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Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation

Thomas Hindré,[1]† Holger Brüggemann,[2] Carmen Buchrieser[2] and Yann Héchard[1]

Correspondence
Yann Héchard
yann.hechard@univ-poitiers.fr

[1]Laboratoire de Chimie de l'Eau et de l'Environnement, UMR 6008, Université de Poitiers, 40 Avenue du Recteur Pineau, 86022 Poitiers Cedex, France

[2]Unité de Génomique des Microorganismes Pathogènes and CNRS URA 2171, Institut Pasteur, 28 Rue du Dr Roux, 75724 Paris, France

In aquatic environments, biofilms constitute an ecological niche where *Legionella pneumophila* persists as sessile cells. However, very little information on the sessile mode of life of *L. pneumophila* is currently available. We report here the development of a model biofilm of *L. pneumophila* strain Lens and the first transcriptome analysis of *L. pneumophila* biofilm cells. Global gene expression analysis of sessile cells as compared to two distinct populations of planktonic cells revealed that a substantial proportion of *L. pneumophila* genes is differentially expressed, as 2.3 % of the 2932 predicted genes exhibited at least a twofold change in gene expression. Comparison with previous results defining the gene expression profile of replicative- and transmissive-phase *Legionella* suggests that sessile cells resemble bacteria in the replicative phase. Further analysis of the most strongly regulated genes in sessile cells identified two induced gene clusters. One contains genes that encode alkyl hydroperoxide reductases known to act against oxidative stress. The second encodes proteins similar to PvcA and PvcB that are involved in siderophore biosynthesis in *Pseudomonas aeruginosa.* Since iron has been reported to modify biofilm formation in other species, we further focused on iron control of gene expression and biofilm formation. Among the genes showing the greatest differences in expression between planktonic cells and biofilm, only *pvcA* and *pvcB* were regulated by iron concentration. A Δ*pvcA L. pneumophila* mutant showed no changes in biofilm formation compared to the wild-type, suggesting that the *pvcA* product is not mandatory for biofilm formation. However, biofilm formation by *L. pneumophila* wild-type and a Δ*pvcA* strain was clearly inhibited in iron-rich conditions.

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INTRODUCTION

Legionella pneumophila is the causative agent of a severe pneumonia known as Legionnaires' disease. When infected aerosols are inhaled by humans, bacteria can infect and multiply within alveolar macrophages. In the environment, *L. pneumophila* is ubiquitously found in natural freshwater as well as in man-made water systems (Steinert *et al.*, 2002), where free-living amoebae constitute an ecological niche allowing bacterial replication (Abu Kwaik *et al.*, 1998). In amoebae, as well as in alveolar macrophages, *L. pneumophila* is able to inhibit phagosome maturation, giving rise to a specific vacuole where bacteria replicate actively. As nutrients become limiting, *L. pneumophila* exits from this replicative phase to enter the transmissive phase, resulting in host cell lysis and dispersion of bacteria (Molofsky & Swanson, 2003). While some of these cells re-establish a replicative niche within a new host cell, others survive in the aqueous environment as planktonic cells and/or within biofilms as sessile cells (Molofsky & Swanson, 2004). Biofilms are microbially derived sessile communities irreversibly attached to a substratum, embedded in a secreted extracellular matrix and exhibiting a specific phenotype (Donlan & Costerton, 2002). In the environment, biofilms are the prevalent microbial lifestyle, probably

†Present address: Institut National de Recherches Agronomiques, Laboratoire de Microbiologie, Centre de Recherche INRA de Clermont Ferrand Theix, 63122 Saint-Genes Champanelle, France.

Abbreviations: Cp, crossing point; CV, crystal violet.

The microarray results for these experiments are accessible on the website http://genoscript.pasteur.fr/ (see text for details).

Two supplementary tables showing genes with significant differences in expression in sessile and planktonic cells and the expression fold change of genes associated with iron homeostasis are available with the online version of this paper.

because matrix-embedded microbes enjoy a number of advantages over their planktonic counterparts (Watnick & Kolter, 2000). Indeed, polymeric matrix not only helps to capture and concentrate environmental nutrients, but also acts as a protective barrier against environmental challenges or antimicrobial factors. In addition, specific phenotypes inherent to the sessile mode of growth reduce susceptibility of biofilm cells to antimicrobial agents (Shirtliff *et al.*, 2002; Hall-Stoodley *et al.*, 2004). *Legionella* biofilm studies that have been conducted on naturally occurring microbial communities in tap water systems have suggested that biofilms allow persistence of *L. pneumophila* whereas its proliferation most likely requires the presence of amoebae for intracellular growth (Murga *et al.*, 2001; Kuiper *et al.*, 2004; Declerck *et al.*, 2007). Recently, two different studies have investigated the ability of *L. pneumophila* to form monospecies biofilms. Piao *et al.* (2006) tested different materials and temperatures for biofilm formation, reporting that on glass and polystyrene biofilms were formed more slowly at 25 °C than at 37 or 42 °C but that they remained more stably attached at 25 °C. At 25 °C biofilms possessed structural features typical of biofilms reported previously (i.e. pillar- and mushroom-like structures) whereas at 37 °C, filamentous, mycelial-mat-like biofilms were the structures observed. Mampel *et al.* (2006) reported that biofilm formation by *L. pneumophila* in complex nutrient-rich media most likely relies on adhesion of bacteria grown in the planktonic phase. Also, the Tat secretion pathway has been shown to be involved in the biofilm formation ability of *L. pneumophila* (De Buck *et al.*, 2005).

Numerous disinfection methods directed against *Legionella* have been described in the literature (Kim *et al.*, 2002). Unfortunately, these treatments generally do not lead to a total eradication of the bacterium, and recolonization occurs as soon as the treatments are interrupted (Thomas *et al.*, 2004). Resistance of *L. pneumophila* to disinfection is due not only to its capacity to enter amoebae, where bacteria could be protected from biocides, but also to its association with biofilms (Abu Kwaik *et al.*, 1998; Thomas *et al.*, 2004; Saby *et al.*, 2005). However, only very little information regarding the physiological state and gene expression of *L. pneumophila* within biofilms is currently available. A better knowledge of the *L. pneumophila* sessile lifestyle may help to reduce the presence of *L. pneumophila* within biofilms and, consequently, decrease its spread in the environment.

The aim of our work was to design a model biofilm for *L. pneumophila* and to study gene expression of sessile and planktonic *L. pneumophila* cells, to learn about the changes occurring in *L. pneumophila* biofilm. Our results give new insights into *L. pneumophila* biofilm formation and point to several genes that may be important for biofilm formation and maintenance.

METHODS

Bacterial strain and growth conditions. *L. pneumophila* Lens was grown without agitation in filter-sterilized BYE (5 g ACES l^{-1}, 10 g yeast extract l^{-1}; pH 6.9) supplemented with L-cysteine (0.4 g l^{-1}) and ferric pyrophosphate (0.25 g l^{-1}). Solid medium was obtained by adding charcoal (2 g l^{-1}) and agar (15 g l^{-1}) to non-sterilized BYE. The resulting medium (BCYE-agar) was autoclaved and then supplemented with L-cysteine and ferric pyrophosphate prior to use.

Microtitre plate assays of biofilm formation. *L. pneumophila* biofilm formation was monitored using polystyrene 96-well microtitre plates (Nunclon MicroWell Plates, Nunc). Three distinct procedures were used in this study. (1) Biofilm formation at different temperatures was assayed using cells grown on BCYE plates at 37 °C, 5 % CO_2 for 3–4 days. Cells were resuspended in BYE at OD_{600} 0.15 and then aliquoted (150 µl) into eight wells of a 96-well microtitre plate, which was incubated at 20, 37 or 42 °C until late stationary phase. Medium and non-adherent cells were then removed by aspiration and the wells were rinsed with BYE (200 µl). Cells that had adhered to the wells were stained with 200 µl 0.3 % crystal violet (CV) at room temperature for 15 min. Excess CV was then removed and the wells were washed three times with water (400 µl). Fixed CV was solubilized in 200 µl 100 % ethanol, 15 min at room temperature. Biofilm formation was estimated by measuring the A_{595} of each well using a microtitre plate reader (Sunrise, Tecan) and calculating the mean from the eight wells. (2) Assays for adhesion were performed using cells grown at 20 °C in BYE liquid cultures, harvested at different growth phases (48, 120 and 192 h of growth for exponential, stationary and late stationary phase, respectively) and resuspended in BYE at OD_{600} 2.0. Cell suspensions were then aliquoted (150 µl) into eight wells of a 96-well microtitre plate, which was incubated at 20 °C for 6 h. Adhesion was estimated by CV staining of adherent cells, as described above. (3) Assays for biofilm growth were done in a similar way using cells grown in BYE at 20 °C until late stationary phase. After 6 h incubation at 20 °C, medium and non-adherent cells were removed and 150 µl BYE were added to the wells. Incubation was continued for 7–14 days. Where indicated, medium and non-adherent cells were removed and replaced by sterile BYE on day 7. After incubation, biofilm formation was quantified by CV staining, as described above.

Biofilm conditions for transcriptome analyses. *L. pneumophila* Lens biofilms were grown at 20 °C in 75 cm^2 cell culture treated flasks (BD-Falcon). BYE (30 ml) was inoculated at an initial OD_{600} of 0.15 with cells from a preculture grown in BYE at 20 °C. The flask was incubated at 20 °C until growth reached late stationary phase. The cell suspension was then removed from the flask and planktonic cells (termed inoculation cells) were recovered by centrifugation (2 min, 3000 ***g***, 4 °C) and immediately stored at −80 °C. Cells that had adhered to the bottom of the flask were covered with 100 ml fresh BYE and the flask was incubated at 20 °C. To avoid cell sedimentation onto the adhered cells, the flask was placed vertically. After 7 days, the culture medium was replaced by sterile BYE and incubation was continued for 7 days. The culture medium containing planktonic cells was then removed and cells (termed suspension cells) were recovered by centrifugation (2 min, 3000 ***g***, 4 °C) and immediately stored at −80 °C. The flask was rinsed twice with BYE, and 1 ml cold BYE was added to the flask. Cells that had adhered to the walls of the flask were detached using a sterile cell scraper and resuspended in cold BYE. The resulting cell suspension was removed and biofilm cells (termed sessile cells) were recovered by centrifugation (2 min, 3000 ***g***, 4 °C) and immediately stored at −80 °C.

Microscopic analyses of *L. pneumophila* biofilm. Two distinct procedures were used for microscopic analyses of *L. pneumophila* biofilms. For electron microscopy, biofilms grown in cell culture flasks were fixed with 2 % glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) for 1 h. Fixed biofilms were scraped from the culture flasks and transferred onto polylysine-coated glass slides. Subsequent dehydration was performed stepwise using 50, 70, 90 and 100 %

acetone in water, 50 % ethanol : 50 % acetone, 100 % ethanol, 50 % ethanol : 50 % trichlorotrifluoroethane and finally 100 % trichlorotrifluoroethane. The samples were coated with gold and examined using a SEM JEOL JSM 840 electron microscope. Analysis of biofilm formation at various iron concentrations was performed using phase-contrast microscopy. *L. pneumophila* Lens was diluted in BYE medium containing 0, 0.25 or 1.25 g l^{-1} iron pyrophosphate to approximately 10^6 c.f.u. ml^{-1}. Then 6 ml of this dilution was used to fill a six-well microtitre plate (Nunclon Delta Surface), to which microscope glass cover slides (18 mm × 18 mm) had been previously added. Biofilms were allowed to form for 2 weeks at 20 °C. The medium was changed after 1 week. At the end of the incubation, medium and non-adherent cells were removed and cells that had adhered to the glass slides were stained with 0.3 % CV at room temperature for 15 min. Glass slides with biofilms on both sides were rinsed with water and cleaned on the bottom side with an alcohol swab. The biofilm attached to the top side was examined by phase-contrast microscopy.

Total RNA isolation, cDNA synthesis and labelling. The FastProtein Blue kit was used with a Fast Prep apparatus (Q-biogene) in order to lyse cells. Total RNA was extracted as described by Milohanic *et al.* (2003). RNA was quantified by A_{260} and A_{280} and its integrity was confirmed on 1 % agarose gels. The total preparation was divided into 10 µg aliquots that were stored at −80 °C until use. Absence of genomic DNA contamination was assayed by PCR amplification using two primers complementary to the *mip* gene (*mip*F, AGCATTGGTGCCGATTTG; and *mip*R, TCTGTCCATCCA-GGGATAAC). Total RNA (10 µg) was used for cDNA synthesis with the Atlas Powerscript Fluorescent labelling kit (BD-Bioscience) and $pd(N)_6$ primers (Roche), according to the manufacturer's recommendations. Two distinct cDNA syntheses were made for each cell sample and obtained cDNA were pooled. One half was then labelled with Cy3 and the other with Cy5.

Array hybridization and data analysis. Hybridizations were performed using 250 pmol Cy3- and Cy5-labelled cDNA following the manufacturer's recommendations (Corning) and using slides described by Brüggemann *et al.* (2006). The cDNA of each cell sample was compared to that of the two others using two distinct hybridizations on slides, including a dye-swap. In addition, a biological replicate was carried out giving a total of 12 slides for the overall experiment. Slides were scanned on a GenePix 4000A scanner (Axon Instruments). Laser power and/or photomultiplier tubes (PMT) were adjusted to balance the two channels. The resulting files were analysed using Genepix Pro 4.0 software. Spots were excluded from analysis in cases of high local background fluorescence, slide abnormalities, or weak intensity. Data normalization and differential analysis were conducted using the R software (Brüggemann *et al.*, 2006; http://www.R-project.org). A Loess normalization (Yang *et al.*, 2002) was performed on a slide-by-slide basis (BioConductor package marray; http://www.bioconductor.org/packages/bioc/html/marray.html). Differential analysis was carried out separately for each comparison between two cell samples, using the VM method [VarMixt package (Delmar *et al.*, 2005)], together with the Benjamini and Yekutieli (Reiner *et al.*, 2003) *P*-value adjustment method. Only genes with significant ($P<0.05$) fold changes in expression were taken into consideration. Empty and flagged spots were excluded, and only genes with no missing values were analysed.

Quantitative RT-PCR. cDNA templates were obtained from 10 µg total RNA using $pd(N)_6$ primer (Roche) and M-MLV, RNase H Minus, Point Mutant reverse transcriptase (Promega) according to the manufacturer's instructions. Quantitative RT-PCR was performed on a LightCycler (Roche) using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) according to the manufacturer's instructions. The 16S rRNA gene was used as a reference gene to normalize gene expression, using primers 16SF (ACCTGG-CCTAATACTGAC) and 16SR (TACGGGACTTAACCCAAC). The level of gene expression was assessed by determining the cycle at which the amplification curve crossed the detection threshold. This is defined as the crossing point (Cp). The relative changes in gene expression were calculated as commonly described (Livak & Schmittgen, 2001) by calculating the $2^{-\Delta\Delta Cp}$, where ΔCp=Cp target gene−Cp reference gene (16S) and ΔΔCp=ΔCp sample 1−ΔCp sample 2.

Mutant construction. Electro-competent cells of *L. pneumophila* Lens were prepared as follows. A bacterial suspension (100 µl, OD_{600} 2) was plated on BCYE and incubated for 24 h at 37 °C. The cells were harvested from the plate surface and suspended in 40 ml 10 % (v/v) glycerol at 4 °C. Bacteria were centrifuged (4000 ***g***, 10 min) and washed twice in 20 ml and 10 ml 10 % glycerol. Finally, cells were resuspended in 10 % glycerol at OD_{600} 100, divided into 50 µl aliquots and frozen at −80 °C. Competent cells were electroporated with the pJS5 plasmid (Allard *et al.*, 2006), which contains a fragment carrying the *pvcA* gene inactivated by the insertion of a kanamycin-resistance gene. The transformants were plated on BCYE with kanamycin and tested by PCR using the PVCA9 F and PVCB6-R primers described previously (Allard *et al.*, 2006). The resulting mutant strain was used to test its ability to form biofilm as described above.

RESULTS

Physiological studies of biofilm formation

The ability of *L. pneumophila* strain Lens to form a monospecies biofilm was investigated at different growth temperatures in order to determine the most favourable conditions for biofilm establishment. Microtitre plates were inoculated with *L. pneumophila* Lens and incubated at 20, 37 or 42 °C. Culture growth was monitored as OD_{595} and the number of sessile cells within the obtained biofilms was estimated by measurement of A_{595} after CV staining, 12–16 h after entry into stationary phase (Fig. 1a). It clearly appeared that biofilm formation by the Lens strain was favoured at 20 °C since A_{595} was at least sixfold higher at this temperature as compared to 37 and 42 °C. This result was not due to growth differences between *L. pneumophila* at the various temperatures as the A_{595} divided by the final OD_{595} of each culture gave similar results (data not shown). We also observed that biofilms formed at 20 °C were more stably attached than those formed at 37 and 42 °C, which became detached during the washing steps prior to CV staining. Accordingly, biofilm formation experiments with the *L. pneumophila* Lens strain were conducted at 20 °C.

In order to optimize biofilm formation, we tested the influence of growth phase on adhesion of *L. pneumophila* Lens. Bacteria were grown at 20 °C, and equivalent numbers of cells from exponential, stationary and late stationary phase were used to inoculate 96-well microtitre plates. After 6 h of incubation at 20 °C, the number of adhered cells was estimated by CV staining (Fig. 1b). The resulting A_{595} values for cells from exponential, stationary and late stationary phase were 0.16, 0.37 and 0.59,

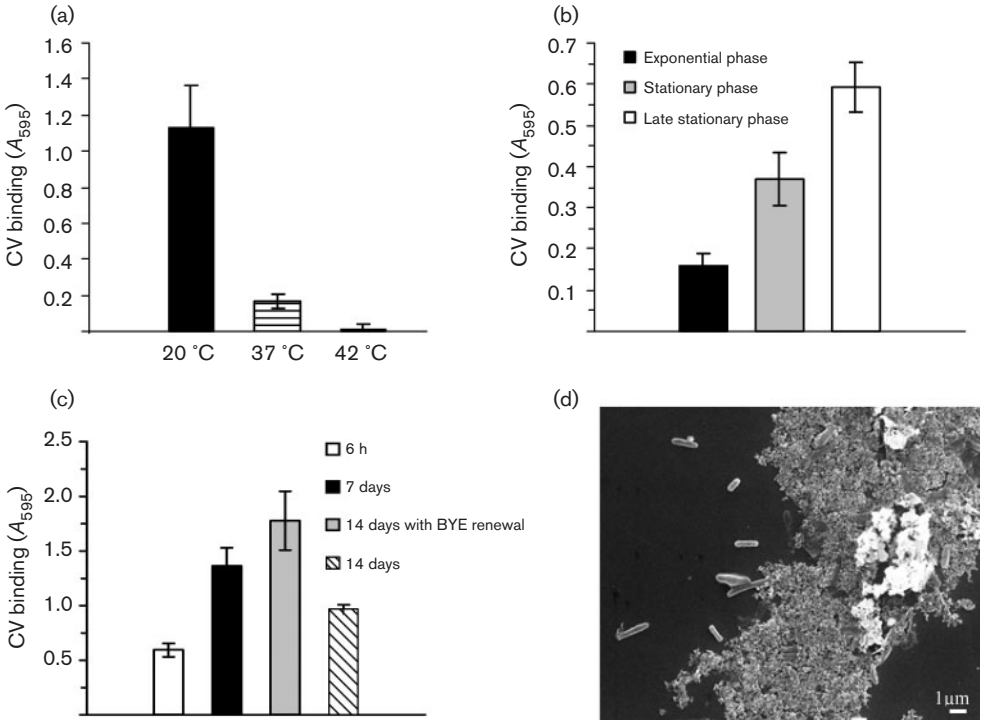

Fig. 1. Biofilm formation by *L. pneumophila* Lens. (a) Effect of temperature on biofilm formation. Cell suspension (initial OD_{600} 0.15) was aliquoted into eight wells of a microtitre plate, which was incubated until late stationary phase. Biofilm formation was then estimated by CV staining assessed by A_{595}. (b) Influence of growth phase on adhesion. Cell suspensions containing equivalent numbers (OD_{600} 2) of cells, harvested at the indicated growth phase, were aliquoted into 96-well microtitre plates and incubated at 20 °C for 6 h prior to CV staining. (c) Effect of incubation time on biofilm formation. Adhered cells from the initial adhesion step were further incubated in fresh BYE for 7 and 14 days. Biofilm formation was then estimated by CV staining. All the A_{595} values presented are mean ± SD of eight wells from two distinct experiments. (d) Scanning electron micrograph of a *L. pneumophila* Lens biofilm. A biofilm formed after 14 days in culture flasks was fixed and detached before staining for electron microscopy.

respectively. This clearly showed that the adhesion ability increases during growth and that, consequently, cells in late stationary phase exhibit the highest capacity for adhesion. Furthermore, wells inoculated with late stationary phase cells were washed after the initial 6 h adhesion step and filled with fresh BYE medium. Growth of the adhered cell community was then followed for 14 days at 20 °C, with or without growth medium replacement on day 7. As estimated by CV staining, the number of adhered cells increased during the first 7 days and medium replacement allowed a further increase of cell density until 14 days (Fig. 1c). Whereas microtitre plate assays are useful for biofilm formation studies, they are not suitable for recovery of large amounts of sessile cells for transcriptome analyses. We therefore decided to use 75 cm^2 cell culture flasks, which offer a larger surface for biofilm development. Based on the above results, biofilm formation was initiated using *L. pneumophila* Lens cells grown until late stationary phase, which were then incubated at 20 °C for 14 days, with medium replacement on day 7. These growth conditions allowed the development of a consistent biofilm where bacteria are embedded in an extracellular matrix (Fig. 1d), as described for other monospecies biofilms. Cells harvested from the walls of the flasks were defined as 'sessile cells'. The two planktonic cell samples used as references were the non-adherent cells from the initial adhesion step, termed 'inoculation cells' and the planktonic cells present in the flask at the end of the 14 day incubation period, were termed 'suspension cells' (see Methods).

Transcriptome analyses

Total RNA of the three different cell samples, namely 'sessile cells', 'suspension cells' and 'inoculation cells' was used for cDNA synthesis, labelling and subsequent hybridization to a DNA oligonucleotide array containing 3823 gene-specific oligonucleotides (Brüggemann *et al.*, 2006). These probes were designed to match every predicted ORF in the three sequenced *L. pneumophila* genomes, of strains Lens, Paris and Philadelphia (Cazalet *et al.* 2004, Chien *et al.*, 2004). Gene expression changes in the three different cell samples were recorded in a two-way comparison, i.e. sessile cells vs inoculation cells, sessile cells vs suspension cells and suspension cells vs inoculation cells.

The full results are accessible on the website http://genoscript.pasteur.fr/ (choose 'Public area', then 'Legionella'). Taking a twofold difference in gene expression between sessile and inoculation cells as a threshold, over 15.5 % of the *L. pneumophila* genes (457 out of 2932 predicted) were altered in their expression (see Supplementary Table S1, available with the online version of this paper). In addition, 11.2 % of the *L. pneumophila* genes (329 out of 2932 predicted) showed at least a twofold change in expression in comparisons of sessile and suspension cells (Table S1). Together, these results clearly indicate major changes between the sessile and planktonic forms. However, it is highly unlikely that all these changes are associated with the sessile mode of life per se. In order to focus our analysis on biofilm-specific genes, we selected genes with at least a twofold altered expression in biofilm cells relative to both planktonic cell populations. This revealed a total of 69 genes (2.3 % of the genome), among which 52 were upregulated and 17 downregulated (Table 1). Searching for gene clusters or genes involved in the same biological pathway highlighted several genes. Five gene clusters with induced expression were identified within this group: *lpl0236–0237*, whose predicted products are similar to pyoverdine synthesis proteins; *lpl0628–0629*, which includes a gene encoding a putative aminochorismate lyase; *lpl2213–2214*, which encodes phosphatase and pseudouridine synthase; *lpl2271–2272*, which encodes alkyl hydroperoxide reductases; and *lpl2916–2917*, which probably encodes ATP synthase subunits. Moreover, virulence-related genes such as *lpl0820*, encoding the global regulator CsrA and *lpl0497*, encoding IcmJ/DotN, which is part of the type IV secretion system of *L. pneumophila*, are also induced in sessile cells. Two other induced genes (*lpl2576* and *lpl2686*) encode ribosomal proteins. Finally a gene cluster showing reduced expression in sessile cells was identified: *lpl1059–1060* that encodes proteins similar to EnhC involved in the entry into host cells and a major facilitator protein. To learn about the global physiological state of sessile cells, genes previously identified as good markers for the transmissive and replicative phases of the *L. pneumophila* life cycle during growth in *Acanthamoeba castellanii* (Bruggemann *et al.*, 2006) (Table 2) were examined for their expression fold change in the sessile cells as compared to the inoculation cells. Actually, inoculation cells constitute a bacterial population in the late stationary phase of growth, whose gene expression profile resembles that of transmissive *L. pneumophila* cells (Bruggemann *et al.*, 2006). Interestingly, sessile cells appeared more related to bacteria in the replicative phase since flagellar genes (*flaA*, *fliA* and *fliS*), genes of the type IV secretion system substrates and their homologues (*ralF*, *sdeA*, *sdcA* and *sidC*) and *enhA*, encoding the enhanced entry protein A, were downregulated in the sessile cells. Accordingly, the key regulator *csrA*, known to repress the expression of transmissive traits, was clearly induced (4.29-fold) in sessile cells as compared to inoculation cells. However, when the more heterogeneous planktonic population constituted by suspension cells was used as reference, the flagellar genes, *sdeA* and *sdcA*, as well as *csrA*, appeared to be upregulated in sessile cells (Table 2).

In order to identify the main pathways involved in the *L. pneumophila* sessile mode of life, we further focused our study on genes with the highest alteration in expression in sessile cells with respect to both planktonic cell samples. Thus, regulated genes showing a significant threefold or higher expression change between biofilm cells and the two planktonic cell samples were selected (i.e. *lpl0660*, *lpl1853*, *lpl0236*, *lpl0237*, *lpl0482*, *lpl1129*, *lpl2032*, *lpl2271*, *lpl2272*, *lpl2462*, *lpl2719*, *lpl2728*, *lpl2780* and *lpl2916*). To confirm their differential regulation, the expression of these 14 genes was measured by qRT-PCR. Genes with at least a threefold change in expression, as revealed by both microarray and qRT-PCR, were further analysed (Table 3). This high-stringency selection was performed to reveal genes whose altered expression is truly specific for the sessile mode of life.

Analysis of biofilm-regulated genes

Among the genes with the most significantly altered expression in sessile cells, seven genes were shown to be 3–80-fold induced in biofilm cells by quantitative RT-PCR (Table 3) and so can be referred to as biofilm-regulated genes. The deduced protein sequences of three of these genes (*lpl0482*, *lpl2462* and *lpl2780*) show no similarity with proteins present in public databases and thus do not allow assignment of a putative function. The remaining four genes with induced expression in the biofilm comprise two distinct clusters. *lpl2271* and *lpl2272*, the most highly induced genes in sessile cells, encode AhpD and AhpC2. These alkyl hydroperoxide reductases have been shown to protect *L. pneumophila* from peroxide challenge (Le Blanc *et al.*, 2006). The second cluster whose gene expression was induced in the sessile cells consists of two genes, *lpl0236* and *lpl0237*. Lpl0236 contains the conserved domain of pyoverdine/dityrosine biosynthesis protein (pfam05141) and is 61 % homologous with PvcA from *Pseudomonas aeruginosa*. Lpl0237 is 66 % homologous with PvcB from *P. aeruginosa* and contains the conserved domain of the TauD, TfdA oxygenase family (pfam02668). In the *P. aeruginosa* genome, *pvcA* and *pvcB* are the first two genes of an operon of four genes (*pvcABCD*), encoding proteins required for the synthesis of the siderophore pyoverdine (Stintzi *et al.*, 1996, 1999). In *L. pneumophila*, the genes downstream of *lpl0237* show no similarity to *pvcC* and *pvcD*. Since it has been shown for *P. aeruginosa* (Musk *et al.*, 2005; Banin *et al.*, 2005) and for *Staphylococcus aureus* (Johnson *et al.*, 2005) that biofilm formation is linked to iron availability, we investigated a probable influence of iron on biofilm formation of *L. pneumophila* as well as the influence of iron on the expression of the putative siderophore-encoding genes (*lpl0236* and *lpl0237*) and the remaining five highly regulated genes within the biofilm.

Table 1. Genes whose mRNA level was significantly changed at least twofold in sessile cells with respect to both inoculation and suspension cells

Gene		Product description	Sessile vs			
			Inoculation		Suspension	
			FC*	*P*-value†	FC*	*P*-value†
Repressed genes						
lpl0183		Similar to hypothetical protein	0.45	1.1×10^{-3}	0.26	1.2×10^{-9}
lpl0660		Similar to hypothetical protein, predicted membrane protein	0.16	0	0.15	7.1×10^{-13}
lpl1030		Unknown	0.49	1.2×10^{-2}	0.40	1.2×10^{-4}
lpl1037		Similar to *Legionella* LvrB protein	0.43	2.5×10^{-4}	0.27	6.3×10^{-10}
lpl1059		Similar to EnhC protein, contains seven sel-1 domains	0.48	1.4×10^{-2}	0.28	4.0×10^{-8}
lpl1060		Similar to major facilitator family transporter	0.44	8.4×10^{-4}	0.32	1.8×10^{-7}
lpl1495		Similar to unknown protein	0.50	1.3×10^{-2}	0.34	7.4×10^{-7}
lpl1651		Some similarity with flagellar hook-length control protein FliK	0.41	2.8×10^{-4}	0.42	4.0×10^{-4}
lpl1837		Similar to putative general secretion pathway protein	0.48	7.6×10^{-3}	0.38	1.2×10^{-5}
lpl1853		Similar to hypothetical protein	0.23	3.0×10^{-10}	0.19	0
lpl1890		Similar to conserved hypothetical protein	0.42	1.2×10^{-3}	0.47	1.1×10^{-3}
lpl2184		Putative membrane protein	0.31	4.9×10^{-7}	0.41	3.9×10^{-5}
lpl2186		Similar to poly(3-hydroxyalkanoate) synthetase	0.46	7.9×10^{-4}	0.28	3.0×10^{-9}
lpl2297		Unknown	0.27	2.1×10^{-8}	0.47	2.0×10^{-3}
lpl2334		Similar to conserved hypothetical protein	0.46	2.5×10^{-3}	0.49	2.6×10^{-3}
lpl2500		Unknown	0.28	1.8×10^{-8}	0.40	5.5×10^{-5}
lpl2849		Similar to ABC transporter permease protein	0.49	1.2×10^{-2}	0.32	1.9×10^{-6}
Induced genes						
lpl0020		Similar to *Legionella* zinc metalloproteinase precursor	2.26	7.8×10^{-4}	2.20	1.5×10^{-3}
lpl0035		Unknown	2.19	9.3×10^{-4}	4.64	2.6×10^{-12}
lpl0061		Unknown	2.35	6.4×10^{-4}	2.53	1.1×10^{-5}
lpl0064		Similar to transposase	3.47	1.6×10^{-8}	2.83	1.5×10^{-5}
lpl0119		Some similarity to *L. pneumophila* IcmL/DotI	2.35	1.2×10^{-3}	4.36	6.6×10^{-12}
lpl0236		Similar to pyoverdine biosynthesis protein PvcA	3.22	1.7×10^{-6}	4.10	9.1×10^{-11}
lpl0237		Similar to pyoverdine biosynthesis protein PvcB	3.95	2.7×10^{-9}	3.71	4.7×10^{-9}
lpl0328		Unknown	2.51	6.4×10^{-5}	4.43	9.3×10^{-12}
lpl0444		Unknown	2.15	4.4×10^{-3}	4.51	3.6×10^{-12}
lpl0479		Ankyrin repeat protein	2.10	1.5×10^{-3}	4.84	0
lpl0482		Unknown	3.87	1.0×10^{-9}	4.03	9.7×10^{-11}
lpl0497	*icmJ/dotN*	Unknown	2.02	2.2×10^{-3}	2.85	7.6×10^{-7}
lpl0588	*prs*	Ribose-phosphate pyrophosphokinase	3.32	7.8×10^{-8}	2.52	1.4×10^{-5}
lpl0606		Unknown	5.94	3.3×10^{-13}	2.74	3.2×10^{-5}
lpl0628		Similar to unknown protein	7.95	0	2.68	2.0×10^{-5}
lpl0629		Similar to aminodeoxychorismate lyase (PabC)	6.72	0	2.39	3.6×10^{-5}
lpl0820	*csrA*	Global regulator CsrA	4.29	3.3×10^{-11}	2.67	7.1×10^{-6}
lpl0843		Similar to other proteins	2.03	6.7×10^{-3}	2.61	9.8×10^{-5}
lpl1001		Hypothetical protein	3.03	1.7×10^{-6}	2.54	5.1×10^{-5}
lpl1010		Similar to single-stranded DNA-binding protein (ssb)	3.02	3.0×10^{-6}	2.98	2.2×10^{-7}
lpl1089		Unknown	2.28	5.8×10^{-4}	2.73	2.4×10^{-5}
lpl1095		Unknown	2.51	9.3×10^{-5}	2.21	4.7×10^{-4}
lpl1126		Unknown	2.67	1.6×10^{-4}	3.47	5.2×10^{-8}
lpl1129		Unknown	3.15	2.7×10^{-6}	6.78	0
lpl1144		Unknown	3.04	3.9×10^{-6}	2.96	5.6×10^{-7}
lpl1163		Similar to aminopeptidase	2.01	2.1×10^{-3}	2.40	6.1×10^{-5}
lpl1450		Hypothetical protein	2.60	3.1×10^{-5}	2.57	1.2×10^{-5}
lpl1583		Similar to transposase	2.78	1.8×10^{-5}	2.21	8.0×10^{-4}
lpl1656		Conserved hypothetical protein	2.08	1.4×10^{-3}	3.92	2.2×10^{-9}
lpl1882		Weakly similar to endoglucanase	2.37	3.1×10^{-4}	3.45	7.5×10^{-9}
lpl1957		Some similarity with eukaryotic proteins	4.03	8.4×10^{-10}	2.85	1.8×10^{-6}
lpl1970	*mltA*	Similar to membrane-bound lytic murein transglycosylase	2.47	7.8×10^{-5}	2.23	1.8×10^{-4}
lpl2013		Similar to transporters	2.05	2.4×10^{-3}	2.04	6.6×10^{-4}

Table 1. cont.

Gene		Product description	Sessile vs			
			Inoculation		Suspension	
			FC*	*P*-value†	FC*	*P*-value†
lpl2032		Similar to transposase	7.25	0	4.05	3.1×10^{-10}
lpl2072		Protein with ankyrin repeat and a F-Box domain	2.03	1.6×10^{-2}	2.61	1.1×10^{-4}
lpl2123		Unknown	2.25	3.9×10^{-4}	3.10	7.7×10^{-7}
lpl2197		Unknown	3.66	2.5×10^{-8}	2.42	3.6×10^{-5}
lpl2213		Similar to phosphatase	2.35	1.4×10^{-4}	2.15	2.8×10^{-4}
lpl2214	*rluC*	Ribosomal large subunit pseudouridine synthase	2.23	5.1×10^{-4}	2.66	1.7×10^{-5}
lpl2271		Similar to alkyl hydroperoxide reductase AhpD	10.74	0	9.78	0
lpl2272		Similar to alkyl hydroperoxide reductase AhpC	30.72	0	23.30	0
lpl2366		Unknown	2.42	2.5×10^{-4}	2.17	8.5×10^{-4}
lpl2462		Unknown	3.08	4.9×10^{-7}	5.44	0
lpl2514		Unknown	2.77	3.7×10^{-5}	3.22	1.4×10^{-7}
lpl2576	*rplU*	50S ribosomal protein L21	6.10	3.3×10^{-13}	2.44	3.3×10^{-5}
lpl2686	*rpsO*	30S ribosomal protein S15	3.21	2.6×10^{-7}	2.10	1.3×10^{-3}
lpl2719		Unknown	3.59	1.7×10^{-8}	4.09	4.8×10^{-11}
lpl2728		Unknown	3.12	8.9×10^{-7}	4.27	2.5×10^{-11}
lpl2780		Unknown	3.15	4.5×10^{-7}	3.46	5.6×10^{-8}
lpl2868		Similar to transposase	2.08	5.1×10^{-3}	2.44	5.3×10^{-4}
lpl2916	*atpB*	Highly similar to H^+-transporting ATP synthase chain a	6.31	0	3.06	2.3×10^{-7}
lpl2917	*atpI*	Similar to ATP synthase subunit i	5.57	0	2.61	1.5×10^{-5}

*FC, expression fold change: ratio of gene expression in sessile cells compared to inoculation or suspension cells.
†Differential analysis was carried out using the VM method [VarMixt package (Delmar *et al.*, 2005)], together with the Benjamini and Yekutieli (Reiner *et al.*, 2003) *P*-value adjustment method.

Effect of iron availability on biofilm-regulated gene expression

In order to determine whether iron availability constitutes a signal regulating gene expression in sessile cells, expression of the seven genes, here identified as biofilm-regulated, was measured by quantitative RT-PCR after growth in BYE broth supplemented with different iron concentrations. The *pvc* genes were positively regulated by iron since their expression under iron-rich conditions was 6–10-fold induced, compared to conditions without iron (Fig. 2a). However, expression of other biofilm-regulated genes did not appear to be significantly altered by iron availability. These results underline a likely involvement of *pvc* gene products in iron homeostasis but suggest that iron does not constitute the only signal triggering biofilm-regulated gene induction in sessile cells. Accordingly, iron-regulated genes in *L. pneumophila* such as *fur*, *frgA*, *iraAB* or *lbtAB* (Cianciotto, 2007) did not appear significantly regulated in our microarray data, except *feoA* (see Supplementary Table S2, available with the online version of this paper). The latter is involved in a ferrous-uptake system (Robey & Cianciotto, 2002), whose expression was 1.99-fold induced ($P=0.003$) in sessile cells as compared to suspension cells.

Iron availability influences biofilm formation

To further examine a possible link between the induction of *pvcA* in sessile cells, iron availability and biofilm formation, the *pvcA*-like gene of *L. pneumophila* Lens was inactivated by insertion of a kanamycin cassette, as described previously (Allard *et al.*, 2006). Biofilm formation by the Δ*pvcA* mutant and the wild-type strain was then compared in BYE supplemented with various iron concentrations. As estimated by our standard microtitre plate assay, high iron pyrophosphate concentration (1.25 vs 0.25 g l^{-1}) was detrimental to biofilm formation by wild-type *L. pneumophila* Lens, with a 6.5-fold reduction in CV binding (data not shown). This result was confirmed by microscopic analysis of biofilms formed on glass slides at various iron concentrations (Fig. 2b) since wild-type *L. pneumophila* Lens established a consistent biofilm with 0 or 0.25 g iron pyrophosphate l^{-1} whereas only a few adhered cells could be observed at higher iron concentrations. Also, biofilms formed by the *L. pneumophila* Lens Δ*pvcA* mutant did not appear significantly different from those of the wild-type strain whatever the conditions tested. The product of *pvcA* alone thus did not appear necessary for biofilm establishment by *L. pneumophila* Lens in standard BYE containing 0.25 g iron pyrophosphate l^{-1}. In

Table 2. Expression profiles of marker genes of the *L. pneumophila* biphasic life cycle in sessile cells

Gene	Sessile vs			
	Inoculation		Suspension	
	FC*	*P*-value†	FC*	*P*-value†
Transmissive phase				
flaA	0.42	4×10^{-4}	3.17	1×10^{-7}
sdeA	NS	NS	3.31	1×10^{-7}
sdcA	0.45	4×10^{-3}	2.09	1×10^{-3}
fliA	0.49	2×10^{-3}	2.06	7×10^{-4}
ralF	0.42	2×10^{-3}	1.93	2×10^{-3}
enhA	0.46	1×10^{-3}	NS	NS
fliS	0.44	6×10^{-4}	NS	NS
sidC	0.49	1×10^{-2}	3.12	6×10^{-7}
Replicative phase				
csrA	4.29	3×10^{-11}	2.67	7×10^{-6}
secE	3.38	1×10^{-7}	NS	NS

*FC, expression fold change: ratio of gene expression in sessile cells compared to inoculation or suspension cells.
†Differential analysis was carried out using the VM method [VarMixt package (Delmar *et al.*, 2005)], together with the Benjamini and Yekutieli (Reiner *et al.*, 2003) *P*-value adjustment method. NS, Non-significant.

addition, disruption of *pvcA* did not modify biofilm formation under iron-rich conditions, suggesting that the deleterious effect of iron on biofilm formation is neither mediated nor counterbalanced by *pvcA* induction in sessile cells.

DISCUSSION

We report here the development of a monospecies model biofilm for *L. pneumophila*, a bacterium known to form biofilms in nature, which affects its distribution and spread. Although these models do not reflect the complexity found in natural environmental biofilms, monospecies biofilms were the first step towards understanding adaptation to the sessile lifestyle for many bacteria, including two recent studies on *L. pneumophila* (Mampel *et al.*, 2006; Piao *et al.*, 2006). These models pave the way for the study of complicated microbial interactions and have proven to be useful for assessing the gene expression profile of sessile cells even if the different cell types present within a biofilm cannot be analysed independently by this approach (Schembri *et al.*, 2003; Beloin *et al.*, 2004; Waite *et al.*, 2005; Whiteley *et al.*, 2001).

Our study has revealed that biofilm formed by *L. pneumophila* is more consistent at 20 °C than at 37 or 42 °C. Piao *et al.* (2006) also reported that the *L. pneumophila* biofilms with structural features typical of biofilms (i.e. pillar- and mushroom-like structures) formed at 25 °C remained more stably attached than filamentous, mycelial-mat-like biofilms formed at 37 and 42 °C. Also, Mampel *et al.* (2006) reported that biofilm formation at 37 °C was 30 % lower than at 23 and 30 °C. Furthermore, we have shown that adhesion is highest for late stationary phase cells. This is expected, as *L. pneumophila* shows increased expression of the flagellum and of type IV pili, both of which have been described in other bacteria to be involved in adhesion (O'Toole & Kolter, 1998; Pratt & Kolter, 1998; Watnick & Kolter, 1999), in stationary phase during low-temperature growth (Heuner *et al.*, 1999; Soderberg *et al.*, 2004). Because stationary phase mimics the transmissive phase in which bacteria are released from

Table 3. Genes whose mRNA level was changed at least threefold in sessile cells with respect to both inoculation and suspension cells

Gene	Product description	Sessile vs					
		Inoculation			Suspension		
		Microarrays		qRT-PCR*	Microarrays		qRT-PCR*
		FC†	*P*-value‡	FC† ± SD	FC†	*P*-value‡	FC† ± SD
lpl0236	Similar to pyoverdine biosynthesis protein PvcA	3.22	1.7×10^{-6}	3.15 ± 0.90	4.10	9.1×10^{-11}	20.49 ± 2.80
lpl0237	Similar to pyoverdine biosynthesis protein PvcB	3.95	2.7×10^{-9}	5.21 ± 0.75	3.71	4.7×10^{-9}	44.30 ± 11.37
lpl0482	Unknown	3.87	1.0×10^{-9}	9.56 ± 0.80	4.03	9.7×10^{-11}	12.53 ± 0.92
lpl2271	Similar to alkyl hydroperoxide reductase AhpD	10.74	0	16.74 ± 0.08	9.78	0	41.39 ± 2.43
lpl2272	Similar to alkyl hydroperoxide reductase AhpC	30.72	0	25.30 ± 1.24	23.30	0	80.19 ± 2.75
lpl2462	Unknown	3.08	4.9×10^{-7}	5.43 ± 0.16	5.44	0	16.78 ± 1.72
lpl2780	Unknown	3.15	4.5×10^{-7}	7.32 ± 0.36	3.46	5.6×10^{-8}	20.79 ± 3.05

*qRT-PCR, quantitative RT-PCR.
†FC, expression fold change: ratio of gene expression in sessile cells compared to inoculation or suspension cells.
‡Differential analysis was carried out using the VM method [VarMixt package (Delmar *et al.*, 2005)], together with the Benjamini and Yekutieli (Reiner *et al.*, 2003) *P*-value adjustment method.

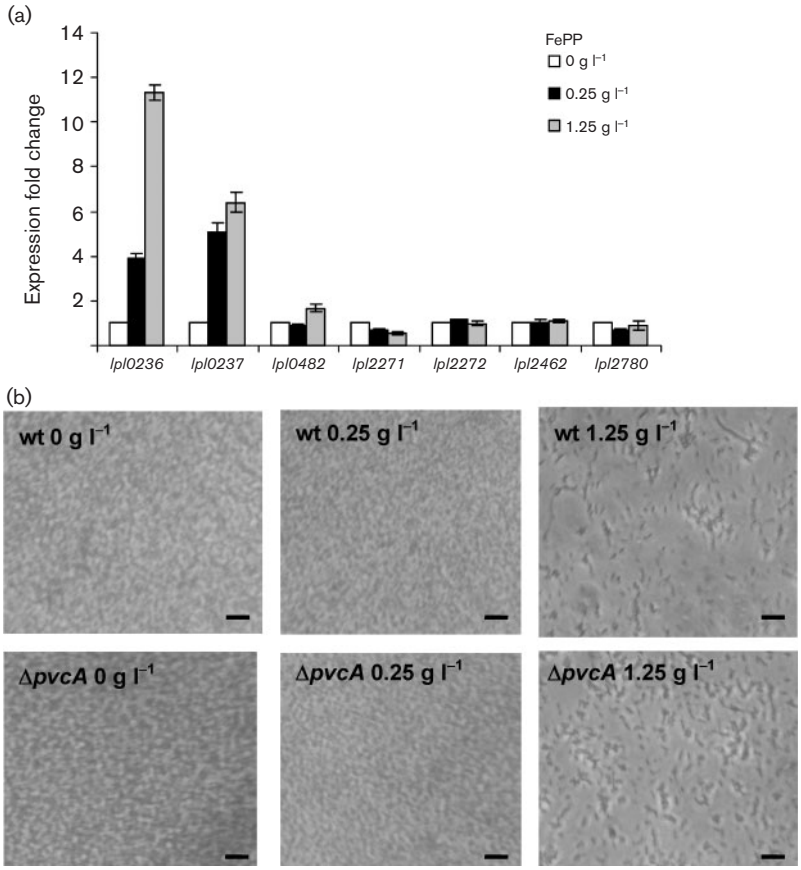

Fig. 2. Effect of iron pyrophosphate on expression of biofilm-regulated genes and biofilm formation by *L. pneumophila* Lens. (a) Bacteria were grown at 20 °C in BYE containing 0, 0.25 or 1.25 g iron pyrophosphate l^{-1} until stationary phase. Expression of biofilm-regulated genes is presented as expression fold change at 0.25 or 1.25 g l^{-1} as compared to expression at 0 g l^{-1} iron pyrophosphate. Values are means ± SD from two experiments. (b) Influence of iron concentration on biofilm formation of *L. pneumophila* Lens wild-type and Δ*pvcA* mutant. Bars, 10 μm.

amoebae (Molofsky & Swanson, 2004), our observations suggest that bacteria that exit host cells in natural low-temperature environments are in an optimal physiological state to adhere to biofilms.

We report here the first transcriptome analysis of *L. pneumophila* biofilm cells. It reveals that expression of 2.3 % (69 genes) out of the 2932 predicted genes was changed at least twofold in biofilm cells with respect to two distinct planktonic cell populations. This is consistent with two previous reports on *Escherichia coli* biofilms that stated that genes whose expression is regulated in the same way when biofilm cells are compared to both exponential and stationary phase cells represent about 1 % of the predicted *E. coli* genes (Schembri *et al.*, 2003; Beloin *et al.*, 2004). The global physiological state of sessile *L. pneumophila* cells was judged by comparing the gene expression pattern with those determined for transmissive- and replicative-phase *L. pneumophila* during growth in *A. castellanii* (Bruggemann *et al.*, 2006). Interestingly, the gene encoding the global regulator, CsrA, involved in repression of transmission and survival traits (Molofsky & Swanson, 2003), was induced in sessile cells. In our study, sessile cells thus seem to more closely resemble replicative- than transmissive-phase bacteria, suggesting that biofilm constitutes a favourable environment for *L. pneumophila*. Surprisingly, the flagellar genes whose expression is induced in the transmissive phase in *A. castellanii* were induced in the sessile cells as compared to the suspension cells. Since CsrA is known to downregulate flagellar gene expression in *L. pneumophila* (Fettes *et al.*, 2001), this observation suggests that the regulatory networks are modified in the biofilm as compared to planktonic life. Such modification may allow expression of *L. pneumophila* traits required for the biofilm lifestyle such as the flagella although CsrA is induced. This observation is in line with a recent report that expression of flagella is maintained throughout all stages of *E. coli* biofilm development (Domka *et al.*, 2007). Searching for genes predicted to encode proteins involved in extracellular matrix generation revealed *lpl2186* encoding a putative poly-β-hydroxyalkanoate (PHA) synthase that is repressed in *L. pneumophila* sessile cells with respect to the planktonic populations. Interestingly, Pham *et al.* (2004) have suggested that PHA accumulation in *P. aeruginosa* is in competition with alginate biosynthesis and that the PHA-negative mutant formed a stable biofilm with large, distinct and differentiated microcolonies characteristic of alginate-overproducing strains of *P. aeruginosa*.

A stringent analysis of the biofilm-regulated genes highlighted seven genes whose expression was at least threefold altered in sessile cells with respect to both planktonic cell populations. Among these genes were the clusters *pvcAB*

and *ahpC2D*. The first encodes proteins homologous to PvcA and PvcB that are required for production of the siderophore pyoverdine in *P. aeruginosa*. However, there are differences between *L. pneumophila* and *P. aeruginosa* regarding the *pvc* genes. First, we demonstrated that their expression in *L. pneumophila* increases in response to high iron concentrations in broth culture, while they are repressed under these conditions in *P. aeruginosa*. Second, the *pvc* locus of *P. aeruginosa* contains four genes, *pvcABCD*, whereas only *pvcA* and *pvcB* are present in the *L. pneumophila* genome (Stintzi *et al.*, 1999). Third, *pvc*-like genes of *L. pneumophila* might not be involved in siderophore activity, since *pvc* mutants showed no difference as compared to the wild-type strain in chrome azurol S assays (Allard *et al.*, 2006). It is thus likely that the function of the *pvcA* and *pvcB* products in *L. pneumophila* is different from that in *P. aeruginosa*. The second gene cluster, including *ahpC2* and *ahpD*, whose products are alkyl hydroperoxide reductases, displayed the highest induction in biofilm cells. These proteins are known to play a role in protection against oxidative stress (Rocha & Smith, 1999; Le Blanc *et al.*, 2006).

Interestingly, iron is known to participate in the production of reactive oxygen intermediates (Andrews *et al.*, 2003). In its soluble, reduced state (Fe^{2+}) iron is toxic; its oxidation by oxygen to Fe^{3+} induces production of hazardous intermediate reactive species. Oxidative stress and iron metabolism are often related. In *Neisseria meningitidis*, an iron-induced operon is required for protection against hydrogen peroxide-mediated killing (Grifantini *et al.*, 2004) and in *Campylobacter jejuni*, induced expression of *ahpC* under iron limitation is believed to counterbalance the intracellular accumulation of iron due to active acquisition systems induced under these conditions (Baillon *et al.*, 1999). Induction of both *pvcAB* and *ahpC2D* genes in sessile cells could thus be related and reflect the need for protection against oxidative stress resulting from high iron concentrations. A possible explanation is that the *pvc* genes, encoding proteins putatively involved in synthesis of siderophore-like molecules, may contribute to iron sequestration and/or uptake in order to maintain iron concentration below a toxic level. Interestingly, proteomic studies on a *P. aeruginosa* biofilm revealed the induction of AhpC, together with L-ornithine 5-monooxygenase, whose gene is located within the *pvd* locus involved in pyoverdine synthesis (Visca *et al.*, 1994; Sauer *et al.*, 2002). In contrast, transcriptome analyses of *P. aeruginosa* biofilms (Whiteley *et al.*, 2001; Waite *et al.*, 2005) revealed no induction of the pyoverdine genes, except when the gene expression profile of biofilm cells was compared to that of cells released from the biofilm (Sauer *et al.*, 2004). However, only *pvcAB* appeared to be iron-regulated during the planktonic lifestyle, suggesting that iron does not constitute the only signal inducing expression of the biofilm-regulated genes. Accordingly, the expression of other iron-regulated genes, as described by Cianciotto (2007), was not significantly altered in sessile cells. In addition, except the *ahp* genes, no other gene encoding proteins related to resistance to oxidative stress (Table S1) was induced in the sessile cells, suggesting that sessile cells undergo a moderate oxidative stress. Nevertheless, high iron concentrations (a fivefold increase in iron pyrophosphate concentration) appear detrimental to the sessile mode of life since our experiments revealed a strong inhibition of biofilm formation under these conditions. Interestingly, it has already been shown that iron salts perturb biofilm formation of *P. aeruginosa* (Musk *et al.*, 2005). It has also been proposed that a critical level of intracellular iron serves as the signal for biofilm development (Singh, 2004; Banin *et al.*, 2005). The *L. pneumophila pvcA* mutant showed no significant difference in biofilm formation whatever the tested iron concentration. The product of *pvcA* alone is thus not essential either for biofilm formation by *L. pneumophila* or for protection of sessile cells against high iron concentrations. Its induction in sessile cells might therefore be triggered by an unknown signal rather than by an iron-rich environment. Alternatively, *pvcA* inactivation may be compensated for by another *L. pneumophila* gene.

In conclusion, these first results on *in vitro* biofilm formation of *L. pneumophila* and the characterization of its transcriptional profile provide the basis for a better understanding of the sessile mode of life of *L. pneumophila*. It will be challenging to explore the fine interactions between iron, oxidative stress and biofilm formation.

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