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New insights into *Legionella pneumophila* biofilm regulation by c-di-GMP signaling

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ABSTRACT

The waterborne pathogen *Legionella pneumophila* grows as a biofilm, freely or inside amoebae. Cyclic-di-GMP (c-di-GMP), a bacterial second messenger frequently implicated in biofilm formation, is synthesized and degraded by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), respectively. To characterize the c-di-GMP-metabolizing enzymes involved in *L. pneumophila* biofilm regulation, the consequences on biofilm formation and the c-di-GMP concentration of each corresponding gene inactivation were assessed in the Lens strain. The results showed that one DGC and two PDEs enhance different aspects of biofilm formation, while two proteins with dual activity (DGC/PDE) inhibit biofilm growth. Surprisingly, only two mutants exhibited a change in global c-di-GMP concentration. This study highlights that specific c-di-GMP pathways control *L. pneumophila* biofilm formation, most likely *via* temporary and/or local modulation of c-di-GMP concentration. Furthermore, Lpl1054 DGC is required to enable the formation of a dense biofilm in response to nitric oxide, a signal for biofilm dispersion in many other species.

KEYWORDS

Biofilm; *Legionella*; c-di-GMP signaling; second messenger; NO response

Introduction

Legionella pneumophila is a waterborne environmental bacterium and the causative agent of legionnaires' disease (LD), a severe pneumonia (Fields et al. 2002). The only mode of transmission is by inhalation. Contaminated water droplets reach pulmonary alveoli where macrophages phagocytize the bacterium. Inside macrophages, *L. pneumophila* is able to resist digestion by evading the endocytic pathway. This mechanism requires a type-4 secretion system (T4SS) that translocates effector proteins into the host cell and leads to the formation of a vacuole where *L. pneumophila* replicates. Similarly, in aquatic environments *L. pneumophila* survives and replicates inside free-living amoebae. These host-cells are therefore considered to be a risk factor for LD when present in a water system (Pagnier et al. 2009; Thomas & Ashbolt 2011). In addition to amoebae, biofilms are also an important niche for *L. pneumophila* in aquatic environments. Several studies have reported the frequent occurrence of *L. pneumophila* in aquatic multispecies biofilms, and the capacity of bacteria to form monospecies biofilms (Mampel et al. 2006; Hindré et al. 2008; Pécastaings et al. 2010; Bigot

et al. 2013). Considering the high tolerance of sessile bacteria to noxious environmental conditions (eg the presence of biocide) and the constant release of bacteria in the circulating water, it is of particular interest to understand the mechanisms controlling biofilm formation by *L. pneumophila*.

Over the last decade, cyclic dimeric diguanylate (c-di-GMP), an intracellular bacterial secondary messenger, was identified as a key component in the regulation of various bacterial processes, including biofilm formation (Tamayo et al. 2007; Hengge 2009; Römling et al. 2013). The involvement of c-di-GMP in the regulation of the transition from the planktonic to the sessile form and/or biofilm formation has been demonstrated in many bacterial species (Ahmad et al. 2011; Newell et al. 2011). Diguanylate cyclases (DGCs) synthesize c-di-GMP from two GTP molecules, and are characterized by a conserved GGDEF domain (Simm et al. 2004). The hydrolysis of c-di-GMP is due to phosphodiesterases (PDEs) that linearize c-di-GMP into pGpG. Conserved EAL or HD-GYP motifs are typically found in PDEs (Simm et al. 2004). The coordination of several signaling pathways is responsible

for the regulation of global or local c-di-GMP concentrations resulting in the control of specific cellular processes at several levels (transcriptional, post-transcriptional, enzymatic activities or protein–protein interactions) (Krasteva et al. 2012). These signaling pathways can be activated or inhibited by environmental signals. GGDEF/EAL proteins often contain or interact with proteins containing sensor domains allowing bacteria to adapt their behavior in response to environmental cues. For example, sensor domains like REC (cheY-homologous Receiver domain) and PAS (Per-Arnt-Sim) are commonly associated with EAL and GGDEF domains in c-di-GMP-metabolizing proteins (Seshasayee et al. 2010). It has also been demonstrated that a wide variety of extracellular signals control bacterial behavior through c-di-GMP-dependent signaling pathways such as the redox state, quorum-sensing molecules, blue light, CO, O₂ or nitric oxide (NO) gases (Mills et al. 2011; Martínková et al. 2013; Plate & Marletta 2013; Winkler et al. 2014).

Depending on the strain, the *L. pneumophila* genome contains 22–24 genes encoding GGDEF/EAL proteins. In addition, *L. pneumophila* contains a particularly high rate (around 13%) of potential c-di-GMP metabolizing enzymes among the proteins containing one of the main signal domains listed in the MiST2 (microbial signal transduction) database (Ulrich & Zhulin 2010; Allombert et al. 2014), suggesting the importance of the c-di-GMP signaling network in the various lifestyles of *L. pneumophila*. Recently, c-di-GMP signaling has been linked to *L. pneumophila* virulence since overproduction of most GGDEF/EAL proteins impact the ability of *L. pneumophila* to replicate inside amoebae or macrophages, and three of them are required to evade endocytic degradation (Levi et al. 2011; Allombert et al. 2014). So far, only one c-di-GMP-metabolizing enzyme, namely the DGC Lpg1057, has been involved in the regulation of *L. pneumophila* biofilm since overproduction of Lpg1057 leads to a hyper-biofilm phenotype (Carlson et al. 2010). Interestingly, Carlson et al. demonstrated that the NO-binding protein H-NOX1, one of the two H-NOX (Heme-Nitric oxide/Oxygen binding) proteins of *L. pneumophila*, inhibits the *in vitro* activity of Lpg1057 in LP02 Philadelphia strain, suggesting a role for NO in c-di-GMP metabolism and biofilm regulation (Carlson et al. 2010).

To better understand the role of the c-di-GMP signaling network in biofilm formation, each GGDEF/EAL-encoding gene was systematically inactivated in *L. pneumophila* Lens strain, and the consequences of these deletions on the sessile population and the architecture of biofilms were evaluated. This work shows that, in addition to *lpl1054* (homolog of *lpg1057* in the Lens strain), two additional genes (*lpl0075* and *lpl1559*) are necessary for biofilm formation, and two other genes (*lpl0329* and *lpl1176*) are required for biofilm inhibition. In these five mutant strains, the correlation between the overall production of c-di-GMP in the cell and

biofilm formation was assessed, as proposed in the literature for many bacteria (Simm et al. 2004; Tischler & Camilli 2004; Kozlova et al. 2011; Fang et al. 2014; Tan et al. 2014). This study provides evidence that five c-di-GMP-metabolizing enzymes specifically contribute to the regulation of monospecies *L. pneumophila* biofilm formation. Finally, the impact of NO on *L. pneumophila* biofilm formation was investigated, and the crucial role played by Lpl1054 in presence of NO was established.

Materials and methods

Planktonic bacterial growth and biofilm formation

The bacterial strains, plasmids and oligonucleotides used in this study are listed in Table S1. *L. pneumophila* growth was achieved on BCYE (buffered charcoal yeast extract) medium (bioMérieux, Marcy l’Etoile, France) or in BYE (buffered yeast extract) broth (Chatfield & Cianciotto 2013) at 37°C with agitation (200 rpm) for five days. The medium was supplemented with chloramphenicol (Cm) (5 µg ml⁻¹) when necessary. In particular, all biofilm experiments were performed with Lens wild-type and mutant strains transformed with pJCL2968 vector (‘pCOMP-’) (Allombert et al. 2014), or derivatives of pJCL2968 vector, carrying a Cm resistance gene (‘pCOMP+’). Biofilms were formed as described previously in SBB-Cm medium in 24-well microplates (BD Falcon, France) at 32.5°C for nine days. SBB-Cm was renewed every three days after rinsing with 1 ml of sterile distilled water (SDW) (Pécastaings et al. 2010). *Escherichia coli* strains were grown at 37°C in LB medium supplemented with ampicillin (100 µg ml⁻¹) and kanamycin (20 µg ml⁻¹).

Inactivation of genes encoding GGDEF/EAL proteins

Genes encoding GGDEF/EAL proteins were inactivated by allelic exchange between an inactivation fragment carried by a suicide plasmid (pAV695) and the wild type chromosome (Allombert et al. 2014). The inactivation fragments consisted of a selection marker (kanamycin (km)-resistance cassette) flanked by the 5’ and 3’ sequences of the targeted genes. For complementation experiments, wild type alleles were cloned in pJCL2968 vector carrying a Cm resistance cassette (Allombert et al. 2014). All the strains used in this study were subsequently transformed either by the empty vector pJCL2968, or by the complementation vector.

Quantification of biofilm bacteria by qPCR (quantitative PCR) and vPCR (viability PCR) (Chang et al. 2009)

Wells containing the biofilms were rinsed with 2 ml of water and scraped for 1 min with a flame-sterilized spatula

after addition of 1 ml of SDW, in order to collect adhered bacteria. The DNA contained in 0.5 ml of this suspension was extracted using the QIAamp DNA Mini kit (QIAGEN, Courtaboeuf, France) following the manufacturer's instructions. For vPCR, 0.5 ml of bacterial suspension was pre-incubated with ethidium monoazide (EMA; 10 $\mu\text{g ml}^{-1}$; Life Technologies, Villebon-sur-Yvette, France) for 5 min at room temperature in the dark and 5 min under a neon light (11 W, $\phi = 0.37$, at 15 cm from experimental tubes) on ice. The latter step (photo-crosslinking) is necessary to covalently link the EMA to the extracellular DNA (or DNA from injured or dead bacteria) in order to prevent its amplification during PCR. This method allows the quantification of viable bacteria only (vPCR). Following the photo-crosslinking step, DNA extraction was performed as previously described.

The qPCR targeting the 16S rRNA gene of *L. pneumophila* was performed in a final volume of 25 μl (including 5 μl of DNA matrix) with iQ™ SYBR Green Supermix 2X (Biorad, Marnes-la-Coquette, France) and forward and reverse primers (LEG427F and LEG880R; 200 nM; Chang et al. 2009, 2009) in a MyiQ™ thermal cycler (Biorad). The initial denaturation step (95°C, 3 min) was followed by 40 PCR cycles (95°C, 15 s; 63.5°C, 30 s; 72°C, 30 s) and a final hybridization step at 55°C for 1 min. Melt curve analysis was then performed (temperature increase of 0.5°C every 10 s up to 95°C). Each sample was analyzed in triplicate.

Observation of biofilms by confocal laser scanning microscopy

Biofilms grown in six-well microplates were stained with Syto9 (Life Technologies), added directly into the wells at a final concentration of 0.28 μM in order to stain total bacteria. Representative observations were made on a spinning disk confocal microscope (PerkinElmer, Villebon-sur-Yvette, France) equipped with a 40 \times /0.8 water objective and two EmCCD cameras (Hamamatsu C9100-13, Hamamatsu Photonics, Massy, France). Volocity software (PerkinElmer) was used for the 3-D reconstitution of images.

Overproduction and purification of GGDEF/EAL proteins

DNA fragments corresponding to the coding sequences of *lpl0075*, *lpl0329*, *lpl1054*, *lpl1176* and *lpl1559* (full-length ORF for *lpl0329* and ORF without sequence encoding the N-terminal transmembrane domains for *lpl0075*, *lpl1054*, *lpl1176* and *lpl1559*) were PCR amplified using genomic DNA of *L. pneumophila* Lens as template, and the following oligonucleotide pairs: JA85-86 (*lpl0075*); JCL412-416 (*lpl0329*); JCL421-424 (*lpl1054*); JA87-88 (*lpl1176*)

and JCL441-442 (*lpl1559*) (Table S1). The amplified DNA fragments were digested with BamHI and Sall and then inserted into pQE30 plasmid (Qiagen). The resulting plasmids (pQE30-*lpl0075*, pQE30-*lpl0329*, pQE30-*lpl0329*, pQE30-*lpl0329* and pQE30-*lpl1118*) allowed the IPTG inducible synthesis of His₆-tagged GGDEF/EAL proteins. The plasmids were introduced in a BL21 *E. coli* strain overproducing GroES and GroEL chaperons favoring the folding and the solubilization of GGDEF/EAL proteins. 100 ml of LB supplemented with ampicillin and kanamycin were inoculated with transformants and incubated at 37°C until cultures reached an OD_{600nm} of 0.8. Gene expressions were induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 3 h at 20°C, and cell lysates were prepared by using a French pressure cell. His₆-recombinant proteins were purified under native conditions by affinity chromatography with magnetic beads (PureProteome Nickel Magnetic Beads, Millipore, Saint-Quentin-en-Yvelines, France), according to the manufacturer's recommendations. The purity of the eluted protein was analyzed by SDS-PAGE. Fractions containing pure proteins were pooled and dialyzed against reaction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, MgCl₂ 20 mM). The concentrations of the dialyzed protein fractions were determined using a Nanodrop 2000 (Thermo Fisher Scientific, Villebon-sur-Yvette, France).

Diguanylate cyclase and phosphodiesterase activity assays

Enzymatic assays were performed with GGDEF/EAL proteins purified as previously described (Allombert et al. 2014). GGDEF- and GGDEF/EAL-containing proteins were incubated in a reaction buffer containing GTP (50 μM) and α -³²P-GTP (0.3 $\mu\text{Ci } \mu\text{l}^{-1}$). For proteins with an EAL domain only, tests were run in the presence of radioactive c-di-GMP synthesized enzymatically by the His₆-tagged DGC Lpl0922EAL* corresponding to the Lpl922 protein whose EAL domain was inactivated by site-directed mutagenesis generating F546V and E547D substitutions (Allombert et al. 2014). Reaction products were separated by thin layer chromatography on polyethyleneimine cellulose (Macherey Nagel, Hoerd, France) in (NH₄)₂SO₄ (4 M, pH 3.6) and KH₂PO₄ (1.5 M, pH 3.6) buffer (1:1.5 v/v) and exposed on a Phosphorimager screen. Frontal references specific to each product allowed the reaction products to be identified.

C-di-GMP extraction (Simm et al. 2004)

Bacteria were grown onto BCYE for five days at 37°C (stationary phase). Bacterial suspensions were made in ultrapure water at a final concentration of 1 \times 10⁹ cells

ml⁻¹ (OD₆₀₀=1.0) and 10 ml were centrifuged (4,000 rpm, 20 min, 15°C). The resulting pellet was suspended in 170 µl⁻¹ of ultrapure water and transferred into a pre-weighed microtube. This suspension was heated (96°C, 15 min), placed on ice and 330 µl of ice-cold ethanol were added in order to extract nucleotides. Cellular debris were precipitated by centrifugation (10,000 rpm, 10 min, 4.0°C). The extraction step was repeated to increase the extraction yield by suspending the pellet in 0.5 ml of ice-cold ethanol (65%) and re-centrifuging. Tubes were maintained in refrigerated racks during the experiment. Final extracts were dried in a SpeedVac (Savant) and maintained at -20°C until UPLC-MS (ultra performance liquid chromatography coupled to mass spectrometry) analysis. The debris pellets were dried at 86°C for five days in order to calculate the dry weight.

Quantification of c-di-GMP in cellular extracts by UPLC-MS (Massie et al. 2012)

Dehydrated nucleotide extracts were suspended in 200 µl of water/methanol (50:50 v/v) and filtered (cutoff 0.2 µm) before analysis. UPLC-MS was achieved on an Acquity Waters device (Waters, Guyancourt, France) equipped with a BEH C18 column (50 × 2.1 mm, particle size 1.7 µm) at 50°C, with a UV diode array UV detector and a simple quadrupole mass detector (SQD, Waters). Eluent A consisted of 15 mM tributylamine, 10 mM acetic acid and 3% methanol in water and eluent B was methanol. The injection volume was 5 µl and the flow rate was 0.3 ml min⁻¹. The gradient was as follows: 99% A and 1% B from 0 to 2.5 min, 80% A and 20% B from 2.5 to 7.0 min, 35% A and 65% B from 7.0 to 7.5 min, 5% A and 95% B from 7.5 to 9.0 min, 99% A and 1% B from 9.0 to 9.5 min. With this method, the retention time of c-di-GMP was 4.67 min. The detection of c-di-GMP was performed by ESI with the following parameters: capillary voltage -3.5 kV, source temperature 135°C, desolvation 450°C, nitrogen (900 l h⁻¹), cone -50 V, gas cone 50 l h⁻¹. The negative-ion mode was used to detect c-di-GMP. Since c-di-GMP molecular weight is 690.4, *m/z* in the negative mode was 689.4. Each sample was analyzed in triplicate.

A standard range (from 0.39 µM to 12.5 µM) was prepared in ultrapure water using a commercial stock of c-di-GMP (BioLog, Bremen, Germany). Areas corresponding to the c-di-GMP peaks were plotted. The correlation coefficient was R² = 0.9997.

Exposure to nitric oxide (NO)

Sodium nitroprusside (SNP) was used as an NO donor (Barraud et al. 2006). A SNP solution (500 mM) was prepared extemporaneously with distilled water and sterilized

by filtration (0.2 µm). Since SNP is photodegradable and not stable in aqueous solution, it was stored in the dark for no more than 8 h. SNP was added to the growth medium daily. Microplates or tubes were kept in the dark at 32.5°C. The release of NO was measured indirectly by quantifying NO₂⁻, a stable and non-volatile breakdown product of NO, in the medium with the Griess reaction (Griess Reagent System, Promega, Charbonnières-les-Bains, France). Briefly, 50 µl of a sulfanilamide solution (1% in 5% phosphoric acid) were added to samples (50 µl) already dispatched in a 96-well microplate. After incubation for 10 min in the dark, 50 µl of *N*-1-naphthylethylenediamine dihydrochloride (NED) solution (0.1%) were added, leading to the production of an azo-compound (purple color) proportional to the quantity of NO₂⁻. After 10 min in the dark, the absorbance at 490 nm was measured with a microplate reader (BMG LabTech, Champagny-sur-Marne, France). A nitrite standard curve was prepared by diluting a nitrite reference solution (100 mM, Promega) in the growth medium used (SBB or BYE). Each sample was analyzed in triplicate. With this method, the quantification limit was between 3.13 and 6.50 µM according to the assays.

Determination of NO cytotoxicity on planktonic bacteria

SNP (500 mM, prepared as previously described) was added to glass tubes containing 4 ml of bacterial suspension (OD₆₀₀ = 0.2) in BYE at concentrations ranging from 63 to 1,000 µM. Tubes were maintained in the dark at 37°C with agitation (200 rpm) for 72 h. SNP was prepared extemporaneously and added into the tubes daily. A sample (100 µl) was taken daily from each tube, diluted in sterile distilled water and spread on BCYE-Cm in order to determine the bacterial concentration.

Results

***Lpl0075*, *Lpl1054* and *Lpl1559* are required for biofilm formation, and *Lpl0329* and *Lpl1176* are required for biofilm repression in *L. pneumophila* Lens**

To assess the role of GGDEF/EAL proteins in biofilm formation, 20 of the 22 genes encoding these proteins were inactivated by allelic exchange using a kanamycin-resistance cassette flanked by the 5' and 3' sequences of the target genes. Two genes could not be inactivated, most probably due to the presence of flanking regions leading to aberrant homologous recombination events. Planktonic growth of mutant strains in rich medium (BYE) was not significantly affected by the deletion of these genes, except

for strain $\Delta lpl0219$, which exhibited a reproducible growth defect in BYE medium (Figure S1). This strain was not included in further experiments to avoid taking indirect effects on biofilm formation into account.

The ability of each strain to form a biofilm was monitored using microplate assays in SBB medium that prevents planktonic growth (Pécastaigns et al. 2010). The total and viable bacteria in the biofilms formed by the mutant strains were evaluated after nine days by qPCR or vPCR targeting the 16S rRNA gene of *L. pneumophila*. vPCR allows the quantification of viable bacteria only. This method has been used successfully for the quantification of viable cells in several bacterial species (Shi et al. 2011; Li & Chen 2013), including viable but not cultivable cells (VBNC), which can make up a high proportion of biofilm cells. The results were systematically compared with those of the WT biofilm by calculating the log—transformed ratio of the population of sessile bacteria in the mutant and the WT strains ($\log(\text{mutant}/\text{WT})$) (Figure S2).

Among the 19 strains, seven ($\Delta lpl0075$, $\Delta lpl0220$, $\Delta lpl0283$, $\Delta lpl0860$, $\Delta lpl1559$, $\Delta lpl2333$, and $\Delta lpl2567$) clearly exhibited fewer adhered bacteria compared to the WT (Figure S2), especially for viable bacteria. More precisely, after a nine-day incubation, the reduction in the total population (measured by qPCR) was between 1.55 ± 0.51 and 4.13 ± 0.16 log, and the reduction in the viable population (measured by vPCR) was between 1.34 ± 0.32 and 4.41 ± 0.28 log. In contrast, two strains ($\Delta lpl0329$ and $\Delta lpl1176$) exhibited an increased sessile population compared to the WT strain, as illustrated by positive log ratios (Figure S2), suggesting a role in biofilm repression. In these cases, the sessile population was 5–10 times higher than observed for the WT. Finally, in these experimental conditions the $\Delta lpl1054$ strain presented only a slight decrease in the sessile population compared with the WT strain (Figure S2). However, since a previous study demonstrated that Lpg1057 (homolog of Lpl1054 in the LP02 strain) was involved in biofilm formation (Carlson et al. 2010), this strain was selected for further assays. Interestingly, the strains deleted for the three genes recently identified for their role in *L. pneumophila* virulence from the same mutant collection (Allombert et al. 2014) are part of the nine strains showing no defect in biofilm formation.

In order to check that these biofilm phenotypes were due to the absence of corresponding GGDEF/EAL proteins, functional complementation assays were carried out by introducing pJCL2968-derivative vectors that expressed the corresponding WT allele in the mutant strains. Quantification of the sessile population was performed as indicated above. Results showed that, for five of the 10 strains ($\Delta lpl0075$, $\Delta lpl1054$, $\Delta lpl1559$, $\Delta lpl0329$ and $\Delta lpl1176$), the introduction of the WT allele allowed the partial or total

complementation of the mutant phenotype (Figure 1A and 1B). In some cases, the overexpression of the complement gene induced an inversion of the phenotype as illustrated in the $\Delta lpl0075/plpl0075$ strain, which exhibited a higher sessile population compared with the WT (Figure 1A). The modifications of biofilm phenotypes in other mutant strains could be caused by possible polar effects of the mutations towards the downstream genes, or by secondary mutations independent of GGDEF/EAL genes deletions. To focus on GGDEF/EAL protein encoding genes involved in biofilm formation in *L. pneumophila*, these strains were not studied further. To sum up, deletions of *lpl0075*, *lpl1054*, *lpl1559*, *lpl0329* and *lpl1176* genes specifically impacted biofilm formation and/or adherent cell growth.

The impact of these deletions on nine-day-old biofilm structures was assessed by confocal microscope observations after staining with Syto9. While the WT strain was able to produce structured biofilms, characterized by dispersed microcolonies on the substratum that could reach a thickness of 250 μm (Figure 1D), $\Delta lpl0075$ strain exhibited thinner biofilms, with only a layer of bacteria homogeneously covering the substratum surface (maximum biofilm thickness of 37 μm), without large microcolonies (Figure 1C). Complementation with the WT gene allowed the recovery of biofilm forming capacities, and thicker microcolonies than the WT strain (maximum biofilm thickness = 390 μm) were observed, probably due to the overproduction of the protein. The defect in the $\Delta lpl1559$ strain was more substantial since only dispersed bacteria were observed on the substratum after nine days of incubation (Figure 1C). Biofilms of the complemented strain exhibited a wild type phenotype, even though the thickness of the microcolonies did not exceed 100 μm (Figure 1C). Interestingly, strain $\Delta lpl1054$, whose sessile bacterial population is very similar to the WT strain (Figure 1A), formed a thinner biofilm (maximum thickness of 180 μm) compared to the WT (Figure 1C) and complementation with the *lpl1054* gene promoted the thickening of the biofilm so that it exceeded the thickness of the biofilm of the wild type strain (maximum thickness of 377 μm) (Figure 1C), in accordance with the hyper-biofilm phenotype described for a strain with a *lpg1057*-containing plasmid (Carlson et al. 2010).

Lastly, observations of $\Delta lpl0329$ biofilm revealed microcolony structures higher and denser (Figure 1D) than the wild type strain. Biofilm of the $\Delta lpl0329$ strain reached up to 500 μm , twice the thickness of the WT biofilm. The thickness of $\Delta lpl1176$ biofilm was not significantly increased. But, as for $\Delta lpl0329$, the introduction of wild type alleles inhibited biofilm formation since the thickness of biofilms reached 198 and 100 μm for strains $\Delta lpl0329/plpl0329$ and $\Delta lpl1176/plpl1176$ respectively (Figure 1D), thus confirming the results obtained by qPCR and vPCR (Figure 1B).

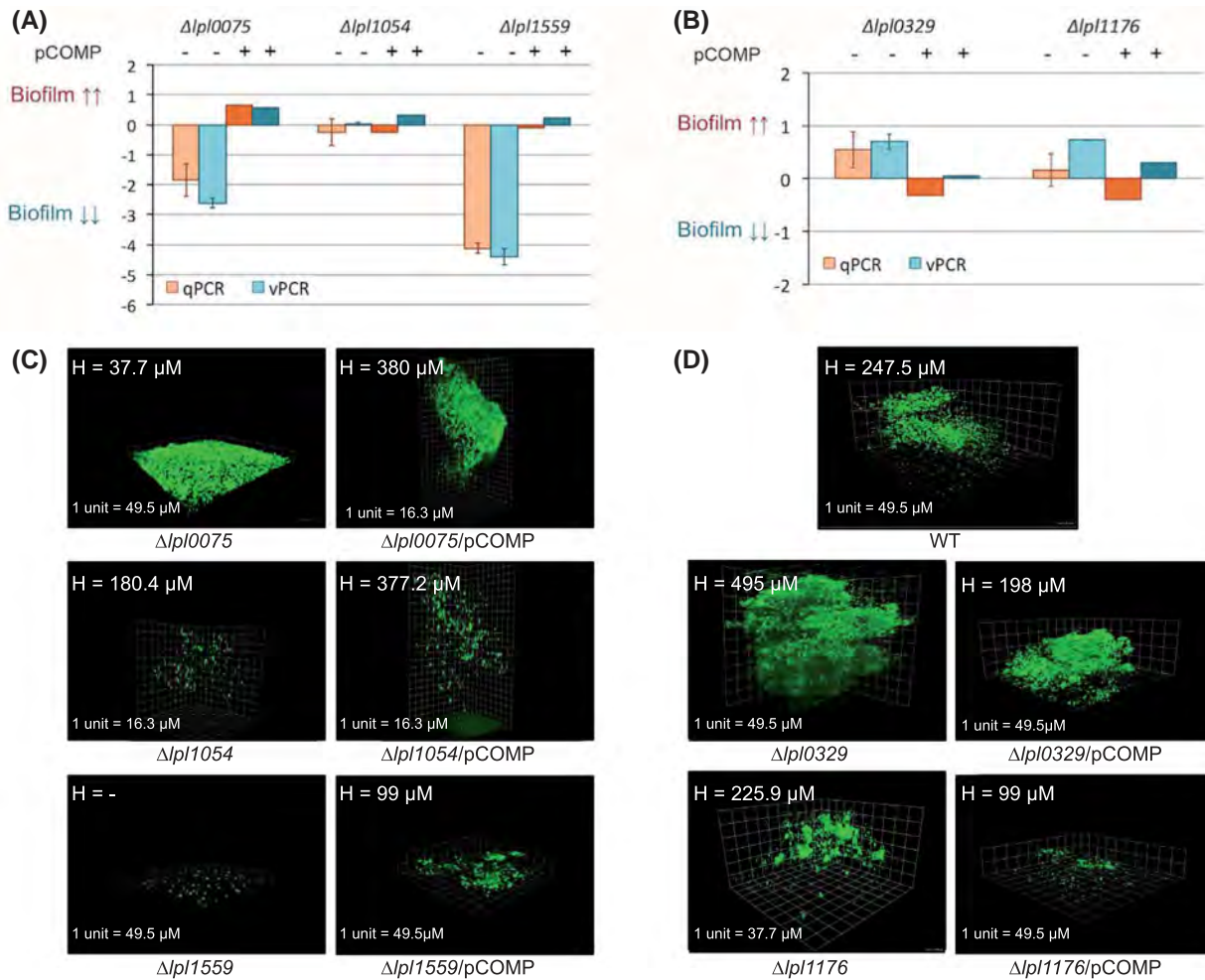


Figure 1. Proteins Lpl0075, Lpl1054 and Lpl1559 are positive regulators, while proteins Lpl0329 and Lpl1176 are negative regulators of biofilm formation in *L. pneumophila*. Biofilms of WT, mutant (with the empty plasmid pJCL2968, 'pCOMP-') and complemented strains (pCOMP+) were grown in SBB and bacterial population was measured after incubation for nine days by qPCR (orange) and vPCR (blue). (A, B) Biofilms of mutant (pCOMP-) and complemented strains (pCOMP+) were compared with the WT biofilm by calculating the log-transformed ratio of the sessile bacterial population in the mutant or complemented to that of the WT strain, in each assay. A negative value indicates a lower sessile population ('Biofilm $\downarrow\downarrow$ ') compared to the WT, and a positive value indicates higher sessile population ('Biofilm $\uparrow\uparrow$ ') compared to the WT ($n = 2$). (C, D) Architecture of nine-day-old biofilms of WT, mutant (pCOMP-) and complemented strains (pCOMP+) observed by confocal microscopy after staining with Syto9. Confocal images were used to build 3-D reconstructions. H is the maximum height of the observed biofilm. The size of one square unit is indicated for each image. The images are representative of at least five observed fields from two independent experiments.

Together, these results suggest that five GGDEF/EAL proteins are specifically involved in biofilm structuring in *L. pneumophila* Lens. Among them, three proteins (Lpl0075, Lpl1054, Lpl1559) appear to promote biofilm formation, whereas Lpl0329 and Lpl1176 seem to inhibit its formation.






GGDEF/EAL proteins associated with aberrant biofilm phenotypes could have antagonistic enzymatic activities

The architecture analysis of proteins on NCBI's Conserved Domain Database (CDD) (Marchler-Bauer et al. 2009b)

indicated that Lpl0075, Lpl0329, Lpl1054, Lpl1176 and Lpl1559 displayed GGDEF and/or EAL domains (Figure 2A). More specifically, domain conservation scores indicated by the CDD gave an estimate of the putative activity of these domains. For example, Lpl0075 contains a highly conserved EAL domain (E-value = 1.54×10^{-75} , Figure 2A) and a more weakly conserved GGDEF domain (E-value = 7.93×10^{19}), suggesting that Lpl0075 could have at least a PDE activity.

The enzymatic activity of two of these proteins was already established: Lpl0329 is a bi-functional enzyme able to synthesize and degrade c-di-GMP according to its phosphorylation status (Levet-Paulo et al. 2011) and

(A)

Gene	Domain organization	Conservation		Enzymatic activity
		Domain	E-value	
<i>lpl0075</i>		QSDEF ESS	7.93×10^{-19} 1.54×10^{-75}	ND
<i>lpl0329</i>		GGDEF EAL	2.11×10^{-75} 9.56×10^{-93}	DGC/PDE ^a
<i>lpl1054</i>		GGDEF EVL	1.46×10^{-60} 2.47×10^{-99}	DGC ^b
<i>lpl1176</i>		GGDEF EIL	1.08×10^{-76} 1.01×10^{-94}	DGC/PDE *
<i>lpl1559</i>		EVL	8.44×10^{-93}	PDE*

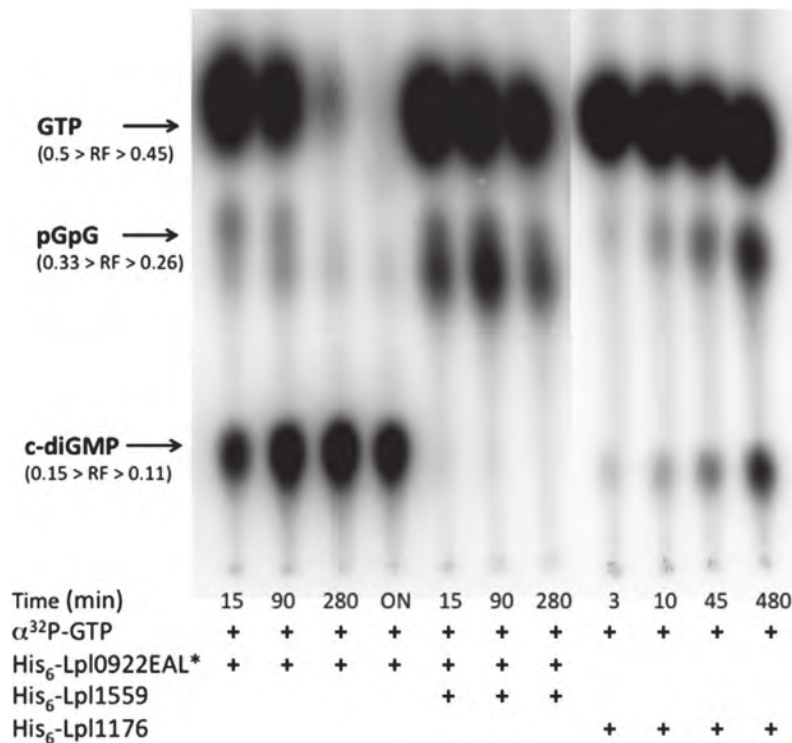
(B)

Figure 2. Domain organization of Lpl0075, Lpl0329, Lpl1054, Lpl1176 and Lpl1559. (A) The arrangement of protein domains is represented in graphs for each protein. The expected values (E-values) of the GGDEF and EAL domains were collected on NCBI's Conserved Domain Database (CDD) (Marchler-Bauer et al. 2009a). Transmembrane domains are represented as black vertical boxes. Low complexity domains are represented as horizontal black boxes. A sensing domain, Per-Arnt-Sim (PAS), and a phosphoacceptor receiver domain (REC) are also present in Lpl0329. Experimentally observed enzymatic activities are indicated for Lpl0329 (Levet-Paulo et al. 2011), Lpl1054 (deduced from DGC activity of LP02 homolog protein Lpg1057 (Carlson et al. 2010)), Lpl1176 and Lpl1559 (*: this study, see B). Enzymatic activity of Lpl0075 was not determined (ND) although a phosphodiesterase activity could be expected from the highly conserved EAL domain (E-value = 1.54×10^{-75}). (B) Lpl1559 is a phosphodiesterase and Lpl1176 is a bifunctional enzyme with diguanylate cyclase and phosphodiesterase activities. Purified His₆-tagged DGC Lpl0922EAL* was incubated with [α -³²P]GTP to produce [α -³²P]c-di-GMP, with or without His₆-tagged protein Lpl1559. Purified His₆-tagged DGC Lpl1176 was incubated with [α -³²P]GTP. Samples were collected at different time points, analyzed on PEI-cellulose TLC, and plates were exposed to a phosphorimager screen. C-di-GMP linearization to pGpG reveals the PDE activity of Lpl1559. Accumulation of both c-di-GMP and pGpG reveals the dual diguanylate cyclase and phosphodiesterase activities of Lpl1176. The frontal references (RF) specific to each product are indicated.

Lpg1057 (homolog of Lpl1054 in LP02 strain) had a DGC activity *in vitro* even though it contains both GGDEF and EAL domains (Carlson et al. 2010).

In an attempt to characterize the enzymatic activities of Lpl0075, Lpl1176 and Lpl1559, genes encoding these proteins were cloned into the pQE30 plasmid, allowing the overproduction of His₆-tagged proteins in *E. coli*. After purification by affinity chromatography, the His₆-Lpl1559 protein and the His₆-Lpl1176 could be obtained in the soluble fraction. Lpl1559 contains only an EAL domain (E-value = 3.70×10^{-94}). To test its PDE activity, His₆-Lpl1559 was incubated with α -³²P-GTP and a DGC (His₆-Lpl0922EAL*) (Allombert et al. 2014). Products were removed at different time intervals and separated by migration on PEI-cellulose thin-layer chromatography (TLC). In the presence of radioactive GTP, His₆-Lpl0922EAL* was able to produce radioactive c-di-GMP. When co-incubated with His₆-Lpl1559, the accumulation of c-di-GMP was no longer visible and instead an accumulation of pGpG, the degradation product of c-di-GMP, was noticeable, thus establishing the PDE activity of Lpl1559 (Figure 2B). As Lpl0329, Lpl1176 harbors strongly conserved GGDEF and EAL domains. When incubated with α -³²P-GTP, His₆-Lpl1559 produced both c-di-GMP and pGpG, demonstrating its bifunctional activity. It is noteworthy that while bifunctional DGC/PDE enzymes are still rarely reported (Liu et al. 2010; Li et al. 2013; Österberg et al. 2013; Römling et al. 2013), it is the third example with Lpl0329 and Lpl0922 of such an enzyme in *L. pneumophila* (Levet-Paulo et al. 2011; Allombert et al. 2014).

In summary, antagonistic activities of c-di-GMP-metabolizing enzymes have different consequences on *L. pneumophila* biofilm, since 1 DGC, 1 PDE and a putative PDE (Lpl1054, Lpl1559 and Lpl0075) enhance different steps of biofilm formation, while two proteins with a dual activity DGC/PDE (Lpl0329 and Lpl1176) inhibit biofilm growth.

Intracellular concentration of c-di-GMP in mutant strains is not systematically linked to their ability to form biofilms

In order to analyze the impact of gene inactivation on the c-di-GMP intracellular pool, quantitative assays were carried out by UPLC-MS (ultra-performance liquid chromatography coupled to mass spectrometry) on bacterial cultures. In the WT strain, the mean c-di-GMP concentration was 0.153 ± 0.038 nmol mg⁻ⁿ dry weight (Figure 3). For strains Δ lpl0075, Δ lpl1054 and Δ lpl1559, the measured concentrations were not significantly different from the WT, although the concentration in the Δ lpl0075 strain seemed slightly higher (0.212 ± 0.028 nmol mg⁻ⁿ,

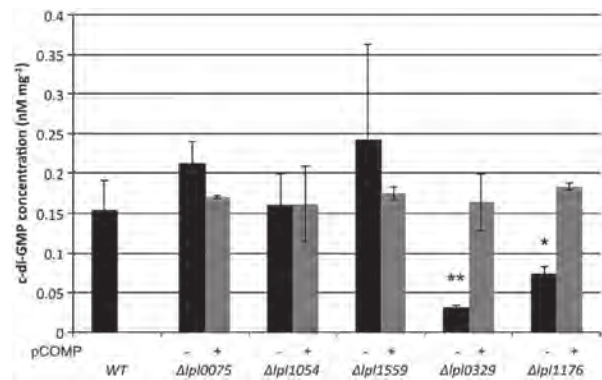


Figure 3. The c-di-GMP concentration in mutant strains is not correlated with biofilm phenotypes. C-di-GMP was extracted in WT, mutant (black bars, pCOMP-) and complemented (gray bars, pCOMP+) strains with 65% ethanol, measured by UPLC-MS and expressed as a function of the dry weight of the bacteria used for extraction ($n = 2$). The results were analyzed statistically with the Student *t*-test (* $p > 0.01$; ** $p > 0.05$).

$p = 0.13$). C-di-GMP levels in the complemented strains were therefore logically not significantly different from the WT strain. The fact that deletion of *lpl0075*, *lpl1054* and *lpl1559* genes impact biofilm formation without major consequences on the intracellular pool of c-di-GMP suggests that the corresponding proteins could act *via* temporary changes of global c-di-GMP concentration, or *via* local modifications of c-di-GMP concentration.

In contrast, Δ lpl0329 and Δ lpl1176 strains exhibited intracellular levels of c-di-GMP 2–3-times lower than measured for the WT strain (0.030 ± 0.004 and 0.073 ± 0.004 nmol mg⁻ⁿ, respectively). The levels of c-di-GMP were restored when the corresponding WT alleles were introduced in the mutant strains (Figure 3). These results indicate that Lpl0329 and Lpl1176 proteins are involved in the overall regulation of the cellular concentration of c-di-GMP and, unexpectedly, that low concentrations of c-di-GMP could be associated with an increase in biofilm growth of *L. pneumophila*.

Lpl1054 is necessary for *L. pneumophila* biofilm maintenance in presence of nitric oxide (NO)

A previous study demonstrated that H-NOX1, an NO sensing protein, interacted with Lpg1057, a GGDEF/EAL protein with a diguanylate cyclase activity and that H-NOX1 inhibited Lpg1057 activity *in vitro* when bound to NO (Carlson et al. 2010). Furthermore, the hyper-biofilm phenotype observed in the absence of H-NOX1 was reverted by the simultaneous deletion of *lpg1057*. In order to study the direct impact of NO on *L. pneumophila* biofilm formation, and more specifically the role of Lpl1054 (Lpg1057 homolog), biofilms were subjected to sodium nitroprusside (SNP), an NO donor. Under the

present study test conditions, the adjunction of SNP at 250 μM allowed the production of NO that is sub-lethal for *L. pneumophila* (Figure S3). In this condition, the concentration of NO could be estimated as around 250 nM, since effective concentrations of NO are estimated to be 1,000 times lower than the concentration of SNP (Barraud, Schleheck, et al. 2009). This level of NO concentration is also considered as a non-toxic environmental signal for other species (Arora et al. 2015).

Therefore, in order to assess the effect of nontoxic concentrations of NO on *L. pneumophila* biofilms, six-day-old biofilms were subjected to SNP (250 μM). After three days, the sessile population was quantified by qPCR and vPCR and the ratio of bacterial populations in biofilms exposed to NO vs in non-exposed biofilms was calculated for each strain. On average, the bacterial population in WT biofilm decreased by a factor of 0.71 and 1.21 log (by vPCR and qPCR respectively) when it was exposed to NO (Figure 4A). Nonetheless, the relative stability of the bacterial population was somewhat unexpected since, in most cases such as in *Pseudomonas aeruginosa* or *Shewanella woodyii*, exposure to non-toxic concentrations of NO (< 1 μM) triggers the transition from the sessile to the planktonic state and thus the release of biofilm bacteria (Barraud et al. 2006; Barraud, Schleheck, et al. 2009; Barraud, Storey, et al. 2009; Liu et al. 2012; Arora et al. 2015).

Interestingly, the decrease in the bacterial population was more substantial in the $\Delta\text{lpl}1054$ strain and was > 2.60 logs for both qPCR and vPCR. This result suggests that Lpl1054 could be necessary for the maintenance of *L. pneumophila* biofilms in presence of NO. The wild type phenotype was restored in the complemented strain.

In order to better understand the effect of NO on biofilm structure, exposed and non-exposed biofilms were stained with Syto9 and observed with confocal microscopy. When exposed to NO, WT strain biofilm exhibited a condensation of microcolonies with no decrease in the thickness (Figure 4B, top images), which is consistent with quantification results obtained by PCR. In contrast, the $\Delta\text{lpl}1054$ strain was strongly affected by the exposure to NO and no biofilm could be observed after three days; only a few bacteria were dispersed on the well surface. Again, functional complementation allowed partial recovery of the WT phenotype; biofilms of $\Delta\text{lpl}1054/\text{plpl}1054$ consisted of densely packed microcolonies, though their thickness did not exceed 50 μm . Together, these results demonstrate that the DGC Lpl1054 promotes the expansion of *L. pneumophila* biofilm in the absence of NO, as shown for Lpg1057 (Lpl1054 homolog in LP02 strain) (Carlson et al. 2010). DGC Lpl1054 is also critical for the persistence of *L. pneumophila* biofilm by increasing biofilm density when NO is present and able to inhibit its DGC activity.

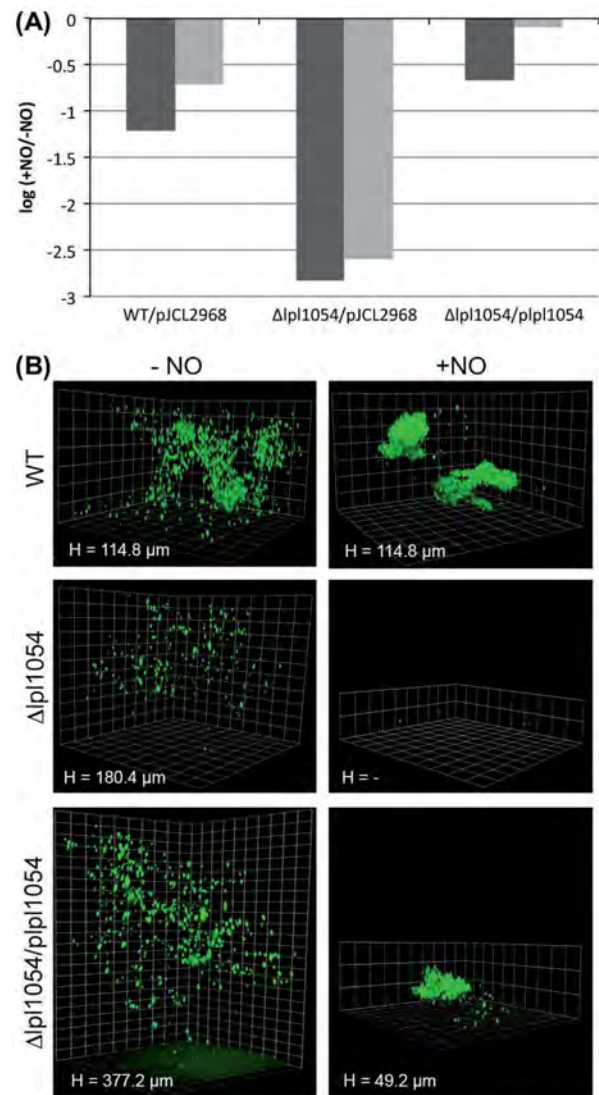


Figure 4. Lpl1054 is necessary to maintain biofilms in the presence of NO (SNP). (A) Six-day-old biofilms of WT, $\Delta\text{lpl}1054$ and $\Delta\text{lpl}1054/\text{plpl}1054$ strains were exposed to 250 μM of SNP for three days. At the end of the experiment, biofilms were sampled and quantified by qPCR (dark gray bars) and vPCR (light bars). The log-transformed ratio of the mean density in exposed biofilms (+NO) to the mean density in non-exposed biofilms (-NO) was calculated in order to determine the loss of bacteria in exposed biofilms ($n = 2$). (B) Architecture of biofilms of WT, $\Delta\text{lpl}1054$ and $\Delta\text{lpl}1054/\text{plpl}1054$ strains, exposed or not to NO (+NO or -NO) strains as observed by confocal microscopy after staining with Syto9. Confocal images were used to build 3-D reconstructions. The size of one square unit is 16.4 μm . The images are representative of at least five observed fields from two independent experiments.

Discussion

It is now known that c-di-GMP plays a key role in the control of the transition from the planktonic to sessile lifestyle in many species (Povolotsky & Hengge 2012; Spurbeck et al. 2012). Moreover, modifications of c-di-GMP

signaling pathways could promote adherence and biofilm formation as illustrated by the 2011 German outbreak of Shiga toxin-producing *E. coli* O104:H4 (Richter et al. 2014). While biofilms constitute an important ecological niche for *L. pneumophila* especially because of the protection they provide against detrimental environmental conditions (Abdel-Nour et al. 2013), little is known about the control of *L. pneumophila* biofilm formation.

In this work, five GGDEF/EAL encoding genes (*lpl0075*, *lpl0329*, *lpl1054*, *lpl1176* and *lpl1559*) were identified whose deletion led to aberrant biofilm phenotypes as revealed by PCR and/or confocal observations. In the five mutant strains, reversion to the wild type phenotype by functional complementation could be observed.

The deletion of three of these genes, *lpl0075*, *lpl1054* and *lpl1559*, led to an overall decrease in the biofilm, although quite differently according to the deletion. Given that Δ *lpl0075* strain is only able to adhere to the well surface, the establishment of the 3-D structure of the *L. pneumophila* biofilm seems to require Lpl0075. Confocal observations of biofilm formed by Δ *lpl1054* and also Δ *lpl1054*/*plpl1054* strains revealed that Lpl1054 enhanced biofilm growth according to previously described biofilm-promoting role of Lpg1057 (Lpl1054 homolog in LP02 strain) (Carlson et al. 2010). Finally, Lpl1559 is required for the first step of biofilm formation, eg adhesion. Concerning the enzymatic activities of these three biofilm-promoting proteins, two of them, Lpl0075 and Lpl1054, contain both GGDEF and EAL domains. Only DGC activity was shown for Lpg1057 (Lpl1054 homolog) (Carlson et al. 2010). In contrast, despite difficulties in purifying Lpl0075, the high E-values for its EAL domains suggest that this enzyme probably functions at least as a PDE. Finally, the results showed PDE activity *in vitro* for the unique EAL domain of Lpl1559. Together, these results indicate that enzymes with antagonistic activities towards c-di-GMP are required to control various steps in biofilm formation. Interestingly, at least one PDE, Lpl1559 (and potentially Lpl0075), appears to be involved in that process. Researchers generally accept that an increase in the cellular pool of c-di-GMP is linked to the promotion of biofilm formation (Povolotsky & Hengge 2012; Römmling et al. 2013) and numerous DGCs are involved in the regulation of processes that increase adhesion, like pili expression, up-regulation of matrix components such as cellulose or inhibition of flagellar motility (Povolotsky & Hengge 2012; Anwar et al. 2014; Hunter et al. 2014). In contrast, to the authors' knowledge, no studies have clearly shown the requirement of PDEs for biofilm formation, since studies have attributed a positive role to EAL-like proteins lacking phosphodiesterase activity, such as STM197 in *Salmonella typhimurium* (Ahmad et al. 2013). The results from this present study help to demonstrate that PDEs (c-di-GMP

degrading enzymes) are not systematically associated with biofilm disruption or inhibition but can also play a role in biofilm formation. The results also show that the absence of the three promoting-biofilm proteins, whether they are PDE or DGC, have no major consequences on the c-di-GMP intracellular concentration measured by UPLC-MS from planktonic bacteria. These results are consistent with other studies showing that the intracellular concentration is not clearly affected by the deletion of genes encoding diguanylate cyclase involved in biofilm formation or surface attachment (Abel et al. 2013; Xu et al. 2013). Attempts have been made also to measure the c-di-GMP-concentration in large amounts of sessile bacteria. The amount of material obtained was insufficient for reliable measurements by UPLC-MS. However, since *L. pneumophila* biofilm formation seems to be under the control of enzymes with antagonistic activities (synthesis and degradation of c-di-GMP), it is very likely that it involves local and/or temporary changes in c-di-GMP concentrations that are difficult to detect, in sessile cells.

The two other identified genes, *lpl0329* and *lpl1176*, encode two GGDEF-EAL proteins inhibiting biofilm formation since their deletion led to an overproduction of biofilm. Surprisingly, in both mutant strains with a hyper-biofilm phenotype (Δ *lpl0329* and Δ *lpl1176* strains) the intracellular c-di-GMP concentration was decreased. As expected, the level of c-di-GMP of the WT was restored in complemented strains. A previous study (Levet-Paulo et al. 2011) and the present results indicate that Lpl0329 and Lpl1176 are bifunctional DGC/PDE. However, the low concentrations of c-di-GMP observed in the corresponding mutants argue in favor of major DGC activity, at least under the present experimental conditions. Again, in spite of what is generally observed in other bacterial species (Povolotsky & Hengge 2012; Römmling et al. 2013), this result suggests that a decrease in the c-di-GMP pool is compatible with an increase of the capacity to form a biofilm in *L. pneumophila*, without excluding the possibility of a local and/or temporary increase of c-di-GMP concentration during biofilm formation.

Interestingly, *lpl0329* and *lpl1176* genes, could be jointly regulated, as transcriptomic assays revealed that they are the only GGDEF/EAL-encoding genes that are clearly repressed in a Δ *fleQ* strain that lacks the master regulator of flagellar biosynthesis (Albert-Weissenberger et al. 2010). These results are consistent with their role highlighted in this study, namely as repressors of biofilm formation, since it is assumed that flagellar synthesis, associated with bacterial motility, is inversely correlated with biofilm formation. Furthermore, it is noteworthy that the concentration of c-di-GMP was significantly different from the WT in strains Δ *lpl0329* and Δ *lpl1176*, but not in Δ *lpl0075*, Δ *lpl1054* and Δ *lpl1559* strains. Most likely,

Lpl0329 and Lpl1176 are able to modify the global pool of cellular c-di-GMP, which suggest activities in the whole cell, whereas Lpl0075, Lpl1054 and Lpl1559 may act more specifically either during a limited time or in a more local manner. These results support the probable existence of a highly specific c-di-GMP signaling pathway, finely controlling temporary changes in the global c-di-GMP pool or the more localized c-di-GMP micro-pool, as recently argued in different studies (Merritt et al. 2010; Hopley et al. 2012; Abel et al. 2013).

As an environmental bacterium, *L. pneumophila* has to be able to sense various extracellular signals in order to adapt its behavior. Among these signals, nitric oxide has been increasingly studied, especially because NO was shown to induce the dispersion of many pathogenic biofilms (eg *E. coli*, *Fusobacterium nucleatum*, *Staphylococcus aureus*, and *Vibrio cholerae*) (Jardeleza et al. 2014; Arora et al. 2015; Barraud et al. 2015), and to increase the sensitivity of biofilm bacteria to antibiotics (Barraud et al. 2006; Barraud, Storey, et al. 2009). However, biofilm formation by *Azospirillum brasilense*, *Vibrio harveyi* and *Shewanella oneidensis* appears to be induced by the addition of NO in the medium (Arruebarrena Di Palma et al. 2013; Plate & Marletta 2012; Henares et al. 2013). Since high concentrations of NO result in the formation of reactive nitrogen species, it can be hypothesized that in these bacterial species, NO triggers responses to nitrosative stress, resulting in the protection of the bacteria, eg the enhancement of biofilm formation.

In the aquatic environment, *L. pneumophila* can be exposed to NO by the potential proximity of denitrifying bacteria (Plate & Marletta 2013) or as an intracellular pathogen to NO produced by macrophages or protozoa (Bogdan et al. 2000; Rojas-Hernandez et al. 2007). The genome of *L. pneumophila* also contains two genes encoding proteins with H-NOX domains which are sensor domains for NO in eukaryotes and bacteria (Plate & Marletta 2013). One of these, H-NOX1, inhibits the *in vitro* DGC activity of Lpg1057 when bound to NO and the $\Delta hnox1$ strain exhibits a hyperbiofilm phenotype (Carlson et al. 2010). Together, these data indicate that NO sensing could play an important role in *L. pneumophila* behavior.

In the present study, SNP was used as the NO donor to assess the impact of NO on *L. pneumophila* biofilm formation. The concentration of 250 μ M was chosen since it is tolerated by *L. pneumophila* and allows a significant production of NO_2^- (formed from NO) in the present experimental conditions. This treatment resulted in only a 1-log reduction in *L. pneumophila* biofilms. Furthermore, the persistence of *L. pneumophila* cells in spite of the presence of NO was correlated with the increase in microcolony biomass, highlighting the ability

of *L. pneumophila* to resist the presence of NO by forming a dense biofilm.

The results also show that, without exposure to NO, the inactivation of *lp1054* led to only a slight modification of the biofilm phenotype with the reduction of biofilm thickness. In contrast, overproduction of Lpl1054 led to a hyper-biofilm phenotype. These results are consistent with the proposed biofilm-promoting role of the DGC Lpg1057 (homolog of Lpl1054 in LP02 strain) (Carlson et al. 2010). Nevertheless, biofilm formed by the $\Delta lp1054$ strain exposed to NO was substantially altered, with a decrease in its bacterial population 100 times greater than for the wild-type strain under the same conditions. It is noteworthy that in $\Delta strain$ biofilms exposed to NO, variations of qPCR and vPCR levels were similar. Increased mortality in the biofilm can be excluded since it should result in a lower vPCR signal and a stable qPCR signal (Delgado-Viscogliosi et al. 2009). Thus, it can be concluded that the exposure to NO led to the dispersal of bacteria from Δ . Thus, biofilms.

Interestingly, these results indicate that Lpl1054 is crucial to maintain a dense biofilm structure and to impair biofilm dispersion in the presence of NO. This role could be performed independently of its DGC activity, as it was shown that Lpg1057 DGC activity is inhibited by NO-bound H-NOX1 (Carlson et al. 2010). Therefore, the present results suggest a new dual role for Lpl1054 based on the availability of NO. In the absence of NO (or at very low concentrations), the role of Lpl1054 could involve DGC activity to enhance 3-D biofilm development, while the second role, in the presence of NO, when DGC activity must be inhibited by H-NOX1, could be to increase the density of the biofilm (avoid dispersion) which could be a physical protection mechanism to NO (Plate & Marletta 2013).

In summary, this work brings new insights into the role of c-di-GMP signaling networks in *L. pneumophila*. While three c-di-GMP-metabolizing enzymes were shown to be involved in the intracellular survival of *L. pneumophila*, especially in the avoidance of endosomal degradation and the orchestration of effector secretion by T4SS (Allombert et al. 2014), five other c-di-GMP-metabolizing enzymes appear to be specifically involved in various steps of biofilm formation. In addition, it was shown that *L. pneumophila* somehow escapes the usual rules for biofilm formation, since (1) c-di-GMP degrading activities could promote biofilm formation, (2) hyper-biofilm phenotypes may be associated with low concentrations of c-di-GMP, and (3) exposure to NO led to the densification of biofilm instead of a release of adhered bacteria. Deciphering the c-di-GMP signaling networks and identifying the specific signals that trigger the different c-di-GMP signaling

pathways are necessary if the behavior of *L. pneumophila* is to be understood in various environments.

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Disclosure statement

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