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Legionella pneumophila in the environment: the occurrence of a fastidious bacterium in oligotrophic conditions

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Abstract

Legionella pneumophila, a micro-organism encountered in aquatic environments, can cause serious intracellular infections among humans. Since the bacterium is ubiquitous in aquatic habitats, it appears to be impossible to prevent *L. pneumophila* from entering man-made water systems. However, many questions concerning the survival and/or growth in the environment, the partners and opponents of *L. pneumophila* remain unanswered. This review focuses on the factors governing the ecology of *L. pneumophila*, since there is considerable divergence and even contradiction in literature on its environmental requirements. A key question to be resolved is the discrepancy between the fastidious nature of *L. pneumophila* in axenic cultures (e.g. 400 mg l^{-1} L-cysteine and 250 mg l^{-1} ferric iron) and the nutritionally poor environments in which it is commonly detected. It is assumed that dense microbial communities, as occurring in sediments and biofilms – but not likely in surface and drinking water, – can provide the necessary growth requirements for *L. pneumophila*. However, most of the studies concerning *L. pneumophila* have led to the general opinion that the organism can only multiply in the aquatic environment as a parasite in certain protozoa. The discovery of the non-classical siderophore legiobactin also indicates that the iron requirement for survival and autonomous growth is not as high as has been assumed. It thus appears that in order to control *Legionella* in the environment, focus should be on the eradication of microbial hotspots in which *L. pneumophila* resides.

1. Introduction

Legionnaires' disease is one of the few respiratory diseases associated with the inhalation of aerosols. Its causative agents are bacteria belonging to the genus *Legionella*, with *Legionella pneumophila* being the most important bacterium within the genus (Reingold et al. 1984). Microbial communities, in which *L. pneumophila* persists, can have a radical impact on human health. Since person-to-person transmission has never been observed, the control measures to prevent the bacterium from spreading usually focus on the elimination of the pathogen from water installations. In this respect, the detection and analysis of *L. pneumophila* in complex environmental consortia have become increasingly important (Fields 1996; Abu Kwaik et al. 1998; Steinert et al. 2002).

Legionella spp. have first been characterised as autonomous organisms. Studies have therefore focused on the identification of their nutritional, physical and chemical requirements for growth (Vogel & Isberg 1999). As from the mid-1980s, the concept of *L. pneumophila* as an intracellular parasite of protozoa, first described by Rowbotham (1980), has been accepted in the scientific

community. *Legionella* spp. are described as being 'protozoonotic', i.e. naturally infecting protozoa. Although it is now generally accepted that *L. pneumophila* primarily exist within biofilms, only a limited number of studies have attempted to characterise the interaction of the bacterium within these complex matrices (Fields 1996; Fliermans 1996; Steinert et al. 2002). Most recent studies pay attention to the pathogenesis, the epidemiology, the clinical microbiology and some molecular aspects of *L. pneumophila*, rather than to the ecology of the bacterium.

The survival or growth of *L. pneumophila* probably depends on the influence of its partners and opponents, since the pathogen mostly persists in microbial hotspots such as multispecies biofilms (Rogers et al. 1994A; Fields 1996; Fliermans 1996; Murga et al. 2001; Steinert et al. 2002). The relation between biofilm formation and the multiplication of *L. pneumophila* has not yet been quantified and the prevention or abatement of biofilm formation has attracted limited attention as a control measure. Preventing the spread of *L. pneumophila* by limiting biofilm formation or the association of *L. pneumophila* in the biofilm requires a systematic and stringent approach to ensure the biosafety of water and materials. However such approach may be a promising control measure to lower the risk for the multiplication of *L. pneumophila* (van der Kooij et al. 2002). The knowledge about factors that contribute to the survival or active growth of *L. pneumophila* in its natural environment and in plumbing systems is very limited and considerably divergent. Therefore, the goal of this review is to scrutinize the current knowledge of the ecology of *L. pneumophila* enabling to take accurate actions concerning the *Legionella* policy.

2. Habitat

Legionella spp. ubiquitously occur in lakes and rivers, although the cell density in these natural habitats is usually very low (less than 1% of the total bacterial population, Fliermans et al. 1981; Atlas 1999). Their presence in natural systems enables them to enter man-made water systems, favouring their multiplication. Human infections occur exclusively by the inhalation of contaminated aerosols which can be produced by air conditioning systems, cooling towers, whirlpools, spas, fountains, ice machines, vegetable misters, dental devices, respiratory therapy equipment and shower heads (Lye et al. 1997; Schwartz et al. 1998; Atlas 1999). Clearly any system which releases small droplets of water ($<$5–10 μm) into the air can be a source of infection, next to some technical risk factors such as the presence of dead-end loops, stagnation in plumbing systems and periods of non-use or construction. Especially those water systems with a water temperature between 25 and 45 °C are a main risk factor for the growth of *Legionella* (Atlas 1999).

Legionella pneumophila in axenic cultures is remarkably fastidious. The primary isolation medium is supplemented with 400 mg l^{-1} L-cysteine and 250 mg l^{-1} ferric pyrophosphate, two

Table 1. Media used to culture *Legionella pneumophila*

Medium	Reference
Primary isolation medium	• Feeley et al. (1978)
	• Weaver and Feeley (1979)
Liquid medium	• Ristroph et al. (1980)
Chemically defined medium	• Pine et al. (1979)
	• Warren and Miller (1979)
	• Ristroph et al. (1981)
	• Reeves et al. (1983)
Semi-selective medium	• Edelstein and Finegold (1979)
	• Rathgeb et al. (1982)
Improved semi-selective medium	• Edelstein (1981)
Selenium-enriched medium	• Smalley et al. (1980)
Blood agar medium	• Dennis et al. (1981)

absolute growth requirements for *L. pneumophila* (Feeley et al. 1978; Weaver & Feeley 1979). Table 1 gives an overview of some of the media used to culture *Legionella pneumophila*. The fastidious nature in axenic cultures is contradictory to the nutritionally poor environment in which the bacterium is often detected (Steinert et al. 2002). There is plenty of evidence that in oligotrophic natural environments *L. pneumophila* is capable of obtaining its necessary supply of amino acids and organic carbon from other organisms such as photosynthetic algae and bacteria (Tison et al. 1980; Wadowsky & Yee 1983), heterotrophic bacteria (Toze et al. 1990) and protozoa (Rowbotham 1980; Tyndall & Dominque 1982). Furthermore, *L. pneumophila* has a fascinating ecology as intracellular parasite of various free-living freshwater protozoa (Abu Kwaik et al. 1998). They are also known to persist in biofilms which develop in building water systems, either with or without these protozoa (Rogers et al. 1994A; Fields 1996; Fliermans 1996; Murga et al. 2001; Steinert et al. 2002; van der Kooij et al. 2002). The survival in biofilms is discussed below. As yet, the only clear-cut preferred partner relationship of *L. pneumophila* is the relation with protozoa, e.g. *Hartmanella* as described in many studies. Inversely, there are indications that some bacteria can inhibit *L. pneumophila*, but the mechanisms have not been unravelled (Zanetti et al. 2000). In view of the potentially positive or negative interactions with other organisms, the important partners and opponents of *L. pneumophila* should be identified.

L. pneumophila can survive extreme ranges of environmental conditions. It has been shown that the bacterium survives in habitats with a wide range of physico-chemical parameters (Fliermans et al. 1981). However, the latter study did not show whether *L. pneumophila* could multiply in these conditions. In the study of Ohno et al. (2003), *L. pneumophila* exhibited survival up to 60 days without loss of cultivability in microcosms with a temperature of 25 °C. This suggested that this genus may have adapted to aqueous environments at low temperatures, although the preferred growth temperature in laboratory medium is 37 °C. Many studies, e.g. Rogers et al. (1994A) and Atlas (1999), have shown that *L. pneumophila* multiplies at temperatures ranging from 20 to 45 °C. Kusnetsov et al. (1996) reported that cell growth and metabolic activity decreased considerably in all strains of *L. pneumophila* at temperatures above 45 °C. However, metabolic activity was retained at 51.6 °C. Beyond the maximum temperature for cell growth, *L. pneumophila* could survive planktonically, retaining its metabolic activity, although its culturability was lost in an environment with a high temperature such as hot spring water (Ohno et al. 2003).

Studies (States et al. 1987; Lye et al. 1997; Schwartz et al. 1998; Atlas 1999; Zanetti et al. 2000; Ohno et al. 2003) have indicated that *Legionella* spp. may inhabit municipal drinking water supplies, the latter serving as pathways for the contamination of plumbing systems in hospitals and other buildings. The difficulties to detect *Legionella* spp. within these systems could be explained by their sporadic and limited occurrence. According to some researchers (Yee & Wadowsky 1982; Rogers et al. 1994A; Murga et al. 2001; Steinert et al. 2002) naturally occurring *L. pneumophila* are able to grow either planktonically or in dense microbial associations such as biofilms. Other studies (Lee & West 1991; Fields 1996; Abu Kwaik 1998) suggest that this opportunistic pathogen only replicates within protozoa or on rich laboratory media. However, most of the studies concerning *L. pneumophila* have led to the general opinion that the organism can only multiply in the aquatic environment as a parasite in certain protozoa.

3. What's for dinner?

3.1. *Sources of carbon, nitrogen and energy*

The knowledge of the growth requirements of *L. pneumophila* can have a major impact on control strategies for the prevention of Legionnaires' disease (Steinert et al. 2002). *Legionella* spp. are particularly metabolically active towards amino acids and their derivatives, which act as carbon and/or energy sources (George et al. 1980; Pine et al. 1986). Due to the absence of nitrate reduction and the probable impossibility to use ammonium, amino acids also serve as nitrogen source (Keen & Hoffman 1984). According to studies on the metabolism of *Legionella*, the genus is been considered fastidious because of its inability to metabolise carbohydrates or to grow on a range of

routine laboratory media (Pine et al. 1979; George et al. 1980; Müller 1981; Tesh et al. 1983; Franzus et al. 1984). It is rather ironic that *Legionella* spp. are referred to as fastidious bacteria, because they may grow luxuriantly in tap water and can multiply in the usually hostile environment of phagocytic cells.

To get more insight in its metabolic potential, a study using the BIOLOG® panel has been conducted to test its substrate utilisation (Mauchline & Keevil 1991). Apart from information about the substrates that were consistently metabolised by all of the *L. pneumophila* strains, the study also revealed that *L. pneumophila* has significant proteolytic activity and possesses esterase activity. The specifications of the aminopeptidases were in partial agreement with the amino acid requirements that previously had been described (Müller 1981). In natural conditions, all of the enzymes may be needed for the degradation of algal extracellular products in aquatic habitats. Also the virulence of *L. pneumophila* could be related to its enzyme activities, i.e. the ability to degrade peptides and proteins of the infected host. In comparison with *L. pneumophila*, other *Legionella* strains generally showed a more narrow metabolic potential in the BIOLOG® panel (Mauchline & Keevil 1991). None of the *Legionella* spp. tested reacted positively with any sugar within the panel. This is not unexpected since reports have indicated that the species is unable to use carbohydrates as carbon and/or energy sources.

The inconsistency in the amino acid requirements of *L. pneumophila* strains among the different studies and the difference in relative aminopeptidase activity with regard to the substrates is puzzling. According to some studies different amino acids are used as the major carbon and/or energy sources: L-cysteine (Pine et al. 1979, 1986), L-Serine and L-Threonine (George et al. 1980) and L-Glutamic acid, L-Serine, L-Threonine and L-Tyrosine (Tesh et al. 1983). Perhaps subtle variations in the media composition, technique or passage history of the strains explain the apparent discrepancy between the different studies (Müller 1981; Nolte et al. 1982; Pine et al. 1986). However, the key question to be resolved is the discrepancy between the fastidious nature of this organism in axenic laboratory cultures (nutrients provided at concentrations in the range of g l^{-1} (Reeves et al. 1983) relative to the poor nutritional environment in which it is commonly detected, e.g. surface waters in which the levels of assimilable organic carbon (AOC) ranges from 40 to 600 μg l^{-1} (Escobar et al. 2001).

3.2. *Suppliers of carbon, nitrogen and energy*

Legionella spp. are generally detected in natural environments of low nutrient content (Steinert et al. 2002). The exogenous supply of amino acids required by *L. pneumophila* implies that the bacterium in these natural habitats obtains these molecules from other micro-organisms producing them in excess or from decaying organic matter. Apparently, *L. pneumophila* can synthesize all other necessary chemical constituents *de novo* and has no vitamin requirements (Pine et al. 1986).

Research has revealed that three groups of micro-organisms promote *L. pneumophila* growth: (i) protozoa (Rowbotham 1980; Tyndall & Dominque 1982), (ii) algae (Tison et al. 1980; Pope et al. 1982; Hume & Hann 1984) and (iii) non-*Legionellaceae* bacteria (Wadowsky & Yee 1983; 1985; States et al. 1987; Kusnetsov et al. 1993). Protozoa, including free-living amoebae such as *Acanthamoeba* sp., *Hartmanella* sp., *Naegleria* sp. (Rowbotham 1980; Tyndall & Dominque 1982; Anand et al. 1993) and the ciliate *Tetrahymenae pyriformis* (Abu Kwaik et al. 1998) are essential for the growth of *L. pneumophila* in natural and man-made environments. Since many clinically relevant pathogens are associated with protozoa in the environment, it has been suggested that these host cells play an important role as a reservoir for these pathogens (Abu Kwaik et al. 1998). The protozoa do not only provide nutrients for the intracellular *L. pneumophila*, but also represent a shelter when environmental conditions become unfavourable. The interaction with protozoa could be the driving force in the evolution of the pathogenicity of *L. pneumophila* (Steinert et al. 2002). A study which has analysed the interaction between *L. pneumophila* and protozoa at the cellular and the molecular level, has shown that *L. pneumophila* possesses type IV pili, designated the competence and adherence-associated pilus, which may be involved in adherence of *L. pneumophila* to host cells or biofilms (Liles et al. 1998; Stone & Abu Kwaik 1998).

Besides protozoa, blue-green algae (Cyanobacteria) may support the growth of *L. pneumophila* in the outdoor aquatic environment. Tison et al. (1980) isolated *L. pneumophila* serogroup (sg) 1 from an algal–bacterial mat community, growing in a man-made thermal effluent. The isolate grew in association with the cyanobacterium *Fischerella* spp. over a pH range of 6.9–7.6 in a mineral salts medium at 45 °C. *L. pneumophila* could apparently use algal extracellular products – normally present in natural habitats – as its carbon and energy sources. Furthermore the study revealed that the growth of *L. pneumophila* depends upon active photosynthesis by *Fischerella* sp. and upon the presumably extracellular release of algal substrates and possible cofactors. The amount of photosynthetic products released extracellularly by the mat community used in this study ranged from < 1 to 6% of the total amount of CO_2 fixed photosynthetically. These observations confirm that the temperature, the pH and the nutritional requirements of *L. pneumophila* are not as stringent as those observed previously when cultured on complex media (Pine et al. 1979). Therefore, the rapid growth rates (mean doubling time of 2.7 h) – twice as rapid as that previously reported for growth on complex or defined media (doubling time 6–8 h), – of *L. pneumophila* in these associations could explain the apparently widespread distribution of the bacterium in natural and man-made habitats (Tison et al. 1980).

In addition, some microbial species in the water may play an important role in the control of *L. pneumophila*. Their influence can be either inhibiting or promoting. Toze et al. (1990) found that up to 32% of heterotrophic bacteria, isolated from chlorinated drinking water, inhibit the growth of *Legionella* spp. There is however evidence that some micro-organisms in natural and plumbing systems favour the growth of *L. pneumophila* by excreting organic compounds (Yee & Wadowsky 1982; Wadowsky & Yee, 1983, 1985). Some of these unidentified non-*Legionellaceae* bacteria which enhanced the growth of *L. pneumophila* have been shown to produce L-cysteine, one of the absolute growth requirements on culture media, or a related compound. Stout et al. (1985) showed the ability of environmental bacteria to provide L-cysteine or metabolic substitutes. The presence of these environmental bacteria, with the most prevalent ones being *Flavobacterium, Pseudomonas, Alcaligenes* and *Acinetobacter*, improved the survival of *L. pneumophila*.

The combination of sediment (scale and organic particulates) and its natural complement of living microbiota can act synergistically to improve the survival of *L. pneumophila* (Stout et al. 1985). The role of sediment in this synergistic effect was determined to be nutritional. Sediment, with a total organic carbon level of 128 mg l^{-1}, apparently stimulated the growth of all bacteria present (from 10^4 CFU ml^{-1} to 4.0×10^5 CFU ml^{-1} after three days of incubation at 37 °C), which in turn stimulated the growth of *L. pneumophila* with a factor 3. *L. pneumophila* did not survive in sediment-free suspension (supernatant) regardless of the presence of the associated environmental microbiota. This finding suggested that the microbiota is not sufficient to promote growth and indicated that *L. pneumophila* can not multiply planktonically. The lack of growth response by *L. pneumophila* in sterile sediment also excluded the direct effect of sediment as a growth promoter. Sediment, which is composed of mineral deposits and decaying plant matter (detritus), can be used as a nutrient source by many prokaryotic and eukaryotic organisms. The data of Stout et al. (1985) indicated however, that *L. pneumophila* is not a saprophytic organism capable of multiplying on dead or decaying organic matter, but that it requires the presence of both the organic matter and the saprophytic microbial association. This supports the hypothesis of *L. pneumophila* as being indigenous to natural microbial hotspots.

Another study, conducted by Wadowsky & Yee (1985), showed that a series of non-*Legionellaceae* bacteria do not support the growth of *L. pneumophila* in tap water. The subculture of *L. pneumophila* on artificial medium may have affected the ability of the organism to multiply in tap water. The differences in the mechanisms for multiplication of naturally occurring and artificial medium-grown bacteria need further examination. It is postulated that environmental isolates may rapidly degenerate under laboratory conditions, thus giving rise to very high nutritional demands. Indeed, it is conceivable that naturally occurring and medium-grown *L. pneumophila* may differ in the efficiency of amino acid

transport across cell membranes. Another explanation may be that the cultivation of non-*Legionellaceae* bacteria on artificial medium reduces their ability to produce or excrete the necessary nutrients required to support the growth of *L. pneumophila*. It is also possible that the non-*Legionellaceae* bacteria, recovered from the water stock culture merely support cell survival of *L. pneumophila* in tap water.

3.3. *Role of iron in survival and its link to pathogenesis*

Iron is thought to be a key requirement for *L. pneumophila*. The mineral plays an important role in microbial pathogenesis and physiology through its participation in diverse biological processes. Gram-negative bacteria usually need 0.3–1.8 μM iron for growth (James et al. 1995).

Iron was found to be a critical nutrient for the growth of *L. pneumophila* with 3.3 μM Fe^{3+} required for optimum growth (James et al. 1995). However, previous reports on the iron requirements of *L. pneumophila* vary considerably. While the primary isolation medium is supplemented with ferric iron, the chemically defined medium contains ferrous iron (Pine et al. 1979; Reeves et al. 1983). As determined on these bacteriological media, the iron requirement for *L. pneumophila* is 3–13 μM for minimal growth and >20 μM for optimal growth, varying with the strain. It has been argued that one potential reason for this unusually high level of iron is that *L. pneumophila* cytoplasm may contain a high concentration of an iron-containing aconitase (James et al. 1995, 1997). Moreover, it appears necessary for *L. pneumophila* to encounter an iron-rich environment prior to aerosolisation in order to induce the expression of a virulent phenotype (Viswanathan et al. 2000). Studies of James et al. (1995, 1997) clearly demonstrate the critical role of iron in modulating the physiology and virulence of *L. pneumophila* and further support the theory that multiple environmental factors participate in the coordinated regulation of the physiology and virulence of this intracellular pathogen.

In the aquatic environment the dominant form of iron, ferric hydroxide, is highly insoluble ($pK_{sp} \approx 38$). The maximum amount of uncomplexed Fe^{3+} in solution at biological pH is probably not higher than 10^{-18} M (Neilands 1995). Given the metallic nature of plumbing systems and the presence of *L. pneumophila* as a common contaminant of plumbing systems, the effects of metals leached from hot-water tanks and pipes on the survival and growth of *L. pneumophila* remain uncertain. However States et al. (1985) showed that lower levels of certain metals such as iron, zinc and potassium enhance growth of naturally occurring *L. pneumophila* in a hot-water tank. These metal plumbing components and associated corrosion products seem to be important factors in the survival and growth in drinking water plumbing systems. *L. pneumophila* survives in these potable water systems despite the presence of chlorine residuals typically found in municipal water supplies (States et al. 1987; Toze et al. 1990).

Little is known about the ability of *L. pneumophila* to scavenge iron from the environment. Moreover, the influence of the nature of iron supply on the physiology and virulence of this pathogen is poorly understood. In order to survive and compete in iron-restricted environments, many microorganisms have developed specific mechanisms for iron acquisition. The most common specific iron uptake system involves the synthesis of relatively low molecular-weight, high-affinity iron chelators called 'siderophores', which scavenge iron from the environment and make the mineral available to the microbial cell (Bossier et al. 1988; Neilands 1995). *L. pneumophila* under iron deprivation stress does not synthesize the common chemical types of siderophores (Reeves et al. 1983). The pathogen can not compete with or use such siderophores for the acquisition of iron when they are present. Tison et al. (1980) have recovered *L. pneumophila* from natural colonies of cyanobacteria. Growth in close association with these colonies could conceivably provide *L. pneumophila* with the required concentrations of the necessary amino acids and trace metals such as iron. This kind of environment would preclude the stress needed to develop a siderophore in this organism (Reeves et al. 1983). However, later it was demonstrated that *L. pneumophila* elaborates a non-hydroxamate, non-phenolate siderophore (legiobactin), the expression of which is subject to a form of growth phase regulation (Liles et al. 2000). The discovery of legiobactin and its promotion of growth in iron-deleted chemically defined medium indicate that the *L. pneumophila* requirement for iron is not as high as has been assumed; it may be even below 1 μM.

L. pneumophila also obtains iron during intracellular growth in the EMB phagosome within mammalian macrophages and within protozoa, the mechanism behind this is not yet known (Abu Kwaik et al. 1998). Investigations by Barker et al. (1993) using cells grown within amoebae and in iron-deficient batch culture failed to detect the induction of specific membrane-associated iron uptake systems. Without this specific ability, the bacterium must depend upon the diffusion of iron carriers to its cell surface (James et al. 1995). *L. pneumophila* can proteolytically degrade transferrin and use the released iron in steady-state, iron-limited cultures (James et al. 1997). However, this indirect method of iron acquisition is unlikely to be relevant for intracellular growth, since the *L. pneumophila* phagosome does not contain transferrin and the bacterium itself does not bind transferrin (Viswanathan et al. 2000). Binding of lactoferrin, which is similar to transferrin, has been detected, but it does not lead to iron assimilation (Pope et al. 1996). Some other important mechanisms for acquiring iron include specific iron acquisition genes, which are regulated by the transcriptional ferric uptake regulator: (i) a methyltransferase *iraA* (Pope et al. 1996; Viswanathan et al. 2000), (ii) a putative iron peptide transporter *iraB* (Viswanathan et al. 2000), (iii) the inner-membrane cytochrome c biogenesis system *ccmC* (Viswanathan et al. 2002), (iv) a locus *hbp* that promotes hemin binding (Pope et al. 1996), (v) two internal ferric reductases (Poch & Johnson 1993) and (vi) genetic loci encoding for a hydroxamate biosynthetic gene and a pyoverdin-like siderophore (Steinert et al. 2002).

As yet, there is no established aetiology between the presence of iron either as metal or as ion and the ecology of *L. pneumophila*. Neither is there a well-understood relation between iron and virulence. It therefore appears of interest to explore the potential that it is not principally the amount of ferric iron available to the bacterium, but the way it is present and rendered available that could be a key feature in the occurrence and virulence of this organism.

4. Environmental persistence

The environmental persistence of *L. pneumophila* is promoted by the ability to adapt to a variety of different ecological niches, either as planktonic cells, as free-living members of complex communities or as intracellular parasites of protozoa (Figure 1). The characteristics and consequences associated with these growth patterns are given in Table 2.

Table 2. Postulated growth patterns of *L. pneumophila*

Growth pattern	Characteristics	Consequences
Autonomous		
Axenic	• Fastidious • Extreme high requirements for certain growth factors, particularly L-cysteine and ferric iron	• Doubling time of 6–8 h
Mixed culture	• Acquiring amino acids and ferric iron from algae (Cyanobacteria), protozoa and/or other heterotrophic bacteria present in microbial hotspots or biofilms	• Growth with phenotypic plasticity • Survival for long periods
Protozoonotic	• Intracellular parasite • Receiving nutrients from host • Enterance by competence and adherence-associated pilus • Shelter against unfavourable environmental conditions	• Inducing virulence • PHB-rich phenotype • Stress-resistant phenotype • Altered morphology

4.1. *Biofilms and microbial hotspots*

Biofilms and microbial hotspots represent microbial life in aggregates. They comprise structured matrix-enclosed communities whose cells express genes in a pattern that differs profoundly from that of their planktonic counterparts. Biofilms can comprise a single or multiple microbial species and are developed on a range of (a) biotic solid–liquid, solid–air or liquid–air interfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surface of medical implants. Different bacterial species, including virulent pathogens may be concentrated and harboured inside biofilms, which then become responsible for a variety of common afflictions (Stoodley et al. 2002).

Within drinking water pipes, accumulations of organisms are living of the meagre nutrients which are available in tap water, e.g. AOC levels in the order of 1–30 μg l^{-1} (Charnock & Kjonno 2000). Although mostly harmless to human health, these accumulations of microbial cells are difficult to remove and nearly impossible to prevent. Various investigators (Rogers et al. 1994A; Fields 1996; Fliermans 1996; Murga et al. 2001; Steinert et al. 2002; van der Kooij et al. 2002) have stated that *L. pneumophila* is able to multiply in such biofilms on the liquid-bathed surfaces of different kinds of water systems with certain protozoa grazing on the biofilm, serving as hosts. These studies have explored whether *L. pneumophila* is able to grow or only to survive in biofilms. Attention is paid to the influence of the surface material (Keevil et al. 1993; Rogers et al. 1994B; Murga et al. 2001), to the water characteristics (Kusnetsov et al. 1993; Zanetti et al. 2000) and to the water-air interface (Preston et al. 2001). In a study of Rogers et al. (1994B) filtersterilised tap water was used to culture a naturally occurring association of microorganisms including virulent *L. pneumophila*. At 20 °C *L. pneumophila* accounted for a low proportion of the biofilm microbiota on polybutylene and chlorinated polyvinyl chloride, but was absent in biofilms attached to copper surfaces. The pathogen was most abundant in biofilms on plastics at 40 °C, where it accounted for up to 50% of the biofilm microbiota based on plate counting. Furthermore, the pathogen was able to survive in biofilms on the surface of the plastic materials at 50 °C. These data support the notion that iron-materials are not essential for the proliferic growth of *L. pneumophila*. In another study, Keevil et al. (1993) studied biofilm formation on various plumbing construction materials using a natural inoculum consisting of a diverse range of bacteria, fungi and protozoa, including principally *Alcaligenes*, *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Methylobacter*, *Vibrio*, *Pseudomonas* spp.,

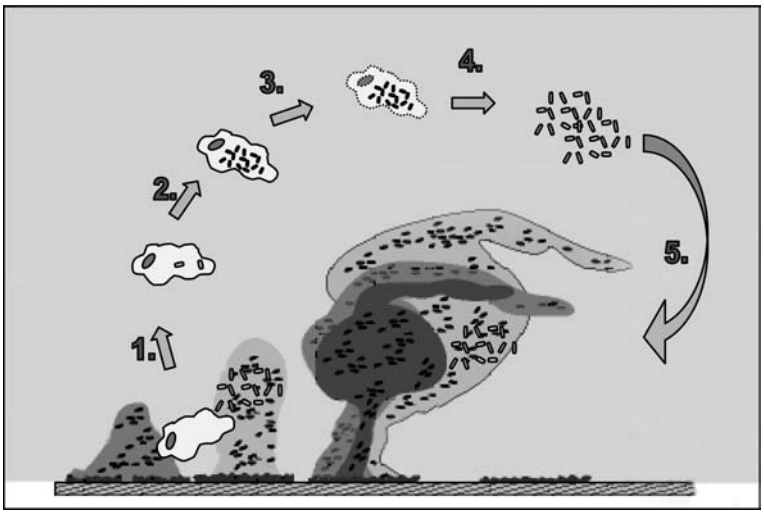

Figure 1. Proposed ecological niches for *L. pneumophila*: (1) *L. pneumophila* infects protozoa grazing on the biofilm in which *L. pneumophila* resides, (2) intracellular replication within protozoa, (3) lysis of the host cell, (4) planktonic survival and (5) free-living members in complex communities such as biofilms and microbial hotspots.

Actinomycetes, amoebae and ciliates. It was concluded that the substratum material can promote the growth of *L. pneumophila* by providing nutrients to the microbial consortium. Murga et al. (2001) showed the presence of *L. pneumophila* in biofilms on stainless steel, although unable to replicate in the absence of protozoa, *L. pneumophila* was able to persist.

Although it is generally accepted that *L. pneumophila* persists in biofilms, there is no direct relationship between the biofilm count and the number of pathogenic cells incorporated in the biofilm (Keevil et al. 1993). Recent studies have focused on dental-unit water systems. In the tubing of these systems *L. pneumophila*, *Mycobacterium* spp., *Candida* spp. and *Pseudomonas* spp. have been detected (Zanetti et al. 2000). The authors have studied the relation between these bacteria and some physical, chemical and microbiological characteristics of the water. *L. pneumophila* is widespread in low densities in natural water but its number increases in artificial habitats (21.8% of the tested dental units were positive for *L. pneumophila* (Zanetti et al. 2000)) due to the protection of the matrix of a biofilm or a microbial hotspot often developed in these systems. However, Kusnetsov et al. (1993) found that the number of bacteria and the nutrient concentrations were generally lower in *L. pneumophila*-positive cooling towers than in -negative systems.

Attention should also be paid to the formation of microbial associations at the water–air interface. Amoebae docked at the water–air interface remain and flourish there (Preston et al. 2001). Since they are important for the intracellular survival of *L. pneumophila*, they could be at the origin of the existence of *L. pneumophila* at such an interface.

In the first step of biofilm growth bacterial cells and a certain kind of exopolymeric substances (EPS) are needed to attach the substratum. The amount of EPS synthesis within the biofilm will greatly depend on the availability of carbon substrates and on the balance between carbon and other limiting nutrients. The presence of one species producing copious amounts of EPS may enhance the stability of other cell types even if they do not synthesise EPS themselves (Sutherland 2001). Thus, although *L. pneumophila* would not be able to synthesise EPS, it can be entrapped in biofilms in association with other micro-organisms.

Besides the EPS production, some specific structural components have been shown to play a critical role in facilitating bacterial interaction with surfaces, including flagella, pili and adhesion molecules. The flagellum of *L. pneumophila* may be considered as a factor that positively affects the early infection process of host cells by the bacterium (Pruckler et al. 1995). Pili and pilus-associated adhesions have been shown to be important for the adherence to and the colonisation of surfaces. Type IV pili are used by bacterial pathogens to attach to surfaces and epithelial cells. However, they can also play a role in the attachment in biofilms. The presence of a type IV pilin gene and its expression by *L. pneumophila* may provide an advantage for the colonisation of lung tissues during Legionnaires' disease, the invasion of amoebae in the environment and the adherence to biofilms (Stone & Abu Kwaik 1998). Next to the type IV secretion system, *L. pneumophila* also contains a type II general secretion pathway required for growth in amoebae (Steinert et al. 2002). Membrane proteins and bacterial extracellular polysaccharides may also influence bacterial attachment processes and initial biofilm development, since these factors contribute to cell surface charge, which affects electrostatic interactions between bacteria and the substratum (Stoodley et al. 2002).

Various protocols have been examined for the control of *L. pneumophila* in potable water supplies including chlorination, heat, copper/silver-ionisation, monochloramine, electrolytic disinfection and ultraviolet light. None of these protocols have shown to be successful, probably because they generally only temporarily decrease microbial growth in biofilms or microbial hotspots. Several biocides (e.g. Bronopol, Kathon) that showed promising results in the laboratory have been less efficacious *in situ* (Kim et al. 2002). Surface-associated bacteria tend to be more resistant to biocides and antibiotics than their corresponding planktonic counterparts. The resistance to antimicrobial killing of sessile bacteria versus their planktonic counterparts arises from their ability to grow in glycocalyx-enclosed micro-colonies (Stoodley et al. 2002). A promising development may be the quenching of the intercellular communication, so-called quorum sensing (Hentzer & Givskov 2003). Although it is still a question whether quorum sensing is a specific component of biofilm or microbial hotspot development, these

findings suggest a possible approach to control biofilm formation. Proof of signal molecule production by *L. pneumophila* species has heretofore been lacking.

4.2. *Protozoa*

One of the most complex environments encountered by facultative intracellular pathogenic bacteria is the intracellular environment of the host cell. Intracellular bacterial pathogens that replicate within phagosomes are simultaneously exposed to multiple signals. They respond to these signals by a global alteration in protein synthesis that involves elevated levels of several stress-induced proteins (Barker et al. 1993).

Outside the amoebal host, *L. pneumophila* encounters stressful environmental conditions, such as limited nutrient availability. Intra-amoebal growth is believed to promote the extracellular survival by inducing a stress-resistant phenotype, characterised by altered morphology and envelope composition and by increased resistance towards biocide inactivation, including chlorine treatment (Barker et al. 1993; Abu Kwaik et al. 1997; James et al. 1999). In the amoebae, *L. pneumophila* can accumulate significant intracellular reserves (between 6 and 18% of cell dry weight) of poly-3-hydroxybutyrate (PHB), which promote its long-term survival up to 600 days under conditions of starvation (James et al. 1999). A study has demonstrated that all *Legionella* strains metabolise the monomer β-hydroxybutyrate (Mauchline & Keevil 1991). This PHB-rich phenotype may play a significant role as carbon and energy storage compound for survival in oligotrophic environments.

5. Survival strategies

5.1. *Viable but non-culturable state*

To adapt to a stressful environment, bacteria often enter a 'temporarily dormant-like non-culturable state', in which they regulate cell differentiation to adapt to different stress conditions and then resuscitate when environmental conditions become favourable for growth. This change is generally referred to as 'viable but non-culturable (VBNC)'. As a number of other Gram-negative bacteria, *L. pneumophila* is able to enter such a state. They can potentially survive as free organisms for long periods up to 600 days by maintaining metabolic activity, but they temporarily lose culturability. Yet, they may require resuscitation by ingestion by free-living amoebae or by injection into embryonated eggs (Ohno et al. 2003). This temporary loss of culturability is a side-effect of an effective strategy for survival in an aqueous environment (Steinert et al. 2002). Thus, it is important to include methods for the detection of VBNC bacteria when testing environmental and clinical samples for purposes of public health safety.

5.2. *Phenotypic plasticity*

Adaptations, resulting in the loss of metabolic activity associated with the loss of culturability, may be affected by the expression of specific stress-related genes. Actually, very little evidence is available indicating that environmental stress signals lead to gene activation or genetic rearrangements facilitating adaptation to environmental changes. Intraclonal polymorphism, sometimes called 'phenotypic plasticity', resulting in changes in fimbrae nature, membrane protein composition and EPS production have been observed. However, hardly any information is available about the signals causing such phenotypic variations (Bossier & Verstraete 1996).

The phenotypic plasticity of *L. pneumophila* contributes significantly to the transmission and virulence of the pathogen (Lüneberg et al. 2001). It may also relate to its high nutritional demands, when grown in axenic cultures. Recently, phase variable expression of a lipopolysaccharide (LPS) epitope in *L. pneumophila* sg 1 strains has been reported to be associated with changes in virulence properties in human macrophage-like cell line HL60 and in *Acanthamoeba castellanii*. The molecular mechanism, responsible for LPS phase variation and loss of virulence, has been attributed to chromosomal insertion and excision of an unstable 30-kb genetic element presumably of phage origin. The selective advantages of phase variation remain to be investigated (Lüneberg et al. 2000, 2001).

Next to phenotypic plasticity, also genetic diversity provides a mechanism for populations to adapt to their ever-changing environment. Cazalet et al. (2004) showed that the genetic mobility may

enhance the versatility of *L. pneumophila*. Numerous genes encode eukaryotic-like proteins or motifs that are predicted to modulate host cell functions to the pathogen's advantage. The genome thus reflects the history and lifestyle of *L. pneumophila*.

5.3. *Pigmentation*

Pigmentation also contributes to the ecological adaptation of *L. pneumophila* (Steinert et al. 1995). In the host *Hartmanella vermiformis*, the pigment legiolysin might exert its protective effect either by serving as a scavenger molecule for oxygen radicals or by damaging the host and thereby eliminating its antimicrobial activities. It is not involved in intracellular multiplication of *L. pneumophila* in *H. vermiformis*. The pigmentation of *L. pneumophila* seems to be important for the survival of cells stressed by light, but does not have any influence on the virulence of *L. pneumophila* cells in guinea pigs or the infection of U937 macrophage-like cells (Steinert et al. 1995).

6. Conclusions and further research questions

There is a discrepancy between the growth requirements of *L. pneumophila* for certain amino acids and the amount of ferric iron in axenic cultures and in the environmental sites in which the bacterium is commonly detected. Moreover, the organism demonstrates a considerable form of phenotypic plasticity, which needs to be better understood. Hence, it is important to focus on the following ecological questions:

- Can one characterise a set of micro-organisms constituting a microbial hotspot, which may provide the niche for *L. pneumophila* by providing its necessary nutrients and growth factors, or inversely which antagonise its cohabitation?
- Can one explore how long the strain has to thrive in an iron-rich environment before inducing virulence? What is the role of other micro-organisms in providing bio-available iron?
- Is the proposed phenotypic plasticity in terms of virulence related to the way the bacterium is provided with nutrients and growth factors? Furthermore, to what extent is the genomic expression related to the presence of other partner micro-organisms?
- Is the control of *L. pneumophila* to be sought in the eradication of the species as such and in the prevention of microbial hotspots in general, as is currently the standard practice? Or alternatively, can more sustainable approaches be developed based on a better understanding of the ecological situations in which the bacterium thrives and becomes subject to induce virulence?

These questions constitute a major challenge, but in view of the major investments currently made in the sanitation and water industry to eradicate this bacterium, they certainly warrant proper consideration.

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