Sessile *Legionella pneumophila* is able to grow on surfaces and generate structured monospecies biofilms

S. Pécastaings[a,b]*, M. Bergé[a], K.M. Dubourg[b] and C. Roques[a]

[a]*LU 49, Adhésion bactérienne et formation de biofilms, UPS; Université de Toulouse, Toulouse, France;* [b]*Institut du Thermalisme, Université Victor Segalen-Bordeaux 2, Dax, France*

Currently, models for studying *Legionella pneumophila* biofilm formation rely on multi-species biofilms with low reproducibility or on growth in rich medium, where planktonic growth is unavoidable. The present study describes a new medium adapted to the growth of *L. pneumophila* monospecies biofilms *in vitro*. A microplate model was used to test several media. After incubation for 6 days in a specific biofilm broth not supporting planktonic growth, biofilms consisted of 5.36 $\pm$ 0.40 log (cfu cm^{-2}) or 5.34 $\pm$ 0.33 log (gu cm^{-2}). The adhered population remained stable for up to 3 weeks after initial inoculation. *In situ* confocal microscope observations revealed a typical biofilm structure, comprising cell clusters ranging up to $\sim$300 μm in height. This model is adapted to growing monospecies *L. pneumophila* biofilms that are structurally different from biofilms formed in a rich medium. High reproducibility and the absence of other microbial species make this model useful for studying genes involved in biofilm formation.

Keywords: *Legionella pneumophila*; biofilm; sessile growth; confocal microscopy

Introduction

The etiological agent of Legionnaire's Disease and Pontiac Fever, *Legionella pneumophila*, is a ubiquist Gram-negative rod frequently isolated in fresh water. Ecological studies have demonstrated that environmental reservoirs for the Legionellaceae are usually freshwater aquatic systems such as lakes and rivers, as well as soil (Fliermans et al. 1981). In the environment, *Legionella pneumophila* adopts a specific life cycle, alternating between a transmissive phase and a replicative phase, that allows the bacterium to colonize and replicate in protozoa. The ability of *L. pneumophila* to replicate in eucaryotic phagocytic cells in the environment most likely accounts for its ability to infect human lung macrophages and cause a potentially lethal pneumonia (Molofsky and Swanson 2004). Because of its ubiquity, *L. pneumophila* also colonizes man-made environments such as water pipes, shower heads, faucets, fountains, spas, air-conditioning systems, and cooling towers. Inhalation of water droplets from these devices can lead to Legionnaire's disease (Bollin et al. 1985). Various parameters favor the presence of *L. pneumophila* in water, such as hardness, temperature (between 25 and 45°C), corrosion, scale, flow regimes (Lasheras et al. 2006; Liu et al. 2006), and biofilms (Murga et al. 2001). Biofilms are fixed microbial communities located at the interface between a biotic or abiotic surface and an aqueous phase (Donlan 2002; Hall-Stoodley et al. 2004) and are considered to be the most important source of water pipe contamination (Berry et al. 2006). One of the many advantages of a biofilm to *L. pneumophila* is its increased resistance to stressful environmental conditions and to biocides (Kim et al. 2002; Borella et al. 2005).

At this time, little is known about surface colonization and biofilm formation by *L. pneumophila*. Previous studies have shown that in oligotrophic conditions *L. pneumophila* is able to colonize multispecies biofilms from water systems, (Rogers and Keevil 1992; Rogers et al. 1994). Some authors argue that other microbial species are indispensable for *L. pneumophila* to multiply in a biofilm in such conditions (Kuiper et al. 2004; Declerck et al. 2007). Hence, these models involve interactions among multiple species, making them poorly reproducible *in vitro* for subsequent analyses.

More recently, *L. pneumophila* biofilm formation was studied in a model using the rich growth medium, Buffered Yeast Extract (Piao et al. 2006; Hindre et al. 2008), to produce monospecies *L. pneumophila* biofilms. However, Mampel et al. (2006) showed that growth was mostly due to planktonic rather than sessile growth. Also, growth conditions used in these studies could have led to a different biofilm structure compared to that developed in less nutritious conditions. The aim of the present study was to design

*Corresponding author. Email: sophie@pecastaings.net

conditions that promote the growth of adherent *L. pneumophila*, not simple cell adhesion, in order to produce a monospecies structured biofilm and to dispose of a more accurate model for *in vitro* purposes.

Materials and methods

Bacterial strains and growth conditions

Four strains of *L. pneumophila* were used. *L. pneumophila* serogroup 1 strain Philadelphia 1 was obtained from the Collection Institut Pasteur, Paris, France (CIP 103854 corresponding to strain ATCC 33152) and was used as a reference strain. Two environmental non-serogroup 1 *L. pneumophila* strains were also used: one was isolated in a hot water system by the Laboratory of Industrial Microbiology, Toulouse, France (HW S2-14), and the other in a mineral water system, by the Institut du Thermalisme in Dax, France (NMW S2-14). The fourth strain was a serogroup 1 environmental isolate from a hot water system, recovered by the Laboratory of Bacteriology and Hygiene of the Hospital of Purpan, Toulouse, France (HW S1). Strains were stored in Eugon broth (bioMérieux, Marcy l'Étoile, France) plus 15% glycerol (VWR International, Fontenay-sous-Bois, France), at $-80°C$. All strains were routinely grown on Buffered Charcoal Yeast Extract agar (BCYE, bioMérieux, Marcy l'Étoile, France), for 72 h at 37°C.

Before assays, bacterial suspensions were made by dispersing *L. pneumophila* colonies from a BCYE Petri dish (from frozen stock) in 10 ml of liquid medium. The optical density at 600 nm (OD_{600}) was adjusted to 0.220, in order to obtain a concentration of $\sim 10^8$ cfu ml^{-1}.

Biofilm formation was tested in various broths. Oligotrophic media consisted of sterile distilled water (SDW, sterilized at 121°C for 15 min), filter-sterilized (0.22 μm) tap water (STW, $Na^+ = 13.0$ mg l^{-1}, $K^+ = 1.1$ mg l^{-1}, $Ca^{2+} = 34.0$ mg l^{-1}, $SO_4^{2-} = 23.0$ mg l^{-1}, $Cl^- = 12.0$ mg l^{-1}, pH 7.9), and filter-sterilized natural mineral water (NMW, $Na^+ = 128.9$ mg l^{-1}, $K^+ = 20.8$ mg l^{-1}, $NH_4^+ = 0.2$ mg l^{-1}, $Ca^{2+} = 127.0$ mg l^{-1}, $Mg^{2+} = 35.0$ mg l^{-1}, $SO_4^{2-} = 432.1$ mg l^{-1}, $Cl^- = 153.9$ mg l^{-1}, $Fe^{2+} = 0.08$ mg l^{-1}, pH 7.4). These oligotrophic media were tested with and without an iron and cystein supplement (*Legionella* supplement, bioMérieux, Marcy l'Étoile, France) that was used pure (1:1) or in a tenfold dilution (1:10).

Two biofilm broths (BBs), already in use for the formation of biofilms of *Pseudomonas aeruginosa, Escherichia coli*, or *Staphylococcus aureus* (Alasri et al. 1992; Pineau et al. 1997; Samrakandi et al. 1997; Campanac et al. 2002; Khalilzadeh et al. 2010), were tested, viz. BB and modified biofilm broth (MBB). The mineral composition of these broths is similar (Table 1), however, they differ in their carbon source. MBB contains only one carbohydrate (glucose), whereas BB contains a carbohydrate (lactose) and amino acids (yeast extract and casamino acids). Supplemented biofilm broth (SBB) and supplemented modified biofilm broth (SMBB) are derivations of BB and MBB respectively that were supplemented with iron and cystein (*Legionella* supplement, bioMérieux, Marcy l'Étoile, France, 1:10 diluted). Broths were initially prepared as 10-fold concentrated solutions and then autoclaved, except for $MgSO_4$, which was prepared and autoclaved separately as a 100-fold concentrated solution. The *Legionella* supplement (bioMérieux, Marcy l'Étoile, France) was reconstituted as recommended by the manufacturer. The solutions and the *Legionella* supplement were then appropriately diluted to obtain the final broths (see composition in Table 1).

In addition, a liquid rich medium, BYE (*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-Buffered Yeast Extract), was used for the tests. This medium, classically used to grow *L. pneumophila* planktonically (Ristroph et al. 1980), has already been used to produce *L. pneumophila* monospecies biofilms (Mampel et al. 2006; Piao et al. 2006; Hindre et al. 2008).

Table 1. Composition of tested biofilm broths.

Composition	Concentration (mg l^{-1})	Biofilm broth (BB)	Supplemented biofilm broth (SBB)	Modified biofilm broth (MBB)	Supplemented modified biofilm broth (SMBB)
$MgSO_4$[a]	200.0	+	+	+	+
$FeSO_4$[a]	0.5	+	+	+	+
Na_2HPO_4[a]	1250.0	+	+	+	+
KH_2PO_4[a]	500.0	+	+	+	+
Casamino acids[b]	100.0	+	+	−	−
Lactose[b]	25.0	+	+	−	−
Yeast extract[c]	100.0	+	+	−	−
Glucose[a]	50.0	−	−	+	+
Legionella supplement[d]	1/10 diluted	−	+	−	+

[a]Sigma-Aldrich, Saint Louis, MO, USA; [b]Difco, Becton Dickinson, Spark, MD, USA; [c]AES, Bruz, France; [d]bioMérieux, Marcy l'Étoile, France.

Biofilm production

Bacterial suspensions of the tested strains were prepared and diluted in each tested medium in order to obtain a concentration of either 10^6 or 10^2 cfu ml^{-1}. Two milliliters of bacterial suspensions were added to the wells of 24-well polystyrene microplates (BD Falcon, San Jose, CA, USA). Microplates were incubated statically at 37°C, for 6–20 days according to assays. During the course of incubation, the medium was renewed every 3 days, after two gentle rinsings, in order to eliminate planktonic bacteria and favor sessile growth. Medium selection was made with a serogroup 1 reference strain (CIP 103854) and an environmental non-serogroup 1 strain (HW S2-14).

In-well biofilm samplings were made at least 2 h after inoculation in order to evaluate initial bacterial adhesion, and then every 3 days. Before biofilm collection, planktonic cells were sampled by pipetting directly 100 μl in the bulk phase. After two successive rinsings with 2 ml of SDW, 1 ml of SDW was added and the well was scraped with a sterilized spatula for 1 min in order to detach biofilm cells. Cell quantification was made by culture on BCYE or quantitative PCR (q-PCR).

In order to quantify viable and culturable *L. pneumophila* (planktonic or adherent), samples were homogenized and serially diluted (10-fold dilutions), and 100 μl of each dilution was spread on BCYE plates and incubated at 37°C for 5 days.

q-PCR was used to measure the total DNA in *L. pneumophila* biofilm samples. DNA was extracted from samples (100 μl), according to the manufacturer's instructions (Genesystems, Bruz, France) described elsewhere (Yaradou et al. 2007). Briefly, DNA was extracted using a Genextract device (Genesystems, Bruz, France), that allows the realization of all extraction steps, and extraction reagents (Genesystems, Bruz, France), by sonication (251 watts, 40 Hz, 20 min) and heat treatment (100°C, 10 min). DNA was purified on a silica column and eluted in order to proceed to q-PCR. DNA samples were analysed in triplicate. A positive (with exogenous DNA) and a negative (without probe) controls were made for each sample. A negative control was made for each extraction batch (with SDW). Calibration curves were made with calibrated *L. pneumophila* (strain ATCC 33152) DNA (Genesystems, Bruz, France). Results are given in terms of genome units (gu).

Control of planktonic growth

Liquid cultures of *L. pneumophila* (CIP 103854 or HW S2-14), were performed in BYE (Ristroph et al. 1980) or in SBB, in a neutral glass tube. Tubes were incubated at 37°C and 250 rpm using an orbital shaker (New Brunswick Scientific, Edison, NJ, USA), for 72 h. Growth was analyzed by measuring OD_{600} at 2, 24, 48, and 72 h.

Confocal laser scanning microscopic (CLSM) observations

For microscopic studies, biofilms were grown as described above, in 6-well polystyrene microplates (BD Falcon, San Jose, CA, USA). Before observing biofilms, 0.5 μl of Syto® 9 (Syto9, 5 mM, Molecular Probe, Invitrogen, Eugene, OR, USA) was added directly into the wells. No rinsing was performed before observation because moving planktonic cells were clearly distinguishable from fixed biofilm cells, and also because the biofilm growth medium did not create any artifacts. Syto9 is a cell-permeant nucleic acid stain that shows significant fluorescent shift when bound to nucleic acids. This dye was sometimes used with Propidium Iodide (PI, LIVE/DEAD© *Bac*Light™ kit, Invitrogen, Eugene, OR, USA), an intercalating nucleic acid dye, as a counterstain. One μl (20 mM in DMSO) of PI was added directly into the wells when necessary. PI is membrane impermeant and stains only cells with damaged membranes or extracellular DNA (eDNA), whereas Syto9 stains damaged and intact cells and also eDNA (Stocks 2004). Cells with damaged membranes and eDNA, stained with both dyes, appear mostly red because of the fading of Syto9 by PI. However, intact cells appear green. Ohno et al. (2003) validated the use of this kit to detect *L. pneumophila* viability in water.

Observations were made with a TCS SPII confocal microscope (Leica, Heidelberg, Germany), using a 40 × (HCX PL APO water, N.A. 0.8) water immersion lens. The 488 nm ray line of an argon laser was used to detect the emission of Syto9 fluorescence, collected in the range between 498 and 533 nm. For PI, an HeNe 543 nm laser was used and the emitted fluorescence was collected in the range between 553 and 623 nm. Samples were scanned in the xy direction with a step size of 0.7 μm. Image stacks are represented as maximal intensity projections (using the Z-project plugin) or volumic reconstructions (using the Volume Viewer plugin), achieved with Image J, a free image analysis program from NIH (Rasband 1997–2009). Each image is representative of the results of two assays.

Statistical analysis

Results obtained at different quantification times or with different quantification methods were compared. When comparing sample means, a Student's t test was performed, using MS Excel. Statistical significance was inferred for $p \leq 0.01$.

Results

Protocol designing for L. pneumophila *biofilm formation*

With the aim of describing conditions that support the sessile growth of *L. pneumophila*, three types of media were tested: (i) oligotrophic media and supplemented oligotrophic media, (ii) BBs and SBBs, and (iii) rich medium. Suspensions (10^6 cfu ml^{-1}) of CIP 103854 and HW S2-14 strains were incubated in 6 well-microplate wells for 6 days before confocal observations. Figure 1 presents results obtained with STW (as a representative result for oligotrophic media and BBs,

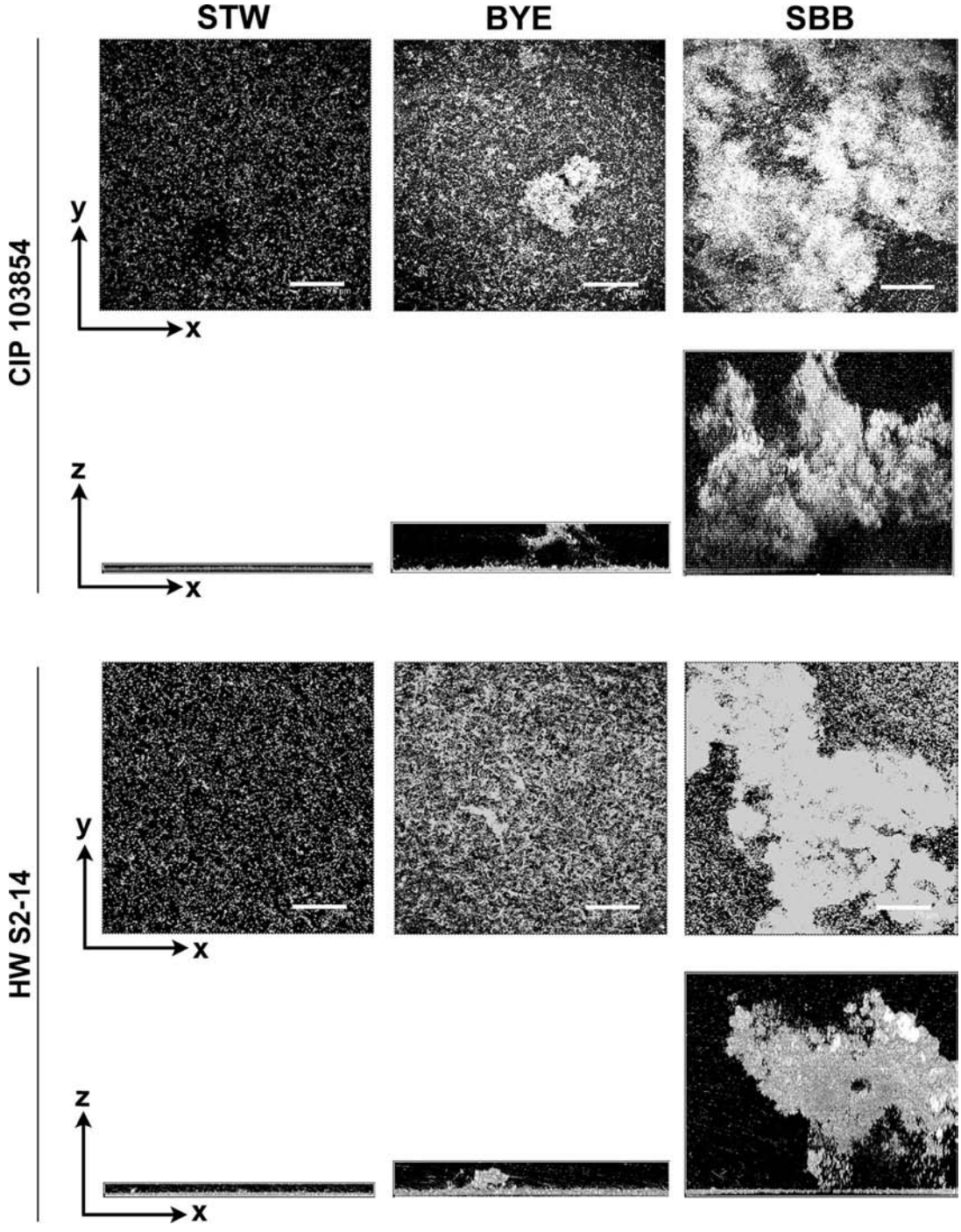

Figure 1. Analyses of 6-day-old biofilms of strain CIP 103854 (top) and HW S2-14 (bottom) produced in sterile tap water (left), buffered yeast extract (center) and supplemented biofilm broth (right) starting from an inoculum of 10^6 cfu ml^{-1}. Biofilms produced in STW are representative of biofilms obtained in oligotrophic media and biofilm broths (except SBB). Images represent maximal fluorescence intensity in top view (x–y) or side view (x–z). Scale bar = 75 μm.

except SBB), BYE and SBB, as maximal intensity projections in the x-y direction (top view) or in the x-z direction (side view).

After 6 days in oligotrophic media or BBs (except SBB), cells adhered to the well surface, as illustrated by Figure 1, and formed a layer of 8.4 $\pm$ 0.5 μm above the well surface.

In the rich medium, cells formed a dense layer on the well surface that reached 15.2 $\pm$ 1.3 μm in thickness (Figure 1). Aggregates of variable size (from 32 to 64 μm) were disseminated on the cell layer. This type of aggregation is typical in BYE medium and has been described by Mampel et al. (2006). According to the images, more cells adhered to the wells containing BYE than to those containing oligotrophic media or BBs (except SBB). The ability of *L. pneumophila* to replicate planktonically in BYE (Ristroph et al. 1980; Mampel et al. 2006) probably led to a greater accumulation of cells on the well surface, thus resulting in a dense cell layer.

The formation of larger aggregates was observed in wells containing SBB. Under these conditions, bacterial aggregates reached a maximum height of 294.0 μm, as shown in Figure 1. Confocal analysis revealed that a layer of 18.0 $\pm$ 5.7 μm was fixed to the well surface. Aggregates were attached by pillars and developed vertically in the bulk medium (see profile images, Figure 1). It was therefore concluded from these experiments that SBB could constitute a good alternative model to produce *L. pneumophila* biofilms. Considering that BYE medium allows biofilm development that is based on planktonic replication (Mampel et al. 2006), planktonic growth in SBB medium was then investigated.

Validation of the growth of L. pneumophila *in biofilms*

Because *L. pneumophila* is a fastidious bacterium, a high number of cfu was initially inoculated in the microplate wells (10^6 cfu ml^{-1}) in order to reduce biofilm formation time. In order to check if biofilm formation originated in the growth of adherent cells or in the deposition of planktonic cells, two assays were performed. First, wells containing SBB were inoculated with low levels of bacteria (10^2 cfu ml^{-1}) of strains CIP 103854 and HW S2-14 and biofilm formation kinetics and planktonic populations were monitored using culture counts.

The results (Figure 2a) indicate that, for a low inoculum, the adhered population increased with incubation time. After 2 h, biofilms consisted of <1 log (cfu cm^{-2}). After 12 days, biofilms consisted of an average of 5.72 $\pm$ 0.10 log (cfu cm^{-2}) for the CIP 103854 strain ($n = 3$) and 6.84 $\pm$ 0.03 log (cfu cm^{-2}) for HW S2-14 ($n = 2$). Confocal analyses made in parallel clearly showed evolution into a typical biofilm structure (Figure 2b). During the adhesion stage (2 h), only a few dispersed cells adhered to the well surface (data not shown), whereas after 6 days a uniform layer of cells was formed and aggregates were evenly distributed on this basal layer for both strains. This structure was stable over a period of 20 days as long as the medium was renewed every 3 days (data not shown). Figure 2a shows that planktonic cells in the liquid medium followed the same development pattern as the biofilm population, even if adherent cells grew on a 7.04 cm^2 surface and planktonic cells developed in 2 ml of medium.

Two hypotheses were possible regarding the origin of the planktonic cells: (i) cells replicated in the planktonic phase and were deposited on the well surface, meaning that growth did not take place in the biofilm, or (ii) cells developed in the biofilm and were released in the planktonic phase. To test these hypotheses, a second test was performed. Suspensions of *L. pneumophila* (ATCC 33125 or HW S2-14) were made in BYE, as a positive control, and in SBB and incubated with agitation in order to limit biofilm development. Planktonic growth was monitored for 3 days, which correspond to the period between medium renewals in microplate wells. The results are shown in Figure 2c. As expected, in BYE, OD_{600} increased from 0.243 $\pm$ 0.018 to 4.100 $\pm$ 0.106 ($n = 3$, $p < 0.01$) for strain CIP 103854 and from 0.234 $\pm$ 0.017 to 2.597 $\pm$ 0.002 ($n = 3$, $p < 0.01$) for HW S2-14, confirming that both strains were able to grow planktonically in this medium. However, the OD_{600} remained steady in SBB for 3 days at 0.199 $\pm$ 0.033 ($n = 6$, for both strains). The absence of growth was also verified on SBB agar (as opposed to growth observed on the BCYE control, data not shown). These results demonstrate that planktonic growth does not occur in SBB, hence biofilms in microplates result only from the growth of adhered bacteria. This also implies that planktonic cells in the microplate wells (Figure 2a) originate in biofilm cells released in the planktonic phase.

Viability of cells in biofilms

Biofilms can contain cells in different physiological states (viable, viable but not culturable – VBNC, dead cells). q-PCR is a method that allows the quantification of total *L. pneumophila* genome units in a sample, derived from viable cells, VBNC, dead cells, and also eDNA. To verify the proportion of total genome units and culturable cells in biofilms formed in SBB, wells were inoculated with HW S2-14 and also with two other environmental strains (NMW S2-14 and HW S1, 10^6 cfu ml^{-1}) and biofilm formation was monitored by

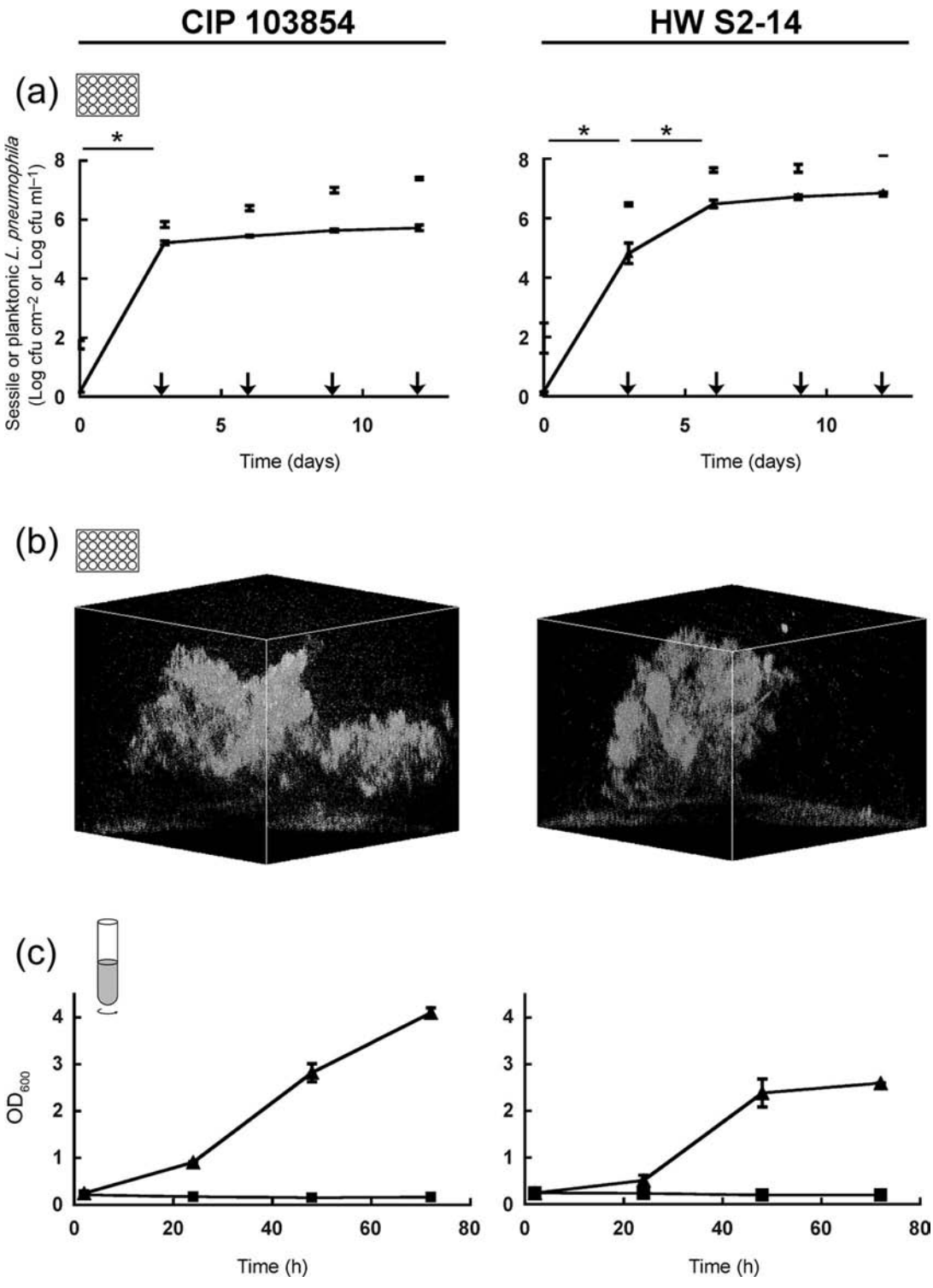

Figure 2. Growth of *L. pneumophila* (CIP 103854 on the left; HW S2-14 on the right) in SBB. (a) Kinetics of *L. pneumophila* biofilm formation (log [cfu cm^{-2}], curve) and corresponding planktonic concentrations (log [cfu ml^{-1}], dots) in microplate wells. Inocula consisted of 10^2 cfu ml^{-1}. Bacteria were first quantified 2 h after inoculation. Arrows, medium renewal in microplate wells; bars, SD ($n = 3$), **p*-values ≤ 0.01 between two biofilm quantification times. (b) *L. pneumophila* biofilm structures after 6 days. Images represent volumic reconstitutions of *L. pneumophila* biofilms observed with CLSM. Parallelepiped size is 375 μm $\times$ 375 μm $\times$ 284 μm for CIP 103854 and 375 μm $\times$ 375 μm $\times$ 265 μm for HW S2-14. (c) Planktonic growth of *L. pneumophila* in BYE (▲) or SBB (■) in tubes. Growth was monitored by the OD at 600 nm. Bars = SD ($n = 3$).

q-PCR and culture counts. The results are given in Figure 3.

Comparison of the data indicated no significant difference between culture counts and q-PCR results for all the tested strains, with the exception of the HW S2-14 strain after 2 h. 3.47 $\pm$ 0.09 log (cfu cm^{-2}) of adhered *L. pneumophila* cells were quantified by cultivation whereas 4.20 $\pm$ 0.08 log (gu cm^{-2}) were detected by q-PCR. This difference may be due to the presence of dead or non-cultivable bacteria in the inoculation suspension added to the wells. The strong correlation between the culture and q-PCR results indicate that the majority of the adhered cells in samples were viable and culturable. These results also mean that the method used to collect adhered biomass, involving well scraping, does not damage or induce a high mortality rate of biofilm cells and is effective in dispersing cells. Also, eDNA was not detected in the biofilm, probably because it was present in very low quantities, if at all.

These results were confirmed by staining a HW S2-14 biofilm with *Bac*Light viability kit. Figure 4 shows the results obtained on a 7 day-old biofilm (1 day after medium renewal). The image exhibits a majority of green cells in the biofilm, demonstrating that the biofilm was constituted mainly of viable cells and confirming the correlation of culture counts and q-PCR enumeration.

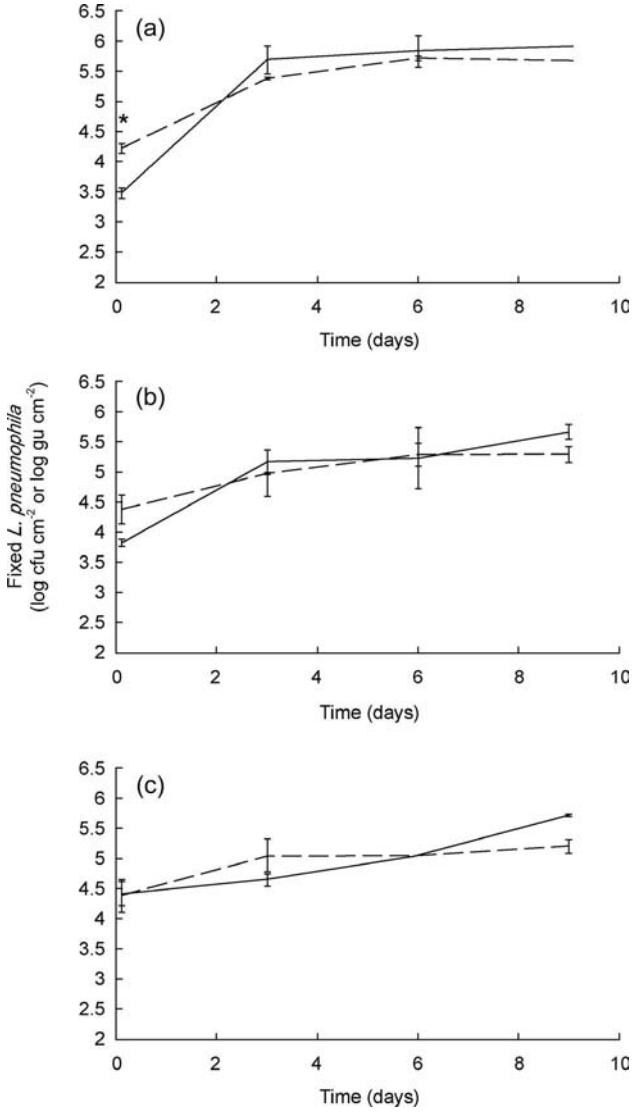

Figure 3. Kinetics of biofilm formation by *L. pneumophila* monitored by culture counts (– – –, log [cfu cm^{-2}]) or q-PCR (—, log [gu cm^{-2}]), starting with an inoculum of 10^6 cfu ml^{-1}. (a) Strain HW S2-14. (b) Strain NMW S2-14. (c) Strain HW S1. Bars = SD ($n = 2$); **p*-values ≤ 0.01 between culture and q-PCR results.

Discussion

L. pneumophila is a fastidious and facultative intracellular bacterium with specific growth needs (Feeley et al. 1978, 1979; Ristroph et al. 1981), hence it is assumed that monospecies proliferation (planktonically or in biofilm) in oligotrophic conditions is limited. In an oligotrophic medium like tap water, mixed species biofilms are considered as initiators for water system colonization by *L. pneumophila*. In the environment, biofilms often contain protozoa in which *L. pneumophila* can develop (Murga et al. 2001; Fields et al. 2002; Kuiper et al. 2004). Some bacterial species (Guerrieri et al. 2008; Declerck 2010) and specific algae are also known to enhance *L. pneumophila* development in aquatic environments (Taylor et al. 2009). In this case, *L. pneumophila* seems to profit from nutrients excreted by other species, and is thus considered as a secondary biofilm colonizer. For instance,

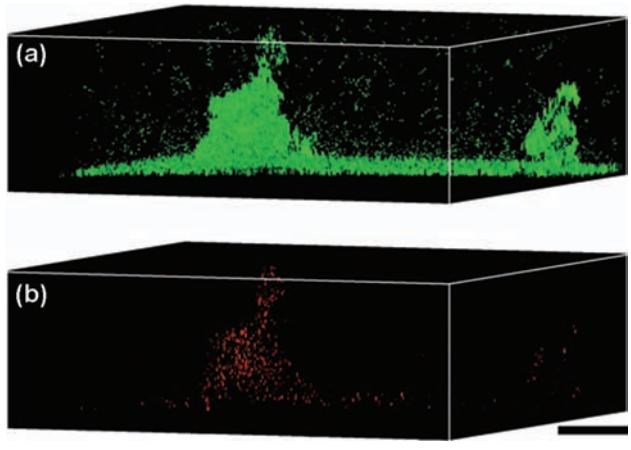

Figure 4. Viability of *L. pneumophila* cells in the biofilm. Images are volumic reconstitutions of a 7-day-old biofilm of strain HW S2-14 (inoculum of 10^6 CFU ml^{-1}) stained with Syto9 (a, total cells and eDNA) and PI (b, dead or damaged cells, eDNA). Scale bar = 50 μm.

Temmerman et al. (2006) demonstrated that *L. pneumophila* could grow in oligotrophic media containing heat-killed bacteria (necrotrophic growth), proving that *L. pneumophila* is able to grow independently of eukaryotes. However, interactions with various protozoan or bacterial species make these models poorly reproducible in studying *L. pneumophila* growth in biofilms. Since tap water alone is not sufficient to support *L. pneumophila* growth *in vitro*, a microplate assay using rich medium (BYE) is now used (Piao et al. 2006; Alleron et al. 2008; Hindre et al. 2008). However, Mampel et al. (2006) demonstrated that biofilms produced in these conditions rely on planktonic multiplication.

In the present study, consistent with the literature (Ohno et al. 2003), no sterile oligotrophic medium (sterile distilled water, mineral water or tap water) allowed the growth of *L. pneumophila* biofilms (Figure 1), even if supplemented with iron and cystein. This implies that the oligotrophic media used here did not contain enough nutrients, or appropriate nutrients, to support the sessile growth of *L. pneumophila*. The same is true for the BBs, except for SBB, a medium containing amino acids as a source of carbon and a supplement of iron and cystein. Among the BBs, only SBB promoted the growth of *L. pneumophila*, more specifically in the biofilm (Figure 2). These results are consistent with previous work showing that the replication of *L. pneumophila* relies on both amino acids as a carbon source (arginine, serine, threonine, cysteine, valine, and methionine (Ristroph et al. 1981)), and on a significant iron concentration (Feeley et al. 1978; Hickey and Cianciotto 1997). In the present study, amino acids were provided by yeast extract, casamino acids, and cystein supplement in SBB, even if their concentration was reduced compared to a rich growth medium like BYE. Hindre et al. (2008) showed that high concentrations of iron (1.25 g l^{-1}) inhibited *L. pneumophila* biofilm formation in rich medium compared to a biofilm grown with 0.25 g l^{-1} of iron. Here, iron concentration was lowered tenfold (25 mg l^{-1}) compared to BYE and this concentration was sufficient to support biofilm growth of *L. pneumophila*.

In the same study, Hindre et al. (2008) showed that the attachment of *L. pneumophila* was better in BYE at low temperatures (20°C) than at higher temperatures (37°C and 42°C). This result is seemingly contradictory with the results of Piao et al. (2006) and with the fact that, in the environment, *L. pneumophila* is most often recovered from warm water systems. In the present study, biofilm production was assayed at different temperatures (22.5, 37.0, and 43.0°C, see Supplementary material [Supplementary material is available *via* a multimedia link on the online article webpage]). It was concluded that, even if biofilms were observed at all temperatures when starting with high levels of bacteria (10^6 cfu ml^{-1}), the growth of adherent cells for low initial inocula (10^2 cfu ml^{-1}) was observed only at 37°C.

The protocol designed here is adapted to the formation of single-species biofilms of *L. pneumophila*, of all tested strains, *via* growth of adherent cells. Under the conditions set up in the present study, rinsing eliminated planktonic bacteria every 3 days. A complementary assay showed that planktonic growth was insignificant in SBB during incubation for 3 days if adhesion did not occur (Figure 2). It can be concluded that, in SBB, cells grow only when adhered and that cells colonizing the planktonic phase of the microplate wells are the result mainly of the release of cells actively growing in the biofilm (Figure 2a). Hence, the biofilm acts as a reservoir that allows colonization of the planktonic phase.

The switch between the planktonic state and the sessile state is tightly controlled in bacteria. Various regulatory pathways involving the second messenger cyclic di-GMP have been elucidated (Krasteva et al. 2010; Simm et al. 2004), some of them being sensitive to environmental cues (like the SinI-SinR system in *Bacillus subtilis* (Bai et al. 1993; Kearns et al. 2005)). Biofilm development in media not supporting planktonic development has already been described, eg for *P. aeruginosa* (Khalilzadeh et al. 2010) or *Xyllela fastidiosa* (Leite et al. 2004), and it can be hypothesized that the concentration of nutrients in the medium and/or the composition of the medium constitute signals for the bacteria to develop in a planktonic or a sessile mode.

According to this information, SBB appears to be a good intermediate solution for forming *L. pneumophila* biofilms. It is an intermediate solution between an oligotrophic medium, where *L. pneumophila* is able to survive but not to grow (Ohno et al. 2003), and a rich medium that favors planktonic growth prior to biofilm formation (Mampel et al. 2006). Using a microplate model to grow *L. pneumophila* Philadelphia-1 in a rich medium, Mampel et al. (2006) observed biofilms with a maximum height of 20 μm after incubation for 5 days at 30°C. Growing *L. pneumophila* strain Knoxville-1 for 6 days in BYE at 37°C led to the formation of unstructured biofilms 72 μm in thickness according to Piao et al. (2006). Biofilms obtained with the protocol described here differed substantially from biofilms produced in a rich medium and displayed characteristic features (Figures 1 and 2b). More specifically, the size of the aggregates reached $\sim$300 μm after incubation for 6 days, independently of the initial quantity of bacteria in the wells (10^2 or 10^6 cfu ml^{-1}). This is an indication that the SBB medium, compared to the

BYE medium, may favor the production of an extracellular matrix, allowing the structuration of cells into aggregates.

Interestingly, the biofilm and planktonic populations of all tested strains reached a maximum level after incubation for 6 days, and then remained stable for 20 days. The culturable biofilm population level stayed constant even though the medium was renewed every 3 days. This result indicates that sessile and planktonic populations of *L. pneumophila* are regulated in some manner, and that nutrient limitation is probably not the sole cause of that regulation. The microplate model described here, with frequent medium renewal, corresponds to quasi-static conditions. Liu et al. (2006) showed that *L. pneumophila* biofilm colonization was promoted by turbulent flows in a model plumbing system. It would be interesting to further analyze the flow effect on the structure of biofilms formed in SBB.

The physiology of *L. pneumophila* (formation of VBNC cells, cell death) is subjected to change when challenged by stresses such as biocide adjunction, temperature variations, low nutrient availability, or an increase in salt concentration (Ohno et al. 2003; Alleron et al. 2008). Moreover, biofilms are often constituted of cells in various physiological conditions (Kim et al. 2009) that can sometimes be recovered with difficulty by culture counts. Promising methods such as q-PCR provide rapid quantification of total DNA in a sample from viable, VBNC, damaged and dead cells (Rompre et al. 2002) and eDNA. q-PCR is currently used to complement standard cultivation methods in enumerating *Legionella* spp. and *L. pneumophila* in water (Wellinghausen et al. 2001; Yaradou et al. 2007). Comparing the results from culture and q-PCR methods also gives physiological information about bacteria. For example, in a study by Alleron et al. (2008), the authors described the diminution of culture count results after formation of VBNC cells in their samples (by the addition of monochloramine), while the q-PCR results remained stable. During the biofilm development observed with the present model, no significant difference was observed between colony counts and genome counts, indicating that most DNA detected by q-PCR originated from culturable cells. This conclusion also implies that biofilms did not contain detectable levels of VBNC cells, damaged cells, dead cells, or eDNA.

Syto9/PI staining is another useful method to detect physiological changes in a bacterial population: cells with an intact membrane (viable cells and VBNC cells) appear green (stained with Syto9), whereas damaged cells and eDNA can be observed in the red channel (stained with PI) (Baum et al. 2009; Giao et al. 2009). This method, combined with CLSM, is often used to visualize *in situ* changes in bacterial biofilms subjected to antimicrobial agents (Chiang et al. 2009; Dynes et al. 2009). In the conditions tested here, confocal observations of *L. pneumophila* biofilms stained with Syto9 and PI confirmed that the majority of the biofilm cells were viable *in situ* (green cells, Figure 4) and that damaged bacteria did not have a specific location in the biofilm. These results confirm quantification results obtained by culture and q-PCR that revealed a majority of viable cells in the biofilm.

In conclusion, the present study describes the use of a new medium, SBB, adapted to growing monospecies *L. pneumophila* biofilms, without planktonic growth. *L. pneumophila* biofilms formed under these conditions contain aggregates of cells that can range up to $\sim 300\ \mu m$ in height, which means that the SBB medium increases biofilm structuration, most likely by favoring the production of an extracellular matrix. This protocol could be useful for further studies concerning sessile *L. pneumophila* cells and the characterization of genes and proteins involved in biofilm formation. CLSM analyses revealed that *L. pneumophila* cells clung together in aggregates but were physically distant from each other (data not shown), compared to a *P. aeruginosa* biofilm formed in similar conditions (Khalilzadeh et al. 2010). Further charaterization of the biofilm matrix composition would provide new insights into *L. pneumophila* biofilm formation and behavior.

Acknowledgements

Sophie Pécastaings is grateful for grants received from the French Association for Thermal Spa Treatments Research (Association Française pour la Recherche Thermale, AFReTh) and the General Council of Les Landes. The authors also express their gratitude to the Regional Council of Midi-Pyrénées and to Genesystems (Bruz, France), for providing a GeneExtract and a GeneDiscCycler device and appropriate reagents for q-PCR analyses. In addition, the authors would like to thank C. Pouzet, Y. Martinez and A. Jauneau from the Microscopy Platform, and the Institut Fédératif de Recherche 40 of CNRS (National Center of Scientific Research) for microscopic analyses.

References

Alasri A, Roques C, Michel G, Cabassud C, Aptel P. 1992. Bactericidal properties of peracetic acid and hydrogen peroxide, alone and in combination, and chlorine and formaldehyde against bacterial water strains. Can J Microbiol 38:635–642.

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

Bai U, Mandic-Mulec I, Smith I. 1993. SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis*, by protein-protein interaction. Genes Dev 7:139–148.

Baum MM, Kainovic A, O'Keeffe T, Pandita R, McDonald K, Wu S, Webster P. 2009. Characterization of structures in biofilms formed by a *Pseudomonas fluorescens* isolated from soil. BMC Microbiol 9:103–115.

Berry D, Xi C, Raskin L. 2006. Microbial ecology of drinking water distribution systems. Curr Opin Biotechnol 17:297–302.

Bollin GE, Plouffe JF, Para MF, Hackman B. 1985. Aerosols containing *Legionella pneumophila* generated by shower heads and hot-water faucets. Appl Environ Microbiol 50:1128–1131.

Borella P, Guerrieri E, Marchesi I, Bondi M, Messi P. 2005. Water ecology of *Legionella* and protozoan: environmental and public health perspectives. Biotechnol Annu Rev 11:355–380.

Campanac C, Pineau L, Payard A, Baziard-Mouysset G, Roques C. 2002. Interactions between biocide cationic agents and bacterial biofilms. Antimicrob Agents Chemother 46:1469–1474.

Chiang WC, Schroll C, Hilbert LR, Moller P, Tolker-Nielsen T. 2009. Silver-palladium surfaces inhibit biofilm formation. Appl Environ Microbiol 75:1674–1678.

Declerck P. 2010. Biofilms: the environmental playground of *Legionella pneumophila*. Environ Microbiol 12:557–566.

Declerck P, Behets J, van Hoef V, Ollevier F. 2007. Replication of *Legionella pneumophila* in floating biofilms. Curr Microbiol 55:435–440.

Donlan RM. 2002. Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890.

Dynes JJ, Lawrence JR, Korber DR, Swerhone GD, Leppard GG, Hitchcock AP. 2009. Morphological and biochemical changes in *Pseudomonas fluorescens* biofilms induced by sub-inhibitory exposure to antimicrobial agents. Can J Microbiol 55:163–178.

Feeley JC, Gorman GW, Weaver RE, Mackel DC, Smith HW. 1978. Primary isolation media for Legionnaires disease bacterium. J Clin Microbiol 8:320–325.

Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB. 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J Clin Microbiol 10:437–441.

Fields BS, Benson RF, Besser RE. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin Microbiol Rev 15:506–526.

Fliermans CB, Cherry WB, Orrison LH, Smith SJ, Tison DL, Pope DH. 1981. Ecological distribution of *Legionella pneumophila*. Appl Environ Microbiol 41:9–16.

Giao MS, Wilks SA, Azevedo NF, Vieira MJ, Keevil CW. 2009. Validation of SYTO 9/propidium iodide uptake for rapid detection of viable but noncultivable *Legionella pneumophila*. Microb Ecol 58:56–62.

Guerrieri E, Bondi M, Sabia C, de Niederhausern S, Borella P, Messi P. 2008. Effect of bacterial interference on biofilm development by *Legionella pneumophila*. Curr Microbiol 57:532–536.

Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95–108.

Hickey EK, Cianciotto NP. 1997. An iron- and fur-repressed *Legionella pneumophila* gene that promotes intracellular infection and encodes a protein with similarity to the *Escherichia coli* aerobactin synthetases. Infect Immun 65:133–143.

Hindre T, Bruggemann H, Buchrieser C, Hechard Y. 2008. Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. Microbiology 154:30–41.

Kearns DB, Chu F, Branda SS, Kolter R, Losick R. 2005. A master regulator for biofilm formation by *Bacillus subtilis*. Mol Microbiol 55:739–749.

Khalilzadeh P, Lajoie B, El Hage S, Furiga A, Baziard G, Bergé M, Roques C. 2010. Growth inhibition of adherent *Pseudomonas aeruginosa* by a N-butanoyl-homoserine lactone analog. Can J Microbiol 56:1–9.

Kim BR, Anderson JE, Mueller SA, Gaines WA, Kendall AM. 2002. Literature review-efficacy of various disinfectants against *Legionella* in water systems. Water Res 36:4433–4444.

Kim J, Hahn JS, Franklin MJ, Stewart PS, Yoon J. 2009. Tolerance of dormant and active cells in *Pseudomonas aeruginosa* PA01 biofilm to antimicrobial agents. J Antimicrob Chemother 63:129–135.

Krasteva PV, Fong JC, Shikuma NJ, Beyhan S, Navarro MV, Yildiz FH, Sondermann H. 2010. *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. Science 327:866–888.

Kuiper MW, Wullings BA, Akkermans AD, Beumer RR, van der Kooij D. 2004. Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride. Appl Environ Microbiol 70:6826–6833.

Lasheras A, Boulestreau H, Rogues AM, Ohayon-Courtes C, Labadie JC, Gachie JP. 2006. Influence of amoebae and physical and chemical characteristics of water on presence and proliferation of *Legionella* species in hospital water systems. Am J Infect Control 34:520–525.

Leite B, Andersen PC, Ishida ML. 2004. Colony aggregation and biofilm formation in xylem chemistry-based media for *Xylella fastidiosa*. FEMS Microbiol Lett 230:283–290.

Liu Z, Lin YE, Stout JE, Hwang CC, Vidic RD, Yu VL. 2006. Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. J Appl Microbiol 101:437–442.

Mampel J, Spirig T, Weber SS, Haagensen JA, Molin S, Hilbi H. 2006. Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. Appl Environ Microbiol 72:2885–2895.

Molofsky AB, Swanson MS. 2004. Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. Mol Microbiol 53:29–40.

Murga R, Forster TS, Brown E, Pruckler JM, Fields BS, Donlan RM. 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. Microbiology 147:3121–3126.

Ohno A, Kato N, Yamada K, Yamaguchi K. 2003. Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. Appl Environ Microbiol 69:2540–2547.

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

Pineau L, Roques C, Luc J, Michel G. 1997. Automatic washer disinfector for flexible endoscopes: a new evaluation process. Endoscopy 29:372–379.

Rasband WS. 1997–2009. ImageJ. Bethesda. Maryland, USA: National Institute of Health.

Ristroph JD, Hedlund KW, Allen RG. 1980. Liquid medium for growth of *Legionella pneumophila*. J Clin Microbiol 11:19–21.

Ristroph JD, Hedlund KW, Gowda S. 1981. Chemically defined medium for *Legionella pneumophila* growth. J Clin Microbiol 13:115–119.

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

Rogers J, Dowsett AB, Dennis PJ, Lee JV, Keevil CW. 1994. Influence of plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water systems. Appl Environ Microbiol 60:1842–1851.

Rompre A, Servais P, Baudart J, de-Roubin MR, Laurent P. 2002. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods 49:31–54.

Samrakandi MM, Roques C, Michel G. 1997. Influence of trophic conditions on exopolysaccharide production: bacterial biofilm susceptibility to chlorine and monochloramine. Can J Microbiol 43:751–758.

Simm R, Morr M, Kader A, Nimtz M, Romling U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol Microbiol 53:1123–1134.

Stocks SM. 2004. Mechanism and use of the commercially available viability stain, BacLight. Cytometry A 61:189–195.

Taylor M, Ross K, Bentham R. 2009. *Legionella*, protozoa, and biofilms: interactions within complex microbial systems. Microb Ecol 58:538–547.

Temmerman R, Vervaeren H, Noseda B, Boon N, Verstraete W. 2006. Necrotrophic growth of *Legionella pneumophila*. Appl Environ Microbiol 72:4323–4328.

Wellinghausen N, Frost C, Marre R. 2001. Detection of legionellae in hospital water samples by quantitative real-time LightCycler PCR. Appl Environ Microbiol 67:3985–3993.

Yaradou DF, Hallier-Soulier S, Moreau S, Poty F, Hillion Y, Reyrolle M, Andre J, Festoc G, Delabre K, Vandenesch F, et al. 2007. Integrated real-time PCR for detection and monitoring of *Legionella pneumophila* in water systems. Appl Environ Microbiol 73:1452–1456.