Chapter

Biofilm, a Cozy Structure for *Legionella pneumophila* Growth and Persistence in the Environment

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Abstract

Legionella pneumophila (*L. pneumophila*) is the causative agent of Legionnaires' disease. Transmission to humans is mediated via inhalation of contaminated water droplets. *L. pneumophila* is widely distributed in man-made water systems, multiple species of protozoa, and nematodes. *L. pneumophila* persist within multi-species biofilms that cover surfaces within water systems. Virulence, spread, and resistance to biocides are associated with survival of *L. pneumophila* within multi-organismal biofilm. Outbreaks of Legionellosis are correlated with the existence of *L. pneumophila* in biofilms, even after the intensive chemical and physical treatments. Several factors negatively or positively modulate the persistence of *L. pneumophila* within the microbial consortium-containing *L. pneumophila*. Biofilm-forming *L. pneumophila* continue to be a public health and economic burden and directly influence the medical and industrial sectors. Diagnosis and hospitalization of patients and prevention protocols cost governments billions of dollars. Dissecting the biological and environmental factors that promote the persistence and physiological adaptation in biofilms can be fundamental to eliminating and preventing the transmission of *L. pneumophila*. Herein, we review different factors that promote persistence of *L. pneumophila* within the biofilm consortium, survival strategies used by the bacteria within biofilm community, gene regulation, and finally challenges associated with biofilm resistance to biocides and *anti-Legionella* treatments.

Keywords: *legionella pneumophila*, biofilm, Legionellosis, protozoa, *Caenorhabditis elegans*

1. Introduction

L. pneumophila, the causative agent of Legionellosis, as being pathogenic to humans was following an outbreak of pneumonia at a convention of the American Legion in Philadelphia, USA in July 1976 [1]. This pathogen causes a severe form of pneumonia termed Legionnaires' disease (LD), and less frequently, Pontiac fever, a self-limited flu-like illness. Approximately 90% of LD cases are caused by *L. pneumophila*. Transmission of *L. pneumophila* occur primarily through the spread of contaminated aerosols present in cooling towers, condensers, faucets, showers, and hot tubs [2, 3]. Although stringent water quality examinations, the

formation of contaminated aerosols remains to be a major problem associated with disease spread [4].

Multiple mechanisms of persistence are harbored by *L. pneumophila* in various environmental conditions and in humans. Following invasion of amoeba or human macrophages, *L. pneumophila* form the *Legionella*-containing vacuole (LCV), which acquires vesicles from early and late endosomes, mitochondria and the endoplasmic reticulum (ER), thus escaping the microbicidal endocytic pathway. Hijacking the endocytic pathway by LCV is fundamental in initiating and maintaining a niche that secure *L. pneumophila* replication [5, 6]. Importantly, a battery of effector proteins produced by the Dot/Icm type IV secretion system of *L. pneumophila.* The Dot/Icm secreted effectors are required for successful intracellular replication of *L. pneumophila* [7–13]. Like other intracellular bacteria, *L. pneumophila* switch between a transmissive (virulent) and replicative (non-virulent) biphasic cycles. This switch is essential to ensure bacterial replication in nutrient starved or rich environments and transmit between different niches [14]. Nutrient rich environment is conducive of the replicative phase, where *L. pneumophila* express few virulence factors. While nutrient deprived environment is promotive of the transmissive phase, especially when the phagosome is unable to support the replication phase of *L. pneumophila*. Hallmark features of the transmissive phase include, increased motility, expression of plethora of virulence factors, resistance to stressors and egress from the infected host [14]. In the environment, *L. pneumophila* survive as free living (planktonic) or form bacterial biofilms with other organisms that adhere to surfaces [15–20]. Moreover, *L. pneumophila* is able to differentiate into inert, cyst-like but extremely infectious mature intracellular form (MIF) [21, 22]. Resilience of *L. pneumophila* extracellularly and under harsh environmental settings is attributed to its ability to exist in viable non-culturable (VBNC) state [23, 24]. Harboring a VBNC mode hinders the detection of many *Legionella* species. In nature, colonization and persistence is promoted via biofilm formation [25], and survival within freshwater amoeba and *C. elegans* [5, 26].

Herein, we review factors that mediate biofilm persistence, strategies utilized by the bacteria to become a member of the biofilm consortium and modes of eradicating *L. pneumophila* biofilm.

1.1 Constituents of *L. pneumophila* biofilm

L. pneumophila is found as sessile cells associated with biofilms in freshwater environments, [19, 27, 28]. Biofilms mediate bacterial attachments to surfaces and to other pre-attached bacterial communities. Attachment is attained via forming an extracellular matrix (ECM) that is composed mainly of water, proteins, exopolysaccharides, lipids, DNA and RNA, and inorganic compounds [29–32]. Three developmental phases are required for biofilm formation. (I) initial attachments to a surface, (II) maturation and extracellular matrix formation, and (III) detachments and dispersion of the bacteria. Biofilms eventually develop into three-dimensional structures containing water channels, which allow bacteria to obtain nutrients, oxygen and get rid of waste products. The behavior of *L. pneumophila* has mainly been studied in the context of mono- or mixed species biofilms, due to the complexity of biofilm formed in natural environment [17–19, 33, 34]. Interestingly, *L. pneumophila* exhibit minor representation among other species in freshwater and environmental biofilms, [27, 28], and the existence of *L. pneumophila* may be influenced by other microorganisms in complex biofilms [35]. Some bacterial species positively or negatively affect the persistence of *L. pneumophila* biofilm [19]. Intriguingly, *Klebsiella pneumoniae (K. pneumoniae)*, *Flavobacterium* sp., *Empedobacter breve, Pseudomonas putida*

and *Pseudomonas fluorescens* positively associated with the long-term persistence of *L. pneumophila* in biofilms [18, 19, 36]. Other species within biofilms seem to be the provider of capsular polysaccharides, extracellular matrix that support the adherence [37–39], or the contributor of growth factors that stimulate growth of *L. pneumophila* [19]. *Pseudomonas aeruginosa* (*P. aeruginosa*), *Aeromonas hydrophila*, *Burkholderia cepacia*, *Acidovorax* sp., and *Sphingomonas* sp. [40] are among species that antagonize the persistence of *L. pneumophila* within the biofilm [19]. Inhibition of *L. pneumophila* biofilm by *P. aeruginosa* could be a consequence of the effect of homoserine lactone quorums sensing (QS) molecule [41], or production of bacteriocin [40]. Interestingly, *L. pneumophila* can coexist in biofilm formed by *P. aeruginosa* and *K. pneumoniae* indicating that the inhibition of *L. pneumophila* biofilm formation by *P. aeruginosa* can be alleviated by the permissive *K. pneumoniae* [19]. The authors suggest that the growth provided by *K. pneumoniae* to promote survival of *L. pneumophila* can at the same time lessen the inhibitory effect by *P. aeruginosa* [19]. Therefore, the identity, number and nature of interactions between bacterial species (commensalism or interference) can directly affect growth of *L. pneumophila* within biofilms.

Biofilm formation of *L. pneumophila* in the laboratory is achieved by growing the bacteria under stringent conditions in nutrient-rich Buffered Yeast Extract medium (BYE) [18, 34]. Different temperatures correlated with different amount, degree of attachment and rate of biofilm formation. Mushroom like structure containing water channels is the hallmark features of biofilms formed at 25°C. In contrast, at 37°C *L. pneumophila* biofilm is thicker and deficient of water channels observed at 25°C. However, filamentous appearance with mat-like structure has been observed with *L. pneumophila* grown at 42°C. Studies in our laboratory showed that in contrast to the *dotA* mutant that lacks the type IV secretion, WT *L. pneumophila* form biofilm when grown statically at 37°C for 7 days.

Our knowledge is lacking regarding the factors encoded by *L. pneumophila* that promote the attachment and persistence within multispecies biofilms created by other bacteria.

1.2 Formation of biofilms as a survival niche in oligotrophic environment

Biofilm is extremely nutritious environment that harbors a mixture of living, dead organisms as well as protozoa and bacteria. To be a productive member of the microbial consortium, *L. pneumophila* has to compete with other bacteria for nutrients in a multispecies biofilm. Therefore, it is essential for *L. pneumophila* to strive in an environment adjacent to bacterial neighbors that best sustains their growth and survival [42]. Given the fastidious and auxotrophic nature of *L. pneumophila*, supplementation of the laboratory media with amino acids and iron is essential for growth [43, 44]. However, the ability of *L. pneumophila* to survive in oligotrophic environments is puzzling and suggests that the bacteria can live on a diet provided by other members in the biofilm community. To overcome the starvation mode in oligotrophic environment, *L. pneumophila* incorporate in two- and multispecies biofilms. Instead of attaching as a primary colonizer, *L. pneumophila* use a strategic mode where they dock to a pre-established biofilm, thus mediating bacterial survival and association in the biofilm community [19, 42].

Obtaining the required carbon, nitrogen, and amino acids for replication of *L. pneumophila* seems to be primarily reliant on necrotrophic feeding on the products of dead bacteria and tissues within the biofilm [35, 36]. Moreover, heterotrophic bacteria support growth of *L. pneumophila* on media that does not usually support growth because it is deficient in L-cysteine and ferric pyrophosphate [45]. Consistent with this, *L. pneumophila* showed satellite colonies around some aquatic

bacteria including *Flavobacterium breve*, *Pseudomonas spp*., *Alcaligenes spp*., and *Acinetobacter spp*. Further, *L. pneumophila* are able to obtain nutrients directly from algae and to grow on the extracellular products produced by cyanobacteria under laboratory conditions [46]. Further, several algae such as *Scenedesmus* spp., *Chlorella* spp., and *Gloeocystis* spp., supported the growth of *L. pneumophila* in basal salt media [28].

The second mechanism by which *L. pneumophila* obtain nutrient in biofilms is through amoeba. Amoeba serve as a secure niche that provides the environmental host for survival and replication of *Legionella* species in the environment [47, 48], and protect the bacteria from antibacterial agents [49]. Importantly, pathogenesis of *L. pneumophila* is correlated with persistence and adaptation of *L. pneumophila* in various amoebal hosts, and the nature of protozoal species can directly affect biofilm colonization with *L. pneumophila* [50, 51]. Indeed, *L. pneumophila* can parasitize more than 20 species of amoebae, three species of ciliated protozoa and one species of slime mold [52, 53]. Further, multiplication within amoeba mediated increase production of polysaccharides by *L. pneumophila,* thus enhancing its capacity to establish biofilm [54]. Further, debris from dead amoeba has been shown to support *L. pneumophila* growth [55], and the biomass of protozoa is directly correlated with outbreaks of *L. pneumophila*. Moreover, absence of amoeba did not result to an increase in the number of biofilm-associated *L. pneumophila*. Instead, *L. pneumophila* can enter the VBNC state to mediate their survival [28]. It has been suggested that metazoan such as the *C. elegans* could provide a natural host for *L. pneumophila* [56, 57]. Moreover, *L. pneumophila* survive within biofilm containing protozoan and *C. elegans* [58]. Therefore, harnessing nutrient from mixed species biofilms as well as survival in the amoeba and *C. elegans* enhances the persistence of *L. pneumophila*. Therefore, diversity of biofilm-associated organisms would provide a various means of nutrient acquisition in oligotrophic environment for such a fastidious organism.

1.3 Factors influencing biofilm formation by *L. pneumophila*

1.3.1 Cyclic-di-GMP

Regulation of bacterial pathogenesis and biofilm formation has been associated with the bacterial second messenger Cyclic-dimeric diguanylate (c-di-GMP) [59–62]. Biofilm regulation for several bacteria has been shown to be reliant on c-di-GMP [63–65]. Two main enzymes have been implicated in regulating the synthesis of the c-di-GMP. (I) A diguanylate cyclases (DGCs) containing GGDEF domain mediates the production of c-di-GMP from two GTPs molecules [66]. (II) A phosphodiesterases (PDEs) proteins containing EAL domain that mediate the degradation of c-di-GMP [66].

The *L. pneumophila* genome encodes for 22–24 GGDEF/EAL-containing proteins that vary between strains, suggesting that c-di-GMP signaling plays a role in the *L. pneumophila* life style [67–69]. Furthermore, *L. pneumophila* replication within amoeba and macrophages as well as virulence is influenced by the expression of GGDEF/EAL-containing proteins [68, 69]. Three GGDEF/EAL-containing proteins positively regulate biofilm formation in *L. pneumophila* Lens, [67]. *L. pneumophila* lacking these proteins showed reduced biofilm formation, however the level of c-di-GMP was not different when compared to the wild type (WT) bacteria [67]. However, two GGDEF/EAL-containing proteins have been shown to negatively regulate biofilm formation and deletion of these proteins resulted in overproduction of biofilm but surprisingly a decrease in the level of the c-di-GMP [67]. Therefore, GGDEF/EAL-containing proteins utilize different mechanisms to regulate biofilm by *L. pneumophila* when compared to other bacteria.

The Haem Nitric oxide/Oxygen (H-NOX) binding domains family of hemoprotein sensors have been demonstrated to play a role in regulating biofilm formation and the c-di-GMP activity [70]. Intriguingly, *L. pneumophila* is the only prokaryote found to encode two H-NOX proteins and show widespread of the H-NOX proteins in their genomes. Hyper-biofilm formation phenotype is attributed to deletion of *hnox1* without influencing growth of *L. pneumophila* in nutrient proficient media (BYE), mouse macrophages or *Acanthamoeba castellanii*. Importantly, a diguanylate cyclase is adjacent to *hnox1* and when overexpressed, *L. pneumophila* exhibits a hyper-biofilm phenotype. Presence of the H-NOX in the NO-bound state inhibited the diguanylate cyclase activity; suggesting that the diguanylate cyclase activity is regulated by NO [70]. Exposure to NO did not result in dispersing the adherent bacteria, but instead the biofilm intensity was increased. The reduced level of c-di-GMP has been associated with the excessive biofilm formation and the c-di-GMP degrading ability could enhance biofilm formation [67]. In the aquatic environment, exposure to NO occurs when *L. pneumophila* is in close contact to denitrifying bacteria, or when exposed to NO produced by macrophages or protozoa. Therefore, biofilm formation can be regulated by NO sensing.

1.3.2 Iron

Even though it is essential for *L. pneumophila* growth and replication [71–73], the concentration of iron must be stringently regulated, to overcome the toxic effect associated with production of reactive oxygen species (ROS), when used in excessive amount [74, 75]. Biofilm formation is inhibited when a fivefold increase in the concentration of iron pyrophosphate was used [17]. In addition, iron salt has been shown to disturb biofilm formation by other bacteria such including *P. aeruginosa* [76]. Recently, the effect of iron pyrophosphate and several iron chelators on the persistence of *L. pneumophila* in mixed biofilm were tested [77]. Chelating ferrous iron dipyridyl, DIP, enhanced the growth of (WT or mutant in iron uptake), suggesting that DIP positively contributes to the persistence of *L. pneumophila* [77]. Interestingly, DIP has no effect on the bacterial population in biofilm or survival of free-living amoeba in the biofilm and is independent of iron acquisition systems as mutants in iron uptake were not affected by DIP. These data suggest that contribution of DIP to the persistence of *L. pneumophila* in biofilm is via protecting *L. pneumophila* from the adverse effects of iron due to a decrease in ROS production [77].

1.3.3 Genetic control

Even though biofilm formation plays a role in the colonization, survival, dissemination and likely the pathogenesis of *L. pneumophila* [78], the genetic factors and molecular mechanisms involved in this process need to be elucidated. Genes that belong to the putative twin-arginine translocation pathway, which is required for transport of folded proteins across the cytoplasmic membrane, have been shown to be required for biofilm formation. Biofilm formation is reduced in mutants with insertional inactivation of the *tatB* and *tatC* genes [79]. Further, biofilm formation in static microtiter plates is impaired in a strain lacking the flagellar sigma factor FliA (σ^{28}) [18]. Expression of genes associated with the transmissive phase of *L. pneumophila* is controlled by FliA [80, 81]. Biofilm-derived *L. pneumophila* down-regulate FliA expression compared to planktonic bacteria in mouse macrophages infection, [82]. Production of flagella is controlled by *L. pneumophila* quorum sensing (Lqs) signaling compound LAI-1(3-hydroxypentadecane-4-one) as well as the stationary phase regulatory network, sensing availability of nutrient

[83]. However, the flagella are not required for attachment and persistence of *L. pneumophila* biofilm formed by *K. pneumonia* [19]. This is consistent with our observation showing the down-regulation of the flagella during biofilm formation in mouse macrophage [82].

Binding to sulfated glycosaminoglycans (CAGs) of the host extracellular matrix is mediated via the *Legionella* collagen-like (LcI) adhesin. Even though LcI is widely distributed in different *L. pneumophila* environmental and clinical isolates, it is lacking in poor biofilm producers; indicating the acquisition of this gene by horizontal gene transfer to *L. pneumophila* [84]. The GC content of *lpg2644* is different from the rest of *L. pneumophila* genome [84], indicating the acquisition of this gene by horizontal gene transfer to *L. pneumophila* [84]. Further, biofilm formation, cell–cell adhesion and cell-matrix interactions is reduced in strains with mutation in *lpg2644* [84]. The *L. pneumophila lpg2644* gene is differentially regulated during growth phases and biofilm formation [41]. Regulation of late stages of biofilm formation is mediated by *P. aeruginosa* quorum sensing (3OC12-HSL). Therefore, regulation of biofilm formation promotes dispersion of bacteria and mediates initiation of another biofilm cycle to another surface [41]. These events are crucial for the proliferation and transmission of *L. pneumophila* [78].

1.3.4 Quorum sensing

In Gram-negative bacteria, gene expression of several bacterial processes, including virulence, sporulation, bioluminescence, competence and biofilm formation is regulated by quorum sensing (QS) [85, 86]. Quorum sensing bacteria are usually identified in man-made water systems and it is well appreciated that QS signaling regulate environmental biofilm production [87]. The LAI-1 (3-hydroxypentadecane-4-one) QS autoinducer is the only (*Legionella* quorum sensing) Lqs system identified up to date [88–91]. The *L. pneumophila* LAI-1 is detected by the Lqs system which is composed of the autoinducer synthase LqsA, the homologous sensor kinases LqsS and the response regulator LqsR [88–90]. The Lqs system of *L. pneumophila* is homologous to the *cqsAS* QS of *Vibrio cholera*, which regulates cell-density, virulence and biofilm formation [85, 92]. Importantly, the *L. pneumophila* biofilm formation is inhibited by the *P. aeruginosa* quorum sensing autoinducer (3-oxo-C12-HSL), which down-regulate the expression of *lqsR* [41, 93]. Therefore, QS could potentially disperse *L. pneumophila* biofilm during later stages.

1.4 Modulation of gene expression in biofilms

Differential gene expression between planktonic and biofilm forming *L. pneumophila* was shown through transcriptomic analysis [17]. The gene expression pattern was compared with the replicative and transmissive phases during growth of *L. pneumophila* in *A. castellanii* [94]. Importantly, gene expression profile of sessile bacteria is similar to the replicative phase of *L. pneumophila*. Furthermore, genes that are involved in repressing the transmissive phase were well expressed in the sessile bacteria [17], suggesting that biofilm is a secure niche for *L. pneumophila* [17]. The *pvcAB* gene cluster (which is regulated by iron) is among the genes that were highly expressed in the sessile form [17]. The *L. pneumophila pvcA* and *pvcB* genes are homologous exhibit homology to the *P. aeruginosa* proteins PvcA and PvcB and are required for the production of the iron binding protein (siderophore). The *pvcA* and *pvcB* in *L. pneumophila* encode for a siderophore-like molecule, which promote iron sequestration at a sub toxic level. The second gene cluster, including *ahpC2* and *ahpD*, encodes for alkyl hydroperoxide reductases and play a role in protection against oxidative stress [95, 96] displayed the highest induction in

biofilm cells [95]. Iron plays a role in the production of reactive oxygen species and the metabolism of iron and oxidative stress is related. Induction of both *pvcAB* and *ahpC2D* genes in sessile cells could be utilized to overcome the toxic environment associated with high iron level concentrations.

Further, examining the expression of the macrophage infectivity potentiator (*mip*) to transcriptionally active *L. pneumophila* infected in cell culture was used to evaluate the virulence of biofilm-associated *L. pneumophila* [16]. Expression of *mip* is required for growth in protozoa and human macrophages [97]. Further, *mip* expression is up-regulated during the transmissive stages of *L. pneumophila* life cycle, but downregulated at early stages of infection [98]. At early stages of biofilm formation, which is similar to the replicative phase, expression of *mip* was constant. However, at later stages of biofilm formation, which is similar to the replicative phase, *mip* expression was predominately up-regulated [16]. Upregulation of *mip* expression could be correlated with the switch to the transmissive phase observed in the planktonic form and suggests that biofilm could protect the replicative form of *L. pneumophila*.

1.5 Biocides treatments of *L. pneumophila* biofilm and bacterial resistance

L. pneumophila survive in biofilms covering environmental and artificial water systems such as ventilation and conditioning systems [78]. In addition, biofilm-containing *L. pneumophila* can become a transient or permanent habitat for other relevant microorganisms. Therefore, biofilm-associated organisms can survive for days, weeks or even months depending on the substratum and the environmental factors that stimulate biofilm formation [99, 100]. To restrict *L. pneumophila* growth, numerous chemical, physical and thermal disinfection methods have been used against *L. pneumophila* [101]. However, these treatments generally do not result in total elimination of the bacterium, and after a lag period, recolonization occurs as quickly as the treatments are discontinued [35]. Biofilm-associated *L. pneumophila* is extremely resistant to disinfectants and biocides [101, 102]. Further, exposure of biofilm-encased bacteria to biocides could lead to entry into a viable non-culturable status [103]. Chlorine and its derivatives are the most common biocides used in disinfection protocols and have been shown to be appropriate in eliminating planktonic *L. pneumophila* but not biofilms [104]. Resistance of *L. pneumophila* to disinfection is due not only to its capacity to survive within biofilm, but also the bacteria exhibit the intra-amoebal life-style [105, 106]. Therefore, amoeba- associated *L. pneumophila* are more resistant to disinfection possibly due to differences in membrane chemistry or life cycle stages of this primitive organism [35, 107]. It has been shown that vesicles containing intracellular *L. pneumophila* released by amoeba are resistant to biocide treatments [108]. Importantly, these vesicles remained viable for few months [109]. Understanding the molecular mechanisms that governs the intra-amoeba related resistance should pave the way for development of new strategies to eradicate *L. pneumophila*.

Other methods have been used to limit *L. pneumophila* such as applying heat which has been shown to be effective in reducing the number of bacteria and protozoan trophozoites, but infective against killing cysts [110, 111]. UV radiation is also effective when the bacteria are in direct contact with the radiation [112]. However, higher UV intensities are required to inactivate the protozoa [113]. Other methods have been proposed to control *L. pneumophila* growth such as controlling the carbon source within anthropogenic water system [114], or addition of phages to control bacterial or specifically *L. pneumophila* growth. The phage is capable of degrading polysaccharides and therefore destabilizing the biofilm [115, 116]. Furthermore, nanoparticles have been shown to be effective in reduction of *L. pneumophila* biofilm

volume and showed some efficacy against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms [117–119]. Moreover, several natural compounds (biosurfactants, antimicrobial peptides, protein and essential oil) have been shown to exhibit anti-*Legionella* properties [120]. Collectively, it is necessary to control *L. pneumophila* growth and their natural hosts to optimize eradication of the bacteria.

2. Conclusions

Several chemical and physical parameters can influence the behavior of *L. pneumophila* in biofilms, including the surface, the temperature, carbon and metal concentrations, and the presence of biocides [17, 18, 34, 114, 121–128]. Biological factors such as being a member of mixed species biofilm or parasitizing free-living amoeba or nematodes influence biofilm formation by *L. pneumophila*. Biofilm-associated *L. pneumophila* is resistant to biocides and Legionellosis outbreaks have been attributed to biofilms. Therefore, it is essential to design new remedies for eradication of *L. pneumophila* biofilm in different environmental settings. Treatment studies should be performed when the bacterium is in its natural host to determine how the bacterium is protected inside the amoeba and if the passages through the natural hosts modify the resistance. Thus, preventing biofilm formation appears as one strategy to reduce water system contamination.

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Conflict of interest

The authors of the manuscript declare that the submitted work was carried out in the absence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

Author contributions

Arwa Abu Khweek wrote the book chapter, and Amal O. Amer edited the manuscript.

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