. pneumophila*.

Patient serum 1 was observed to bind to the LPS antigens of broth grown and intra-monocytic grown *L. pneumophila*, but not to the LPS of intra-amoebic grown legionellae (Figure 6.14). Patient serum 2 did not respond to the LPS antigens of any of the *Legionella* examined. In contrast, patient serum 3 bound to the LPS antigens of all the legionellae (Figure 6.15, Lanes 1-4). The response towards the lower region of each LPS profile was very weak relative to that of the upper region. No binding to the band which previously remained unstained by silver in the LPS profiles was observed. No variations in antibody response to antigens in the upper region of each LPS profile was noted. The antibody responses of patient sera 1 and 3 were weak in comparison to that of rabbit raised anti-legionellae sera. This could be due to the relatively low antibody titres in the patient sera.

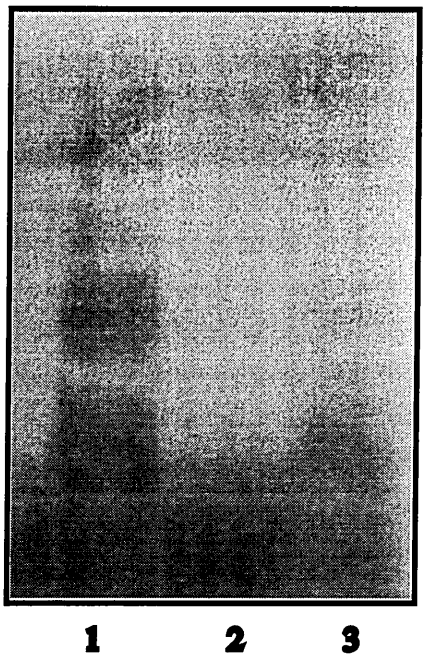


Figure 6.14 Immunoblot analysis of proteinase K extracted LPS of variously grown *L. pneumophila* reacted with patient serum 1. Lane 1, YE broth grown (exponential phase); Lane 2, intra-amoebic grown; Lane 3, YE broth grown (stationary phase).

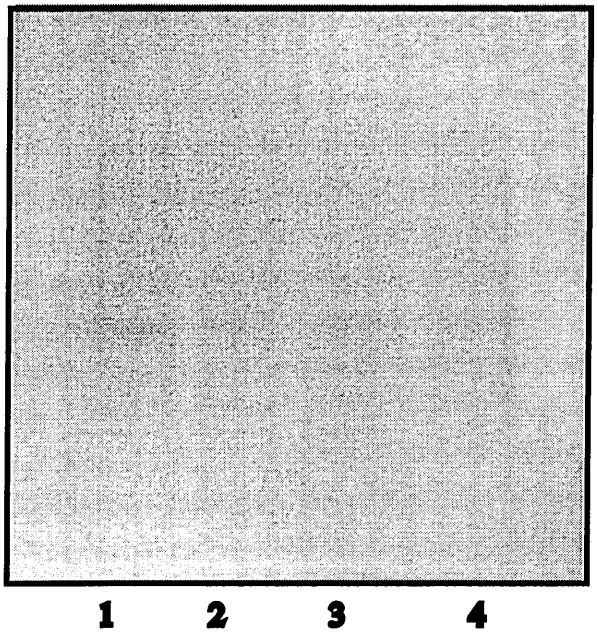


Figure 6.15. Immunoblot analysis of proteinase K extracted LPS of variously grown *L. pneumophila* reacted with patient serum 3. Lane 1, YE broth grown (exponential phase); Lane 2, YE broth grown (stationary phase); Lane 3, intra-amoebic grown; Lane 4, intra-monocytic grown.

6.5 DISCUSSION.

It has been reported that about 10 % of the total mass of *Legionella* sp. is localised in the outer membrane (OM) of the bacteria (Hindahl and Iglewski, 1984). Although OMPs are of potential use for the purposes of bacterial identification, they are also of considerable interest as they play a role in pathogenesis. OM antigens come into primary contact with the inflammatory cells and immune system of the host. The host immune system produces antibodies which bind specifically to the respective antigens (Dowling *et al*, 1992). The OM of Gram negative bacteria *per se* also constitutes a permeability barrier (Nikaido and Nakae, 1979; Lugtenberg and van Alpen, 1983 and Hancock, 1984). There is evidence to suggest that the composition of the macromolecules of the OM of Gram negative bacteria show considerable variation and is dependent in part on growth rates and specific nutrient availability. This variation can affect the microorganism's response to antibiotics and disinfectants as well as host cell defences (Brown, 1977; Al-Hitti and Gilbert 1980; Harder *et al.*, 1981, Cozens *et al.*, 1983; Ombaka *et al.*, 1983; Ikeda *et al.*, 1984 and Brown and Williams, 1985a).

SDS-PAGE analysis carried out in the current investigation has shown a total of 12 proteins to be associated with the cytoplasmic and outer membranes of *L. pneumophila*. Of these only five, have been shown to be present in the outer membrane and to be sarkosyl insoluble. All the *L. pneumophila* examined have been shown to possess a 28 kDa sarkosyl insoluble OMP. A number of researchers have demonstrated that the major outer membrane protein (MOMP) of *L. pneumophila* has a molecular weight between 24 and 29 kDa (Hindahl and Iglewski, 1984; 1986; Butler *et al.*, 1985; Ehret and Ruchdeschel, 1985; Gabay *et al.*, 1985; Gabay and Horwitz, 1985 and Barker *et al.*, 1993). Ehret and Ruchdeschel (1985) reported the presence of a 29 kDa OMP in all ten serogroups of *L. pneumophila* examined. It is possible, therefore, that the 28 kDa protein shown to be present in each form of *L. pneumophila* examined in the current study represents the MOMP of these bacteria. The small differences betwen the MOMP molecular mass found in this work and the range reported in the literature may be a function of the methods of outer membrane

preparation. Investigators using HEPES buffer reported a 29 kDa protein (Ehret and Ruchdeschel, 1985 and Hindahl and Iglewski, 1986), whereas those using Tris buffer reported a 24 kDa protein (Butler *et al.*, 1985 and Gabay *et al.*, 1985). The MOMP has been shown to be bound to the peptidoglycan layer by disulphide bonds (Hindahl and Iglewski, 1984) and to be exposed to the cell surface. Gabay *et al.*, (1985) demonstrated that the MOMP of *L. pneumophila* is a cation-selective porin. Porins are a class of bacterial proteins that form channels through which ions can pass. Horwitz (1988) has suggested that one mechanism by which *L. pneumophila* Philadelphia 1 might inhibit acidification of the monocyte phagosome is by the insertion of a porin ionophore, namely the cation selective MOMP porin into the monocyte membrane. Yamamoto *et al.* (1994) reported that binding of the MOMP to the monocyte membrane increases the production of cellular cytokines. It is possible that these cytokines may encourage binding of the phagosome with the endoplasmic reticulum thereby inhibiting acidification. MOMPs, which act as porins, have also been identified in other Gram negative pathogenic bacteria which are known to survive intracellularly. Examples of these are *Rickettsia prowazeki* (29 kDa), *P. aeruginosa* (29 kDa), *S. typhimurium* (29 kDa) and *E. coli* (32.8 kDa) (Winkler and Turco, 1988).

It has been shown in this study that growth phase has no effect on the outer membrane protein profile of broth grown *L. pneumophila*. Miyamoto *et al.* (1993) also examined the protein profiles of both mid-exponential and early stationary phase broth grown *L. pneumophila* and found no differences between them. They also reported that broth grown *L. pneumophila* have the same protein profile as those grown on agar. The protein profiles of broth grown *L. pneumophila* examined in the current study show the presence of sarkosyl insoluble OMPs including one of *ca.* 42 kDa. Several OMPs with similar molecular weights to the latter have been reported. Elliott and Johnson (1981) showed *L. pneumophila* flagella to have a filament subunit of 47 kDa. These authors separated the flagella from whole *Legionella* by passage through a narrow gauge needle followed by centrifugation. The flagella were harvested from the resulting

supernatant by high speed centrifugation and were analysed by SDS-PAGE, following the addition of 2-mercaptoethanol to the resulting pellet. Amano and Williams (1983) on the other hand, showed the presence of a 39 kDa OMP, which is apparently released from peptidoglycan after treatment with lysozyme. As the OM preparation techniques used in this study also includes the use of lysozyme, it is highly probable that the 42 kDa protein observed in the broth grown *Legionella* is the same as the 39 kDa OMP identified by Amano and Williams (1983). This protein was not observed in the absence of added lysozyme (Figure A1, Lanes 2-8). High molecular weight proteins have also been shown to be present in the broth grown legionellae, *viz* 106, 98 and 78 kDa. Butler *et al.* (1985) reported a 95 kDa protein which they suggested may represent a porin consisting of four disulphide cross-linked sub-units of the MOMP and that this complex is bound to the peptidoglycan *in situ*. Whilst it is probable that the 98 kDa OMP found in the current work is the same as that reported by Butler *et al.* (1985), no explanation can be offered at this stage as to the origins of the 106 kDa and 78 kDa proteins.

The current investigation has shown that intra-monocytic grown legionellae contain only one other sarkosyl insoluble OMP in addition to the 28 kDa MOMP. This appears to have a molecular weight of 24 kDa. The origin of this protein is unclear, but evidence has been obtained in this study which strongly suggests that it is bacterial and not host cell in origin. It has been shown that the 24 kDa protein is absent from the OMP profile of the inoculum broth grown *Legionella*, but present in the profile of the bacteria after replication within U937 monocytic cells. In addition, the 24 kDa protein 'disappears' from the OMP profile of intra-monocytic grown *L. pneumophila* upon 'ageing'. These observations strongly suggest expression of the protein during intracellular replication and repression during 'ageing' when the *Legionella* undergo a morphological change. The U937 monocytic cell has been shown by this study to also contain a 24 kDa protein. It could be argued that this is the source of the novel protein, and that the *L. pneumophila* have become 'coated' with it during intracellular replication. A similar arguement was used by Barker *et al.* (1993) to account for the

presence of a novel protein of mass 15 kDa in the OMP profile of *L. pneumophila* following intra-amoebic replication. If the 24 kDa protein is merely mechanically attached ('coated on') to the intra-monocytic grown *L. pneumophila*, then its absence from the OMP profile of the bacteria after 'ageing' must be explained by its loss into the supernatant. No evidence for this has been found during the current study. It is remotely possible, however, that the presence of proteins in the supernatant profile was masked by the general smearing due to LPS. Finally, immunoblotting has shown that the sarkosyl insoluble OMPs of intra-monocytic grown *L. pneumophila* are not recognised by antibodies raised in rabbits against uninfected U937 monocytes, but are recognised, albeit weakly, by those raised against intra-monocytic grown *L. pneumophila.*

The expression of a 24 kDa protein during intra-monocytic replication has also been described in the literature. Miyamoto *et al.* (1993) reported the expression of a 24 kDa protein by *L. pneumophila* serogroup 1 (sub-group Philadelphia) after replication in peritoneal macrophages of guinea pigs, A/J mice and hamsters. Unlike the current study, which examined only sarkosyl insoluble OMPs, Miyamoto *et al.* (1993) investigated the total protein profile of intra-macrophage grown *L. pneumophila.* Western blotting was used to confirm that the isolated protein was not the macrophage initiator protein (Mip), which is reported to be an OMP of 24.8 kDa (Rechnitzer *et al.*, 1994). Southern blotting was also performed to confirm that the 24 kDa protein was bacterial in origin and not arising from host cell debris. After sequencing the protein Miyamoto *et al.* (1993) found the protein to have a calculated molecular weight of 20 kDa. More recently, Abu Kwaik (1998) have shown the induction of a 20 kDa protein by *L. pneumophila* upon replication in U937 monocytic cells. This protein was shown not to be induced by broth grown legionellae when exposed to environmental stresses. Abu Kwaik *et al.* (1993) have also shown by metabolic labeling that more than 35 *Legionella* whole cell proteins were induced and 32 repressed during intracellular replication in U937 monocytic cells.

Information gained during the currrent study has shown that intra-amoebic grown legionellae contain only one sarkosyl insoluble OMP in addition to the 28 kDa MOMP. This appears to have a molecular mass of *ca.* 15 kDa. As in the case of the 24 kDa protein of the intra-monocytic grown legionellae, there is strong indirect evidence to suggest that the15 kDa protein is bacterial in origin and not amoebic in spite of the presence of such a protein within amoebae being demonstrated by this study and reported in the literature (Korn & Wright, 1973; Thompson & Pauls, 1980; Clarke *et al.*, 1988 and Barker *et al.*, 1993). The 15 kDa protein was found to be absent from the OMP profile of the intra-amoebic grown *Legionella* after 'ageing' and from the corresponding supernatant profile. Similarly, immunoblotting showed that the sarkosyl insoluble OMPs of intra-amoebic grown *L. pneumophila* are not recognised by antibodies raised against *A. polyphaga*, but are recognised by those raised against intra-amoebic grown *Legionella*.

The presence of a 15 kDa protein within the sarkosyl insoluble OMP profile of intra-amoebic grown *L. pneumophila* has been reported previously by Barker *et al.* (1993). Steps were taken by these workers to demonstrate that their observations were not due to amoebic debris. Barker *et al.* (1993) incubated broth grown *L. pneumophila* with amoebic debris for 24 h. prior to harvesting and OMP preparation. The protein profile of these bacteria did not exhibit the 15 kDa protein. These workers concluded that 15 kDa protein observed in the intra-amoebic *L. pneumophila* profile was not amoebic debris in origin. In the current study, the intracellular grown legionellae were washed until no pellet formed on low speed centrifugation. Barker *et al.* (1993) performed a similar investigation using commercial anti-acanthamoebae serum. These workers reported that the 15 kDa OMP of neither the sarkosyl preparations of *A. polyphaga* trophozoites nor intra-amoebic grown *L. pneumophila* were recognised by the anti-serum. Despite these observations, Barker *et al.* (1993) proposed that the source of the 15 kDa OMP isolated from intra-amoebic grown *L. pneumophila* was amoebic in origin. It was suggested that the transfer of protein arises as a result of the physical conditions imposed by the coculture growth system; ie. the generation and lysis of the

infected amoebae releases membraneous material into the environment where it becomes attached to the surface of the legionellae. Furthermore, these workers earlier speculated that the 'coating' of *Legionella* with amoebic proteins is involved in the marked resistance of intra-amoebic grown bacteria towards biocides (Barker *et al.*, 1992).

SDS-PAGE analysis during this study has shown differences between the sarkosyl insoluble OMP profiles of broth and intracellular grown *L. pneumophila*. Immunoblotting using antibodies raised in rabbits against the variously grown bacteria was carried out to determine the antigenic potential of the *Legionella* proteins identified during the analysis. It was shown that antibodies raised against the variously grown *L. pneumophila* recognise OM antigens with the exception of the MOMP. The pattern of banding was smeared suggesting that the antibodies were responding to LPS antigens within the OM preparation and not specific OMPs. The lack of binding specificity was confirmed during the ELISA investigation. As the antibodies were raised against whole bacteria it can be anticipated that the majority will act against the *L. pneumophila* major antigen. This has been reported as being the LPS (Gabay and Horwitz, 1985; Ciesielski *et al.*, 1986; Conlan and Ashworth, 1986 and Barker *et al*, 1993) and is located at the outermost surface of the bacteria.

The SDS-PAGE profiles of the OM preparation supernatant revealed smeared patterns when stained with Coomassie blue. The smeared patterns were recognised by each of the rabbit raised anti-legionellae antibodies. The appearence of the antibody binding would suggest the presence of LPS antigens in the supernatant. Ideally, no LPS should be present in the supernatant as it is tightly bound to the OM. However, it is possible that during the preparation of the sarkosyl insoluble OM small fragments of LPS became detached from the outer membrane and remained in the supernatant after the final centrifugation.

Several investigators have established that LPS can be separated by SDS-PAGE according to the number of repeating units in the O-side chain and that smooth type LPS yields a ladder-like array of bands (Goldman and Leive, 1980; Palva and Makla, 1980 and Hitchcock and Brown, 1983). Gabay and Horwitz (1985) have reported the LPS profile of *L. pneumophila* to be markedly different to the patterns observed for either the rough or smooth LPS of *E. coli*. Commercially extracted smooth *E. coli* 0.111 LPS was used as a reference during the current studies. The ladder-like banding pattern observed in the lower region of *L. pneumophila* LPS was more compact and the bands narrower than that of *E. coli*. Conlan and Ashworth (1986) and Nolte *et al.* (1986) have compared the LPS banding patterns of *L. pneumophila* serogroup 1 with that of *Salmonella* sp. which have similar patterns to *E. coli*. Nolte *et al.* (1986) reported that the LPS profile of *L. pneumophila* contains 3-4 bands for each band present in the LPS profile of *Salmonella* sp. The characteristic narrow distance between individual bands of the ladder-like pattern in the lower region of the *L. pneumophila* LPS profiles is indicative of a small monosaccharide repeating unit in the polysaccharide chain. Knirel *et al.* (1996) have determined that *L. pneumophila* LPS contains a core oligosaccharide which is a highly O-acetylated heptasaccharide. To date, the latter has not been reported in the LPS of other Gram negative bacteria. The O-acetylated groups endow the outer region of the LPS core with a characteristic hydrophobicity. The latter is also a characteristic of the attached polysaccharide chain. The presence of highly O-acetylated groups in the LPS has been shown to induce a high immunogenic response from polyclonal antibodies (Knirel *et al.*, 1996).

The current studies have shown that growth conditions can affect the LPS profile of *L. pneumophila* serogroup 1. Each profile was characterised by an unstained or a slightly stained band which separated it into upper and lower regions. The LPS profile of intra-amoebic grown legionellae consisted of distinct bands, whereas those of broth grown and intra-monocytic grown legionellae showed diffuse bands. The lower region of each of the profiles contained 8 narrow, compact bands in a ladder-like pattern. These bands represent short chains of LPS with low numbers of sugar repeats. Barker

et al. (1993), have reported differences in the LPS profiles of broth grown and intra-amoebic grown *L. pneumophila,* which were confined to the lower region of the LPS profile. These workers reported that the LPS profile of broth grown *L. pneumophila* contains 5-8 bands in the lower region and that intra-amoebic grown legionellae has 10 bands. No bands were reported in the upper region or an unstained band. Nolte *et al.* (1986) on the other hand, showed the LPS profile of broth grown *L. pneumophila* serogroup 1 (sub-group Knoxville) to contain 12 discrete bands in the lower region, whilst Gabay and Horwitz (1985) reported *ca.* 10 bands. None of these groups of workers reported the presence of bands in the upper region of the LPS profile. The source of the bands in the upper region LPS profiles observed in the current study is at present unknown. It is possible, however, that the bands are due to long chains containing large numbers of sugar repeats (Chart, 1994).

The differences between the LPS profile observed in the current study and those reported in the literature could be due in part to the modified method of LPS preparation employed. Proteinase K was used in the current study to extract LPS from previously obtained sarkosyl insoluble OM preparations. This reduced considerably the quantity of protein requiring degradation. In contrast, Nolte *et al.* (1986) and Barker *et al.* (1993) both extracted LPS from whole cell bacterial lysates under identical conditions using the same concentration of proteinase K employed in the current study. The method of these workers resulted in poor resolution of bands in the upper region of the LPS profiles. This would suggest that the technique of proteinase K extraction of previously prepared OM reduces protein contamination of the LPS, resulting in better resolution of bands in the upper region of the LPS profile. Chart (1994) reported that smearing of LPS profiles was due to insufficient proteinase K digestion. Conlan and Ashworth (1986) extracted LPS from *L. pneumophila* serogroup 1 (sub-group Corby) by the phenol-water procedure of Westphal and Jann (1965), followed by the independent dialysis of the aqueous and phenol phases. This resulted in a ladder-like pattern typical of smooth LPS being observed throughout the profile. Conlan and Ashworth (1986) suggested the absence of higher molecular weight bands in the

profiles reported by Gabay and Horwitz (1985) may have been due to these workers running too small a sample, or using an extraction technique which possibly selects only low molecular weight species of LPS. Further comparative studies are required to establish whether proteinase K extraction of whole bacteria is a satisfactory method for revealing the LPS ladder pattern upon analysis by SDS-PAGE, or whether extraction of the sarkosyl insoluble OM would give more defined profiles, that is with less protein contamination.

It is possible that the unstained band, or slightly stained band, observed in the LPS profiles of variously grown *L. pneumophila* is due to the presence of MOMP. Hindahl and Iglewski (1986) have reported similar profiles upon silver staining of proteinase K extracted LPS of broth grown *L. pneumophila* serogroup 1. These workers also observed an unstained band in a similar position and molecular weight as that observed in the current study. When an equivalent LPS profile was stained with Coomassie blue, the band which was unstained by silver, was intensely stained indicating the presence of a protein. Hindahl and Iglewski, (1986) determined the band as the MOMP. Proteinase K resistant proteins have also been reported in association with the glycan backbone of *L. pneumophila* peptidoglycan (Butler *et al.*, 1985 and Hindahl and Iglewski 1986). The MOMP has been reported to be tightly associated with the LPS of the OM. Gabay and Horwitz (1985) reported that the only contaminant of the LPS in their work was due to one protein, i.e the MOMP. This tight association between LPS and the MOMP has also been demonstrated in *Neisseria gonorrhoeae* and *Haemophilus influenzae* (Gulig *et al.*, 1983 and Barthe *et al.*, 1988). It is possible, therefore, that the MOMPs of *L. pneumophila* and other Gram negative bacteria are resistant to degradation by proteinase K. The reasons for this abnormal resistance are unclear but it may arise from the observed tight association of the MOMP and LPS of these bacteria.

Immunoblotting of anti-*Legionella* antibodies raised in rabbits against the sarkosyl-insoluble OMP antigens and supernatant antigens suggested that the major antigen of *L. pneumophila* is located in the LPS. Immunoblotting of the antibodies against the

LPS antigens of the variously grown *Legionella* confirm this finding. Strong binding occurred in the lower region of all the LPS profiles with the ladder-like banding pattern being clearly defined. Binding of the antibodies to the upper regions of the profiles was less defined with no specific banding. Similar antibody binding behaviour has been reported by Conlan and Ashworth (1986) and Nolte *et al.* (1986). Nolte *et al.* (1986) reported that rabbit antiserum raised against *L. pneumophila* serogroup 1 (sub-group Knoxville) binds in a similar manner to the LPS profiles of nine *L. pneumophila* serogroup 1 strains. Conlan and Ashworth (1986) investigated the relationship between LPS and serogroup specificity of *L. pneumophila*. Antisera to serogroups 1 and 3 antigens were prepared by immunising rabbits. The lower region of the LPS profile was specifically targetted by the rabbit raised antibodies with well defined bands in evidence. The authors concluded that the staining of the immunoblots demonstrated that the serogroup specificity of *L. pneumophila* is in fact the O-specificity of its LPS. They also suggested that the absence of antibody response towards OMP bands of high molecular weight may be due to either inefficient transfer to the membrane or to a lack of sensitivity arising from a low antigen concentration in this area. No binding of antibodies to the unstained band of the LPS or the MOMP band of the OMP profile of the variously grown *L. pneumophila* was observed in the current study. This suggests that the MOMP is not an antigen recognised by the anti-legionellae antibodies raised in rabbits during this investigation.

The ELISA analysis of antisera from patients infected with *L. pneumophila* serogroup 1 conducted as part of this study showed that each antisera recognised the LPS antigens as the major antigen within the OM preparation. The binding was not, however, as strong as that of rabbit raised sera and possibly due to a lower antibody titre in the patient sera. Unfortunately, these results were not confirmed by the immunoblotting analysis. The inconclusive nature of these results may be due to a number of factors. It is possible that the patient sera recognise different antigens of the LPS to those by the rabbit sera. They could also be a reflection of the source of the sera. The rabbit sera was raised in laboratory bred rabbits against legionellae and

amoebae and U937 monocytic cells grown under carefully controlled conditions. Thus the antibodies raised in this manner can be considered to be highly specific in nature. Patient sera, on the other hand , would probably contain antibodies against many different bacterial infections. Some of those could influence the binding behaviour of patient raised anti-*Legionella* antibodies with the LPS antigens of the variously grown *L. pneumophila*. For example, cross reactions have been reported in patients with anti-*Campylobacter* antibodies and *Legionella* antigens, possibly because of similar LPS antigens (Personal communication, J. Barker). It may also be possible that the results were inconclusive due to the small quantities of patient sera made available for the work, and the consequential non-standard method of immunoblotting which had to be employed.

There have been very few reports on the interaction of patient sera with OMP and LPS antigens. Sampson *et al.* (1986) investigated the response of sera from patients with confirmed *L. pneumophila* serogroup 1 disease towards whole cell protein antigens of 14 legionellae species or serogroups. It was suggested that a 58 kDa protein antigen found in all *Legionella* sp. was especially prominant after immunoblotting and would be a prime candidate as a specific antigen for the serologic confirmation of legionellosis. However, these workers appear not to have considered the LPS as a possible source of the antigen.

Gabay and Horwitz (1985) investigated the response of patient sera towards the outer membrane and LPS antigens of broth grown *L. pneumophila* serogoup 1 (sub-group Philadelphia). These workers studied the sera of six patients who had laboratory evidence of infection by *L. pneumophila* serogroup 1. To investigate what proportion of patient anti-*L. pneumophila* antibodies were directed against the two major components of the bacterial outer membrane, the MOMP and LPS, Gabay and Horwitz (1985) assessed the capacity of purified MOMP and LPS to inhibit fluorescence in an Indirect Fluorescence Antibody (IFA) assay. The IFA titre of each patient sera was determined before and after incubation with excess amounts of LPS and MOMP. These

authors found that pre-incubation with excess amounts of MOMP had no influence on the IFA titre. In contrast, an excess amount of LPS reduced the IFA titre by > 98 %. These authors concluded the great majority of patient antibodies that recognise *L. pneumophila* serogroup 1 are directed against the LPS of this strain. Whilst this is in agreement with the general findings of the current investigation, the experimental design used in their acquisition is possibly flawed. Gabay and Horwitz (1985) determined the response of patient anti-*L. pneumophila* antibodies against the MOMP and LPS of broth grown *Legionella.* It is highly probable that the original bacteria inhaled by the patient would be intra-amoebic grown *L. pneumophila.* Thus the patient antibodies would have been initially raised against intra-amoebic grown *L. pneumophila* and subsequently against intra-monocytic grown *Legionella.* Immunoblotting should, therefore, have been carried out against the MOMP and LPS of intra-amoebic and intra-monocytic grown *L. pneumophila* and not those of broth grown *Legionella.*

6.6 CONCLUSIONS

A preliminary investigation into the effect of growth conditions on the surface structure of *L. pneumophila* (serogroup 1) has been carried out.

The following conclusions can be drawn from observations made during the above work:

- Growth conditions influence the surface composition of *L. pneumophila* (serogroup 1).
- The OMP profiles of intra-amoebic and intra-monocytic grown *L. pneumophila* each contained one novel protein in addition to the MOMP. Broth grown legionellae contained four additional sarkosyl insoluble OMPs.
- 'Ageing' of intracellular grown *L. pneumophila* results in the loss of the novel protein.

CHAPTER 7. GENERAL DISCUSSION.

A comprehensive study has been undertaken to examine the hypothesis that intracellular replication of *L. pneumophila* induces enhanced stress resistance relative to legionellae grown exclusively *in vitro.* The work presented in this thesis has shown that this hypothesis is correct. Intracellular replication, in either *A. polyphaga* or U937 monocytes, induced an enhanced resistance to all the stress conditions studied, relative to that of YE broth grown legionellae. It has also been shown that the growth phase of broth grown legionellae has an effect on resistance to stress. Bacteria in the stationary phase of growth exhibited a consistently higher resistance to stress than those in the exponential phase. 'Ageing' of intracellular grown *L. pneumophila* induced a fall in stress resistance to a level similar to that of broth grown bacteria in stationary phase.

Microscopical studies have shown that growth conditions also had a marked effect on the morphology and motility of *L. pneumophila*. Broth grown legionellae were rod-shaped and non-motile. Intracellular grown legionellae were small, rounded and highly motile. If allowed to remain in coculture for more than 24 h. after release from the host cell ('ageing'), the intracellular grown legionellae were observed to elongate into rod-shapes and to lose their motility.

A preliminary examination of the surface composition of the variously grown *L. pneumophila* revealed that freshly harvested intra-amoebic and intra-monocytic grown *Legionella* each had one extra sarkosyl insoluble OMP, in addition to the 28 kDa MOMP, common to all *L. pneumophila*. Intra-amoebic grown legionellae had a unique 15 kDa OMP, whereas intra-monocytic grown bacteria had an OMP of 24 kDa. Upon 'ageing' of the intracellular grown bacteria, the 15 kDa and 24 kDa were lost from the protein profiles. Broth grown *L. pneumophila* were observed to contain four OMPs of 42, 78, 98 and 106 kDa, in addition to the MOMP.

The above observations suggest that the enhanced resistance to environmental stress of intracellular grown *L. pneumophila*, relative to that of broth grown *Legionella*, could be due, at least in part, to a difference in the surface proteins of the bacteria. It is widely accepted that an intracellular environment is considerably 'harsher', with respect to a bacterium, than that of most media used for *in vitro* growth (Welch et al., 1991). It may be suggested, therefore, that successful intracellular replication necessitates the simultaneous adaptation of the bacteria to a multitude of stress parameters and that this 'pre-adapts' them to subsequent external stress stimuli. An examination of the intracellular environment and its influence upon the replication of *L. pneumophila* is outside the remit of this thesis . It is possible, however, to speculate upon the processes involved, and their effect on the subsequent response of *L. pneumophila* to external stress stimuli, by consideration of the published literature.

Infection of host cells, whether protozoan or mammalian, by bacteria is a complex dynamic interaction. It is characterised by a series of recognition events and phenotypic alterations during which both the host cell and the microbe undergo a process of mutual recognition and adaptation. This process is often referred to as the 'adaptive response'. The adaptive response of bacteria involves the switching on / off of a series of genes which are located in specific, well defined regulatory networks that respond to environmental stress. A typical regulatory mechanism of these networks involves the modification of sigma factors, whose primary role is to bind the core RNA polymerase, conferring gene promoter specificity. Many networks , such as the heat shock response, are associated with σ^{32}. The mechanism of induction of the heat shock response by bacteria is well documented (Dougan, 1989; DiRita and Mekalanos, 1989; Finlay and Falkow, 1989 and Smith, 1990). It consists of three phases, that in concert, rapidly elevate the level of σ^{32} upon a rise in temperature : (i) decreased degradation of the sigma factor, (ii) increased synthesis of messenger RNA, and (iii) increased translation as compared to that at 'normal' temperature. This leads to the increased synthesis of bacterial heat shock proteins (HSP).

The selective synthesis of heat shock proteins during growth is a common feature of all intracellular bacteria, including *S. typhimurium* (Buchmeier and Heffron, 1990), *M. tuberculosis* (Lathigra *et al.*, 1991) and *B. abortus* (Lin and Ficht, 1995). All *Legionella* sp. and serogroups have been reported as expressing increased quantities of a HSP of mass 58-60 kDa during intracellular replication (Hoffman *et al.*, 1989; 1990 and Sampson *et al.*, 1990). The presence of increased quantities of HSP in intracellular grown *L. pneumophila* could account for the greater resistance to elevated temperatures of these bacteria, as reported in this thesis. It may also account for the increased resistance of intra-amoebic grown *L. pneumophila* to heat-, osmotic-, oxidative- and acid-shock reported by Abu Kwaik *et al.* (1997). The HSP60 has been shown to be located in the cytoplasmic fraction (Gabay and Horwitz, 1985) but may localise to the bacterial cell surface when the bacteria are intracellular (Plikaytis *et al.*, 1987; Hoffman *et al.*, 1989; 1990 and Sampson *et al.*, 1990). The current study failed to isolate the 60 kDa HSP. However, the extraction method used was aimed at producing a general sarkosyl insoluble protein profile of the OM and not the isolation of a specific protein. Plikaytis *et al* (1987) and Hoffman *et al.* (1989) extracted the 60 kDa HSP from whole *Legionella* cells and purified the protein using density gradients and ammonium sulphate precipitation.

It has been reported that HSPs act as chaperones protecting the bacteria from the potential disruptive effects of unfolded peptides (Lathigra *et al.*, 1991). The unfolding of peptides represents an important threat to bacterial survival, especially within host cells. A corresponding increase in HSP synthesis, in order to neutralise any increase in concentrations of unfolded polypeptides, is essential for bacterial survival. It can be argued that any stress which results in increased levels of unfolded polypeptides by, for example, altering patterns of protein synthesis, translocation, or causing denaturation of proteins within the cell, will be countered by induction of HSPs. It follows, therefore, that the presence of HSPs will be increased by parameters other than a rise in temperature. For example, bacteria which enter host phagocytes will be exposed to a variety of reactive oxygen metabolites that will cause the unfolding of peptides. The

ability of bacteria to survive such an exposure may play an important role in the infective process. Part of the survival strategy would be the activation of various regulatory networks which are induced by different forms of oxidative damage; for example, the *oxyR* network (hydrogen peroxide), the *soxRS* network (superoxide), the *fur* network (iron uptake regulation) and the *rpoS* network (hydrogen peroxide, exonucleotides). The latter network has been reported as inducing a DNA binding protein to protect the bacteria against oxidative damage (Eisenstark *et al.*, 1995). It has also been reported that oxidative agents and certain antibiotics can induce each others regulatory networks within bacteria. For example, H_2O_2 can induce both the *soxRS* and *mar* regulatory network, the latter regulates genes for antibiotic resistance in *E. coli.* In a similar manner, certain antibiotics can induce synthesis of proteins under *soxRS* regulation (Cohen *et al.*, 1993 and Ariza *et al.*, 1994).

The composition of intracellular environments are, as yet, largely unknown. However, it is generally accepted that they are nutrient depleted in comparison with most of the media used for *in vitro* growth. Exposure of a bacterium to a nutrient depleted environment induces a further general regulatory network response, often termed the 'stringent response'. The stringent response is characterised by many factors, including adaptation by the bacterium of a smaller, more rounded morphology (Torella and Morita, 1981; Roszak and Colwell, 1987 and Barer, 1993), modification of the fatty acid composition (Malmcrona-Friberg *et al.*, 1986) and the onset of resistance to a variety of stress conditions (Nystrom *et al.* 1992 and Holmquist and Kjelleberg, 1993).

Morphological studies reported in this thesis have shown that broth grown and 'aged' intracellular grown *L. pneumophila* become smaller and more rounded upon starvation. This suggests that these bacteria exhibit a stringent response in an endeavour to survive in the absence of nutrients. The enhanced survival of intracellular grown legionellae during exposure to starvation, and the apparent lack of morphological change during this period, suggests that prior exposure to a nutrient depleted intracellular environment has primed the bacteria for survival. That is, the requisite regulatory

networks have been activated and the proteins necessary for starvation resistance have been induced during intracellular growth.

Further evidence of the role of the stringent response in determining the resistance of *L. pneumophila* to stress may be found in the observation that broth grown bacteria in stationary phase are always more resistant to external stress stimuli than those in exponential phase. The stationary phase of growth of *L. pneumophila* in broth cultures is brought about by, amongst other factors, nutrient depletion. It can be suggested, therefore, that the onset of this growth phase is accompanied by a limited form of stringent response. Activation of the stringent response regulatory networks would lead to the synthesis of a number of stress proteins. It is suggested that these proteins impart an enhanced resistance to external stress stimuli upon the *L. pneumophila* relative to bacteria in the exponential phase of growth.

It has been shown that the enhanced resistance of intracellular grown *L. pneumophila* to external stress stimuli is lost upon 'ageing'. The 'ageing' process is characterised by the apparent 'disappearance' of the novel proteins arising from intracellular replication and a change in morphology to one more typical of *in vitro* growth. Evidence presented in this thesis, and re-examination of the data published by Barker *et al.* (1992) leads to the suggestion that *L. pneumophila* may scavenge cell debris immediately after host cell lysis, in the absence of other viable host cells. It is tentatively suggested that, relative to the intracellular environment, the lysed host cell debris represents a nutrient rich environment and that the bacteria adapt accordingly. Some, or all, of the stress proteins expressed as a result of the intracellular environment would be expected to be repressed with a concomitant loss of resistance to stress. Adaptation to a richer nutrient environment would also be accompanied by a change in morphology to one more typical of *in vitro* grown bacteria. The OMP profiles of the 'aged' bacteria did not contain the high molecular weight proteins observed in the profiles of broth grown *L. pneumophila*. This would suggest that, although the

morphology of 'aged' bacteria is similar to that of broth grown legionellae, the two forms of *L. pneumophila* have different surface compositions.

The concept of a single network regulating the entire response of a bacterium to stress, the global stress response, has been debated for many years. However, it is only recently that a small number of genes with this capacity have been identified. *RpoS* (also termed σ^{38}, σ^{S}, *katF*) has now been identified as such a gene in *E. coli* and *S. typhimurium* (Jishage and Ishihawa, 1995; Soo Bang *et al.*, 1995; Soo Lee *et al.*, 1995; Eraso *et al.*, 1996 and Levinthal and Pownder, 1996). Early studies indicated that the main function of *rpoS* is to regulate genes that protect cells during dormancy (McCann *et al.*, 1991 and Loewen and Hengge-Aronis, 1994). Subsequent studies showed that, for example, in *E. coli rpoS* may regulate 30 or more other genes. The proteins synthesised by these regulated genes cover many aspects of cellular activity, (Figure 7.1). *RpoS* has recently been reported in *Legionella* sp. (Personal communication J. Barker) but its role has not been elucidated. It may be speculated that its role will be similar to that of *rpoS* in *E. coli* and *S. typhimurium*.

A global regulator operating in a similar manner to that proposed for *rpoS* in *E. coli* has been identified in *L. pneumophila* by Abu Kwaik *et al.* (1994; 1997). These workers have described a protein with a molecular weight of 19 kDa which is induced primarily during intracellular replication and to a lesser extent by a wide variety of *in vitro* stress stimuli. This they termed the 'global stress protein', GspA. The *gspA* gene is reported as being regulated by two overlapping promoters, the heat shock transcriptional factor, σ^{32}, and the oxidative stress transcription factor, σ^{70}. It is possible that there is a coordinated regulatory linkage between the two factors, similar to that between *rpoS* (σ^{38}) and *oxyR* (σ^{70}) in *E. coli* (Eisenstark *et al.*, 1995). The major advantage for *gspA* and other genes having multiple promoters is that each promoter can be regulated differently. This would allow the bacteria to control the induction of regulatory networks through either promoter, in response to certain environmental stress stimuli. A detailed investigation into the role of *gspA* in the stress

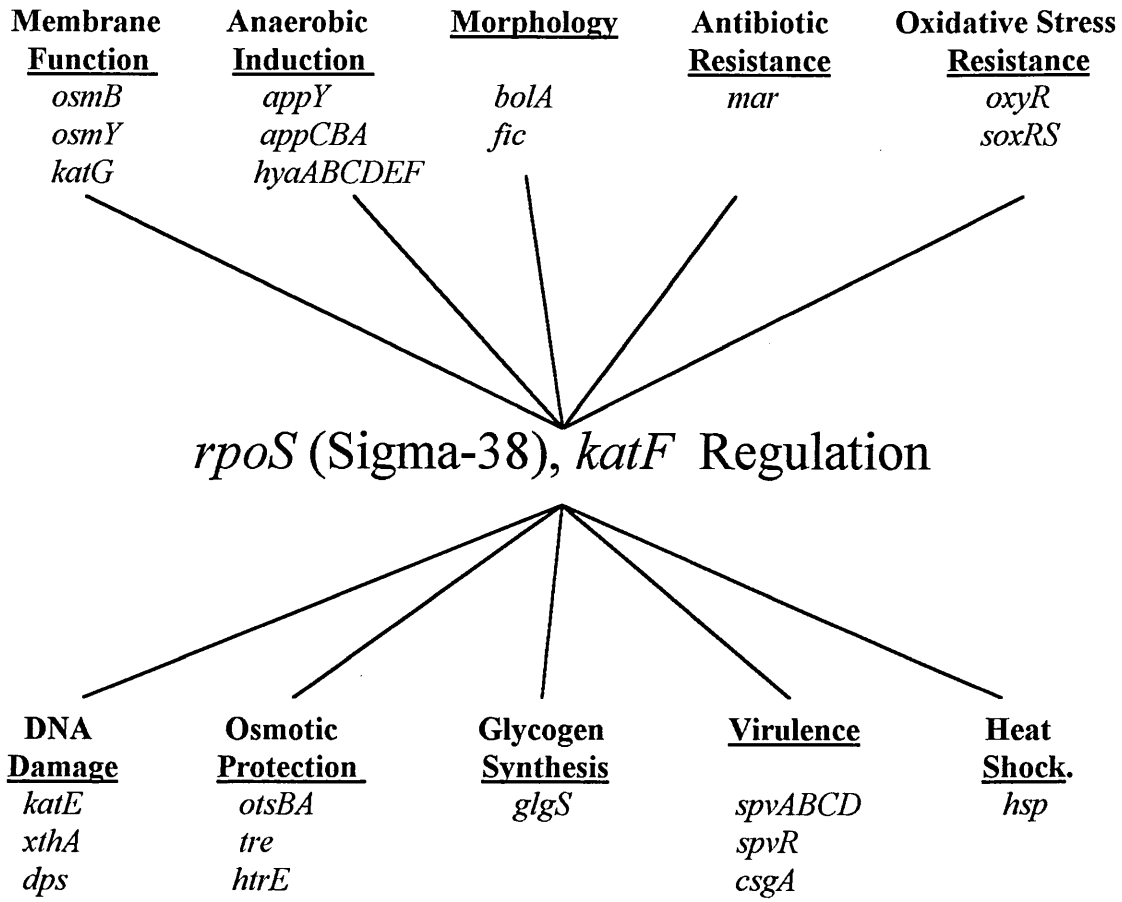


Figure 7.1. Genes regulated by *rpoS* in *Escherichia coli.*

resistance of *L. pneumophila* has been reported by Abu Kwaik *et al.* (1997). These authors showed that the level of *gspA* present in *L. pneumophila* throughout intracellular replication was many times greater than that induced within broth grown *Legionella* by any single *in vitro* stress stimulus. To test the hypothesis that *gspA* expression imparts an almost universal stress resistance, this group of workers formed *gspA* insertion mutants within broth grown *L. pneumophila*. The stress resistance of these mutants was found to be considerably less than that of the wild-type bacteria. Restoration of the gene restored stress resistance.

Abu Kwaik *et al.* (1997) suggested that *gspA* is constitutively induced throughout intracellular infection, but they do not present any evidence to support this claim. It is well known that intracellular replication of legionellae involves the generation of very high numbers of bacteria which fill the phagosome of the host cell. It may be suggested, therefore, that *gspA* expression during intracellular replication of *L. pneumophila,* and thus the acquisition of enhanced stress resistance, is controlled by a quorum sensing mechanism similar to that identified in a number of other Gram negative bacteria including *S. typhimurium, E. coli, P. aeruginosa* and *Erwinia carotovora* (Jones *et al.*, 1993 and Surette and Bassler, 1998). It is also possible that the onset of a variety of responses, including motility and the acquisition of the novel OMP proteins by *L. pneumophila* during intracellular replication, are also triggered by a quorum sensing effect. Quorum sensing is a cell density dependent mechanism of autoinduction of regulatory networks within a population of replicating bacteria. Replicating bacteria secrete small signalling molecules, or pheromones, which are detected by neighbouring bacteria. Once a bacterium senses more than a critical level of the signalling molecule in its immediate environment, it triggers the induction of stress regulatory networks. This results in the secretion of further signalling molecules, which in turn leads to the induction of neighbouring bacteria. This creates a positive feedback effect that can generate a large and rapid coordinated response by the bacterial population to a small initial stimulus.

Referring back to the aims of the project, evidence has been gained which clearly indicates that intracellular replication within *A. polyphaga* trophozoites and U937 monocytic cells results in a form of *L. pneumophila* whose phenotype is more resistant to external stress than that of legionellae grown in broth culture. The enhanced resistance properties of the intracellular bacteria are lost upon 'ageing'. A preliminary analysis of the OMP profiles of the variously grown bacteria has revealed the presence of a novel OMP in each of the freshly harvested intracellular grown *L. pneumophila*. The loss in stress resistance by these bacteria upon 'ageing' is concomitant with a loss of these proteins. Further work must, therefore, consider the origin of these novel proteins and their role in the enhanced resistance to stress of intracellular grown *L. pneumophila*. In this respect it is suggested that the following work be undertaken :

- The preliminary surface composition analysis reported in this thesis only examined the sarkosyl insoluble OMP profile and the LPS of each type of *L. pneumophila*. This should be repeated and extended to include an analysis of the whole cell protein profile and the fatty acid composition using standard laboratory techniques. By comparing and contrasting these results, it should be possible to identify structural components which induce enhanced stress resistance in intracellular grown *L. pneumophila.*

- The work presented in this thesis suggests that the enhanced stress resistance of intracellular grown *L. pneumophila* is associated with novel proteins. If it is assumed that this is correct, then the origin of these proteins must be established. Evidence has been presented which suggests two possible sources for the novel proteins, expression by the bacteria itself during intracellular replication or from the host cell. A possible approach to identify the origin of the novel proteins would involve the determination of the amino acid sequence of similar sized proteins extracted from both the bacteria and the host cell. Comparison of the sequences should indicate whether or not the proteins have a common origin. The sequences would be obtained by extraction of the protein band from the respective profile

followed by sequencing using a microsequencer. Further evidence would be obtained by synthesising oligonucleotides from the amino acid sequences and using them as DNA probes during Southern blot hybridisations.

- If the novel proteins were proved to be bacterial in origin, then it would be necessary to establish, beyond all reasonable doubt, that the enhanced stress resistance is due to these proteins alone. The experimental approach would be similar to that of Abu Kwaik *et al.* (1997). The genes encoding the novel proteins would be cloned and mutant *L. pneumophila,* which did not have the capacity to form the novel proteins, derived. The stress assays would be repeated and the responses of the mutants compared to the wildtypes.

- If the enhanced stress resistance of intracellular grown *L. pneumophila* is shown to be due to the presence of the novel proteins, then the regulatory network determining their expression must be identified. A recent approach by Abu Kwaik *et al.* (1997) has identified a global stress protein (GspA) within intra-amoebic grown *L. pneumophila* which regulated, in part, the enhanced stress resistance of these bacteria. This work would be repeated using the same techniques and include both intra-amoebic and intra-monocytic grown *L. pneumophila.*

- Irrespective of whether the novel proteins are bacterial or host cell in origin, the point at which the *L. pneumophila* acquires the protein must be determined. Theoretically this should be relatively easy. Infected host cells would be lysed at specific time intervals and the protein profiles of the bacteria determined. In practice, however, such an investigation would be very difficult. Infection of host cells by *L. pneumophila* in coculture is an asynchronous process. Thus it would be very difficult to obtain bacterial populations which were all at the same point of replication.

CHAPTER 8. REFERENCES.

Abshire, K. Z. and Neidhardt, F. C. (1993). Analysis of proteins synthesised by *Salmonella typhimurium* during growth within host macrophage. *Journal of Bacteriology*. **175**, 3734-3743.

Abu Kwaik, Y. (1996). The phagosome containing *Legionella pneumophila* within *Hartmannella vermiformis* is surrounded by the endoplasmic reticulum. *Applied and Environmental Microbiology*. **62**, 2022-2028.

Abu Kwaik, Y. (1998). Induced expression of the *Legionella pneumophila* gene encoding a 20-kilodalton protein during intracellular infection. *Infection and Immunity*. **66,** 203-212.

Abu Kwaik, Y. and Engleberg, N. C. (1994). Cloning and molecular charcterisation of an *Legionella pneumophila* gene induced by intracellular infection and by various *in vitro* stress conditions. *Molecular Microbiology*. **13** 243-251.

Abu Kwaik, Y., Eisenstein, B. I. and Engleberg, N. C. (1993). Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. *Infection and Immunity*. **61,** 1320-1329.

Abu Kwaik, Y., Gao, L., Harb, O. S. and Stone, B. J. (1997). Transcriptional regulation of the macrophage-induced gene (*gspA*) of *Legionella pneumophila* and phenotypic characterisation of a null mutant. *Molecular Microbiology*. **24,** 629-642.

Abu Kwaik, Y., Gao, L., Stone, B. J., Venkataraman, C. and Harb, O. S. (1998). Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. *Applied and Environmental Microbiology*. **64,** 3127-3133.

Al-Hiti, M. M. A. and Gilbert, P. (1980). Changes in preservative sensitivity of the USP antimicrobial agents effectiveness test for microorganisms. *Journal of Applied Bacteriology*. **49**, 119-126.

Allen-Austin, D., Austin, B. and Colwell, R. R. (1984). Survival of *Aeromonas salmonicida* in river water. *FEMS Microbiology Letters*. **21,** 143-146.

Amano, K. and Williams, J. (1983). The partial characterisation of peptidoglycan - associated proteins of *Legionella pneumophila. Journal of Biochemistry*. **94,** 601-606.

Ames, G. F. (1974). Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. *Journal of Biological Chemistry*. **249,** 634-644.

Anand, C. M., Skinner, A. R., Malic, A. and Kurtz, J. B. (1983). Interaction of *Legionella pneumophila* and a free living amoeba (*Acanthamoeba palestinesis*). *Journal of Hygiene*. **91,** 167-178

Anwar, H., Brown, M. R. W., Day, A. and Weller, P. H. (1984). Outer membrane antigens of mucoid *Pseudomonas aeruginosa* isolated directly from the sputum of a cystic fibrosis patient. *FEMS Microbiology Letters*. **24**, 235-239.

Anwar, H., Shand, G. H., Ward, K. H., Brown, M. R. W., Alpar, K. E. and Gower, J. (1985). Antibody response to acute *Pseudomonas aeruginosa* infection in a burn wound. *FEMS Microbiology Letters*. **29,** 225-230.

Anwar, H., Dasgupta, M. K. and Costerton, J. W. (1990). Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrobial Agents and Chemotherapy*. **34,** 2043-2046.

Ariza, R. R., Cohen, S. P., Bachhawat, N., Levy, S. B. and Demple, B. (1994). Repressor mutations in the *marRAB* operon that activate oxidative stress genes and

multiple antibiotic resistance in *Escherichia coli. Journal of Bacteriology.* **176,** 143-148.

Armon, R. and Payment, P. (1990). A transparent medium for isolation of *Legionella pneumophila* from environmental water sources. *Journal of Microbiological Methods.* **11,** 65-71.

Badenoch, J. P. (1986). First report of the Committee of Inquiry into the outbreak of Legionnaires' disease in Stafford in April 1985. Her Majesty's Stationary Office, London.

Bakken, L. and Olsen, R. (1987). The relationship between cell size and viability of soil bacteria. *Microbial Ecology.* **13,** 103-114.

Baltch A., Smith, R. P. and Ritz, W. (1995). Inhibitory and bactericidal activities of levofloxacin, ofloxacin, erythromycin and rifampicin used singly and in combination against *Legionella pneumophila. Antimicrobial Agents and Chemotherapy.* **39,** 1661-1666

Bangsborg, J. M., Shand, G., Pearlman, E. and Hoiby, N. (1991). Cross-reactive *Legionella* antigens and the antibody response during infection. *APMIS.* **99,** 854-865.

Bantroch, S., Buhler, T. and Lam, J. S. (1994). Appropriate coating methods and other conditions for enzyme-linked immunosorbent assay of smooth, rough and neutral lipopolysaccharides of *Pseudomonas aeruginosa. Clinical and Diagnostic Laboratory Immunology.* **1,** 55-62.

Barbaree, J. M., Fields, B.S., Feeley, J. C., Gorman, G. W. and Martin, W. T. (1986). Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila. Applied and Environmental Microbiology.* **51**, 422-424.

Barer, M. R. (1997). Viable but non-culturable and dormant bacteria: time to resolve an oxymoron and a misnomer? *Journal of Medical Microbiology*. **46**, 629-631.

Barer M. R., Gribbon, L. T., Harwood, C. R. and Nwoguh, C. E. (1993). The viable but non culturable hypothesis and medical bacteriology. *Reviews in Medical Microbiology*. **4,**183-191.

Barker, J. and Farrell, I. D. (1990). The effects of single and combined antibiotics on the growth of *Legionella pneumophila* using time kill studies. *Journal of Antimicrobial Chemotherapy*. **26,** 45-53.

Barker J. and Brown, M. R. W. (1994). Trojan horses of the microbial world : Protozoa and the survival of bacterial pathogens in the environment. *Microbiology*. **140,** 1253-1259.

Barker, J. and Brown, M. R. W. (1995). Speculation on the influence of the phenotype on virulence and antibiotic susceptibilty of *Legionella pneumophila*. *Journal of Antimicrobial Chemotherapy*. **36,** 7-21.

Barker J., Farrell, I. D. and Hutchinson, J. G. P. (1986). Factors affecting growth of *Legionella pneumophila* in liquid media. *Journal of Medical Microbiology*. **22,** 97-100.

Barker, J., Brown, M. R. W., Collier, P. J, Farrell, I. D. and Gilbert, P. (1992). Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga* : Physiological status and susceptibility to chemical inactivation. *Applied and Environmental Microbiology*. **58,** 2420-2425.

Barker J., Lambert, P. A. and Brown, M. R. W. (1993). Influence of intra-amoebic and other growth conditions on the surface properties of *Legionella pneumophila*. *Infection and Immunity*. **61,** 3503-3510.

Barthe, C., Joly, J R., Ramsay, D., Boissinot, M. and Benhamou, N. (1988). Common epitope on the lipopolysaccharide of *Legionella pneumophila* recognised by a monoclonal antibody. *Journal Clinical Microbiology*. **26,** 1016-1023.

Bartlett, C. L. R. and Bibby, L. F. (1983). Epidemic legionellosis in England and Wales 1979-1982. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene I. Abteilung: Originale A.* **255**, 64-70.

Bartlett, C. L. R, Kurtz, J. B. Hutchinson, J. G. P., Turner, G. C. and Wright, A. E. (1983). *Legionella* in hospitals and hot water supplies. *Lancet i.* 1389-1390.

Bartlett, C. L. R., Swann, R. A., Casal, J., Canada-Royo, L. and Taylor, A. G. (1984). Recurrent Legionnaires' disease from a hotel water system. In *Legionella. Proceedings of the 2nd International Symposium.* (Eds. Thornsberry, C., Barlows, A., Feeley, J. C. and Jakubowski, W.) pp237-239. American Society for Microbiology, Washington D C, USA.

Bartlett, C. L. R., Macrae, A. D. and Macfarlane, J. T. (1986). *Legionella* infections. Edward Arnold, London, UK.

Bates, J. H., Campbell, G. D. and Baron, A. L. (1992). Microbial etiology of acute pneumonia in hospitalised patients. *Chest.* **101**, 1005-1012.

Beaty, H. N., Miller, A. A., Broome, C. V., Goings, S. and Phillips, C. A. (1978). Legionnaires' disease in Vermont, May to October, 1977. *Journal of American Medical Association.* **240,** 127-131.

Bellinger-Kawahara, C. and Horwitz, M. A. (1990). Complement component C3 fixes selectively to the major outer membrane protein (MOMP) of *Legionella pneumophila* and mediates phagocytosis of liposome-MOMP complexes by human monocytes. *Journal of Experimental Medicine*. **172,** 1201-1210.

Bender, L., Ott, M., Debes, A., Rdest, U., Hessemann, J. and Hacker, J. (1991). Distribution, expression, and long-range mapping of legiolysin gene (*lly*)-specific DNA sequences in legionellae. *Infection and Immunity*. **59**, 3333-3336.

Berger, K. H. and Isberg, R. R. (1993). Two Distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila. Molecular Microbiology* **7**, 7-19.

Berger, K. H., Merriam, J. J. and Isberg, R. R. (1994). Altered intracellular targetting properties asscoiated with mutations in the *Legionella pneumophila dotA* gene. *Molecular Microbiology.* **14,** 809-822.

Berk, S. G., Ting, R. S., Turner, G. W. and Ashburn, R. J. (1998). Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* sp. *Applied and Environmental Microbiology.* **64,** 279-286.

Beveridge, T. J. (1988). The bacterial surface : General considerations towards design and function. *Canadian Journal of Microbiology.* **34,** 363-372.

Beveridge, T. J. and Koval, S. F. (1981). Binding of metals to cell envelopes of *Escherichia coli* K-12. *Applied and Environmental Microbiology*. **42,** 325-335.

Bhardwaj, N., Nash, T. W. and Horwitz, M. A. (1986). Interferon-gamma-activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila. Journal of Immunology*. **137,** 2662-2669.

Bhopal., R. S., Fallon, R. J., Buist, E. C., Black, R. J. and Urquhart, J. D. (1991). Proximity of the home to a cooling tower and the risk of non-outbreak Legionnaires' disease. *British Medical Journal*. **302**, 378-383.

Biddick, C. J., Rogers, L. H. and Brown, T. J. (1984). Viability of pathogenic and non-pathogenic free-living amoebae in long-term storage at a range of temperatures. *Applied and Environmental Microbiology*. **48**, 859-860.

Bitton, G., Koopman, B., Jung, K., Voiland, G. and Kotob, M. (1993). Modifications of the standard epifluorescence microscopic method for total bacterial counts in environmental samples. *Water Research*. **27,** 1109-1112.

Blackmon, J. A., Hicklin, M. D. and Chandler, F. W. (1978). Legionnaires' disease. Pathological and historical aspects of a "new" disease. *Archives of Pathological Laboratory Medicine*. **102**, 337-343.

Blanchard, D. K., Klein, T. W., Friedman, H. and Stewart, W. E. (1985). Kinetics and characterisation of interferon production by murine spleen cells stimulated with *Legionella pneumophila* antigens. *Infection and Immunity*. **49,** 719-725.

Blanchard, D. K., Djeu, J. Y., Klein, T. W., Friedman, H. and Stewart, W. E. (1987a). Induction of tumour necrosis factor by *Legionella pneumophila*. *Infection and Immunity*. **55,** 433-437.

Blanchard, D. K., Stewart, W. E., Klein, T. W., Friedman, H. and Djeu, J. Y. (1987b). Cytolytic activity of human peripheral blood leukocytes against *Legionella pneumophila*-infected monocytes: characterisation of the effector cell and augmentation by interleukin II. *Journal of Immunology*. **139,** 551-556.

Blander, S. J., Szeto, L., Shuman, H. A. and Horwitz, M. A. (1990). An immunoprotective molecule, the major secretory protein of *Legionella pneumophila,* is not a virulence factor in a guinea pig model of Legionnaires' disease. *Journal of Clinical Investigation*. **86,** 817-824.

Bloomfield, S. F., Stewart, G. S. A. B., Dodd, C. E. R., Booth, I. R and Power, E. G. M. (1998). The viable but non-culturable phenomenon explained? *Microbiology.* **144,** 1-3.

Boulanger, C. A. and Edelstein, P. H. (1995). Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation. *Applied and Environmental Microbiology.* **61,** 1805-1809.

Boulanger, J., Faulds, D., Eddy, E. M. and Lingwood, C. A. (1995). Members of the 70 kDa heat shock protein family specifically recognise sulfoglycolipids: role in gamete recognition and mycoplasma-related infertility. *Journal of Cell Physiology.* **165,** 7-17.

Bozue, J. A. and Johnson, W. (1996). Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii* : Uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. *Infection and Immunity.* **64,** 668-673.

Brand, B. C., Sadowsky, A. B. and Shuman, H. A. (1994). The *Legionella pneumophila icm* locus : a set of genes required for intracellular multiplication in human macrophages. *Molecular Microbiology.* **14,** 797-808.

Breiman, R. F. (1993). Modes of transmission in epidemic and nonepidemic *Legionella* infection: directions of further study. In *Legionella : Current Status and Emerging Perspectives* (Eds., Barbaree, J. M., Breiman, R. F. and Dufour, A. P.) pp. 30-35. American Society of Microbiology, Washington, DC. USA.

Breiman, R. F., Fields, B. S., Sanden, G. N., Volmer, L. J. Meier, A. and Spika, J. (1990). Association of shower use with Legionnaires' disease. Possible role of amoebae. *Journal of the American Medical Association.* **263,** 2924-2926.

Brenner, D. J. (1986). Classification of Legionellaceae. Current status and remaining questions. *Israel Journal of Medical Science.* **22,** 655-661.

Brenner, D. J., Steigerwalt, A. G. and McDade, J. E. (1979). Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family Legionellaceae, familia nova. *Annals of Internal Medicine*. **90,** 656-658.

Brieland, J., McClain, M., Heath, L., Chrisp, C., Huffnagle, G., LeGendre, M., Hurley, M., Fantone, J. and Engleberg, C. (1996). Coinoculation with *Hartmanella vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine model of Legionnaires' disease. *Infection and Immunity*. **64**, 2449-2456.

Brieland, J., McClain, M., LeGendre, M. and Engleberg, C. (1997). Intrapulmonary *Hartmannella vermiformis* : a potential niche for *Legionella pneumophila* replication in a murine model of legionellosis. *Infection and Immunity*. **65,** 4892-4896.

Brown, M. R. W. (1975). The role of the envelope in resistance. In *Resistance of Pseudomonas aeruginosa*. (Brown, M. R. W. Eds) John Wiley and Sons, London.

Brown, M. R. W. (1977). Nutrient depletion and antibiotic susceptibility. *Journal of Antimicrobial Chemotherapy*. **3,** 198-201.

Brown, M. R. W. and Melling, J. (1969). Role of divalent cations in the action of polymyxin B and EDTA on *Pseudomonas aeruginosa*. *Journal of General Microbiology*. **59**, 263-274.

Brown, M. R. W. and Williams, P. (1985a). Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. *Journal of Antimicrobial Chemotherapy*. **15,** (Suppl. A), 7-14.

Brown, M. R. W. and Williams, P. (1985b). The influence of environment on the envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology*. **39,** 527-556.

Brown, M. R. W., Anwar, H, and Lambert, P. A. (1984). Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. *FEMS Microbiology Letters.* **21**, 113-117.

Brown, M. R. W., Collier, P. J. and Gilbert, P. (1990). Influence of growth rate on susceptibility to antimicrobial agents: Modification of the cell envelope and batch and continuous culture studies. *Antimicrobial Agents and Chemotherapy.* **34,** 1623-1628.

Buchanan, C. E. and Sowell, M. O. (1982). Synthesis of penicillin-binding protein 6 by stationary phase *Escherichia coli. Journal of Bacteriology.* **151**, 491-494.

Buchmeier, N. A. and Heffron, F. (1990). Induction of *Salmonella* stress proteins upon infection of macrophages. *Science.* **248**, 730-732.

Butler, C. A. and Hoffman, P. S. (1990). Characterisation of a major 31-kDa peptidoglycan-bound protein of *Legionella pneumophila. Journal of Bacteriology.* **172,** 2401-2407.

Butler, C. A., Street, E. D., Hatch, T. P. and Hoffman, P. S. (1985). Disulphide-bonded outer membrane proteins in the genus *Legionella. Infection and Immunity.* **48,** 14-18.

Casida, L. E. (1965). Abundant microorganisms in soil. *Applied Microbiology.* **13,** 327-334.

Catrenich, C. E and Johnson, W. (1988). Virulence conversion of *Legionella pneumophila* : a one way phenomenon. *Infection and Immunity.* **56**, 3121-3125.

Catrenich, C. E. and Johnson, W. (1989). Characterisation of the selective inhibition of growth of virulent *Legionella pneumophila* by supplemented Mueller-Hinton medium. *Infection and Immunity.* **57**, 1862-1864.

Chandler, F. W., Hicklin, M. D. and Blackmon, J. A. (1977). Demonstration of the agent of Legionnaires' disease in tissue. *New England Journal of Medicine*. **297,** 1218-1220.

Chart, H. (1994). Lipopolysaccharide : isolation and characterisation. In *Methods in practical laboratory bacteriology*. (Eds. Chart, H.) pp.11-20. CRC Press Inc., London.

Chen. S. C. A., Paul, M. L. and Gilbert, G. L. (1993). Susceptibility of *Legionella* species to antimicrobial agents. *Pathology.* **25,** 180-183.

Chidiac, C. and Mouton, Y. (1991). Quinolones in the treatment of lower respiratory tract infections caused by intracellular pathogens. *Infection.* **19,** (Suppl. 7), 365-371.

Cianciotto, N. P., Eisenstein, B. I., Mody, C. H., Toews, G. B. and Engleberg, N. C. (1989). A *Legionella pneumophila* gene encoding a species specific surface protein potentiates initiation of intracellular infection. *Infection and Immunity*. **157,** 1255-1262.

Cianciotto, N. P., Eisenstein, B. I., Mody, C. H. and Engleberg, N. C. (1990a). A mutation in the *mip* gene results in an attenuation of *Legionella pneumophila* virulence. *Journal of Infectious Diseases.* **162,** 121-126.

Cianciotto, N. P., Bangsborg, J. M., Eisenstein, B. I. and Engleberg, N. C. (1990b). Identification of *mip*-like genes in the genus *Legionella. Infection and Immunity*. **58,**. 2912-2918.

Ciesielski, C. A., Blaser, M. J. and Wang, W. L. (1984). Role of stagnation and obstruction of water flow in isolation of *Legionella pneumophila* from hospital plumbing. *Applied and Environmental Microbiology*. **48,** 984-987.

Ciesielski, C. A., Blaser, M. J. and Wang, W. L. (1986). Serogroup specificity of *Legionella pneumophila* is related to lipopolysaccharide characteristics. *Infection and Immunity.* **51,** 397-404.

Cirillo, J. D., Falkow, S. and Tompkins, L. S. (1994). Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infection and Immunity.* **62,** 3254-3261.

Clarke, B. J., Hohman, T. C. and Blowers, B. (1988). Purification of plasma membrane from *Acanthamoeba castellanii. Journal of Protozoology.* **35,** 408-413.

Clemens, D. L. (1996). Characterisation of the *Mycobacterium tuberculosis* phagosome. *Trends in Microbiology.* **4,** 113-118.

Clemens, D. L. and Horwitz, M. A. (1995). Characterisation of the *Mycobacterium tuberculosis* phagosome and evidence that phagosme maturation is inhibited. *Journal of Experimental Medicine.* **181,** 257-270.

Cochrane, D. M. G., Anwar, H., Brown, M. R. W., Lam, K. and Costerton, J. W. (1987). Iron deprivation *in vivo*: surface protein antigens of *Pseudomonas aeruginosa* in infection. *Antibiotics and Chemotherapy.* **39**, 125-135.

Cohen, S. P., Levy, S. B., Foulds, J. and Rosner, J. L. (1993). Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and a *mar*-independent pathway. *Journal of Bacteriology.* **175**, 7856-7862.

Colbourne, J. L S. and Dennis, P. J. (1989). The ecology and survival of *Legionella pneumophila. Journal of International Water and Environmental Management.* **3,** 345-350.

Colbourne, J. L. S., Pratt, D. J., Smith, M. G., Fisher-Hoch, S. P. and Harper, D. (1984). Water fittings as a source of *Legionella pneumophila* in a hospital plumbing system. *Lancet i.* **28,** 210-213.

Colwell R. R. (1993). Nonculturable but still viable and potentially pathogenic. *Zentralblatt fur Bakteriologie und Hygiene.* **279,** 154-156.

Colwell, R. R., Brayton, P. R., Grimes, D. J., Roszak, D. B., Huq, S. A. and Palmer, L. M. (1985). Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for the release of genetically-engineered microorganisms. *Bio/technology.* **3**, 817-820.

Conlan J. W. and Ashworth, L. A. E. (1986). The relationship between the serogroup antigen and the lipopolysaccharide of *Legionella pneumophila. Journal of Hygiene.* **96,** 39-48.

Conn, H. J. (1948). The most abundant groups of bacteria in soil. *Bacteriological Reviews.* **12,** 257-273.

Coon, S. L., Kotob, S., Jarvis, B. B., Wang, S., Fuqua, W. C. and Weiner, R. M. (1994). Homogenistic acid is the product of MelA, which mediates melanogenesis in the marine bacterium *Shewanella collwelliana* D. *Applied and Environmental Microbiology.* **60,** 3006-3010.

Cozens, R. M., Klemperer, R. M. and Brown, M. R. W. (1983). The influence of cell envelope composition on antibiotic activity. In *Antibiotics : assessment of antimicrobial activity and resistance*. (Eds., Russell, A. D. and Quesnel, L. B.) Academic Press, Inc. (London) Ltd., London, UK.

De Chastellier, C., Frehel, C., Offredo, C. and Skamene, C. (1993). Implication of phagosome-lysosome fusion in restriction of *Mycobacterium avium* growth in bone

marrow macrophages from genetically resistant mice. *Infection and Immunity.* **61**, 3775-3784.

De Chastellier, C., Lang, T. and Thilo, L. (1995). Phagocytic processing of the macrophage endoparasite *Mycobacterium avium* in comparison to phagosomes with *Bacillus* or latex beads. *European Journal of Cell Biology.* **68**, 167-182.

De La Rosa, E. J., De Pedro, M. A. and Vasquez, D. (1982). Modification of penicillin binding proteins of *Escherichia coli* associated with changes in the state of growth of the cells. *FEMS Microbiology Letters.* **14,** 91-94.

Di Rita, V. J. and Mekalanos, J. J. (1989). Genetic regulation of bacterial virulence. *Annual Review of Genetics.* **23,** 455-482.

Dalhoff, A. (1985). Differences between bacteria grown *in vitro* and *in vivo*. *Journal of Antimicrobial Chemotherapy.* **15,** (Suppl. A) 175-195.

Dawes, E. A. (1984). Stress of unbalanced growth and starvation in microorganisms. *Society for Applied Bacteriology Symposium Series* 12, pp.19-42, Academic Press Inc.(London) Ltd. London.

Dawson, M. P., Humphrey, B. A. and Marshall, K. C. (1981). Adhesion: a tactic in the survival strategy of a marine *Vibrio* during starvation. *Current Microbiology.* **6**, 195-199.

Dean, A. C. R. (1972). Influence of environment on the control of enzyme synthesis. *Journal of Applied Chemistry and Biotechnology.* **22,** 245-259.

Dean, A. C. R., Ellwood, J., Melling, J. and Robinson, A. (1976). The action of antibacterial agents on bacteria grown in continuous culture, In *Continuous culture 6 : applications and new fields.* (Eds., Dean, A. C. R, Ellwood, D. C., Evans, C. G. T. and Melling, J.) Ellis Horwood, Chichester, UK.

Dennis P. J. and Lee, J. V. (1988). Differences in aerosol survival between pathogenic and non-pathogenic strains of *Legionella pneumophila* serogroup 1. *Journal of Applied Bacteriology.* **65,** 135-141.

Dennis, P. J., Taylor, J. A. and Barrow, G. T. (1981). Phosphate buffered, low sodium chloride blood agar medium for *Legionella pneumophila. Lancet ii.* 636.

Dennis, P. J., Taylor, J. A., Fitzgeorge, R. B., Bartlett, C. L. R. and Barrow, G. I. (1982). *Legionella* in water plumbing systems. *Lancet* **i,** 949-951.

Dennis P. J., Green, D. and Jones, B. P. (1984). A note on temperature tolerance of *Legionella. Journal of Applied Bacteriology.* **56,** 349-350.

Desplaces, N., Nahapetian, K. and Dournon, E. (1984). Inventiare des *Legionella* dans le environnment parisien. *Implications pratiques. Presse med.* **13,** 1875-1879.

Dougan, G. (1989). Molecular characterisation of bacterial virulence factors and the consequence for vaccine design. *Journal of General Microbiology.* **135,** 1397-1406.

Dournon, E., Mayaud, C., Wolff, M., Schlemmer, B., Samual, D. M., Sollett, J. P. and Levasseur-Rajagopalan, P. (1990). Comparison of the activity of three antibiotic regimens in severe Legionnaires' disease. *Journal of Antimicrobial Chemotherapy.* **26,** (Suppl. B) 129-139.

Dowling J. N., Saha, A. K and Glew, R. H. (1992). Virulence factors of the family *Legionellaceae. Microbiological Reviews.* **56,** 32-60.

Dufour, A. P. and Jakubowski, W. (1982). Drinking water and Legionnaires' disease. *American Water Works Association Journal.* **74,** 631-637.

Edelstein, P. H. (1981). Improved semi-selective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *Journal of Clinical Microbiology*. **14,** 298-303.

Edelstein, P. H. (1982). Comparative study of selective media for isolation of *Legionella pneumophila* from potable water. *Journal of Clinical Microbiology*. **16,** 697-699.

Edelstein, P. H. (1991). Rifampicin resistance of *Legionella pneumophila* is not increased during therapy for experimental Legionnaires' disease: study of rifampicin resistance using a guinea pig model of Legionnaires' disease. *Antimicrobial Agents and Chemotherapy*. **35,** 5-9.

Edelstein, P. H. (1993). Legionnaires' disease. *Clinical Infectious Diseases.* **16,** 741-749.

Edelstein, P. H. and Edelstein, M. A. C. (1989). WIN 57273 is bactericidal for *Legionella pneumophila* grown in alveolar maceophages. *Antimicrobial Agents and Chemotherapy*. **33,** 2132-2136.

Edelstein, P. H. and Edelstein, M. A. C. (1994). *In vitro* extracellular and intracellular activities of clavulanic acid and those of piperacillin and ceftriaxome alone and in combination with tazobactam against clinical isolates of *Legionella* species. *Antimicrobial Agents and Chemotherapy*. **38,** 200-204.

Edelstein, P. H., Meyer, R. D. and Finegold, S. M. (1979). Isolation of *Legionella pneumophila* from blood. *Lancet i.* 750-751.

Ehret, W. and Ruckdeschel, G. (1985). Membrane proteins of Legionellaceae. I. Membrane proteins of different strains and serogroups of *Legionella pneumophila*. *Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene*. Reihe A. **259,** 433-435.

Eisenstark, A., Yallaly, P., Ivanova, A. and Miller, C. (1995). Genetic mechanisms involved in cellular recovery from oxidative stress. *Archives of Insect Biochemistry and Physiology*. **29,** 159-173.

Elliott, J. A. and Johnson, W. (1981). Immunological and biochemical relationships among flagella isolated from *Legionella pneumophila* serogroups 1, 2 and 3. *Infection and Immunity*. **33,** 602-610.

Elliott, J. A. and Winn, W. C. (1986). Treatment of alveolar macrophage with cytochalasin D inhibits uptake and subsequent growth of *Legionella pneumophila. Infection and Immunity.* **51,** 31-36.

Ellwood, D. C. and Tempest, D. W. (1972). Effects of environment on bacterial wall content and composition. *Advances in Microbial Physiology*. **7,** 83-117.

England, A. C., Fraser, D. W., Plikaytis, B. D., Tsai, T. F., Storch, G. and Broome, C. V. (1981). Sporadic legionellosis in the United States: the first thousand cases. *Annals of Internal Medicine.* **94,** 164-170.

Engleberg, N. C., Carter, C., Weber, D. R., Cianciotto, M. P. and Eisenstein, B. I. (1989). DNA sequence of mip, a *Legionella pneumophila* gene associated with macrophage infectivity. *Infection and Immunity*. **57,** 1263-1270.

Ensign, J. C. (1970). Long term starvation of rod and spherical cells of *Arthrobacter crystallopoietes. Journal of Bacteriology.* **103**, 569-577.

Eraso, J. M., Chidambaram, M. and Weinstock, G. M. (1996). Increased production of colicin E1 in stationary phase. *Journal. of Bacteriology*. **178,** 1928-1935.

Farrell, I. D., Barker J. E., Miles, E. P. and Hutchinson, J. G. P. (1990). A field study of *Legionella pneumophila* in a hospital water system. *Epidemiology and Infection.* **104**, 381-387.

Feeley, J. C., Gorman, G. W., Weaver, D., Mackel, D. C. and Smith, H. W. (1978). Primary isolation media for Legionnaires' disease bacterium. *Journal of Clinical Microbiology*. **8**, 320-325.

Feeley, J. C., Gibson, R. J., Gorman, G. W., Langford, N. C., Rasheed, J. K., Mackel, D. C. and Baine, W. B. (1979). Charcoal Yeast Extract Agar : primary isolation medium for *Legionella pneumophila. Journal. of Clinical Microbiology.* **10,** 437-441.

Ferris, F. G. and Beveridge, T. J. (1984). Binding of a paramagnetic metal cation to *Escherichia coli* K-12 outer membrane vesicles. *FEMS Microbiology Letters.* **24,** 43-46.

Ferris, F. G. and Beveridge, T. J. (1986). Physicochemical roles of soluble metal cations in the outer membrane of *Escherichia coli*. *Canadian Journal of Microbiology.* **32**, 594-601.

Fields, B. S. (1996). The molecular ecology of legionellae. *Trends in Microbiology.* **4,** 286-290

Fields, B. S., Sanden, G. N., Barbaree, J. M., Morrill, W. E., Wadowsky, R. M., White, E. H. and Feeley, J. C. (1989). Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. *Current Microbiology*. **18**, 131-137.

Fields, B. S., Barbaree, J. M., Sanden, G. N. and Morrill, W. E. (1990). Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using

protozoan, cell culture, and guinea pig models. *Infection and Immunity.* **58,** 3139-3142.

Fields, B. S., Utley Fields, S. R., Chin Loy, J. N., White, E. H., Steffens, W. L.and Shotts, E. B. (1993). Attachment and entry of *Legionella pneumophila* in *Hartmannella vermiformis. Journal of Infectious Diseases.* **167,** 1146-1150.

Finlay, B. B. and Falkow, S. (1989). Common themes in microbial pathogenicity. *Microbiology Reviews.* **53**, 210-230.

Fitzgeorge, R. B., Gibson, D. H., Jepras, R. and Baskerville, A. (1985). Studies on ciprofloxacin therapy of experimental Legionnaires' disease. *Journal of Infection.* **10,** 194-203.

Fitzgeorge, R. B, Baskerville, A. and Featherstone, A. S. R. (1986). Treatment of experimental Legionnaires' disease by aerosol administration of rifampicin, ciprofloxacin and erythromycin. *Lancet i.* 502-503.

Flesher, A. R., Ito, S, Mansheim, B. and Kasper, D. L. (1979). The cell envelope of the Legionnaires' Disease bacterium: morphologic and biochemical characteristics. *Annals of Internal Medicine.* **90,** 628-630.

Fliermans, C. B. (1983). Autoecology of *Legionella pneumophila. Zentralblatt fur Bakteriologie und Hygiene. Orig. A.* **255**, 58-63.

Fliermans, C. B., Cherry, W. B., Orrison, L. H. and Thacker, L. (1979). Isolation of *Legionella pneumophila* from non-epidemic-related aquatic habitats. *Applied and Environmental Microbiology.* **37**, 1239-1242.

Fliermans, C. B., Cherry, W. B., Orrison, L. H., Smith, S. J., Tison, D. L. and Pope, D. H. (1981). Ecological Distribution of *Legionella pneumophila. Applied and Environmental Microbiology.* **41**, 9-16.

Fraser, D. W. (1980). Legionellosis: evidence of airborne transmission. *Annals of the New York Academy of Science.* **353,** 61-66.

Fraser, D. W. (1984). Sources of legionellosis. In *Legionella. Proceedings of the Second International Symposium.* Washington D. C. (Eds. Thornsberry, C., Barlows, A., Feeley, J. C. and Jakubowski, W.). pp. 277-280. American Society for Microbiology.

Fraser, D. W., Tsai, T. R., Orenstein, W., Parkin, E., Beecham, H. J., Sharrar, R. G., Harris, J., Mallison, G. F., Martin, S. M., McDade, J. E., Shepard, C. C.and Brachman, P.S. (1977). Legionnaires' disease: description of an epidemic of pneumonia. *New England Journal of Medicine.* **297,** 1189-1197.

Frehel, C., De Chastellier, C., Lang, T. and Rastogi, N. (1986). Evidence for inhibition of fusion of lysosomal and pre-lysosomal compartments with phagosomes in macrophages infected with pathogenic *Mycobacterium avium. Infection and Immunity.* **52**, 252-262.

Friedman, R. L., Iglewski, B. H. and Miller. R. D. (1980). Identification of a cytotoxin produced by *Legionella pneumophila. Infection and Immunity.* **29**, 271-274.

Fuqua, W. C., Coyne, V. E., Stein, D. C., Lin, C. M. and Weiner, R. M. (1991). Characterisation of *melA* : a gene encoding melanin biosynthesis from the marine bacterium *Shewanella colwelliana. Gene.* **109**, 131-136.

Gabay J. E. and Horwitz M. A. (1985). Isolation and characterisation of the cytoplasmic and outer membrane of the Legionnaires' disease bacterium (*Legionella pneumophila*). *Journal of Experimental Medicine.* **161,** 409-422.

Gabay J. E., Blake, M., Niles, W. D. and Horwitz, M. A. (1985). Purification of *Legionella pneumophila* major outer membrane protein and demonstration that it's a porin. *Journal of Bacteriology.* **162,** 85-91.

George, J. R., Pine, L., Reeves, M. W. and Harrell, W. K. (1980). Amino acid requirements of *Legionella pneumophila. Journal of Clinical Microbiology.* **11,** 286-291.

Gibson, D. H., Fitzgeorge, R. B. and Baskerville, A. (1983). Antibiotic therapy of experimental airborne Legionnaires' disease. *Journal of Infection.* 7, 210-217.

Gibson, F. C., Tzianabos, A. O. and Rodgers, F. G. (1994). Adherence of *Legionella pneumophila* to U937 cells, guinea pig alveolar macrophage, and MRC5 cells by a novel, complement-independent binding mechanism. *Canadian Journal of Microbiology.* **40**, 865-872.

Gilbert, P. and Brown, M. R. W. (1978). Influence of growth rate and nutrient limitation on the gross cellular composition of *Pseudomonas aeruginosa* and its resistance to 3- and 4-chlorophenol. *Journal of Bacteriology.* **133**, 1066-1072.

Gilbert, P. and Brown, M. R. W. (1980). Cell-wall mediated changes in sensitivity of *Bacillus megaterium* to chlorhexidine and 2-phenoxyethanol, associated with the growth rate and nutrient limitation. *Journal of Applied Bacteriology.* **48,** 223-230.

Gilbert, P. and Wright, N. E. (1986). Non-plasmidic resistance towards preservatives in pharmaceutical and cosmetic products. *Society of Applied Bacteriology Technical Series.* **22,** 255-279.

Glick, T. H., Gregg, M. B., Berman, B., Mallison, G., Rhodes Jr., W. W. and Kassanoff, I. (1978). Pontiac fever. An epidemic of unknown etiology in a health department. I. Clinical and epidemiologic aspects. *American Journal of Epidemiology.* **107,** 149-160.

Goebel, W. (1988). Intracellular bacteria. *Current Topics in Microbiology and Immunology.* **138**, 1-2

Goldman, R. C. and Leire, L. (1980). Heterogenicity of antigenic-side-chain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. *European Journal of Biochemistry.* **107**, 145-153.

Griffiths, E., Stevenson, P. and Joyce, P. (1983). Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*. *FEMS Microbiology Letters.* **16,** 95-99.

Groothuis, D. G. and Veenendaal, H. R. (1983). Heat treatment as an aid for isolation of *Legionella pneumophila* from clinical and environmental samples. *Zentralblatt fur Bakteriologie und Hygiene* Abt. 1 Originale A. **255,** 39-43.

Groothuis, D. G., Veenendaal, H. R. and Dijkstra, H. L. (1985). Influence of temperature on the number of *Legionella pneumophila* in hot water systems. *Journal of Applied Bacteriology.* **59**, 529-536.

Gulig, P. A., Frisch, C. F. and Hansen, E. J. (1983). A set of two monoclonal antibodies specific for the cell surface-exposed 39 kDa major outer membrane protein of *Haemophilus influenzae* type b defines all strains of this pathogen. *Infection and Immunity.* **42,** 516-524.

Gump, D. W., Frank, R. O., Winn, W. C., Foster, R. S., Broome, C. V.and Cherry, W. B. (1979). Legionnaires' disease in patients with associated serious disease. *Annals of Internal Medicine.* **90,** 538-542.

Habich, W. A. and Muller, H. E. (1988). Occurrence and parameters of frequency of *Legionella* in warm water systems of hospitals and hotels in Lower Saxony. *Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene I. Abteilung: Originale A.* **255**, 64-70.

Hackstadt, T. and Williams, J. C. (1981). Biochemical strategem for obligate parasitism of eukaryotic cells by *Coxiella burnetii. Proceedings of the National Academy of Science*. **78**, 3240-3244.

Halablab, M. A., Richards, L. and Bazin, M. J. (1990). Phagocytosis of *Legionella pneumophila. Journal of Medical Microbiology.* **33,** 75-83.

Haldane, D. J. M., Bezanson, G. S., Burbridge, S. M., Kuehn, R. D. and Marrie, T. J. (1993). Resistance of a model hot water system to colonisation by *Legionella pneumophila.* In *Legionella* : *Current Status and Emerging Perspectives* (Eds., Barbaree, J. M., Breiman, R. F. and Dufour, A. P.) pp. 245-247. American Society of Microbiology, Washington, DC. USA.

Hambleton, P., Broster, M. G., Dennis, P. J., Henstridge, R., Fitzgeorge, R. and Conlan, J. W. (1983). Survival of virulent *Legionella pneumophila* in aerosols. *Journal of Hygiene.* **90**, 451-460.

Hancock, R. E. W. (1984). Alterations in outer membrane permeabiliy. *Annual Review of Microbiology*. **38**, 237-264.

Hand, W. L., Corwin, R. W., Steinberg, T. H. and Grossman, G. D. (1984). Uptake of antibiotics by human alveolar macrophages. *American Review of Respiratory Disease*. **129**, 933-937.

Harder, K. J., Nikaido, H. and Matsuhashi, M. (1981). Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the ompF porin. *Antimicrobial Agents and Chemotherapy*. **20,** 548-552.

Harf, C. (1993). Free-living amoeba : interactions with environmental pathogenic bacteria. *Endocytobiosis and Cell Research.* **10,** 167-183.

Harf, C. and Monteil, H. (1989). Pathogen microorganisms in environmental waters: a potential risk for human health. *Water International.* **14,** 75-79.

Harley, V. S, Draser, B. S. and Tovey, G. (1997). The ultrastructure of stressed *Legionella pneumophila. Microbios.* **91,** 73-78.

Hatch, T. P., Allan, I and Pearce, J. H. (1984). Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of *Chlamydia. Journal of Bacteriology.* **157,** 13-20.

Havlichek, D., Saravolatz, . and Pohlod, D. (1987). Effect of quinolones and other antimicrobial agents on cell-associated *Legionella pneumophila. Antimicrobial Agents and Chemotherapy.* **31,** 901-908.

Hay, J., Seal, D. V., Billcliffe, B. and Freer, J. H. (1995). Non-culturable *Legionella pneumophila* associated with *Acanthamoeba castellanii* : detection of the bacterium using DNA amplification and hybridisation. *Journal of Applied Bacteriology.* **78**, 61-65.

Hebert, G. A., Steigerwalt, A. G. and Brenner, D. J. (1980). *Legionella micdadei* species nova: classification of a third species of *Legionella* associated with human pneumonia. *Current Microbiology.* **3,** 255-257.

Hedlund, K. W. (1981) Legionella toxin. *Pharmacological Therapy.* **15,** 123-130

Heinmets, F., Taylor, W. W. and Lehman, J. J. (1953). The use of metabolites in the restoration of the viability of heat and chemically inactivated *Escherichia coli. Journal of Bacteriology.* **67,** 5-14.

Heller, R., Holler, C., Submuth, R. and Gundermann, K. O. (1998). Effect of salt concentration and temperature on survival of *Legionella pneumophila. Letters in Applied Microbiology.* 26, 64-68.

Henke, M. and Seidel, K. (1986). Association between *Legionella pneumophila* and amoebae in water. *Israel Journal of Medical Sciences.* **22,** 690-695.

Hickey, E. K. and Cianciotto, N. P. (1994). Cloning and sequencing of the *Legionella pneumophila fur* gene. *Gene*. **143,** 117-121.

Higa, F., Saito, J., Inadome, J., Kusano, N. Kitsukawa, K. (1993). Influence of methylprednisolone on the intracellular antimicrobial activity of erythromycin and clindamycin against *Legionella pneumophila. Journal of Antimicrobial Chemotherapy.* **31,** 901-908.

Higa, F., Kusano, N., Tateyama, M., Shinzato, T., Arakaki, N. Kawakami, K. and Saito, A. (1998). Simplified quantitative assay system for measuring activities of drugs against intracellular *Legionella pneumophila. Journal of Clinical Microbiology.* **36,** 1392-1398.

Hindahl, M. S. and Iglewski, B. H. (1984). Isolation and characterisation of the *Legionella pneumophila* outer membrane. *Journal of Bacteriology.* **159,** 107-113.

Hindahl, M. S. and Iglewski, B. H. (1986). Outer membrane proteins from *Legionella pneumophila* serogroups and other *Legionella* species. *Infection and Immunity.* **51,** 94-101.

Hindersson, P., Hoiby, N. and Bangsborg, J. (1991). Sequence analysis of the *Legionella micdadei groEL* operon. *FEMS Microbiology Letters.* **77,** 31-38.

Hitchcock, P. J. and Brown, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology.* **154,** 269-277.

Hobbie, J. E., Daley, R. and Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*. **33,** 1225-1228.

Hodinka, R. L. and Wyrick, P. B. (1986). Ultrastructural study of the mode of entry of *Chlamydia psittaci* into L-929 cells. *Infection and Immunity*. **54,** 855-863.

Hoffman, P. S. (1984). Bacterial physiology. In *Legionella, Proceedings of the 2nd International Symposium*. (Eds., Thornsberry, C., Balows, A., Feeley, J. C. and Jakubowski W.) pp. 61-67. American Society of Microbiology, Washington DC, USA.

Hoffman, P. S., Pine, L. and Bell, S. (1983). Production of superoxide and hydrogen peroxide in medium used to culture *Legionella pneumophila* : Catalytic decomposition by charcoal. *Applied and Environmental Microbiology*. **45**, 784-791.

Hoffman, P. S., Butler, C. A. and Quinn, F. D. (1989). Cloning and temperature-dependent expression in *Escherichia coli* of a *Legionella pneumophila* gene coding for a genus-common 60 kDa antigen. *Infection and Immunity*. **57**, 1731-1739.

Hoffman, P. S., Houston, L. and Butler, C. A. (1990). *Legionella pneumophila htaB* heat shock operon: nucleotide sequence and expression of the 60 kDa antigen in *L. pneumophila* infected Hela cells. *Infection and Immunity*. **58,** 3380-3387.

Hoffman, P. S., Seyer, J. H. and Butler, C. A. (1992). Molecular characterisation of the 28 and 31 kilodalton subunits of the *Legionella pneumophila* outer membrane protein. *Journal of Bacteriology*. **174,** 908-913.

Holden, E. P., Winkler, H. H., Wood, D. O. and Leinbach, E. D. (1984). Intracellular growth of *Legionella pneumophila* within *Acanthamoeba castellanii* Neff. *Infection and Immunity*. **45**, 18-24.

Holme, T. (1972). Influence of environment on the content and composition of bacterial envelopes. *Journal of Applied Chemistry and Biotechnology*. **22**, 391-399.

Holmquist, L. and Kjelleberg, S. (1993). Changes in viability, respiratory activity and morphology of the marine *Vibrio* sp. strain S14 during starvation of individual nutrients and subsequent recovery. *FEMS Microbiology Ecology*. **102,** 195-199.

Hooper, T. L., Gould, F. K. and Swinbourne, C. R. (1988). Ciprofloxacin : a preferred treatment for *Legionella* infections in patients receiving cyclosporin A. *Journal of Antimicrobial Chemotherapy*. **22**, 952-953.

Horwitz, M. A. (1982). Cell-mediated immunity in Legionnaires' disease. *Journal of Clinical Investigation*. **71,** 1686-1697.

Horwitz, M. A. (1983). The Legionnaires' Disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *Journal of Experimental Medicine*. **158,** 2108-2126.

Horwitz, M. A. (1984). Phagocytosis of the Legionnaires' Disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: Engulfment with a pseudopod coil. *Cell*. **36,** 27-33.

Horwitz, M. A. (1988). Phagocytosis and intracellular biology of *Legionella pneumophila*. In *Bacteria-host cell interaction*. (Horwitz, M. A. Ed) p 283-302. Alan R. Liss, Inc., New York, USA.

Horwitz, M. A. (1993). Towards an understanding of host and bacterial molecules mediating *Legionella pneumophila* pathogenesis. In *Legionella* : *Current Status and Emerging Perspectives* (Eds., Barbaree, J. M., Breiman, R. F. and Dufour, A. P.) pp. 55-62. American Society of Microbiology, Washington, DC. USA.

Horwitz, M. A. and Maxfield, F. R. (1984). *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *Journal of Cell Biology.* **99,** 1936-1943.

Horwitz, M. A. and Silverstein, S. C. (1980). Legionnaires' Disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *Journal of Clinical Investigation.* **66,** 441-450.

Horwitz, M. A. and Silverstein, S. C. (1981). Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. I. *L. pneumophila* resists killing by polymorphonuclear leukocytes, antibody, and complement. *Journal of Experimental Medicine*. **153,** 386-397.

Horwitz M. A. and Silverstein, S. C. (1983). Intracellular multiplication of Legionnaires' Disease bacterium (*Legionella pneumophila*) in human monocytes is reversibly inhibited by erythromycin and rifampicin. *Journal of Clinical Investigation.* **71,** 15-26.

Hoyle, B. and Beveridge, T. J. (1983). Binding of metallic ions to the outer membrane of *Escherichia coli. Applied and Environmental Microbiology.* **46,** 749-752.

Hoyle, B. and Beveridge, T. J. (1984). Metal binding by the peptidoglycan sacculus of *Escherichia coli* K-12. *Canadian Journal of Microbiology.* **30**, 204-211.

Hurst, A. (1977). Bacterial injury: a review. *Canadian Journal of Microbiology.* **23,** 935-944.

Husmann, L. K. and Johnson, W. (1994). Cytotoxicity of extracellular *Legionella pneumophila. Infection and Immunity.* **62,** 2111-2114.

Hussong, D., Colwell, R. R., O'Brien, M., Weiss, E., Pearson, A. D., Weiner, R. M. and Burge, W. D. (1987). Viable *Legionella pneumophila* not detectable by culture on agar media. *Bio/technology.* **5,** 947-950.

Ikeda, T. A., Ledwith, A., Bamford, C. H. and Hann, R. A. (1984). Interaction of a polymeric biguanide with phospholipid membranes. *Biochemica et Biophysica Acta.* **769,** 57-66.

Jishage, M. and Isihihama, A. (1995). Regulation of RNA polymerase sigma sub-unit synthesis in *Escherichia coli* : Intracellular levels of σ^{70} and σ^{38}. *Journal of Bacteriology.* **177,** 6832-6835.

Johnson, D. M., Erwin, M. E., Barrett, M. S., Gooding, B. B. and Jones, R. N. (1992). Antimicrobial activity of ten macrolide, lincosamine and streptogramin drugs tested against *Legionella* species. *European Journal of Clinical Microbiology and Infectious Diseases.* **11,** 751-755.

Johnson, S. R., Schalla, W. O., Wong, K. H. and Perkins, G. H. (1982). Simple, transparent medium for study of legionellae. *Journal of Clinical Microbiology.* **15,** 342-344.

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J. R., Golby, P., Reeves, P. J., Stephens, S., Winson, M. K., Stewart, G. S. A. B. and Williams, P. (1993). The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa. The EMBO Journal.* **12,** 2477-2482.

Kaprelyants, A. S, Gottschal, J. C. and Kell, D. B. (1993). Dormancy in non-sporulating bacteria. *FEMS Microbiology Reviews.* **104,** 271-286.

Keel, J. A., Finnerty, W. R. and Feeley, J. C. (1979). Fine structure of the Legionnaires' Disease bacterium : *In vitro* and *in vivo* studies of four isolates. *Annals of Internal Medicine.* **90,** 652-655.

Keen, M. G. and Hoffman, P. S. (1989). Characterisation of a *Legionella pneumophila* extracellular protease exhibiting haemolytic and cytotoxic activities. *Infection and Immunity.* **57,** 732-738.

Kilvington, S. and Price, J. (1990). Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *Journal of Applied Bacteriology.* **68,** 519-525.

King, C. H. and Shotts, E. B., Jr. (1988). Enhancement of *Edwardsiella tarda* and *Aeromonas salmonicida* through ingestion by the ciliated protozoan *Tetrahymena pyriformis*. *FEMS Microbiology Letters.* **51,** 95-100.

Kirby, B. D., Snyder, K. M., Meyer, R. D. and Finegold, S. M. (1980). Legionnaires' disease: report of sixty-five nosocomially acquired cases, a review of the literature. *Medicine (Baltimore).* **59,** 188-205.

Kirby, J. E., Vogel, J. P., Andrews, H. L. and Isberg, R. R. (1998). Evidence for pore-forming ability by *Legionella pneumophila*. *Molecular Microbiology.* **27,** 323-336.

Kitsukawa, K., Hara, J. and Saito, A. (1991). Inhibition of *Legionella pneumophila* in guinea pig peritoneal macrophages by new quinolone, macrolide and other antimicrobial agents. *Journal of Antimicrobial Chemotherapy.* **27**, 343-353.

Kjelleberg, S., Humphrey, B. A. and Marshall, K. C. (1983). Initial phases of starvation and activity of bacteria at surfaces. *Applied and Environmental Microbiology.* **46,** 978-984.

Kjelleberg, S., Hermansson, M. and Marden, K. C. (1987). The transient phase between growth and non-growth of heterotrophic bacteria, with emphasis on the marine environment. *Annual Review of Microbiology.* **41,** 25-49.

Kjelleberg, S., Albertson, N., Flardh, K, Holmquist, L., Jouper-Jaan, A, Marouga, R., Ostling, J., Svenblad, B., and Weichart, D. (1993). How do non-differentiating bacteria adapt to starvation? *Antonie Van Leeuvenhoek.* **63,** 333-341.

Knirel, Y. A., Moll, H. and Zahringer, U. (1996). Structural study of a highly acetylated core of *Legionella pneumophila* serogroup 1 lipopolysaccharide. *Carbohydrate Research.* **293,** 223-234.

Kogure, K., Simidu, U. and Taga, N. (1979). A tentative direct microscopic method for counting living marine bacteria. *Canadian Journal of Microbiology.* **25,** 415-420.

Kondo, K., Takade, A. and Amako, K. (1994). Morphology of the viable but non-culturable *Vibrio cholerae* as determined by freeze fixation technique. *FEMS Microbiology Letters.* **123,** 170-184.

Konings, W. M. and Veldkamp, H. (1980). Phenotypic responses to environmental change. In *Contemporary microbial ecology.* (Eds. Ellwood, D. C., Latham, M. J., Hedger, J. N., Lynch, J. M. and Slater, J. H. pp. 161-191. Academic Press, Inc. (London). Ltd., London.

Korn, E. D. and Wright, P. L. (1973). Macromolecular composition of an amoeba plasma membrane. *Journal of Biological Chemistry.* **248,** 439-447.

Lambert, P. A. (1984). In *Continuous Culture 8, Biotechnology, Medicine and the Environment.* (Eds., Dean, A. C. R. , Ellwood, D. C. and Evans, C. G. T.) pp. 38 Ellis Horwood, Chichester, UK.

Lambert, P. A. (1988a). The bacterial cell surface and antimicrobial resistance. *Progress in Drug Research.* **32,** 149-174.

Lambert, P. A. (1988b). Isolation and purification of outer membrane proteins from Gram negative bacteria. In *Bacterial cell surface techniques* (Eds., Hancock, I and Poxton, I.) pp. 110-118. John Wiley and Sons, Chichester, UK

Lathigra, R. B., Butcher, P. D., Garbe, T. R. and Young, D. B. (1991). Heat shock proteins as virulence factors of pathogens. *Current Topics in Microbiology and Immunology.* **167,** 125-145.

Leduc, M., Frehel, C., Siegel, E. and van Heijenoort, J. (1989). Multilayered distribution of peptidoglycan in the periplasmic space of *Escherichia coli. Journal of General Microbiology.* **135,** 1243-1254.

Lee, J. V. and West, A. A. (1991). Survival and growth of *Legionella* species in the environment. *Journal of Applied Bacteriology* (symposium). **70,** 121S-129S.

Lee, T. C., Stout, J. E. and Yu, V. L. (1988). Factors predisposing to *Legionella pneumophila* colonisation in residential water systems. *Archives of Environmental Health.* **43,** 59-62.

Levinthal, M. and Pownder, T. (1996). *hns, rpoS* and *lrp* mutations affect stationary-phase survival at high osmolarity. *Research in Microbiology.* **147,** 333-342.

Liebers, D. M., Baltch, A. L., Smith, R. P., Hammer, M. C. and Conroy, J. V. (1989). Susceptibility of *Legionella pneumophila* to eight antimicrobial agents including four macrolides under different assay conditions. *Journal of Antimicrobial Chemotherapy.* **23,** 37-41.

Lin, J. and Ficht, T. A. (1995). Protein synthesis in *Brucella abortus* induced during macrophage infection. *Infection and Immunity.* **63,** 1409-1414.

Lind, K. (1983). Serological investigations of *Legionella* infections in Denmark. *Acta Pathologie, Microbiologie Immunologie.* (Scandanavia Section B). **91,** 209-213.

Loewen, P. C. and Hengge-Aronis, R. (1994). The role of the sigma factor σ^{S} (*katF*) in bacterial global regulation. *Annual Reviews in Microbiology.* **48,** 53-80.

Ludwig, B., Schmidt, A., Marre, R. and Hacker, J. (1991). Cloning, genetic analysis, and nucleotide sequence of a determinant coding for a 19 kDa peptidoglycan-associated protein (Ppl) of *Legionella pneumophila. Infection and Immunity.* **59**, 2515-2521.

Lugtenberg, B. and van Alpen, L. (1983). Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram negative bacteria. *Biochemica et Biophysica Acta.* **737**, 51-115.

Ly, T. M. C. and Muller, H. E. (1990a). Ingested *Listeria monocytogenes* survive and multiply in protozoa. *Journal of Medical Microbiology.* **33**, 51-54.

Ly, T. M. C. and Muller, H. E. (1990b). Interactions of *Listeria monocytogenes, Listeria seeligeri* and *Listeria innocua* with protozoans. *Journal of General Applied Microbiology.* **36,** 143-150.

MacDonnell, M. and Hood, M. (1982). Isolation and characterisation of ultramicrobacteria from a gulf coast estuary. *Applied and Environmental Microbiology.* **43,** 566-571.

McCann, M. P., Kidwell, J. P. and Matin, A. (1991). The putative sigma factor *katF* has a central role in development of starvation mediated general resistance in *Escherichia coli*. *Journal of Bacteriology.* **173,** 4188-4194.

McDade, J. E., Shepard, C. C., Fraser, D. W., Tsai, T. R., Redus, M. A. and Dowdle, W. R. (1977). Legionnaires' disease : isolation of a bacterium and demonstration of its role in other respiratory disease. *New England Journal of Medicine*. **297,** 1197-1203.

McDade, J. E., Brenner, D. J. and Bozeman, F. M. (1979). Legionnaires' disease bacterium isolated in 1947. *Annals of Internal Medicine.* **90,**. 659-661.

Macfarlane, J. T. (1987). Treatment of lower respiratory infections. *Lancet ii.* 1446-1449.

Makin, T. and Hart, C. A. (1991). The effect of a self regulating trace heating element on *Legionella* within a shower. *Journal of Applied Bacteriology.* **70,** 258-264.

Malmcrona-Friberg, K., Tunlid, A, Marden, P. and Kjelleberg, S. (1986). Chemical changes in the envelope and poly-β-hydroxbutyrate during short term starvation of a marine bacterial isolate. *Archives of Microbiology*. **144**, 340-345.

Marra, A., Horwitz, M. A. and Shuman, H. A. (1990). The HL-60 model for the interaction of human macrophages with the Legionnaires' Disease bacterium. *Journal of Immunology*. **144,** 2738-2744.

Marra, A., Blander, S., Horwitz, M. A. and Shuman, H. A. (1992). Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. *Proceedings of the National Academy of Science*. **89,** 9607-9611.

Marshak, E. M. and Tocanne, J. F. (1980). Polymyxin B-phosphatidylglycerol interactions. A monolayer ($\pi\Delta v$) study. *Biochemica et Biophysica Acta.* **596,** 165-179.

Mason, C. A., Hamer, G. and Bryers, J. (1986). The death and lysis of microorganisms in environmental processes. *FEMS Microbiology Reviews.* **39,** 373-401.

Mauchline, W. S., Araujo, R., Fitzgeorge, R. B., Dennis, P. J. and Keevil, C. W. (1993). Environmental regulation of the virulence and physiology of *Legionella pneumophila.* In *Legionella : Current Status and Emerging Perspectives* (Barbaree, J. M., Breiman, R. F. and Dufour, A. P., Eds) pp. 262-264. American Society of Microbiology, Washington, DC. USA.

Mauchline, W. S., James, B. W., Fitzgeorge, R. B., Dennis, P. J. and Keevil, C. W. (1994). Growth temperature reversibly modulates the virulence of *Legionella pneumophila. Infection and Immunity.* **62,** 2995-2997.

Meenhorst, P. L., van Cronenburg, B. J. and van Furth, R. (1983). De betekenis van leidingwater besmet *Legionella pneumophila* voor het onstaan van legionella-pneumonie als ziekenhuisinfectie. *Nederlands Tijdschrift voor Geneeskunde.* **127,** 327-332.

Meers, J. L. and Tempest, D. W. (1970). The influence of growth limiting substrate and medium NaCl concentration on the synthesis of magnesium binding sites in the walls of *Bacillus subtilis* var. niger. *Journal of General Microbiology.* **63,** 325-331.

Mekalanos, J. J. (1992). Environmental signals controlling expression of virulence determinants in bacteria. *Journal of Bacteriology.* **174,** 1-7.

Mengaud, J. M. and Horwitz, M. A. (1993). The major iron-containing protein of *Legionella pneumophila* is an aconitase homologous with the human iron-responsive element-binding protein. *Journal of Bacteriology.* **175,** 5666-5676.

Meyer, R. D. (1989). Activity of the quinolones versus legionellae. *Revista Espanola de Quimioterapia.* **3**, 215-221.

Miller, A. C. (1981). Erythromycin in Legionnaires' disease : a reappraisal. *Journal of Antimicrobial Chemotherapy.* **7,** 217-222.

Miller, J. F., Mekalanos, J. J. and Falkow, S. (1989). Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science.* **243,** 916-922.

Miller, V. L., Finlay, B. B. and Falkow, S. (1988). Factors essential for the penetration of mammalian cells by *Yersinia. Current Topics in Microbiology and Immunology*. **138,** 15-41

Minnikin, D. E., Abdulrahimzadeh, H. and Baddiley, J. (1971). The interaction of phosphatidylethanolamine and glycosyldiglycerides in bacterial membranes. *Biochemical Journal.* **124,** 447-448.

Mintz, C. S., Chen, J. and Shuman, H. A. (1988). Isolation and characterisation of auxotrophic mutants of *Legionella pneumophila* that fail to multiply in human monocytes. *Infection and Immunity*. **56,** 1449-1455.

Miyamoto, H., Yoshida, S., Taniguchi, H., Qin, M. H., Fujio, H. and Mizuguchi, Y. (1993). Protein profiles of *Legionella pneumophila* Philadelphia-1 grown in macrophages and characterisation of a gene encoding a novel 24 kDa *Legionella* protein. *Microbial Pathogenesis*. **15,** 469-484.

Moffat, J. F. and Tompkins, L. S. (1992). A quantitative model of intracellular growth of *Legionella pneumophila* in *Acanthamoeba castellanii. Infection and Immunity.* **60,** 296-301.

Moffie, B. G. and Mouton, R. P. (1988). Sensitivity and resistance of *Legionella pneumophila* to some antibiotics and combinations of antibiotics. *Journal of Antimicrobial Chemotherapy.* **22,** 457-462.

Monteil, H., Harf, C., Meunier, O. and Jehl, F. (1992). *Xanthomonas maltophilia*, bacterie opportuniste. *Reviews in French Laboratories.* **236,** 22-26.

Morita, R. Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. *Canadian Journal of Microbiology.* **34,** 436-441.

Muder, R. R., Yu, V. L. and Woo, A. H. (1986). Mode of transmission of *Legionella pneumophila*: a critical review. *Archives of Internal Medicine.* **146,** 1607-1612.

Muller, A., Hacker, J. and Brand, B. C. (1996). Evidence for apoptosis of human macrophage-like HL-60 cells by *Legionella pneumophila* infection. *Infection and Immunity.* **64,** 4900-4906.

Murphy, T. F. (1994). Antigenic variation of surface proteins as a survival strategy for bacterial pathogens. *Trends in Microbiology.* **2,** 427-429.

Navratil, J. S., Palmer, R. H., States, S., Kuchta, J. M., Wadowsky, R. M. and Yee, R. B. (1990). Increased chlorine resistance of *Legionella pneumophila* released after growth in the amoeba *Hartmannella vermiformis.* Q-82, p. 302. Abstr. 90th Annual Meeting American Society of Microbiology, Washington, D.C.

Neidhardt, F.C., Ingraham, J. L. and, Schaechter, M. (1990). Regulation of gene expression: multigene systems and global regulation. In *Physiology of the bacterial*

cell. A molecular approach. pp. 351-389. Sinauer Associates Inc. Sunderland, Massachusetts, USA.

Neill, A. M., Gorman, G. W., Gibert, C., Roussel, A., Hightower, A. W., McKinney, R. M. and Broome, C. V. (1985). Nosocomial legionellosis, Paris, France. *American Journal of Medicine*. **78,** 581-588.

Newhall, W. J. and Jones, R. B. (1983). Disulphide-linked oligomers of the major outer membrane protein of *Chlamydia. Journal of Bacteriology*. **154,** 998-1001.

Nichols, W. W., Evans, M. J., Slack, M. P. E. and Walmsley, H. L. (1989). The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa. Journal of General Microbiology.* **135**, 1291-1303.

Nikaido, H. (1973). Biosynthesis and assembly of lipopolysaccharide. In *Bacterial Membranes and Walls.* (Ed. Dekker, M.). L. Leive, New York.

Nikaido, H. (1976). Outer membranes of *Salmonella typhimurium:* transmembrane diffusion of some hydrophobic substances. *Biochemica et Biophysica Acta.* **433,** 118-132.

Nikaido, H. and Nakae, T. (1979). The outer-membrane of Gram negative bacteria. *Advances in Microbial Physiology.* **20,** 163-250.

Nilsson, L., Oliver, J. D. and Kjellleberg, S. (1991). Resuscitation of *Vibrio vulnificus* from the viable but non-culturable state. *Journal of Bacteriology*. **173,** 5054-5059.

Nimmo, G. R. and Bull, J. Z. (1995). Comparative susceptibility of *Legionella pneumophila* and *Legionella longbeachae* to 12 antimicrobial agents. *Journal of Antimicrobial Chemotherapy*. **36,** 219-223.

Nolte, F. S., Conlin, C. A. and Motley, M. A. (1986). Electrophoretic and serological characterisation of the lipopolysaccharide of *Legionella pneumophila. Infection and Immunity.* **52,** 676-681.

Novitsky, J. A.and Morita, R. Y. (1978). Possible strategy for the survival of marine bacteria under starvation conditions. *Marine Biology.* **48,** 289-295.

Nystrom, T., Olsson, R. M. and Kjelleberg, S. (1992). Survival, stress resistance, and alterations in protein expression in the marine *Vibrio* sp. strain S14 during starvation for different individual nutrients. *Applied and Environmental Microbiology.* **58**, 55-65.

O'Brien, S. J. and Bhopal, R. S. (1993). Legionnaires' Disease : The infective dose paradox. *Lancet i.* **342,** 5-6.

O'Mahony, M. C., Stanwell-Smith, R. E., Tillett, H. E., Harper, D., Hutchison, J. G. P., Farrell, I. D., Hutchinson, D. N., Lee, J. V., Dennis, P. J., Duggal, H. V., Skully, J. A. and Denne, C. (1990). The Stafford outbreak of Legionnaires' disease. *Epidemiology and Infection.* **104,** 361-380.

Oldham, L. J. and Rodgers, F. G. (1985). Adhesion, penetration and intracellular replication of *Legionella pneumophila* on *in vitro* model of pathogenesis. *Journal of General Microbiology.* **131**, 697-706.

Ombaka, A., Cozens, R. M. and Brown, M. R. W. (1983). Influence of nutrient limitation of growth on stability and production of virulence factors of mucoid and non-mucoid *Pseudomonas aeruginosa. Reviews in Infectious Disease.* **5,** 5880-5888.

Onody, C., Matsiota-Bernard, P. and Nauciel, C. (1997). Lack of resistance to erythromycin, rifampicin and ciprofloxacin in 98 clinical isolates of *Legionella pneumophila. Journal of Antimicrobial Chemotherapy.* **39,** 815-816.

Ortiz-Roque, C. M. and Hazen, T. C. (1987). Abundance and distribution of Legionellaceae in Puerto Rican waters. *Applied and Environmental Microbiology.* **53,** 2231-2236.

Osterholm, M. T., Chin, T. D. Y., Osborne, D. O., Dull, H. B., Dean, A. G., Fraser, D. W., Hayes, P. S. and Hall, W. N. (1983). A 1957 outbreak of Legionnaires' disease associated with a meat packing plant. *American Journal of Epidemiology.* **117**, 60-67.

Otten, S., Iyer, S., Johnson, W. and Montgomery, R. (1986). Serospecific antigens of *Legionella pneumophila. Journal of Bacteriology.* **167**, 893-904.

Palva, E. T. and Makla, P. H. (1980). Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analysed by sodium dodecyl sulphate polyarylamide gel electrophoresis. *European Journal of Biochemistry.* **107,** 137-143.

Pasculle, A. W., Feeley, J. C., Gobson, R. C., Cordes, L. C., Myerowitz, R. L., Patton, C. M., Gorman, G. W., Carmack, C. L., Ezzell, J. W. and Dowling, J. N. (1980). Pittsburgh pneumonia agent : direct isolation from human lung tissue. *Journal of Infectious Disease.* **141**, 727-732.

Paszko-Kolva, C., Shahamat, M., Yamamoto, H., Sawyer, T., Vives-Rego, J. and Colwell, R. R. (1991). Survival of *Legionella pneumophila* in the aquatic environment. *Microbial Ecology.* **22,** 75-83.

Paszko-Kolva, C., Shahamat, M. and Colwell, R. R. (1992). Long-term survival of *Legionella pneumophila* serogroup 1 under low nutrient conditions of associated morphological changes. *FEMS Microbiology Ecology.* **102,** 45-55.

Paszko-Kolva, C., Shahamat, M. and Colwell, R. R. (1993). Effect of temperature on the survival of *Legionella pneumophila* in the aquatic environment. *Microbial Releases.* **2,** 73-79.

Payne, N. R. and Horwitz, M. A. (1987). Phagocytosis of *Legionella pneumophila* is mediated by a human monocyte complement receptors. *Journal of Experimental Medicine*. **166,** 1377-1389.

Pearlman, E., Jiwa, A. H., Engleberg, N. C. and Eisenstein B. I. (1988). Growth of *Legionella pneumophila* in human macrophage-like U937 cell line. *Microbial Pathogenesis.* **5,** 87-95.

Pine, L., George, J. R., Reeves, M. W. and Harrell, W. K. (1979). Development of a chemically defined liquid medium for growth of *Legionella pneumophila. Journal of Clinical Microbiology.* **9,** 615-626.

Pine, L., Franzus, M. J. and Malcolm, G. B., (1986). Guanine is a growth factor for *Legionella* species. *Journal of Clinical Microbiology*. **23**, 163-169.

Plikaytis, B. B., Carlone, G. M., Pau, C.-P. and Wilkinson, H. W. (1987). Purified 60 kDa *Legionella* protein antigen with *Legionella*-specific and non-specific epitopes. *Journal of Clinical Microbiology*. **25,** 2080-2084.

Plouffe, J. F., Webster, L. R. and Hackman, B. (1983). Relationship between colonisation of hospital buildings with *Legionella pneumophila* and hot water temperatures. *Applied and Environmental Microbiology.* **46,** 769-770.

Poindexter, J. S. (1981). Oligotrophy, fast and famine existence. *Advances in Microbial Ecology.* **5,** 63-89.

Pruckler, J. M., Benson, R. F., Moyenuddin, M., Martin, W. T. and Fields, B. S. (1995). Association of flagellum expression and intracellular growth of *Legionella pneumophila. Infection and Immunity.* **63,** 4928-4932.

Quinn, F. D., Keen, M. G. and Tompkins, L. S. (1989). Genetic, immunological, and cytotoxic comparisons of *Legionella* proteolytic activities. *Infection and Immunity.* **57**, 2719-2729.

Rajagopalan-Levasseur, P., Dournon, E., Dameron, G., Vilde, J. L. and Pocidalo, J. J. (1990). Comparative post-antibacterial activities of pefloxacin, ciprofloxacin, and ofloxacin against intracellular multiplication of *Legionella pneumophila* serogroup 1. *Antimicrobial Agents and Chemotherapy.* **34,** 1733-1738.

Ramirez, J. A., Summersgill, J. T., Miller, R. D., Meyers, T. L. and Raff M. J. (1992). Bactericidal activity of imipenem compared to erythromycin against intracellular *Legionella pneumophila.* Drugs and Experimental Clinical Research. XVIII **10,** 407-410.

Rathman, M., Sjaastad, M. D. and Falkow, S. (1996). Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infection and Immunity.* **64,** 2765-2773.

Rdest, U., Wintermeyer, E., Ludwig, B. and Hacker, J. (1991). Legiolysin, a new haemolysin from *Legionella pneumophila. Zentralblatt fur Bakteriologie und Hygiene. Abt. I Orig. A.* **274**, 471-474.

Rechnitzer, C. (1994) Pathogenic aspects of Legionnaires' disease : interaction of *Legionella pneumophila* with cellular host defences. *APMIS* Suppl. 43. **102,** 5-43.

Reda, C., Quaresima, T. and Castellani-Pastoris, M. (1994). *In vitro* activity of 6 intracellular antibiotics against *Legionella pneumophila* strains of human and environmental origin. *Journal of Antimicrobial Chemotherapy.* **33,** 757-764.

Reingold, A. L., Thomason, B. J., Brake, L., Thacker, H. W. and Kuritsky, J. N. (1984). *Legionella* pneumonia in the United States: the distribution of serogroups and species causing human illness. *Journal of Infectious Disease.* **149**, 819.

Resnick, M. N., Roguel, N., Bercovier, H., Enk, C., Frankenberg, S. and Kedar, E. (1988). Lysis of murine macrophages infected with intracellular pathogens by interleukin II-activated killer (LAK) cells *in vitro*. *Cell Immunology*. **113,** 214-219.

Roantree, R. J., Kuo, T. T. and MacPhee, D. G. (1977). The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium*. *Journal l,* **103,** 223-224.

Rodgers, F. G. and Gibson, F. C. (1993) Opsonin-independent adherence and intracellular development of *Legionella pneumophila* within U937 cells. *Canadian Journal of Microbiology*. **39,** 718-722.

Rodriguez-Zaragoza, S., Rivera, F., Bonilla, P., Ramirez, E., Gallegos, E., Calderon, A., Ortiz, R. and Hernandez, D. (1993) Amoebological study of the atmosphere of San Luis Potosi, SLP, Mexico. *Journal of Experimental and Analytical Environmental Epidemiology*. **3,** (Suppl. 1). 229-235.

Rogers, J., Dowsett, A. B., Dennis, P. J., Lee, J. V. and Keevil, C. W (1994a). Influence of plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water. *Applied and Environmental Microbiology*. **60,** 1842-1851.

Rogers, J., Dowsett, A. B., Dennis, P. J., Lee, J. V. and Keevil, C. W. (1994b). Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Applied and Environmental Microbiology* **60,** 1585-1592.

Rollins, D. M. and Colwell, R. R. (1986). Viable but non-culturable stages of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and Environmental Microbiology*. **52,** 531-538.

Roszak D. B. and Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews.* **51,** 365-379.

Roszak, D. B., Grimes, D. J. and Colwell, R. R. (1984). Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. *Canadian Journal of Microbiology.* **30,** 334-338.

Rowbotham, T. J. (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for fresh water and soil amoebae. *Journal of Clinical Pathology* **33,** 1179-1183.

Rowbotham, T. J. (1986) Current views on the relationships between amoebae, legionellae and man. *Israel Journal of Medical Sciences,* **22,** 678-689.

Rowbotham, T. J. (1992). Little and large particle theory for legionellosis acquired from polymicrobial sources. Proceedings of the International Symposium on *Legionella*, Florida. pp. 32. American Society Microbiology III.

Roy, C. R. and Isberg, R. R. (1997). Topology of *Legionella pneumophila* DotA: an inner membrane protein required for replication in macrophages. *Infection and Immunity.* **65**, 571-578.

St. John, G and Steinman, H. M. (1996). Periplasmic copper-zinc superoxide dismutase of *Legionella pneumophila* : role in stationary-phase survival. *Journal of Bacteriology.* **178,** 1578-1584.

Sadowsky, A. B., Wiater, L. A. and Shuman, H. A. (1993). Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infection and Immunity* **61**, 5361-5373.

Sahney, N. N., Summersgill, J. T. and Miller, R. D. (1993). Inhibition of oxidative burst and chemotaxis in human phagocytes by *Legionella pneumophila* protease. In

Legionella : *Current Status and Emerging Perspectives* (Eds., Barbaree, J. M., Breiman, R. F. and Dufour, A. P.) pp. 111-112. American Society of Microbiology, Washington, DC. USA

Salyers, A. A. and Whitt, D. D. (1994). Host defences against bacterial pathogens. In *Bacterial Pathogenesis. A Molecular Approach.* (Eds. Whitt, D. D.) pp.16-30. American Society for Microbiology Press, Washington, USA.

Sampson, J. S., Plikaytis, B. B. and Wilkinson, H. W. (1986). Immunologic response of patients with legionellosis against major protein-containing antigens of *Legionella pneumophila* serogroup 1 as shown by immunoblot analysis. *Journal of Clinical Microbiology.* **23,** 92-99.

Sampson, J. S., O'Connor, S. P., Holloway, B. P., Plikaytis, B. B., Carlone, G. M. and Mayer, L. W. (1990). Nucleotide sequence of *htpB*, the *Legionella pneumophila* gene encoding the 58 kDa common antigen, formally designated the 60 kDa common antigen. *Infection and Immunity.* **58,** 3154-3157.

Sansonetti, P. J., Ryter, A., Clerc, P., Maurelli, A. T. and Mounier, J. (1986). Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact haemolysis. *Infection and Immunity.* **51,** 461-469.

Schachter, J. (1988). The intracellular life of *Chlamydia. Current Topics in Microbiology and Immunology.* **138,** 109-134.

Schlegal, H. G. (1988). Production of secondary metabolites. In *General Microbiology.* (Eds. Schmidt, K. and Kogut, M.). pp. 339-350.Cambridge University Press. Cambridge, UK.

Schulze-Robbecke, S., Rodder, M. and Exner, M. (1987). Vermehrungs-und abotungstemperaturen naturlich vorkommender *Legionellen. Zentralblatt fur Bakteriologie und Hygiene*, Serie B. **184,** 495-500.

Segreti, J, Meyer, P and Kapell, K (1996). *In vitro* activity of macrolides against intracellular *Legionella pneumophila. Diagnostic Microbiology and Infectious Disease.* **25,** 123-126.

Selchon, J. G., Chiarella, C. and Horadam, A. F. (1984). Mathematical modelling and the preparation of industrial mathematics. In *Teaching and applying mathematical modelling.* (Eds. Berry, J. S., Burghes, D. D., Huntley, I., James, D. J. G. and Moscardini, A. O.) pp.235-246. Ellis-Horwod Ltd. London.

Shand, K. N., Ho, J. L., Meyer, R. D., Gorman, G. W., Edelstein, P. H., Mallison, G. F., Finegold, S. M. and Fraser, D. W. (1985). Potable water as a source of Legionnaires' disease. *Journal of American Medical Association.* **253**, 1412-1416.

Shinnick, T. M. (1991). Heat shock proteins as antigens of bacterial and parasitic pathogens. *Current Topics in Microbiology and Immunology.* **167**, 145-161.

Shuman, H. A., Purcell, M., Segal, G., Hales, L. and Wiater, L. A. (1998). Intracellular multiplication of *Legionella pneumophila* : Human pathogen or accidental tourist? *Current Topics in Microbiology and Immunology.* **225,** 99-112.

Sieburth, J. (1979). *Sea microbes.* Oxford University Press, New York.

Skaliy, P. and McEachern, H. V. (1979). Survival of the Legionnaires' disease bacterium in water. *Annals of Internal Medicine.* **90,** 662-663.

Skoog, D. A. and Leary, J. J. (1992). Induced-coupled plasma analysis. In Principles of instrumental analysis. (Skoog, D. A. and Leary, J. J.) pp. 234-237. Saunders College Publishing, London.

Smith, G. M., Abbott, K. H. and Beale A. S. (1995). Combined therapy with co-amoxiclav and erythromycin against intracellular *Legionella pneumophila* in cell culture and in an experimental respiratory infection in neutropenic rats. *Journal of Antimicrobial Chemotherapy*. **35,** 889-893.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olsen, B. J. Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*. **150,** 76-85

Smith, R. P., Baltch, A. L., Franke, M., Hioe, W., Ritz, W. and Michelson, P., (1997). Effect of levofloxacin, erythromycin or rifampicin pre-treatment on growth of *Legionella pneumophila* in human monocytes. *Journal of Antimicrobial Chemotherapy*. **40**, 673-678.

Sonensson, A., Jantzen, E., Bryn, K., Larsson, L. and Eng, J. (1989). Chemical composition of a lipopolysaccharide from *Legionella pneumophila. Archives in Microbiology*. **153,** 72-78.

Soo Bang, I., Soo Lee, I., Nok Lee, Y., Park, Y. K. (1995). Identification of the genes involved in stationary phase specific acid resistance of *Salmonella typhimurium. Journal of Microbiology.* **33,** 21-27.

Soo Lee, I., Lin, J., Hall, H., Bearson, B. and Foster, J. W. (1995). The stationary phase sigma factor *rpoS* is required for a sustained acid tolerance response in virulent *Salmonella typhimurium. Molecular Microbiology.* **17,** 155-167.

Sorger, K. (1991). Heat shock factor and heat shock response. *Cell.* **65,** 363-366.

States, S. J., Podorski, J. A., Conley, L. F., Young, W. D., Wadowsky, R. M., Dowling, J. N., Kuchta, J. M., Navratil, J. S. and Yee, R. B. (1993). Temperature and the survival and multiplication of *Legionella pneumophila* associated with

Hartmannella vermiformis. In *Legionella : Current Status and Emerging Perspectives* (Eds., Barbaree, J. M., Breiman, R. F. and Dufour, A. P.) pp. 147-149. American Society of Microbiology, Washington, DC. USA.

Steinert, M., Ott, M., Luck, P. C., Tannich, E. and Hacker, J. (1994). Studies on the uptake and intracellular replication of *Legionella pneumophila* in protozoa and in macrophage-like cells. *FEMS Microbiology Ecology.* **15,** 299-308.

Steinert, M., Engelhard, H., Flugel, M., Wintermeyer, E. and Hacker, J. (1995). The Lly protein protects *Legionella pneumophila* from light but does not directly influence its intracellular survival in *Hartmannella vermiformis. Applied and Environmental Microbiology.* **61,** 2428-2430.

Steinert, M., Emody, L., Amann, R., Hacker, J. (1997). Resuscitation of viable but non-culturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii. Applied and Environmental Microbiology.* **63,** 2047-2053.

Steinert, M., Birkness, K., White, E., Fields, B. S. and Quinn, F. (1998). *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Applied and Environmental Microbiology.* **64**, 2256-2261.

Steinman, H. M. (1992). Construction of an *Escherichia coli* K-12 strain deleted for manganese and iron superoxide dismutase genes and its use in cloning the iron superoxide dismutase of *Legionella pneumophila. Molecular Genetics.* **232,** 427-430.

Steinmetz, I., Rheinheimer, C., Hubner, I. and Bitter-Suermann, D. (1991). Genus specific epitope on the 60 kilodalton *Legionella* heat shock protein recognised by a monoclonal antibody. *Journal of Clinical Microbiology.* **29,** 346-354.

Stevens, D. R. and Moulton, J. E. (1978). Ultrastructural and immunological aspects of the phagocytosis of *Trypanosoma brucei* by mouse peritoneal macrophage. *Infection and Immunity.* **19,** 972-982.

Stevenson, L. H. (1978). A case for bacterial dormancy in aquatic systems. *Microbial Ecology.* **4,** 127-133.

Stokes, D. H., Slocombe, B. and Sutherland, R. (1989). Bactericidal effects of amoxycillin / clavulanic acid against *Legionella pneumophila. Journal of Antimicrobial Chemotherapy.* **23,** 43-51.

Stout, J. E., Yu, V. L., Vickers, R. M., Zuravleff, J., Best, M., Yee, R. B. and Wadowsky, R. (1982). Pneumophila in the water supply of a hospital with endemic Legionnaires' disease. *New England Journal of Medicine.* **306,** 466-468.

Stout, J. E., Best, M. G. and Yu, V. L. (1986). Susceptibility of members of the family Legionellaceae to thermal stress : Implications for heat eradication methods in water distribution systems. *Applied and Environmental Microbiology.* **52,** 396-399

Surette, M. G. and Bassler, B. L. (1998). Quorum sensing *in Escherichia coli* and *Salmonella typhimurium. Proceedings of the National Academy of Sciences.* **95,** 7046-7050.

Susa, M., Hacker, J. and Marre, R. (1996). De Novo synthesis of *Legionella pneumophila* antigens during intracellular growth in phagocytic cells. *Infection and Immunity.* **64,** 1679-1684.

Swanson, M. S. and Isberg, R. R. (1995). Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infection and Immunity.* **63,** 3609-3620.

Szczepanski, A. and Fleit, H. B. (1978). Interaction between *Borrelia burgdorferi* and polymorphonuclear leukocytes. Phagocytosis and the induction of the respiratory burst. *Annals of the New York Academy of Science.* **539**, 425-428.

Szeto, L. and Shuman, H. A. (1990). The *Legionella pneumophila* major secretory protein, a protease, is not required for intracellular growth or cell killing. *Infection and Immunity.* **58**, 2585-2592

Tabor, P. S., Ohwada, K. and Colwell, R. R. (1981). Filterable marine bacteria found in the deep sea: distribution, taxonomy and response to starvation. *Microbial Ecology.* **7**, 67-83.

Takasuna, K., Kasai, Y., Usui, C., Takahashi, M., Hirohashi, Tamura, K. and Takayama, S. (1992). General pharmacology of the new quinolone antibacterial agent levofloxacin. *Drug Research.* **42,** 408-418.

Tempest, D. W., Dicks, J. W. and Ellwood, D. C. (1968). Influence of growth conditions on the concentration of potassium in *Bacillus subtilis* var. niger and its positive relationship to cellular ribonuceic acid, teichoic acid and techuronic acid. *Biochemical Journal.* **106**, 237-243.

Tesh, M. J. and Miller, R. D. (1982). Growth of *Legionella pneumophila* in defined media : requirement for magnesium and potassium. *Canadian Journal of Microbiology.* **28**, 1055-1058.

Teuber, M. and Bader, J. (1976). Action of polymyxin B on bacterial membranes: phosphatidylglycerol- and cardiolipin-induced susceptibility to polymyxin b in *Acholeplasma laidlawii* B. *Antimicrobial Agents and Chemotherapy.* **9**, 26-35

Thom, S., Warhurst, D. and Drasar, B. S. (1992). Association of *Vibrio cholerae* with fresh water amoebae. *Journal of Medical Microbiology.* **36**, 303-306.

Thompson, J. E. and Pauls, K. P. (1980). Membranes of small amoebae. In *Biochemistry and physiology of protozoa* (Levandowsky, M. and Hunter, S. H. Eds 2nd Ed., vol. 3.) pp. 207-253. Academic Press, Inc., New York, USA

Tobin, J. O., Beare, J., Dunnill, M. S., Fisher-Hoch, S., French, M., Mitchell, R. G., Morris, P. J. and Muers, M. F. (1980). Legionnaires' disease in a transplant unit: isolation of the causative agent from shower baths. *Lancet ii.* 118-121.

Tobin, J. O., Swann, R. A. and Bartlett, C. L. R. (1981). Isolation of *Legionella pneumophila* from water systems : methods and preliminary results. *British Medical Journal.* **282**, 515-517.

Torrella, F. and Morita, R. Y. (1981). Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in sea water. *Applied and Environmental Microbiology.* **41**, 518-527.

Toumenen, E., Cozens, R., Tosch, W., Zak, O. and Tomasz, A. (1986). The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. *Journal of General Microbiology.* **132,** 1297-1304.

Tsai, C. and Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry.* **119,** 115-119.

Tyndall, R. L. and Domingue, E. L. (1982). Cocultivation of *Legionella pneumophila* and free living amoeba. *Applied and Environmental Microbiology.* **44,** 954-959.

Tyndall, R. L., Soloman, J. A. and Christenson, S. W. (1985). Legionnaires' disease bacteria in power plant cooling systems: downtime report. Oak Ridge National Laboratory, Tennesse.

Unertl, K. E., Lenhart, F. P., Forst, H., Vogler, G., Wilm, V., Ehret, W. and Ruchdeschel, G. (1989). Ciprofloxacin in the treatment of legionellosis in critically ill patients, including cases unresponsive to erythromycin. *American Journal of Medicine*. **87,** (Suppl. 5A), 128-131.

Van den Broek, P. J. (1991). Activity of antibiotics against microorganisms ingested by mononuclear phagocytes. *European Journal of Clinical Microbiology and Infectious Disease.* **10,** 114-118

Van der Linden, M. P. G., de Haan, L., Hoyer, M. A. and Keck, W. (1992). Possible role of *Escherichia coli* penicillin-binding protein 6 in stabilisation of stationary phase peptidoglycan. *Journal of Bacteriology*. **174**, 7572-7578.

Venkataraman, C. B., Haack, B. J., Bondada, S. and Abu Kwaik, Y. (1997). Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires' disease bacterium, *Legionella pneumophila. Journal of Experimental Medicine*. **186**, 537-547.

Vickers, R. M., Yu, V. L., Hanna, S. S., Muraca, P., Diven, W., Carmen, N. and Taylor, F. B. (1987). Determinants of *Legionella pneumophila* contamination of water distribution systems : 15-hospital prospective study. *Infection Control.* **8**, 357-363

Vilde, J. L., Dournon, E. and Rajagopalan, P. (1986). Inhibition of *Legionella pneumophila* multiplication within human macrophages by antimicrobial agents. *Antimicrobial Agents and Chemotherapy.* **30**, 743-748.

Wadowsky, R. M., Yee, R. B., Mezmar, L., Wing, E. J. and Dowling, J. N. (1982) Hot water systems as sources of *Legionella pneumophila* in hospital and non-hospital plumbing fixtures. *Applied and Environmental Microbiology.* **43**, 1104-1110.

Wadowsky, R. M., Wolford, R., McNamara, A. M. and Yee, R. B. (1985). Effect of temperature, pH and oxygen level on the multiplication of naturally occuring *Legionella pneumophila* in potable water. *Applied and Environmental Microbiology.* **49,** 1197-1205.

Wadowsky, R. M., Butler, L. J., Cook, M. K., Verma, S. M., Paul, M. A., Fields, B. S., Keleti, G., Sykora, J. L. and Yee, R. B. (1988). Growth supporting activity for *Legionella pneumophila* in tap water cultures and implication of *Hartmannellid* amoeba as growth factors. *Applied and Environmental Microbiology.* **54**, 2677-2682.

Warren, W. J. and Miller, R. D. (1979). Growth of Legionnaires' Disease bacterium (*Legionella pneumophila*) in chemically defined medium. *Journal of Clinical Microbiology.* **10,** 50-55.

Weichart, D. and Kjelleberg, S. (1996). Stress resistance and recovery potential of culturable and viable but non-culturable cells of *Vibrio vulnificus. Microbiology.* **42,** 845-853.

Weiss, E. and Westfall, H. N. (1984). Substrate utilisation by *Legionella* cells after cryopreservation in phosphate buffer. *Applied and Environmental Microbiology.* **48,** 380-385.

Welch, W. J., Kang, H. S., Beckmann, R. P. and Mizzen, L. A. (1991). Response of mammalian cells to metabolic stress; changes in cell physiology and structure / function of stress proteins. *Current Topics in Microbiology and Immunology.* **167,** 31-57.

West, A. A., Rogers, J., Lee, J. V. and Keevil, C. W. (1993). Lack of dormancy in *Legionella pneumophila*? In *Legionella* : *Current Status and Emerging Perspectives* (Eds., Barbaree, J. M., Breiman, R. F. and Dufour, A. P.) pp. 201-203. American Society of Microbiology, Washington, DC. USA.

Westphal, O. and Jann, K. (1965). Bacterial lipopolysaccharide extraction with phenol-water and further applications of the procedure. In *Methods in carbohydrate chemistry,* volume 5. (R. L. Whistler Ed) Academic Press Inc., New York, USA.

Williams, P. (1988). Role of the cell envelope in bacterial adaption to growth *in vivo* in infections. *Biochimie.* **70**, 987-1011.

Winkler, H. H. and Turco, J. (1988). *Rickettsia prowazekii* and the host cell: entry, growth and control of the parasite. *Current Topics in Microbiology and Immunology.* **138**, 81-109.

Winn, W. (1988). Legionnaires' Disease : Historical perspective *Clinical Microbiological Reviews.* **1,** 60-81.

Winn, W. C. and Myerowitz, R. L. (1981). The pathology of the *Legionella* pneumonias. A review of 74 cases and the literature. *Human Pathology.* **12,** 401-422.

Winter, J. H., McCartney, C., Bingham, J., Telfer, M., White, L. O. and Fallon, R. J. (1988). Ciprofloxacin in the treatment of severe Legionnaires' disease. *Reviews in Infectious Disease.* **10,** (Suppl. 1) 218-219.

Wintermeyer, E., Rdest, U., Ludwig, B., Debes, A. and Hacker, J. (1991). Characterisation of legiolysin (*lly*), responsible for haemolytic activity, colour production and fluorescence of *Legionella pneumophila. Molecular Microbiology.* **5,** 1135-1143.

Wintermeyer, E., Flugel, M., Ott, M., Steinert, M., Rdest, U., Mann, K. and Hacker, J. (1994). Sequence determination and mutational analysis of the *lly* locus of *Legionella pneumophila. Infection and Immunity.* **62,** 1109-1117.

Wyrick, P. B. and Brownridge, E. A. (1978). Growth of *Chlamydia psittaci* in macrophages. *Infection and Immunity.* **19,** 1054-1060.

Xu, S., Roberts, N., Singleton, F. L., Atwell, R. W., Grimes, D. J. and Colwell, R. R. (1982). Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the esturine and marine environment. *Microbial Ecology.* **8,** 313-323.

Xu, S., Cooper, A., Strurgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P. and Russell, D. G. (1994). Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. *Journal of Immunology.* **153,** 2568-2578.

Yamamoto, Y., Okubo, S., Klein, T. W., Onozaki, K., Saito, T. and Friedman, H. (1994). Binding of *Legionella pneumophila* to macrophages increases cellular cytokine mRNA. *Infection and Immunity.* **62,** 3947-3956.

Yee, R. B. and Wadowsky, R. M. (1982). Multiplication of *Legionella pneumophila* in unsterilised tap water. *Applied and Environmental Microbiology.* **43,** 1330-1334.

Zacheus, O. M. and Martikainen, P. J. (1996). Effect of heat flushing on the concentrations of *Legionella pneumophila* and other heterotrophic microbes in hot water systems of apartment buildings. *Canadian Journal of Microbiology.* **42,** 811-818.

Zeilstra-Ryalls, J., Fayet, O. and Georgopoulos, C. (1991). The universally conserved GroE (Hsp60) chaperonins. *Annual Review of Microbiology.* **45**, 301-325.

Zhu, W. H. and Loh, T. T. (1996). Differential effects of phorbal ester on apoptosis in HL-60 promyelocytic leukemic cells. *Biochemistry and Pharmocology.* **51**, 1229-1236.

CHAPTER 9. APPENDICES.

9.1 APPENDIX 1. STATISTICAL ANALYSIS.

SECTION A VIABLE PLATE COUNT

(i) Accuracy and reproducibility of viable plate counts (Section 3.1.3).

Plate / Dilution	1	2	3	4
1	4.07	4.00	5.33	4.67
2	3.60	4.00	4.67	5.33
3	3.57	3.33	4.07	4.57
4	4.00	4.67	3.97	4.07
5	4.07	4.57	3.67	3.60
Total (T)	19.31	20.57	21.71	21.67

Table A1 Viable counts (x 10^6 cfu/ml) determined for 4 serial ten-fold dilutions of the suspension aliquoted on to 5 plates. Accuracy of serial ten-fold dilutions.

n = number of observations per count = 4

m = number of counts = 5

nm = total number of observations = 20

T = $\sum x$

(i) $\sum x^2 = 361.8624$

(ii) $\sum T^2/n = 347.12294$

(iii) $(\sum T)^2/m.n = 343.3040445$

SOURCE OF VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO (F)
Between counts	(2)-(3) 3.8189	m-1 4	0.95473	1.0267
Within counts	(1)-(2) 14.7031	nm-m 15	0.98021	

Table A2. Analysis of variance of 5 replicate dilution series

Significance of "F"

The calculated "F" value = 1.0267. The critical values of "F" for 4/15 degrees of freedom at probability levels of 5 % and 1 % levels are 3.05 and 4.90, respectively. Since the calculated "F" is less than the critical F in both cases (1.0267 < 3.05, 1.0267 < 4.90) it may be concluded that at a probability of 1 % the observed differences between the five groups are not significant. Thus the null hypothesis is retained.

(ii) Accuracy of plating serially ten-fold diluted suspensions of *L. pneumophila* on to BCYE agar

Plate / Dilution	1	2	3	4
1	5.33	4.67	4.07	3.33
2	3.33	4.00	3.60	2.67
3	4.10	3.97	3.13	4.33
4	4.67	3.67	4.47	2.67
5	5.07	3.57	3.97	3.57
Total (T)	22.05	18.88	19.24	16.33

Table A3. Viable counts (x 10^6 cfu/ml) determined for 4 serial ten-fold dilutions of the suspension aliquoted on to 5 plates. Accuracy of plating suspension on to BCYE agar.

n = number of observations per count = 4

m = number of counts = 5

nm = total number of observations = 20

$T = \sum x$

(i) $\sum x^2 = 309.1693$

(ii) $\sum T^2/n = 299.91018$

(iii) $(\sum T)^2/m.n = 296.0651$

SOURCE OF VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO (F)
Between counts	(2)-(3) 3.84506	m-1 4	0.961265	1.5583
Within counts	(1)-(2) 9.25282	nm-m 15	0.61685	

Table A4. Analysis of variance of five replicate platings

Significance of "F"

The calculated "F" value = 1.5583. The critical values of "F" for 4/15 degrees of freedom at 5 % and 1 % levels are 3.05 and 4.90, respectively. Since the calculated "F" is less than the critical "F" in both cases (1.5583 < 3.05, 1.5583 < 4.90) it may be concluded that at a probability of 1 % the observed differences between the five groups are not significant. Thus the null hypothesis is retained.

SECTION B SPREAD PLATE TECHNIQUE

(i) Accuracy of spreading 100 µl volumes of *L. pneumophila* suspension on to BCYE agar

Plate / Dilution	1	2	3	4	5
1	196	148	144	190	157
2	205	215	133	168	185
3	201	167	214	196	172
4	182	183	196	218	158
5	211	189	202	170	188
Total (T)	995	902	889	942	860

Table A5 Colony counts per plate for five replicate counts

n = number of observations per count = 5

m = number of counts = 5

nm = total number of observations = 25

$T = \sum x$

(i) $\sum x^2 = 855086$

(ii) $\sum T^2/n = 8441182.6$

(iii) $(\sum T)^2/m.n = 841989.76$

The results in Table A5 were subjected to analysis of variance as shown in Table A6.

SOURCE OF VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN VARIANCE SQUARES	RATIO (F)
Between counts	(2)-(3) 2192.84	m-1 4	548.21	1.006
Within counts	(1)-(2) 13096.24	nm-m 20	545.17	

Table A6 Analysis of variance of five replicate counts

Significance of "F"

The calculated value of "F" = 1.006. The critical values of "F" for 4/20 degrees of freedom at 5 % and 1 % levels are 2.87 and 4.43, respectively. Therefore, the variation between counts is not significantly greater than the variation within counts. Since the calculated "F" is less than the critical "F" in both cases ($1.006 < 2.87$, $1.006 < 4.43$) it may be concluded that at a probability of 1 % the observed differences between the five groups are not significant. Thus the null hypothesis is retained.

SECTION C HAEMOCYTOMETER COUNTING

(i) The accuracy of haemocytometer counting (Section 3.1.5.1)

.	1	2	3	4	5
1	27	27	27	29	25
2	23	23	15	23	25
3	16	21	20	16	26
4	24	20	17	22	17
5	21	17	22	15	21
Total (T)	114	108	101	105	114

Table A7 Accuracy of five counts of five identical suspensions stained with equal amounts of trypan blue.

n = number of observations per count = 5

m = number of counts = 5

nm = total number of observations = 25

$T = \sum x$

(I) $\sum x^2 = 12037$

(ii) $\sum T^2/n = 11776.4$

(iii) $(\sum T)^2/m.n = 11750.56$

SOURCE OF VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO (F)
Between counts	(2)-(3) 25.84	m-1 4	6.46	2.017
Within counts	(1)-(2) 260.6	nm-m 20	13.03	

Table A8 Analysis of variance of five replicate dilution series

Significance of "F"

The calculated value = 2.017. The critical values of "F" for 4/20 degrees of freedom at 5 % and 1 % levels are 2.87 and 4.43, respectively. Therefore, the variation between counts is not significantly greater than the variation within counts. Since the calculated "F" is less than the critical "F" in both cases (2.017 < 2.87, 2.017 < 4.43) it may be concluded that at a probability of 1 % the observed differences between the five groups are not significant. Thus the null hypothesis is retained.

SECTION D TOTAL COUNT

The accuracy of total counting using DAPI stain and Epifluorescence Microscopy (Section 3.2.4.3)

	1	2	3	4	5
1.	14	14	18	10	15
2	14	9	19	15	19
3	13	15	8	14	18
4	14	15	13	19	18
5	14	16	10	16	15
6	21	16	12	13	11
7	12	12	16	10	12
8	12	16	18	12	14
9	11	13	20	10	10
10	18	15	18	10	11
11	13	11	10	16	11
12	16	19	14	18	13
13	17	13	17	13	19
14	19	18	13	10	13
15	16	12	13	15	17
Total	224	214	219	201	216

Table A9 Accuracy of data from fifteen counts of five identical membrane slides prepared from the same sample of microcosm water.

The results in Table A9 were subjected to analysis of variance as shown in Table A10.

n = number of observations per count = 15

m = number of counts = 5

nm = total number of observations = 75

$T = \sum x$

(I) $\sum x^2 = 16074$

(ii) $\sum T^2/n = 15399.334$

(iii) $(\sum T)^2/m.n = 15379.68$

SOURCE OF .VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO (F)
Between counts	(2)-(3) 19.654	m-1 4	4.9135	2.2884
Within counts	(1)-(2) 674.666	nm-m 60	11.244	

Table A10 Analysis of variance of five replicate dilution series

Significance of "F"

The calculated value of "F" = 2.2884. The critical values of "F" for 4/60 degrees of freedom at 5 % and 1 % levels are 2.53 and 3.65, respectively. Therefore, the variation between the counts is not significantly greater than the variation within the counts. Since the calculated "F" is less than the critical "F" in both cases ($2.2884 < 2.53$, $2.2884 < 3.65$) it may be concluded that at a probability of 1 % the observed differences between the five groups are not significant. Thus the null hypothesis is retained.

9.2 APPENDIX 2. VITAL STAINING

The vital staining kit employed during the microcosm studies was a Molecular Probes' Live/Dead BacLight bacterial viability kit. This kit provided a novel two-colour fluorescence assay of bacterial viability. A mixture of SYTO 9 green fluorescent nucleic acid (solution A) and the red fluorescent nucleic acid stain, propidium iodide (solution B). These stains differ both in their spectral characteristics and in their ability to penetrate healthy cells.

When used alone, the SYTO 9 stain generally labels all bacteria in a population; those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, competing with SYTO 9 stain for nucleic acid binding sites when both dyes are present. Thus, with an appropriate mixture of SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation / emission maxima for these dyes are about 480 nm / 500 nm for SYTO 9 stain and 490 nm / 635 nm for propidium iodide. The background remains virtually non-fluorescent. The use of uv light and the appropriate filters enables both the red and green fluorescing bacteria to be visualised independently.

9.3 APPENDIX 3. DEVELOPMENT OF THE OMP PREPARATION

Method 1 Standard Method

Details of the standard method are given in Section 3.3.3.1

Method 2 Optimising the sonication period

(a) The number of cycles of sonication was increased from 10 to 20 and 50, each cycle consisting of a 10 sec. pulse and 20 sec. for cooling.

(b) The length of the pulse period was increased to 20 sec. and 30 sec. The cooling period was extended to 30 sec. The longer pulse periods caused evaporation of the sample. Changes in the sonication process did not result in clear protein bands on separation by SDS-PAGE.

Method 3 Optimising the incubation period with sarkosyl

The original method described in Section 3.3.3.1 was modified in that the incubation period after the addition of sarkosyl was extended to 2 h. No improvement in the separation of the protein bands was achieved.

Method 4 The removal of DNA

The presence of DNA is known to cause 'smearing' of protein bands on separation by SDS-PAGE (Personal communication P. Lambert).In an endeavour to overcome this potential problem, 1 µl of DNAse and 5 µl 500 mM $MgCl_2$ were added to the sample after the addition of sarkosyl. The sample was incubated at room temperature for 1 h. No improvement in band clarity was observed.

Method 5

The method of OMP preparation as described by Barker *et al.* (1993) was investigated. A 500 µl sample of *L. pneumophila* was thawed at room temperature for 30 min. prior to dilution 1 in 4 in amoebic saline. The suspension was sonicated in a clean glass

bijoux held in a beaker of ice for 10 cycles of 10 sec. pulses with 20 sec. pauses for cooling. A MSE Soniprep sonic disintegrator with a 9 mm diameter probe was used at maximum power and an amplitude of 14 μm. (MSE Scientific Instruments). Half the sonicated suspension was placed in each of two eppendorf tubes. Sarkosyl (150 μl, 20 %, w/v) was added to each sample immediately prior to centrifugation at 11 600 x g for 5 min.. The supernatants were removed and placed in polyallomer tubes prior to centrifugation at 11,600 x g for 90 min. at 4°C. Each pellet was resuspended in 25 μl of ddH_2O and pooled together. The pellet suspension and supernatants were stored at 4°C prior to separation by SDS-PAGE. This method failed to produce distinct bands on separation by SDS-PAGE.

Method 6 Effect of final centrifugation speed

It was suggested that the cause of the 'smear' produced when the OMP preparation was separated by SDS-PAGE was due to cell debris being deposited along with the OMPs during the high speed centrifugation. Method 1 was repeated except the final centrifugation was performed for 90 min. at a low speed, 11,600 x g, (Barker *et al.*, 1993) or a medium speed, 50,000 x g. (*cf.* 100,000 x g). Centrifugation speed had no apparent effect on the separation of the protein bands.

Method 7 Addition of Lysozyme

The method of OMP preparation as described by Barthe *et al.* (1988) was investigated. Sarkosyl (125 μl; 10 % w/v) was added to a 500 μl sample of *L. pneumophila* suspension prior to incubation at room temperature for 1 h. The sample was sonicated in an ice bucket for 20 cycles of 10 sec. pulses with 20 sec. pauses for cooling. A 500 μl volume of 50 mM Tris, pH 7.2, containing lysozyme (1 mg/ml) and 2 % (w/v) sarkosyl was added to the sonicated suspension prior to centrifugation at 5,800 x g for 10 min.. The pellet was removed and the supernatant centrifuged for a further 5 min.. The resulting supernatant was placed in a polyallomer tube and centrifuged at 100,000 x g for 30 min. at 4°C. After centrifugation, the pellet formed was separated from the supernatant, which was stored at 4°C prior to separation by SDS-PAGE. The pellet

was resuspended in 500 µl of 50 mM Tris, pH 7.2, containing 2 % (w/v) sarkosyl. The centrifugation was repeated at 100,000 x g for 30 min. at 4°C. The resulting supernatant was removed and the pellet stored at 4°C.

This method was found to produce a clear protein band upon separation of the pellet by SDS-PAGE. An example of the separation of a pellet prepared by this method is shown in Figure A1 together with examples of the results from the methods described above. The corresponding supernatants prepared by each method are shown in Figure A2.

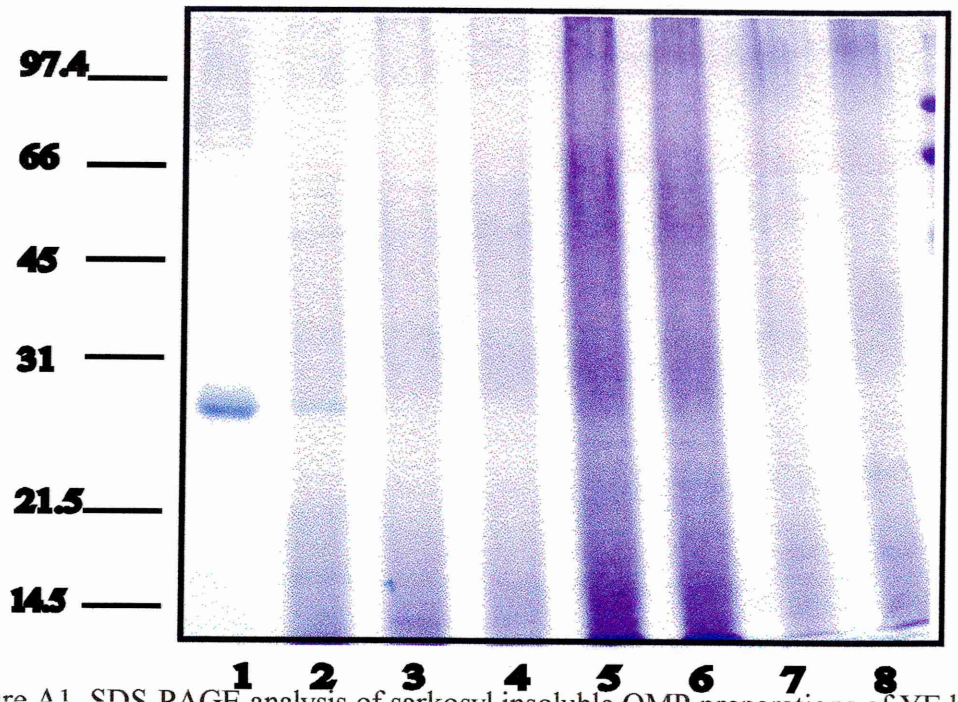


Figure A1. SDS-PAGE analysis of sarkosyl insoluble OMP preparations of YE broth grown *L. pneumophila* as prepared by various methods. Lane 1, method 7, Lane 2, method 6, Lane 3, method 5, Lane 4, method 4, Lane 5, method 3, Lane 6, method 2, Lane 7 and 8, method 1.

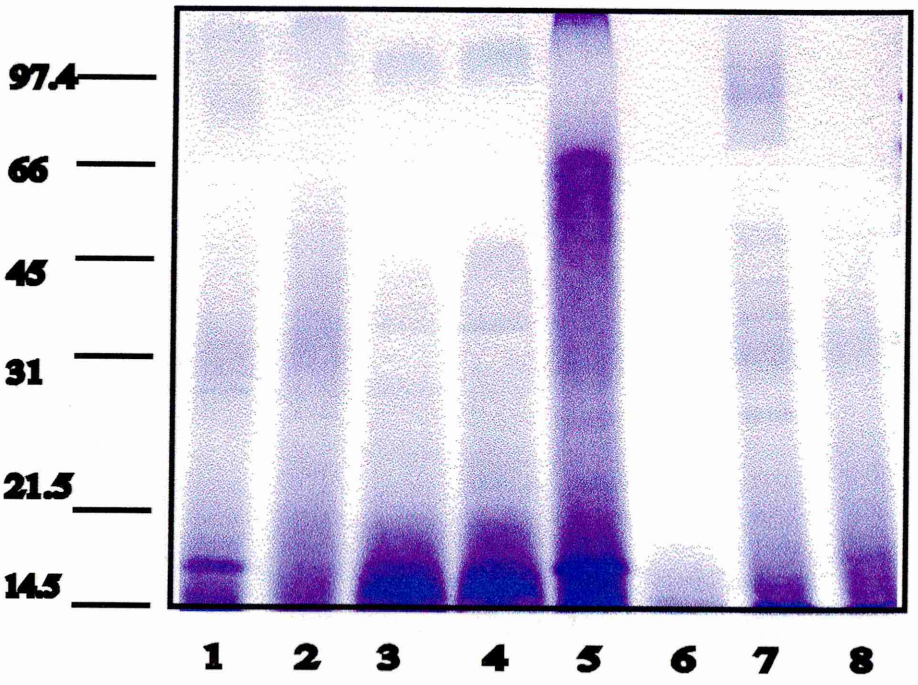


Figure A2. SDS-PAGE analysis of the corresponding supernatants of sarkosyl insoluble OMP preparations of YE broth grown *L. pneumophila* as prepared by various methods. Lane 1, method 7, Lane 2, method 6, Lane 3, method 5, Lane 4, method 4, Lane 5, method 3, Lane 6, method 2, Lane 7 and 8, method 1.

9.4 APPENDIX 4. ELECTROPHORETIC TRANSFER

Semi-dry electrophoretic transfer with single buffer system

The semi-dry electrophoretic transfer was repeated as described in Section 3.4.4.1 with the exception that a single buffer was employed. The gel-membrane sandwich was prepared using two stacks of three pieces of 3MM absorbent soaked in transfer buffer. Transfer buffer consisted of 48 mM Tris, 39 mM glycine, 0.037 % (w/v) SDS and 20 % (v/v) methanol. Both the nitrocellulose membrane and gel were wetted with ddH_2O prior to formation of the gel-membrane sandwich.

Wet electrophoretic transfer

Wet electrophoretic transfer involves the complete immersion of the gel-membrane sandwich in buffer held in a tank (Figure A3). The sandwich was formed by cutting two pieces of 3MM absorbent paper and one piece of nitrocellulose membrane to the size of the gel. Each was soaked in conducting buffer (25 mM Tris, 192 mM glycine and 20 % (v/v) methanol). The gel-membrane sandwich was prepared as outlined in Figure A4. The cassette assembly was placed in the tank transverse to the electric fields and submerged under conducting buffer. An ice block was placed in the tank which was held on ice throughout the transfer period to prevent over-heating. The transfer was carried out at 100 V, 6 mA for 1 h. before the gel was stained for proteins or LPS accordingly, and the membrane blocked (Section 3.4.4.2).

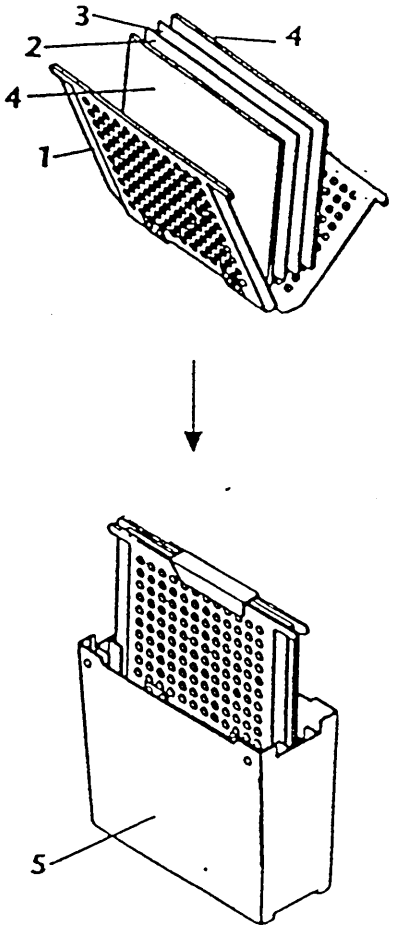


Figure A3. Wet blotting apparatus - exploded view. 1, Cassette, 2, gel, 3, nitrocellulose membrane, 4, fibre pads and absorbant paper soaked in buffer provide a positive contact within the gel-membrane sandwich. 5, Buffer tank. The gel cassette is inserted vertically in the buffer tank.

9.5 APPENDIX 5. COMPARISON OF COUNTING TECHNIQUES.

A comparison of bacterial total count, recoverability on BCYE agar and the number of membrane intact bacteria as determined by vital staining / epifluorescence microscopy, was made for each microcosm. A typical example has been shown in Figure 5.17. The data for each remaining microcosm is shown below (Figure A4; a-g).

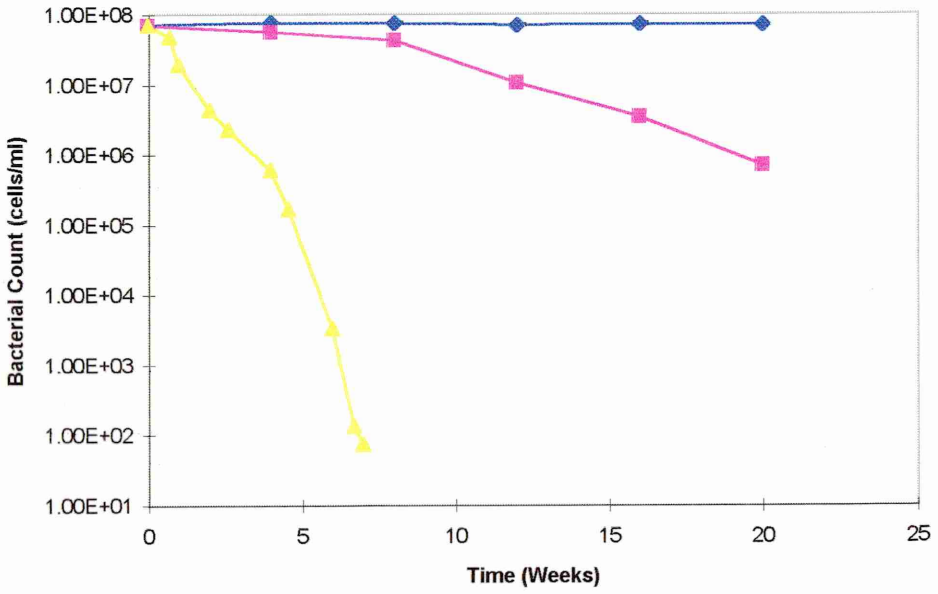


Figure A4 (a) Comparison of vital staining and plate count methods to determine bacterial survival. The total count (◆), viable count by vital staining, (■) and viable count on agar (▲) of YE broth grown *L. pneumophila* (stationary phase) in deionised water.

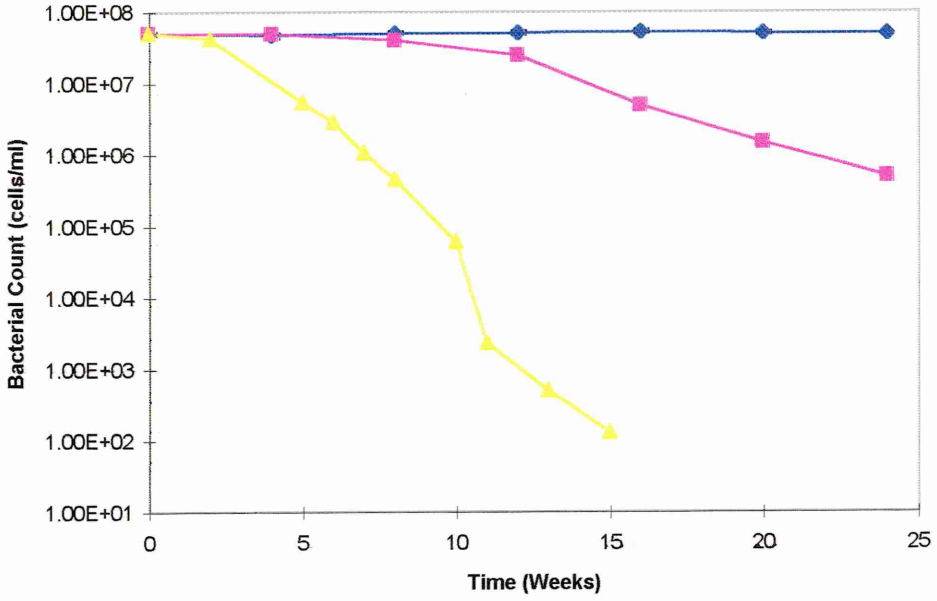


Figure A4 (b) Comparison of vital staining and plate count methods to determine bacterial survival. The total count (◆), viable count by vital staining, (■) and viable counton agar (▲)of intra-amoebic grown *L. pneumophila* in deionised water.

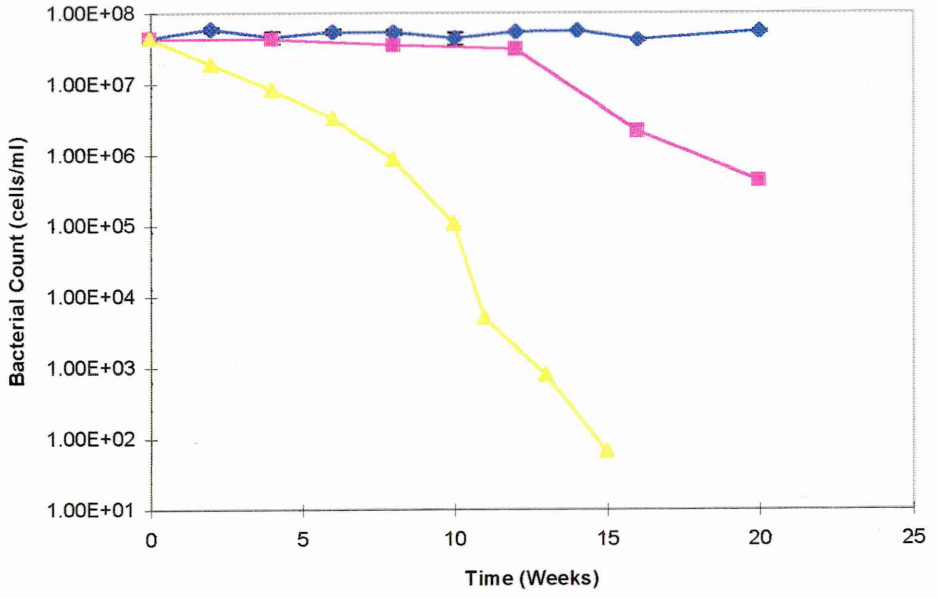


Figure A4 (c) Comparison of vital staining and plate count methods to determine bacterial survival. The total count (◆), viable count by vital staining, (■) and viable count on agar (▲) of intra-monocytic grown *L. pneumophila* (stationary phase) in deionised water.

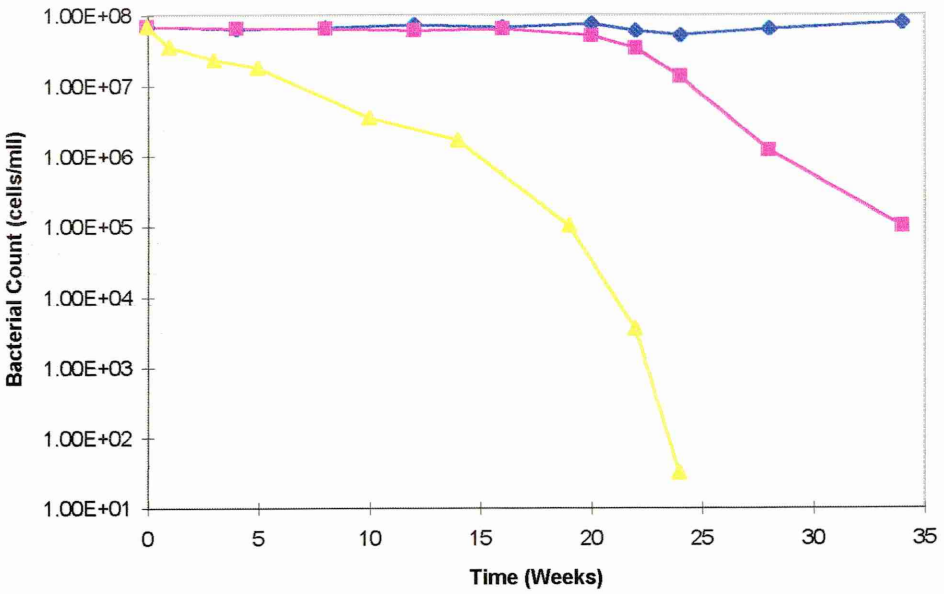


Figure A4 (d) Comparison of vital staining and plate count methods to determine bacterial survival. The total count (◆), viable count by vital staining, (■) and viable count on agar (▲)of YE broth grown *L. pneumophila* (exponential phase) in tap water.

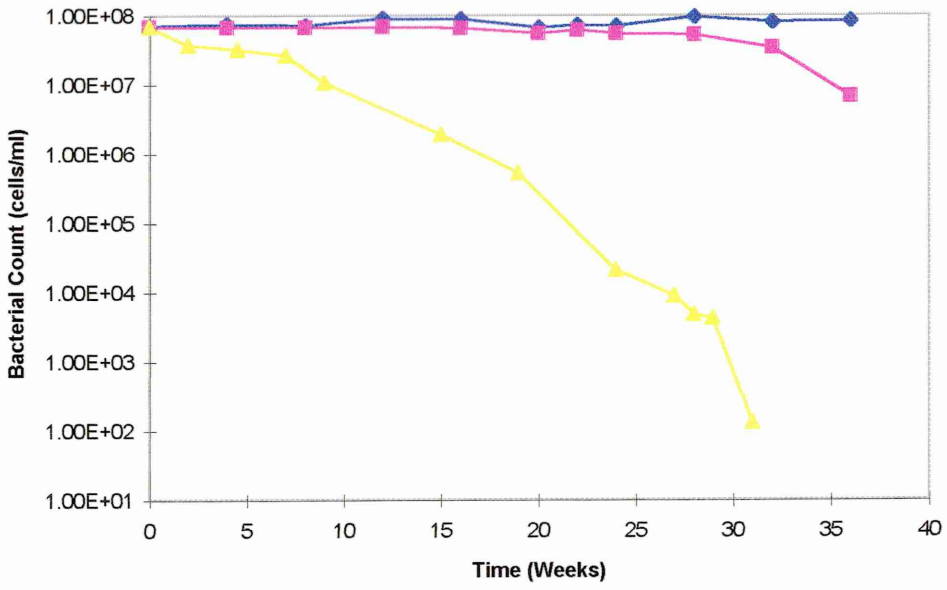


Figure A4 (e) Comparison of vital staining and plate count methods to determine bacterial survival. The total count (◆), viable count by vital staining, (■) and viable count on agar (▲) of YE broth grown *L. pneumophila* (stationary phase) in tap water.

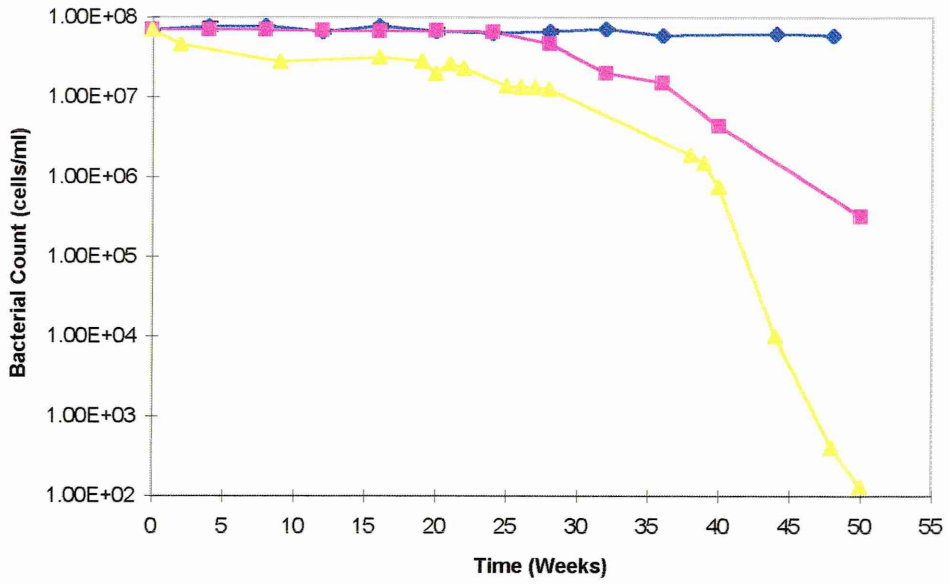


Figure A4 (f) Comparison of vital staining and plate count methods to determine bacterial survival. The total count (◆), viable count by vital staining, (■) and viable count on agar (▲) of intra-amoebic grown *L. pneumophila* in tap water.

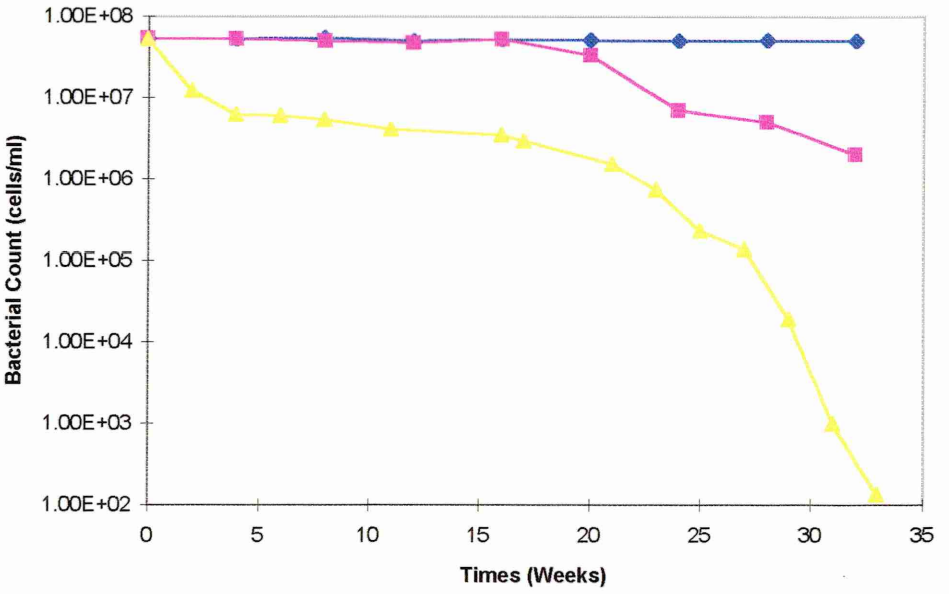


Figure A4 (g) Comparison of vital staining and plate count methods to determine bacterial survival. The total count (◆), viable count by vital staining, (■) and viable count on agar (▲) of 'aged' intra-amoebic grown *L. pneumophila* in tap water.