Legionella pneumophila filamentous morphological form and the generation of novel spherical form upon interaction with protozoan hosts

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LEGIONELLA PNEUMOPHILA
FILAMENTOUS MORPHOLOGICAL FORM AND
THE GENERATION OF NOVEL SPHERICAL FORM
UPON INTERACTION WITH PROTOZOAN HOSTS

CHAN SOCK HOAI
SCHOOL OF BIOLOGICAL SCIENCES
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ABSTRACT

The filamentous form of *Legionella pneumophila* is acknowledged to be an alternative morphological form of the bacterium in nature, but its characteristics and ecological relevance in the life cycle of *L. pneumophila* is poorly understood. In this study, we explored the environmental factors that can trigger formation of the filamentous form as well as the interaction of this morphological form with *L. pneumophila*'s natural protozoan hosts. Our investigation showed that filamentation cannot be neatly pegged to a single dominant factor but is promoted under a concerted influence of environmental conditions leaning towards (i) higher than ambient temperatures, at 37-42°C, (ii) static fluid movement, and (iii) with at least a minimal organic content of 5% buffered yeast extract, or equivalent. In the course of investigating interaction of the filamentous form with protozoan hosts, a spherical form was discovered. Ultrastructural analysis confirmed the cellular nature of this novel entity and revealed morphological variations that are suggestive of a transitional relationship between the filamentous and the spherical forms. *In vitro* characterization demonstrated that emergence of the spherical form is (i) preceded by the filamentous form, (ii) rapidly triggered by some soluble factors secreted by protozoa, and (iii) independent of *de novo* protein synthesis. Exposure of a filament-rich bacterial population to secreted factors of protozoa resulted in the decline in abundance of the filamentous form that is paralleled by a transient surge in the spherical form and a gradual prevalence of the bacillary form; indicating that the spherical form may be an intermediate in the filament-to-rod morphological transition in *L. pneumophila*. Collectively, these findings suggest that under an intricate balance of environmental factors, the predominantly bacillary *L. pneumophila* is capable of differentiating into the filamentous form, which upon interaction with protozoan hosts may be triggered to differentiate rapidly back into the bacillary form via a spherical intermediate.

LIST OF PUBLICATIONS

Journal papers

Sock Hoai CHAN and Chun Chau SZE. Environmental relevance of the filamentous form of *Legionella pneumophila* – conditions for occurrence and proliferative potential in amoebic host (manuscript submitted)

Sock Hoai CHAN, Rafael A. GARDUÑO and Chun Chau SZE. Filamentous cells of *Legionella pneumophila* morphologically differentiate into spherical forms during the pre-internalization phase of protozoan interaction (manuscript submitted)

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Sock Hoai CHAN and Chun Chau SZE (2009) *In vitro* analysis of interaction of *Acanthamoeba castellanii* with *Legionella pneumophila* of the filamentous morphological form. Presented as poster at the 7th International Conference on *Legionella*, Paris, France. Abstract P83.

Sock Hoai CHAN and Chun Chau SZE (2008) Screening of *Legionella pneumophila* random mutant libraries using fluorescence-activated cell sorter (FACS). Presented as poster at the 3rd Korea-Singapore International Conference on Bioscience and Biotechnology, Singapore.

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LIST OF ABBREVIATIONS

Abbreviation	In full
<i>A. castellanii</i>	<i>Acanthamoeba castellanii</i>
<i>A. polyphaga</i>	<i>Acanthamoeba polyphaga</i>
AC buffer	<i>Acanthamoeba castellanii</i> buffer
ACES	N-(2-Acetamido)-2-Aminoethanesulfonic acid
ATCC	American Type Culture Collection
BYE	buffered yeast extract
BYECA	buffered yeast extract charcoal agar
CFU	colony forming unit
CLSM	confocal laser scanning microscope
cm	centimeter
Cm	chloramphenicol
Cys	L-cysteine
ddH ₂ O	distilled deionized water
DIC	differential interference contrast
EB	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
EP	exponential phase (form)
FACS	fluorescence-activated cell sorter
FePP	ferric pyrophosphate
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	gram
GC-FC	gonococcal-ferric cysteine
GFP	green fluorescence protein
<i>gfp</i>	gene of green fluorescent protein mutant
xg	relative centrifugal force
h	hour
kb	kilobase
kV	kilovolt

Km	kanamycin
Km ^r	kanamycin-resistant
L	litre
<i>L. pneumophila</i>	<i>Legionella pneumophila</i>
LB	Luria-Bertani
M	molar
MIC	minimum inhibitory concentration
MIF	mature intracellular form
μF	microFaraday
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
ms	millisecond
nm	nanometer
OD ₆₀₀	optical density at 600nm
PE	phycoerythrin
PI	propidium iodide
PMT	photomultiplier
PYG	peptone yeast extract glucose
RF	replicative form
rpm	revolutions per minute
RT	room temperature
s	second
SD	standard deviation
SP	stationary phase (form)
SSC	side scatter
<i>T. pyriformis</i>	<i>Tetrahymena pyriformis</i>

TBE	Tris/Borate/EDTA
TEM	Transmission electron microscopy
UV	ultraviolet
VBNC	viable but nonculturable
w/v	weight per volume
v/v	volume per volume

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. The genus *Legionella*

Members of the genus *Legionella* (Class γ -proteobacteria, Order Legionellales, Family Legionellaceae) are Gram-negative, aerobic and bacillary. They can be ubiquitously found in freshwater environments (Campodonico and Roy, 2009), but are a cause for concern if sufficiently accumulated in man-made water systems. Comprising of as many as 50 species with at least 73 serogroups to date (Lück, 2008), the genus is infamously associated with Legionnaires' disease, a severe atypical pneumonia, and Pontiac fever, a milder flu-like illness (Fields et al., 2002). These diseases are collectively known as legionellosis. While most species of this genus may be suspected to be capable of causing Legionnaires' disease under permissive conditions, *L. pneumophila* is still regarded as the principal aetiological agent of Legionnaires' disease, accounting for 91.5% of the sporadic community-acquired infections by *Legionella* surveyed internationally (Yu et al., 2002) and 79.5% of Legionnaires' disease cases between 2007-2008 in Europe alone (Joseph and Ricketts, 2010). Transmission of the disease occurs via inhalation of aerosolized water bearing the bacteria, and primarily affects susceptible human hosts, e.g. the elderly and the immunocompromised. In the historic outbreak which gave Legionnaires' disease its name in the summer of 1976, at a convention of the American Legion in Philadelphia, the source of infection was traced to the air-conditioning system at the convention site which was found to be contaminated with high levels of *L. pneumophila* (Campodonico and Roy, 2009; Fields, 1996; Fraser et al., 1977). Since then, man-

made water systems – such as cooling towers, air-conditioning systems, hot spring spas, decorative fountains, and even ice machines – have been recognized as sources capable of harbouring *Legionella* and aerosolizing them into the open environment (Steinert et al., 2002). These man-made sources are usually fed with potable water drawn from natural aquatic reservoirs, e.g. lakes and rivers, where *Legionella* inhabits; thus an accidental breach during any of the water purification stages can bring the bacteria to close proximity with the human population. However, under normal circumstances, the density of *Legionella* in the water system is likely to be too low to pose any significant health hazard. It is only when environmental conditions become permissive for replication – such as elevation of the water temperature to one favourable for proliferation (Fields, 2007), increased nutrient availability or the significant presence of its natural host e.g. amoeba (Devos et al., 2005; Steinert et al., 2002) – that *Legionella* can amplify its population to an infectious dose within artificial water systems. This then greatly increases the chances of bacteria-human contact and heightens the risk of an Legionnaires' disease outbreak.

Given the ominous threat, much work has been done to understand *Legionella* from multiple angles – epidemiology, mechanism of pathogenesis, genomics, clinical microbiology and ecology in its natural environment, in hopes of achieving better management and control of the bacteria and legionellosis. An extensive volume of these studies centres on *L. pneumophila* – the most clinically important species of *Legionella*. Understanding the ecological relevance of *L. pneumophila* through its life cycle is therefore a pertinent component within this frame of work. Section 1.2 below will attempt to present what is currently known about the life cycle of *L. pneumophila*. As with most well-studied models, the foundation laid through their studies can be the

basis on which inference is drawn for similar organisms – in this case, for other members of the genus *Legionella*.

1.2. The *L. pneumophila* life cycle

L. pneumophila is a fastidious bacterium that uses amino acids as carbon and energy sources but does not metabolize carbohydrates (Devos et al., 2005; Steinert et al., 2002). Despite its finicky nature, it is ironic that *L. pneumophila* is ubiquitous in freshwater environments where concentration of nutrients is low (Devos et al., 2005). The ability of the bacterium to replicate within protozoan hosts such as amoebae and to persist in environmental biofilms has been attributed to the bacterium's continued presence in the natural environment (Fields et al., 2002). These modes of intracellular and extracellular existence are integral to the life cycle of *L. pneumophila*. Figure 1.1 illustrates the life cycle of *L. pneumophila*, with the free-living rod-shaped form anchoring the centre of the spread. The planktonic rod-shaped cell of *L. pneumophila* is known to exist in various forms, which will be elaborated in the later section (Section 1.3.2). For now, the perspective of *L. pneumophila* life cycle as fundamentally comprising of the intracellular (within host, Figure 1.1 A & B) cycle and the extracellular (outside host, Figure 1.1 C & D) cycle will be presented.

Figure 1.1 A diagrammatic representation of *L. pneumophila* life cycle as composed of the intracellular and extracellular cycles. (A) Intracellular cycle within protozoan hosts. Internalization and establishment of intracellular replication by *L. pneumophila* in the host leads to amplification of the bacterium in the environment. (B) Intracellular cycle within human host. Infection of human lung macrophages via inhalation of *L. pneumophila*-contaminated aerosols generated by man-made water systems leads to legionellosis in susceptible individuals. This usually constitutes an epidemiological dead-end since person-to-person transmission has never been documented. (C) Extracellular cycle in biofilm. *L. pneumophila* is known to persist and perhaps proliferate in multispecies biofilms in the environment. (pink circles and yellow rectangles represent other environmental bacterial species) (D) *In vitro* cycle. *L. pneumophila* can be cultured in laboratory in growth medium supplemented with cysteine and iron.

1.2.1. The intracellular life cycle

Shortly after its identification in 1977 (McDade et al., 1977), *L. pneumophila* was demonstrated to be pathogenic not only to humans, but also to certain freshwater and soil amoebae (Rowbotham, 1980).

In the environment, free-living protozoa naturally feed on bacteria as part of the ecological association. In fact, predation by protozoa is known to be an important mechanism for controlling microbial communities (Pernthaler, 2005). The population dynamics as well as the structure and organization of microbial communities, e.g. bacterial biofilms, can be significantly influenced by protozoan grazing (Huws et al., 2005; Jurgens et al., 1999). For instance, it has been shown that grazing by protozoa altered the morphological and taxonomic compositions of the bacterial assemblages of a freshwater pond, resulting in the development of a dominating population of grazing-resistant filamentous bacteria (Jurgens et al., 1999). In addition, predation by protozoa also plays a role in nutrient recycling, especially of nitrogen and phosphorus, because it helps to release into the environment nutrients that would otherwise be immobilized in the bacterial biomass (Pernthaler, 2005). However, some bacteria have evolved mechanisms to survive protozoan predation and even to exploit the predators as hosts for proliferation, (Greub and Raoult, 2004; Molmeret et al., 2005; Pernthaler, 2005). Many of these bacteria are also human pathogens, such as *L. pneumophila*, *Coxiella burnetii*, *Listeria monocytogenes*, *Mycobacterium avium*, and *Burkholderia cepacia* (Holden et al., 1984; Landers et al., 2000; Ly and Müller, 1990; Scola and Raoult, 2001; Steinert et al., 1998). Hence ubiquitous, free-living protozoa

can act as reservoirs for bacterial pathogens in the environment (Barker and Brown, 1994; Greub and Raoult, 2004). In the case of *L. pneumophila*, observation of amoebal vacuoles containing many *L. pneumophila* cells by Rowbotham (Rowbotham, 1980) indicated the ability of the bacteria to survive digestion by and establish intracellular growth within amoeba. Since then, extensive research on the pathogenicity of *L. pneumophila* had been carried out, and relentless efforts by many laboratories have resulted in a greater understanding of *L. pneumophila*'s intracellular life cycle which is critical to its pathogenesis. It is now indisputably established that *L. pneumophila* is able to multiply within protozoan cells as well as mammalian macrophages (Fields et al., 2002).

Multiplication of *L. pneumophila* in the environment hinges most on the support of its natural protozoan host (Devos et al., 2005; Fields, 1996) because in the free-living form, *L. pneumophila*'s fastidious nutritional needs make it easily out-competed by other faster-growing bacteria. Protozoa are therefore central to the life cycle of *L. pneumophila* in that they provide nutrition and shelter (Steinert et al., 2002) i.e. favourable conditions for the bacterium's replication, which are rarely encountered in the environment. The intracellular cycle will start with the initial attachment of *L. pneumophila* cells to the protozoan host and engulfment of the bacteria by phagocytosis, resulting in enclosure of these cells into a phagosome (Figure 1.1, A). The bacterium alters the property of the phagosome and evades its fusion to the host lysosomes, effectively resisting digestion by the host. Inside this protected enclosure, bacterial replication is initiated and *L. pneumophila* reproduces large numbers of virulent progenies before exiting the host cell by inducing its cytolysis (Abu Kwaik et al., 1998; Harb et al., 2000; Molmeret et al., 2007). At least 14 species of amoebae

and 2 species of ciliates have been demonstrated to support the intracellular life cycle of *L. pneumophila*, including those from the genera *Acanthamoeba*, *Hartmanella*, *Dictyostelium*, and *Tetrahymena* (Fields et al., 1984; Hagele et al., 2000; Molmeret et al., 2007; Steinert et al., 2002).

While the protozoa-*Legionella* relationship is a natural interaction in the ecosystem, the bacterial relationship with human macrophages has been the consequence of accidental proximity. Inhalation of aerosolized *L. pneumophila*-laden water, unwittingly accumulated within man-made water systems, sets up the opportunity for the infection of human alveolar macrophages (Figure 1.1, B). Upon uptake by these human phagocytes, the intracellular pathway of *L. pneumophila* follows essentially one that is similar to that in the protozoan host: enclosure of the bacteria in the phagosome, subversion of host lytic enzyme digestion, initiation of replication and the subsequent egress of virulent bacteria from wasted host cells undergoing apoptosis and/or necrosis (Harb et al., 2000). Although the completion of one bacterium's infection cycle in human macrophages will lead to the dispersal of its progenies and subsequent infection of other macrophage cells within the same human host, this infection process can be considered an epidemiological dead-end (Albert-Weissenberger et al., 2007; Garduño, 2007). Person-to-person transmission has never been observed – apparently, replicated bacteria are contained within the infected individual and not circulated into the environment. In contrast, the dispersal of virulent *L. pneumophila* from one protozoan cell to other protozoan cells in the environment is assured of a continuity of spread.

1.2.2. The extracellular life cycle

Although it thrives within protozoan hosts, *L. pneumophila* can also exist as free-living entities (Steinert et al., 1997). *In vitro* cultivation of axenic culture is supported in buffered yeast extract broth supplemented with L-cysteine and iron (Figure 1.1, D). The batch culture of *L. pneumophila* exhibits a typical bacterial growth curve characterized by the exponential and stationary phases. Like other bacterial species, *L. pneumophila* in these two phases are phenotypically dissimilar (Byrne and Swanson, 1998), which will be discussed in Section 1.3.2.2.1.

In contrast to the *in vitro* culture conditions, nutrient availability is usually low and physical conditions harsh in natural or man-made freshwater environments. One of the means through which *L. pneumophila* persists in this oligotrophic milieu is by associating with biofilms (Devos et al., 2005; Fields, 2007). In the natural environment, biofilm is the prevailing microbial lifestyle (Watnick and Kolter, 2000). Biofilm is defined as “a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription” (Donlan and Costerton, 2002). Biofilms can grow on a myriad of surfaces, including medical devices, pipings of industrial or potable water system, and in natural aquatic systems. This is a public health concern because of the propensity for these natural biofilms to harbour pathogens of infectious diseases (Donlan, 2002; Parsek and Singh, 2003; Wingender and Flemming, 2011). It is recognized and acknowledged that *L.*

pneumophila can persist within such environmental biofilms (Declerck et al., 2007a; Fields, 2007; Storey et al., 2004).

In general, biofilm formation begins with the irreversible attachment of the planktonic bacterium to the substratum followed by its multiplication to form microcolonies.

Cells would secrete extracellular polymeric substances that encase the microcolonies as the bacteria continue multiplying in number. As the biofilm matures, the microbial community becomes increasingly organized, develops complex architectures and culminates in the dispersal of bacterial cells from the biofilm when environmental conditions become unfavourable (Hall-Stoodley et al., 2004; Watnick and Kolter, 2000). Although biofilm structures can be quite heterogenous, they are typically characterized by mushroom- or pillar-shaped structures interspersed with water channels (Donlan, 2002; Tolker-Nielsen and Molin, 2000). It is proposed that bacteria develop biofilms for several reasons: (i) as a form of stress response because the organization of a biofilm makes it resistant to physical forces, chemical insults as well as predation, (ii) as a mechanism to remain in a favourable niche, and (iii) to take advantage as a community with members in close physical association, benefitting from sharing of metabolic burden and exchange of genetic material via gene transfer. It has also been proposed that perhaps biofilm, instead of planktonic cell, is the default bacterial mode of growth (Jefferson, 2004).

Environmental biofilms, whether in nature or in artificial systems, are predominantly multispecies in composition (Watnick and Kolter, 2000; Yang et al., 2011). Although *L. pneumophila* is commonly detected in biofilms found in plumbing fixtures (Colbourne et al., 1984; Wadowsky et al., 1982; Wright et al., 1989), few studies have

investigated *L. pneumophila* in the context of these multispecies communities. The limited number of studies have looked into the influence of protozoan hosts as well as the impact of abiotic factors, e.g. temperature and plumbing material, on *L. pneumophila* growth in mixed-species biofilms (Kuiper et al., 2004; Murga et al., 2001; Rogers et al., 1994a, b; van der Kooij et al., 2005). To date, they have presented rather divergent views in terms of the replication status of *L. pneumophila* in the biofilms. On the one hand, *L. pneumophila* has been shown to merely persist in multispecies biofilms, and because replication does not seem to occur unless in the presence of protozoan hosts, the route of intracellular replication has been implied (Declerck et al., 2007b; Kuiper et al., 2004; Mampel et al., 2006; Murga et al., 2001). On the other hand, there have been reports indicating that growth of *L. pneumophila* in the multispecies biofilm is not exclusively conditional upon intracellular replication (Rogers et al., 1994b; Rogers and Keevil, 1992; Surman et al., 2002). It remains unresolved whether *L. pneumophila* is capable of replicating extracellularly per se in mixed population biofilms in the environment. Nevertheless, these studies demonstrated that in the extracellular environment, *L. pneumophila* can survive by taking refuge in, and enjoying the benefits of associating with, multispecies biofilms. A limited number of studies have also shown that *L. pneumophila* is itself able to form monospecies biofilms *in vitro* (Hindre et al., 2008; Konishi et al., 2006; Pecastaings et al., 2010; Piao et al., 2006). This implies that *L. pneumophila* in the environment has the potential to engage in active development of biofilms, should conditions become permissive for extracellular replication.

The developmental cycle of the biofilm includes a stage in which bacterial cells undergo dispersal from the existing biofilm as dictated by environmental conditions

(Hall-Stoodley et al., 2004; Watnick and Kolter, 2000), and consequently planktonic cells are released into the environment. The demise of protozoan host cells at the end of an infection cycle also results in the liberation of bacterial cells, which will remain free-living until they are taken up by another host. The planktonic cells of *L. pneumophila* can therefore be expected to emerge from both sources described, making up part of the bacterium's extracellular population.

1.3. Morphological plasticity – The many faces of *L. pneumophila*

1.3.1. Bacterial morphology in general

Bacteria are known to exist in an array of shapes and sizes (Young, 2006). They adopt certain cell shapes to cope with their environment; thus cell shape is strongly influenced by selective pressures (e.g. competition for nutrient, predation) and physical constraints (e.g. diffusion limitation, turgor pressure) as experienced by the bacteria in its environment (Margolin, 2009; Young, 2007). Having different morphologies allows bacteria to display different physical features to the environment. Therefore, for a bacterium to adopt a particular cell shape, there must be a survival value to that morphology in coping with its specific environmental conditions (Young, 2007). For instance, for two bacterial cells of equal cell volume, the one with the rod-shaped morphology may confer several practical advantages, such as a higher surface-to-volume ratio for efficient diffusion of nutrients and a greater area for substratum contact in combating high shear forces, over the one with the coccal morphology (Young, 2006). Since cell shape influences their chances of survival in the

environment, it is not surprising that most bacteria adopt a particular predominant morphology and invest in stringent mechanisms to preserve consistent cell shapes.

However, there are conditions in which the bacterial cell shape has been found to deviate from the predominant morphology. Morphological aberrations could be a manifestation of stress response due to chemical insults such as antimicrobials (DeLoney and Schiller, 1999; Rodgers et al., 1990) or mutation in important genes that govern cell division or cell wall synthesis such as *mreB* and *rodA* in *Escherichia coli* (Cabeen and Jacobs-Wagner, 2005; Scheffers and Pinho, 2005). For example, the rod-shaped *E. coli* is known to form filament when exposed to β -lactams such as ampicillin and form spheroplasts, i.e. spherical forms with compromised cell walls, when treated with high concentrations of amoxicillin (Comber et al., 1977). On the other hand, deviations from the predominant morphology may also be a form of bacterial adaptation to fluctuating environmental conditions, such as to gain better nutrient access in low-nutrient conditions or to escape death by predation (Young, 2006). For instance, the length of *Caulobacter crescentus* prosthecae stalk (a thin appendage extruding from the cell), which has been suggested to play a role in phosphate uptake, was found to increase under conditions of low phosphate concentrations (Poindexter, 1984; Young, 2006). More recently, uropathogenic *E. coli* was observed to form filament in a bid to resist killing by phagocytes (Horvath Jr et al., 2011). Therefore, the departure of bacterial cell shape from the predominant morphology may not always be an abnormality due to stress but could be a form of morphological plasticity in ensuring survival of the bacteria in the changing environment.

1.3.2. Morphological differentiation of *L. pneumophila*

L. pneumophila is known to be a pleomorphic bacterium and has been described in a spectrum of shapes (Katz, 1978; Katz et al., 1984). Although predominantly bacillary (i.e. rods) in form, the accumulated knowledge from investigations into the life cycle of *L. pneumophila* has also helped shed light on the morphological plasticity of the bacterium (Faulkner and Garduño, 2002; Garduño, 2007).

Figure 1.2 (courtesy of Garduño (Garduño et al., 2008)) succinctly illustrates the *L. pneumophila* life cycle in the context of several differentiated forms associated with specific stages. In terms of gross morphology, most of the forms can be classified as bacillary “rods”, and only one form deviates clearly from that: the “filamentous” form. However, the various differentiated forms of rods can be distinguished from one another through finer morphological features and aspects of physiology.

The curious display of multiple differentiated forms – probably one of the most numerous for a bacterium – by *L. pneumophila* may be a consequence of its sensitivity to the fluctuation of resources in its environment. The regulated responses to adapt to a wide range of environmental conditions probably consist of a suite of changes not confined to metabolic switching but also including morphological switching. At the point in time when this project was started, we really did not know what direct advantage this morphological plasticity presents to *L. pneumophila*, other than to speculate that it helps to maximize every resource and opportunity available in ensuring the survival of *L. pneumophila* in various environments.

Figure 1.2 Diagram depicting the relationships amongst various morphological forms in two distinct developmental stages of *L. pneumophila*: the intracellular (within host) and extracellular (outside host) stages of its life cycle. Diagram is cited from *Developmental Cycle – Differentiation of Legionella pneumophila* by Garduño et. al in *Legionella: Molecular Microbiology* published by Caister Academic Press. (Garduño et al., 2008)

1.3.2.1. Morphological forms in the intracellular life cycle

In the intracellular environment, *L. pneumophila* follows a developmental cycle that can be viewed as an alternation between two discrete stages: the replicative phase and the transmissive phase (Campodonico and Roy, 2009). The primary distinction between these two stages is that the bacteria is metabolically active and undergoes cell division in the replicative phase, but not in the transmissive phase. Currently, at least two differentiated forms are associated with these two phases of the intracellular

developmental cycle: the replicative form (RF) in the replicative phase and the postreplicative form or mature intracellular form (MIF) in the transmissive phase (Garduño, 2007; Greub and Raoult, 2003; Molofsky and Swanson, 2004).

These two forms differ distinctively in varying aspects of physical, physiological and virulence characteristics. In terms of physical features, the RF is bacillary in shape, stains green with Giménez (Garduño et al., 2002a), lacks flagella (Byrne and Swanson, 1998), and exhibits the typical Gram-negative envelope (defined by the inner and outer membrane separated by a periplasmic space) (Figure 1.3, A), whereas the MIF is shorter in size although still bacillary, stains red with Giménez (Garduño et al., 2002a), is highly motile (Garduño, 2007), characterized by a complex laminated envelope that is unlike the typical Gram-negative envelope, and has pronounced intracellular inclusions (Faulkner and Garduño, 2002; Greub and Raoult, 2003) (Figure 1.3, B). It should be noted here that the MIF is technically not a single form, but a distinct morphological group of the postreplicative forms identified by long invaginations of the cytoplasmic membrane and an electron-dense laminated outer membrane structure or prominent stacks of intracytoplasmic membrane (Faulkner and Garduño, 2002; Garduño et al., 2002b) (Figure 1.3, C). It has been observed that many postreplicative *L. pneumophila* cells in macrophages, although showing traits of maturation such as prominent intracellular inclusions and non-wavy outer membrane, lack the typical envelope complexity associated with MIF (Garduño et al., 2002b), suggesting that MIF may be the fully differentiated postreplicative form (Garduño, 2007). Physiologically, RF and MIF differs in their capacity for replication and environmental fitness; the RF actively undergoes cell division and does not persist in the extracellular environment (Garduño, 2007), whereas the MIF does not replicate

(Faulkner and Garduño, 2002; Garduño et al., 2002a), but displays enhanced resistance to antibiotics, increased environmental fitness and virulence to mammalian and protozoan cells (Garduño, 2007).

It is now known that upon internalization by the host, *L. pneumophila* establishes an intracellular life cycle by differentiating into the RF, undergoes multiple cell divisions to generate a large number of progenies, enters the postreplicative phase, in which it differentiates into the environmentally resilient transmissive MIF. The MIF then exits the host cell into the environment, where *L. pneumophila* may encounter a new host to re-establish an intracellular cycle, or persist in the environment, possibly by differentiating into other extracellular forms (Garduño et al., 2008).

Figure 1.3 Electron micrographs showing the ultrastructural morphologies of the replicative form (RF) and mature intracellular form (MIF) of *L. pneumophila*. (A) RFs located within the amoeba *Hartmannella vermiformis* shown here to have typical Gram negative envelope ultrastructure. (B) MIFs within vesicles expelled from *H. vermiformis*. (C) Higher magnification of a MIF exhibiting a distinctive cell envelope ultrastructure characterized by prominent stacks of intracytoplasmic membrane. Images are adapted from (A, B) Greub et. al (Greub and Raoult, 2003) and (C) Garduno et. al (Garduño et al., 2002b). Size bars are (A, B) 0.5 μm and (C) 0.1 μm .

1.3.2.2. Morphological forms in the extracellular life cycle

Compared to the intracellular life cycle, more differentiated forms of *L. pneumophila* have been reported for its extracellular life cycle (Figure 1.2) (Garduño et al., 2008). Of these forms, the bacillary forms of the *in vitro* cycle are perhaps the most studied and best characterized.

1.3.2.2.1. *In vitro* bacillary forms

Similar to the developmental cycle observed in the intracellular environment, *L. pneumophila* in the *in vitro* cycle alternates between the replicative or exponential phase and the postreplicative or stationary phase, and these phases are associated with two differentiated bacillary forms: the exponential phase (EP) form and the stationary phase (SP) form (Edwards and Swanson, 2007; Garduño, 2007). These *in vitro* EP and SP forms share many similar features with their corresponding intracellular RF and MIF counterparts. For instance, like the intracellular RF, the EP form is actively replicative and is not as environmentally fit nor virulent as the SP form which, like the MIF, exhibits greater stress resistance (e.g. osmotic and thermal tolerance) and increased cytotoxicity to mammalian cells and protozoa (Byrne and Swanson, 1998; Cirillo et al., 1994; Garduño, 2007; Molofsky and Swanson, 2004).

It is noteworthy that while the *in vitro* SP form and intracellular MIF share similar traits, they have been demonstrated to be two distinct forms of *L. pneumophila*, differing in some aspects of morphology and physiology. For instance, the characteristic electron-dense laminated outer membrane and long cytoplasmic invaginations associated with MIF is not found in the SP form, which has the cell wall ultrastructure of a typical Gram-negative bacterium (Figure 1.4) (Garduño et al.,

2002b). In addition, the MIF is more infectious to mammalian cells and demonstrated greater resistance to antibiotics than the SP form (Garduño et al., 2002b). Interestingly, it has been reported that the SP form can directly differentiate into the MIF during transitory residence in food vacuoles of the ciliate host *Tetrahymena tropicalis* (Faulkner et al., 2008), suggesting the SP form and MIF may comprise a differentiation continuum and that the SP form may be an incomplete intermediate of the fully differentiated postreplicative form, the MIF (Garduño et al., 2008).

Figure 1.4 Electron micrograph showing the ultrastructure of a stationary phase (SP) form of *L. pneumophila*. The SP form displays a typical Gram negative cell wall ultrastructure with wavy outer membrane. Image is adapted from Garduno et. al (Garduño et al., 2002b). Size bar is 0.1 μm .

1.3.2.2.2. Viable but nonculturable form (VBNC)

When present for an extended period of time in environmental water samples, e.g. tap water and creek water microcosms, *L. pneumophila* has been shown to be capable of entering the non-replicative viable but nonculturable (VBNC) state (Hussong et al., 1987; Paszko-Kolva et al., 1992; Steinert et al., 1997). The nutrient level in these microcosms is usually too low to support multiplication and the bacterium may survive these growth-limiting conditions by differentiation into the VBNC form. *L. pneumophila* in the VBNC state is characterized by the loss of culturability on agar

media but can be resuscitated to the culturable state by its natural amoebic host *Acanthamoeba castellanii* (Paszko-Kolva et al., 1992; Steinert et al., 1997). However, very little has been investigated of the VBNC form, thus, details regarding the morphology and physiology of this *L. pneumophila* extracellular form currently remains unknown.

1.3.2.2.3. Filamentous form

Of special interest and relevance to my study is the filamentous form in the extracellular life cycle of *L. pneumophila*. Filaments have been documented in passing since the identification of the bacterium (Katz, 1978; Rodgers, 1979; Warren and Miller, 1979) but never quite studied in detail. Perhaps due to the irregularity and scarcity in their appearance, they have often been dismissed as outliers and not given much attention. Since the filamentous form of *L. pneumophila* will constitute the subject of this thesis, the topic of filamentation will be further discussed below (Section 1.3.3).

1.3.3. The filamentous morphology

1.3.3.1. Bacterial filamentation

Within the breadth of cell morphologies exhibited by bacteria is the filamentous form, characterized by an elongated cell length that may range from 20-30 times that of the width of the cell or even longer. While some bacterial species are filamentous by default, e.g. the green nonsulfur bacterium *Chloroflexus aurantiacus* (Tang et al., 2011) and the cyanobacterium *Anabaena* sp. (Kaneko et al., 2001), some others exhibit conversion from another form such as rods, to the filamentous one, as in the

case of *L. pneumophila*. Such filamentation most often occurs as a result of cell growth proceeding in the absence of cell division; thus the elongation of the bacterial cell is accompanied by the generation of multiple nucleoids (Justice et al., 2008).

A possible advantage for bacteria to adopt the filamentous morphology is to help cope with nutrient deprived situations by rapidly increasing the total surface area (for substrate intake) while maintaining the surface area-to-volume ratio, without expending energy for cell division. For example, *Pseudomonas aeruginosa* and *E. coli* were observed to filament when cultured under nutrient-deprived conditions (Steinberger et al., 2002; Wainwright et al., 1999). Filamentation may particularly benefit bacterial cells that are attached to substratum because it increases the surface area available for contact with the solid medium, as well as enabling cells to access niches of higher nutrient that would be mechanically out of reach for cells of the bacillary or coccoid morphologies, thereby increasing the chances of acquiring nutrients (Young, 2006). Bacteria may also filament as a way to counter predation in the environment because filamentous cells are usually too large to be captured or digested by predators (Young, 2006). Murine macrophages have been observed to preferentially kill bacillary forms of uropathogenic *E. coli* over the filamentous forms because internalization of the bacterial filaments was comparatively unproductive (Horvath Jr et al., 2011; Justice et al., 2006).

Nevertheless, not all instances of bacterial filamentation are adaptive responses to changes in the environment. Filamentation is also associated with bacterial stress response, one that occurs as a result of disruption in bacterial cell division. For instance, filamentation by *E. coli* in response to stressful levels of hydrostatic pressure

is a consequence of inhibition in the formation of the FtsZ ring during the fission of two dividing daughter cells (Ishii et al., 2004). Treatment of *E. coli* with antimicrobials, such as β -lactam antibiotics that inhibit proteins involved in synthesis of new peptidoglycan chains required for cell division, can also lead to filamentation (Nelson and Young, 2000; Spratt, 1975).

1.3.3.2. *L. pneumophila* filamentous form

The filamentous form of *L. pneumophila* was first reported along with the more predominant rod-shaped form during the identification of *L. pneumophila* as the aetiological agent of Legionnaires' disease (McDade et al., 1977). It was subsequently noted in ultrastructural characterization studies of the bacterium (Katz, 1978; Rodgers, 1979; Rodgers et al., 1978), in the course of formulating chemically defined medium for the *in vitro* cultivation of *Legionella* (Pine et al., 1979; Warren and Miller, 1979), in the earlier studies concerning the pathogenesis of *L. pneumophila* as well as its survival in nutrient-limiting conditions (Berg et al., 1985; Mauchline et al., 1992; Paszko-Kolva et al., 1992; Rowbotham, 1980; Wong et al., 1980), and also in the assessment of antibiotic susceptibilities and efficacy of diagnostic methods (Edelstein and Finegold, 1979; Elliott and Rodgers, 1985; Faine et al., 1979; Smalley et al., 1980; Watson and Sun, 1981). More recently, filaments were reported in monospecies biofilms of *L. pneumophila* cultivated at higher than ambient temperatures (Konishi et al., 2006; Piao et al., 2006). However, none of these studies were addressing fundamental questions relating to the filamentous form of *L. pneumophila*. These investigations were focused on other aspects of *L. pneumophila* physiology and ecology; the occurrence of the filamentous form was mostly mentioned in passing.

Nevertheless, a few key observations can be gathered from these sporadic reports. First, the *L. pneumophila* filamentous form is multi-nucleoid, aseptate and can elongate up to over 100 µm (Konishi et al., 2006; Piao et al., 2006), indicating that cell growth and DNA replication have proceeded without cell division. Second, occurrence of the filamentous form was seen often at higher-than-ambient growth temperatures, ranging approximately between 37-42°C (Konishi et al., 2006; Mauchline et al., 1992; Piao et al., 2006), and in surface-associated cultivation conditions, e.g. on agar and in biofilms, more than in conditions with fluid movement (Garduño et al., 2008; Konishi et al., 2011; Paszko-Kolva et al., 1992; Rodgers, 1979). Third, filamentation has been documented in experiments using nutrient-rich laboratory growth media (Konishi et al., 2006; Piao et al., 2006) as well as nutrient-limiting conditions (Paszko-Kolva et al., 1992; Warren and Miller, 1979). However when transferred to fresh rich medium under agitation, filaments did not persist and rod-shaped cells appeared instead, suggestive of filament-to-rod conversion, leading to the speculations that filamentation may be a form of *L. pneumophila* adaptive response employed to facilitate rapid proliferation and dissemination of the bacterium (in bacillary form) when environmental conditions become favourable for growth (Paszko-Kolva et al., 1992; Piao et al., 2006). Finally, the filamentous form has been suggested to be less virulent to guinea pigs than the bacillary form (Nowicki et al., 1987; Paszko-Kolva et al., 1992). However, very little of that experiment has actually been described and even less is known of other physiological characteristics of the filament.

In the study demonstrating that *L. pneumophila* has the ability to form extensively filamentous monospecies biofilms (Piao et al., 2006), the possibility of filamentation in *L. pneumophila* being a stress-induced aberration of cell division was eliminated by the authors based on the fact that the optimal conditions of 37°C and nutrient-rich medium had been used; leading to the authors' speculation that filamentation is a temperature-regulated response to adapt to its surroundings. The recent proposal by Justice et al. (2008) that morphological plasticity of pathogenic bacteria may be a direct and adaptive response to the sensing of environmental changes – yielding fitter generations to survive multitude forms of environmental stress (Justice et al., 2008) – is beginning to be substantiated with emerging experimental evidences obtained from environmental and clinically important bacteria (Corno and Jurgens, 2006; Giotis et al., 2007; Horvath Jr et al., 2011). This has further encouraged us to consider *L. pneumophila* filamentation in this light.

If, indeed, filamentation by *L. pneumophila* is a regulated adaptation strategy in response to specific environmental stimuli, we have yet to understand what these stimuli may be, how the regulation is effected, and how the adaptation impacts on the bacterium in terms of survival advantage, e.g. in free-living form versus in interaction with protozoan hosts. It is in this rather humbling situation that, four years ago, I entered the uncharted territory of *L. pneumophila* filamentation.

1.4. Aim of the study

1.4.1. Relevance of understanding *L. pneumophila* filamentation

Thermally altered man-made water distribution systems, e.g. hot spring spas, municipal hot water supplies and cooling towers have frequently been implicated as sources of infection in large community outbreaks of Legionnaires' disease involving fatalities and hospitalization of hundreds of patients (Okada et al., 2005; Tartakovskiy et al., 2009). The more frequent mentions of filaments under conditions of elevated temperatures or surface-associated cultivation observed in earlier studies (Mauchline et al., 1992; Paszko-Kolva et al., 1992; Piao et al., 2006) suggests that filamentation by *L. pneumophila* may be common in the artificial water distribution systems discussed above. Eradication of *L. pneumophila* from these water systems has been difficult despite rigorous disinfection measures. The tenacity of *L. pneumophila* within these environments has been attributed in part to *Legionella*'s ability to exist as biofilms (Fields, 2007) and to differentiate into the VBNC form (Alleron et al., 2008) – modes of existence in the bacterium's extracellular life cycle which are generally believed to confer survival advantages under adverse conditions. It will be pertinent to attempt to find out whether adaptation into the filamentous form, another player in the *L. pneumophila* extracellular life cycle, is linked to the persistence and colonization of the bacterium in such man-made water systems.

Since systematic characterization studies on the filamentous form of *L. pneumophila* was very much lacking, it was unclear how the implicated environmental factors, e.g. temperature and nutrient availability, specifically influence filamentation. Therefore, this study firstly aimed to investigate the environmental factors that can trigger the

formation of filaments by *L. pneumophila*. A greater understanding of the triggering factors may provide clues on the environmental incidence of the *L. pneumophila* filament, and perhaps evidence to discern if filamentation by *L. pneumophila* is indeed a morphological adaptation offering specific advantages for survival.

It has been implied that water environments with higher-than-ambient temperatures can upset the normal balance in the interaction of *L. pneumophila* with its free-living protozoan hosts, leading to rapid proliferation of the pathogen and consequently, the risk of an Legionnaires' disease outbreak (Fields, 2007). Therefore, the second aim of this study was to investigate how the filamentous form of *L. pneumophila* interacts with the ubiquitous free-living protozoan hosts and how this protozoa-bacteria relationship compares with that of the bacillary form, which has been better characterized (Albert-Weissenberger et al., 2007; Campodonico and Roy, 2009; Molmeret et al., 2007). Although *L. pneumophila* filaments have been indicated to be avirulent *in vivo* (Nowicki et al., 1987; Paszko-Kolva et al., 1992), infectivity of the filamentous form to natural protozoan hosts was not known. An understanding of the filamentous form from the perspective of its relationship with the natural environmental hosts may provide insights on the potential role of *L. pneumophila* filaments in the colonization of water distribution systems and in the risk of Legionnaires' disease outbreaks.

1.4.2. Exploring the filamentous form

While many strains of *L. pneumophila* have been known to be responsible for Legionnaires' disease, *L. pneumophila* strain Philadelphia-1 was opted as the model

strain for my study since it is the type strain, as well as belonging to serogroup 1, the group most often implicated in Legionnaires' disease cases. In addition, there exist a large volume of references for this strain including its full genome (Russo and Morozova, 2004).

As gathered from earlier studies, filamentation by *L. pneumophila* was mostly observed under conditions of surface-associated cultivation, such as biofilms (Konishi et al., 2006; Piao et al., 2006). In addition, certain environmental factors such as temperature (Konishi et al., 2006; Mauchline et al., 1992) and nutrient status (Warren and Miller, 1979) have also been linked to the formation of filaments. Therefore, as a starting point, I would be adopting the biofilm experimental model in order to replicate, in the strain Philadelphia-1, the temperature-dependent filamentation observed in *L. pneumophila* strain Knoxville-1 biofilms by Piao et. al (Piao et al., 2006). The ability of *L. pneumophila* strain Philadelphia-1 to filament under varying status of nutrient availability would then be explored by growing the bacteria in media varying in concentration and nutrient components.

To evaluate the interaction of the filamentous form with protozoan hosts, *L. pneumophila* strain Philadelphia-1 filaments will be co-incubated with three species of protozoa that are widely used in *Legionella* studies: the amoeba (*Acanthamoeba castellanii*, *Acanthamoeba polyphaga*) and the ciliate (*Tetrahymena pyriformis*). The amoeba and the ciliate differ in their feeding mechanisms – the amoeba is a surface grazer that crawls on surfaces and feeds via phagocytosis, whereas the ciliate is a suspension feeder that swims via the beating of its cilia and ingests bacteria by sweeping them into its oral groove (Bozue and Johnson, 1996; Fenchel, 1980).

Therefore, in addition to assessing the ability of the filamentous form of *L. pneumophila* to infect these hosts, it is also possible to evaluate the commonalities and differences between the two categories of protozoa with respect to their association with this alternative form of *L. pneumophila* in the environment.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1. Materials

2.1.1. List of strains and plasmids

2.1.1.1. List of bacterial strains

Strain	Description	Source
<i>L. pneumophila</i> LP1	Philadelphia-1, type strain	ATCC 33152
<i>L. pneumophila</i> LP3	Philadelphia-1 carrying pPZ1 plasmid, constitutively GFP-producing, Km ^r	This work
<i>L. pneumophila</i> JR32	Philadelphia-1, salt sensitive, streptomycin-resistant, restriction-deficient, modification-positive isolate	Marra et. al, 1992 (gift from Howard Shuman)
<i>L. pneumophila</i> LP14	JR32 carrying pPZ1 plasmid, constitutively GFP-producing, Km ^r	This work
<i>Escherichia coli</i> ECS121	strain DH5 carrying pPZ1 plasmid, constitutively GFP-producing, Km ^r	Laboratory stock

Km^r : kanamycin-resistant

Table 2.1 List of bacterial strains.

2.1.1.2. List of protozoan strains

Strain	Description	Source
<i>Acanthamoeba castellanii</i> OS4-7B/Neff	amoeba, Neff strain, soil-isolated from Pacific Grove, CA	ATCC 50373
<i>Acanthamoeba polyphaga</i> JAC/S2	amoeba, soil-isolated from Japan	ATCC 50372
<i>Tetrahymena pyriformis</i>	ciliate, environmental isolate	gift from Diane McDougald, UNSW, Australia

Table 2.2 List of protozoan strains.

2.1.1.3. List of plasmids

Plasmid	Relevant description	Source
pPZ1	Km ^r ; RSF1010-based broad-host-range vector carrying <i>gfp-mut3*</i> under the control of constitutive P _{A1/04/03} promoter	Piao et. al, 2006

Table 2.3 List of plasmids.

2.1.2. List of materials

2.1.2.1 Laboratory stock solutions

Reagent	Constituent / Remarks
10x TBE	0.9 M Tris, 0.9 M boric acid, 20mM Na ₂ EDTA (pH 8.0)
6x loading dye	From Fermentas
10% (v/v) glycerol solution	Autoclaved and stored at Room Temperature (RT)
60% (v/v) glycerol solution	Autoclaved and stored at RT
0.1 M sodium cacodylate buffer, pH 7.0	Stored at 4°C
2.5% (v/v) glutaraldehyde solution	Diluted in 0.1 M sodium cacodylate buffer, stored at 4°C
Lugol's iodine	0.5 gL ⁻¹ I ₂ , 1 gL ⁻¹ KI

Table 2.4 List of laboratory stock solutions.

2.1.2.2 Commercial kits

Kit	Source
Nucelospin® Plasmid	Macherey-Nagel, Germany
NucleoBond PC500	Macherey-Nagel, Germany
LIVE/DEAD® BacLight™ Bacterial Viability Kit	Invitrogen, USA

Table 2.5 List of commercial kits.

2.1.2.3 Media

2.1.2.3.1 Bacterial culture

Media	Composition / Remarks
Buffered yeast extract (BYE) broth	1% (w/v) yeast extract (BD Difco, USA) 1% N-(2-Acetamido)-2-Aminoethanesulfonic acid (ACES) buffer pH 6.9 0.4 gL ⁻¹ L-cysteine ^a 0.25 gL ⁻¹ ferric pyrophosphate ^a
BYE charcoal agar (BYECA)	BYE broth 1.5% (w/v) Bacto agar (BD Difco, USA) 0.2% (w/v) activated charcoal 0.4 gL ⁻¹ L-cysteine ^a 0.25 gL ⁻¹ ferric pyrophosphate ^a
M63 minimal medium	100mM KH ₂ PO ₄ 15mM (NH ₄) ₂ SO ₄ 1.7μM FeSO ₄ ·7H ₂ O Preparation was dissolved in sterile distilled deionized water (ddH ₂ O), adjusted to pH 7 using KOH, autoclaved and cooled before addition of 0.1% (v/v) 1 M MgSO ₄ ^a .

Table 2.6 List of bacterial culture media. All media preparations were sterilized by autoclaving at 121°C for 20 min, unless stated otherwise. ^a Heat-sensitive components were individually sterilized by filtration through 0.22 μm filter then added into autoclaved media preparations.

2.1.2.3.2 Protozoan culture

Media	Composition / Remarks
Peptone yeast glucose (PYG) broth	<p>2% (w/v) proteose peptone no.3 (BD Difco, USA)</p> <p>0.1% (w/v) yeast extract (BD Difco, USA)</p> <p>0.1M glucose</p> <p>Preparation was autoclaved and cooled before addition of the salts of AC buffer. Adjusted pH to 6.5 using HCl, and finally sterilized by filtration through 0.22 µm filter (Nalgene, USA).</p>
AC buffer	<p>4mM MgSO₄</p> <p>0.4M CaCl₂.2H₂O</p> <p>0.05mM Fe(NH₄)₂(SO₄)₂.6H₂O</p> <p>2.5mM Na₂HPO₄.7H₂O</p> <p>2.5mM KH₂PO₄</p> <p>3.4mM C₆H₅Na₃O₇.2H₂O</p> <p>Preparation was dissolved in sterile ddH₂O, adjusted to pH 6.5 and finally sterilized by filtration through 0.22 µm filter.</p>

Table 2.7 List of protozoan culture media. All media preparations were sterilized by autoclaving at 121°C for 20 min, unless stated otherwise.

2.1.2.4 Media supplements and antibiotics

Supplement / Antibiotic	Preparation
100 gL ⁻¹ L-cysteine (Cys)	Dissolved in ddH ₂ O and sterilized by filtration through 0.22 µm filter.
62.5 gL ⁻¹ ferric pyrophosphate (FePP)	Dissolved in ddH ₂ O and sterilized by filtration through 0.22 µm filter.

30 mgml ⁻¹ chloramphenicol (Cm)	Prepared in ethanol.
100 mgml ⁻¹ kanamycin (Km)	Prepared in ddH ₂ O and sterilized by filtration through 0.22µm filter.

Table 2.8 List of media supplements and antibiotics.

2.2. Methods

2.2.1. Molecular techniques

2.2.1.1. Plasmid extraction

For small scale extractions of plasmid pPZ1, 5 ml LB broth supplemented with 25 µg/ml kanamycin was inoculated with one colony of the *E. coli* strain bearing the required plasmid. Inoculation was incubated at 37°C for 14-16 h with agitation at 250 rpm (MRC, LOM-150). The extraction and purification was thereafter performed using Nucleospin® Plasmid Kit (Macherey-Nagel, Germany). For large scale plasmid extractions, NucleoBond PC500 Kit (Macherey-Nagel, Germany) was used in replacement. All kits were used according to manufacturer's instructions.

2.2.1.2. Agarose gel electrophoresis

Gel of 0.7% agarose was casted for verification of plasmid DNA extracted. Agarose (USB Corporation, USA) was boiled in 1x TBE, cooled for 30 min and then casted in a gel apparatus (Bio-Rad). After 30 min, the solidified gel was submerged ~2 mm below the surface in 1x TBE. Plasmid DNA extractions were mixed with 1x loading dye and loaded into the wells. An electric field of approximately 10 volts/cm was applied. Following electrophoresis, DNA was stained in 1 µg/ml ethidium bromide (EB) solution. The fluorescing of the DNA/EB under UV light (302 nm, UV transilluminator TM-20, UVP) was captured with a Mitsubishi video copy processor.

2.2.1.3. DNA quantification

DNA concentration was determined either by comparing the intensity of gel bands with a standard 1 kb DNA ladder (GeneRuler™, Fermentas) or by spectrophotometric reading at 260 nm using quartz cuvettes (Hellma®, Germany) on a Biospec-mini spectrophotometer (Shimadzu, Japan).

2.2.1.4. Electro-competent cell preparation

L. pneumophila inoculated from colonies were cultured to saturation (10 ml) at 37°C for 24 h with agitation at 250 rpm, diluted in a flask with 100 ml pre-warmed BYE broth and further shaken at 37°C until the OD₆₀₀ of 0.6-1.2 was reached. Culture was transferred into 250 ml centrifuge bottles, chilled on ice for at least 5 min and then cells were harvested by centrifugation at 4°C for 10 min at 5000 x g with Avanti™ J-25 centrifuge (Beckman Coulter™). Pellet was transferred to 30 ml centrifuge bottles, rinsed twice in ice-cold sterile ddH₂O (5 ml), once in ice-cold 10% glycerol solution (2.5 ml) and finally resuspended in 2 ml ice-cold 10% glycerol solution. Electro-competent cells were stored in 45 µl aliquots at -80°C.

2.2.1.5. Transformation of plasmid pPZ1 into *L. pneumophila* via electroporation

For each electroporation, an aliquot of 45 µl *L. pneumophila* electrocompetent cells were thawed, transferred to an ice-cold 2 mm electroporation cuvette (Bio-Rad) and mixed with approximately 0.2 µg of plasmid pPZ1. The cuvettes were gently tapped to mix and to ensure the absence of air bubbles, and then chilled on ice for at least 5 min. Gene Pulser (Bio-Rad) apparatus was set to 25 µF, 200 Ω and a voltage of 2.5 kV. A time constant of > 4.5 ms was taken as an indication of high electroporation efficiency. Immediately after electroporation, 1ml of BYE was added into the cuvette

and the mixture is transferred into a sterile tube for recovery at 37°C with agitation (150 rpm) for 4 h. The suspension was spread onto BYECA plates supplemented with kanamycin at 25 µg/ml.

2.2.2. Culture conditions

2.2.2.1. Bacteria

2.2.2.1.1. Colony culture

Frozen -80°C stocks of *L. pneumophila* strains were routinely cultured on BYECA, supplemented with appropriate antibiotics where necessary, at 37°C in a humidified Incucell incubator (MMM Group, Germany) for 3-4 days. For cultivation of plasmid pPZ1-carrying *L. pneumophila* strains (LP3, LP14), BYECA was supplemented with 25 µg/ml Km.

2.2.2.1.2. Storage of bacterial strains

A single colony was inoculated into 3 ml BYE broth, supplemented with appropriate antibiotics where necessary, and incubated at 37°C with agitation for 24 h. The stationary culture was stored in 20% glycerol solution at -80°C in 2 ml screw-cap cryotubes.

2.2.2.1.3. Planktonic cultures

2.2.2.1.3.1. Cultivation for seed culture

Unless otherwise stated, planktonic cultures were routinely inoculated with *L. pneumophila* colonies in 3 ml BYE broth, supplemented with appropriate antibiotics where necessary, in 15 ml snap-cap tubes (Falcon 352059, Becton Dickinson) and incubated at 37°C or 42°C for 24 h with agitation at 250 rpm to obtain bacillary (95-

100% bacillary) or filament-rich (76-91% filamentous, refer also to Section 3.2.1 on FACS) planktonic cultures, respectively. Cell morphologies were confirmed by brightfield or fluorescence microscopy examination, as described under Section 2.2.5.2. For cultivation of *L. pneumophila* strains carrying plasmid pPZ1 (LP3, LP14), BYE broth was supplemented with 25 µg/ml Km.

2.2.2.1.3.2. Cultivation for filamentation in various nutritional combinations

To survey the influence of nutritional components on filamentation, the growth medium was made variable by two components: (a) BYE, and (b) growth supplements (Cys and FePP). Media variations were prepared by diluting, with sterile ddH₂O, the following stock media to 50%, 20%, 10%, 5% and 1% of the original (100%) concentration: (i) BYE supplemented with both Cys and FePP, (ii) BYE supplemented with only Cys, (iii) BYE supplemented with FePP, and (iv) unsupplemented BYE.

To assess filamentation under static cultivation, *L. pneumophila* bacillary cells (obtained from planktonic cultures incubated at 37°C for 24 h with shaking at 250 rpm) were inoculated at a cell density of 3×10^6 cells/ml in 200 µl of each medium per well, in 96-well clear-bottom microplates (Iwaki 3860-096, Japan) and incubated statically at 25°C, 37°C and 42°C in a humidified Incucell incubator for up to 7 days.

To assess filamentation under shaking cultivation, *L. pneumophila* bacillary cells were inoculated at a cell density of 1×10^8 cells/ml in 3 ml of each medium in 15 ml inoculation tubes and incubated with orbital shaking at 250 rpm at 25°C, 37°C and

42°C for up to 4 days. Cell morphologies were monitored daily by fluorescence microscopy, as described in Section 2.2.5.2.

2.2.2.1.4. Biofilm cultures

2.2.2.1.4.1. General cultivation

Biofilms were cultivated on glass coverslips (24x50 mm, CellPath) tilted at a slight angle in thin-layer chromatography glass tanks (Figure 2.1) as described by Piao et al (Piao et al., 2006). Briefly, *L. pneumophila* colonies were inoculated in BYE broth, supplemented with 25 µg/ml Km where necessary, at 37°C for 24 h to obtain bacillary cultures, and adjusted to an OD₆₀₀ of 0.25 (cell density of approximately 1x10⁸ cells/ml) in the same medium. The diluted culture was transferred into glass tanks in aliquots of 30 ml, and tanks were statically incubated in humidified Incucell incubators at 25°C, 37°C or 42°C, where appropriate, for up to 18 days, unless indicated otherwise. Biofilms were observed at three-day intervals under confocal laser scanning microscope (CLSM), unless otherwise stated.

To prepare biofilms for CLSM, coverslips were carefully removed from glass tanks and the medium-immersed region was gently dipped into sterile ddH₂O to rinse off planktonic cells. One side of the coverslip was swabbed clean with 70% ethanol and the biofilm-intact side was mounted on water-filled grooves of flow chambers (Bjarke Bak Christensen, Technical University of Denmark) to keep the biofilms hydrated for CLSM.

Figure 2.1 Schematic diagram depicting the tank setup used for cultivation of *L. pneumophila* biofilms. (A) A front view of the chromatography glass tank holding 8 glass coverslips, which were placed vertically with a slight tilt and were immersed in the bacterial culture. (B) The top of coverslip was taped to stabilize the standing position of the coverslip in the tank. Prominent biofilm lines would be formed at the air-liquid interface on the surface of the coverslip, as indicated by the bold arrow. A continuous layer of film was also observed in the medium-immersed region of the glass coverslip.

2.2.2.1.4.2. Cultivation of biofilms in nutrient-replenished conditions

To assess the profile of biofilms in nutrient-replenished conditions, *L. pneumophila* biofilms were similarly cultivated as described above (Section 2.2.2.1.4.1) except that at every 24 h over the course of observation, the glass coverslips supporting biofilm growth were gently transferred into fresh glass tanks containing 30 ml fresh BYE broth.

Since this transfer would also involve the removal of planktonic cells in the growth medium, a control ('planktonic-free nutrient-depleting' condition) was included; instead of replacing the growth medium with fresh BYE broth, the spent BYE was taken from the tank at every 24 h and filtered through 0.22 μm pores to remove planktonic cells before returning into the tank.

2.2.2.2. Protozoa

Amoebae were routinely passaged in 5 ml PYG broth in 25 cm² tissue culture flasks (Nunc 156340, Denmark) and statically incubated at 28°C in a hydrated incubator (Nüve EN500, Turkey). *Tetrahymena* was similarly cultured and passaged, except that a volume of 10 ml PYG broth was used instead, and incubated at RT (~19-20°C).

2.2.3. Quantification of cell density

2.2.3.1. Bacteria

2.2.3.1.1. Enumeration of colony forming unit (CFU)

Density of viable cells was determined by enumeration of CFU. Bacterial cultures were serially diluted in 10-fold by transferring 200 µl cultures to 1800 µl sterile ddH₂O in 5 ml snap-cap tubes (Falcon 352058, Becton Dickinson). Colony forming units per ml (CFU/ml) was quantified by spreading 100 µl of appropriately diluted cultures on BYECA plates, in triplicates. Plates were incubated at 37°C in a humid incubator space for 3-4 days. Only plates of dilutions with CFUs in the range of 30-300 were enumerated.

2.2.3.1.2. OD₆₀₀-cell density correlation

Cell density of stationary-phase *L. pneumophila* cultures were approximated by correlation of OD₆₀₀ at stationary-phase with enumeration of CFU. Colonies of *L. pneumophila* were inoculated into BYE broth (supplemented with 25 µg/ml Km where required) and incubated at 37°C with agitation for 24 h to obtain stationary-phase cultures. OD₆₀₀ of the cultures was recorded, cultures serially diluted, spread on BYECA and incubated for 3 days for CFU count. Cell densities (CFU/ml) determined

by enumeration of CFU were correlated to the correspondingly recorded OD₆₀₀ values. The cell density for OD₆₀₀ = 1 was calculated to be 1x10⁹ cells/ml and used for approximation of stationary-phase cell density in the seed cultures used in various experiments.

2.2.3.1.3. Counting chamber

L. pneumophila planktonic cultures were 10-fold serially diluted in 1.5 ml microcentrifuge tubes (Axygen MCT-150-C) sterile ddH₂O to achieve the appropriate density for enumeration. A 2 µl aliquot of the countable dilution was dropped on a Petroff bacterial counting chamber (10 µm depth, Marienfeld), covered with a glass coverslip (22x22 mm, CellPath) and counted under brightfield and/or fluorescence microscope. Bacterial cell density was calculated using the following formula:

$$\text{Cell density (cells/ml)} = (\text{bacterial count in chamber}) \times (\text{dilution factor}) \times 10^5$$

2.2.3.2. Protozoa

Adherent amoebae in tissue culture flasks were brought to suspension by physically rapping the flask. Amoeba number was enumerated by dispensing 10 µl of the resuspended culture in the chamber of a C-Chip Neubauer-improved hemocytometer (Digital Bio Technology, Korea) and counted under an inverted brightfield microscope. Cell density was calculated using the formula:

$$\text{Cell density (cells/ml)} = (\text{amoeba count in chamber}) \times (\text{dilution factor}) \times 10^4$$

Tetrahymena was similarly enumerated, except the ciliate was immobilized with Lugol's iodine (in 1:4 volume ratio of iodine solution:ciliate culture) prior to counting.

2.2.4. Protozoa infection assay

2.2.4.1. Preparation of bacteria

Stationary phase *L. pneumophila* bacillary and filament-rich planktonic cultures were obtained described in Section 2.2.2.1.3.1. Because a minor population of the bacillary form could not be completely removed, only filament-rich planktonic cultures comprising at least 82% filamentous cells were used to represent the filamentous form in the infection assays, microscopically checked as described in Section 2.2.5.2. Cell densities of the bacillary and filament-rich cultures were quantified (as described in Section 2.2.3.1.3) and adjusted to 1.5×10^8 cells/ml in AC buffer pre-warmed to 37°C for infection.

2.2.4.2. Preparation of protozoa and infection

Amoebae, grown to confluence in 5 ml PYG broth at 28°C for 3 days, were brought to suspension by rapping the tissue culture flask. Cell density was adjusted to 3×10^5 cells/ml in PYG broth and dispensed into 24-well tissue culture plates (Nunc 142475, Denmark) in aliquots of 0.5 ml per well. Plates were incubated at 37°C for 1 h for amoeba adherence, after which the wells were rinsed once with pre-warmed AC buffer to remove non-adherent cells and finally immersed with fresh AC buffer in preparation for infection.

Tetrahymena cultures were cultivated in 10 ml PYG broth in tissue culture flask (Nunc 156340) and statically incubated at RT for 3 days. Cells were harvested by transferring the culture into 15 ml centrifuge tubes (Greiner 188271, Germany) and centrifuging at 700 xg for 10 min at RT with a Sorvall Legend Mach 1.6R Centrifuge

(Thermo Electron Corporation). The pellet was resuspended in AC buffer pre-warmed to 37°C, cell density adjusted to 3×10^5 cells/ml and the suspension was dispensed into 24-well tissue culture plates in aliquots of 0.5 ml per well for infection.

Infection assay was performed according to Moffat et. al (Moffat and Tompkins, 1992), with modifications. Unless stated otherwise, bacillary or filament-rich cultures of *L. pneumophila* (prepared as described in Section 2.2.4.1) were inoculated at a multiplicity of infection (MOI) of 10 to amoeba or *Tetrahymena* by dispensing 10 μ l of the bacterial cultures at 1.5×10^8 cells/ml into each well containing 1.5×10^5 protozoa in AC buffer in the tissue culture plates. The point of bacterial inoculation was denoted as time 0 h. Plates were incubated at 37°C in a humid incubator space and the dynamics of infection were monitored over a period of 3-5 days via brightfield and fluorescence microscopy. In the assay described by Moffat et. al, gentamycin was added at 1 h post-infection to kill all extracellular bacteria and amoebae were subsequently lysed in order to quantify intracellularly growing bacteria (Moffat and Tompkins, 1992). This treatment was not performed in our infection assay since it does not correspond to our purpose to qualify the interaction between *L. pneumophila* and protozoa.

2.2.4.3. Preparation of protozoa-conditioned buffer and inoculation

2.2.4.3.1. To assess the impact of protozoa secreted products on *L. pneumophila* morphology

For preparation of protozoa-conditioned buffers, *Acanthamoeba* or *Tetrahymena* in AC buffer were incubated at 3×10^5 cells/ml for 4 h at 37°C, unless otherwise stated. To remove the protozoa, *Acanthamoeba*-conditioned buffers were harvested by centrifugation at 11000 xg for 10 min at RT, whereas *Tetrahymena*-conditioned

buffers were harvested by filtration through sterile 0.22 µm filters (Pall Corporation). Protozoa-conditioned buffers were dispensed in aliquots of 0.5 ml in each well of 24-well tissue culture plates and inoculated with either bacillary or filament-rich *L. pneumophila* cultures (as described in Section 2.2.4.1). Plates were incubated at 37°C and the morphology of *L. pneumophila* cells was monitored for up to 5 days under fluorescence microscope.

2.2.4.3.2. To assess the impact of protein inhibition on generation of spheres

To test the effect of protein inhibition on generation of the spherical form in *L. pneumophila*, Chloramphenicol (Cm) was added at 100 µg/ml to protozoa-conditioned buffers prepared as described above (Section 2.2.4.3.1). The conditioned buffers were inoculated with either bacillary or filament-rich *L. pneumophila* cultures, incubated at 37°C and the morphology of *L. pneumophila* cells was monitored daily for 24 h under fluorescence microscope.

2.2.5. Microscopy

2.2.5.1. Staining bacterial cells for microscopy

Staining of *L. pneumophila* cells with the LIVE/DEAD® BacLight™ Bacterial Viability kit (Invitrogen) was performed as recommended by the manufacturer. Briefly, bacterial cells were harvested by centrifugation of approximately 1 ml culture/infection suspension at 11000 xg for 10 min at RT, followed by staining with 30 µl of the double stain mixture for 15-20 min in the dark. The stain mixture was prepared by adding 0.5 µl SYTO9: 0.5 µl PI (propidium iodide) to 300 µl sterile ddH₂O. Cells were then observed via fluorescence microscopy.

2.2.5.2. Brightfield and fluorescence microscopy

To view bacterial cells from planktonic cultures, a small drop of culture was wet-mounted on microscope slides (CellPath), covered with glass coverslip (22x22 mm, CellPath) and viewed under 40x-60x oil immersion objectives on an Eclipse 80i upright brightfield microscope (Nikon). Monitoring of bacterial morphology as well as protozoa infection over an extended period of incubation was carried out using Eclipse 80i inverted brightfield microscope with 20-40x objectives (Nikon) in 24 h intervals, unless otherwise stated. Both upright and inverted microscopes were equipped with differential interference contrast (DIC) as well as Intensilight C-HGFI mercury lamp and filters (blue light: Ex 450-490/ DM 505/ BA 520, green light: Ex 510-560/ DM 575/ BA 590) for viewing green and red fluorescence respectively. Images were captured with DS-U2 Digital Sight camera (Nikon) and presented using ImagePro Plus 6.2 software (Media Cybernetics Inc.).

The cell dimensions (width, length, diameter) of *L. pneumophila* bacillary, filamentous and spherical forms captured in fluorescence images were evaluated using ImagePro Plus 6.2. The relative densities of each morphological type in the images were quantified with the help of the ImageJ program (Wayne Rasband, NIH), by calculating the percentage of each morphological form over the total number of bacterial cells counted per field of view.

2.2.5.3. Confocal Laser Scanning Microscopy (CLSM)

Biofilm structure was evaluated under an Eclipse 90i CLSM (Nikon) equipped with a 488 nm argon laser. For visualization of green fluorescence, the channel was configured to 488 nm excitation and 515/30 nm emission. For observation, 10x and

60x (with oil) objectives were used for macro view of the biofilm structure and magnification of bacterial cell morphology respectively. Microscope controls were manipulated using iControl software (Nikon). Images were captured and processed with EZ-C1 3.2 and EZ-C1 3.2 FreeViewer softwares (Nikon) respectively.

Optical image stacks of the biofilm three-dimensional structures captured with CLSM were quantified using the COMSTAT software (Heydorn et al., 2000), which runs on the MATLAB (The MathWorks Inc.) platform. Quantification includes variables such as biomass, average thickness, substratum coverage and surface area-to-volume ratio, of which only the former two were presented and discussed in this thesis.

2.2.5.4. Transmission Electron Microscopy (TEM)

The spherical form of *L. pneumophila* was obtained for TEM by harvesting the infection buffer from the infection assay of *A. castellanii* with filament-rich cultures of *L. pneumophila* (described in Section 2.2.4.2) at 4 h or 7 h post-infection, and prepared for TEM as recommended by our collaborator (Garduño R., personal communication). Briefly, the infection buffer containing *L. pneumophila* cells was centrifuged at 11000 xg for 10 min at RT, supernatant discarded and pellet rinsed once with equal volume of 0.1 M sodium cacodylate buffer followed by fixation with 2.5% glutaraldehyde at RT for 2 h. Fixed cells were centrifuged to remove the fixative and the pellet was resuspended in 0.1 M sodium cacodylate buffer in 1.5 ml microcentrifuge tubes. Fixed samples were then shipped to our collaborator (R. Garduño, Dalhousie University, Canada) for TEM examination, using Philips EM300 transmission electron microscope (Garduño et al., 2002b).

2.2.6. Separation of bacillary and filamentous forms

2.2.6.1. By fluorescence-activated cell sorting (FACS)

Previous optimization studies done in our laboratory showed that to achieve analysis and sorting resolution approximating 1 event = 1 bacterial cell, the density of cultures should be maintained at 10^7 cells/ml. To explore the utility of FACS for separating the bacillary and filamentous forms of *L. pneumophila*, stationary phase rods and filament-rich planktonic cultures (as described in Section 2.2.2.1.3.1) were diluted in sterile ddH₂O to a suspension with the density of 10^7 cells/ml in 5 ml snap cap tubes.

Flow cytometric analyses were performed on FACS Aria™ Cell Sorter (Becton Dickinson), which is equipped with air-cooled argon lasers at 488 nm. The flow rate for FACS Aria™ was maintained at the minimum value of 1 and the threshold for an event to be counted was set using side scatter (SSC) at 200. The photomultiplier (PMT) voltage settings were configured as follows: forward scatter (FSC) 300, side scatter (SSC) 300, FITC 500, and PE 650. These values were based on previous work done in our laboratory, in which FACS Aria™ was optimized for detection and sorting of bacterial cells. For each sample, 10000 events were analyzed in triplicates. Data were processed with FACSDIVA (BD Biosciences) and FlowJo (Tree Star) softwares.

2.2.6.2. By critical culture dilution

Filament-rich planktonic cultures of *L. pneumophila* (as described in section 2.2.2.1.3.1) were adjusted to a density of 1.5×10^8 cells/ml in AC buffer in a 1.5 ml microcentrifuge tube and dispensed into a single column of wells in a 96-well or 384-well clear-bottom microplate (Iwaki, Japan). A series of 10-fold dilutions was then performed by systematically and serially diluting the original bacterial culture with

AC buffer into the subsequent columns of the microplate so as to generate cell densities in the range of $1.5 \times 10^2 - 1.5 \times 10^8$ cells/ml. Microplates were viewed under the inverted microscope to identify the cell density with wells containing only the filamentous form.

CHAPTER 3

THE *L. PNEUMOPHILA* FILAMENTOUS FORM

3. The *L. pneumophila* Filamentous Form

Very early characterization studies of *L. pneumophila* have documented that the bacterium is capable of filamentation (Berg et al., 1985; Pine et al., 1979; Rodgers, 1979; Warren and Miller, 1979), but for a long time, there lacked further investigation of this form specifically. Although it has been acknowledged to be an alternative morphology of *L. pneumophila* in the environment and appeared to be avirulent *in vivo* (Paszko-Kolva et al., 1992), many fundamental questions are still unsatisfactorily addressed, if they were investigated at all. As the appearance of filaments was noted by chance circumstances in these earlier studies, instead of through designs of the experiments, biological implications mostly remained merely implications and nothing more.

At the time that I started this project in 2007, studies by Konishi et al and Piao et al, both reported in 2006, were perhaps the most recent documentation available which showed some systematic attempts to investigate the filamentous form. Both were using biofilms as experimental models. Hence the approach that I took was to first start with a broad exploratory examination of the filamentous form of *L. pneumophila* in the biofilm context, followed by investigations with more specific evaluation criteria on the factors triggering filamentation. There are potentially a great number of environmental conditions that may contribute to the formation of filaments in the *Legionella* life cycle. Among the more commonly implicated factors are the growth temperatures (Konishi et al., 2006; Mauchline et al., 1992; Piao et al., 2006) and the

status of nutrition (Warren and Miller, 1979), so these also served as my starting points.

3.1. Factors affecting formation of filaments in *L. pneumophila* biofilms

3.1.1. High temperature promotes filamentation

It has been reported that *L. pneumophila* strain Knoxville-1 biofilms, which were grown on glass under nutrient-rich conditions, were filamentous and dense, to the point of being “mat-like”, at elevated temperatures of 37°C and 42°C, but not when cultivated at the ambient temperature of 25°C (Piao et al., 2006). To ascertain whether the same morphological profile is reproducible in the *L. pneumophila* type strain Philadelphia-1, these conditions for biofilm growth (detailed in Materials and Methods section 2.2.2.1.4.1) were replicated in our laboratory. For ease of observation, a GFP-expressing derivative of the *L. pneumophila* strain Philadelphia-1 was used, which is referred to as strain LP3. It was generated via transformation of the plasmid pPZ1 (refer Materials and Methods section 2.2.1) provided by Piao et al. (2006), who has previously established that the pPZ1-encoded GFP-expression does not result in artefacts with respect to filamentation.

As shown in Figure 3.1, formation of biofilms by this strain at the air-liquid interface was observed across all of the three growth temperatures (Figure 3.1, A-C). Filaments can be seen only in the 37°C and 42°C biofilms – strands of elongated cells were enmeshed together with a minor proportion of rod-shaped cells (Figure 3.1, E and F). This is contrasted with the 25°C biofilm, which was comprised almost exclusively of rods (Figure 3.1, D). In particular, the biofilms cultivated at 42°C were extensively

filamentous. These morphological distributions of the Philadelphia-1 derivative strain LP3 according to the growth temperatures were consistent with that reported in the Knoxville-1 counterpart by Piao et al (Piao et al., 2006). Hence, we were assured that, under this rich nutrient condition of fully supplemented BYE medium, cells in the biofilms of *L. pneumophila* strain Philadelphia-1 also (i) exhibit filamentation at 37°C and 42°C, and (ii) remain rod-shaped at the lower temperature of 25°C.

Figure 3.1 CLSM micrographs of *L. pneumophila* strain Philadelphia-1 (LP3) biofilms cultivated at ambient and elevated temperatures. LP3 biofilms were cultivated on glass coverslips in BYE growth medium at 25°C (A, D), 37°C (B, E), 42°C (C, F). Captured here are biofilms at the air-liquid interface in their matured state, indicated at the bottom by the number of days incubated at the respective temperatures. Shown in each micrograph are the x-y, x-z (horizontal section) and y-z planes (vertical section), corresponding to the faint red line indicated in the respective perpendicular sections. All micrographs are presented in the air-liquid orientation indicated below micrograph A. (Upper panel) Macro view of biofilm line, size bar is 50 μm . (Lower panel) Magnified view of the cell morphology composed in the corresponding biofilm line, size bar is 10 μm .

The biofilms were examined further to obtain more clues about the nature of the filamentous form. Similar to the Knoxville-1 strain, there exist, for the Philadelphia-1 derivative, some temperature-dependent differences in (i) the speed of biofilm formation and (ii) the biofilms' adherence stability. At 25°C, biofilms took longer to form, exhibiting well-developed films after 9-14 days of incubation, and appearing stably adherent. On the other hand, biofilms formed quickly at 37°C and 42°C – taking only 3-6 days – but were seen to dislodge more easily upon disturbance. Incidentally, the temperature-dependent adherence stability has also been documented for *L. pneumophila* strain Lens biofilms although filamentation per se was not checked for (Hindre et al., 2008). Thus, between the two morphological forms of the same strain, the rod form often appears to be more adherent relative to the filamentous form.

A quantitative analysis of the biofilm images revealed that, at 42°C, biofilms of the strain LP3 were thinner and have significantly less biomass compared to those at 25°C and 37°C (Table 3.1). This is in contrast to the profile described for the Knoxville-1 biofilms, in which the 42°C biofilm was thicker than the 25°C counterpart. The average thickness of 37°C LP3 biofilms was also much less ($9.3 \pm 3.7 \mu\text{m}$) than that reported in Knoxville-1 ($\sim 72 \mu\text{m}$) (Piao et al., 2006). Although filaments were observed at 37°C, the LP3 biofilm was comparatively more porous than the Knoxville-1 counterpart, and not dense and mat-like in structure. These differences demonstrate that even though the filamentous form of *L. pneumophila* was induced in both strains at higher temperatures, it did not result in similar biofilm properties. This serves to caution us against generalization of the filamentous form based on the subsequent characterization of the Philadelphia-1 strain.

Temperature (°C)	Day ^a	Average thickness ^b (μm)	Biomass ^b (μm ³ /μm ²)
25	13	9.9 ± 1.7	7.6 ± 2.7
37	5	9.3 ± 3.7	5.9 ± 2.2
42	5	2.8 ± 0.7	2.1 ± 0.9

Table 3.1 Thickness and biomass of the matured biofilms of GFP-expressing *L. pneumophila* Philadelphia-1 strain LP3. ^a Due to slower growth rate, mature 25°C biofilms are observed on Day 13 instead of Day 5. ^b Values were obtained using COMSTAT (Materials and Methods Section 2.2.5.3) and presented as mean ± SD of three to four samples.

3.1.2. Status of nutrition influences formation of filaments

3.1.2.1. Impact of global nutrient depletion

The experimental model for biofilm cultivation described in Section 3.1.1 was such that the growth medium was not changed in the entire observation period, i.e. the bacterial cells started with a rich medium but this would be expected to be progressively nutrient-depleted over the days. In this situation, it is conceivable that *L. pneumophila* has filamented as a general starvation stress response. To address this potential link between filamentation in biofilms and nutrient limitation, the Philadelphia-1 derivative LP3 biofilm at 37°C under the nutrient-depleting condition (Figure 3.2, top panel) identical to that used in the previous experiment (Section 3.1.1) was compared against those under a nutrient-replenished condition whereby the BYE growth medium supporting biofilm development was replaced every 24 h with a batch of fresh medium, over the course of cultivation (Figure 3.2, bottom panel). A variation of the nutrient-depleting condition was also included, in which spent BYE was taken from the biofilm tank every 24 h and filtered through 0.2 μm pores to

remove the planktonic cells, before being returned to the tank ('planktonic-free nutrient-depleting' condition, Figure 3.2, middle panel). This is to serve as a control for the fact that in the test sample (Figure 3.2, bottom panel), planktonic cells would be removed during each 24-hourly nutrient replenishment. The profiles of biofilms from these three conditions were monitored everyday and compared for cell morphology and biofilm structure (Figures 3.3 and 3.4). If nutrient limitation is indeed a necessary factor triggering filamentation in biofilms, then the condition in which the culture medium was constantly refreshed would be expected to show no filamentation.

Figure 3.2 Schematic diagram illustrating the model of biofilm cultivation improvised to investigate the occurrence of filaments in biofilms grown under nutrient-replenished and nutrient-depleting conditions. *L. pneumophila* strain LP3 were cultivated at 37°C for 9 days in the respective conditions.

As shown in Figure 3.3, LP3 biofilms in all three conditions of nutrient availability exhibited formation of filaments in the region immediately below the air-liquid interface. By day 6, biofilm growth which extended beyond the air-liquid interface into the liquid-immersed region of the glass substratum had also become apparent (Figure 3.4) and likewise showed filaments under all three nutritional status tested (representatively shown in Figure 3.2). This implies that filamentation in Philadelphia-1 biofilm is unlikely to be a generic starvation response.

Figure 3.3 CLSM micrographs showing filaments in LP3 biofilms cultivated at 37°C under nutrient-depleting and nutrient-replenished conditions. Captured here are biofilms at the air-liquid interface at Day 4 of incubation. Shown in each micrograph are the x-y, x-z (horizontal section) and y-z planes (vertical section), corresponding to the faint red line indicated in the respective perpendicular sections. All micrographs are presented in the air-liquid orientation indicated below micrograph A. Size bar is 10 μm .

Figure 3.4 CLSM micrographs showing a macro view of LP3 biofilms cultivated at 37°C under nutrient-depleting and nutrient-replenished conditions. Biofilms were observed to extend from the air-liquid interface towards the liquid-immersed region of the glass coverslip. Captured here are biofilms at Day 6 of incubation. A magnified view of cell morphology at the air-liquid interface (white circle) and liquid-immersed (white square) regions are presented in the following Figures (3.7 and 3.8) respectively. The x-y, x-z (horizontal section) and y-z planes (vertical section) of each micrograph correspond to the faint red line indicated in the respective perpendicular sections. Micrographs are presented in the air-liquid orientation indicated below micrograph A. Size bar is 50 μm .

3.1.2.2. Survey of nutritional components and their influence on filamentation

Since the inocula for the biofilm cultures shown above were rod-shaped planktonic stationary phase cells, conversion from the rod to the filamentous form must have occurred during the development of biofilms under our static incubation condition. To dissect for the influence of a particular nutritional component, stationary phase LP3 rods were inoculated in a series of media with varying nutrient composition in duplicates in 96-well microplates. These cells (Figure 3.5, A) were then monitored every 24 h for signs of conversion to filaments as they were incubated statically at 37°C. Components that were made variable included: (a) the organic component (BYE) of the growth medium and (b) growth supplements L-cysteine (essential amino acid) and ferric pyrophosphate (for iron which is necessary for the bacterium's initial rapid growth) (Ristroph et al., 1981). A spectrum of media with BYE removed or in decreasing concentrations was supplemented with both of the growth supplements or none at all (Table 3.2). Two minimal media, AC buffer and M63, and sterile distilled deionized water (ddH₂O) were also included to expand the range of nutrient-poor conditions tested. A control in the fully supplemented 100% BYE growth medium (as used in the biofilm experiments) served as the reference (Table 3.2).

At 37°C, filamentation was observed to occur in the nutrient-rich fully supplemented, undiluted BYE control sample, as expected (Table 3.2, Column '37°C with supplement', Row '100% BYE'). However, only one other cluster of nutrient conditions showed filamentation, i.e. unsupplemented media containing only 5-10% BYE (Table 3.2, Column '37°C without supplement', Rows '5-10 % BYE'). Under

these conditions, long filaments appeared at about four days post-inoculation of rods (Figure 3.5). Curiously, cells in the lower (1%) and higher (20-50%) BYE concentration ranges also unsupplemented with L-cysteine and ferric pyrophosphate did not filament, implying that filamentation is not determined by a “threshold” of the BYE concentration. LP3 rod-shaped cells in all other media variations with or without BYE and growth supplements showed no changes in their cell morphology.

Temperature		25°C		37°C		42°C	
Supplement		Yes	No	Yes	No	Yes	No
+ BYE	100 % BYE	-	-	+	-	+	+
	50 % BYE	-	-	-	-	+	+
	20 % BYE	-	-	-	-	+	+
	10 % BYE	-	-	-	+	+	+
	5 % BYE	-	-	-	+	+	+
	1 % BYE	-	-	-	-	-	-
- BYE	Sterile water	-	-	-	-	-	-
	AC buffer	-	-	-	-	-	-
	M63	-	-	-	-	-	-

Table 3.2 Occurrence of filaments in media varying in organic nutrient (BYE) and supplement (L-cysteine and ferric pyrophosphate) components at 25°C, 37°C and 42°C. Each medium variation was inoculated with stationary phase *L. pneumophila* strain LP3 rods in duplicate wells, incubated statically in 96-well clear-bottom microplates for 168 h and monitored for filamentation using fluorescence microscope. Samples were identified as positive for filamentation when cells longer than 5 rods' length appeared at any time point within the observation period. Result tabulated is a representation of two replicate experiments. 'Yes' and 'No' indicates the presence or absence of supplements L-cysteine and ferric pyrophosphate. + : Filamentation positive. - : Filamentation negative.

Figure 3.5 Representative fluorescence microscope images illustrating formation of filaments, or the lack of, by *L. pneumophila* strain LP3 rods inoculated in the spectrum of media variants detailed in Table 3.2. (A) Rods at 5 h post-inoculation. (B) Formation of filaments observed in permissive nutrient conditions (refer Table 3.2) at about Day 4 post-inoculation. (C) Cells in nutrient conditions that did not support formation of filaments remained rods for the entire observation period. Size bar is 10 μm .

However, when the same range of media variation was tested at two other growth temperatures, 25°C and 42°C, a different pattern was observed. At 25°C, filamentation was completely absent regardless of nutrient availability (Table 3.2, Column 25°C). On the other hand, at 42°C, filaments were observed to occur above a BYE concentration threshold of 5%, regardless of the availability of supplements (Table 3.2, Column 42°C). It appears from the data in Table 3.2 that under static cultivation conditions, the influence of nutritional components (organic nutrient and growth supplements) on filamentation varies with temperature.

It is interesting to note that the availability of growth supplements (L-cysteine and ferric pyrophosphate) appeared to affect the formation of filaments only at the cultivation temperature of 37°C (Table 3.2, Column '37°C'). To investigate how each supplement may individually influence filamentation, the spectrum of test media was further varied by supplementing with only either L-cysteine or ferric pyrophosphate, both, or none at all (Table 3.3). Stationary phase *L. pneumophila* strain LP3 rods were then inoculated in these media in 96-well microplates, incubated statically at 37°C and monitored for filamentation.

As shown in Table 3.3, neither L-cysteine nor ferric pyrophosphate – present individually or in combined presence/absence – appeared to have a clear-cut pattern of influence on the occurrence of filaments at 37°C. Although the incidence of filamentation was higher in L-cysteine-deprived conditions, this was true only in the BYE concentration range of 5-10 %.

Collectively, it seemed that there was no absolute or exclusive association of filamentation with any one particular nutritional component based on these two sets of data.

Supplement	L-cysteine	Yes	Yes	No	No
	Ferric pyrophosphate	Yes	No	Yes	No
+ BYE	100 % BYE	+	-	-	-
	50 % BYE	-	-	-	-
	20 % BYE	-	-	-	-
	10 % BYE	-	-	+	+
	5 % BYE	-	-	+	+
	1 % BYE	-	-	-	-
- BYE	Sterile water	-	-	-	-
	AC buffer	-	-	-	-
	M63	-	-	-	-

Table 3.3 Influence of growth supplements, L-cysteine and ferric pyrophosphate, on filamentation by *L. pneumophila* strain LP3 incubated statically at 37°C. Stationary phase LP3 rods obtained from planktonic culture grown for 24 h at 37°C were inoculated in the media in 96-well clear-bottom microplates, incubated statically at 37°C for 168 h and monitored for filamentation using fluorescence microscope. Samples were identified as positive for filamentation when cells longer than 5 rods' length appeared at any time point within the observation period. Result tabulated is a representation of two replicate experiments. 'Yes' and 'No' indicates the presence or absence of each supplement. + : Filamentation positive. - : Filamentation negative.

3.1.3. Microscale oxygen gradient in biofilm may affect formation of filaments

The biofilms, although showing no difference in filamentation status across the three nutrient conditions tested in section 3.1.2.1 (Figure 3.3), did reveal some subtle differences between the air-liquid interface and the liquid-immersed regions in terms of the localization of filaments (Figure 3.4). Biofilm studies in other bacteria have clearly shown that there is microscale heterogeneity in nutrient concentrations within and around biofilms (Stewart and Franklin, 2008), and physiological heterogeneity

displayed by biofilm bacteria can be correlated to the differences in local concentrations of nutrients such as oxygen (Xu et al., 1998). Microscale gradient of oxygen is expected to occur along the vertical axis of our biofilm set up – the local concentration of oxygen near the air-liquid interface is likely to be higher than that in the liquid-immersed region. With this in mind, the subtle differences in these two regions were scrutinized to glean possible information on the possible influence of oxygen on *L. pneumophila* filamentation. Since the observation was similar across all three conditions (Figure 3.3 and 3.4), only the nutrient-replenished sample will be shown representatively (Figure 3.6–3.8).

At the air-liquid interface, filaments were enmeshed with an abundance of rods almost homogeneously across the entire cross-section of the biofilm. Filaments were observed at the substratum layer as well as the layers of cells above (Figure 3.7, A and D). However, in the liquid-immersed biofilms, the layers appeared distinctly separated (Figure 3.8, B and C), sometimes ‘peeling off’ neatly when disturbed (Figure 3.6). Close examination of the cell morphology revealed that the layer directly on the substratum consisted almost exclusively of rods (Figure 3.8, D) while the peel-able biofilm layer above the substratum was very filamentous (Figures 3.6 and 3.8A). This agrees with our earlier observation that the adherence of filaments seemed lower than the rods (Section 3.1.1, page 51). The fact that this peel-able layer was much more filamentous than the air-liquid interface region suggests that the relative reduction in oxygen in the liquid-immersed region may be more conducive for filamentation. At this point, it should be clarified that although oxygen is our greatest suspect, possible influence of other micro-chemical gradients cannot be entirely taken out of the picture.

Figure 3.6 CLSM micrograph of a three-dimensional side view (x-z plane) depicting the two distinct layers of a liquid-immersed LP3 biofilm cultivated at 37°C under nutrient-replete conditions. Size bar is 50 μm .

Figure 3.7 CLSM micrographs comparing distribution of filaments and rods at the layer above substratum (A) with the layer of substratum (D) of an air-liquid interface of LP3 biofilm cultivated at 37°C under nutrient-replete conditions. The x-z (horizontal section) and y-z (vertical section) planes are shown in B and C respectively. * The z-position corresponding to the x-y plane shown in A. # The z-position corresponding to the x-y plane shown in D. Size bar is 10 μm .

Figure 3.8 CLSM micrographs comparing distribution of filaments and rods at the layer above substratum (A) with the layer of substratum (D) of a liquid-immersed region of LP3 biofilm cultivated at 37°C under nutrient-replete conditions. The x-z (horizontal section) and y-z (vertical section) planes are shown in B and C respectively. * The z-position corresponding to the x-y plane shown in A. # The z-position corresponding to the x-y plane shown in D. Size bar is 10 μm.

3.1.4. The effects of homogenizing agitation on filamentation in planktonic cultures

Since static incubation for biofilm cultivation likely created a gradient of oxygen (or other compounds) that might have resulted in the heterogeneous distribution of filaments in the afore-mentioned two regions, the effect of incubation with agitation (ensuring greater homogeneity) was tested. To compare against the static incubation of biofilm cultures earlier described (Section 3.1.1), *L. pneumophila* Philadelphia-1 strain LP3 cells were incubated with agitation of 250 rpm in 14ml inoculation tubes with fully supplemented BYE medium at the temperatures 25°C, 37°C and 42°C. During exponential phase, the cells in the cultures remained rod shaped, but at stationary phase (cultivation for 24 h and beyond), filamentation occurred at 42°C, but never at 37°C or 25°C (Figure 3.9). Hence, this homogenizing agitation resulted in no filamentation at 37°C, in contrast to the clear filamentation under static incubation conditions, either as surface-attached cells in biofilms (Figure 3.1) or as discrete cells settled at the bottom of microplate wells (Figure 3.5 and Table 3.2).

Even though these data suggest the existence of microscale gradients to be favourable for filamentation in *L. pneumophila* strain LP3, there is a qualitative distinction when the temperature factor was layered on. Between the temperatures 37°C and 42°C – which both showed filamentation under static incubation – at 37°C, agitation abolished filamentation but at 42°C, filamentation occurred regardless of the agitation (Figure 3.9).

Figure 3.9 Filamentation in stationary-phase planktonic cultures of *L. pneumophila* strain LP3 upon incubation at 42°C but not 37°C or 25°C. LP3 cultures were predominantly rods when cultivated at 25°C or 37°C (A) but richly filamentous at 42°C (B). These morphologies were observed with 100% consistency in at least 25 replicates for each temperature. Planktonic cultures were grown to stationary-phase in BYE growth medium with shaking at 250 rpm and observed between 24-48 h post-inoculation. Size bar is 10 μ m.

Since it was observed that homogenizing agitation had an impact on filamentation of *L. pneumophila* strain LP3 in fully supplemented BYE medium at 37°C, I wondered whether it will also affect the incidence of filamentation in other media varying in the nutritional composition (organic component, BYE, and supplements, L-cysteine as well as ferric pyrophosphate). To assess, planktonic cultures of *L. pneumophila* strain LP3 cells were inoculated in the spectrum of media used in Section 3.1.2.2 and incubated with agitation at 25°C, 37°C and 42°C. While the tendency to filament was unchanged at 25°C, homogenizing agitation was observed to affect filamentation in some media variations at 37°C and 42°C (Table 3.4). At 37°C, cultivation with agitation in unsupplemented media with BYE concentration between 20-100% resulted in filamentation (Table 3.4), which was not observed during static incubation (Table 3.2). Curiously, the tendency for filamentation was abolished with

homogenizing agitation at 42°C in supplemented media with a concentration of BYE between 20-50% (compare Tables 3.2 and 3.4).

Temperature		25°C		37°C		42°C	
Supplement		Yes	No	Yes	No	Yes	No
+ BYE	100% BYE	-	-	-	+	+	+
	50% BYE	-	-	-	+	-	+
	20% BYE	-	-	-	+	-	+
	10% BYE	-	-	-	+	+	+
	5% BYE	-	-	-	-	+	+
	1% BYE	-	-	-	-	-	-
- BYE	Sterile water	-	-	-	-	-	-
	AC buffer	-	-	-	-	-	-
	M63	-	-	-	-	-	-

Table 3.4 Filamentation by *L. pneumophila* strain LP3 in media of varying organic (BYE) and supplement (L-cysteine and ferric pyrophosphate) content under conditions of homogenizing agitation at 25°C, 37°C and 42°C. Each medium variation was inoculated with LP3 rods in inoculation tubes and incubated with agitation at 250 rpm for 96 h. Samples were identified as positive for filamentation when cells longer than 5 rods' length appeared at any time point within the observation period. Data tabulated were consistently obtained from two independent replicate experiments. 'Yes' and 'No' indicates the presence or absence of supplements L-cysteine and ferric pyrophosphate. + : Filamentation positive. - : Filamentation negative.

The fact that homogenizing agitation appears to influence the incidence of filamentation in different nutritional environments at different temperatures again indicated that it is not one factor that determines the filamentation status of *L. pneumophila* Philadelphia-1 but rather, the interweaving of several factors. This necessitates that we review the data obtained while investigating each category separately (Sections 3.1.1-3.1.4) and integrate them for an overview in the following section.

On a side note, performing this series of experiments had led to the decision that culturing stationary phase cells at 37°C and 42°C with agitation in the fully supplemented 100% BYE medium would be a reliable method to obtain bacillary and filamentous cells, respectively, for experiments in the subsequent part of my project, which required specific morphologies of *L. pneumophila* Philadelphia-1 (or its derivative LP3) as biological materials. This had turned out to be a good decision as among the 67 inoculations at 37°C and the 204 inoculations at 42°C performed during the course of my study, rods and filaments were obtainable with 100% reliability.

3.1.5. Overview

As an initial step towards understanding the environmental conditions that trigger formation of filaments in *L. pneumophila*, two conditions frequently associated with bacterial filamentation – temperature and nutrient status – were studied. In the course of the exploration, a possible influence of micro-chemical gradients, most probably that of oxygen, was surfaced. It was also revealed that the effect of each condition was not always independent of one another – the compounding effect appears to determine the occurrence, or not, of filamentation. The differential combinations of these factors had led to varying outcomes, and Tables 3.5 and 3.6 summarize these findings as far as is feasible.

Temperature (°C)	Cultivation mode			
	Static		Shaken ^c	
	Biofilm ^a	Microplate ^b	Exponential ^d	Stationary ^e
25	-	-	-	-
37	+	+	-	-
42	+	+	-	+

Table 3.5 Filamentation by *L. pneumophila* strain LP3 relative to cultivation mode and temperature. ^a Biofilms were cultivated on glass coverslips in 30 ml BYE medium and incubated statically for over 15 days. ^b Stationary phase bacillary cells were inoculated in 200 µl BYE medium in 96-well clear-bottom microplates incubated statically for over 7 days. ^c Stationary phase bacillary cells were inoculated in 14ml inoculation tubes with 3 ml BYE medium and incubated with shaking at 250 rpm for 4 days. ^d Exponential phase was sampled post-inoculation at 18 h for 25°C culture, 12 h for 37°C and 42°C cultures. ^e Stationary phase was sampled post-inoculation at 96 h for 25°C culture, 24 h for 37°C and 42°C cultures + : Filamentation positive. - : Filamentation negative.

Clearly, regardless of the cultivation mode (Table 3.5) or status of nutrient availability (Table 3.6), filaments were never observed at 25°C. Higher temperatures of 37-42°C appeared to promote filamentation, but the incidence was additionally affected by the nutritional component present as well as the mode of cultivation (static or shaken). Nevertheless, filamentation is more likely to occur at 42°C than 37°C under all possible combinations of cultivation mode and nutrient status.

Temperature (°C)	Static ^a							Shaken ^b							Cys-Fe
	+ BYE						- BYE	+ BYE						- BYE	
	100%	50%	20%	10%	5%	1%		100%	50%	20%	10%	5%	1%		
25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Yes
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No
37	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Yes
	-	-	-	+	+	-	-	+	+	+	+	-	-	-	No
42	+	+	+	+	+	-	-	+	-	-	+	+	-	-	Yes
	+	+	+	+	+	-	-	+	+	+	+	+	-	-	No

Table 3.6 An overview of the influences of temperature, cultivation mode and nutritional components on filamentation by *L. pneumophila* strain LP3.

Media without BYE (-BYE) includes sterile water, AC buffer and M63 minimal media. ‘Yes’ and ‘No’ indicates the presence or absence of supplements.

Cys-Fe : supplements L-cysteine and ferric pyrophosphate. + : Filamentation positive. - : Filamentation negative. ^{a, b} Cultivation conditions correspond to Static-Microplate and Shaken-Stationary cultivation modes in Table 3.5 respectively.

Occurrence of filaments in biofilms under both nutrient-replenished and nutrient-depleting conditions (Section 3.1.2.1, Figure 3.3) indicated that filamentation by *L. pneumophila* is unlikely to be a general starvation response. This was in agreement with the subsequent observations from the survey investigating the influence of nutritional components on filamentation; filaments were found to occur in fully supplemented undiluted BYE media at 37°C and 42°C under static cultivation (Table 3.6). Moreover, filamentation at 37°C and 42°C appeared to be more likely to happen in various nutrient combinations comprising of an organic component of at least 5% BYE and never observed to occur at nutrient combinations with 1% BYE or less (Table 3.6), suggesting that formation of filaments may require the presence of a minimal concentration of organic nutrient. However, it appears that filamentation is conditional to particular combinations of nutrient; for instance, under static cultivation at 37°C, filaments were observed only either in fully supplemented undiluted BYE or in a narrow range of 5-10% BYE concentration in the absence of cysteine and iron supplements (Table 3.6).

In addition, the mode of cultivation, either static or shaken, has an impact on the outcome of filamentation, especially at 37°C. Filaments were observed in fully supplemented undiluted BYE medium at 37°C in the static, but not the shaken, cultivation mode (Table 3.5). The combinations of nutrients at 37°C in which filamentation was found to occur were not the same under static or shaken cultivation (Table 3.6, Row '37°C', compare columns 'Static' and 'Shaken'). This difference could probably happen as a consequence of variation in microscale solute chemistry due to the difference in the fluid dynamics of the two cultivation modes. A steeper gradient of solutes such as dissolved oxygen or metabolites exists under conditions of

static cultivation than in continuous shaking and this could affect the cell morphology. However, at a higher temperature of 42°C, the influence of static or shaken mode of cultivation seemed less prominent than that observed at 37°C (Tables 3.5 and 3.6).

Based on these data, it was not possible to clearly attribute the occurrence of filamentation to a dominant triggering factor. Instead, it is likely that the influence of each of the factors of temperature, nutritional status and mode of cultivation is neither exclusive nor over-arching but instead, it is the specific interplay of these factors that determines the incidence of filamentation by *L. pneumophila*.

It is interesting to note that a number of observations of the filamentous form mentioned in the earlier reports of *L. pneumophila* were associated with cultivation on agar (Edelstein and Finegold, 1979; Katz et al., 1984; Konishi et al., 2006; Nowicki et al., 1987; Paszko-Kolva et al., 1992; Piao et al., 2006; Rodgers, 1979). Indeed, some of the more recent reports also noted more frequent occurrences of filaments on agar than in liquid cultures (Garduño et al., 2008; Piao et al., 2006). The greater tendency for filamentation of cells cultured on agar could either be due to the more pronounced microscale gradients in the solid medium, or alternatively, because cells are in association with surfaces.

To summarize, filamentation in *L. pneumophila* is not controlled exclusively by any single factor, but is under the influence of multiple parameters acting in concert. It should be noted that the combinations of parameters tested here are not exhaustive, and there could be other factors, such as surface association, affecting filamentation that were not considered in this study. Nevertheless, the collective data obtained

(Tables 3.5 and 3.6) clearly showed that filamentation by *L. pneumophila* is more likely to occur under conditions of (i) higher than ambient temperatures of 37-42°C, (ii) in static cultivation than with agitation, and (iii) when an organic content of at least 5% BYE (500 mgL⁻¹ yeast extract) or equivalent is present. To my knowledge, results from the systematic assay of temperature, nutrient and fluid flow in this study is the first to demonstrate that manifestation of the *L. pneumophila* filamentous morphology is not driven by a single dominant factor but the interplay of multiple environmental factors.

When this series of experiments was initiated, it was hoped that a specific class of environmental triggers could be identified to be clearly dominant over others, from which more in-depth investigations could be oriented. Since the interplay of factors turned out to be complex, sometimes with very narrow (and frankly, puzzling) matrix of parameters, I did not pursue this line of study further. However, in analysing the data from this series of experiments, the diversity of circumstances from which *Legionella* filaments had been observed now made greater sense to me.

3.1.6. Discussions

Amongst normally bacillary bacteria, filamentation is commonly associated with aberrations in cell division as a consequence of stress, e.g. DNA damage, mutation, exposure to antimicrobial agents (Healy et al., 2007; Janion et al., 2002; Nelson and Young, 2000; Schapiro et al., 2003). However, bacteria have also been described to filament in response to changes in their environment. For example, *Pseudomonas putida* was observed to filament when oxygen became a limiting factor during growth

in liquid media (Jensen and Woolfolk, 1985). In *L. pneumophila*, although the formation of filaments has been implicated in varying growth conditions, such as low-nutrient (Paszko-Kolva et al., 1992) as well as high temperature (Konishi et al., 2006; Piao et al., 2006), a systematic profile was lacking that encompasses the multiple factors that could influence filamentation. In this study, I attempted to systematically assess the impact of temperature and nutrient status on the occurrence of the filamentous morphology in *L. pneumophila*, and the results revealed that the phenomenon is influenced by a complex interplay of factors not limited to temperature and nutrient status. As presented in 3.1.5 Overview, the mode of cultivation, e.g. in static or shaking cultivation, appears to also influence the occurrence of filaments in *L. pneumophila*.

3.1.6.1. Temperature dependence of filamentation may be a manifestation of a regulated response

It has been previously reported that filamentation by *L. pneumophila* appeared to be temperature-dependent, as greater incidence of filaments was observed at higher-than-ambient growth temperatures (Konishi et al., 2006; Mauchline et al., 1992; Piao et al., 2006). In my study, the occurrence of filaments by *L. pneumophila* was indeed correlated to the higher temperatures of 37-42°C. However, the induction of filamentation within this range of high temperature was not uniform across all nutrient and cultivation conditions investigated. For instance, in static cultivation at 37°C, filamentation was observed only either in fully supplemented undiluted BYE medium or in unsupplemented media with 5-10% BYE content (Table 3.6). Since the influence of high temperature is not universal across all other nutrient and cultivation conditions, it appears unlikely that the observed filamentation is a simple consequence of global

temperature stress, but more an adaptive response in which the key molecular players are regulated by temperature in addition to other factors such as nutrient concentration. Incidentally, it was recently reported that over-expression of the heat shock-induced chaperonin HtpB in *L. pneumophila* was correlated with filamentation (Garduno et al., 2011). This may be a potential candidate playing a role in the regulation of filamentation based on temperature fluctuations in the environment.

3.1.6.2. Certain nutrient concentrations may favour filamentation by influencing bacterial growth rate

Although nutrient starvation has been associated with the formation of filaments in some rod-shaped bacteria (Steinberger et al., 2002; Wainwright et al., 1999), such a link has historically been ambiguous in *L. pneumophila*. On the one hand, there has been observation of extensive filamentation under nutrient-limiting growth conditions such as creek water microcosm (Paszko-Kolva et al., 1992), or in cultures of chemically defined medium but not in nutritionally richer gonococcal-ferric cysteine (GC-FC) complex medium (Warren and Miller, 1979), suggesting that filamentation may be a consequence of nutrient limitation. On the other hand, observation of *L. pneumophila* filaments in a higher nutrient condition of 0.8% casein hydrolysate semisynthetic broth, but not at a lower concentration of 0.4%, suggested otherwise (Pine et al., 1979).

This diversity of nutrient conditions in which filaments could be observed was also reflected in my study. The fact that filaments occurred in varying nutrient combinations but always in those with a BYE concentration of at least 5% and never in media with 1% BYE content or less (Table 3.6) suggested that nutrient limitation

cannot be the trigger of filamentation. In an earlier study assessing the effect of growth rate on the physiology and morphology of *L. pneumophila*, it was demonstrated that the subpopulations of bacillary and filamentous forms of *L. pneumophila* predominate at different bacterial growth rates, leading the authors to propose that the occurrence of filaments may be dependent on the bacterial growth rate (which in turn, is affected by availability of the array of nutrients) (Berg et al., 1985). Based on the complex interplay of influence observed in this study between nutritional status and other factors, e.g. temperature and mode of cultivation, it is highly likely that the growth rate of *L. pneumophila* is modulated, in part, by the specific combinations of nutrients, consequently affecting other bacterial processes that regulates cell morphology, and thus resulting in filamentation.

3.1.6.3. Effect of diffusion and chemical heterogeneity of microenvironment on filamentation

The fact that *L. pneumophila* filaments were more likely to be found under conditions of static rather than shaking cultivation (Table 3.5) suggests that filamentation is influenced by the state of liquid flow in the environment. Interestingly, a very recent study reported similar findings, whereby *L. pneumophila* filamentation was observed in biofilms cultivated under static liquid but not liquid flow conditions (Konishi et al., 2011). The effect of liquid flow on filamentation has also been demonstrated in other bacteria, e.g. *P. putida*, which formed filaments under slow, but not vigorous, shaking cultivation (Jensen and Woolfolk, 1985).

The state of liquid flow affects diffusion of molecules, such as nutrients or dissolved oxygen or metabolites, in the environment; thus, under conditions of static or slow

shaking cultivation, distribution of these molecules is expected to be more heterogenous than under conditions of dynamic liquid flow. The uneven distribution of these molecules of substrates or by-products could generate chemical gradients in the microenvironment directly experienced by the bacteria which may influence bacterial activity or alter gene expression, leading to modifications in bacterial phenotype as a means of adaptation (Marshall and Goodman, 1994). For instance, the violacein pigment-producing marine bacterium *Pseudoalteromonas luteoviolacea* was observed to increase violacein production under static or slow shaking cultivation, probably in response to a gradient in the concentration of dissolved oxygen (Yang et al., 2007).

The influence of a heterogeneous distribution of nutrients and metabolites on bacterial activity may be even more pronounced on solid medium such as agar, whereby molecular diffusion is even more restricted compared to static liquid medium (Lorian, 1989). This could account for the higher frequency of reported observations of *L. pneumophila* filaments on agar than in liquid cultures by other investigators (Garduño et al., 2008; Nowicki et al., 1987; Paszko-Kolva et al., 1992; Piao et al., 2006; Rodgers, 1979). *L. pneumophila* may be able to sense and respond to gradients of its chemical microenvironment, which are more prominent under static or solid cultivations, and this may result in filamentation. Alternatively, since very specific combinations of nutrient components are found to be conducive to filamentation, niches fulfilling such nutritional combinations may be more readily created by gradients under static and solid conditions, resulting in a higher incidence of the filamentous form.

3.1.6.4. *L. pneumophila* filamentous form: A morphological form that should not be neglected

The influence from a complex interplay of temperature, nutrient and fluid flow factors on *L. pneumophila* filamentation seen in this study suggests that within certain environmental niches, the filamentous form may predominate over the bacillary form. To survive in the environment, bacteria are physiologically and morphologically malleable to fluctuating conditions (Roszak and Colwell, 1987). Shown to be capable of demonstrating at least six major differentiated forms in its life cycle (Garduño et al., 2008), *L. pneumophila* may take advantage of its inherent ability to adopt from a suite of cell shapes and differentiate into a morphology that is most strategic to cope with its dynamic environment. Although the data in this study thus far have not been able to pin-point the adaptive advantage for *L. pneumophila* in converting to the filamentous form, the specific combinations of temperature, fluid movement status and nutritional components which dictate whether *L. pneumophila* filamentation occurs suggest that the occurrence of the filamentous form may be fairly common and should not be neglected. Its ecological relevance would therefore have to be investigated in order to obtain a more holistic view of the extracellular phase of *L. pneumophila* in the environment.

3.2. Interaction of *L. pneumophila* filamentous form with protozoan hosts

It has been established from earlier studies that *Legionella* bacterium parasitizes on protozoa in the natural environment (Rowbotham, 1980). Since then, a large volume of work has been dedicated to the characterization of this host-pathogen relationship, and since the most predominant morphology of *Legionella* is the bacillus, all infection models to date have made use of forms of *L. pneumophila* that are rod-shaped.

Interaction between the filamentous form of *L. pneumophila* and protozoa has never been described, even though filaments are acknowledged to be an alternative form of the bacterium in the environment (Garduño et al., 2008; Paszko-Kolva et al., 1992). In order to explore this dimension of host-pathogen relationship as a component of this project, infection profiles of the filamentous form were examined, using the rod-shaped form as a reference for comparison.

3.2.1. Obtaining purely filamentous cultures – A major technical challenge

In my attempt to investigate the interaction of the filamentous form of *L. pneumophila* with protozoa, the first technical obstacle I encountered was the difficulty in obtaining filamentous bacterial cultures that are considered homogeneous, minimally (less than 5%) ‘contaminated’ by rod-shaped cells, for infection assays. The rod-shaped culture counterpart was more easily obtained because the routine laboratory cultivation of broth cultures at 37°C with agitation could generate morphologically homogeneous bacillary culture, with 95-100% of the cells being rod-shaped. On the other hand, the

most reproducibly and abundantly filamentous culture that could be harvested, based on my earlier study (Section 3.1.4), was from broth cultures grown to stationary phase with agitation at 42°C. In these broth cultures cultivated at 42°C, about 76-91% of the cell population were filamentous, while the remaining cells were rod-shaped in morphology. The average abundance of the filaments in such cultures was 81.2% (± 8.9) of the cell population. The presence of a minor but significant population of rods amongst filaments was perceived as a potential problem as it may affect the manifestation of interaction when infection of the protozoa by the filamentous versus the rod-shaped morphology were compared, possibly complicating the interpretation of data.

To obtain morphologically pure filamentous cultures, attempts were first made to separate filaments from rods using the fluorescence-activated cell sorter (FACS). The light scattering properties of bacteria, quantified by FACS as the forward scatter (FSC) and the side scatter (SSC) parameters, are indicative of particle size and complexity respectively, and these profiles may be useful to distinguish between the filaments and the rods. Greater cell length and more complex spatial arrangement of filaments (e.g. folding back of the filament due to its longer length) may increase light scattering, hence registering higher values in FSC and SSC parameters when compared to the rod morphology. By virtue of different light scattering profiles, it may be possible to rapidly make the distinction between the two forms and then exploit the cell sorting function of FACS to separate the filaments from the rods. Furthermore, the ability of FACS to analyse and sort tens of thousands of cells within seconds could potentially lead to more efficient harvesting of large amounts of filaments in minimal time and with reduced labour.

The initial stages of flow cytometric analyses were promising because comparison of the contour-dot plot profiles of rod-shaped cell cultures with filament-rich cultures revealed two distinct types of contour peaks (Figure 3.10, red arrows 1 and 2). The peak corresponding to higher FSC and SSC values (Figure 3.10 B, red arrow 2) were observed only with the filament-rich cultures, and hence very likely represented the filamentous subpopulation. However, in the subsequent stages of manipulation for cell sorting, I was unable to determine the gates (the range of FSC and SSC values that defines the subpopulation) corresponding to the rod and filamentous population, because of batch-to-batch variability and even intra-batch shifts of profiles, even though qualitatively, the two distinct peaks were always observed. As I could not always confidently define gates that reproducibly distinguish the rods from the filaments, in order not to compromise the purity of the sorted cell populations, considerably longer FACS analysis and manipulation time had to be spent to complete the cell sorting. This lag in time (from the point of harvest of cells) prompted us to re-evaluate the merits and demerits of using FACS. In the end, it was decided that this method would not be efficient and reliable enough for the harvest of morphologically homogeneous filamentous cultures for infection experiments, since there may be changes in the physiology of the filamentous cells not only due to the considerable time lag but also possible stress from exposure to laser and electric charges during FACS analysis and sorting. Separation of the *L. pneumophila* morphologies by FACS was therefore not further pursued.

Figure 3.10 Representative fluorescence microscope images and the respective contour-dot plot profiles of predominantly rod-shaped (A) and filament-rich (B) planktonic cultures of the GFP-expressing *L. pneumophila* strain LP3 analysed using FACS. Each dot-plot corresponds to 10,000 events analysed. Arrows 1 and 2 refer to contour peaks mentioned in text (page 82).

A few other methods were also explored, such as the enrichment of the filamentous form by low-speed centrifugation and separation of the cell morphologies by serial dilution of bacterial culture, but without much success. To avoid further stalling in the study because of this technical hurdle, I decided to balance the compromising factor of rod-subpopulation by only selecting for use in the protozoa infection experiments those 42°C stationary-phase broth cultures with an abundance of filaments greater than the mean value of 81.2% (refer page 81). Furthermore, in acknowledgement of the presence of some rods in these filament-rich cultures, the data generated were interpreted with appropriate caution.

3.2.2. Infectivity of the filamentous form to protozoan hosts

The rod forms of *L. pneumophila* have long been known to be infectious to many different types of protozoa, in particular the amoebae, and to some extent, the ciliates (Abu Kwaik et al., 1998; Fields et al., 1984; Hagele et al., 2000; Molmeret et al., 2005; Molmeret et al., 2007). To investigate whether the filamentous form is also infectious to protozoa, filament-rich cultures of the GFP-expressing derivative of *L. pneumophila* Philadelphia-1, i.e. strain LP3, were co-incubated with a few species of protozoan hosts which are commonly used for studying *Legionella*-host interaction: two amoebae, *Acanthamoeba castellanii* and *Acanthamoeba polyphaga*, and one ciliate, *Tetrahymena pyriformis*. As a control, a parallel infection assay was performed using the rod-shaped stationary phase culture of the same strain.

As shown in Figure 3.11, all three species of protozoa infected with the filament-rich cultures appeared to harbour substantial quantities of the green fluorescent LP3 cells intracellularly after three days of co-incubation. Infection of both *A. castellanii* and *A. polyphaga* with the filament-rich cultures ultimately resulted in the amoebic cells being overwhelmed by LP3, and the release of highly motile LP3 rods from the wasted host cells – which are characteristic of *L. pneumophila* intracellular replication progressing to completion in amoebae (Garduño et al., 2008). On the other hand, infection of *T. pyriformis* did not result in the release of motile LP3 rods but the bacterial cells were observed to persist intracellularly instead of being digested. These profiles (Figure 3.11) were similar to what was observed in controls infected with rod-shaped LP3 cultures (data not shown). Since a minor subpopulation of rods were present in the filament-rich culture, the possibility that the protozoa's infections were

a consequence of their interaction with the rods instead of the filaments could not be discounted. To gain further insight, the gross infection dynamics of protozoa infection by the two morphological forms were compared for possible differences.

Figure 3.11 Fluorescent light microscope images depicting three different species of protozoan hosts (*A. castellanii*, *A. polyphaga*, *T. pyriformis*) before and after infection by *L. pneumophila* strain LP3 filament-rich cultures. *A. castellanii* (A, B), *A. polyphaga* (C, D), and *T. pyriformis* (E, F) were co-incubated with *L. pneumophila* LP3 at a MOI of 10 in AC buffer at 37°C for three days and the images captured. Size bar is 10 µm.

3.2.3. Gross infection dynamics of the filamentous form

To observe the infection dynamics of the filamentous form of *L. pneumophila* LP3, the amoeba *A. castellanii* was co-incubated with the filament-rich LP3 culture.

Progression of the infection of amoeba was monitored periodically under the microscope. As in the previous experiment, a control infection of *A. castellanii* with the bacillary culture was carried out in parallel to facilitate comparison.

The general progression of amoeba infection by the filament-rich culture was similar to that of the bacillary culture control (Figure 3.13). Amoebae infected with filament-rich *L. pneumophila* culture showed evident signs of infection (bright fluorescent green vacuoles along the perimeter of the amoeba that expanded into the cytoplasm at the later stages) after approximately 18 h into infection (Figure 3.13, J), which was about the same duration observed for the infection using the rod-shaped control (Figure 3.13, D). By 47 h, most of the amoebae under both infection conditions were very heavily infected (Figure 3.13, F and L).

As a gross measure of infection dynamics, the time taken to reach the phase of release of highly motile *L. pneumophila* cells from wasted amoeba was tracked and compared between amoeba infection by the filamentous and the rod-shaped forms. As mentioned briefly in the earlier section (Section 3.2.2, page 84), the appearance of actively motile *L. pneumophila* cells being released into the extracellular environment from the infected host is characteristic of the bacteria replicating intracellularly and reaching the completion of one infection cycle. Sampling from a sum of 18 independent infection samples, it was observed that the time taken for the exit of

highly motile *L. pneumophila* from amoeba infected with the filamentous form was not significantly different from that of the rod-shaped control, both clocking a range of 30-48 h (Figure 3.12). However, microscopic monitoring of the progression of infection revealed a distinct feature observed only in the co-incubation of amoeba with the filament-rich *L. pneumophila* culture, which will be described in the next section.

Figure 3.12 Comparison of the time taken for completion of one infection cycle in *A. castellanii* samples infected with *L. pneumophila* strain LP3 of the filament and the rod morphology. Each point (X) represents time score obtained from one infection sample. Presented are data from 11 filament infection samples and 7 rod infection samples.

Figure 3.13 Comparison of the infection of *A. castellanii* by *L. pneumophila* strain LP3 bacillary (A-F) and filament-rich (E-L) cultures in AC buffer at 37°C, observed at five time points (0 h, 1 h, 8 h, 18 h, 26 h, 47 h) under fluorescence microscope. Point of infection is denoted t = 0. White arrows indicate spheres. Size bar is 20 μm. Images were enhanced for contrast using Photoshop CS5 and Microsoft Powerpoint.

3.2.4. Emergence of a novel spherical form

During the progression of infection of *A. castellanii* (Figure 3.13) and *A. polyphaga* (data not shown) by the filament-rich *L. pneumophila* culture, numerous spherical entities (which are green fluorescent) appeared as early as 1 h post-infection (Figure 3.13 H, white arrows) and were seen to remain present for over 24 h post-infection (Figure 3.13, K), although their quantity varied over time. A magnified view of these spheres (as the spherical entities will henceforth be referred to) indicates that they are unlikely to be optical distortions of the rod morphology, especially since the diameter of the spheres appeared significantly larger than the width of the rods (Figure 3.14). This spherical form was completely absent in controls of amoeba infection with the homogeneously rod-shaped *L. pneumophila* cultures (Figure 3.13, A-F), suggesting that the filamentous form may be a pre-requisite for the emergence of this spherical form.

The appearance of spheres in the co-incubation of amoeba with the filament-rich *L. pneumophila* culture was reproducibly observed in at least ten subsequent infections. The spheres were noted to be originally absent from the filament-rich *L. pneumophila* culture itself, thus the spherical form had not been passively introduced into the infection assays but apparently emerged as a consequence of the co-incubation of amoeba with the filament-rich *L. pneumophila* culture.

Figure 3.14 Fluorescent light microscope image showing a magnified view of *L. pneumophila* LP3 spheres. Spheres were observed at (A) 19 h post-inoculation of *L. pneumophila* LP3 filaments to amoeba *A. castellanii* in AC buffer at 37°C, and (B) 3 h post-inoculation of LP3 filaments to ciliates in AC buffer at 30°C. R: LP3 rod, S: LP3 sphere, Ac: uninfected amoeba, Tp: uninfected ciliate. Size bar is 10 µm. Images were enhanced for contrast using Photoshop.

3.2.4.1. Spheres are not artefacts of GFP expression

The amoeba infection assays in which the spheres were observed to occur were performed using our GFP-tagged *L. pneumophila* strain LP3. Expression of GFP is enabled by the constitutively expressed *gfp-mut3** gene carried on the plasmid pPZ1 (Piao et al., 2006) which was transformed into *L. pneumophila* Philadelphia-1. The GFP expression from pPZ1 had previously been verified to have no observable impact on biofilm development with respect to the parental strain (Piao et al., 2006). In relevance to our focus, I decided to also check that the spheres observed were not artefacts due to some unexpected physiological abnormality arising from GFP expression.

To validate that the expression of GFP was not the cause of the occurrence of the spherical form, i.e. the spheres were not a consequence of physiological aberration accumulated due to GFP expression, the amoeba infection with the filament-rich culture was repeated using the wild type parent strain *L. pneumophila* Philadelphia-1. Bacterial cells were harvested and visualized under the microscope with the aid of the SYTO 9–propidium iodide double stain (see also Section 4.1.2). As shown in Figure 3.15, spherical forms of similar sizes to the spheres of the GFP-tagged strain LP3 (Figure 3.14) were observed, confirming that the spherical form also occurs in the interaction of the amoeba with the wild type *L. pneumophila* Philadelphia-1 and that the expression of GFP in strain LP3 was not the cause of the emergence of the spheres.

Figure 3.15 Spherical form (white arrows) was also observed in amoeba infection assay performed with wild type *L. pneumophila* Philadelphia-1. Cells were double-stained with SYTO 9 and PI fluorescence dyes. Size bar is 5 μ m.

3.2.4.2. The spherical form is not specific only to strain Philadelphia-1

Infection of *A. castellanii* using the filament-rich cultures of the *L. pneumophila* strain JR32, a commonly studied strain, and its GFP-expressing derivative were also performed. Both strains resulted in the generation of these spheres in association with

the amoeba (data not shown), similarly to strains Philadelphia-1 and LP3. Hence the emergence of the spherical form appeared not to be a phenomenon specific only to the strain Philadelphia-1.

3.2.5. Overview and Discussions

In the environment, *L. pneumophila*'s interaction with protozoa is central to the bacterium's life cycle because the intracellular milieu of protozoan hosts provides shelter and nutrition that enables the continuous propagation of the bacterium. Given that the filamentous form is one of the morphologies *L. pneumophila* adopt in the environment, information on its interaction with the protozoan hosts would facilitate better understanding of the ecological relevance of the filamentous form.

3.2.5.1. Protozoa and filamentous *Legionella*: An unexplored relationship

Unlike the various rod morphological forms, the relationship between the filamentous form of *L. pneumophila* and protozoa is, to date, still completely unknown. While the capability of the rod-shaped forms of *L. pneumophila*, such as the MIF, the RF, etc, to infect protozoan hosts is well characterized, this potential has not been correspondingly investigated for the filamentous form.

The apparent lack of attention on the interaction between *L. pneumophila* filaments and the bacterium's natural protozoan hosts may have its cause in part to the seemingly sporadic nature of the appearance of the filamentous form and the subsequent difficulty in obtaining homogeneously filamentous cultures for

experimentation. The first half of this chapter (Section 3.1) has attempted to provide a more systematic view of the conditions that can induce or promote filamentation. However, it was noted during these experiments that when filamentation occurred, the percentage of abundance of the filamentous form tended to show a wide spread, rather than a narrow spectrum, under any one condition that was tested. For example, under static incubation in microplates, samples in the complete BYE medium showed filamentation abundance covering a range from 60 to 95%. Likewise, under agitated cultivation, the 42°C stationary phase culture showed 76-91% abundance of the filamentous form. The latter cultivation condition had been chosen to provide filamentous samples for the infection experiments due to practicality and reliability, and most importantly, because it is a logical counterpart to the stationary phase bacillary form often used and reported in amoeba infection studies (Bozue and Johnson, 1996; Greub and Raoult, 2003; Molmeret et al., 2004). Inevitably, a minor but significant proportion of the rod-shaped cells would be present in these *L. pneumophila* planktonic cultures cultivated for filaments. This was expected to complicate data interpretation when investigating the interaction of protozoa with the filamentous form, since the impact of the rod minority within the system could not be discounted. Unfortunately, attempts to efficiently harvest for purely filamentous culture by physical separation using FACS, serial dilution and enrichment via low-speed centrifugation did not meet with much success. Cell separation methods using density gradient centrifugation, such as high-speed centrifugation in Percoll performed for purification of intracellular *L. pneumophila* from host cells (Faulkner and Garduño, 2002), has not been tested and the success of these methods for isolating the filamentous form remains to be validated. However, in view of my main intention of studying the infective potential of the filamentous form, this technique

has been eliminated from consideration in the initial exploration as the manipulation may affect the physiology of the filamentous cells and hence the interaction with the protozoan hosts. The lack of reports by other groups on successful attempts to separate *L. pneumophila* filaments from the rod-shaped cells could be due to a genuine lack of interest, or it may be that others have faced the same obstacle, hampering studies on the association of the filamentous form with protozoan hosts. In pursuing my investigation, I adopted a criterion in which 42°C stationary phase cultures comprising of at least 81% filaments (i.e. at mean abundance level or above) were used to assess the interaction of protozoa with the filamentous form of *L. pneumophila*. When the initial series of data (Sections 3.2.3 – 3.2.4) revealed differences that were indisputably unique to the presence of filaments, it was decided that my subsequent studies (Chapter 4) would follow up on that specific line of investigation, since the exclusivity of the phenomenon due to the presence of the filaments could minimize the ambiguity caused by the existence of the minor subpopulation of bacillary cells.

Apart from the experimental difficulties in obtaining samples, a lack of interest in the filamentous morphology as a differentiated form in the environment may have also been prevalent, and consequently a lack of studies on its relationship with the protozoan hosts. Under most ambient conditions, the bacterium predominantly exists in the bacillary form (Section 3.1.5). It may have led to an apparent scarcity of the filamentous form in environmental samples and the perception that the likelihood of its interaction with the protozoan hosts is low. This could have sufficiently dampened interests in the host-pathogen relationship of the filamentous form as it may be perceived as less ecologically relevant than the rod-shaped counterparts.

However, my work in the first half of the chapter (Section 3.1) has highlighted that, given the convergence of specific conditions, niches in the environment that favour the existence of *L. pneumophila* in the filamentous form may be more easily formed than previously assumed. These niches should not be neglected, since there may be greater incidences of interaction of the filamentous form with the resident protozoan community. For example, it has been reported that *L. pneumophila* survives in hot spring water at 42°C (Ohno et al., 2003), a temperature which was found to favour the formation of the filamentous morphology in other investigations (Konishi et al., 2006; Piao et al., 2006) and further validated in this study (Section 3.1.5). Aquatic environments with higher than ambient water temperatures, such as hot spring spas, hot water distribution systems and air-conditioning cooling towers, may potentially harbour a sizeable number of *L. pneumophila* in the filamentous form and thereby increase the probability of protozoa-filament encounters, especially since protozoa can colonize hot water systems (Ohata et al., 2006; Rohr et al., 1998). Since man-made aquatic systems with thermally altered waters are ubiquitous in our daily lives and have been commonly implicated in outbreaks of Legionnaires' disease (Fields, 2007), it would be relevant to understand the *Legionella*-protozoa interaction of the filamentous form as much as that of the bacillary form.

3.2.5.2. Interaction between protozoa and the filamentous form involves a novel spherical form

In exploring the interaction between the filamentous form of *L. pneumophila* with protozoa, I started by addressing the question of whether this form has the capacity for infecting protozoa. Many studies have demonstrated the infectivity of *L.*

pneumophila to a wide variety of protozoan and mammalian host cells and have greatly detailed the mechanism of infection to the genetic level (Molmeret et al., 2007; Newton et al., 2010), but these were investigated with respect to the bacillary forms of the bacterium. Although the filamentous form was briefly mentioned to be less virulent than the bacillary forms when infected to chick embryo and guinea pigs (Nowicki et al., 1987; Paszko-Kolva et al., 1992), neither of these were clearly demonstrated. As for the infectivity of the filamentous form to protozoa, this aspect has yet to be reported and was completely unknown at the point when I have started this study.

Qualitative comparison was made between the bacillary and the filament-rich *L. pneumophila* cultures, with respect to the infectivity and the gross infection dynamics based on two types of protozoan hosts – the amoeba and the ciliate (Section 3.2.2). The infection by filament-rich culture was found to show no significant difference from the bacillary counterpart (Figure 3.12). Although the presence of a minor population of rods in the filament-rich culture meant that the contribution of this minority to the infection profile could not be negated, an unusual phenomenon, never observed in the control infection by the purely bacillary culture, was revealed during infection by the filament-rich *L. pneumophila*.

This unique characteristic, observed only in the interaction of the protozoa with the filament-rich culture of *L. pneumophila* and hence clearly “filament-driven”, was the appearance of a novel spherical entity. The spheres appeared as early as 1 h upon the introduction of the filament-rich *L. pneumophila* culture to the amoeba (Figure 3.13) and were distinctively larger than the bacterium’s rod-shaped cells (Figure 3.14).

These were established to be not artefacts caused by the expression of GFP (Section 3.2.4.1), and could be observed in another strain of *L. pneumophila*, JR32, other than strain Philadelphia-1 (Section 3.2.4.2). Infection of the amoeba with the rod morphology was never seen to generate these large spheres in my study, nor has it been reported elsewhere. Therefore it may be that the filament is the pre-requisite for the appearance of the spherical form.

The fact that the spheres were not present within the filament-rich planktonic *L. pneumophila* cultures used for the infection assays, but appeared only upon co-incubation with the amoeba suggests that the manifestation of the spherical form is dependent on the bacterium's association with the amoeba. In the past, changes in bacterial morphology apparently induced by the presence of bacterivorous protists have been reported, and were postulated to be a consequence of the bacteria's exposure to predatory pressure (Hahn et al., 1999; McArthur, 2006; Pernthaler et al., 1997). For instance, in the presence of the flagellate protist *Bodo saltans*, rod-shaped bacteria of the class β -proteobacteria were found to form filaments within 24 h of co-incubation (Pernthaler et al., 1997). An increase in mean bacterial cell width, but not length, was observed in a mixed bacterial community to which a ciliate bacterial predator, *Cyclidium glaucoma*, was added (Posch et al., 2001). However, the shift in morphology as a consequence of interaction with the protozoa described in most of these reports occurred over a longer period of time (in days to weeks) and usually investigated within a continuous culture system. The spherical form of *L. pneumophila* was observed as soon as 1 h post-introduction of the filament-rich culture to the protozoa, unlike the adaptive morphological change at the population level attributed to predatory pressure described in these studies. Moreover, a

morphological shift from the *L. pneumophila* filamentous to spherical form is not intuitively congruent with the notion of increasing bacterial cell shape to escape predation as implied in the other cases; on the contrary, it seems to hint at the possibility of a deliberate sizing down of the cell dimension to facilitate uptake by the protozoan hosts.

Discovery of the spheres led to a multitude of questions on this novel entity.

Therefore, in the following chapter I will report on my exploration of the unique phenomenon and the characteristics of this *L. pneumophila* spherical form.

CHAPTER 4

CHARACTERISTICS OF THE *L. PNEUMOPHILA* NOVEL SPHERICAL FORM

4. Characteristics of the *L. pneumophila* Novel

Spherical Form

Although *L. pneumophila* is known to be capable of adopting variable morphologies (Garduño et al., 2008), the spherical or coccoid form of *L. pneumophila* is scarcely reported. A rare mention of the coccoid morphology was found in a few early reports on the characterization of the numerous morphologies of *L. pneumophila* cells which had been propagated in yolk sac tissues and on bacteriological media (Katz, 1978; Katz et al., 1984), but these coccoid forms of the bacterium has never been reported in any interaction with the bacterium's natural protozoan hosts. In view of the lack of literature on and the novelty of the spherical entity reported in the previous chapter, I began the study of this form by examining its physical characteristics.

4.1 Physical characteristics of the spherical form

4.1.1. Physical dimensions of the spheres

Spheres of both the wild type *L. pneumophila* Philadelphia-1 and the GFP-expressing strain LP3 observed by fluorescence microscopy exhibited diameters within the range of 0.9 – 4.4 μm (mean $\pm$ SD: 2.0 $\pm$ 0.9 ; sample size, n=30). In comparison, the rods (n=33) and filaments (n=45) measured at 0.3 – 0.5 μm (0.4 $\pm$ 0.03) in width, and 1.2 – 5.1 μm (2.4 $\pm$ 0.8) and 8.4 – 61.8 μm (24.5 $\pm$ 11.2) in length, respectively (Table 4.1). The dimension of spheres was later also obtained from transmission electron

microscopy (TEM) analyses (detailed in Section 4.1.4 below) and found to be fairly consistent with that from fluorescence microscopy; diameters measured between 0.8 – 2.1 μm , averaging at 1.2 μm (± 0.3 , $n=36$). This is threefold the average width of rods ($n=30$) and filaments ($n=30$) of 0.4 μm (± 0.01), indicating that the spheres are distinct forms and unlikely to be the latitudinal cross-sections of a rod or filament that had been mistakenly interpreted.

Morphology	Rod	Filament	Sphere
Fluorescence image
Width (μm)	0.3 - 0.5 (0.4 ± 0.03)	0.3 - 0.5 (0.4 ± 0.03)	-
Length (μm)	1.2 - 5.1 (2.4 ± 0.8)	8.4 - 61.8 (24.5 ± 11.2)	-
Diameter (μm)	-	-	0.9 - 4.4 (2.0 ± 0.9)
TEM micrograph
Width (μm)	0.3 - 0.5 (0.4 ± 0.01)	0.3 - 0.5 (0.4 ± 0.01)	-
Diameter (μm)	-	-	0.8 - 2.1 (1.2 ± 0.3)

Table 4.1 Fluorescence/TEM micrographs and physical dimensions of the sphere compared with the rod and filament forms. Mean $\pm$ SD of physical dimensions is indicated in parentheses. Fluorescence image size bar is 10μm. Images of spherical form presented were captured from (C) LP3 axenic culture, and (G) infection of *A. castellanii* with *L. pneumophila* Philadelphia-1.

4.1.2. Cell wall permeability of the spheres to SYTO/PI nucleic acid stains

The green fluorescence of the spheres found in the infection of amoeba by *L. pneumophila* strain LP3 (Figure 3.14) suggests that the spherical entities had originated from the green fluorescent *L. pneumophila*. To assess the status of the spheres in terms of overall physical integrity (if indeed they are cellular in nature), the extracellular medium harvested from the infection of *A. castellanii* by the filament-rich culture of the wild type strain *L. pneumophila* Philadelphia-1, containing a mixture of *L. pneumophila* filaments, rods and spheres, were stained with the Live/Dead[®] BacLight[™] Bacterial Viability Kit (Molecular Probes, Invitrogen) comprising of two nucleic acid-binding fluorescent dyes, SYTO 9 and propidium iodide (PI). The utility of the BacLight kit lies in the differential ability of the two dyes to penetrate bacterial cells: SYTO 9 stains all bacterial cells green whereas PI penetrates only bacteria with damaged membranes, staining them red while partially displacing SYTO 9 and quenching the remaining SYTO 9 emissions via fluorescence resonance energy transfer (MolecularProbes, 2004; Stocks, 2004).

The SYTO 9/PI stained spheres of *L. pneumophila* Philadelphia-1 were found to display a wide variation in staining (Figures 3.15 and 4.1). The spheres were predominantly stained red (Figure 4.1, D), but some were stained green (Figure 4.1, B). There were also some that did not stain completely red or green but exhibited a mixed staining appearance in which the central mass of the spheres was stained orange while the perimeter was green (Figure 4.1, C). Contrastingly, the filamentous cells were mostly stained green, a minor few stained red, and a few showed mixed

staining (Figure 4.1 A, open arrows). Such spectra of staining and intermediate colours exhibited by the spheres and the filaments of *L. pneumophila* from SYTO 9/PI double staining have been reported in other bacteria too and were frequently attributed to variation in the cells' permeability to PI as a result of varying degrees of damage present in the cell membranes and cell walls (Berney et al., 2007; Boulos et al., 1999; Giao et al., 2008). The assortment of staining profiles displayed by *L. pneumophila* spheres strongly suggests that alterations in cell membrane/wall structure have occurred in stages, resulting in a spectrum of cell wall permeability to the PI stain. This could arise either from cell death preceded by varying levels of damages to cell membrane/wall, or from viable cells exhibiting significant transition in the structures of cell membrane/wall.

Figure 4.1 The sphere form of wild type *L. pneumophila* strain Philadelphia-1 (LP1) stained with SYTO9 and PI showed varying staining profiles and cell arrangements. Cells were harvested from samples of *A. castellanii* infection by LP1 filaments at 4-7 h post-infection and stained with SYTO and PI (Live/Dead® BacLight staining kit) for 15-20 mins at RT. Spheres and filaments stained both red (R) and green (G), sometimes showing mixed (M) staining. Block arrows indicate spheres, open arrows indicate filaments. Size bar is 5 μ m.

4.1.3. Spatial arrangement of the spheres

Apart from demonstrating a heterogeneous staining profile, SYTO 9/PI double staining of the wild type *L. pneumophila* Philadelphia-1 spheres also showed that they varied in arrangements relative to each other. The spheres did not always appear as discrete singles; many were found in clusters of two's or three's (Figure 4.1, C and D). Spheres of the GFP-expressing *L. pneumophila* strain LP3 viewed under fluorescence microscope were also found to occur in clusters (Figure 4.2, A), and quite frequently in pairs of a large and a small spheres. Occasionally, grape-like clusters and chains of spheres were observed (Figure 4.2, B). Spheres within such clusters were also noted to vary in diameter (Figure 4.2, C)

Figure 4.2 Fluorescence microscope images of LP3 spheres varying in size and in spatial arrangement. (A) Many spheres appeared in clusters of two's or three's. Pairs consisting of a large and a small sphere (P) were also frequently observed. (B) Spheres in chains (X) and grape-like clusters (Y). (C) Juxtaposition of large and small spheres demonstrating heterogeneity in diameter. Spheres were harvested from (A) co-incubation of LP3 filament-rich culture with *A. castellanii*, and (B, C) LP3 filament-rich axenic cultures. Size bar is 10 μm .

4.1.4. Morphological features revealed by TEM analysis

Next, since it had been possible for the different forms of *L. pneumophila* to be distinguished by ultrastructural features visible under the electron microscope (Faulkner and Garduño, 2002; Garduño et al., 2002b; Greub and Raoult, 2003), the spherical form was accordingly examined using the TEM. Cultures of the wild type *L. pneumophila* Philadelphia-1 containing spheres were processed and analysed by TEM with the help of our collaborator (R. Garduño, Dalhousie University, Canada). As the harvested bacterial culture was a heterogeneous mix of bacillary, filamentous and spherical forms, the cellular dimensions, which were earlier documented via fluorescence microscopy analysis (reported in Section 4.1.1), were used as guidelines to identify the corresponding forms when viewing the TEM fields.

4.1.4.1. Ultrastructural variation among the spheres

As shown in the electron micrographs in Table 4.1 (panels D-G), the sphere could indeed be visualized as a distinct form with a circular shape (panel G) and diameters with two to five fold greater dimensions than the widths of the rods and filaments (panels D and E). Similarly to the rod-shaped and filamentous cells, within the spherical form, the presence of cytoplasmic material could be clearly observed, enclosed by a Gram negative envelope – defined by distinct inner and outer cell membranes separated by a conspicuous, translucent periplasmic space. Amongst the spherical cells, several variations with respect to the periplasmic layer could be found: (i) a typical compact periplasmic space of thickness approximating 10 nm covering the inner membrane-bound protoplasm (Table 4.1, panel G), (ii) an expanded

periplasmic space with loose peptidoglycan surrounding an inner membrane-bound spherical protoplasm (Figure 4.3 A, B) and more intriguingly, (iii) an expanded periplasmic space with considerably less dense peptidoglycan surrounding an inner membrane-bound but irregularly shaped protoplasm (Figure 4.3 C, D). To my knowledge, the latter two features, both showing curious ultrastructures involving an unusually expanded periplasmic space, have never been reported for other *L. pneumophila* morphological forms and may be distinct features associated with the novel spherical form.

The outer membranes covering the expanded periplasmic space tended to be wrinkled (Figure 4.3 A, C, D), although the overall cell shapes remained spherical. Of special interest is one of the variants that showed a protoplasm resembling a folded bacillary-shaped cell, located within the spacious periplasmic space but nevertheless limited by a spherically-shaped outer membrane (Figure 4.3, D). It is likely that the peptidoglycan layer had remained intact and maintained the spherical cell shape (Garduño R., personal communication), but by differing degrees among the variants, the connections holding the outer membrane in place could have broken away, giving a ‘loose’ profile and an enlarged periplasm. This unusual cell wall structure could be responsible in part for the spectrum of staining suggestive of either cellular damages or transition in cell membrane/wall architecture, observed via SYTO 9/PI double stain earlier (Figures 4.1).

Figure 4.3 TEM micrographs depicting morphological variations of the spherical form exhibiting a loose outer membrane and expanded periplasmic space. The inner membrane-bound cytoplasmic material held in spherical shape surrounded by an enlarged periplasmic space, apparently created by the detachment of outer membrane from the inner membrane (A-C). In a few specimens, spherical cells with expanded periplasmic space contained cytoplasmic material that was not circular but appeared like folded rods (D). Spheres were harvested from (A, B) LP3 filament-rich axenic cultures, and (C, D) co-incubation of filament-rich *L. pneumophila* Philadelphia-1 culture with *A. castellanii*. Block arrows: inner membrane, open arrows: outer membrane. PS: periplasmic space.

4.1.4.2. Morphological variants of *L. pneumophila* exhibiting spherical components

In addition, TEM analysis revealed several morphological variations with rounded components falling within the dimension of our ‘spheres’, that could account for the variety of arrangements of spheres (pairs, clusters etc) observed by fluorescence microscopy earlier (Section 4.1.3). Twin-lobular (Figure 4.4, A) and spherically bulging (Figure 4.4, B) structures could possibly correspond to the paired arrangement (Figures 4.1 C and 4.2 A), while other irregularly-shaped variations (Figure 4.4, C-F) may account for the clusters of spheres (Figure 4.2) observed under the fluorescence microscope.

Figure 4.4 TEM micrographs illustrating morphological variations of the spherical form.

(A) Two spheres that are still fused together by a cytoplasmic bridge. (B) Sphere that appears as a bulge at a rod-like terminal. (C, D) Variants, possibly filamentous cells, exhibiting lobular bulges. (E) Irregularly-shaped form with lobular bulges. (F) A spherical variant with short cytoplasmic projections. Size bar is 500 nm.

4.1.5. Overview and Discussions

4.1.5.1. The spherical entity is a distinct morphological form

Fluorescence microscopic visualization of the spheres in the harvested cultures of the GFP-expressing *L. pneumophila* strain LP3 and the SYTO 9/PI-fluorescent stained *L. pneumophila* strain Philadelphia-1 showed that the spherical form is distinct from the rod and filamentous forms in its shape and larger diameter of 0.8 – 4.4 μm (Section 4.1.1). The sizes of these spheres had at first raised the speculation if they were the respirable vesicles of the amoeba containing clusters of live *L. pneumophila* (Berk et al., 1998) or – as was recently called – “pellets”, similar in nature to those excreted by *Tetrahymena* spp. (Berk et al., 2008). However, this possibility was ruled out fairly early in our investigation as each spherical entity appeared to be a single mass instead of a conglomerate of many bacillary or filamentous cells. TEM analysis was able to verify these microscopic observations at a higher resolution (Section 4.1.4); spherical cells with diameters within the abovementioned range were found to indeed exist as discrete units. They were also found to contain cytoplasmic material, enclosed within a Gram negative cell envelope structure with either the typical compact periplasm (Table 4.1, panel G) or the unusually expanded ones (Figure 4.3). These microscopic findings were able to demonstrate that the spherical form is cellular in nature and therefore, can be regarded as a distinct morphological form of *L. pneumophila*.

The closest relatives to the spheres, among the numerous differentiated forms of *L. pneumophila* which have been reported thus far, are perhaps the coccoid and spore-like structures described by Katz (Katz, 1978). The coccoid structures were cytoplasm-containing round bodies with cell walls exhibiting wavy external contours

but their dimensions did not exceed 0.6 μm (Figure 4.5, B), whereas the spore-like entities were round structures with an electron-dense rim and they could measure up to a maximum size of 4 μm (Figure 4.5, C). Hence, apart from similarity in terms of their spherical shapes, neither of these structures are likely to be the same form as the spheres observed in this study, since the coccoid structures were smaller in size than our spherical form, and the spore-like form's characteristic electron-dense envelope ultrastructure had not been observed among our spheres. Incidentally, these forms described by Katz were found either intracellularly in infected yolk sac tissues or in 5-day-old cultures cultivated in bacteriological (supplemented Mueller-Hinton) medium – conditions that did not involve *Legionella*-amoeba association. All in all, it appears that the spheres observed in this study has never been reported to date, and may represent a stage in the bacterium's life cycle during the filamentous form's association with the amoeba.

Figure 4.5 Comparison of our spherical form (A) with coccoid (B) and spore-like (C) structures reported by Katz. Size bar is 0.5 μm . Images B and C adapted from Katz.(Katz, 1978).

4.1.5.2. Spheres may be a manifestation of cellular damage or transitional structures

Although the ultrastructure and overall morphology of the spherical form do not coincide with any of the other differentiated forms of *L. pneumophila*, it was possible to assign to it some degree of semblance to the aberrant state of the bacterium under the assault of antibiotics, documented in a few of the early studies (Chan et al., 1987; Rodgers et al., 1990). For example, the spherical form which exhibits the typical compact periplasm shares a degree of morphological similarity to some “spheroplasts” observed after the exposure of *L. pneumophila* to methicillin (Chan et al., 1987; Rodgers et al., 1990). However, unlike the spheroplasts which proceeded to “collapse” after 6 h even in osmotically balanced culture broths, leading to a complete loss of cytoplasmic material by 24 h (Chan et al., 1987; Rodgers et al., 1990), the spheres appeared resilient in the medium of *A. castellanii* assay buffer, remaining physically intact and numerous at least up to 19 h (Figure 3.14).

In the case of the more intriguing variant of spherical cells which has a greatly expanded periplasmic space (Figure 4.6, A), this ultrastructure has also never been described in any other *L. pneumophila* differentiated forms. However, treatment with rifampicin (Rodgers et al., 1990) and methicillin (Chan et al., 1987; Rodgers et al., 1990) could result in cell membrane damages that manifest as a separation of the outer membrane from the remaining cell wall, giving an appearance of an expanded periplasm (Figure 4.6, C-D). Association of this unusual cell wall structure with bacterial injury was also indicated in an ultrastructural study of *Ehrlichia chaffeensis*, an obligate intracellular pathogen from the family *Rickettsiaceae*. Upon the infection of *E. chaffeensis* on its host cells, some bacteria with enlarged periplasmic space were

Figure 4.6 Comparison of the spherical variant observed in our study with other bacterial forms reported to exhibit the unusual cell wall ultrastructure. (A) A morphological variant of the sphere with an expanded periplasmic space and loose outer membrane observed in this study. (B) Elementary bodies (EBs) of *Chlamydia* sp. strain TWAR were characterized by pear-shaped cells with large periplasmic space lined by the outer membrane (Chi et al., 1987). Detachment of outer membrane from the remaining cell wall was observed in *L. pneumophila* treated with (C) rifampicin at 20 times the MIC for 24h (Rodgers et al., 1990) and (D) methicillin at the MIC for 5h (Chan et al., 1987). Images were adapted from the respectively cited publications. Size bars are (A- C) 0.5 μm and (D) 0.2 μm .

observed and this was suggested to be an injured form of the bacterium exhibiting structural abnormalities (Popov et al., 1995). The similarities of these cells to our spheres are nevertheless only partial, since for the spherical form of *L. pneumophila*, the degree of expansion of the periplasm was of far greater magnitude, and the peptidoglycan materials, although visibly less dense, had remained substantial (Figure

4.6, A). Most of the spherical cells in our study have rounded protoplasts, and a rare few showed irregularities (Figure 4.4, C-D) – but not of the kind typically associated with dying cells e.g. cytoplasmic leakage or loss. These variants will be re-visited after the next phase of my work has been presented (Section 4.2).

The partial ultrastructural resemblance of the spheres with these various manifestation of cell membrane/wall damages during antibiotic stress could be the basis for the predominantly “red-biased” staining (conventionally assumed to imply cell membrane/wall damage or death) of the sphere population by the SYTO 9/PI dye set (Figure 4.1). However, as highlighted earlier, there were also considerable differences between the spherical form and the antibiotic-damaged cells suggestive of a greater integrity of the structure of the spherical form. Hence, the unusual ultrastructure of the spherical form could also be viewed as a viable cell harbouring features of “structural transition” as part of its characteristic properties. An example of this possibility can be found in *Chlamydia* sp. (Chi et al., 1987), an obligate intracellular pathogen of the family *Chlamydiaceae* and the causative agent of *Chlamydia* infections in humans (Abdelrahman and Belland, 2005). In its developmental cycle, *Chlamydia* alternates between two morphological forms; the smaller extracellular form known as elementary body (EB) are internalized by host cells and differentiates into the larger replicating intracellular form, the reticulate body (RB), which undergoes binary fission followed by secondary differentiation back to EBs (Abdelrahman and Belland, 2005; Kuo et al., 1995). Ultrastructural analysis of the TWAR strains of *Chlamydia* was able to demonstrate that pear-shaped EB has a large periplasmic space limited by the outer membrane (Figure 4.6, B), a structure not present in the replicative RBs but only observed in mature EBs (Chi et al., 1987), the transmissive form of the

bacterium. It has been suggested that the loose outer membrane of these EBs could be more dynamic in membrane fluidity than other chlamydiae and may facilitate the attachment of TWAR to its host cells (Kuo et al., 1988). This illustrates that the expanded periplasmic ultrastructural feature may well have biological functions specific to the bacterium's life style, and should not be perceived merely as an indicator of cellular damage.

At this point in time, whether the expanded periplasm of the spherical form of *L. pneumophila* is fulfilling a biological role, or is simply a manifestation of cellular damage, has not been conclusively established. Nevertheless, it should be remembered that the appearance of the spheres in this study had not been induced by harsh treatments akin to antibiotics exposure, but instead, had occurred upon the interaction of *L. pneumophila* cells with its natural protozoan host – a beneficial interaction for *L. pneumophila* in the environmental phase of its life cycle. Hence it is more likely that transitional structural changes in the bacteria had been induced, rather than simply damages to the bacterial cell envelope. The possibilities would be followed up in the later part of my work.

4.1.5.3. Peculiar variations of the spherical form

The appearance of spheres in chains and clusters were noted when the infection samples were viewed under the fluorescence microscope (Figure 4.2) and subsequent TEM analysis were able to reveal some morphological variants that are likely to correspond to these former observations (Figure 4.4). For instance, there were TEM images of cells with a bulbous terminal or 2-3 spherical lobes (Figure 4.4, A-D),

which may account for the clustered appearance of spheres as seen by fluorescence microscopy (Figure 4.2). These variations in cell arrangement and cell shape are suggestive of morphological transition of *L. pneumophila* from the filamentous form into the spherical form, or alternatively, to other forms (e.g. bacillary form) via spheres as an intermediate.

So far, investigation of the physical characteristics of the spherical form has demonstrated that this form can be defined by its distinct shape and dimension and to some extent, the cell envelope ultrastructure (Section 4.1.4.1). The multi-lobular appearance of some cellular entities revealed by TEM (Section 4.1.4.2), in addition to the observation that spheres appeared only in the infection of amoeba by the filament-rich *L. pneumophila* culture but never by the bacillary control culture (Section 3.2.4), suggests a possible morphological transition from the filamentous to the spherical form that is triggered by the presence of the protozoan hosts. If that is indeed the case, it may explain why some of the spheres have cell wall features which are suggestive of structural transition. To gain further insight on this possibility as well as other characteristics of this novel morphology, the dynamics in the appearance of the spherical form was explored in the subsequent phase of my work.

4.2. Biological characterization of the spherical form

In the earlier amoeba infection experiments (Section 3.2.4), two important observations were noted. First, the appearance of large spheres in amoeba infection assays was reproducibly demonstrated to occur only in association with the filament-

rich *L. pneumophila* cultures, i.e. never with the bacillary control culture – suggesting that the filamentous form may be a pre-requisite for the emergence of the spherical form. Second, since the filament-rich *L. pneumophila* cultures used for the amoeba infection assays did not itself carry the spherical form, the emergence of the spheres was likely to be attributed to the presence of amoeba. This section will focus on the studies conducted following these two leads, in order to better understand the biology of this novel phenomenon.

4.2.1. Incidence of spheres in axenic culture and in co-culture with amoeba

The data from my earlier experiment (Section 3.2.3, Figure 3.13), whereby either the bacillary or the filament-rich cultures of *L. pneumophila* strain LP3 were co-incubated with *A. castellanii*, were quantified for the frequency of incidence (percentage of samples exhibiting the presence of spheres) as well as the abundance of the spheres (percentage of spheres in the *L. pneumophila* population within the fields of view) and tabulated in Table 4.2. As previously described only in qualitative terms, all bacillary cultures were unable to generate spheres when co-incubated with the amoeba for infection (% incidence = 0, Table 4.2), in contrast to the filament-rich cultures (% incidence = 100). To obtain further evidence that the emergence of spheres is biased towards a filament-rich inoculum, I surveyed the general occurrence of spheres in a range of axenic culture conditions which had been used in Chapter 3 to profile for factors which could promote filamentation (Section 3.1, Table 3.6). A significant number of independent *L. pneumophila* strain LP3 cultures (n = 136) were sampled from a collection of conditions which were inoculated with rod-shaped LP3 cells, and

found to either remain bacillary (n = 66) or become filamentous (n = 70). Samples were screened for spheres via fluorescence microscopy and the frequency of incidence as well as the abundance of the spheres were quantified (Table 4.2).

Culture conditions	Appearance of spheres			
	Incidence			Abundance
	n	S	%	% Spheres / f.o.v. ^e
Co-culture with <i>A. castellanii</i>^{a, f}				
Bacillary culture	7	0	0.0	0.0 ± 0.0
Filament-rich culture	11	11	100.0	36.5 ± 13.4
Axenic culture^{b, g}				
Conditions that generate bacillary population ^c	66	3	4.5	1.4 ± 0.8
Conditions that generate filament-rich population ^d	70	20	28.6	2.1 ± 1.6

Table 4.2 Statistics of appearance of spheres of *L. pneumophila* strain LP3 in co-culture with *A. castellanii* and in axenic culture conditions. n: number of independent cultures sampled, S: number of sampled cultures scoring positive for the appearance of spheres, f.o.v.: field of view. ^a *A. castellanii* was co-incubated with LP3 bacillary or filament-rich cultures in AC buffer at 37°C and scored for spheres at 1 h post-infection. ^b LP3 was cultivated in BYE growth medium. ^c Bacterial culture is homogeneously bacillary. ^d Bacterial culture is a predominantly filamentous with a minor population of bacillary cells. ^e Values are mean ± standard deviation of the percentage of spheres over total number of cells per field of view, sampled over 5-10 fields. ^f Abundance of spheres is significantly more in co-culture with filament-rich LP3 cultures (P -value = 3.86E-06). ^g No significant difference in abundance of spheres (P -value = 0.28) is observed between bacillary and filament-rich axenic cultures.

Amongst the 136 independent cultures sampled, only 23 cultures (Table 4.2, under Incidence column ‘S’), or 17%, were found to exhibit the occurrence of spheres. These incidences seemed skewed towards the category of cultivation condition that favours the filamentation of *L. pneumophila* – within the 17% of samples documented

positive for the appearance of spheres, 20 out of 23 samples, or 87%, were from cultivation conditions that caused filamentation (Table 4.2). The propensity of spheres to occur under axenic conditions correlated with the filamentation of *L. pneumophila* reinforces the notion that the presence of filaments may be a prerequisite for the appearance of the spherical form.

However, under these axenic conditions where the spherical form's presence was noted, the abundance of spheres had been consistently very low (Figure 4.7), with an average of 2.1% or less over the total number of cells viewed (Table 4.2, under Abundance column '% Spheres/f.o.v.'). This is in contrast to the significant abundance observed when the filament-rich cultures were co-incubated with amoeba (Table 4.2). Under such amoeba-associated conditions, it was found that the abundance of spheres averaged at 36.5% – a level that was never encountered under the axenic conditions. This trend was verified with a larger sample size of 23 (Table 4.3, row 'With amoeba'), which showed a 100% incidence and an average abundance of 36%. When filament-rich cultures were incubated in the infection buffer without amoeba, a high incidence (25 out of 26 samples) but very low, if not zero, abundance of the spherical form (Table 4.3, row 'Without amoeba') were noted, concurring with the observations made for the axenic cultures (Table 4.2).

Collectively, these data not only provided strong evidence that the presence of filaments is a prerequisite for the generation of spheres, but also demonstrated that the abundance of the spheres is augmented by the presence of the amoeba.

Incubation condition of filaments	Appearance of spheres			
	Incidence			Abundance
	n	S	%	% Spheres / f.o.v. ^a
Without amoeba	26	25	96	0.0 ± 0.0
With amoeba	23	23	100	36.0 ± 11.5

Table 4.3 Statistics of appearance of spheres in the presence or absence of amoeba *A. castellanii*. Filament-rich *L. pneumophila* strain LP3 culture were inoculated in AC buffer at 37°C and scored for the appearance of spheres at 1 h post-inoculation. n: sampling number, S: number of samples scoring positive for the appearance of spheres, f.o.v.: field of view. ^a Values are mean ± standard deviation of the percentage of spheres over total number of cells per field of view, sampled over 10-30 fields.

Figure 4.7 Fluorescence microscopy images illustrating the low abundance of spheres in axenic culture conditions that generate filaments. (A) *L. pneumophila* strain LP3 in BYE planktonic culture at 42°C cultivated statically for four days. (B) CLSM micrograph of LP3 biofilm cultivated in BYE medium for three days at 37°C, red boxes highlight the sphere viewed from x-z (horizontal) and y-z (vertical) planes respectively. White arrows indicate spheres. Size bar is 10 µm.

4.2.2. Secretion by protozoan hosts can trigger the appearance of spheres

The fact that *L. pneumophila* spheres had emerged in significantly greater abundance upon interaction with the amoeba raised the question of whether it was the physical presence of the amoeba, or the changes to the assay buffer mediated by the amoeba, e.g. through the secretion of soluble factors, that augmented the occurrence of the spherical form.

To evaluate the latter possibility, amoeba-conditioned assay buffers were prepared by incubating the amoeba *A. castellanii* in infection buffer and subsequently removing the amoeba cells. A second set of infection buffers in which *A. castellanii* had been co-incubated with *L. pneumophila* strain LP3 filaments were similarly harvested in order to include the possible influence of the filamentous cells, e.g. inducing the amoeba to secrete soluble factors or by other additional modifications to the assay buffer. These two types of cell-free pre-conditioned buffers were then inoculated with filament-rich cultures of *L. pneumophila* strain LP3 and monitored for the appearance of spheres.

In both types of pre-conditioned assays buffers, the spherical form was found to occur in significant abundance, similar to that of the control condition in which *A. castellanii* was physically present (Table 4.4, Figure 4.8). At 1 h post-inoculation of the filament-rich cultures in these pre-conditioned buffers, the spherical form was found to constitute 26-27% of total bacterial cells (observed in the fields of view), which is comparable to the 36% obtained in the physical presence of amoeba. This was in contrast to the complete absence of spheres in the negative control, i.e. the

unconditioned buffer (Table 4.4). The comparable abundance of spheres in the pre-conditioned buffers with that observed in the physical presence of amoeba indicates that alterations to the assay buffer by the amoeba, probably via secretion of soluble factor(s), is sufficient to result in the augmented occurrence of *L. pneumophila* spheres.

Interestingly, pre-conditioned buffers prepared by co-incubation of *A. castellanii* with or without *L. pneumophila* filaments were both equally capable of triggering the appearance of spheres and at similar abundance (Table 4.4, Figure 4.8, red and pink bars), suggesting that the prior presence of *L. pneumophila* was not essential to influence *A. castellanii* in the enhancement of the sphere subpopulation.

Incubation condition of filaments	Abundance of spheres at 1 h (% Spheres / f.o.v.)
Co-cultured with amoeba	36.0 ± 11.5
Buffer pre-conditioned with amoeba	26.0 ± 1.4
Buffer pre-conditioned with amoeba & filaments	27.7 ± 8.6
Unconditioned buffer	0.0 ± 0.0

Table 4.4 Abundance of spheres at 1 h post-inoculation of filament-rich *L. pneumophila* strain LP3 cultures co-cultured with *A. castellanii* or inoculated in buffers pre-conditioned with the amoeba. Filament-rich LP3 cultures were inoculated in the respective conditions at 37°C and monitored for spheres via fluorescence microscopy. Abundance of spheres is scored as a percentage of total bacterial cell number viewed per field of view and expressed as mean ± standard deviation of at least 6 independent fields. f.o.v: field of view.

Figure 4.8 Appearance of *L. pneumophila* strain LP3 spheres in assay buffers pre-conditioned by three different protozoan hosts with/without filaments of *L. pneumophila* strain LP3. The pre-conditioned buffers were inoculated with filament-rich cultures of LP3 at 37°C and monitored for the abundance of spheres up to 72 h. For reference, the abundance of spheres in the positive control (Ac (physically present): buffer with the physical presence of *A. castellanii*) and negative control (Unconditioned buffer) were included. Ac: *A. castellanii*, Ap: *A. polyphaga*, Tp: *T. pyriformis*. Abundance of spheres is scored as the percentage over total bacterial cells viewed per field of view, and expressed as mean $\pm$ standard deviation of at least 6 independent fields from 2-3 replicate wells.

When the abundance of the spherical forms in the pre-conditioned buffers was monitored over a period of 72 h, several interesting patterns could be noted (Figure 4.8). First, after the initial surge at 1 h, the abundance of spheres decreased over time across all assays performed – those in the two types of pre-conditioned buffers (Figure 4.8, red and pink bars) or in the control infection with the physical presence of *A. castellanii* (Figure 4.8, blue bars). The rate at which the spherical population decreased, however, was more rapid in the physical presence of amoeba, whereby the proportion of spheres decreased by eightfold within 24 h after the initial scoring at 1 h

post-inoculation and was no longer detectable by 72 h (blue bars). In assays performed with the pre-conditioned buffers, the population of spheres was maintained at between 26-35% of the total number of bacterial cells observed per field, from the initial scoring at 1 h up to 24 h post-inoculation, and an eight- to tenfold reduction to 2-3% was observed only at 72 h post-inoculation of the *L. pneumophila* filament-rich culture (red and pink bars). The faster disappearance of the spheres may conceivably be due to their uptake by the physically present amoeba, although instances of uptake of spheres by the amoeba has yet to be directly visualized.

Second, a corresponding assessment using assay buffers pre-conditioned with another species of amoeba, *A. polyphaga*, and the ciliate, *T. pyriformis* has also revealed that they had the ability to trigger the appearance of the spherical form (Figure 4.8, green, light green and maroon bars) in appreciably greater abundance than the control of the unconditioned buffer (purple bars). The extent of the abundance of spheres observed was varied among the three different species of protozoa, with that of *T. pyriformis* being the highest.

It should be mentioned that monitoring for the appearance of spheres had been particularly challenging for samples with the physical presence of *T. pyriformis*. Scoring of spheres was unreliable due to the rapid ingestion of the bacteria by this suspension feeding ciliate, unlike the surface grazing amoebae, *A. castellanii* and *A. polyphaga*, which demonstrated a slower feeding. However, the appearance of spheres at significant abundance (59%) within 1 h after the inoculation of *L. pneumophila* filaments in *T. pyriformis*-conditioned buffer (Figure 4.8, maroon bars) showed that the ciliate is equally capable of inducing the emergence of spheres. Thus,

the ability to trigger an augmented occurrence of the spherical form by the conditioning of external medium appears to be not only specific to *Acanthamoeba spp.*, but also common to the ciliate *Tetrahymena*.

Third, the abundance of spheres over time in *A. polyphaga*- and *T. pyriformis*-conditioned buffers displayed a decreasing trend similar to that observed in *A. castellanii*-based assessments (Figure 4.8). It is interesting to note that the magnitude of decrease in *T. pyriformis*-conditioned buffer (refer maroon bars) more closely resembled the pattern observed in the control infection mixture where *A. castellanii* was physically present (refer blue bars) ; the population of spheres reduced by over fivefold within 24 h after the initial scoring at 1 h and was nearly nonexistent by 72 h. Since this large decline in the proportion of spheres within the first 24 h occurred in the absence of the actively feeding ciliate hosts, the rapidity of disappearance of the spheres in this instance could not be explained as due to ingestion by the protozoan host, but may reflect the quantitative difference in the two protozoa's influence on the filamentous and spherical forms of *L. pneumophila*.

Collectively, the rapid but transient appearance of spheres induced by protozoa directly or through its conditioning of external medium is suggestive of a role for the spherical form in the interaction between the filamentous form of *L. pneumophila* and its protozoan hosts, for instance in the infection of the host by the bacterium.

4.2.3. Abundance of spheres is inversely correlated with the emergence of infected amoeba

To survey the possible associations between the transient appearance of the spheres and the infection process of protozoa, the abundance of spheres during co-incubation with *A. castellanii* (Figure 4.9, filled circles) was compared against the population of infected *A. castellanii* (Figure 4.9, vacant circles) at the corresponding time intervals throughout the course of infection. Infected amoebae were identified by the presence of fluorescent green vacuoles, a sign of replicating *L. pneumophila* strain LP3 within the amoeba, and were quantified in terms of the percentage over total amoeba cells viewed per field of view.

During the first eight hours of infection whereby the population of spheres nearly halved from its initial abundance of 36% (at 1 h) to 19%, the population of infected amoeba had remained low at under 2% (Figure 4.9). Subsequently, as the abundance of spheres continue to further decrease to less than 5% at 26 h and became undetectable by 48 h, the population of infected amoeba rose steadily – accounting for nearly 20% of the total amoebic population at 26 h and 74% by 48 h. This concomitant increase of infected amoeba cells as the population of spheres gradually decreased was similarly observed in the infection with *A. polyphaga* (data not shown). In the case of *T. pyriformis*, due to its rapid speed of ingestion of *L. pneumophila* cells, it was challenging to track the population of spherical cells, thus the correlation between the abundance of spheres with infected *T. pyriformis* was not tested.

Figure 4.9 Correlation between the abundance of *L. pneumophila* strain LP3 spheres and the emergence of infected *A. castellanii* monitored over 48 h. Abundance of spheres (filled circles) and infected amoeba (vacant circles) are expressed as percentage over total bacterial or amoeba cells, respectively, per field of view under fluorescence microscope. Each data point represents mean $\pm$ standard deviation of at least 26 fields quantified across three replicate infections samples. Note that at time 0 h, the abundance of spheres and infected amoebae are both valued at zero (data points not shown).

The steady increase of infected subpopulation of amoeba during the transient existence of the spheres suggests a potential role for the spherical form in the infection process of amoeba. The disappearing population of spheres and the concomitant rise in infected amoeba could be attributed to the internalization of the spheres by amoeba as proposed earlier (page 123-124), although this direct ingestion process has not yet been captured visually under microscope or by TEM analysis and remains speculative. However, it was noticed in the course of the above experiment that during the transient appearance of the spheres, the abundance of the filaments

visibly decreased while that of the rods, which had hitherto remained low, significantly increased. Interestingly, this was also observed in assays performed with the protozoa-conditioned buffers (Section 4.2.2). The dynamic relationship between the three forms hints at the possibility that a morphological transition, from the filamentous to the bacillary form via an intermediate in the spherical form, may be triggered under the influence of the protozoa. To explore this possibility, I followed up by tracking the dynamics of all three morphological forms in the protozoa-conditioned buffer.

4.2.4. Dynamics of the relative abundance of spheres, filaments and rods

While the dynamics of the three morphological forms (spherical, bacillary and filamentous) of *L. pneumophila* described above were noted in both conditions – either when co-incubated with the amoeba, or in the amoeba-conditioned buffer – it was difficult to assess the possibility of a morphological transition in the physical presence of the amoeba. This is because the changes in the relative abundance of each morphological form are also additionally affected by (i) the amoebic feeding, (ii) the possibility of preferential ingestion of one morphological form over others, as well as (iii) the massive amounts of intracellular bacillary cells that are released upon lysis of the infected amoebae, which would be indistinguishable from the bacillary cells that could arise from the hypothesized morphological transition. Therefore, in order to track for changes in the distribution of bacterial forms that may reflect the occurrence of a morphological transition, amoeba-conditioned buffers inoculated with the filament-rich cultures of *L. pneumophila* were used, since this condition is equally

capable of generating the spherical form but would circumvent the above-mentioned complications arising from the presence of amoeba.

Figure 4.10 shows the distribution of the abundance of all three morphological forms monitored over a period of 120 h upon inoculation of the filament-rich culture of *L. pneumophila* strain LP3 in the assay buffer conditioned with the amoeba *A. castellanii*. This was compared with the control of the same bacterial culture in the unconditioned assay buffer. The bacterial culture started out as being predominantly filamentous, comprising of 79% filaments, 21% rods and a complete absence of spheres (Figure 4.10, 0 h). In the amoeba-conditioned buffer, as early as 1 h into incubation, a sharp rise in the subpopulation of the spheres from 0% to 26% and a concomitant decrease in that of filaments from 79% to 54% was observed while that of rods remained constant. Over the next 23 h, the proportion of spheres stayed within the range of 26-31% whilst the subpopulation of filaments steadily decreased and that of rods gradually increased. By 24 h, more than half (52%) of the bacterial population was comprised of rods, whereas the initially predominant filamentous form was reduced to 22%. In the subsequent duration of observation, the rods continued to rise, becoming the major morphological form that accounted for 95% of the population at 120 h, while the proportion of filaments further shrunk to 3% and the spheres decreased to a mere 2%. This profile is suggestive of a morphological transition involving two phases: filament-to-sphere, followed by sphere-to-rod, i.e. the sphere is an intermediate form in the filament-to-rod transition.

A concern had been deliberated over, prior to this experiment, about the possible participation by the minor but significant subpopulation of rods in the filament-rich

culture. Therefore, several controls were set up, whereby pure bacillary cultures were inoculated in (i) *A. castellanii*-conditioned buffer, (ii) unconditioned buffer and (iii) a buffer conditioned with *A. castellanii* and filament-rich *L. pneumophila*, and incubated for over 120 h. The rod-shaped cells were unable to replicate in all three types of buffers (data not shown), establishing that any increase in the subpopulation of rods are unlikely to be due to the proliferation of the existing rods, supported by the assay buffer and/or any amoeba-secreted or filament-secreted factor(s). Hence the eventual dominance of the rod subpopulation in the amoeba-conditioned buffer (Figure 4.10) has its origin in the filamentous and spherical forms, and the most probable scenario would be the filament-to-rod transition via the sphere intermediate.

Contrastingly, the control of unconditioned buffer carrying the same inoculum showed a distribution of morphological forms that remained relatively unchanged up to 71 h post-inoculation (Figure 4.10, Unconditioned buffer); the filaments were consistently dominant at 75-85% of the bacterial population whereas the rod subpopulation fluctuated within a narrow range between 14-21%. The spheres only made a low level negligible appearance from 6 h onwards, which increased slightly over time but accounted for no more than 3.5% of the population. A significant change in the relative distribution was eventually observed at 120 h – the proportion of rods had almost increased to 47%, almost equaling that of the filaments (48%), while the percentage of spheres had risen to 5%.

From the above, we can see that without the conditioning influence of the amoeba, the increase in the rod subpopulation from 21% did not occur even as late as 71 h, and only reached a relative abundance of 47% at 120 h. In contrast, the *L. pneumophila*

culture in amoeba-conditioned buffer had managed to enhance its rod subpopulation to a similar level of 52% at a much earlier time point of 24 h. To verify that this was indeed reflective of an actual increase in the number of rods, the absolute counts of the rods were checked for the respective samples (24 h for amoeba-conditioned, 120 h for unconditioned), and found to be comparable, both exhibiting approximately 6-7 fold increase from the initial count at 0 h. Therefore, filament-to-rod transition indeed occurred significantly more rapidly when the intermediate of sphere was involved (under amoebic influence) than when filament-to-rod transition was direct (no amoebic influence).

Figure 4.10 Distribution of *L. pneumophila* cell morphologies over 120 h with/without the influence of *A. castellanii*. Abundance of each morphological form, expressed in percentage over total bacterial cells counted per field of view, is a mean $\pm$ standard deviation of at least six fields obtained from two to three replicate samples.

When the assay was repeated in the buffer which had been conditioned with the ciliate *T. pyriformis*, the dynamics in the distribution of the three forms was observed to be similar to that in the *A. castellanii*-conditioned buffer with some quantitative variation (Figure 4.11, Tp-conditioned buffer). The filament-rich starting bacterial culture (83% filaments, 17% rods, 0% spheres) initially saw an upsurge of spheres (59%) and a drastic drop in the filament subpopulation (25%) while a constant proportion of rods was maintained (16%). By 24 h, the rods had clearly become the predominant morphology (73%), as the proportion of spheres plunged to 11% and the filaments further decreased to 5.6%. The rods remained as the majority at 72 h while the filaments and spheres subpopulation continue to reduce in abundance (4% filaments, 95.6% rods, 0.4% spheres).

Hence the temporal trend in the distribution of the morphological forms of *L. pneumophila* is not specific only to the influence by the amoeba *A. castellanii*, but can also occur in association with its ciliate host, *T. pyriformis*.

Figure 4.11 Distribution of *L. pneumophila* strain LP3 morphological forms over 72 h under the influence of amoeba *A. castellanii* and ciliate *T. pyriformis* compared to control without protozoa influence. Abundance of each morphological form, expressed in percentage over total bacterial cells counted per field of view, is a mean $\pm$ standard deviation of at least six fields obtained from two to three replicate samples. The set of data for *A. castellanii*-conditioned buffer shown here was adapted from Figure 4.10.

4.2.5. Generation of spheres does not involve de novo protein synthesis

An intriguing feature that I have noted about the emergence of the spheres was the rapid manner in which the large quantity of spheres appeared within the initial 1 h, and maintained over at least 6 hours of exposure to protozoa or protozoa-conditioned medium. To obtain an indication of whether this rapid morphological change involve protein synthesis, chloramphenicol (Cm), an inhibitor of prokaryotic protein synthesis, was added at a minimal inhibitory concentration (MIC) of 100 $\mu\text{g/ml}$ to the filament-rich culture of *L. pneumophila* strain LP3 inoculated in *A. castellanii*-conditioned

buffers. The appearance of spheres up to 24 h post-inoculation was compared against a control inoculation untreated with chloramphenicol.

As shown in Figure 4.12, the appearance and abundance of spheres was unaffected by the addition of Cm; the initial abundance of the sphere subpopulation at 34% was comparable to that in the untreated control condition, at 35% of total bacterial cells viewed per field (Figure 4.12, bars at 2 h). The abundance of spheres in the Cm-treated condition was very similar to that of the control up to 24 h of observation. The presence of chloramphenicol therefore had no apparent influence on the occurrence of spheres. This suggests that the generation of the spherical form is independent of *de novo* protein synthesis.

Figure 4.12 Presence of chloramphenicol (Cm), an inhibitor of protein synthesis, had no effect on the appearance of *L. pneumophila* strain LP3 spheres in *A. castellanii*-conditioned assay buffer. Cm was added at 100 µg/ml. The abundance of spheres is expressed as the percentage over total bacterial cells counted per field of view and represented as mean $\pm$ standard deviation of at least five fields from triplicate samples.

4.2.6. Protozoan cell density influences the distribution dynamics of spheres

So far, it has been demonstrated that both the amoeba and the ciliate, from the genera of *Acanthamoeba* and *Tetrahymena* respectively, have significant influence on (i) the augmentation of the spherical form's appearance from the filament-rich culture of *L. pneumophila*, and (ii) the dynamics of the three morphological forms with respect to their relative abundance. This led to the question of whether a certain threshold density of protozoa is required to effect the observed changes. To find out, the appearance of spheres was investigated in a series of assay buffers pre-conditioned with varying cell densities of protozoa. Since the initial appearance of spheres more than doubled under the influence of *T. pyriformis* conditioned buffer as compared to that of *A. castellanii* (Figure 4.8 and 4.11, data at 1 h), quantitative difference due to changes in cell density, if any, may be more clearly manifested in the former. Hence the test was performed using *T. pyriformis*-conditioned buffers. The original ciliate concentration at 10^5 cells per ml was subjected to 10-fold serial dilution to generate a series of assay buffers conditioned by lesser densities of *T. pyriformis*, ranging from 10 to 10^5 cells per ml. Filament-rich cultures of *L. pneumophila* strain LP3 were inoculated in these assay buffers and the appearance of spheres was monitored over a period of 72 h, and scored for the percentage of abundance.

Figure 4.13 compares these profiles obtained from the series of assay buffers pre-conditioned with the indicated range of *T. pyriformis* cell densities. The profile previously observed – appearance of a large amount (56-59%) of spheres early in the assay, at 1 h post-inoculation, followed by a significant decrease to 11-18% within the next 24 h and a gradual reduction over the remaining time – was reproduced in the

Figure 4.13 Abundance of spheres in assay buffers pre-conditioned with varying concentrations of *T. pyriformis* monitored over 72 h. Filament-rich *L. pneumophila* strain LP3 cultures were inoculated in assay buffers conditioned with *T. pyriformis* of densities in the range of 10 to 10⁵ cells/ml at 37°C for 72 h. Abundance of spheres is expressed as the percentage over total bacterial cells counted per field of view. Data points represent mean ± standard deviation of at least three fields viewed per time interval.

buffer conditioned with the original density of 10⁵ ciliates per ml, and similarly observed in buffers conditioned with 10⁴ cells per ml of *T. pyriformis* (Figure 4.13, filled circles and squares). However, buffers exposed to ciliates at the concentration of 10³ cells per ml exhibited an almost reversed pattern; the initial abundance of spheres was very low at 1.3%, slowly increased over the first 24h to 20% after which it remained unchanged up to 72 h (Figure 4.13, vacant triangles). In buffers conditioned with lower ciliate densities of 10² cells per ml and below, the appearance of spheres was negligible, with an abundance of lower than 2.2% throughout the entire duration of assay (Figure 4.13, vacant squares and circles), akin to the unconditioned buffer

control (Figure 4.13, cross symbol). This relationship between the occurrence of the spheres and the *T. pyriformis* cell density indicates that a cell density of at least 10^3 ciliates per ml is required to trigger the appearance of the spherical form to significant (defined here as $\geq 20\%$) abundance.

The cell density of *T. pyriformis* also affected the distribution dynamics of the filamentous and bacillary forms in tandem with the observed changes in the abundance of the spherical form (Figure 4.14). It has been demonstrated earlier that the appearance of a large subpopulation of spheres was concomitant with a reduction in the filamentous subpopulation and subsequent predomination of the bacillary form (Figure 4.11). This pattern was consistently observed in assays performed with buffers conditioned with *T. pyriformis* at a cell density of 10^3 cells per ml or higher which had resulted in $\geq 20\%$ abundance of spheres (Figure 4.14). At 10^2 ciliates per ml and below, whereby the abundance of spheres was lower than 2.2%, the filamentous subpopulation remained dominant at more than 60% throughout the duration of 72 h. This suggests that a minimal threshold density of ciliate (10^3 cells per ml) is required to influence the filament-rich *L. pneumophila* culture towards morphological transition.

Figure 4.14 Distribution of *L. pneumophila* strain LP3 morphological forms in assay buffers conditioned with *T. pyriformis* at varying cell densities monitored over 72 h.

Abundance of each morphological form, expressed as percentage of total bacterial cells per field of view, is a mean $\pm$ standard deviation of at least three fields of view. For visual simplicity, 48 h data were not represented in the bar chart because values were similar to 72 h.

4.2.7. Overview and Discussions

My exploration into the biological characteristics of the spherical form of *L. pneumophila*, which was observed to be generated when the filament-rich cultures of the bacterium was co-incubated with amoebic hosts, affirmed the two initial leads: that (i) the filamentous form of *L. pneumophila* is a prerequisite for the appearance of spheres, and (ii) protozoan hosts, such as amoeba, can trigger the generation of spheres. Additionally, the results have revealed that the influence by protozoa to induce the appearance of spheres is possibly mediated through soluble secreted component(s), and that the emergence of spheres is likely to be the prelude to a morphological transition process.

4.2.7.1. Filamentous form is a precursor of the spherical form

Through the survey of a large number of samples of *L. pneumophila* cultures, it was possible to demonstrate the link between the presence of the filamentous form and the emergence of the spherical form, as well as to confirm the effect that amoeba has in bringing about the appearance of large quantities of spheres (Section 4.2.1). When the filament-rich *L. pneumophila* culture was exposed to protozoan influence and the dynamics in the relative abundance of the morphological forms were traced, it was observed that during the initial phase (1-6 h), the emergence of spheres was paralleled by a decline in the subpopulation of filaments, while that of the rods remained constant (Section 4.2.4, Figure 4.11). The most apparent possibility to explain this phenomenon is that some of the filamentous cells had converted to spherical cells, i.e. there was a morphological transition. This brings to mind our earlier discussion based on the data from TEM analysis, that the spherical form exhibited ultrastructural features in the cell envelope which are suggestive of structures in transition (Section 4.1.5.2).

However, due to the presence of the approximately 20% of rods in the starting filament-rich culture, a second possibility that could also account for the above observation exists – that under the influence of the filaments, the bacillary cells had converted to spheres, and concurrently, some filaments had converted to rods (hence the rod subpopulation remain constant by means of this dynamic equilibrium). Indeed, cases of conversion of filamentous to bacillary cells have been suggested previously (Piao et al., 2006; Pine et al., 1979). Nevertheless, we consider this scenario to be less

likely, for three reasons. The first is based on our data from the TEM analysis, which provided a sampling of cells present during the early phase (4-7 h post-infection, refer Section 2.2.5.4 of Materials and Methods) when the spheres were in greatest abundance (1-6 h, refer Figure 4.8). There were irregularly-shaped long cells carrying multiple lobular segments (Figure 4.4), the dimensions of which were consistent with what could be best envisaged as a filament in the midst of converting into a cluster of spheres. The second is that the emergence of spheres was rapid and did not appear to involve *de novo* protein synthesis (Section 4.2.5, Figure 4.12), which is not compatible with the fact that for a bacillary cell to attain the size of a sphere, it will have to increase its cellular material considerably through biosynthesis. The third reason is targeted at the hypothetical “influence” that the filaments may exert on the rods to induce it to convert into spheres. Preliminary data has shown that bacillary cultures exposed to cell-free supernatants from filament-rich cultures were unable to generate any spheres even when amoebic stimulation was provided (data not shown). In view of these arguments, the rod-to-sphere scenario would be considered less likely than the filament-to-sphere scenario.

4.2.7.2. The spherical form may be an intermediate in filament-to-rod morphological transition

Influence by the protozoa (either directly in the physical presence of, or through their conditioned buffer) is required to augment the abundance of the spherical form (Table 4.4). This implies that the sphere may have a role to play in the *Legionella*-protozoa association, and the fact that the transient existence of the spheres is correlated with the increase in the population of infected amoebae (Figure 4.9) suggests a potential role in the infection of protozoan hosts. The filament-to-sphere morphological

transition as discussed in the previous section was at first hypothesized to be for the facilitation of amoebic uptake, i.e. by sizing down the filaments to the more palatable dimension of the spheres (Section 3.2.5.2, page 97-98) – it has often been reported that shorter cells were preferentially phagocytosed over the filamentous ones (Horvath Jr et al., 2011; Justice et al., 2006; Young, 2006).

However, following the dynamics of the relative abundance of the three morphological forms in protozoa-conditioned buffer for a longer period of time (beyond 6 h, to 120 h, Figure 4.10) suggested otherwise. The entire process can be viewed as two phases in continuum. The initial phase, as has already been discussed in the previous section, shows (i) the filamentous form declining in quantity over time, (ii) paralleled by a surge in the spherical form, which is transient. This is followed by the next phase, in which (iii) an increase in the bacillary form finally results in it being as the dominant form as (iv) both the filamentous and spherical forms dwindle. Control experiments were able to establish that the eventual dominance of the rod subpopulation has its origin in the filamentous and spherical forms, and not in the replication of the rod subpopulation existing at the start of the culture (discussed in Section 4.2.4). Under these circumstances, the most probable scenario would be a filament-to-rod transition via an intermediate – the sphere.

Interestingly, in the absence of protozoa influence (unconditioned buffer) and hence absence of a subpopulation of spheres, the originally filament-rich culture was also able to increase its bacillary subpopulation (Figure 4.10). However, the process was vastly slower, requiring 3 times longer time than the culture in protozoa-conditioned buffer to accrue a comparable quantity of rods (discussed in Section 4.2.4, page 131).

This set of data firstly corroborated the claims made by others that filament-to-rod transition can occur (Piao et al., 2006; Pine et al., 1979). Because each filament is multi-nucleoid, it can be expected that one filament will generate numerous rods. Therefore, in terms of environmental survival, filament-to-rod transition may lead to strategic proliferative and disseminative outcomes. With the presence of protozoa, it now appears that there is an added advantage in transiting to the rod morphology – that of ease of uptake by the protozoan host. From my data, it is clear that by triggering the filament-sphere-rod transition route through protozoan influence, the end point of the rod morphology can be reached more rapidly. The survival advantage that is offered is apparent.

A two-stage morphological transition such as the one described thus far would be expected to be mechanistically complex in the sense that orchestration of more players would be required. Yet two facts exist that seem contrary to this expectation of the two-stage transition – it was rapid, and one of the stages (filament-to-sphere) did not require protein synthesis. The secret may well lie in the structural changes that occur during the filament-to-sphere transition. The ultrastructure of the spherical cells showing features bordering on what may be construed as “cellular damages” (Figures 4.1 and 4.3) seems indeed to be signs of morphological transition as discussed (Section 4.1.5.2). In particular, the intriguing variants of the spherical form with irregularly shaped protoplasts (Figure 4.4) may be in the later stage of transition, i.e. in the process of moving on to the bacillary morphology, as one of the examples (Figure 4.3, D) exhibited a rod-like protoplasm.

Recently, it has been reported that the wild-type *Lactococcus lactis*, a Gram-positive ovococcus lactic acid bacterium used for food fermentation, naturally exhibits a morphological transition from coccus to rod then filaments during exponential growth before resolving back to coccus at a later growth stage (Pérez-Núñez et al., 2011). The morphological differentiation was attributed, in part, to a transitory inhibition in the activity of penicillin-binding protein 2x (PBP2x) – a transpeptidase involved in septal peptidoglycan synthesis – during cell division, resulting in a temporary arrest in septation which would resume upon release of inhibition of PBP2x, thus resolving the filaments back to cocci (Pérez-Núñez et al., 2011). Comparison of the *L. lactis* PBP2x protein sequence against *L. pneumophila* Philadelphia-1 genomic database (LegioList, <http://genolist.pasteur.fr/LegioList/>) revealed that the putative penicillin-binding protein 3 (PBP3), a transpeptidase known to be specifically involved in septal peptidoglycan synthesis in rod-shaped bacteria (Den Blaauwen et al., 2008; Sauvage et al., 2008), displayed the closest sequence similarity. As the TEM analysis of the spherical form of *L. pneumophila* has revealed (Section 4.1.4.1), the unusually extended periplasmic structure may play a major role in the morphological transition. Further investigation will have to be conducted in order to discern how much commonality there is between the *L. lactis* mechanism and that of *L. pneumophila*.

4.2.7.3. Induction of morphological change by protozoa-secreted signals

In the initial investigations of interaction between the filamentous form with protozoan hosts, the spherical form was not detected in the filament-rich *L. pneumophila* seed culture but appeared in large numbers upon co-incubation with the amoeba *A. castellanii* (Section 3.2.4), indicating that the amoeba may influence the

appearance of spheres. When incubation of the filamentous culture under axenic culture conditions was contrasted with incubation in the presence of amoeba, it was clear that the abundance of the spherical form is significantly influenced by the presence of the protozoan host (Table 4.3). Additionally, it was found that the physical presence of the protozoa was not essential for the effect; similar incidence and abundance of the spherical form was observed when the filamentous form was incubated in medium that had been pre-conditioned with protozoa and the protozoan host had then been removed (Table 4.1, Figure 4.8). These observations collectively affirm that protozoan hosts can trigger generation of the spherical form and that the trigger is likely a (or possibly more) secreted factor(s).

Morphological adaptation by bacteria in response to secreted products by protozoa has been previously reported in a study investigating the dynamics of predation of a bacterivorous flagellated protozoa *Ochromonas* on a strain of polymorphic freshwater bacterium *Flectobacillus* sp (Corno and Jurgens, 2006). It was demonstrated that predation by *Ochromonas* on a population of rod-shaped *Flectobacillus* caused a shift in bacterial size distribution, resulting in a predominating population of filamentous *Flectobacillus* cells *even* when the protozoa and bacteria were physically separated by a dialysis bag within the experimental setup. This led the authors to propose that the morphological adaptation exhibited by *Flectobacillus* sp. might be induced by chemical signals, such as excretory products, from its protozoan predator *Ochromonas* (Corno and Jurgens, 2006). More recently, it was shown that the bacterium *Francisella philomiragia*, a facultative pathogen capable of infecting protozoa such as amoeba, exhibited a reduced ability for biofilm formation but an increased bacterial growth when cultured in *A. castellanii*-conditioned medium

(Verhoeven et al., 2010); further substantiating the notion that secreted signals from protozoa may significantly influence changes in bacterial characteristics.

The fact that presence of filamentous *L. pneumophila* during conditioning of the medium with *A. castellanii* or *A. polyphaga* had no significant effect on the abundance of spherical form generated by the conditioned medium (Figure 4.8, Ac buffer versus Ac + filament buffer) suggests that the amoeba secreted factor(s) causing the morphological change is unlikely to be specifically induced by *L. pneumophila*. Moreover, the ability of *T. pyriformis*-conditioned medium to similarly elicit formation of spheres indicate that the induction of appearance of spherical form by secreted factor(s) is not a response specific to amoebae, but may be common with other protozoan hosts. Thus, it is possible that the factor may be a common excretory product of the protozoa such as a metabolite, or a chemical cue released by protozoa to help forage for food.

The rapid formation of spheres upon exposure to secreted factors by protozoan hosts invites the question of what benefit could *L. pneumophila* possibly reap from the rapid morphological response. A correlated increase in the population of infected amoebae with the transient appearance of spheres (Figure 4.9) implied a possible relevance of this morphological change to infection of the protozoan host. Furthermore, the time taken for completion of one infection cycle within the amoeba *A. castellanii* by a filamentous culture of *L. pneumophila* was not significantly different from that of a bacillary control culture (Figure 3.12), which was a surprising observation given that bacteria in the filamentous form have been associated with resistance towards ingestion by their predators (Justice et al., 2008; Pernthaler, 2005). This ability to

effectively infect amoeba by *L. pneumophila* filaments could be possible with the more ingestible form of the spheres or rods.

The filamentous form of *L. pneumophila* apparently responds to factor(s) secreted by its protozoan hosts by undergoing a morphological transition as described in the previous section, that may facilitate its uptake by the hosts. While visual evidence of the morphological transition would incontrovertibly substantiate this hypothesis, the collective observations in this study have demonstrated that secreted factor(s) from amoebae (*Acanthamoeba* spp.) and ciliate (*T. pyriformis*), directly or indirectly via modification of the medium, can induce the formation of spherical forms from a filamentous culture of *L. pneumophila* and start a morphological transition process.

4.2.7.4. Conclusion

Thus far, it has been clearly demonstrated that the spherical form initially observed in the infection of amoeba with filamentous culture of *L. pneumophila* is not an artefact but a distinct morphological form which can be generated in significant abundance from a filamentous culture, upon exposure to secreted factors by protozoan hosts. The transient nature of its appearance with the concomitant accrue of bacillary form suggests that the spherical form may represent an intermediate in the morphological transition of the filamentous to bacillary form of *L. pneumophila*.

On the basis of these findings, Figure 4.15 illustrates a simplified proposal to account for the spherical form in the morphological cycle of *L. pneumophila* in relation to the filamentous and bacillary forms.

Figure 4.15 Schematic representation of the proposed morphological transition involving the bacillary, filamentous and spherical forms of *L. pneumophila*. Under permissive conditions, the extracellular bacillary form may elongate into filaments (A). Changes in the environment may trigger the differentiation of the filamentous form back to rods via a spherical intermediate (B) as observed in this study, or directly without any intermediates (C) as implied by previous reports (Piao et al., 2006; Pine et al., 1979). The bacillary form can be ingested by amoebic hosts and establish intracellular multiplication that concludes in the release of replicated rods by lysis of the host (D); the similar capacity by the spherical form (E) remains to be substantiated, although its physical dimensions suggests ingestion by amoebic hosts is possible.

In sum, the novel spherical form of *L. pneumophila* described in this chapter is a transitional morphology that is distinctive to the interaction of the filamentous form of *L. pneumophila* with protozoan hosts such as amoeba and ciliate, with a potential role in host infection. In the following chapter, I shall discuss the ecological relevance of this unique feature in *Legionella*-host relationship and conclude with future perspectives pertinent to this thesis.

CHAPTER 5

DISCUSSIONS AND FUTURE PERSPECTIVES

5. Discussion and Future Perspectives

The life cycle of the bacterium *L. pneumophila*, as depicted in Figure 5.1, can be viewed as an alternation between existence in the intracellular (e.g. within protozoan or mammalian hosts) and the extracellular (as free-living planktonic cells, in natural biofilm communities, or cultivated *in vitro*) environments (Campodonico and Roy, 2009) (Garduño, 2007). Although it is predominantly seen in the bacillary state, *L. pneumophila* is also known to exist in the filamentous morphological form (Berg et al., 1985; Katz et al., 1984; Paszko-Kolva et al., 1992; Rodgers, 1979). In the 32 years since the identification of *L. pneumophila*, much had been studied on this bacterium in relation to its life cycle and forms of existence. However, most work were concentrated on the bacillary morphology of *L. pneumophila* (including the differentiated forms), resulting in a detailed picture of its life story in the bacillary form but less is known about its existence in the filamentous mode.

Unlike the bacillary form, the filamentous morphology had not been a subject of systematic investigation and was mentioned only sporadically, until it was highlighted by a few recent studies to be involved in the formation of biofilms at elevated temperatures (Konishi et al., 2006; Piao et al., 2006). These reports linked the fact that the bacterium could thrive in thermally-altered static aquatic environments – one that is commonly found in man-made water systems and have been implied in outbreaks of legionellosis – to the possibility that it survives better in the filamentous form. Guided by this, I ventured to characterize the lesser studied filamentous form and the

results of my investigation led to the demonstration of a further capacity of *L. pneumophila* for morphological adaptations in its life cycle (Figure 5.1).

Figure 5.1 Diagrammatic representation depicting the relationships of morphological forms in the biphasic life cycle of *L. pneumophila*. Extracellular bacillary forms conceptually represent all known differentiated bacillary forms that occur outside of host cells, such as the stationary phase form and viable-but-non-culturable (VBNC) form. Solid arrows represent relationships that have been studied and reported by other investigators; dotted arrows represent relationships that are implicated to be plausible in this thesis. Modified from *Developmental Cycle – Differentiation of Legionella pneumophila* by Garduno et. al in *Legionella: Molecular Microbiology* published by Caister Academic Press (Garduño et al., 2008).

5.1. Summary of study

The work of this thesis attempts to further understand the filamentous form of *L. pneumophila* and its ecological relevance as an alternative morphological form in the extracellular life cycle of the bacterium. Exploration to identify environmental conditions that might trigger formation of filaments by *L. pneumophila* revealed that the occurrence of filamentation cannot be specifically pinned to a single dominant environmental factor, but is affected by a concerted influence of factors including, but not limited to, temperature, nutritional components and fluid movement which impacts the micro-scale solute gradients surrounding the bacteria in the environment. Dissection of these factors demonstrated that filamentation of *L. pneumophila* is not simply a manifestation of generic starvation or heat-shock response; rather, it is the consequence of a complex interplay of environmental factors. The investigation clearly demonstrated that the incidence of filamentation is promoted under conditions (i) of higher than ambient temperatures of 37-42°C, (ii) of static cultivation more so than in agitated fluid movement, and (iii) that carry at least a minimal organic content of 5% BYE (500 mgL⁻¹ yeast extract) equivalent (Table 3.6)

Efforts were also made to evaluate the ecological relevance of this filamentous form in terms of interaction with *L. pneumophila*'s natural hosts – the protozoa – by looking at the filamentous form's infective capacity. While assessing the potential of a population primarily consisting of *L. pneumophila* filaments to infect amoeba (*Acanthamoeba*) and ciliate (*Tetrahymena*) hosts, a spherical form was discovered, the emergence of which appeared to be unique to the interaction of the filamentous form with protozoan hosts.

Transmission electron microscopy (TEM) analyses verified that this novel spherical entity contains cytoplasmic material encased within a Gram negative cell envelope. It is therefore a distinct morphological form of *L. pneumophila*, with cellular dimension measuring two to five times the average width of the bacillary or filamentous form.

Ultrastructural variations in the periplasmic layer could be identified amongst the spheres, ranging from the typically compact periplasm, to ones with greatly expanded periplasmic space yet sufficient integrity to maintain the spherical shape of the cells (Figure 4.3). The possibility of such features representing cells undergoing structural transition, as well as the presence of multi-lobular cells with sphere-like parts joined by thinner segments (Figure 4.4), hinted at a possible transitional link between the filamentous and the spherical forms.

This speculation later gained supportive evidence from two sets of data. First, there was indeed a strong correlation between the incidence of spheres and the prior presence of filaments (Section 4.2.1, Table 4.2) although it required the influence of protozoa to enhance the abundance of the spheres to a significant level (Table 4.3). Second, upon exposure of the filamentous *L. pneumophila* cultures to protozoan influence, the transient surge in the abundance of spheres was consistently paralleled by a decline in the filamentous subpopulation (Section 4.2.4, Figure 4.11), suggesting that morphological transition from the filamentous to the spherical form may have occurred. It was noted that this transition is rapid, occurring as early as 1 h post-exposure to protozoan influence, and does not appear to involve *de novo* protein synthesis (Section 4.2.5, Figure 4.12).

Although the existence of the filamentous form needs to precede the emergence of the spheres, influence by the protozoa (either directly in the physical presence of, or through their conditioned buffer) is required to augment the abundance of the spherical form, underscoring the potential role the sphere may play in *Legionella*-protozoa association (Table 4.4). A correlation between the transient existence of the spheres and the increase in the population of infected amoebae (Figure 4.9) further implicates the spherical form in the infection process. Filament-to-sphere transition which can rapidly size down the filaments was initially hypothesized to facilitate amoebic intake, but following that first phase, it was observed that accruiement of the bacillary form would set in until almost the entire population of cells became rod-shaped (Section 4.2.4. Figure 4.11). Hence, a model whereby filament-to-rod transition occurs via the intermediate step of the sphere can be envisaged instead.

It has also been established in this work that the physical presence of the protozoan hosts is not essential for influencing the emergence of spheres, as protozoa-conditioned buffers were equally capable of eliciting the appearance of this form. The generation of the spherical form is therefore likely to be triggered by some soluble factors secreted by protozoan hosts. Survey of the filamentous culture in buffers conditioned with varying densities of *T. pyriformis* showed that a threshold density of host cells is required to bring about changes in the buffer to enhance the abundance of spheres significantly, suggesting that a minimum concentration of the secreted factor(s) must be reached to effect this enhancement.

Collectively, these findings suggest that under an intricate balance of environmental factors e.g. temperature, nutritional composition and fluid movement, the predominantly bacillary *L. pneumophila* is capable of differentiating into the filamentous form, which upon interaction with protozoan hosts, such as amoeba and ciliate, may be triggered by secreted factors of the hosts to differentiate rapidly back into the bacillary form via a spherical intermediate. The rod-shaped *L. pneumophila* has the capacity to adapt to changes to the abiotic (temperature, nutritional components, fluid movement) and the biotic (presence of protozoan hosts) factors in its habitat by morphological differentiation into the filamentous and spherical forms. Hence the persistence of *L. pneumophila* in the extracellular environment could be attributed to survival strategies involving multiple morphological forms and these non-bacillary forms may be of greater ecological relevance than previously acknowledged.

5.2. Ecological relevance of the filamentous and spherical forms

L. pneumophila is able to filament under conditions defined by certain combinations of factors including high temperature, static or dynamic fluid environment, minimal or rich organic content, while remaining as bacillary cells in other conditions (Section 3.1.5). This capacity of *L. pneumophila* to switch its morphology according to a wide spectrum of environmental conditions suggests that specific adaptive advantages for survival are associated with each of its morphological forms. In most cases of

Legionnaires' disease outbreak, thermally altered man-made water systems such as communal hot water supplies and cooling towers, where the water temperature is higher than the ambient temperature, have been implicated as the source of infection (Fields, 2007; Joseph and Ricketts, 2010). Large community outbreaks of Legionnaires' disease resulting in hospitalization of hundreds of patients and numerous fatalities have been linked to hot spring spas and municipal hot water supplies harbouring high concentrations of *L. pneumophila* (Okada et al., 2005; Tartakovskiy et al., 2009). Accumulation of organic matter and formation of biofilms in stagnated regions of heat-treated water installations, e.g. water storage tanks and dead-end plumbings in water distribution structures, have also been indicated to promote the persistence of *L. pneumophila* within the water systems and the eventual colonization of these man-made aquatic environments by the bacteria upon encountering permissive growth conditions (Temmerman et al., 2006; Vervaeren et al., 2006). High levels of *L. pneumophila* in the distributed water would increase the risk of human infection, hence heightening the threat of Legionnaires' disease outbreaks.

Within these man-made water systems, there are likely to exist a considerable number of niches that are defined by combinations of factors which favour the filamentation of *L. pneumophila*; for example, the base of the water holding tank of a hot water distribution system. A combination of static fluid environment at high temperature with sufficient nutrient from sedimented organic matter may promote the formation of filamentous biofilm by *L. pneumophila*, allowing the bacterium to persist within the water installation. The afore-described example is only one of the many plausible "filamentation-friendly" niches that can be found in thermally altered man-made water systems. It is conceivable that the filamentous form may be fairly widespread in

these water distribution systems and may confer ecological advantages, such as resistance to antimicrobials (Miller et al., 2004) or facilitating attachment to surfaces in favourable niches (Young, 2006). Another proposed survival advantage that filamentation may offer is the potentially rapid dissemination of *L. pneumophila* in the environment once conversion of the multi-nucleoid filament into multiple rods occurs in response to changes in growth conditions (Paszko-Kolva et al., 1992; Piao et al., 2006). The likelihood of a high prevalence of the filamentous form in man-made water systems may therefore translate to a greater risk for *L. pneumophila* to rapidly colonize water distribution systems, thereby compromising the quality of potable water and increasing the risk of human infection and thus, outbreaks.

Proliferation of *L. pneumophila* in natural water bodies as well as artificial water systems has been mostly attributed to its parasitic stage involving protozoan hosts because the levels of nutrients required for replication is not readily available in the freshwater environment (Fields, 2007). Free-living amoebae, such as *Acanthamoeba*, *Hartmanella* and *Naegleria*, commonly found in natural and man-made environments are known to prey on *L. pneumophila*, which has evolved mechanisms to circumvent digestion by its hosts and to multiply intracellularly (Fields et al., 2002; Molmeret et al., 2007; Steinert et al., 2002). However, this host-pathogen relationship is well studied for *L. pneumophila* only in the context of the bacillary form; it was unknown whether the filamentous form can similarly infect protozoan hosts to establish intracellular multiplication. Work in this thesis has indicated the potential of a filament-rich culture to infect protozoan hosts, probably through a morphological differentiation of the filamentous form into the bacillary form via an intermediate in the spherical form. The filamentous form may therefore be viewed as a “detour”

phase from the bacillary mode of existence in the extracellular life cycle of *L. pneumophila*; a morphological adaptation in certain niches to facilitate survival of the bacterium until environmental conditions become favourable for replication, e.g. when protozoan hosts become available. In this particular case, reversion to the bacillary form may be more strategic to effect the infection of the host in order to proliferate by intracellular replication.

Existence of *L. pneumophila* in the spherical form has never been associated as part of its natural life cycle previously. It has been reported that the bacterium forms swollen spheroplasts, i.e. circular cells with disrupted cell walls, upon treatment with antimicrobials such as ampicillin and methicillin (Chan et al., 1987; Elliott and Rodgers, 1985); however, these are artificially-induced through chemical assault, and not naturally occurring forms. Coccoid forms of *L. pneumophila* were briefly mentioned in an early ultrastructural study of the bacterium (Katz, 1978; Katz et al., 1984), but since it was not followed up with characterization studies, it remains unclear whether the coccoid forms were true differentiated forms in the life cycle of *L. pneumophila*. Our observation of a spherical form generated in transient abundance upon the interaction of the filamentous form with the protozoan hosts implied that a differentiated spherical form of *L. pneumophila* may occur in its extracellular life cycle, probably as part of a morphological transition process from the filamentous form that is triggered in response to the presence of protozoan hosts. Morphological adaptation induced by bacterivorous protozoa has been proposed as a strategy for bacteria to survive against predation in the environment (Corno and Jurgens, 2006; Justice et al., 2008; Pernthaler, 2005); in these situations, the filamentous form holds the advantage of resisting predation. However, for intracellular pathogens of protozoa

such as *L. pneumophila*, providing ease for protozoan uptake would conceivably be preferred as a means to survive and proliferate in unfavourable environmental conditions. Hence, while the same strategy of morphological adaptation may be adopted, the direction of morphological transition would likely be different. While direct filament-to-rod transition is possible, differentiation into the spherical form as an intermediate appears to be a more rapid route to transit from the filamentous to the bacillary morphology (Sections 4.2.4, and 4.2.7.2). This probably enables *L. pneumophila* to better seize the opportunity of a sheltered and nutrient-rich intracellular environment in protozoa to proliferate and perpetuate in the environment.

This apparent versatility of *L. pneumophila* to switch among different morphological forms in response to changes in the environment may underlie its capacity to persist in a wide range of conditions, even in some seemingly inhospitable ones. In the same way that the bacterium has evolved mechanisms to parasitize and multiply intracellularly in protozoan hosts, it may have developed strategies to survive in the dynamic extracellular environment. Broadly, this possibility has important implications on the prevention and control measures for *L. pneumophila* and Legionnaires' disease. For instance, currently practiced measures for controlling colonization and growth of legionellae in water distribution systems include manipulation of the environment, e.g. controlling temperature and nutrient levels, as well as the use of disinfectants (Bartram et al., 2007; Lin et al., 2011). Definition of the parameters for these control measures, e.g. the range of water temperature or nutrient levels or disinfectant concentration which are effectively bacteriostatic if not bactericidal, may vary depending on the predominating morphological mode of existence of *L. pneumophila* at different parts of the water distribution systems.

Additionally, this morphological versatility of *L. pneumophila* should also be taken into account in the choice of disinfection methods (Kim et al., 2002) and even the design of the water distribution systems (e.g. minimizing areas of poor flow such as dead legs) (Merchat and Deumier, 2006; Surman-Lee and Bentham, 2006) as there is a risk of creating niches that may promote filamentation which could facilitate its persistence or that may increase the interaction between filaments and protozoa. The exact impact of these differences will require focused investigation with a healthy dose of respect for the hitherto neglected morphological forms of *L. pneumophila*.

Given its tenacious nature, the complete eradication of *L. pneumophila* from our man-made water distribution systems is difficult if not impossible. However, understanding how the bacterium can persist in a wide spectrum of environmental conditions will enable us to better strategize our approach towards controlling its colonization of our water systems and prevention of disease outbreaks. The ability of *L. pneumophila* to alternate among the filamentous, bacillary and spherical forms under the influence of varying abiotic and biotic factors investigated in this study may provide additional clues to the bacterium's repertoire of strategies for surviving dynamic environments. This in turn may lead us to possibilities which we can apply to better counter this pathogen in the prevention of Legionnaires' disease.

5.3. Future Perspectives

5.3.1. Further characterization of the spherical form

One of the very exciting findings in my work is the discovery of a distinctly spherical form of *L. pneumophila*. Since there had been no recent (up to last two decades) report on *L. pneumophila* in the spherical morphology, investigation of this novel form was very much akin to treading an unknown terrain by trial-and-errors with many failures as well as little victories. As much as the collective observations in this thesis have provided valuable clues about this morphological form, they also opened up more questions regarding the biology and ecology of *L. pneumophila*.

While data obtained from co-incubation assays of filament-rich *L. pneumophila* strain LP3 cultures with protozoa-conditioned media implied that the spherical form could be an intermediate in a morphological transition from the filamentous to bacillary form, some of the experimental limitations encountered in the assays have hampered validation of this hypothesis. For instance, it was uncertain how the outcome of the observed data may have been affected by the presence of a minor population of rods in the filament-rich cultures used to perform the assay. Hence appropriate control experiments had to be incorporated to eliminate certain possibilities and fine-tune the interpretation, but those attempts might not have been exhaustive enough. Visual tracking of this morphological transformation in real time using microscopic time-lapse imaging would not only be able to incontrovertibly demonstrate the morphological differentiation, but also to reveal greater details of the interactive process with protozoa.

Furthering on this possibility of filament-to-spheres-then-rods morphological transition, it is of curiosity what significance the spherical form may hold to the end-point bacillary cell, compared to the bacillary cell that had been converted from filaments directly. Would they be similar in terms of infectivity, osmotolerance and other physiology? From the mechanistic point of view, assessing the molecular players that are involved in cell wall structure reorganization as well as septation may help shed light on this puzzle. Clearly, the rapidity with which the filament-to-rod transition occurs when the intermediate form (sphere) is involved is a point of interest as the mechanism must offer considerable metabolic cost-effectiveness under oligotrophic environmental conditions. Leads to this morphological transition in question will help enrich our knowledge on the relevance of having multiple differentiated forms in *L. pneumophila* extracellular life cycle.

It has long been known that *L. pneumophila* is able to infect and multiply within mammalian cells, e.g. human lung macrophages, by similar mechanisms used to infect its natural protozoan hosts (Fields, 1996; Fields et al., 2002; Horwitz and Silverstein, 1980). The fact that interaction of the filamentous form with amoebae can result in the establishment of intracellular replication and lysis of the wasted host begs the question of whether contact with mammalian cells will lead to the similar outcome. In addition, it would be interesting to find out whether the pathogen-mammalian host interaction can also trigger the transient appearance of a significant abundance of the spherical form. Would inhalation of aerosols bearing these spherical forms by susceptible human hosts increase the risk for legionellosis? Answers to these questions may provide valuable insights to the virulence of the non-bacillary forms with respect to mammalian cells, which may in turn have implications in our

perspectives of *L. pneumophila* pathogenesis and our approach to legionellosis prevention.

5.3.2. Identification of protozoan secretory signals involved in generation of the spherical form

An intriguing characteristic that surfaced in the course of probing the *L. pneumophila* spherical form is the fact that massive quantity of it can be triggered by protozoa in a very short time (approximately 1 h) *even in the absence* of any physical contact between the bacterium and the protozoa (Section 4.2.2). The fact that protozoa-conditioned medium (from both *Acanthamoeba spp.* and *T. pyriformis*) was equally capable of generating significant abundance of the spherical form hints at protozoan secretory products that may serve as molecular signals prompting morphological differentiation in *L. pneumophila*. Trailing this lead, one of the many questions open for investigation is the nature of these suspected secreted products: are they protein or chemical molecules, metabolic wastes or signaling factors? It was recently reported that the ability for biofilm formation by *F. philomiragia* is inhibited by amoeba-conditioned medium and a secreted product of protein nature is likely to be responsible for the inhibition (Verhoeven et al., 2010). The fact that *L. pneumophila* spheres were generated rapidly upon exposure to protozoa-conditioned medium, also raises mechanistic questions, such as how and what signaling pathways these secreted products may trigger to elicit the rapid response, keeping in mind that *de novo* protein synthesis by *L. pneumophila* does not appear to be required for this response (Section 4.2.5.). Isolation, identification and characterization of the protozoan secreted products that may be responsible for the generation of the *L. pneumophila* spherical

form will probably reveal interesting clues to the mechanism of this host-pathogen interaction.

5.3.3. Formation of filamentous and spherical forms by other *Legionella* strains and species

Within the species *L. pneumophila*, there are hundreds of strains categorized into multiple serogroups (of which serogroup 1 is predominantly associated with Legionnaires' disease) that have been shown to be genetically diverse (Cazalet et al., 2008). Different strains of *L. pneumophila* have been reported to display variation in manifestation of phenotypic characteristics, such as biofilm formation and intracellular survival within amoebae (Piao et al., 2006; Samrakandi et al., 2002). Indeed, slight differences in the conditions required for forming dense, mat-like filamentous biofilms was observed between *L. pneumophila* strains Philadelphia-1 and Knoxville-1 (Piao et al., 2006) (Section 3.1.1, page 51), suggesting that certain attributes, e.g. surface adherence stability of the filamentous form, could be strain-specific. Since data in this study were obtained for *L. pneumophila* strain Philadelphia-1, a member of serogroup 1, it would be interesting to evaluate whether other strains within this as well as of other serogroups can also filament and generate the spherical form under the similar conditions studied. This aspect of *L. pneumophila* JR32 (Marra et al., 1992), a strain popularly used because of its genetic amenability (Mampel et al., 2006; Samrakandi et al., 2002; Steinert et al., 1997; Steinert et al., 1995), has been tested in this work and found to respond similarly to strain Philadelphia-1 (Section 3.2.4.2). Comparison amongst more strains would provide clues as to whether manifestation of these characteristics is a common feature of *L.*

pneumophila or is specific to certain subgroups (e.g. of serogroup 1). Juxtaposition of the phenotypic information with genomic data may also shed light on possible correlations between the virulence of the bacterium with its propensity for switching between the different morphological forms.

5.4. Conclusion

The formation of filamentous and spherical forms by *L. pneumophila* documented in this thesis attests to the ability of this bacterium to seamlessly respond to its dynamic environment by assuming a repertoire of morphological forms. This fascinating display of versatility is but a gentle reminder of how much we have yet find out about the life cycle and ecology of this pleomorphic accidental pathogen.

CHAPTER 6

REFERENCES

6. References

Abdelrahman, Y.M., and Belland, R.J. (2005). The chlamydial developmental cycle. *FEMS Microbiol Rev* 29, 949-959.

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

Albert-Weissenberger, C., Cazalet, C., and Buchrieser, C. (2007). *Legionella pneumophila* — a human pathogen that co-evolved with fresh water protozoa. *Cellular and Molecular Life Sciences* 64, 432-448.

Alleron, L., Merlet, N., Lacombe, C., and Frere, J. (2008). Long-term survival of *Legionella pneumophila* in the viable but nonculturable state after monochloramine treatment. *Curr Microbiol* 57, 497-502.

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.

Bartram, J., Chartier, Y., Lee, J.V., Pond, K., and Surman-Lee, S., eds. (2007). *Legionella and the prevention of legionellosis* (Geneva, Switzerland, World Health Organization Press).

Berg, J.D., Hoff, J.C., Roberts, P.V., and Matin, A. (1985). Growth of *Legionella pneumophila* in continuous culture. *Appl Environ Microbiol* 49, 1534-1537.

Berk, S.G., Faulkner, G., Garduno, E., Joy, M.C., Ortiz-Jimenez, M.A., and Garduño, R.A. (2008). Packaging of Live *Legionella pneumophila* into Pellets Expelled by *Tetrahymena* spp. Does Not Require Bacterial Replication and Depends on a Dot/Icm-Mediated Survival Mechanism. *Appl Environ Microbiol* 74, 2187-2199.

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* spp. *Appl Environ Microbiol* 64, 279-286.

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.U., and Egli, T. (2007). Assessment and interpretation of bacterial viability by using the LIVE/DEAD

BacLight Kit in combination with flow cytometry. *Appl Environ Microbiol* 73, 3283-3290.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J., and Desjardins, R. (1999). LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* 37, 77-86.

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

Byrne, B., and Swanson, M.S. (1998). Expression of *Legionella pneumophila* Virulence Traits in Response to Growth Conditions. *Infect Immun* 66, 3029-3034.

Cabeen, M.T., and Jacobs-Wagner, C. (2005). Bacterial cell shape. *Nature reviews Microbiology* 3, 601-610.

Campodonico, E.M., and Roy, C.R. (2009). The Genus *Legionella*. In *Practical Handbook of Microbiology*, E. Goldman, and L. Green, eds. (CRC Press), pp. 505-517.

Cazalet, C., Jarraud, S., Ghavi-Helm, Y., Kunst, F., Glaser, P., Etienne, J., and Buchrieser, C. (2008). Multigenome analysis identifies a worldwide distributed epidemic *Legionella pneumophila* clone that emerged within a highly diverse species. *Genome Res* 18, 431-441.

Chan, E.L., Harris, R.C., and Dalton, H.P. (1987). The effect of antibiotics on the cell morphology of *Legionella pneumophila*. *J Med Microbiol* 23, 149-154.

Chi, E.Y., Kuo, C.C., and Grayston, J.T. (1987). Unique ultrastructure in the elementary body of *Chlamydia* sp. strain TWAR. *J Bacteriol* 169, 3757-3763.

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

Colbourne, J.S., Pratt, D.J., Smith, M.G., Fisher-Hoch, S.P., and Harper, D. (1984). Water fittings as sources of *Legionella pneumophila* in a hospital plumbing system. *The Lancet* 323, 210-213.

- Comber, K.R., Boon, R.J., and Sutherland, R. (1977). Comparative Effects of Amoxycillin and Ampicillin on the Morphology of *Escherichia coli* In Vivo and Correlation with Activity. *Antimicrob Agents Chemother* 12, 736-744.
- Corno, G., and Jurgens, K. (2006). Direct and Indirect Effects of Protist Predation on Population Size Structure of a Bacterial Strain with High Phenotypic Plasticity. *Appl Environ Microbiol* 72, 78-86.
- Declerck, P., Behets, J., Hoef, V.v., and Ollevier, F. (2007a). Detection of *Legionella* spp. and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. *Water Res* 41, 3159-3167.
- Declerck, P., Behets, J., van Hoef, V., and Ollevier, F. (2007b). Replication of *Legionella pneumophila* in floating biofilms. *Curr Microbiol* 55, 435-440.
- DeLoney, C.R., and Schiller, N.L. (1999). Competition of Various beta -Lactam Antibiotics for the Major Penicillin-Binding Proteins of *Helicobacter pylori*: Antibacterial Activity and Effects on Bacterial Morphology. *Antimicrob Agents Chemother* 43, 2702-2709.
- Den Blaauwen, T., De Pedro, M.A., Nguyen-Distèche, M., and Ayala, J.A. (2008). Morphogenesis of rod-shaped sacculi. *FEMS Microbiology Reviews* 32, 321-344.
- Devos, L., Boon, N., and Verstraete, W. (2005). *Legionella pneumophila* in the environment: the occurrence of a fastidious bacterium in oligotrophic conditions. *Reviews in Environmental Science and Biotechnology* 4, 61-74.
- Donlan, R.M. (2002). Biofilms: microbial life on surfaces. *Emerg Infect Dis* 8, 881-890.
- Donlan, R.M., and Costerton, J.W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15, 167-193.
- Edelstein, P.H., and Finegold, S.M. (1979). Isolation of *Legionella pneumophila* from a transtracheal aspirate. *J Clin Microbiol* 9, 457-458.
- Edwards, R.L., and Swanson, M.S. (2007). Regulation of the *Legionella pneumophila* Life Cycle. In *Legionella pneumophila*, pp. 95-111.
- Elliott, T.S.J., and Rodgers, F.G. (1985). Morphological response and growth characteristics of *Legionella pneumophila* exposed to ampicillin and erythromycin. *J Med Microbiol* 19, 383-390.

Faine, S., Edelstein, P., Kirby, B.D., and Finegold, S.M. (1979). Rapid presumptive bacteriological diagnosis of Legionnaires' disease. *J Clin Microbiol* 10, 104-105.

Faulkner, G., Berk, S.G., Garduno, E., Ortiz-Jimenez, M.A., and Garduño, R.A. (2008). Passage through *Tetrahymena tropicalis* Triggers a Rapid Morphological Differentiation in *Legionella pneumophila*. *J Bacteriol* 190, 7728-7738.

Faulkner, G., and Garduño, R.A. (2002). Ultrastructural analysis of differentiation in *Legionella pneumophila*. *J Bacteriol* 184, 7025-7041.

Fenchel, T. (1980). Suspension feeding in ciliated protozoa: Functional response and particle size selection. *Microbial Ecology* 6, 1-11.

Fields, B.S. (1996). The molecular ecology of *legionellae*. *Trends Microbiol* 4, 286-290.

Fields, B.S. (2007). *Legionella* in the Environment. In *Legionella pneumophila: Pathogenesis and Immunity*, P. Hoffman, H. Friedman, and M. Bendinelli, eds. (Springer US), pp. 85-94.

Fields, B.S., Benson, R.F., and Besser, R.E. (2002). *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev* 15, 506-526.

Fields, B.S., Shotts, E.B., Jr., Feeley, J.C., Gorman, G.W., and Martin, W.T. (1984). Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*. *Appl Environ Microbiol* 47, 467-471.

Fraser, D.W., Tsai, T.R., Orenstein, W., Parkin, W.E., Beecham, H.J., Sharrar, R.G., Harris, J., Mallison, G.F., Martin, S.M., McDade, J.E., *et al.* (1977). Legionnaires' disease: description of an epidemic of pneumonia. *N Engl J Med* 297, 1189-1197.

Garduño, R.A. (2007). Life Cycle, Growth Cycles and Developmental Cycle of *Legionella pneumophila*. In *Legionella pneumophila*, pp. 65-84.

Garduño, R.A., Chong, A., and Faulkner, G. (2008). Developmental Cycle - Differentiation of *Legionella pneumophila*. In *Legionella: Molecular Microbiology*, K. Heuner, and S.S. Michele, eds. (Norfolk, UK, Caister Academic Press), pp. 55-73.

Garduno, R.A., Chong, A., Nasrallah, G.K., and Allan, D.S. (2011). The *Legionella pneumophila* Chaperonin - An Unusual Multifunctional Protein in Unusual Locations. *Front Microbiol* 2, 122.

Garduño, R.A., Garduño, E., Hiltz, M., Allan, D., and Hoffman, P.S. (2002a). Morphological and Physiological Evidence for a Developmental Cycle in *Legionella pneumophila*. In *Legionella*, R. Marre, Y.A. Kwaik, C. Bartlett, N.P. Cianciotto, B.S. Fields, M. Frosch, J. Hacker, and P.C. Lück, eds. (Washington DC, ASM Press, American Society for Microbiology), pp. 82-85.

Garduño, R.A., Garduño, E., Hiltz, M., and Hoffman, P.S. (2002b). Intracellular growth of *Legionella pneumophila* gives rise to a differentiated form dissimilar to stationary-phase forms. *Infect Immun* 70, 6273-6283.

Giao, M.S., Wilks, S.A., Azevedo, N.F., Vieira, M.J., and Keevil, C.W. (2008). Validation of SYTO 9/Propidium Iodide Uptake for Rapid Detection of Viable but Noncultivable *Legionella pneumophila*. *Microb Ecol*.

Giotis, E.S., Blair, I.S., and McDowell, D.A. (2007). Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. *International Journal of Food Microbiology* 120, 250-258.

Greub, G., and Raoult, D. (2003). Morphology of *Legionella pneumophila* according to their location within *Hartmannella vermiformis*. *Res Microbiol* 154, 619-621.

Greub, G., and Raoult, D. (2004). Microorganisms Resistant to Free-Living Amoebae. *Clin Microbiol Rev* 17, 413-433.

Hagele, S., Kohler, R., Merkert, H., Schleicher, M., Hacker, J., and Steinert, M. (2000). *Dictyostelium discoideum*: a new host model system for intracellular pathogens of the genus *Legionella*. *Cell Microbiol* 2, 165-171.

Hahn, M.W., Moore, E.R., and Hofle, M.G. (1999). Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. *Appl Environ Microbiol* 65, 25-35.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2, 95-108.

Harb, O.S., Gao, L.Y., and Abu Kwaik, Y. (2000). From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. *Environ Microbiol* 2, 251-265.

Healy, D.P., Gardner, J.C., Puthoff, B.K., Kagan, R.J., and Neely, A.N. (2007). Antibiotic-mediated bacterial filamentation: A potentially important laboratory phenomenon. *Clinical Microbiology Newsletter* 29, 22-24.

- Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B.K., and Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146, 2395-2407.
- Hindre, T., Bruggemann, H., Buchrieser, C., and Hechard, Y. (2008). Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. *Microbiology* 154, 30-41.
- Holden, E.P., Winkler, H.H., Wood, D.O., and Leinbach, E.D. (1984). Intracellular growth of *Legionella pneumophila* within *Acanthamoeba castellanii* Neff. *Infect Immun* 45, 18-24.
- Horvath Jr, D.J., Li, B., Casper, T., Partida-Sanchez, S., Hunstad, D.A., Hultgren, S.J., and Justice, S.S. (2011). Morphological plasticity promotes resistance to phagocyte killing of uropathogenic *Escherichia coli*. *Microbes and Infection* 13, 426-437.
- Horwitz, M.A., and Silverstein, S.C. (1980). Legionnaires' disease bacterium (*Legionella pneumophila*) multiples intracellularly in human monocytes. *J Clin Invest* 66, 441-450.
- 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. *Nat Biotech* 5, 947-950.
- Huws, S.A., McBain, A.J., and Gilbert, P. (2005). Protozoan grazing and its impact upon population dynamics in biofilm communities. *J Appl Microbiol* 98, 238-244.
- Ishii, A., Sato, T., Wachi, M., Nagai, K., and Kato, C. (2004). Effects of high hydrostatic pressure on bacterial cytoskeleton FtsZ polymers *in vivo* and *in vitro*. *Microbiology* 150, 1965-1972.
- Janion, C., Sikora, A., Nowosielska, A., and Grzesiuk, E. (2002). Induction of the SOS response in starved *Escherichia coli*. *Environmental and Molecular Mutagenesis* 40, 129-133.
- Jefferson, K.K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiology Letters* 236, 163-173.
- Jensen, R.H., and Woolfolk, C.A. (1985). Formation of Filaments by *Pseudomonas putida*. *Appl Environ Microbiol* 50, 364-372.

Joseph, C.A., and Ricketts, K.D. (2010). Legionnaires' disease in Europe 2007-2008. *Euro Surveill* 15, 19493.

Jurgens, K., Pernthaler, J., Schalla, S., and Amann, R. (1999). Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. *Appl Environ Microbiol* 65, 1241-1250.

Justice, S.S., Hunstad, D.A., Cegelski, L., and Hultgren, S.J. (2008). Morphological plasticity as a bacterial survival strategy. *Nat Rev Micro* 6, 162-168.

Justice, S.S., Hunstad, D.A., Seed, P.C., and Hultgren, S.J. (2006). Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infection. *Proceedings of the National Academy of Sciences* 103, 19884-19889.

Kaneko, T., Nakamura, Y., Wolk, C.P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ishikawa, A., Kawashima, K., Kimura, T., *et al.* (2001). Complete Genomic Sequence of the Filamentous Nitrogen-fixing Cyanobacterium *Anabaena* sp. Strain PCC 7120. *DNA Research* 8, 205-213.

Katz, S.M. (1978). The morphology of the Legionnaires' disease organism. *Am J Pathol* 90, 701-722.

Katz, S.M., Hashemi, S., Brown, K.R., Habib, W.A., and Hammel, J.M. (1984). Pleomorphism of *Legionella pneumophila*. *Ultrastruct Pathol* 6, 117-129.

Kim, B.R., Anderson, J.E., Mueller, S.A., Gaines, W.A., and Kendall, A.M. (2002). Literature review - efficacy of various disinfectants against *Legionella* in water systems. *Water Research* 36, 4433-4444.

Konishi, T., Nishizono, A., Yamashiro, T., and Koide, M. (2011). Comparison of *Legionella* biofilm formations at three different temperatures in liquid flow, in static liquid and on agar plate. *Journal of Biomechanical Science and Engineering* 6, 160-172.

Konishi, T., Yamashiro, T., Koide, M., and Nishizono, A. (2006). Influence of temperature on growth of *Legionella pneumophila* biofilm determined by precise temperature gradient incubator. *J Biosci Bioeng* 101, 478-484.

Kuiper, M.W., Wullings, B.A., Akkermans, A.D.L., Beumer, R.R., and van der Kooij, D. (2004). Intracellular Proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in Aquatic Biofilms Grown on Plasticized Polyvinyl Chloride. *Appl Environ Microbiol* 70, 6826-6833.

Kuo, C.C., Chi, E.Y., and Grayston, J.T. (1988). Ultrastructural study of entry of *Chlamydia* strain TWAR into HeLa cells. *Infection and Immunity* 56, 1668-1672.

Kuo, C.C., Jackson, L.A., Campbell, L.A., and Grayston, J.T. (1995). *Chlamydia pneumoniae* (TWAR). *Clinical Microbiology Reviews* 8, 451-461.

Landers, P., Kerr, K.G., Rowbotham, T.J., Tipper, J.L., Keig, P.M., Ingham, E., and Denton, M. (2000). Survival and Growth of *Burkholderia cepacia* Within the Free-Living Amoeba *Acanthamoeba polyphaga*. *European Journal of Clinical Microbiology & Infectious Diseases* 19, 121-123.

Lin, Y.E., Stout, J.E., and Yu, V.L. (2011). Controlling *Legionella* in hospital drinking water: an evidence-based review of disinfection methods. *Infect Control Hosp Epidemiol* 32, 166-173.

Lorian, V. (1989). *In vitro* simulation of *in vivo* conditions: physical state of the culture medium. *J Clin Microbiol* 27, 2403-2406.

Lück, P.C. (2008). Diagnostics and Clinical Disease Treatment. In *Legionella: Molecular Microbiology*, K. Heuner, and M. Swanson, eds. (Norfolk, UK, Caister Academic Press), pp. 19-34.

Ly, T.M.C., and Müller, H.E. (1990). Ingested *Listeria monocytogenes* survive and multiply in protozoa. *Journal of Medical Microbiology* 33, 51-54.

Mampel, J., Spirig, T., Weber, S.S., Haagenzen, J.A.J., Molin, S., and Hilbi, H. (2006). Planktonic Replication Is Essential for Biofilm Formation by *Legionella pneumophila* in a Complex Medium under Static and Dynamic Flow Conditions. *Appl Environ Microbiol* 72, 2885-2895.

Margolin, W. (2009). Sculpting the Bacterial Cell. *Current biology : CB* 19, R812-R822.

Marra, A., Blander, S.J., Horwitz, M.A., and Shuman, H.A. (1992). Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. *Proc Natl Acad Sci U S A* 89, 9607-9611.

Marshall, K.C., and Goodman, A.E. (1994). Effects of adhesion on microbial cell physiology. *Colloids and Surfaces B: Biointerfaces* 2, 1-7.

Mauchline, W.S., Araujo, R., Wait, R., Dowsett, A.B., Dennis, P.J., and Keevil, C.W. (1992). Physiology and morphology of *Legionella pneumophila* in continuous culture at low oxygen concentration. *J Gen Microbiol* 138, 2371-2380.

McArthur, J.V. (2006). Species interactions and processes. In *Microbial Ecology: An evolutionary approach* (London, Academic Press), pp. 297-343.

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. *N Engl J Med* 297, 1197-1203.

Merchat, M., and Deumier, A. (2006). Control of *Legionella* Proliferation Risk in Cooling Water Systems. In *Legionella: State of The Art 30 Years After Its Recognition*, N.P. Cianciotto, Y.A. Kwaik, P.H. Edelstein, B.S. Fields, D.F. Geary, T.G. Harrison, C.A. Joseph, R.M. Ratcliff, J.E. Stout, and M.S. Swanson, eds. (Washington D.C., ASM Press), pp. 522-525.

Miller, C., Thomsen, L.E., Gaggero, C., Mosseri, R., Ingmer, H., and Cohen, S.N. (2004). SOS Response Induction by β -Lactams and Bacterial Defense Against Antibiotic Lethality. *Science* 305, 1629-1631.

Moffat, J.F., and Tompkins, L.S. (1992). A quantitative model of intracellular growth of *Legionella pneumophila* in *Acanthamoeba castellanii*. *Infect Immun* 60, 296-301.

MolecularProbes (2004). LIVE/DEAD[®] BacLight[™] Bacterial Viability Kits - Product Information, pp. 1-8.

Molmeret, M., Bitar, D.M., Han, L., and Kwaik, Y.A. (2004). Disruption of the phagosomal membrane and egress of *Legionella pneumophila* into the cytoplasm during the last stages of intracellular infection of macrophages and *Acanthamoeba polyphaga*. *Infect Immun* 72, 4040-4051.

Molmeret, M., Horn, M., Wagner, M., Santic, M., and Abu Kwaik, Y. (2005). Amoebae as training grounds for intracellular bacterial pathogens. *Appl Environ Microbiol* 71, 20-28.

Molmeret, M., Santic, M., and Kwaik, Y.A. (2007). Interaction of *Legionella pneumophila* with Amoeba. In *Legionella pneumophila: Pathogenesis and Immunity*, P. Hoffman, H. Friedman, and M. Bendinelli, eds. (Springer US), pp. 185-202.

Molofsky, A.B., and Swanson, M.S. (2004). Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. *Mol Microbiol* 53, 29-40.

- Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S., and Donlan, R.M. (2001). Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. *Microbiology* 147, 3121-3126.
- Nelson, D.E., and Young, K.D. (2000). Penicillin Binding Protein 5 Affects Cell Diameter, Contour, and Morphology of *Escherichia coli*. *J Bacteriol* 182, 1714-1721.
- Newton, H.J., Ang, D.K.Y., van Driel, I.R., and Hartland, E.L. (2010). Molecular Pathogenesis of Infections Caused by *Legionella pneumophila*. *Clin Microbiol Rev* 23, 274-298.
- Nowicki, M., Bornstein, N., Paucod, J.C., Binder, P., and Fleurette, J. (1987). Effect of culture medium on morphology and virulence of *Legionella pneumophila* serogroup 1. *Zentralbl Bakteriell Mikrobiol Hyg A* 264, 167-177.
- Ohata, K., Sugiyama, K., Suzuki, M., Shimogawara, R., Izumiyama, S., Yagita, K., and Endo, T. (2006). Growth of *Legionella* in Nonsterilized, Naturally Contaminated Bathing Water in A System That Circulates The Water. In *Legionella: State of The Art 30 Years After Its Recognition*, N.P. Cianciotto, Y.A. Kwaik, P.H. Edelstein, B.S. Fields, D.F. Geary, T.G. Harrison, C.A. Joseph, R.M. Ratcliff, J.E. Stout, and M.S. Swanson, eds. (Washington D.C., ASM Press), pp. 431-435.
- Ohno, A., Kato, N., Yamada, K., and Yamaguchi, K. (2003). Factors Influencing Survival of *Legionella pneumophila* Serotype 1 in Hot Spring Water and Tap Water. *Appl Environ Microbiol* 69, 2540-2547.
- Okada, M., Kawano, K., Kura, F., Amemura-Maekawa, J., Watanabe, H., Yagita, K., Endo, T., and Suzuki, S. (2005). [The largest outbreak of legionellosis in Japan associated with spa baths: epidemic curve and environmental investigation]. *Kansenshogaku Zasshi* 79, 365-374.
- Parsek, M.R., and Singh, P.K. (2003). Bacterial Biofilms: An Emerging Link to Disease Pathogenesis. *Annual Review of Microbiology* 57, 677-701.
- Paszko-Kolva, C., Shahamat, M., and Colwell, R.R. (1992). Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. *FEMS Microbiology Letters* 102, 45-55.
- Pecastaings, S., Berge, M., Dubourg, K.M., and Roques, C. (2010). Sessile *Legionella pneumophila* is able to grow on surfaces and generate structured monospecies biofilms. *Biofouling* 26, 809-819.

Pérez-Núñez, D., Briandet, R., David, B., Gautier, C., Renault, P., Hallet, B., Hols, P., Carballido-López, R., and Guédon, E. (2011). A new morphogenesis pathway in bacteria: unbalanced activity of cell wall synthesis machineries leads to coccus-to-rod transition and filamentation in ovococci. *Molecular Microbiology* 79, 759-771.

Pernthaler, J. (2005). Predation on prokaryotes in the water column and its ecological implications. *Nat Rev Micro* 3, 537-546.

Pernthaler, J., Posch, T., Simek, K., Vrba, J., Amann, R., and Psenner, R. (1997). Contrasting Bacterial Strategies To Coexist with a Flagellate Predator in an Experimental Microbial Assemblage. *Appl Environ Microbiol* 63, 596-601.

Piao, Z., Sze, C.C., Barysheva, O., Iida, K., and Yoshida, S. (2006). Temperature-regulated formation of mycelial mat-like biofilms by *Legionella pneumophila*. *Appl Environ Microbiol* 72, 1613-1622.

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*. *J Clin Microbiol* 9, 615-626.

Poindexter, J.S. (1984). The role of calcium in stalk development and in phosphate acquisition in *Caulobacter crescentus*. *Archives of Microbiology* 138, 140-152.

Popov, V.L., Chen, S.-M., Feng, H.-M., and Walker, O.H. (1995). Ultrastructural variation of cultured *Ehrlichia chaffeensis*. *J Med Microbiol* 43, 411-421.

Posch, T., Jezbera, J., Vrba, J., Simek, K., Pernthaler, J., Andreatta, S., and Sonntag, B. (2001). Size Selective Feeding in *Cyclidium glaucoma* (Ciliophora, Scuticociliatida) and Its Effects on Bacterial Community Structure: A Study from a Continuous Cultivation System. *Microbial Ecology* 42, 217-227.

Ristroph, J.D., Hedlund, K.W., and Gowda, S. (1981). Chemically defined medium for *Legionella pneumophila* growth. *J Clin Microbiol* 13, 115-119.

Rodgers, F.G. (1979). Ultrastructure of *Legionella pneumophila*. *J Clin Pathol* 32, 1195-1202.

Rodgers, F.G., Macrae, A.D., and Lewis, M.J. (1978). Electron microscopy of the organism of Legionnaires' disease. *Nature* 272, 825-826.

Rodgers, F.G., Tzianabos, A.O., and Elliott, T.S. (1990). The effect of antibiotics that inhibit cell-wall, protein, and DNA synthesis on the growth and morphology of *Legionella pneumophila*. *Journal of Medical Microbiology* 31, 37-44.

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 Systems. *Appl Environ Microbiol* 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. *Appl Environ Microbiol* 60, 1585-1592.

Rogers, J., and Keevil, C.W. (1992). Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. *Appl Environ Microbiol* 58, 2326-2330.

Rohr, U., Weber, S., Michel, R., Selenka, F., and Wilhelm, M. (1998). Comparison of free-living amoebae in hot water systems of hospitals with isolates from moist sanitary areas by identifying genera and determining temperature tolerance. *Appl Environ Microbiol* 64, 1822-1824.

Roszak, D.B., and Colwell, R.R. (1987). Survival strategies of bacteria in the natural environment. *Microbiol Rev* 51, 365-379.

Rowbotham, T.J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J Clin Pathol* 33, 1179-1183.

Russo, J.J., and Morozova, I. (2004). *Legionella* Genome Project. In <http://legionellacu-genomeorg/> (Columbia Genome Center), pp. <http://legionella.cu-genome.org/>.

Samrakandi, M.M., Cirillo, S.L., Ridenour, D.A., Bermudez, L.E., and Cirillo, J.D. (2002). Genetic and phenotypic differences between *Legionella pneumophila* strains. *J Clin Microbiol* 40, 1352-1362.

Sauvage, E., Kerff, F., Terrak, M., Ayala, J.A., and Charlier, P. (2008). The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews* 32, 234-258.

Schapiro, J.M., Libby, S.J., and Fang, F.C. (2003). Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. *Proceedings of the National Academy of Sciences* 100, 8496-8501.

Scheffers, D.-J., and Pinho, M.G. (2005). Bacterial Cell Wall Synthesis: New Insights from Localization Studies. *Microbiol Mol Biol Rev* 69, 585-607.

Scola, B.L., and Raoult, D. (2001). Survival of *Coxiella burnetii* within free-living amoeba *Acanthamoeba castellanii*. *Clinical Microbiology and Infection* 7, 75-79.

Smalley, D.L., Jaquess, P.A., Ourth, D.D., and Layne, J.S. (1980). Antibiotic-induced filament formation of *Legionella pneumophila*. *Am J Clin Pathol* 74, 852.

Spratt, B.G. (1975). Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. *Proceedings of the National Academy of Sciences* 72, 2999-3003.

Steinberger, R.E., Allen, A.R., Hansma, H.G., and Holden, P.A. (2002). Elongation Correlates with Nutrient Deprivation in *Pseudomonas aeruginosa* Unsaturated Biofilms. *Microbial Ecology* 43, 416-423.

Steinert, M., Birkness, K., White, E., Fields, B., and Quinn, F. (1998). *Mycobacterium avium* Bacilli Grow Saprozoically in Coculture with *Acanthamoeba polyphaga* and Survive within Cyst Walls. *Appl Environ Microbiol* 64, 2256-2261.

Steinert, M., Emody, L., Amann, R., and Hacker, J. (1997). Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. *Appl Environ Microbiol* 63, 2047-2053.

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*. *Appl Environ Microbiol* 61, 2428-2430.

Steinert, M., Hentschel, U., and Hacker, J. (2002). *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol Rev* 26, 149-162.

Stewart, P.S., and Franklin, M.J. (2008). Physiological heterogeneity in biofilms. *Nat Rev Micro* 6, 199-210.

Stocks, S.M. (2004). Mechanism and use of the commercially available viability stain, BacLight. *Cytometry A* 61, 189-195.

Storey, M.V., Langmark, J., Ashbolt, N.J., and Stenstrom, T.A. (2004). The fate of *Legionellae* within distribution pipe biofilms: measurement of their persistence, inactivation and detachment. *Water Sci Technol* 49, 269-275.

Surman-Lee, S., and Bentham, R. (2006). Environmental Sampling Data to Determine Risk: A United Kingdom Perspective. In *Legionella: State of The Art 30 Years After Its Recognition*, N.P. Cianciotto, Y.A. Kwaik, P.H. Edelstein, B.S. Fields, D.F. Geary, T.G. Harrison, C.A. Joseph, R.M. Ratcliff, J.E. Stout, and M.S. Swanson, eds. (Washington D.C., ASM Press), pp. 543-548.

Surman, S., Morton, G., Keevil, B., and Fitzgeorge, R. (2002). *Legionella pneumophila* proliferation is not dependent on intracellular replication. In *Legionella*, R. Marre, Y.A. Kwaik, C. Bartlett, N.P. Cianciotto, B.S. Fields, M. Frosch, J. Hacker, and P.C. Lück, eds. (Washington DC, ASM Press, American Society for Microbiology), pp. 86-89.

Tang, K.-H., Barry, K., Chertkov, O., Dalin, E., Han, C., Hauser, L., Honchak, B., Karbach, L., Land, M., Lapidus, A., *et al.* (2011). Complete genome sequence of the filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus*. *BMC Genomics* 12, 334.

Tartakovskiy, I.S., Demina, Y., Voronina, O.L., Alyapkina, Y.S., and Karpova, T.I. (2009). From large community outbreak in Verhnaya Pyhma to effective prevention of legionellosis in Russia. Paper presented at: *Legionella* 2009 (Paris, France).

Temmerman, R., Vervaeren, H., Nosedá, B., Boon, N., and Verstraete, W. (2006). Necrotrophic Growth of *Legionella pneumophila*. *Applied and Environmental Microbiology* 72, 4323-4328.

Tolker-Nielsen, T., and Molin, S. (2000). Spatial Organization of Microbial Biofilm Communities. *Microbial Ecology* 40, 75-84.

van der Kooij, D., Veenendaal, H.R., and Scheffer, W.J. (2005). Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. *Water Res* 39, 2789-2798.

Verhoeven, A.B., Durham-Colleran, M.W., Pierson, T., Boswell, W.T., and Van Hoek, M.L. (2010). *Francisella philomiragia* biofilm formation and interaction with the aquatic protist *Acanthamoeba castellanii*. *Biol Bull* 219, 178-188.

Vervaeren, H., Temmerman, R., Devos, L., Boon, N., and Verstraete, W. (2006). Introduction of a boost of *Legionella pneumophila* into a stagnant-water model by heat treatment. *FEMS Microbiology Ecology* 58, 583-592.

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 nonhospital plumbing fixtures. *Applied and environmental microbiology* 43, 1104-1110.

- Wainwright, M., Canham, L.T., al-Wajeih, K., and Reeves, C.L. (1999). Morphological changes (including filamentation) in *Escherichia coli* grown under starvation conditions on silicon wafers and other surfaces. *Lett Appl Microbiol* 29, 224-227.
- Warren, W.J., and Miller, R.D. (1979). Growth of Legionnaires' disease bacterium (*Legionella pneumophila*) in chemically defined medium. *J Clin Microbiol* 10, 50-55.
- Watnick, P., and Kolter, R. (2000). Biofilm, city of microbes. *J Bacteriol* 182, 2675-2679.
- Watson, J.H., and Sun, C.N. (1981). Scanning electron microscopy of *Legionella pneumophila* in clinical tissue. *Scan Electron Microsc*, 55-64.
- Wingender, J., and Flemming, H.C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. *Int J Hyg Environ Health* 214, 417-423.
- Wong, M.C., Ewing, E.P., Jr, Callaway, C.S., and Peacock, W.L., Jr (1980). Intracellular multiplication of *Legionella pneumophila* in cultured human embryonic lung fibroblasts. *Infect Immun* 28, 1014-1018.
- Wright, J.B., Ruseska, I., Athar, M.A., Corbett, S., and Costerton, J.W. (1989). *Legionella pneumophila* grows adherent to surfaces in vitro and in situ. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* 10, 408-415.
- Xu, K.D., Stewart, P.S., Xia, F., Huang, C.T., and McFeters, G.A. (1998). Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl Environ Microbiol* 64, 4035 - 4039.
- Yang, L., Liu, Y., Wu, H., Hoiby, N., Molin, S., and Song, Z.J. (2011). Current understanding of multi-species biofilms. *Int J Oral Sci* 3, 74-81.
- Yang, L.H., Xiong, H., Lee, O.O., Qi, S.H., and Qian, P.Y. (2007). Effect of agitation on violacein production in *Pseudoalteromonas luteoviolacea* isolated from a marine sponge. *Letters in Applied Microbiology* 44, 625-630.
- Young, K.D. (2006). The Selective Value of Bacterial Shape. *Microbiol Mol Biol Rev* 70, 660-703.
- Young, K.D. (2007). Bacterial morphology: why have different shapes? *Current Opinion in Microbiology* 10, 596-600.

Yu, V.L., Plouffe, J.F., Pastoris, M.C., Stout, J.E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C.M., Paterson, D.L., *et al.* (2002). Distribution of *Legionella* Species and Serogroups Isolated by Culture in Patients with Sporadic Community-Acquired Legionellosis: An International Collaborative Survey. *Journal of Infectious Diseases* 186, 127-128.