Role of the LuxR family transcriptional regulator Lpg2524 in the survival of *Legionella pneumophila* in water

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Role of the LuxR family transcriptional regulator Lpg2524 in the survival of *Legionella pneumophila* in water

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Abstract

The water-borne Gram-negative bacterium *Legionella pneumophila* (*Lp*) is the causative agent of Legionnaires’ disease. *Lp* is typically transmitted to human from water systems, where it grows inside amoebae. Survival of *Lp* in water is central to its transmission to humans. A transcriptomic study previously identified many genes induced by *Lp* in water. One of such gene, *lpg2524*, encodes a putative LuxR family transcriptional regulator. It was hypothesized that this gene could be involved in the survival of *Lp* in water. Deletion of *lpg2524* does not affect the growth of *Lp* in rich medium, in the amoeba *Acanthamoeba castellanii* or in human macrophage-like THP-1 cells, showing that Lpg2524 is not required for growth *in vitro* and *in vivo*. Nevertheless, deletion of *lpg2524* results in a faster CFU reduction in an artificial freshwater medium, Fraquil, indicating that Lpg2524 is important for *Lp* to survive in water. Over-expression of Lpg2524 also results in a survival defect, suggesting that a precise level of this transcriptional regulator is essential for its function. However, our result shows that Lpg2524 is dispensable for survival in water when *Lp* is at a high cell density (10⁹ CFU ml⁻¹), suggesting that its regulon is regulated by another regulator activated at high cell density.

*Key words:* *Legionella pneumophila*, freshwater, survival, LuxR family protein, cell density
Résumé

La bactérie Gram-négative *Legionella pneumophila* (*Lp*) est l’agent étiologique de la maladie du Légyonnaire. *Lp* est transmis à l’humain à partir de systèmes hydriques dans lesquels il se multiplie à l’intérieur d’amibes. La survie de *Lp* dans l’eau est essentielle à sa transmission à l’humain. Une étude transcriptionnelle a permis d’identifier plusieurs gènes induits par *Lp* dans l’eau, comme *lpg2524* qui code pour un présumé régulateur transcriptionnel de la famille de LuxR. L’hypothèse a été émise que ce gène pourrait être impliqué dans la survie de *Lp* dans l’eau. La délétion de *lpg2524* n’affecte pas la croissance de *Lp* dans un milieu riche, dans l’amibe *Acanthamoeba castellanii* ou dans les macrophages humains en culture THP-1, démontrant que *Lpg2524* est dispensable pour la croissance in vitro et in vivo. Cependant, la délétion de ce gène engendre un défaut de survie dans l’eau, révélant son rôle dans la survie de *Lp* dans l’eau. La surexpression de ce gène engendre aussi un défaut de survie, probablement parce qu’un niveau d’expression précis est requis. De plus, la délétion de *Lpg2524* n’a pas d’effet sur la survie de *Lp* lorsque la densité bactérienne est élevée (10^9 CFU ml^{-1}), ce qui suggère que son régulon est régulé par un autre régulateur activé lorsque la densité bactérienne est élevé.

*Mots-clés:* *Legionella pneumophila*, eau, survie, protéine de la famille de LuxR, densité cellulaire
Introduction

*Legionella pneumophila* (*Lp*) is an opportunistic human pathogen that can infect the alveolar macrophages of susceptible individuals, resulting in a severe pneumonia called Legionnaires’ disease (Fields et al. 2002). As a bacterium inhabiting freshwater environments, *Lp* is frequently exposed to fluctuating conditions and experienced various stresses such as nutrient limitation, temperature and pH. Bacteria tend to reorganize their transcriptomic profile to better adapt to environmental changes (Leaphart et al. 2006; Stintzi 2003). Such changes in overall gene expression are usually achieved by the modulation of transcriptional and post-transcriptional regulators, which control a pool of genes.

Several transcriptional regulators in *Lp* have been well studied due to their significant contribution to the regulation of virulence genes, such as the two-component systems CpxRA, PmrAB, LetAS as well as LqsRS (Gal-Mor and Segal 2003; Hammer et al. 2002; Spirig et al. 2008; Tanner et al. 2016; Zusman et al. 2007). Together, they regulate the expression of over 70 genes encoding Type IVB secretion system and effector proteins, which are essential for intracellular growth (reviewed by Segal 2013). Moreover, the alternative sigma factor RpoS modulates the expression of 739 genes, which is approximately one-fourth of the total annotated genes in *Lp*. (Hovel-Miner et al. 2009). Apart from controlling multiple pathways associated with intracellular growth, RpoS is also important for the survival of *Lp* in water (Trigui et al. 2014). Our previous transcriptomic studies have showed that RpoS positively regulates two genes significantly induced in water, *bdhA* and *lpg1659* (*lasM*), that contribute to the long-term survival of *Lp* in water (Li and Faucher 2016; Li et al. 2015).

Our previous microarray analysis showed that another gene, *lpg2524*, was also significantly induced in *Lp* after 6 hours (Log₂ ratio =1.52) and 24 hours (Log₂ ratio =1.73) in
water when compared to the control grown in rich medium (Li and Faucher 2016). The expression of this gene is positively regulated by RpoS in the post-exponential phase of growth (Hovel-Miner et al. 2009). The protein encoded by \( lpg2524 \) is composed of 287 aa and is annotated as a LuxR family transcriptional regulator in the NCBI database due to the presence of a DNA-binding domain similar to LuxR-like protein at the C-terminal. LuxR is a transcriptional regulator in the LuxIR quorum sensing (QS) system that regulates bioluminescence in \textit{Vibrio fischeri} (Engebrecht and Silverman 1984). Briefly, LuxI is the autoinducer synthase that synthesizes acylated homoserine lactone (AHL), while LuxR is activated by AHL and then binds to the regulatory region of target genes to alter gene expression (Nasser and Reverchon 2007). In Proteobacteria, incomplete QS systems were also found, with a LuxR family sensor/regulator but no cognate LuxI family synthase (Case et al. 2008). These unpaired LuxR family proteins, also called LuxR solos, may respond to endogenous or exogenous AHLs with a lower specificity (Subramoni and Venturi 2009). For instance, the LuxR solo QscR of \textit{Pseudomonas aeruginosa} interacts with two different AHLs produced by LasI, namely 3-oxo-decanoyl-homoserine lactone (3OC10-HSL) and 3-oxododecanoyl-homoserine lactone (3OC12-HSL), and regulates genes involved in metabolism, transport and virulence (Lee et al. 2006; Lequette et al. 2006). LuxR solos may also respond to non-AHL molecules (Subramoni and Venturi 2009). For example, \textit{Xanthomonas campestris} possesses a LuxR solo named XccR, which responds to a plant factor (Zhang et al. 2007).

In \textit{Lp}, there are another four proteins that show sequence similarity to LuxR family transcriptional regulators, including LpnR1 (Lpg2557), LpnR2 (Lpg1946), LpnR3 (Lpg1448) and LetA (Lpg2646) (Hammer et al. 2002; Lebeau et al. 2004). Their importance on host invasion and intracellular multiplication has been characterized, but the function of \( lpg2524 \) has
not been studied yet. Since \textit{lpg2524} is induced in water and positively regulated by RpoS, we hypothesize that this gene is important for \textit{Lp} to survive in water. In this study, we investigate the function of Lpg2524 on the growth of \textit{Lp in vitro} and \textit{in vivo} as well as its survival in water.

\section*{Materials and methods}

\subsection*{Bacterial strains and culture conditions}

The wild-type (WT) strain we used for constructing the mutant and over-expression strains was KS79, which is a constitutively competent strain derived from \textit{Lp} Philadelphia-1 strain JR32 (de Felipe et al. 2008; Sadosky et al. 1993). \textit{Lp} was grown on charcoal yeast extract (CYE) agar with 0.25 mg ml\textsuperscript{-1} ferric pyrophosphate, 0.4 mg ml\textsuperscript{-1} L-cysteine and 0.1% α-ketoglutarate at 37°C for three days (Edelstein 1981; Feeley et al. 1979). Liquid cultures were grown in ACES-buffered yeast extract (AYE) broth (Horwitz and Silverstein 1983). If needed, this medium was further supplemented with 5 µg ml\textsuperscript{-1} chloramphenicol or 25 µg ml\textsuperscript{-1} kanamycin. The strains of \textit{Escherichia coli} were derived from DH5α. They were grown overnight on Luria-Bertani agar at 37°C and the medium was supplemented with 25 µg ml\textsuperscript{-1} chloramphenicol if needed. Bacterial strains used in this study are described in Table 1.

\subsection*{Construction of mutant, complemented and over-expression strains}

For the construction of deletion mutant SPF258 (\textit{Δlpg2524}), 1 kb of the sequence upstream of \textit{lpg2524} was amplified from KS79 by PCR using Taq polymerase (Invitrogen) and the primer set 2524\_UpF/2524\_UpR, and 1 kb of the sequence downstream of \textit{lpg2524} was amplified using the primer set 2524\_DownF/2524\_DownR. A kanamycin cassette was then amplified using pSF6 as template and Kn-F/Kn-R as primers. Using the purified kanamycin
cassette as template, a 1 kb kanamycin fragment with 5’ end complementary to the 3’ end of upstream fragment and 3’ end complementary to the 5’ end of downstream fragment was amplified using the primer set 2524_KnF/2524_KnR. Subsequently, a 3 kb mutant allele was amplified from a mixture of the three 1 kb fragments using the primer set 2524_UpF/2524_DownR and Phusion DNA polymerase (NEB). The amplicon was purified and introduced into KS79 through natural transformation (de Felipe et al. 2008). Successful recombinants in which \( \text{lpg2524} \) is replaced by the kanamycin cassette were confirmed by kanamycin resistance and PCR.

For the construction of plasmid pSF84 (\( \text{plpg2524} \)) for complementation, the \( \text{lpg2524} \) gene and a 500 bp upstream sequence was amplified using KS79 as template and Com2524F_Sacl/Com2524R_XbaI as primers. Both the plasmid pXDC39 and purified amplicon were digested with SacI and XbaI (NEB), following by ligation using T4 DNA ligase (NEB). Ligation mixture was then transformed into \( \text{E. coli} \) DH5\( \alpha \) and transformants were selected based on resistance against chloramphenicol. The plasmid was extracted from the transformant and correct insertion was confirmed by PCR using pXDC39-F/Com2524R_XbaI as primers. Subsequently, the plasmid was introduced into the deletion mutant \( \Delta \text{lpg2524} \) by electroporation, as described previously (Chen et al. 2006), to construct the complemented strain SPF295 (\( \Delta \text{lpg2524} + \text{plpg2524} \)). Successful recombinants were confirmed by kanamycin and chloramphenicol resistance as well as PCR.

For the construction of plasmid pSF93 (\( \text{plpg2524i} \)) for over-expression of \( \text{lpg2524} \), the gene was amplified using KS79 as template and Com2524F2_Sacl/Com2524R_XbaI as primers. Both the plasmid pMMB207c and purified amplicon were digested with SacI and XbaI (NEB), following by ligation using T4 DNA ligase (NEB). Ligation mixture was then transformed into \( \text{E. coli} \).
coli DH5α and the transformants were selected based on resistance against chloramphenicol. The plasmid was extracted from the transformant and correct insertion (lpg2524) was confirmed by PCR using PromF/Com2524R_XbaI as primers. Subsequently, the plasmid was introduced into KS79 by electroporation to construct the over-expression strain SPF311 (WT+p/lpg2524i). Successful recombinants were confirmed by kanamycin and chloramphenicol resistance as well as PCR validation. The sequence of all primers is listed in Table 2.

**Extracellular growth assay**

The WT strain KS79 and the deletion mutant Δlpg2524 were suspended at an OD$_{600}$ of 0.1 in AYE broth. Twenty-five ml of each culture, with three replicates per culture, was grown in 125 ml Erlenmeyer flask at 37°C shaking (250 rpm). The optical density at 600 nm (OD$_{600}$) of each culture was monitored for 32 hours at intervals of 4 hours.

**Cell lines and infection assays**

The amoeba Acanthamoeba castellanii was first grown in 20 ml of peptone yeast glucose (PYG) broth at 30°C in a 75 cm$^2$ tissue culture flask (Sarstedt). PYG broth is composed of 2% proteose peptone, 0.1% yeast extract, 0.1% sodium citrate dihydrate, 0.4 M CaCl$_2$, 0.1 M glucose, 4 mM MgSO$_4$, 2.5 mM NaH$_2$PO$_3$, 2.5 mM K$_2$HPO$_3$ and 0.05 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ (Moffat and Tompkins 1992). When the culture became confluent, the spent medium with any non-adherent amoebae was discarded. Ten ml of fresh PYG broth was added into the flask, which was then shaken sharply to allow detachment of the adherent amoebae from the inner surface of the flask. Subsequently, the suspension of amoebae was diluted to 5×10$^5$ cells ml$^{-1}$ and 1 ml was added to the wells of a 24-well plate (Sarstedt). The plate was settled for two hours to allow adhesion of
amoebae. Then, the medium was replaced with Ac buffer that does not support the growth of \( Lp \) (Moffat and Tompkins 1992) and the plate was settled for another two hours.

To prepare the human monocyte-like cell line THP-1 for infection, cells were first grown in 30 ml of RPMI 1640 (Life Technologies) with 2 mM glutamine and 10% fetal bovine serum at 37°C under 5% CO\(_2\) (Kim et al. 2009). The culture was diluted to \( 5 \times 10^5 \) cells ml\(^{-1} \) and \( 1 \times 10^{-7} \) M phorbol 12-myristate 13-acetate (PMA) (Fisher Scientific) was added. One ml of this culture was added to the wells of a 24-well plate (Sarstedt) and the plate was incubated for three days to allow differentiation of monocytes into adherent macrophage-like cells. Fresh medium was used to replace the spent medium two hours before infection.

To start the infection, the strains KS79, ∆lpg2524 and the \( dotA \) mutant were first suspended in AYE broth at an OD\(_{600}\) of 0.1 and diluted 10 times (to approximately \( 2.5 \times 10^6 \) cells ml\(^{-1} \)). The \( dotA \) mutant is a negative control known to be defective in intracellular growth (Roy and Isberg 1997). Two µl of each bacterial culture was added to three replicate wells in the 24-well plate containing either \( A. \) castellanii or THP-1 cells (i.e. MOI of 0.1). The plate with \( A. \) castellanii was incubated at 30°C and the plate with THP-1 cells was incubated at 37°C under 5% CO\(_2\). The growth of each bacterial strain was monitored every 24 hours by CFU counts and their intracellular growth was determined by comparing the CFU on individual day with the initial CFU.

Survival assays in water

In the survival assays, an artificial freshwater medium Fraquil, which does not support the growth but allows long-term survival of \( Lp \) (Mendis et al. 2015), was used. Fraquil contains 0.25 µM CaCl\(_2\), 0.15 µM MgSO\(_4\), 0.15 µM NaHCO\(_3\), 0.1 µM NaNO\(_3\), 23 nM MnCl\(_2\), 10 nM
K$_2$HPO$_4$, 10 nM FeCl$_3$, 4 nM ZnSO$_4$, 2.5 nM CoCl$_2$, 1 nM CuSO$_4$ and 0.22 nM (NH$_4$)$_6$Mo$_7$O$_{24}$ dissolved in ultra-pure Milli-Q water (Morel et al. 1975). Strains grown on CYE agar were first washed with Fraquil for three times before suspending in fresh Fraquil at an OD$_{600}$ of 0.1. One ml of suspension was mixed with 4 ml of fresh Fraquil (to obtain an OD$_{600}$ of approximately 0.02) and then incubated in a 25 cm$^2$ plastic flask (Sarstedt). Three biological replicates were prepared for each strain and they were incubated at 25°C, 37°C and 42°C. CFU of each sample was monitored once per three weeks, once per two weeks and once per week, respectively. For the samples incubated at 42°C, Live/Dead staining and flow cytometry were used to determine the membrane integrity of $Lp$ after 7 weeks as described previously (Li et al. 2015). Freshly grown KS79 was used as the control for viable cells and KS79 boiled in a water bath for 10 minutes was used as the control for dead cells. Both the samples and controls were diluted to an OD$_{600}$ of 0.01 in Fraquil. One ml of the dilution was then stained with 1.5 µl of SYTO 9 at 3.34 mM and 1.5 µl of propidium iodide at 20 mM. The Guava easyCyte flow cytometer (EMD Millipore) was used for fluorescence signal measurement (green fluorescence from SYTO 9 and red fluorescence from propidium iodide). Unstained cells and stained Fraquil were used as a reference for instrument setting to eliminate background signals. For data analysis, the guavaSoft 2.7 software was used. The sample cells that followed the fluorescence profile of viable control and dead control were considered as viable cells and dead cells, respectively. Noteworthy, the cells that fell in the small overlap region between the profiles of the two controls were considered as undefined. To study the effects of high cell density on the survival of $Lp$ in water, the strains were suspended in Fraquil at an OD$_{600}$ of 1.0 instead of 0.02 before incubating at 42°C.
Results

Deletion of \textit{lpg2524} does not affect the growth of \textit{Lp} \textit{in vitro}

Since transcriptional regulator controls the expression of other genes, \textit{Lpg2524} could be a critical protein involved in various functions such as growth, infection and survival in water. To study the importance of \textit{lpg2524} in those functions, we constructed a mutant by allelic exchange (\textit{\Delta lpg2524}). No significant differences were observed between the growth of WT strain and the mutant \textit{\Delta lpg2524} in rich medium (Fig. 1). Both strains showed exponential growth between 8 to 20 hours and their growth curve remained similar for 32 hours. This result indicates that \textit{lpg2524} is dispensable for optimal growth of \textit{Lp} in complex medium \textit{in vitro} at 37°C.

Deletion of \textit{lpg2524} does not affect the growth of \textit{Lp} \textit{in vivo}

Next, we tested if the deletion of \textit{lpg2524} would alter the ability of \textit{Lp} to infect host cells and to grow intracellularly. Since \textit{Lp} infects amoebae in the natural environment and infects human macrophages on occasion (Fields et al. 2002), the amoeba \textit{A. castellanii} and human macrophage-like THP-1 cells were used as host cells. In both infection assays, the WT strain and \textit{\Delta lpg2524} showed a similar increase in CFU ratio, while the negative control (\textit{dotA} mutant) showed a decreasing ratio as expected (Fig. 2a and 2b). There were no significant differences between the WT and \textit{\Delta lpg2524} in both assays, suggesting that \textit{Lp} does not require \textit{lpg2524} for host infection and intracellular growth.

\textit{lpg2524} is important for \textit{Lp} to survive in water at warm temperatures

Since \textit{Lp} induces expression of \textit{lpg2524} in water (Li et al. 2015), we hypothesized that this gene could be important for survival in water and thus that the deletion of \textit{lpg2524} could
result in a survival defect. Therefore, we exposed the WT strain, mutant strain Δlpg2524 and complemented strain Δlpg2524+pplg2524 to water at different temperatures and monitored their survival by CFU count.

At 25°C, no significant reductions in CFU were detected within 24 weeks (Fig. 3a). At 37°C, the CFU of all strains decreased faster than at 25°C and eventually became undetectable after 22 weeks of exposure in water (Fig. 3b). The mutant Δlpg2524 showed a faster CFU reduction than the WT and significant differences in CFU between the two strains were observed since the 14th week. In contrast, the complemented strain maintained similar CFU as the WT, indicating that lpg2524 is important for the survival of Lp in water. At 42°C, the trend was similar to that at 37°C, though the survival defect of Δlpg2524 was only partially recovered in the complemented strain (Fig. 3c). Also, the CFU of all strains dropped quicker than at 37°C and became undetectable after 5 weeks of exposure to water. Taken together, the results suggest that lpg2524 is important for Lp to survive in water at temperatures of 37°C and above.

The viability of the WT, mutant and complemented strains was then analyzed using Live/Dead staining and flow cytometry. For all three strains, over 90% of the cells were viable and less than 8% were dead (Fig. 3d). Since the CFU of all strains already dropped below detection limit at this time point (the 7th week in water at 42°C), the result suggests that most cells became viable but non-culturable (VBNC).

**Over-expression of Lpg2524 affects the survival of Lp in water**

Recently, we have shown that LasM (Lpg1659) is important for Lp to survive in water and that over-expression of this gene further promotes the survival (Li and Faucher 2016). Therefore, we tested if the over-expression of lpg2524 would have a beneficial effect on the
survival of Lp in water. To test this, a plasmid containing an inducible Ptac promoter upstream of the lpg2524 ORF was introduced into the WT strain (WT+plpg2524i). The WT strain and WT+plpg2524i ON/OFF were then exposed to water at 42°C. In contrast to our initial hypothesis, the CFU became undetectable two weeks earlier than the WT when the over-expression of Lpg2524 was induced with IPTG (WT+plpg2524i ON) (Fig. 4). When Lpg2524 was not over-expressed (WT+plasMi OFF), the CFU dropped faster than the WT and became undetectable one week earlier, probably due to the leaky expression of Ptac. These results indicate that a higher level of Lpg2524 interferes with the survival of Lp in water.

**Lp does not require lpg2524 for survival in water at high cell density**

Since LuxR family proteins are sometimes involved in QS-mediated regulation (Nasser and Reverchon 2007), we hypothesized that Lpg2524 could have such a role in Lp. As QS involves the regulation of gene expression as a function of cell density (Nasser and Reverchon 2007), bacteria at different densities may show distinct phenotypes. Lpg2524 may be needed to regulate the genes for survival in water only when Lp is at a particular cell density. Therefore, we suspended the WT strain, mutant strain Δlpg2524 and complemented strain Δlpg2524+plpg2524 in water at a cell density 100 times superior to what was tested in Fig. 3 (i.e. 10⁹ cells ml⁻¹, an OD₆₀₀ of 1.0) and monitored their survival at 42°C. This temperature was used because the survival defect previously observed in Δlpg2524 was the greatest.

At a higher cell density, the CFU of all strains dropped below detection limit after 7 weeks of water exposure at 42°C (Fig. 5). This is 2 weeks longer than the suspensions at a lower cell density (10⁷ cells ml⁻¹, an OD₆₀₀ of 0.02) (Fig. 3c), which was expected for a larger population. All strains showed a similar survival curve and the mutant Δlpg2524 did not show a
survival defect even at this temperature (Fig. 5), demonstrating that \textit{lpg2524} is no longer important for \textit{Lp} to survive in water at 42°C when at high cell density.

\textbf{Discussion}

LuxR family proteins are usually composed of around 250 aa with two functional domains, a N-terminal domain for AHL-binding and a C-terminal helix-turn-helix DNA-binding domain for transcriptional regulation (Nasser and Reverchon 2007). These LuxR family transcriptional regulators can manipulate the expression of downstream genes in order to regulate certain functions, such as LuxR regulating bioluminescence in \textit{V. fischeri} (Engebrecht and Silverman 1984). In \textit{Lp}, the LuxR solo LetA regulates virulence in \textit{Lp} and the shift between the replicative and transmissive phases (Hammer et al. 2002). Here, we revealed the functions of a previously unknown LuxR family transcriptional regulator, Lpg2524, on the survival of \textit{Lp} in water.

First, we found that the deletion of \textit{lpg2524} does not affect the growth of \textit{Lp in vitro}. This is consistent with a previous study showing that the insertion of transposon in this gene did not result in growth advantages or disadvantages in rich medium (O’Connor et al. 2011). Also, \textit{lpg2524} is dispensable for invasion and intracellular growth in amoeba \textit{A. castellanii} and human macrophage-like THP-1 cells. Noteworthy, the other four LuxR family proteins in \textit{Lp} (LpnR1, LpnR2, LpnR3 and LetA) were found to be involved in virulence to some extent. It is known that LpnR2, LpnR3 and LetA promote the expression of flagellin, which is needed for efficient host cell infection (Hammer et al. 2002; Lebeau et al. 2004; Pruckler et al. 1995). All three LpnR proteins are dispensable for intracellular multiplication in human macrophage-like U937 cells, but LpnR2 is needed for invasion and LpnR3 is needed for intracellular multiplication in \textit{A.
*A. castellanii* (Lebeau et al. 2004). Furthermore, LetA is needed for *Lp* to evade phagosome-lysosome fusion, but not important for intracellular multiplication in bone marrow derived macrophages from A/J mouse (Hammer et al. 2002). Considering our result, it is possible that: 1) Lpg2524 is not involved in the regulation of virulence at all; 2) due to functional redundancy, deletion of *lpg2524* would not result in any observable effects; and 3) the importance of Lpg2524 is host-specific, so the mutant ∆*lpg2524* may have a defect in other host cells, such as primary macrophages.

Survival defect was observed in ∆*lpg2524* exposed to water at 37°C and 42°C, but not at 25°C, suggesting that *lpg2524* is important for *Lp* to survive in warm water. Similar results were found for *bdhA* and *lasM*, which are required for survival in water only when the temperature is above 25°C (Li and Faucher 2016; Li et al. 2015). Since ∆*lpg2524* did not show a defect in water during short-term exposure at 55°C when compared to the WT (data not shown), it is unlikely that the defects we observed at 37°C and 42°C were due to a lower tolerance to high temperature. Therefore, it is possible that ∆*lpg2524* could have a survival defect at 25°C over longer period of time.

In addition, the result of Live/Dead staining showed that the major population of ∆*lpg2524* did not die after exposure to water at 42°C for 7 weeks. Instead, it entered a VBNC state earlier than the WT. VBNC cells are known to be cells in a quiescent status, waiting for revival or transitioning to death (Li et al. 2014). For example, VBNC cells of *Lp* induced by starvation could be resuscitated by co-inoculation with *A. castellanii* (Steinert et al. 1997). However, those induced by monochloramine treatment could not be resuscitated using the same method (Alleron et al. 2013). Since we were unable to resuscitate the VBNC cells in our samples and they remained non-infectious (data not shown), we consider the cells to be dying, indicating
that Δlpg2524 started dying in water at an earlier time point than the WT.

Although Lpg2524 is important for Lp to survive in water, the precise level of this transcriptional regulator seems to be critical for optimal survival. Over-expression of Lpg2524 induced by IPTG greatly hindered the survival of Lp in water at 42°C, whereas slight increase in the level of Lpg2524 caused by the leaky expression of Ptac also resulted in a mild but significant survival defect. It is known that over-expression of transcriptional regulators may disrupt normal pathways and activities in bacteria (reviewed by MacRitchie et al. 2008). For example, RpoS is important for the virulence of Lp (Hovel-Miner et al. 2009), but its over-expression was found to repress LetAS-dependent virulence traits, including motility, cytotoxicity and infectivity (Bachman and Swanson 2004). In fact, most of the LuxR family transcriptional regulators act as activators by recruiting RNA polymerase to the promoter of their target genes (Nasser and Reverchon 2007). It is likely that Lpg2524 could activate genes required for Lp to survive in water. Therefore, the deletion mutant had a survival defect, probably due to its inability to activate critical genes, whereas the over-expression strain also had a survival defect, perhaps due to excessive quantity of Lpg2524 that bind non-specifically to genes that should not be activated when Lp is under nutrient limitation in water (e.g. genes involved in replication and translation).

Noteworthy, the importance of Lpg2524 to the survival of Lp in water appears to be dependent on cell density. Lpg2524 is needed for the survival of Lp when at a cell density of 10⁷ CFU ml⁻¹ (OD₆₀₀ of 0.02) but is dispensable when at a cell density of 10⁹ CFU ml⁻¹ (OD₆₀₀ of 1.0). In the natural environment, it is more likely to find Lp at a lower cell density than at cell density as high as 10⁹ CFU ml⁻¹. Previous studies found 10⁶ CFU ml⁻¹ of Lp in water basin of cooling towers and 10⁴ CFU ml⁻¹ of Lp in hot-water tank (Stout et al. 1985; Türetgen et al. 2005).
However, it is possible that the cell density reaches a higher level in biofilm or during intracellular multiplication. In both cases, *Lp* may not require Lpg2524 to maintain survival because it is not in direct contact with water.

The fact that Lpg2524 belongs to the LuxR family and is important for the survival of *Lp* at low cell density suggests a link with a QS system. Such systems have been observed in both Gram-positive and Gram-negative bacteria to allow interspecies and intraspecies communication to synchronize group behavior through controlling gene expression (Nasser and Reverchon 2007). Most QS systems involve an autoinducer synthase and a cognate response regulator, while there are exceptions that involve an extra component, a sensor kinase, which helps signal detection and transmission (Tiaden et al. 2007). For example, based on the gene cluster homology to *cqsAS* QS system in *Vibrio cholerae*, the first QS system identified in *Lp* includes an autoinducer synthase LqsA, a response regulator LqsR and a sensor kinase LqsS (Tiaden et al. 2007). In this system, LqsA produces the α-hydroxyketone autoinducer LAI-1 that does not bind to the regulator directly but to the sensor, whereas the sensor transfers the phosphoryl group to the regulator to activate the regulation of target genes (reviewed by Schell et al. 2016).

Nevertheless, Lpg2524 is required at low cell density, which would suggest a model where Lpg2524 binds to and activates transcription of target genes when the autoinducer is absent or at a low concentration. At high cell density, the binding of the autoinducer to Lpg2524 may render it inactive. This would result in a similar phenotype between the WT and the mutant strains at high cell density. Since there are no LuxI homologues in *Lp*, it is not clear what signal Lpg2524 senses. None of the five LuxR family proteins (LpnR1, LpnR2, LpnR3, LetA and Lpg2524) have been shown to interact with autoinducers and it is not clear if they are genuine QS regulators. One possibility is that Lpg2524 senses an as yet unknown metabolic product that
accumulates extracellularly as the cell density increases. This signaling molecule could be an as yet unknown molecule or LAI-1 produced by the Lqs system, since LuxR solos are known to bind to autoinducer produced by other systems (Subramoni and Venturi 2009). Since \textit{Lp} is not growing in water, but merely surviving, it is not clear if an autoinducer could be produced and accumulated extracellularly in sufficient quantity to bind to the response regulators. It is known that \textit{Lp} is metabolically active in water since deletion of \textit{bdhA}, a gene involved in the utilization of energy reserves, result in a survival defect in water starting at 6\textsuperscript{th} week (Li et al. 2015). However, transcription is drastically reduced after 24 hours in water, which would require that Lpg2524 inhibition by autoinducers occurs within a few days to have an impact on induction of genetic program required for survival in water, which is likely improbable. Therefore, it is unlikely that Lpg2524 is involved in typical QS-mediated regulation of genes involved in survival of water, thus we do not favor this model.

Alternatively, it is possible that another regulator is activated at high cell density, which bypasses the function of Lpg2524. In this case, Lpg2524 would not be, directly, involved in QS. This other regulator might not be necessarily a typical QS regulator since other molecules that accumulate extracellularly can also play a role in sensing the density of the population. In \textit{P. aeruginosa}, the siderophore pyoverdine regulates the production of exotoxin A and the PrpL protease in a dose-dependent manner (Lamont et al. 2002). Such mechanism would be more consistent with the situation presented here. In water, \textit{Lp} could produce a siderophore, a secreted proteins or another type of molecule that accumulates extracellularly. At high density, this molecule could accumulate to a concentration level sufficient to initiate a response and bypass the requirement for Lpg2524. It is unlikely that this molecule is a siderophore since the genes involved in the production of legiobactin and HGA-melanin (Cianciotto 2015) are repressed in
water at high cell density (Li et al. 2015). More likely, it is possible that a micronutrient is exhausted faster at high cell density, which leads to activation of the alternative regulator that bypasses the requirement for Lpg2524. Previously, we have shown that the putative transporter LasM is required for \textit{Lp} to survive in water and that supplementation with 10 times the normal amount of trace metal rescues this requirement (Li and Faucher 2016). Hence, it is possible that exhaustion of trace metals at high cell density serves as a signal during survival of \textit{Lp} in water.

In conclusion, this study shows that the novel LuxR family transcriptional regulator, Lpg2524, is important for \textit{Lp} to survive in water at warm temperature. Absence of Lpg2524 does not affect the growth of \textit{Lp}, both \textit{in vitro} and \textit{in vivo}. However, both the deletion and over-expression of \textit{lpg2524} result in a survival defect in water, showing that precise level of Lpg2524 is required for maintaining the survival of \textit{Lp}. Such requirement of Lpg2524 for \textit{Lp} to survive in water seems to depend on cell density, as the absence of this protein only cause a survival defect at low cell density but not at high cell density. Further investigations of the target genes of Lpg2524 could provide some insight on the underlying regulatory mechanisms.

**Acknowledgments**

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References


Edelstein, P.H. 1981. Improved semiselective medium for isolation of *Legionella pneumophila*


10.1007/s10661-005-7058-3.


Figure captions

**Fig. 1.** Optical density at 600 nm of the WT strain and deletion mutant Δlpg2524 grown in rich medium for 32 hours. Data shown are the mean and SD of three biological replicates.

**Fig. 2.** Growth of the WT strain, mutant Δlpg2524 and negative control dotA mutant inside (a) A. castellanii or (b) THP-1 macrophages. CFU was monitored daily and the log ratio between the CFU on individual day (CFUT) and the initial CFU (CFU0) was calculated. Data shown are the mean and SD of three biological replicates.

**Fig. 3.** Survival of the WT strain, mutant strain Δlpg2524 and complemented strain Δlpg2524+plpg2524 suspended in water at (a) 25°C, (b) 37°C and (c) 42°C. The initial OD600 of all samples was approximately 0.02. Significant differences between WT and other strains were determined by one-tailed unpaired Student’s t test (*p<0.05; **p<0.005; ***p<0.0005). DL indicates detection limit. (d) Percentage of viable and dead cells in different strains exposed to water at 42°C for 7 weeks. Live/Dead staining together with flow cytometry was used to analyze 5000 cells in each replicate. Data shown are the mean (and SD) of three biological replicates.

**Fig. 4.** Survival of the WT strain and the over-expression strain WT+plpg2524i in water at 42°C. WT+plpg2524i OFF means the over-expression of lpg2524 was not induced, whereas WT+plpg2524i ON means the over-expression of lpg2524 was induced with 1 mM IPTG. Data shown are the mean and SD of three biological replicates. One-tailed unpaired Student’s t test was used to assess significant differences against WT (*p<0.05; **p<0.005; ***p<0.0005). DL indicates detection limit.
**Fig. 5.** Survival of the WT strain, mutant strain Δ*lpg2524* and complemented strain Δ*lpg2524*+plpg2524 in water at 42°C at an OD$_{600}$ of 1.0. Data shown are the mean and SD of three biological replicates. Significant differences between WT and other strains were determined by one-tailed unpaired Student’s t test (*$p<0.05$). DL indicates detection limit.
### Tables

**Table 1.** Bacterial strains used in this study

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<th>Name</th>
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<th>Reference</th>
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<td>JR32</td>
<td>r·m⁺, Sm⁹⁹⁹</td>
<td>(Sadosky et al. 1993)</td>
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<td>KS79 (WT)</td>
<td>JR32 ΔcomR</td>
<td>(de Felipe et al. 2008)</td>
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<tr>
<td>LELA3118 (<em>dotA</em> mutant)</td>
<td>JR32 <em>dotA::Tn903dIIlacZ</em></td>
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<tr>
<td>LM1376 (<em>rpoS</em> mutant)</td>
<td>JR32 *rpoS::Tn903dGent, Gm⁹⁹⁹</td>
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<td>SPF176 (<em>prpoS</em>)</td>
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<td>SPF248 (<em>ΔlasM</em>)</td>
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<td><em>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
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<td>pMMB207C</td>
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<td>Xavier Charpentier</td>
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Table 2. Primer sequences used in this study

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<td>2524_UpR</td>
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<td>Com2524F2_SacI</td>
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<tr>
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<tr>
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<tr>
<td>PromF</td>
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* The underlined bases indicate restriction sites.
Optical density at 600 nm of the WT strain and deletion mutant Δlpg2524 grown in rich medium for 32 hours. Data shown are the mean and SD of three biological replicates.

Fig. 1
73x42mm (300 x 300 DPI)
Growth of the WT strain, mutant Δlp2524 and negative control dotA mutant inside (a) *A. castellanii* or (b) THP-1 macrophages. CFU was monitored daily and the log ratio between the CFU on individual day (CFU_T) and the initial CFU (CFU_0) was calculated. Data shown are the mean and SD of three biological replicates.

Fig. 2

152x169mm (300 x 300 DPI)
Survival of the WT strain, mutant strain Δlpg2524 and complemented strain Δlpg2524+p/lpg2524 suspended in water at (a) 25°C, (b) 37°C and (c) 42°C. The initial OD$_{600}$ of all samples was approximately 0.02. Significant differences between WT and other strains were determined by one-tailed unpaired Student’s t test (* $p<0.05$; ** $p<0.005$; *** $p<0.0005$). DL indicates detection limit. (d) Percentage of viable and dead cells in different strains exposed to water at 42°C for 7 weeks. Live/Dead staining together with flow cytometry was used to analyze 5000 cells in each replicate. Data shown are the mean (and SD) of three biological replicates.

Fig. 3
Survival of the WT strain and the over-expression strain WT+plpg2524i in water at 42°C. WT+plpg2524i OFF means the over-expression of lpg2524 was not induced, whereas WT+plpg2524i ON means the over-expression of lpg2524 was induced with 1 mM IPTG. Data shown are the mean and SD of three biological replicates. One-tailed unpaired Student’s t test was used to assess significant differences against WT (* p<0.05; ** p<0.005; *** p<0.0005). DL indicates detection limit.

Fig. 4
72x36mm (300 x 300 DPI)
Survival of the WT strain, mutant strain Δlp2524 and complemented strain Δlp2524+p/lpg2524 in water at 42°C at an OD600 of 1.0. Data shown are the mean and SD of three biological replicates. Significant differences between WT and other strains were determined by one-tailed unpaired Student’s t test (* p<0.05). DL indicates detection limit.

Fig. 5
72x36mm (300 x 300 DPI)