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FEMS Microbiology Reviews 26 (2002) 149–162

FEMS MICROBIOLOGY Reviews

www.fems-microbiology.org

Legionella pneumophila: an aquatic microbe goes astray

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Received 30 October 2001; received in revised form 19 February 2002; accepted 27 February 2002

First published online 24 April 2002

Abstract

Legionella pneumophila is naturally found in fresh water were the bacteria parasitize within protozoa. It also survives planctonically in water or biofilms. Upon aerosol formation via man-made water systems, *L. pneumophila* can enter the human lung and cause a severe form of pneumonia, called Legionnaires' disease. The pathogenesis of Legionnaires' disease is largely due to the ability of *L. pneumophila* to invade and grow within macrophages. An important characteristic of the intracellular survival strategy is the replication within the host vacuole that does not fuse with endosomes or lysosomes. In recent times a great number of bacterial virulence factors which affect growth of *L. pneumophila* in both macrophages and protozoa have been identified. The ongoing *Legionella* genome project and the use of genetically tractable surrogate hosts are expected to significantly contribute to the understanding of bacterium–host interactions and the regulation of virulence traits during the infection cycle. Since person-to-person transmission of legionellosis has never been observed, the measures for disease prevention have concentrated on eliminating the pathogen from water supplies. In this respect detection and analysis of *Legionella* in complex environmental consortia become increasingly important. With the availability of new molecular tools this area of applied research has gained new momentum.

Keywords: Environment; Survival in protozoa; Fitness; Virulence; Detection; *Legionella pneumophila*

Contents

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0168-6445/02/$22.00
PII: S0168-6445(02)00093-1

1. Introduction

The transmission of pathogens by water is mostly the result of fecal pollution [1]. However, this is not the case for *Legionella pneumophila*, a common etiological agent of severe bacterial pneumonia, called Legionnaires' disease [2]. This environmental bacterium inhabits fresh waters, where it parasitizes intracellularly within free living protozoa [3,4]. Upon transfer from natural aquatic habitats into drinking water systems *Legionella* can impose life threatening health risks. Especially warm water systems provide an ideal habitat for massive growth of the bacterium [5]. Due to a higher percentage of elderly and immunocompromised people in the human society the number of people who are particularly susceptible to *Legionella* infection has increased. The investigation of a number of epidemic and sporadic cases has shown that *L. pneumophila* is a common cause of both community-acquired and nosocomial pneumonia [6]. Since the first documented outbreak of legionellosis in Philadelphia in 1976, more than 42 *Legionella* species have been described [7]. *L. pneumophila* which has originally been isolated during this outbreak is still responsible for most of the infections. However, 17 additional species have been associated with disease. Intensive efforts in subtyping and virulence testing revealed differences in infectivity even for different strains of the same serotype. Results from epidemiological studies showed that infection control is only possible by interference with the transmission at many points of the infection route [8]. Therefore, an integrated view of *Legionella* ecology together with clinical and genetic aspects appear to be necessary to establish effective prevention measures.

2. Ecology of *Legionella*

Studies on the distribution of *Legionella* show that the Gram-negative, aerobic, monopolarly flagellated rod that measures 0.5 μm in width and 2 μm in length is part of the natural aquatic environment [2]. They use amino acids as carbon and energy sources and do not oxidize or ferment carbohydrates [9]. It can not be excluded that legionellae grow planctonically or in biofilms. However, a number of studies suggest that this pathogen only replicates within protozoa or on laboratory media [4,10]. Since *Legionella* is ubiquitous in aquatic habitats it appears to be impossible to prevent *Legionella* from entering man-made water systems. It is without doubt that the exact knowledge of the growth requirements of *Legionella* will have great impact on strategies for disease control.

2.1. Natural and man-made habitats

Legionellae occur ubiquitary in lakes and rivers, although the concentration of *Legionella* in these natural habitats is usually low. Especially, aquatic biofilms are widespread ecological niches in which *Legionella* proliferates. Elevated temperature, inorganic and organic contents of the water and the presence of host protozoa play key roles in their growth and spreading [11]. The concerted influence of these factors may explain why *Legionella* increases in density in artificial habitats such as man-made warm water systems. The highest numbers of *Legionella* are found in water samples with temperatures of 30–40°C [12]. Human infection occurs exclusively by inhalation of contaminated aerosols which can be produced by air conditioning systems, cooling towers, whirlpools, spas, fountains, ice machines, vegetable misters, dental devices and shower heads [13]. In addition the presence of dead-end loops, stagnation in plumbing systems and periods of nonuse or construction have been shown to be technical risk factors [13,14]. Also the material of the piping system has been shown to influence the occurrence of high bacterial concentrations. In this respect the use of copper as plumbing material may help to minimize the risk of Legionnaires' disease whereas plastic materials support high numbers of *L. pneumophila* [15].

2.2. Legionella–protozoa interactions

Protozoa species, which can be distinctive in a variety of environmental settings, are essential for the growth of *Legionella* in natural and man-made environments [4]. Therefore, the presence of *Legionella* in these environments also appears to depend on the spectrum of host protozoa that can be utilized. However, the knowledge of host specificity of most *Legionella* species and the respective growth requirements of the host protozoa is still very limited. *Acanthamoeba*, *Hartmannella*, and *Naegleria* are most commonly isolated from *Legionella*-contaminated plumbing systems. Other species affiliated with *Legionella* are *Saccamoeba*, *Vexillifera* and *Platyamoeba*. *Tetrahymena pyriformis* appears to be important for *Legionella longbeachae* which is also found in soil [4,16]. Whether the adaptation to a specific host correlates with virulence and the epidemiologic prevalence of *L. pneumophila* remains an interesting question still to be answered. Since many clin-

ically relevant pathogens (*Listeria*, *Mycobacterium*, *Chlamydia*, *Vibrio*, *Burkholderia*, Rickettsiales and certain coliforms) are associated with protozoa in the environment it has been suggested that protozoa play an important role as reservoirs for these pathogens [17–20].

Protozoa do not only provide nutrients for the intracellular legionellae, but also represent a shelter when environmental conditions become unfavorable. Particularly inside *Acanthamoeba* cysts the bacteria are able to survive high temperatures, disinfection procedures and drying [21–23]. Similar effects have been observed with expelled vesicles from *Acanthamoeba* containing live cells of *L. pneumophila* [24]. Similar to a number of other Gram-negative bacteria *Legionella* is able to enter a viable but non-culturable (VBNC) state [25]. In laboratory microcosms it could be demonstrated that re-entry of *Legionella* into the culturable state can occur after the uptake by the natural host *Acanthamoeba castellanii* [26].

Beyond protection and reactivation from dormancy *Legionella* may also use protozoa to colonize new habitats. In this regard inhaled protozoa seem to represent also a vehicle for effective transmission to humans [27,28]. By using various model systems it was shown that the interaction of *Legionella* and protozoa contributes to the infection process itself. After intracellular replication within protozoa *L. pneumophila* exhibits a higher stress resistance [3,29]. Mice inoculated with *Legionella* and *Hartmannella* develop more severe symptoms than those infected with either the bacterium or the amoeba alone [29]. However, the underlying mechanisms of this phenomenon are not yet understood. It has been hypothesized that the physiological adaptation to the intracellular environment and also the adherence of amoebic constituents to the bacterial surface may contribute to this effect [30].

The interaction of *L. pneumophila* and protozoa has been analyzed at the cellular and the molecular level. The results show that *L. pneumophila* possesses type IV pili, designated the competence and adherence-associated pilus (CAP), which may be involved in adherence of *Legionella* to host cells or biofilms [31]. However, coculture assays have shown that entry rather than attachment appears to be the limiting step in the infection of *Hartmannella* by *L. pneumophila* [32]. Treatment of *Hartmannella vermiformis* with an inhibitor of microfilament-dependent phagocytosis (cytochalasin D) does not inhibit uptake of *L. pneumophila*. However, bacterial uptake was strongly reduced by methylamine, an inhibitor of adsorptive pinocytosis, and by cycloheximide, an inhibitor of eukaryotic protein synthesis. In contrast to previously published results obtained with *Acanthamoeba polyphaga* [33] data reported recently suggest the requirement for host protein synthesis during bacterial uptake by *A. castellanii* [34]. This reduction in uptake was enhanced by simultaneous addition of cytochalasin D. These findings confirm the proposed heterogeneity of uptake mechanisms by different protozoan hosts [27,35,36]. Apparently *A. castellanii* uses multiple mechanisms for bacterial uptake, while *H. vermiformis* may use only receptor-mediated pinocytosis.

The signal transduction in the protozoan hosts which follows the attachment and invasion of *Legionella* still remains an interesting research topic. It has been described that attachment of *Legionella* to *Hartmannella* is mediated by a 170-kDa galactose/*N*-acetylgalactosamine-inhibitable lectin [37]. Upon attachment a very fast dephosphorylation of tyrosine-phosphorylated proteins including the 170-kDa receptor itself and cytoskeletal-associated proteins occur [10,38]. After internalization, the intracellular bacteria reprogram the endosomal–lysosomal degradation pathway of the host. The multiplication of *Legionella* within a maturation-blocked vacuole that fail to acidify and to fuse with lysosomes shows many similarities to the infection of human phagocytic cells. This includes the recruitment of rough endoplasmic reticulum which surrounds the membrane-bound vacuole [3,39,40]. Therefore, it has been suggested that the interaction with protozoa is the driving force in the evolution of the pathogenicity of *Legionella*.

3. Legionellosis

The diseases caused by *Legionella* are collectively termed legionellosis. Legionnaires' disease is the pneumonic form of legionellosis with an incubation time of 2–10 days, while the benign flu-like form is called Pontiac fever. It is estimated that legionellosis affects 25 000–100 000 persons annually in the United States [41]. In a series of studies from North America and Western Europe, 1–13% of all pneumoniae were associated with this pathogen [6]. Because of the difficulty in distinguishing these diseases from other forms of pneumonia and influenza, many cases go probably unreported. This assumption is supported by serologic surveys which show that many persons in an apparently healthy population have antibodies against legionellae [42]. The infection route of *L. pneumophila* is summarized in Fig. 1.

3.1. Epidemiology, symptoms and clinical manifestations

Epidemiological studies indicate that *Legionella* is an opportunistic pathogen. The case-mortality rate of adequately treated Legionnaires' disease varies from 7% to 24%, with elderly and immuno-compromised patients being most susceptible [8]. The observed differences in host susceptibility and bacterial virulence make it difficult to clearly define an infectious dose.

Legionnaires' disease begins with a mild cough, malaise, muscle aches, low fever and gastrointestinal symptoms. The later manifestations of disease are high fever, alveolitis and bronchiolitis. Considerable lung damage with patchy infiltrated regions can be observed by X-ray ra-

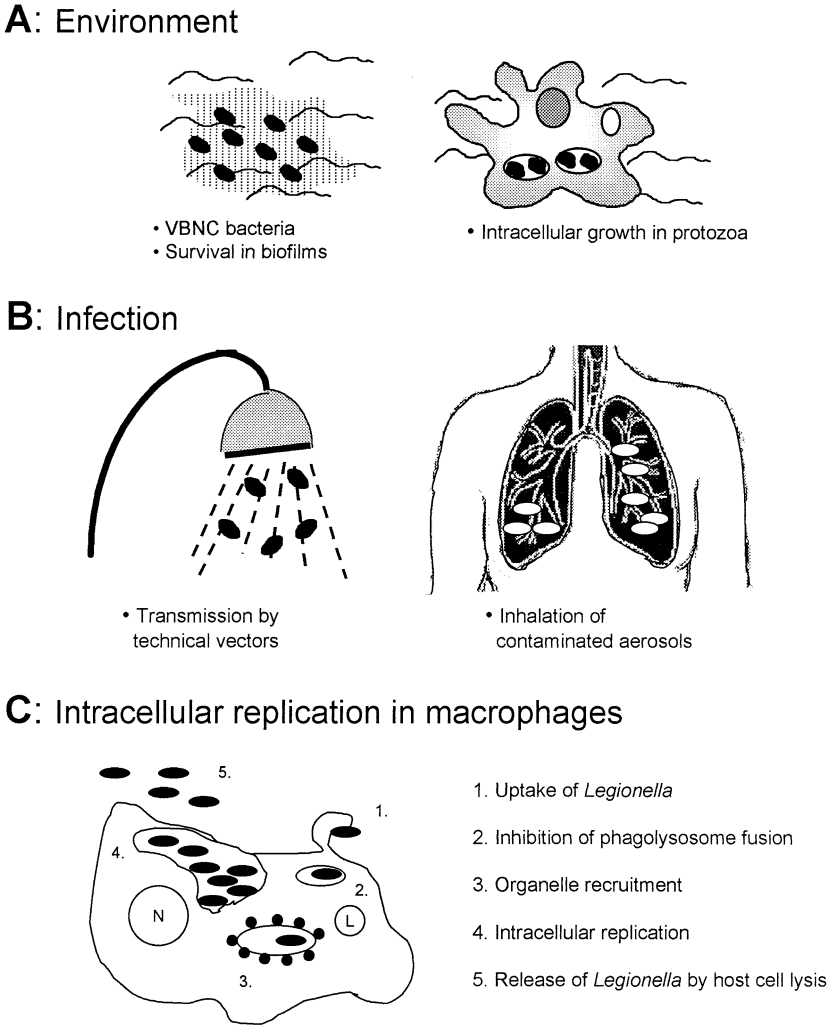

Fig. 1. Infection route of *L. pneumophila*. A: In the environment *Legionella* is able to enter a viable but non-culturable (VBNC) state. The bacteria persist in biofilms and grow within protozoa. B: Upon transmission by technical vectors (showers, air conditioning systems, cooling towers, etc.) *Legionella* colonizes the human respiratory tract. C: After uptake by macrophages *Legionella* replicates within a maturation-blocked vacuole. Finally the bacteria are released by host cell lysis. Abbreviations: N, nucleus; L, lysosome.

diography [2]. Histological reports describe intra- and extracellular bacteria in phagocytes, fibroblasts and epithelial cells [4].

The clinically distinct, self-limited and non-pneumonic Pontiac fever is a milder, influenza-like form of disease [43]. Pontiac fever patients seroconvert to *Legionella*, however the microbe has never been isolated [44]. Therefore it has been speculated that Pontiac fever is caused by VBNC forms of *Legionella* [26]. Other hypotheses to explain Pontiac fever include changes in virulence factors, toxic or hypersensitivity reactions [45].

3.2. Cellular and molecular features of Legionnaires' disease

Ultrastructural and molecular studies contributed significantly to our current understanding of how *L. pneumophila* establishes infection [10]. Two modes of entry have been observed: 'coiling' and 'conventional phagocytosis'. Coiling phagocytosis, in which a long pseudopod coil around the bacterium appears to be an occasional finding. The conventional phagocytosis of the bacteria into macrophages is mediated by opsonization with complement components C3 and C3bi on the bacterial cell surface protein MOMP (major outer-membrane protein) [46]. Virulent *L. pneumophila* strains are resistant to complement-mediated lysis and the bacteria bind to the macrophage complement receptors CR1 and CR3 which results in phagocytosis. This mode of entry limits the oxidative burst of the phagocyte and it has been proposed that *Legionella* inhibits superoxide generation via down-modulation of α and β protein kinase C isotypes [47]. Coating of *L. pneumophila* with specific antibodies results in an immunoglobulin Fc receptor-mediated phagocytosis. However, only about half of the bacteria taken up by this mechanism survive and replicate intracellularly [46]. Moreover, a less well-characterized opsonin-independent mode of entry has been described [31,48].

After entry, the bacteria reprogram the maturation pathway of the phagosome. The *Legionella*-harboring nascent phagosome sequentially recruits smooth vesicles, mitochondria and rough endoplasmic reticulum (rER) and does not fuse with lysosomes. Furthermore, the vacuolar acidification is reduced (for review see [49]). Early phagosomes (5 min post-infection) lack major histocompatibility complex (MHC) class I and class II molecules, alkine phosphatases and other membrane proteins. Additional cellular markers such as CD63, LAMP-1, LAMP-2, lysosomal cathepsin D, transferrin receptors and Rab7 are excluded from the phagosome during the course of intravacuolar growth of *Legionella*. At mid-log phase *Legionella* replicates by binary fission with a doubling time of approximately 2 h. This results in a host cell that is filled with bacteria. During the late replicative phase the *Legionella* phagosome merges with lysosomes without detrimental consequences for the enclosed bacteria (for review see [50]). After the exploitation of the host *Legionella* enters the post-exponential phase of growth in which motility and virulence traits that promote transmission to a new host are expressed.

The host defense responses are obviously triggered by chemokines and cytokines which are released by infected macrophages. Cultured IFN-γ-activated human monocytes inhibit replication of *L. pneumophila* and it has been shown that this effect can be reversed when the cells are supplemented with iron transferrin [51]. The killing and lysis of macrophages, monocytes and epithelial cells has been shown to occur in two phases. During the early stage of infection *Legionella* induces apoptosis [52]. This programmed cell death, which is mediated by the activation of caspase-3, is characterized by condensation of chromatin at the nuclear boundary and interchromosomal DNA cleavage [53]. In the post-exponential phase of growth *Legionella* causes necrosis of its host cell, appearingly by inducing pore formation [54,55].

4. Phenotypic plasticity

L. pneumophila exhibits a remarkable phenotypic plasticity. The VBNC state and two distinguishable phenotypes during the infection cycle have been described [26,50]. Meanwhile it is generally accepted that the virulence of *L. pneumophila* corresponds to sequential growth phases of the bacterium. Post-exponential phase bacteria which are released from a depleted host cell are short, thick, flagellated and highly motile. In addition, this phenotype is more resistant to biocides, antibiotics and it is more invasive and virulent in different infection models [21,56]. Within the reprogrammed, maturation-blocked vacuole, *L. pneumophila* alters its physiology and converts to a replicative form (exponential growth phase). These replicative bacteria are more sodium resistant, do not express flagella and display reduced cytotoxicity [50]. Without lessening the importance of specific virulence genes, it becomes increasingly evident that the phenotypic plasticity of *Legionella* contributes significantly to the transmission and virulence of the pathogen. Therefore, one has to consider that it is not only the expression of specific virulence factors of *L. pneumophila* which are responsible for the prevalence of pneumophila species in disease, but also certain specificities in physiology and gene regulation.

4.1. Stringent response and alternative sigma factors

Amino acid depletion and low temperature lead to the transition from the replicative to the infectious phase (Fig. 2). The conversion involves a stringent response-like mechanism in which uncharged tRNAs activate RelA, a guanosine 3′,5′-bispyrophosphate synthetase [50,57]. The following accumulation of ppGpp then coordinates the entry of bacteria into stationary and infectious phase. By analogy to *Escherichia coli* it has also been speculated that the accumulation of ppGpp increases the amount of the alternative sigma factor RpoS. In support of this hypothesis it has been observed that the expression of RpoS increases during the stationary phase of *Legionella* and apparently coordinates the expression of virulence traits [50,58].

The complex flagella assembly seems to be coordinatively regulated with other virulence-associated traits during the late stage of infection. Consistent with the need of *Legionella* to infect protozoan host cells in natural aquatic

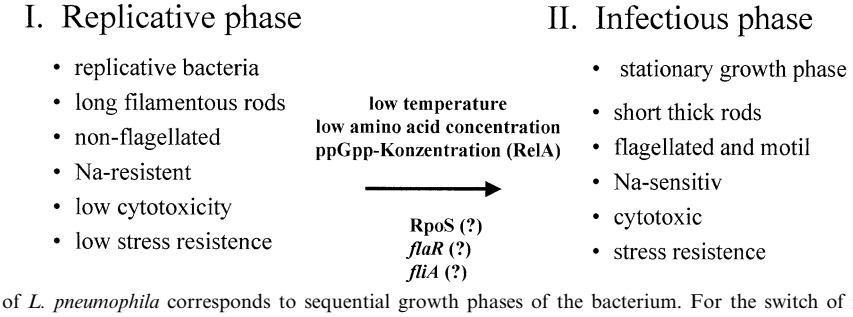

Fig. 2. The infection cycle of *L. pneumophila* corresponds to sequential growth phases of the bacterium. For the switch of replicative *Legionella* (I) during infection or exponential growth on artificial media to infectious stationary phase bacteria (II) a stringent response model has been proposed. Low temperature, amino acid starvation and accumulation of ppGpp appear to trigger the transition. The alternative sigma factors RpoS and FliA as well as the DNA binding protein FlaR may also contribute to this process.

habitats, flagellation, motility and piliation are optimal at temperatures below 37°C. The expression of the flagellar major subunit (*flaA*) gene which is influenced by different environmental factors is regulated at the transcriptional level by the alternative sigma-28 factor FliA and probably by FlaR, a regulator of the LysR family [59–62]. Phenotypically the overexpression of the *csrA* (carbon storage regulator) gene of *L. pneumophila* results in a reduction of flagellation, pigmentation and an altered cell morphology [63]. On a genetic level *csrA* overproduction was associated with a reduction of fliA and flaA transcripts. This suggests that *csrA* destabilizes the corresponding mRNA similarly to the *csrA* homolog in *E. coli* and the *rsmA* homolog in *Erwinia carotovora*.

4.2. Phase variation

Lipopolysaccharide (LPS) produced by *L. pneumophila* is a major immunogenic cell surface determinant that can activate both classical and alternative complement pathways [64,65]. Recently phase variable expression of a LPS epitope in *L. pneumophila* serogroup 1 strains has been reported to be associated with changes in virulence properties in the human macrophage-like cell line HL60 and in *A. castellanii* [66,67]. 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. In the virulent wild-type strain the 30-kb element is located on the chromosome whereas excision from the chromosome and replication as a high copy plasmid resulted in the non-virulent mutant phenotype. Excision from the chromosome is enhanced under in vivo conditions in the guinea pig model and upon serum incubation. However, the selective advantage of phase variation remains to be investigated.

5. Virulence factors and genome structure of *Legionella*

In addition to the description of phenotypic and physiologic changes as well as the implication of regulators of gene expression, much progress have been made toward identifying specific virulence factors, iron acquisition determinants and secretion systems [49]. The corresponding genes have traditionally been analyzed by mutagenesis. Now, with the advent of whole-genome sequencing the exhaustive identification of putative virulence genes becomes possible. Accordingly, the comparison of phylogenetically distantly and closely related genomes will be useful for the description of variations between strains and evolutionary processes.

5.1. Surrogate host systems

The epidemiologic comparison of environmental strains with those associated with disease may be the first step in the analysis of bacterial virulence. However, in order to analyze a particular virulence factor model systems of legionellosis are indispensable. The first isolation of *Legio-*

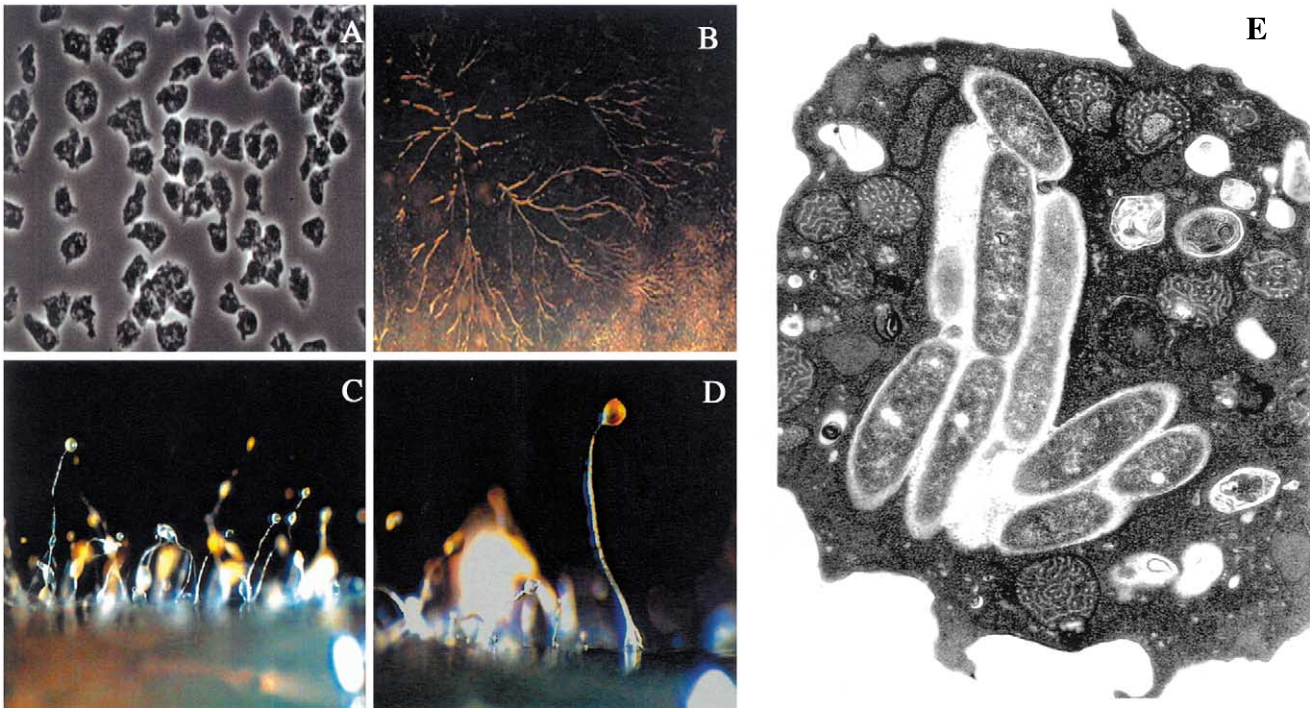

Fig. 3. *D. discoideum*, a new host model system to study *Legionella* pathogenicity. A–D: Development of uninfected *Dictyostelium* cells. A: Upon starvation single cells aggregate (B) and differentiate (C) into mature pluricellular fruiting bodies (D). This morphogenesis does not occur in *Dictyostelium* cells which are infected with *Legionella*. The transmission electron micrograph shows *L. pneumohila* within a vacuole of *D. discoideum* (E).

nella succeeded with the inoculation of guinea pigs and it was demonstrated that *Legionella* can be transferred to yolk sacs of embryonated hen eggs [68]. Although *Legionella* can infect other animals like mice, rats and hamsters, the guinea pig remains the most susceptible animal known. Infected guinea pigs exhibit symptoms like weight loss, fever, bronchopneumonia and death which closely resembles human disease [69,70].

In order to study the cellular and subcellular aspects of Legionnaires' disease polymorphonuclear neutrophils, alveolar macrophages, peripheral blood monocytes and epithelial cells are widely used. Also cell lines derived from human leukemias like the phagocytic U937 and HL60 cells, non-phagocytic HeLa, Vero and McCoy cells and axenically grown *Acanthamoeba*, *Hartmannella*, *Naegleria* have proven to be good model systems [4,71].

Recently it was found that simple model organisms like *Caenorhabditis elegans* and *Drosophila melanogaster* can reveal how bacteria infect cells [72,73]. This encouraged to initiate studies on developing genetically manipulatable host systems for *Legionella*. By using single cell stages of the amoeba *Dictyostelium discoideum* as host cells we and others have begun a molecular analysis of host cell functions and targets during *Legionella* infection [74–76]. *Dictyostelium* feeds on bacteria and upon starvation aggregates and differentiates into pluricellular fruiting bodies (Fig. 3). Besides its amenability to genetic manipulation, *Dictyostelium* expresses highly conserved cellular markers, and cell signaling pathways are well characterized. Moreover, the complete genome sequence will be available in the year 2002. Consequently, future strategies with the *Legionella–Dictyostelium* model will rely on a two-sided genetic approach.

5.2. Surface factors

Various surface structures including LPS (see Section 4.2) contribute to the pathogenicity of *Legionella*. The MOMP protein which is encoded by *ompS* binds C3 and C3bi of the complement system and mediates the uptake of *L. pneumophila* via the CR1 and CR3 receptors of the macrophage. Further attachment factors are the type IV pili which may mediate a complement-independent attachment to mammalian and amoebal host cells [31] and the 60-kDa heat shock protein Hsp60 [77]. The flagellum of *L. pneumophila* also positively affects the establishment of infection. However, this effect is not due to an improved attachment but on the positive effect for the encounter of the host cell as well as by enhancing the invasion capacity [78]. Growth kinetics with the *flaA*-negative mutant and the non-flagellated wild-type bacteria during the exponential growth phase demonstrated that the flagellum is not required for intracellular replication within host cells per se.

Immunogold techniques have shown that the Mip protein (macrophage infectivity potentiator) of *L. pneumophila* is exposed on the cell surface of extracellular grown bacteria. In *Acanthamoeba* infected with *Legionella* the Mip protein was also detected on host membranes which exhibited a multilamellar structure [79]. The 24-kDa Mip

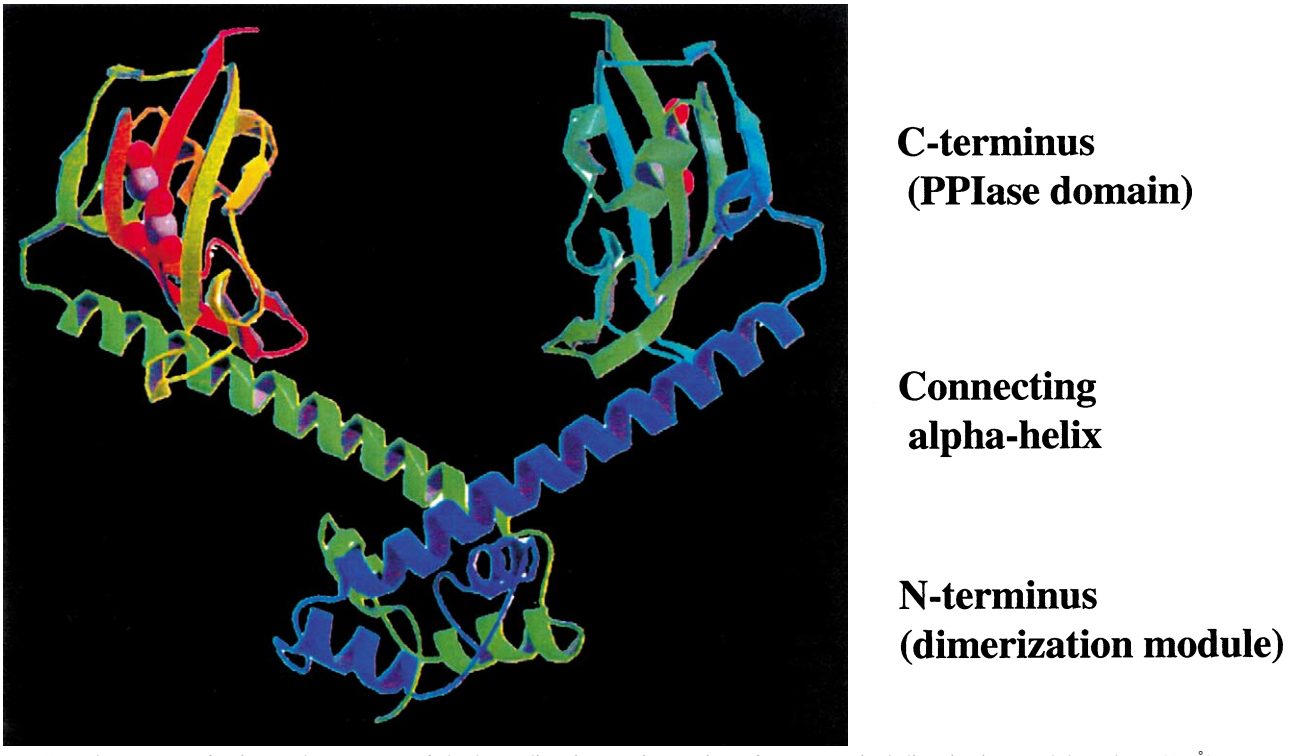

Fig. 4. Crystal structure of Mip. Each monomer of the homodimeric protein consists of an N-terminal dimerization module, a long (65-Å) connecting α-helix and a C-terminal domain, which exhibits the peptidyl-prolyl *cis*/*trans* isomerase (PPIase) activity. Provided by R. Hilgenfeld, Jena, Germany.

is constitutively expressed and the 2.4-Å crystal structure has recently been described (Fig. 4). Each monomer of the homodimeric protein consists of an N-terminal dimerization module, a long 65-Å connecting α-helix and a C-terminal peptidyl-prolyl *cis*/*trans* isomerase (PPIase) domain [80]. The homodimeric protein has been shown to contribute to intracellular survival of *Legionella* in macrophages, epithelial cells, protozoan hosts and guinea pigs. Mip belongs to the enzyme family of FK-506 binding proteins that exhibit PPIase activity [34,79,81]. Although the PPIase activity is not required in monocellular amoeba hosts, it significantly influences the infection processes in guinea pigs (Köhler et al., unpublished).

5.3. Secreted factors

Legionella secretes several enzymes, toxic compounds and pigments. Macroscopically obvious is the browning of the culture medium which is mediated by the Lly protein [82]. Mutagenesis of the *lly* gene does not affect intracellular replication in amoebal hosts or in macrophage-like cells. However, the Lly-negative mutant shows a markedly decreased resistance to light, indicating a contribution of the Lly protein to ecological adaptation of *Legionella* [83]. The detection of homogentisic acid (HGA) and the data analysis of the deduced amino acid sequence of the *lly* gene indicate that the *lly* locus codes for a *p*-hydroxyphenylpyruvate dioxygenase. This enzyme catalyzes the transformation of *p*-hydroxyphenylpyruvate into HGA, which subsequently oxidizes and polymerizes into a melanin-like pigment [84].

An increasing number of studies strengthen the view that the establishment of the intracellular niche of *L. pneumophila* requires a membrane-bound secretion apparatus similar to the type IV conjugational transfer systems [85,86]. This apparatus, which is encoded by a set of *dot* (defective in organelle trafficking) and *icm* (intracellular multiplication) genes, exports virulence factors that inhibit the phago-lysosome fusion and reprogram the *Legionella*-bearing vacuole. Once the vacuole provides conditions for the bacteria to grow, functional genes of the *dot*/*icm* family become dispensable [87]. The effector molecules secreted by this system remain to be identified. Interestingly, *L. pneumophila* contains a second type IV secretion apparatus, that is distinct from the *icm*/*dot* system. This *lvh* (*Legionella* vir homologs) system is not important for pathogenicity. However, it was found to be partially required for conjugation of the plasmid RSF1010 [86,88].

A secretion system of type II is known to transport two phosphatases, an RNAse, a zinc metalloprotease, mono-, di- and triacylglycerol lipases, phospholipase A, a lysophospholipase A and a *p*-nitrophenyl phosphorylcholine hydrolase [89–92]. This secretion system is dependent on the *pilBCD* locus which is involved in the biogenesis of type IV pili and on the *lsp* (*Legionella* secretion pathway) *FGHIJK* locus. The mutation of the implicated prepilin peptidase (*pilD*) dramatically reduces the ability of *L. pneumophila* to infect macrophages, amoebae and guinea pigs while the growth of a *lspGH* mutant is only impaired to grow in amoebae [90,92].

5.4. Other virulence-associated loci

Other genomic regions and factors currently under investigation are the *pmi* (protozoa and macrophage infectivity), the *mil* (macrophage infectivity loci), the *eml* (early stage macrophage-induced locus) and the *enh* (enhanced entry) locus [40,49,93,94]. Although the *enh* and the *eml* loci and the *milA* gene are known to be important for entry and the reprogramming of the endosomal pathway, the exact roles of the corresponding proteins remain to be determined. The recently identified *rtxA* (repeats in toxin) gene from the *enh1* locus and genes from the *rib* (release of intracellular bacteria) locus seem to be involved in pore formation [54,95].

5.5. Iron acquisition

The growth of *L. pneumophila* within human monocytes is iron dependent. In the case of an aberrantly low expression of transferrin receptor in human monocytes, no infection by *Legionella* occurs [51]. However, *Legionella* does not use transferrin or lactoferrin directly [96,97]. Instead the pathogen utilizes secreted and cell-associated factors as well as heme-containing compounds of the host as iron sources [98]. The iron acquisition genes are regulated by the transcriptional regulator Fur [99]. The *L. pneumophila*-specific Fur-regulated *frgA* gene encodes a protein which has homology with the aerobactin synthetases IucA and IucC (iron uptake chelate) of *E. coli*. A *frgA* mutant exhibited a 80-fold reduced intracellular growth in U937 cells [99]. The non-classical siderophore legiobactin as well as a methyltransferase (*iraA*), a putative iron peptide transporter (*iraB*), the inner-membrane cytochrome *c* biogenesis system (*ccmC*), periplasmic and cytoplasmic Fe^{3+} reductases are known to contribute to iron assimilation [100–105]. In addition, genetic loci encoding for a hydroxymate biosynthetic gene and a pyoverdin-like siderophore have been described [99,102].

5.6. L. pneumophila-specific factors

The heterogeneity in intracellular replication and cytopathogenicity of *L. pneumophila* and *Legionella micdadei* in mammalian and protozoan cells [49] indicate that species-specific genes may modulate virulence. Recently a number of *L. pneumophila*-specific genes were described. Sequencing of *mip* flanking regions revealed the *L. pneumophila* infectivity gene *ligA*. The deletion of this gene resulted in sodium resistance, decreased cytotoxicity, decreased hemolytic activity and avirulence in *A. castellanii* [106]. The 16-kDa pneumophila-specific outer-membrane

protein is a putative adhesin that probably contributes to the initial uptake of *L. pneumophila*. *frgA* is a further *L. pneumophila*-specific gene which is involved in iron uptake [99,107]. The previously described FlaR (Section 4.1) and the adjacent coding regions of this transcriptional factor are also specific to *L. pneumophila* [61,108]. Therefore, it has been speculated that the whole region might be a *L. pneumophila*-specific island.

5.7. Genomics and Legionella pathogenesis

By using pulsed field gel electrophoresis, previous studies revealed that the genome size of *L. pneumophila* is approximately 3.9 Mb [109]. Until now it is not known with certainty whether the *Legionella* chromosome exists as a closed circular or linear molecule. Information about the *Legionella* genome which is currently being sequenced is publically available (at http://genome3.cpmc.columbia.edu/~legion/) [110]. 63% of the 1100 putative genes which have been identified so far display homologies to proteins with known or putative functions. Since the whole-genome shotgun approach is supplemented with BAC (bacterial artificial chromosome)-based shotgun experiments, many complete operons have already been identified. As this genome project proceeds it will be possible to correlate gene variation with strain pathogenicity. Additionally, the analysis of mRNA levels (microarray technique) at different times during the intracellular life cycle should reveal much about expression patterns and function of the corresponding proteins.

6. Prevention of legionellosis

In order to establish links between *Legionella* isolates from patients and environmental sources discriminatory subtyping methods during epidemiological investigations have proven useful. Among the 42 species of the genus *Legionella*, *L. pneumophila* is the most commonly isolated one associated with disease. Although erythromycin, rifampin and ciprofloxacin are effective drugs for antimicrobial therapy of legionellosis, lethal treatment failures are well documented [111]. Since person-to-person transmission has never been observed the priority of prevention of *Legionella* infections concentrates on the elimination of the pathogen from water supplies.

6.1. Detection and disinfection

In high-risk areas, such as intensive care units, regular monitoring of *Legionella* concentrations is mandatory. Cultivation of *Legionella* remains the standard method of detection. The most widely used growth medium is buffered charcoal yeast extract agar which is supplemented with cysteine, iron salts and α-ketoglutarate. However, a number of factors, including other bacteria, can interfere with growth of *Legionella*, even on selective media [112,113]. Also serology-based methods are not regarded to be the gold standard anymore since the progressive characterization of new species has shown that antigen cross-reactivity limits specificity [114]. Further routine methods rely on pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), arbitrarily primed and nested PCR [115]. Additionally, gas chromatographic mass spectrometry based on the unique 3-hydroxy and 2,3-dihydroxy fatty acids of the *Legionella* LPS has been described for complex microbial consortia [116].

After detection of unacceptable high levels of legionellae effective decontamination and maintenance of water are critical for prevention of outbreaks of legionellosis. In general, actions need to be taken when the concentration of *Legionella* exceeds 1 CFU ml^{-1}. More restrictive standards apply for high-risk areas, including intensive care and transplantation units [117]. In the recent years a number of methods for controlling the growth of legionellae in drinking water supply systems (heat flushing, ultraviolet light irradiation, ozonation, metal ionization, chlorination) and cooling towers (biocides) have been described [118]. Unfortunately, the decreased heat transfer and biocide penetration into biofilms as well as unused pipes of the water system often interfere with disinfection attempts. Also the interaction of legionellae with amoebae hampers the disinfection in man-made water systems. This is complicated by the fact that protozoa may adapt to biocides [14,119,120].

6.2. Improvements in detection by application of molecular tools

The mortality rate, especially for nosocomial cases, continues to be high. Therefore, prompt detection of infection sources and treatment of infected patients are critical. Almost all current research efforts in this respect focus on molecular approaches. Fluorescence in situ hybridization (FISH) using probes targeting regions of the 16S rRNA molecule, has been reported to be a valuable diagnostic tool for rapid and specific detection (Fig. 5) [121–124]. This method allows in addition to a superb spatial resolution that the bacteria can be detected without the need of cultivation. Therefore, this time-saving method also makes it possible to detect VBNC legionellae, which represent a large portion of the total *Legionella* population and may constitute an unrecognized reservoir for disease [26]. Since FISH can also be used to detect the protozoa hosts, it is expected that this method will improve the knowledge of the conditions that are conducive to *Legionella* growth [121].

Further targets for detection and classification of *Legionella* appear to be protein-encoding genes. The analysis of the *Legionella* 16S rRNA gene and the virulence gene *mip* revealed a higher sequence variation for the *mip* gene (56%

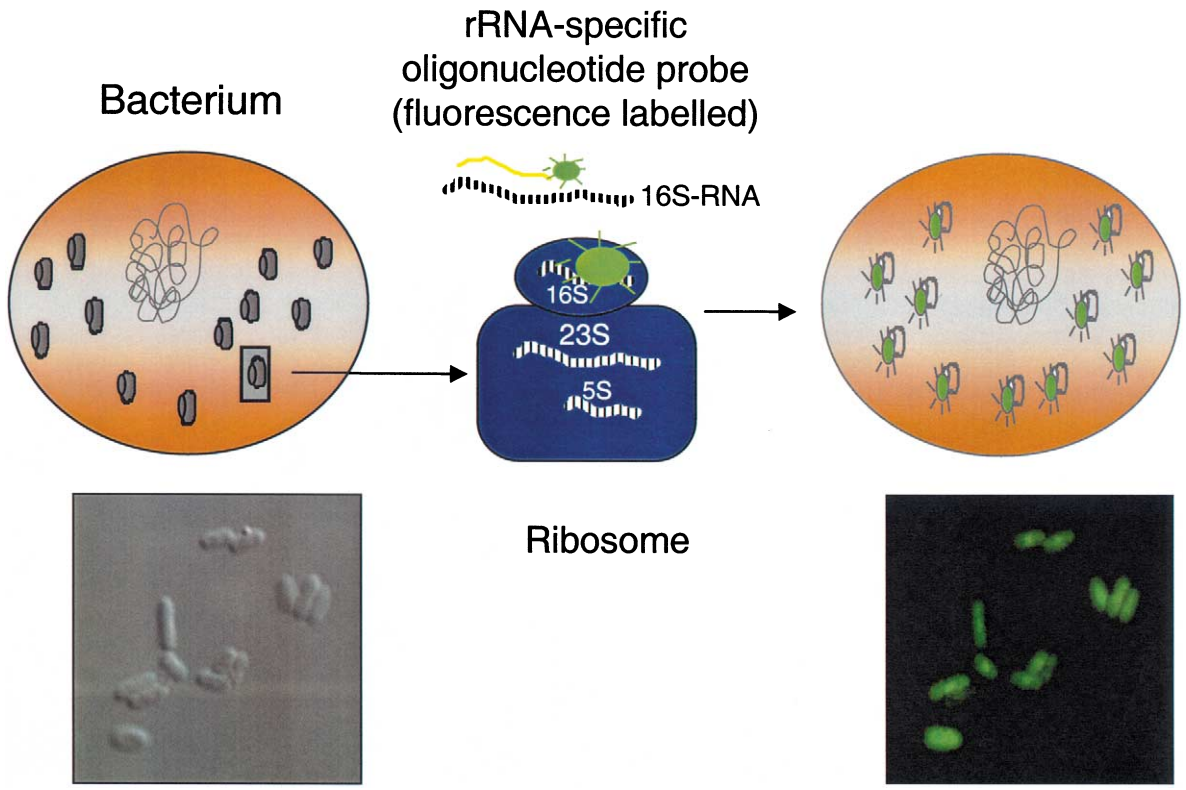

Fig. 5. Fluorescent in situ hybridization (FISH) of whole cells with 16S rRNA-targeted oligonucleotide probes. The comparison of the phase-contrast (left) and epifluorescence (right) micrographs shows that the probe detects single cells of *L. pneumophila*.

versus 23% of base sites) at the DNA level [125,126]. Therefore, it was suggested that the *mip* gene is three times more discriminatory than the 16S rRNA gene for identification of *Legionella* at the species level.

7. Conclusions and perspectives

L. pneumophila per se is not adapted to the human host. However, novel man-made environmental niches and changes in human behavior have led to legionellosis as a new public health risk. Therefore, *Legionella* can be viewed as an aquatic microbe that goes astray. The broad protozoal host spectrum in the environment and the exploitation of very basic cellular mechanisms of eukaryotes obviously allow *Legionella* to infect human cells. Consequently, it has been suggested that protozoa are the driving force in the evolution of the pathogenicity of *Legionella* [127,128].

Answers to fundamental questions concerning the biology of *Legionella* are expected from ongoing *Legionella* genome sequencing projects. Information on new genes and mobile genetic elements, which may play an important role in the evolution of pathogenic interactions, will be available soon [110]. The unstable genetic element that is responsible for *Legionella* phase variation, the plasmid pA5H5 from *L. longbeachae*, which encodes a putative transcriptional regulator (LrpR), and the higher G+C content of the *lvh* genes have encouraged speculations that gene transfer processes have occurred in *Legionella* [66,86,129].

In addition to comparative genomics, the advent of proteomics research with technical refinements in two-dimensional gel electrophoresis, mass spectrometry and software algorithms will help to systematically identify and study proteins of the pathogen and the host. In combination with functional genomics these tools will allow to elucidate regulatory circuits and signals that trigger the delivery of effector molecules by secretion systems. Moreover, a battery of monoclonal antibodies against phagosome components will help to analyze the modulation of the endosome maturation machinery, which appears to be the key virulence trait of *Legionella*. In this respect new insights are expected from systems where both, bacterial and host factors, can be manipulated. The infection of the genetically amenable haploid amoeba *D. discoideum* with *Legionella* allows this approach [74,75]. Recent infection studies with single cell stages of *Dictyostelium* have shown that this model system parallels the *Legionella* infection of human macrophages, *Hartmannella* and *Acanthamoeba*. The possibility of a two-sided genetic approach, the sequencing of the *Dictyostelium* genome, the availability of cellular markers and the knowledge of intracellular signalling pathways hold great promise for the analysis of *Legionella*–host interactions [76,130].

Legionella-free drinking water remains a challenge. To

date, population dynamics of *Legionella* have only been attributed to physical and chemical parameters as well as to the availability and susceptibility of host cells. However, from other bacterial species it is known that cell signalling and quorum sensing dramatically influence microbial communities. Such communication systems have not yet been studied for the genus *Legionella* [131]. In the long run this field of research may reveal new targets for therapeutic intervention and preventive measures in man-made habitats.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (STE 838/3-1) and the Graduiertenkolleg Infektiologie.

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