The *Legionella* Lqs-LvbR regulatory network controls

temperature-dependent growth onset and bacterial cell density

Ramon Hochstrasser[1] and Hubert Hilbi[1]*

[1]*Institute of Medical Microbiology, University of Zürich, Gloriastrasse 30, 8006 Zürich,*

Switzerland.

Running title: Growth regulation by the Lqs-LvbR regulatory network

Key words: amoeba, bacterial physiology, biofilm, cell-cell communication, *Legionella*,

metabolism, transcription factor, quorum sensing.

Abbreviations: Icm/Dot, intracellular multiplication/defective organelle trafficking; LAI-1,

Legionella autoinducer-1; LCV, *Legionella*-containing vacuole; Lqs, *Legionella* quorum

sensing; LvbR, *Legionella* virulence and biofilm regulator, c-di-GMP, cyclic di-guanosine

monophosphate; T4SS, type IV secretion system.

24 ***Correspondence:** E-mail hilbi@imm.uzh.ch,

25   Tel.: +41 (0)44 634 2650, Fax: +41 (0)44 634 4906

Abstract

Legionella species are facultative intracellular pathogens, which can cause a life-threatening pneumonia termed Legionnaires' disease. *Legionella pneumophila* employs the *Legionella* quorum sensing (Lqs)-LvbR network to regulate virulence and motility, but its role for growth in media is ill-defined. Compared to the parental *L. pneumophila* strain JR32, a Δ*lqsR* mutant showed a reduced lag phase at 30°C and reached a higher cell density at 45°C, while the Δ*lqsA*, Δ*lqsS* and Δ*lqsT* mutants exhibited a longer lag phase and reached only a lower cell density. A Δ*lvbR* mutant resumed growth like the parental strain at 30°C, but exhibited a substantially reduced cell density at 45°C. Thus, LvbR is an important cell density regulator at elevated temperatures. A quantitative analysis of temperature-dependent growth characteristics of environmental and clinical strains revealed that *L. pneumophila* strains grew in AYE medium after distinct lag phases with similar rates at 30°C, reached different cell densities at the optimal growth temperature of 40°C, and no longer grew at 50°C. *Legionella longbeachae* reached a rather low cell density at 40°C and did not grow at and beyond 45°C. Genes encoding components of the Lqs-LvbR network were present in the genomes of the environmental and clinical *L. pneumophila* isolates, and the P_{lqsR}, P_{lqsA}, P_{lqsS} and P_{lvbR} promoters from strain JR32 were active in these strains. Taken together, our results indicate that the Lqs-LvbR network governs the temperature-dependent growth onset and cell density of the *L. pneumophila* reference strain JR32, and possibly also of environmental and clinical *L. pneumophila* isolates.

Importance

Environmental bacteria of the genus *Legionella* are the causative agents of the severe pneumonia Legionnaires' disease, the incidence of which is worldwide on the rise. *Legionella pneumophila* and *Legionella longbeachae* are the clinically most relevant species. The opportunistic pathogens are inhaled through contaminated aerosols and replicate in human

lung macrophages with a similar mechanism as in their natural hosts, free-living amoebae.

Given their prevalence in natural and technical water systems, an efficient control of

Legionella spp. by physical, chemical or biological means will reduce the incidence of

Legionnaires' disease. Here we report that the *Legionella* quorum sensing (Lqs) system and

the pleiotropic transcription factor LvbR govern the temperature-dependent growth onset and

cell density of bacterial cultures. Hence, the growth of *L. pneumophila* in water systems is not

only determined by the temperature and nutrient availability, but also by quorum sensing, i.e.,

density- and signaling molecule-dependent gene regulation.

Introduction

Environmental bacteria of the genus *Legionella* are the causative agents of Legionnaires'

disease (1, 2). This life-threatening pneumonia almost exclusively affects elderly people, can

occur in epidemics of several hundreds of cases and is globally on the rise. A total of 65

Legionella species has been identified to date (3), of which at least 24 have been linked to

human disease (1). However, more than 90% of the Legionnaires' disease cases diagnosed is

caused by *Legionella pneumophila*, which together with *Legionella longbeachae* account for

ca. 95% of all *Legionella* infections (1).

L. pneumophila parasitizes free-living amoebae and other protozoa (4-6), wherein which it

forms a unique replication-permissive compartment, the *Legionella*-containing vacuole (LCV)

(7-12). *L. pneumophila* is a facultative intracellular bacterium, which can also grow

extracellularly in complex and defined media as well as in biofilms (13, 14). For intra- and

extracellular growth, *L. pneumophila* requires micronutrients such as iron (15, 16). The

analysis of the carbon and energy sources of *L. pneumophila* revealed that this fastidious,

obligate aerobic bacterium mainly metabolizes amino acids (such as serine), but also uses

carbohydrates and polysaccharides (reviewed in (17)). Accordingly, *L. pneumophila* utilizes

glucose (18, 19) and inositol (20), or glycerol (21) as well as glycerolipids (22), and possibly

polysaccharides (glycogen, starch, cellulose, chitin) (23). In general, *L. pneumophila* employs

a bi-partite metabolism (24), as amino acids (e.g., serine) are preferentially used catabolically

as energy supply, and carbohydrates (e.g., glucose) as well as glycerol are primarily utilized

for anabolic processes.

L. pneumophila employs a bi-phasic life cycle, where growth and virulence are

reciprocally linked: growing bacteria are non-virulent, while non-growing bacteria are

virulent, motile and stress resistant (transmissive) (17, 25). The bi-phasic life cycle is

controlled by the RNA-binding, post-transcriptional global regulator CsrA (carbon storage

regulator A), by the two-component systems LetAS, PmrAB and CpxRA (26), as well as by

the *Legionella* quorum sensing (Lqs) system (27, 28). The Lqs system promotes bacterial

density-dependent gene regulation, and produces, detects and responds to the low molecular

weight signaling molecule LAI-1 (*Legionella* autoinducer-1, 3-hydroxypentadecane-4-one)

(29, 30). The components of the Lqs system are encoded in a cluster (*lqsA–lqsR–hdeD–lqsS*)

(31, 32) and at a distant site in the *L. pneumophila* genome (*lqsT*) (33), and they are all

expressed from distinct promoters (34).

The constituents of the Lqs system comprise the pyridoxal-5-phosphate-dependent

autoinducer synthase LqsA (35), the membrane-bound homologous sensor histidine kinases

LqsS and LqsT (33, 35), and the prototypic response regulator LqsR (36) (**Fig. 1**). The latter

contains a canonical phosphate receiver domain, dimerizes upon sensor kinase-mediated

phosphorylation, and harbors an output domain structurally related to nucleotide-binding

proteins (37, 38). Intriguingly, the Lqs system is linked to c-di-GMP signaling through the

pleiotropic transcription factor LvbR, which negatively regulates *hnox1* expression (39, 40),

and itself is negatively regulated by LqsS (35, 39) (**Fig. 1**). The Hnox1-Lpg1057 system

comprises the diguanylate cyclase inhibitor Hnox1 and the GGDEF/EAL domain-containing

diguanylate cyclase Lpg1057 (41, 42).

The Lqs-LvbR regulatory network controls pathogen-host cell interactions, bacterial

motility, filament production, natural competence for DNA uptake, and biofilm architecture

as well as the expression of a 133 kb genomic "fitness island" (39, 40, 43) (**Fig. 1**). The Lqs

system and synthetic LAI-1 not only regulate flagellum production and motility of *L.*

pneumophila (30), but also the migration of eukaryotic cells, and accordingly, the bacterial

signaling compound promotes inter-kingdom signaling (44). Recent studies revealed that the

Lqs system also determines phenotypic heterogeneity and the occurrence of functionally

different *L. pneumophila* subpopulations in infected amoebae and under sessile/biofilm

conditions (45-47). In this work, we show that the Lqs-LvbR system governs the temperature-

dependent growth onset and cell density of an *L. pneumophila* reference strain and possibly

also of environmental and clinical *L. pneumophila* isolates.

Results

The Lqs-LvbR network regulates *L. pneumophila* growth and density in AYE medium.

The *L. pneumophila* Lqs-LvbR regulatory network controls a number of bacterial traits,

including virulence and motility, natural competence and biofilm architecture (40), but its role

for planktonic bacterial growth and temperature dependency of replication has not been

studied in detail. To address this question, we compared growth of the parental strain JR32

with the Δ*lqsR*, Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT* or Δ*lvbR* mutant strains in AYE medium at

different temperatures. We chose a temperature range of 30-45°C, which is close to the

laboratory standard (and optimal) growth temperature of 37°C.

At 30°C all strains grew with similar maximal rates, but their lag phases varied greatly

(**Fig. 2A**). While the Δ*lqsS*-Δ*lqsT* and Δ*lvbR* mutant strains showed the same lag phase as the

parental strain JR32, the Δ*lqsR* mutant exhibited a shortened lag phase, and the Δ*lqsA*, Δ*lqsS*

and Δ*lqsT* mutants showed a prolonged lag phase. The growth initiation phenotypes of the

Δ*lqsR* and Δ*lqsA* mutant strains (shortened or prolonged lag phase, respectively) were

complemented at least partially by the corresponding plasmid-borne genes, and thus, are caused by the absence of these genes (**Fig. S1A)** (36). All *lqs* mutant strains as well as the *lvbR* mutant used have been complemented previously for other phenotypes, including defects in uptake by phagocytes, intracellular growth and cytotoxicity, as well as hyper-competence for DNA uptake (33, 35, 36, 39).

Upon growth at 40°C, the strains showed essentially the same pattern as at 30°C, except that the differences between the lag phase lengths were smaller (**Fig. 2B**). Interestingly, however, at 45°C, some strains grew with different rates and reached vastly different final cell densities (**Fig. 2C**). At 45°C, the Δ*lqsR*, Δ*lqsA*, Δ*lqsS*, Δ*lqsT* and Δ*lqsS*-Δ*lqsT* mutant strains grew with a similar doubling time as the parental strain JR32 (ca. 2.1-2.2 h), while the Δ*lvbR* mutant showed a longer doubling time (ca. 2.6 h), and therefore, grew significantly slower than the parental strain (**Fig. S2**). At 45°C, the Δ*lqsR* and Δ*lqsS*-Δ*lqsT* mutant strains reached a higher cell density compared to JR32, and the Δ*lqsA*, Δ*lqsS* and Δ*lqsT* mutant reached a lower cell density. Strikingly, the final cell density of the Δ*lvbR* mutant was less than 1/3 than that of the parental strain JR32 (**Fig. 2C**). The pronounced cell density phenotype of the Δ*lvbR* mutant strain was complemented to the full extent by the corresponding plasmid-borne gene, and thus, is caused by the absence of this gene (**Fig. S1B**). In summary, compared to the parental strain JR32, the *lqs* and the Δ*lvbR* mutant strains showed different lag phase lengths upon growth initiation in AYE medium at 30°C, and they reached different final cell densities at 45°C. In particular, the autoinducer synthase LqsA and the transcription factor LvbR positively regulate the cell density of *L. pneumophila* at higher temperatures.

The Lqs-LvbR network regulates *L. pneumophila* growth and density in minimal defined medium. Next, we assessed the role of the Lqs-LvbR regulatory network for growth in minimal defined medium (MDM) (21). To this end, we compared growth of the parental strain JR32 with the Δ*lqsR*, Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT* or Δ*lvbR* mutant strains in

MDM at 30-45°C. At 30°C all strains grew with similar maximal rates, but – similar to

growth in AYE medium – the lag phases of the Δ*lqsA*, Δ*lqsS* and Δ*lqsT* mutants were

prolonged (**Fig. 2D**). Hence, the differences in the lag phases of the mutant strains are

observed in the nutrient-rich and complex AYE medium, as well as in the nutrient-poorer and

defined MDM. The Δ*lqsR* and Δ*lqsS*-Δ*lqsT* as well as the Δ*lvbR* mutant strains showed the

same lag phase as the parental strain JR32.

Upon growth at 40°C, the strains showed a similar pattern, but only the lag phase of the

Δ*lqsA* and Δ*lqsS* mutants was prolonged (**Fig. 2E**). At 45°C, growth of all strains was

dramatically reduced, if not abolished (**Fig. 2F**), suggesting that compared to AYE broth, the

strains cope less successfully with higher temperatures under nutrient poor conditions. Taken

together, in MDM the Δ*lqsA*, Δ*lqsS* and Δ*lqsT* mutants initiated growth later than the parental

strain JR32 at 30°C, and – like the other *lqs* mutants as well as the parental strain – did not

grow at all at 45°C. The differences in the lag phases of the mutant strains were observed in

the nutrient-rich AYE medium, as well as in the nutrient-poorer MDM and accordingly, this

trait seems to be independent of the nutrients available.

Temperature-dependent growth of environmental and clinical *Legionella* isolates. With

the aim to assess the detailed growth characteristics and a role for the Lqs-LvbR system for

environmental and clinical *Legionella* strains, we initially started out with a total of 19

isolates, comprising 15 *L. pneumophila* strains [serogroup (sg) 1, 5, and 6], 2 *L. longbeachae*

strains and 2 *L. micdadei* strains (**Fig. S3**). Growth of these *Legionella* strains was

quantitatively assessed by measuring the optical density at 600 nm (OD_{600}) in AYE medium

at 37°C with a 96-well microplate reader. Using this approach, we observed the following

robust patterns: (i) All *L. pneumophila* strains grew with a similar doubling time (ca. 2.3-3.2

h) with the exception of the slower growing sg 6 clinical strain 526 (4.7 h), but the lag phase

of the strains was different and in some cases shorter than that of the sg 1 reference strain

JR32. (ii) The doubling time of the *L. longbeachae* strains (2.3 h) was similar to the *L.*

pneumophila strains, but the final density was lower. (iii) The doubling time of the *L.*

micdadei strains (3.3-3.6 h) was considerably higher than that of the *L. pneumophila* or *L.*

longbeachae strains, and their final density was 3-4 times lower.

Based on the observed growth patterns, we picked representative strains for a detailed

analysis of growth characteristics, i.e., the *L. pneumophila* environmental isolates 500 and 529

(both sg 1), 525 (sg 6) and the clinical isolate 509 (sg 1), as well as the clinical reference

strains *L. pneumophila* JR32 (sg 1) and *L. longbeachae* NSW150. Like *L. pneumophila*, *L.*

micdadei harbors the Lqs system (48); yet, due to the poor growth of the *L. micdadei* strains

in AYE broth, they were not studied further. *L. longbeachae* lacks the Lqs system (49), and

thus, we included this species for comparison. The selected *Legionella* strains grew with a

larger doubling time and to a lower density in diluted AYE at 37°C (**Fig. S4**), indicating that

the growth characteristics indeed depend on nutrient availability.

Following the growth rate of the selected *L. pneumophila* and *L. longbeachae* strains in

AYE medium over a temperature range of 18°C to 50°C revealed that the growth optimum for

all strains is approximately 40°C, except for strain 529, which optimally grew at 45°C (**Fig.**

3A). *L. pneumophila* strain 509 and *L. longbeachae* NSW150 were most temperature-

sensitive, and at or beyond 50°C (*L. pneumophila*) or 45°C (*L. longbeachae*) the strains did

not grow anymore. At the same time, the doubling times decreased from ca. 15-20 h at 18°C

to ca. 2 h at 40°C (**Fig. S5A**). Intriguingly, the growth rate curve was not symmetrical: while

the growth rates increased linearly in the temperature range of 18-40°C, the growth rate

dropped to zero between 40-50°C (**Fig. 3A**).

The growth rates of the *L. pneumophila* strains were ca. 3-fold lower at a given

temperature in MDM, but the temperature range was similar to growth in AYE broth (**Fig.**

3B). Under these conditions, the doubling times decreased from ca. 38-52 h at 18°C to ca. 5-8

h at 40°C for most strains (**Fig. S5B**). *L. pneumophila* strain 525 (sg 6) grew exceptionally

slow in MDM with a doubling time of ca. 210 h at 18°C and ca. 25 h at 40°C, and *L.*

longbeachae NSW150 did not grow at all. Taken together, the doubling times of selected *L.*

pneumophila and *L. longbeachae* strains decreased ca. 5- to 10-fold between growth at 18°C

and the optimum of 40°C, and the doubling times of the *L. pneumophila* strains were ca. 3-

fold shorter upon growth in AYE medium as compared to MDM.

213 Growth onset and cell density of environmental and clinical *Legionella* strains in AYE

214 medium. To compare growth of the selected environmental and clinical *Legionella* strains to

the laboratory strain JR32 and isogenic *lqs-lvbR* mutants (**Fig. 2**), we further assessed in detail

the growth characteristics at 30-45°C (**Fig. 4**). Upon growth in AYE medium at 30°C the

doubling time of the 5 *L. pneumophila* strains was similar (ca. 4.0-4.6 h; **Table 1**), but the lag

phase length was different (**Fig. 4A**), similar to the initially observed pattern (**Fig. S3**). While

the strains 500, 525 and 529 showed a shorter lag phase compared to strain JR32, the lag

phase of strain 509 was longer.

Upon growth at 40°C the doubling time of the 5 *L. pneumophila* strains was reduced

compared to 30°C (ca. 2.2-2.7 h; **Table 1**), the lag phase length was different, and the final

cell density of *L. pneumophila* strain 500 was reduced (**Fig. 4B**). At 45°C the *L. pneumophila*

strains 525, 529 and JR32 grew fastest with doubling times of 2.0-2.6 h (**Table 1**), while the

L. pneumophila strains 500 and 509 grew slower and to much lower density (**Fig. 4C**).

Intriguingly, the *L. pneumophila* strains showed a bi-phasic growth pattern, in particular at

40°C and to a somewhat lesser extent at 45°C. Finally, *L. longbeachae* NSW150 grew

similarly to *L. pneumophila* (at 30°C), less densely (at 40°C) or not at all (at 45°C) (**Fig. 4A-**

C, Table 1). Taken together, environmental and clinical *L. pneumophila* and *L. longbeachae*

strains grow similarly in AYE medium after distinct lag phases at 30°C, and the strains reach

different cell densities at the optimal temperature of 40°C and in particular at an elevated

temperature of 45°C.

Growth onset and cell density of environmental and clinical *Legionella* strains in minimal defined medium. Next, we assessed the growth characteristics of the 6 selected *Legionella* strains in MDM at 30-45°C. Upon growth in MDM at 30°C the *L. pneumophila* strains JR32, 509 and 529 grew with the shortest doubling time (7.9-9.1 h; **Table 1**), and strain JR32 reached the highest density (**Fig. 4D**). The *L. pneumophila* strains 500 (sg 1) and 525 (sg 6) grew much less densely, and *L. longbeachae* NSW150 did not grow at all. Compared to the nutrient-rich AYE broth, only the *L. pneumophila* strain 509 grew with a comparable pattern in MDM (log phase elongated), while the other environmental and clinical strains showed a considerably different growth pattern and also grew less densely.

Upon growth at 40°C, the growth characteristics of the strains were largely the same as at 30°C (**Fig. 4E**), but the *L. pneumophila* strains JR32, 509 and 529 grew faster with doubling times of 5.0-7.3 h (**Table 1**). At 45°C the *L. pneumophila* strains JR32 and 529 grew with doubling times similar to what was observed at 30°C, and the other strains grew barely or not at all (**Fig. 4F**). However, under these conditions, the final cell density of the strains JR32 and 529 was considerably lower, suggesting that compared to AYE broth, the strains cope less successfully with higher temperatures under nutrient poor conditions. Similar to growth in AYE medium, many *L. pneumophila* strains also showed a bi- if not tri-phasic growth in MDM, in particular the strains JR32, 500, 509 and 529 at 40°C. Taken together, the growth characteristics of the *L. pneumophila* strains JR32, 509 and 529 in MDM were qualitatively similar to AYE medium, but the final cell densities were much lower. *L. pneumophila* 525 (sg 6) barely grew, and *L. longbeachae* NSW150 did not grow at all. Since the growth pattern of same environmental and clinical strains was different in AYE broth and MDM, it appears to be dependent on the nutrients available.

Presence and regulation of *lqs* and *lvbR* genes in environmental and clinical *Legionella* isolates. In order to possibly implicate the *lqs* and *lvbR* genes in the growth characteristics of the selected environmental and clinical *Legionella* strains, we first assessed the presence of the genes in these strains. To this end, the regions comprising P_{lqsR}-*lqsR*, P_{lqsA}-*lqsA*, P_{lqsS}-*lqsS* or P_{lvbR}-*lvbR* were amplified by PCR in the *L. pneumophila* strains JR32 (positive control), 500, 509, 525, 529 and the *L. longbeachae* strain NSW150 (negative control) (**Fig. 5A**). Using genomic DNA of these strains as a template, fragments comprising P_{lqsR}-*lqsR* (1727 bp), P_{lqsA}-*lqsA* (1936 bp) and P_{lqsS}-*lqsS* (1984 bp) were amplified from all *L. pneumophila* strains tested, but not from *L. longbeachae* NSW150, in agreement with the absence of the *lqs* genes in the genome of the latter (49).

The fragments P_{lvbR}-*lvbR* (948 bp), *lvbR* (264 bp) and P_{hnox1}-*hnox1* (1344 bp) were amplified in the *L. pneumophila* strains JR32, 500, 509, and 529, but neither in *L. pneumophila* 525 nor in *L. longbeachae* NSW150 (**Fig. 5B**). However, the fragments *lvbR*_1-201 (201 bp), LvbR_BS (LvbR binding site (39); 347 bp) and LvbR_BS-*hnox1* (969 bp) were amplified in all *L. pneumophila* strains tested including strain 525, indicating that the *lvbR* as well as the *lvbR-hnox1* intergenic region and *hnox1* are indeed present in the genome of the latter strain. Strain 525 harbors an LvbR_BS region, which is shorter than in the other strains tested, and an *lvbR* gene, which appears to be a more distantly related homologue. Sequencing of the LvbR_BS intergenic region and part of the *lvbR* and *hnox1* genes of the strains JR32, 500, 509, 525 and 529 revealed the presence of almost identical *lvbR* and *hnox1* genes and an LvbR_BS intergenic region of strain 525, which is indeed 80 nucleotides shorter (**Fig. S6**). *L. longbeachae* NSW150 likely does not harbor an *lvbR* homologue, and the most closely related protein is a putative YdnC-like transcription factor (29% identity of 55/178 amino acids; E-value $7 \times e^{-4}$). Taken together, these results revealed that the *lqsR*, *lqsA*, *lqsS* and *lvbR* genes are present in all *L. pneumophila* strains analyzed in detail in this study.

Next, we tested the regulation of the *lqs* and *lvbR* genes in environmental and clinical *L.*

pneumophila isolates. The genome sequences for these strains are not available, and therefore,

we used the corresponding promoters of strain JR32. Accordingly, we transformed the strains

JR32, 500, 509, 525 and 529 with reporter constructs comprising transcriptional fusions of

different promoters from strain JR32 and *gfp* (P_{lqsR}-*gfp*, P_{lqsA}-*gfp*, P_{lqsS}-*gfp*, P_{lvbR}-*gfp* or P_{hnox1}-

gfp) and quantified GFP production by fluorescence (**Fig. 6**). This approach revealed that all

environmental and clinical *L. pneumophila* isolates tested expressed P_{lqsR}-*gfp* (**Fig. 6A**), P_{lqsA}-

gfp (**Fig. 6B**), P_{lqsS}-*gfp* (**Fig. 6C**), P_{lvbR}-*gfp* (**Fig. 6D**) and P_{hnox1}-*gfp* (**Fig. 6E**). The expression

pattern of P_{lqsR}, P_{lqsS} and P_{lvbR} , as well as of P_{lqsA} and P_{hnox1}, were similar in the environmental

and clinical *L. pneumophila* isolates. Interestingly, some environmental and clinical *L.*

pneumophila isolates expressed P_{lvbR} with high intensity, as opposed to the parental strain

JR32, which did not express P_{lvbR} (**Fig. 6D**), as observed previously (39). Since all of the

environmental and clinical strains harbor *lqsS* (**Fig. 5A**), and therefore presumably produce

the negative regulator of LvbR, LqsS (35, 39), another mechanism likely accounts for the

observation that the strains indeed express P_{lvbR}.

The expression of P_{lqsR}, P_{lqsS} and P_{lvbR} in the environmental and clinical *L. pneumophila*

isolates peaked at different times (**Fig. 6**), which precisely correspond to the different lag

phase lengths of the strains, and accordingly, to their transition from the exponential to the

stationary growth phase (**Fig. 7**). On the other hand, P_{lqsA} and P_{hnox1} showed a similar, but

more complex expression profile, which was not exclusively linked to the transition from the

exponential to the stationary growth phase (**Fig. 7**).

The *L. pneumophila* strains JR32, 500, 509, 525, and 529 harboring the empty plasmid

pMMB207C-RBS showed very low fluorescence (**Fig. S7A**). The growth profile of these

strains at 30°C (**Fig. S7B**) was very similar to the growth profiles of the strains harboring no

plasmid (**Fig. 4A**), but distinct from the growth profiles of strain 525 harboring one of the

promoter reporters producing GFP (**Fig. 7A-E**). Therefore, GFP production rather than the

presence of the plasmid itself seems to account for the delayed growth of strain 525, but not

the other *L. pneumophila* strains tested. In summary, all environmental and clinical *L.*

pneumophila isolates tested expressed P_{lqsR}, P_{lqsS} and P_{lvbR} of strain JR32 with a single peak in

the post-exponential growth phase, and the strains expressed P_{lqsA} and P_{hnox1} of strain JR32

with a more complex expression profile. These results suggest that the Lqs-LvbR network is

active in the environmental and clinical *L. pneumophila* strains.

Discussion

In this study we analyzed in detail the growth characteristics and the role of the Lqs-LvbR

network for temperature-dependent growth rate and cell density of a *L. pneumophila* reference

and mutant strains, as well as of select environmental and clinical *L. pneumophila* and *L.*

longbeachae isolates. For the *L. pneumophila* reference strain, we implicated components of

the Lqs system in growth onset, doubling time and cell density (**Fig. 2**). Hence, the Lqs

system not only regulates *L. pneumophila* virulence, motility, competence, and a genomic

fitness island, but also growth characteristics of planktonic bacteria (**Fig. 1**). Compared to the

parental strain JR32, a Δ*lqsR* mutant strain lacking the response regulator LqsR showed a

reduced lag phase at 30°C in AYE medium and reached a higher cell density at 45°C (**Fig. 2**).

The Δ*lqsA*, Δ*lqsS* and Δ*lqsT* mutants lacking the autoinducer synthase LqsA or one of the

homologous sensor kinases, LqsS or LqsT, respectively, exhibited a longer lag phase and

reached only a lower cell density. Intriguingly, the components of the Lqs system regulated

growth resumption of sessile *L. pneumophila* in a similar manner: compared to the parental

strain JR32, the percentage of Δ*lqsA*, Δ*lqsS* or Δ*lqsT* mutant strains resuming growth was

lower, while the percentage of Δ*lqsR* and the Δ*lqsS*-Δ*lqsT* double mutant was higher (46).

Intriguingly, growth resumption and final cell density of the *lqs* and *lvbR* mutant strains is

not correlated to their virulence and transmission phenotypes. Both the Δ*lqsR* and the Δ*lvbR*

mutant strains are severely impaired for virulence (36, 39), yet growth resumption

characteristics and final cell density at the temperatures tested are different (**Fig. 2**).

Analogously, the Δ*lqsA* mutant does not show a severe virulence phenotype compared to the

parental strain JR32 (35), yet growth resumption and final cell density of the two strains are

distinct (**Fig. 2**). Finally, the Δ*lqsS*, Δ*lqsT* and Δ*lqsS*-Δ*lqsT* double mutant are all impaired for

virulence (33, 35), yet the individual sensor kinase mutants reached a lower cell density than

the parental strain at 45°C, while the Δ*lqsS*-Δ*lqsT* double mutant reached a higher cell density.

A possible explanation for the latter observation is that LqsS and LqsT regulate many genes in

a complex and reciprocal manner (33), and hence, the Δ*lqsS*-Δ*lqsT* double mutant might

cancel out or revert phenotypes of the single mutants. In any case, the effects of *L.*

pneumophila Lqs-LvbR components on planktonic growth resumption and final cell density

are apparently not directly linked to the virulence phenotypes of the corresponding mutant

strains.

The Lqs system and the α-hydroxyketone autoinducer LAI-1 are not strictly conserved

among *Legionella* species, but α-hydroxyketone-mediated signaling and cell-cell

communication seem to be widely distributed among environmental bacteria (27). A recent

bioinformatics analysis for the presence of the *lqs* cluster in 58 *Legionella* genomes revealed

three categories of species (48): 19 harbored a complete *lqs* cluster, 20 did not possess *lqsA*

but maintained the receptor *lqsR* and/or the sensor kinase *lqsS*, and 19 did not have any of the

lqs genes. These results are in agreement with the notion that the Lqs system functions in

intra- as well as inter-species communication. In agreement with a broad distribution of the

Lqs-LvbR network are the findings that components are not only present in the reference

strain JR32, but also in the genomes of the clinical and environmental *L. pneumophila* strains

analyzed in detail here (**Fig. 5**). Interestingly, the P_{lqsR}, P_{lqsA} and P_{lqsS} promoters from strain

JR32 are expressed to a similar if not higher degree in the clinical and environmental *L.*

pneumophila strains, and P_{lvbR} is highly expressed in these strains, but not in JR32 (**Fig. 6**),

confirming previous findings (39).

In addition to the Lqs system, we found that the pleiotropic transcription factor LvbR plays

a major role for doubling time and cell density of *L. pneumophila* (**Fig. 2**). In AYE medium,

the Δ*lvbR* mutant strain resumed growth like the parental strain at 30°C (**Fig. 2A**), but

exhibited a dramatically reduced cell density at 45°C (**Fig. 2C**). In MDM, the Δ*lvbR* mutant

strain behaved similarly to the parental strain JR32 (**Figs. 2D-F**). Hence, preferentially under

nutrient rich conditions and at elevated temperatures, the LvbR transcription factor functions

as an important regulator of *L. pneumophila* cell density. The low final cell density reached by

the Δ*lvbR* mutant strain in AYE medium might be reflected in the different transcriptomes of

the mutant compared to the parental strain JR32 (39). An inspection of genes differentially

produced in the Δ*lvbR* mutant versus the parental strain revealed that many flagellar genes are

up-regulated, while genes implicated in the metabolism of amino acids, lipids and

carbohydrates are indeed down-regulated.

In the laboratory strain JR32, LvbR is negatively regulated by LqsS (35, 39) (**Fig. 6D**). All

environmental and clinical strains tested harbor *lqsS* (**Fig. 5A**) as well as *lvbR* (**Fig. 5B**, S**6B**).

Based on expression studies with the corresponding promoters of strain JR32, *lqsS* as well as

lvbR are expressed in the environmental and clinical strains (**Fig. 6CD**). Therefore, *lvbR* does

not seem to be negatively regulated by *lqsS* in these strains, and the production of LvbR might

not be linked to the Lqs system.

In *L. pneumophila* strain JR32, LvbR links the Lqs system to c-di-GMP signaling (39, 40),

and therefore, it is likely that the second messenger c-di-GMP controls temperature-dependent

growth rate and cell density of the pathogen. *L. pneumophila* JR32 harbors 22 genes predicted

to encode proteins with domains implicated in c-di-GMP synthesis, hydrolysis and

recognition (50, 51). However, only a few components of the c-di-GMP regulatory network

have been characterized, including the Hnox1-Lpg1057 system, comprising the diguanylate

cyclase inhibitor Hnox1 and the GGDEF/EAL domain-containing diguanylate cyclase

Lpg1057 (41, 42) (**Fig. 1**). It is currently not known, which components of the c-di-GMP

regulatory network play a role in the temperature-dependent control of *L. pneumophila*

growth rate and cell density under given conditions.

Recently, we analyzed the growth of sessile *L. pneumophila* on surfaces and in biofilm

using single cell techniques (46). We found that sessile *L. pneumophila* exhibits phenotypic

heterogeneity and adopts growing and non-growing ("dormant") states in biofilms and

microcolonies formed in AYE medium. The establishment of phenotypic heterogeneity was

controlled by the temperature, the Lqs system and LvbR. The Lqs system and LvbR were

found to determine the ratio between growing and non-growing ("persister") sessile

subpopulations, as well as the frequency of growth resumption ("resuscitation") and

microcolony formation of individual bacteria. The microcolony growth rate was the same for

the parental strain JR32 and *lqs* mutant strains, similar to what we observed for planktonic

growth in AYE medium at 30°C or 40°C (**Figs. 2A, B**). LvbR regulated the growth

resumption of sessile *L. pneumophila* (46), but not the lag phase length of planktonic *L.*

pneumophila growing at 30°C or 40°C (**Figs. 2A, B**), and therefore, substantial differences

between the LvbR-dependent growth regulation of sessile and planktonic bacteria exist.

Other regulatory systems implicated in the survival of *L. pneumophila* in aqueous

environments are the "stringent response" and the alternative sigma factor RpoS (σ^S/σ^{38}) (52),

as well as the two-component system (TCS) LetAS (53). The stringent response is triggered

upon production of the second messenger guanosine 3',5'-bispyrophosphate (ppGpp) by the

RelA and SpoT synthases (54-56), which in turn are activated by amino acid starvation (57)

and inhibition of fatty acid biosynthesis (56). In response to ppGpp the stationary phase sigma

factor RpoS controls the expression of *L. pneumophila* virulence and transmission (54, 58),

and the LetAS TCS also regulates these traits (28, 59-61). RpoS and to a lesser extent LetA

control the production of LqsR in stationary growth phase (36), and thus, the stringent

response is linked to the Lqs system. For planktonic growth of *L. pneumophila* in broth,

minimal media and aqueous environments this implicates that nutrient starvation likely

upregulates the Lqs system, and hence, promotes density-dependent regulation.

In addition to the role of the intrinsic Lqs-LvbR system for growth onset, doubling time

and cell density of *L. pneumophila*, we also assessed the role of the extrinsic (environmental)

cues temperature and nutrient availability for growth of *Legionella* spp. (**Figs. 3, 4, S3-5**).

Compared to *L. pneumophila*, *L. longbeachae* appeared to be more susceptible to elevated

temperatures in AYE medium (**Fig. 3, 4**). While *L. pneumophila* strains grew up to 47°C and

ceased to grow at 50°C, *L. longbeachae* reached only a lower final cell density upon growth at

40°C and ceased to grow already at 45°C (**Fig. 3, 4**). The most temperature-sensitive *L.*

pneumophila strains were the environmental isolate 500 and the clinical isolate 509 (both sg

1), which at a growth temperature of 45°C reached only a 2-3-fold lower cell density in AYE

medium compared to the reference strain JR32 (**Fig. 4C**). The final cell density of these

strains did not correlate with the differences in the lag phase length, since compared to JR32

strain 500 or 509 showed a shortened or extended lag phase, respectively, at 30°C. In general,

the temperature-dependent differences in the final cell density became even more pronounced

in MDM. Under these conditions, only strain JR32 and the environmental isolate 529 grew,

albeit to only a low density, while all other *L. pneumophila* strains no longer grew (**Fig. 4D-**

F). The environmental and clinical *L. pneumophila* strains all harbored the *lqsR*, *lqsA*, *lqsS*

and *lvbR* genes in their genomes, and thus, the Lqs-LvbR network might play a role for the

temperature-dependent growth patterns, but not for the differences observed among the

strains. Accordingly, the reason(s) for the vast differences in growth resumption and final

density of natural *Legionella* isolates in response to temperature are unknown.

Overall, the results documented in this study are in agreement with a general role for

quorum sensing (Lqs) and c-di-GMP signaling (LvbR) for growth onset, doubling time and

final cell density of *L. pneumophila* under different conditions. Future studies will identify the

"downstream" components implicated in the temperature-, Lqs- and LvbR-dependent

regulation of *L. pneumophila* growth under different extra- and intracellular conditions.

Material and Methods

Growth of *Legionella* strains. The *Legionella* strains used in this study are listed in **Table 2**.

Legionella strains were grown on CYE agar plates (62) and in liquid cultures with *N*-(2-

acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (AYE) medium (63)

for 23-24 h or in minimal defined medium (MDM) (21) for 28-29 h at 37°C on a wheel (80

rpm). To obtain growth curves, *Legionella* strains were diluted from cultures grown in AYE

medium to an initial OD_{600} of 0.1 or 0.2 with AYE medium or MDM into 96-well microplates

(100 μl/well, polystyrene, 353072, Falcon) and incubated at the temperatures indicated in the

figure legends while orbitally shaking. Bacterial growth was monitored over time in triplicates

by measuring the optical density at 600 nm (OD_{600}) using a microplate reader (Synergy H1 or

Cytation5 Hybrid Multi-Mode Reader, BioTek). *L. pneumophila* strains harboring empty

control plasmids (pTS10 or pNT28) or complementation plasmids (pAK18, pTS02, pTS04)

were cultivated and analyzed in AYE medium supplemented with chloramphenicol (Cm; 5

μg/ml).

The experiments and the statistics were performed in biological triplicates, as defined by

individual bacterial cultures growing in separate wells of a microtiter plate. The biological

triplicates were repeated at least twice in independent experiments, as defined as bacterial

cultures originating from different pre-cultures, which yielded practically identical results. In

most cases, the experiments were independently repeated 3-5 times. The resulting growth

curves were analyzed with the software GraphPad Prism (Version 5.04). Growth rates

(maximum) and doubling times (minimum) were calculated using a curve fit with a nonlinear

regression model, considering only the fastest exponential growth as defined in semi-

logarithmic plots.

463   **Plasmid construction.** Cloning was performed according to standard protocols, and the

464  primers used for PCR reactions are listed in **Table 3**. Plasmids were amplified and isolated

465  from *E. coli* TOP10 (Invitrogen) using commercially available kits (Macherey-Nagel).

466  Reporter plasmids pRH037, pRH038 and pRH051 containing a transcriptional P_{lqsR}-, P_{lqsA}-, or

467  P_{lqsS}-*gfp* (ASV) fusion were constructed by amplifying the insert by PCR using the primer

468  pair oRH188/189, oRH190/191 or oRH223/224, respectively, and genomic JR32 DNA as a

469  template. PCR products were cloned with the NEBuilder HiFi DNA Assembly reaction

470  (NEB) into the *Sac*I and *Xba*I sites of pCM009 (30), thereby replacing P_{flaA}. The resulting

471  constructs were verified by DNA sequencing.

472   **Genomic DNA extraction, PCR amplification and sequencing.** Genomic DNA was

473  extracted from *Legionella* strains cultured for 18 h in AYE medium using the GenElute^{TM}

474  Bacterial Genomic DNA Kit (NA2100, Sigma-Aldrich) and used as template for PCR

475  reactions. Genes and putative promoter regions were amplified by standard PCR protocols

476  using the primer pairs oRH178/179 (P_{lqsR}-*lqsR*), oRH199/200 (P_{lqsA}-*lqsA*), oRH219/220 (P_{lqsS}-

477  *lqsS*), or oRH201/202 (P_{lvbR}-*lvbR*), oRH208/209 (*lvbR*), oRH208/068 (*lvbR*_1-201),

478  oRH051/069 (LvbR_BS), oRH221/202 (P_{hnox1}-*hnox1*) or oRH221/051 (LvbR_BS-*hnox1*), and

479  the PCR fragments were subsequently analyzed by agarose gel electrophoresis. DNA bands

480  were visualized with MaestroSafe staining (Labgene Scientific SA) and imaged using a

481  FluorChem SP imager (Alpha Innotech). For sequence analysis, the *lvbR-hnox1* gene regions

482  were amplified from genomic DNA by PCR using primer pair oRH104/068. PCR products

483  were analyzed by agarose gel electrophoresis, purified (gel and PCR clean-up kit, Macherey-

484  Nagel) and subsequently sequenced by the use of primers oRH068, 069 or 104 (Sanger

485  sequencing performed by Microsynth). An alignment was created with the resulting

486  sequences by the program CLC Main Workbench 7.

487   **GFP reporter assays.** *L. pneumophila* JR32, 500, 509, 525 or 529 strains harboring P_{lqsR}-,

488  P_{lqsA}-, P_{lqsS}-, P_{lvbR}- or P_{hnox1}-*gfp* (ASV) fusion reporter plasmids (pRH037, pRH038, pRH051,

pRH023 or pRH026) or empty pMMB207C plasmids (pTS10) were grown for 23-24 h at

37°C in AYE medium supplemented with chloramphenicol (Cm; 5 µg/ml). Strains were

subsequently inoculated from these cultures at an initial OD_{600} of 0.2 in AYE medium/Cm

into a black clear bottom 96-well plate (100 µl/well, polystyrene, 353219, Falcon) and

incubated at 30°C while orbitally shaking. GFP production and bacterial growth were

monitored in triplicates by measuring fluorescence (excitation, 485 nm; emission, 528 nm;

gain, 50) and the OD_{600} with a microplate reader (Synergy H1 or Cytation5 Hybrid Multi-

Mode Reader, BioTek). Blank value (AYE medium) was subtracted from all values, and

numbers are expressed as relative fluorescence units (RFU) or OD_{600}.

Acknowledgements

We would like to thank Barbara Borer and Arnd Gildemeister (Geberit International AG) for

stimulating discussions. The project was funded by Geberit International AG. Work in the

group of H.H. was supported by the Swiss National Science Foundation (31003A_175557,

310030_200706), a research grant from the University of Zürich awarded to R.H., and the

Institute of Medical Microbiology.

The funders had no role in study design, data collection and analysis, decision to publish,

or preparation of the manuscript. The authors declare no conflict of interest.

References

1. Newton HJ, Ang DK, van Driel IR, Hartland EL. 2010. Molecular pathogenesis of

infections caused by *Legionella pneumophila*. Clin Microbiol Rev 23:274-98.

2. Hilbi H, Hoffmann C, Harrison CF. 2011. *Legionella* spp. outdoors: colonization,

communication and persistence. Environ Microbiol Rep 3:286–296.

3. Parte AC. 2018. LPSN - List of prokaryotic names with standing in nomenclature

(bacterio.net), 20 years on. Int J Syst Evol Microbiol 68:1825-1829.

4.   Boamah DK, Zhou G, Ensminger AW, O'Connor TJ. 2017. From many hosts, one

accidental pathogen: the diverse protozoan hosts of *Legionella*. Front Cell Infect

Microbiol 7:477.

5.   Hoffmann C, Harrison CF, Hilbi H. 2014. The natural alternative: protozoa as cellular

models for *Legionella* infection. Cell Microbiol 16:15-26.

6.   Swart AL, Harrison CF, Eichinger L, Steinert M, Hilbi H. 2018. *Acanthamoeba* and

Dictyostelium as cellular models for *Legionella* infection. Front Cell Infect Microbiol

8:61.

7.   Asrat S, de Jesus DA, Hempstead AD, Ramabhadran V, Isberg RR. 2014. Bacterial

pathogen manipulation of host membrane trafficking. Annu Rev Cell Dev Biol 30:79-

109.

8.   Escoll P, Mondino S, Rolando M, Buchrieser C. 2016. Targeting of host organelles by

pathogenic bacteria: a sophisticated subversion strategy. Nat Rev Microbiol 14:5-19.

9.   Personnic N, Bärlocher K, Finsel I, Hilbi H. 2016. Subversion of retrograde trafficking

by translocated pathogen effectors. Trends Microbiol 24:450-62.

10.   Sherwood RK, Roy CR. 2016. Autophagy evasion and endoplasmic reticulum

subversion: the yin and yang of *Legionella* intracellular infection. Annu Rev Microbiol

70:413-33.

11.   Steiner B, Weber S, Hilbi H. 2018. Formation of the *Legionella*-containing vacuole:

phosphoinositide conversion, GTPase modulation and ER dynamics. Int J Med Microbiol

308:49-57.

12.   Qiu J, Luo ZQ. 2017. *Legionella* and *Coxiella* effectors: strength in diversity and activity.

Nat Rev Microbiol 15:591-605.

13.   Declerck P. 2010. Biofilms: the environmental playground of *Legionella pneumophila*.

Environ Microbiol 12:557-66.

14. Abdel-Nour M, Duncan C, Low DE, Guyard C. 2013. Biofilms: the stronghold of

Legionella pneumophila. Int J Mol Sci 14:21660-75.

15. Hindré T, Brüggemann H, Buchrieser C, Héchard Y. 2008. Transcriptional profiling of

Legionella pneumophila biofilm cells and the influence of iron on biofilm formation.

Microbiology 154:30-41.

16. Cianciotto NP. 2015. An update on iron acquisition by *Legionella pneumophila*: new

pathways for siderophore uptake and ferric iron reduction. Future Microbiol 10:841-51.

17. Manske C, Hilbi H. 2014. Metabolism of the vacuolar pathogen *Legionella* and

implications for virulence. Front Cell Infect Microbiol 4:125.

18. Eylert E, Herrmann V, Jules M, Gillmaier N, Lautner M, Buchrieser C, Eisenreich W,

Heuner K. 2010. Isotopologue profiling of *Legionella pneumophila*: role of serine and

glucose as carbon substrates. J Biol Chem 285:22232-43.

19. Schunder E, Gillmaier N, Kutzner E, Eisenreich W, Herrmann V, Lautner M, Heuner K.

2014. Amino acid uptake and metabolism of *Legionella pneumophila* hosted by

Acanthamoeba castellanii. J Biol Chem 289:21040-54.

20. Manske C, Schell U, Hilbi H. 2016. Metabolism of *myo*-inositol by *Legionella*

pneumophila promotes infection of amoebae and macrophages. Appl Environ Microbiol

82:5000-5014.

21. Häuslein I, Manske C, Goebel W, Eisenreich W, Hilbi H. 2016. Pathway analysis using

(13)C-glycerol and other carbon tracers reveals a bipartite metabolism of *Legionella*

pneumophila. Mol Microbiol 100:229-46.

22. Häuslein I, Sahr T, Escoll P, Klausner N, Eisenreich W, Buchrieser C. 2017. *Legionella*

pneumophila CsrA regulates a metabolic switch from amino acid to glycerolipid

metabolism. Open Biol 7:e170149.

23. Herrmann V, Eidner A, Rydzewski K, Blädel I, Jules M, Buchrieser C, Eisenreich W, Heuner K. 2011. GamA is a eukaryotic-like glucoamylase responsible for glycogen- and starch-degrading activity of *Legionella pneumophila*. Int J Med Microbiol 301:133-9.

24. Eisenreich W, Heuner K. 2016. The life stage-specific pathometabolism of *Legionella pneumophila*. FEBS Lett 590:3868-3886.

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

26. Segal G. 2013. The *Legionella pneumophila* two-component regulatory systems that participate in the regulation of Icm/Dot effectors. Curr Top Microbiol Immunol 376:35-52.

27. Tiaden A, Spirig T, Hilbi H. 2010. Bacterial gene regulation by α-hydroxyketone signaling. Trends Microbiol 18:288-297.

28. Hochstrasser R, Hilbi H. 2017. Intra-species and inter-kingdom signaling of *Legionella pneumophila*. Front Microbiol 8:79.

29. Spirig T, Tiaden A, Kiefer P, Buchrieser C, Vorholt JA, Hilbi H. 2008. The *Legionella* autoinducer synthase LqsA produces an α-hydroxyketone signaling molecule. J Biol Chem 283:18113-23.

30. Schell U, Simon S, Sahr T, Hager D, Albers MF, Kessler A, Fahrnbauer F, Trauner D, Hedberg C, Buchrieser C, Hilbi H. 2016. The α-hydroxyketone LAI-1 regulates motility, Lqs-dependent phosphorylation signalling and gene expression of *Legionella pneumophila*. Mol Microbiol 99:778-93.

31. Tiaden A, Spirig T, Carranza P, Brüggemann H, Riedel K, Eberl L, Buchrieser C, Hilbi H. 2008. Synergistic contribution of the *Legionella pneumophila lqs* genes to pathogen-host interactions. J Bacteriol 190:7532-47.

32. Tiaden A, Hilbi H. 2012. α-Hydroxyketone synthesis and sensing by *Legionella* and
Vibrio. Sensors 12:2899-2919.

33. Kessler A, Schell U, Sahr T, Tiaden A, Harrison C, Buchrieser C, Hilbi H. 2013. The
Legionella pneumophila orphan sensor kinase LqsT regulates competence and pathogen-
host interactions as a component of the LAI-1 circuit. Environ Microbiol 15:646-62.

34. Sahr T, Rusniok C, Dervins-Ravault D, Sismeiro O, Coppee JY, Buchrieser C. 2012.
Deep sequencing defines the transcriptional map of *L. pneumophila* and identifies growth
phase-dependent regulated ncRNAs implicated in virulence. RNA Biol 9:503-519.

35. Tiaden A, Spirig T, Sahr T, Wälti MA, Boucke K, Buchrieser C, Hilbi H. 2010. The
autoinducer synthase LqsA and putative sensor kinase LqsS regulate phagocyte
interactions, extracellular filaments and a genomic island of *Legionella pneumophila*.
Environ Microbiol 12:1243-1259.

36. Tiaden A, Spirig T, Weber SS, Brüggemann H, Bosshard R, Buchrieser C, Hilbi H. 2007.
The *Legionella pneumophila* response regulator LqsR promotes host cell interactions as
an element of the virulence regulatory network controlled by RpoS and LetA. Cell
Microbiol 9:2903-20.

37. Schell U, Kessler A, Hilbi H. 2014. Phosphorylation signalling through the *Legionella*
quorum sensing histidine kinases LqsS and LqsT converges on the response regulator
LqsR. Mol Microbiol 92:1039-55.

38. Hochstrasser R, Hutter CAJ, Arnold FM, Bärlocher K, Seeger MA, Hilbi H. 2020. The
structure of the *Legionella* response regulator LqsR reveals amino acids critical for
phosphorylation and dimerization. Mol Microbiol 113:1070-1084.

39. Hochstrasser R, Kessler A, Sahr T, Simon S, Schell U, Gomez-Valero L, Buchrieser C,
Hilbi H. 2019. The pleiotropic *Legionella* transcription factor LvbR links the Lqs and c-
di-GMP regulatory networks to control biofilm architecture and virulence. Environ
Microbiol 21:1035-1053.

40. Hochstrasser R, Hilbi H. 2020. *Legionella* quorum sensing meets cyclic-di-GMP

signaling. Curr Opin Microbiol 55:9-16.

41. Carlson HK, Vance RE, Marletta MA. 2010. H-NOX regulation of c-di-GMP metabolism

and biofilm formation in *Legionella pneumophila*. Mol Microbiol 77:930-42.

42. Pécastaings S, Allombert J, Lajoie B, Doublet P, Roques C, Vianney A. 2016. New

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

Biofouling 32:935-48.

43. Personnic N, Striednig B, Hilbi H. 2018. *Legionella* quorum sensing and its role in

pathogen-host interactions. Curr Opin Microbiol 41:29-35.

44. Simon S, Schell U, Heuer N, Hager D, Albers MF, Matthias J, Fahrnbauer F, Trauner D,

Eichinger L, Hedberg C, Hilbi H. 2015. Inter-kingdom signaling by the *Legionella*

quorum sensing molecule LAI-1 modulates cell migration through an IQGAP1-Cdc42-

ARHGEF9-dependent pathway. PLoS Pathog 11:e1005307.

45. Personnic N, Striednig B, Lezan E, Manske C, Welin A, Schmidt A, Hilbi H. 2019.

Quorum sensing modulates the formation of virulent *Legionella* persisters within infected

cells. Nat Commun 10:5216.

46. Personnic N, Striednig B, Hilbi H. 2021. Quorum sensing controls persistence,

resuscitation, and virulence of *Legionella* subpopulations in biofilms. ISME J 15:196-

210.

47. Striednig B, Lanner U, Niggli S, Katic A, Vormittag S, Brülisauer S, Hochstrasser R,

Kaech A, Welin A, Flieger A, Ziegler U, Schmidt A, Hilbi H, Personnic N. 2021.

Quorum sensing governs a transmissive *Legionella* subpopulation at the pathogen

vacuole periphery. EMBO Rep 22:e52972.

48. Herran B, Greve P, Berjeaud JM, Bertaux J, Crepin A. 2021. *Legionella* spp. all ears?

The broad occurrence of quorum sensing elements outside *Legionella pneumophila*.

Genome Biol Evol 13:evab032.

49. Cazalet C, Gomez-Valero L, Rusniok C, Lomma M, Dervins-Ravault D, Newton HJ,

Sansom FM, Jarraud S, Zidane N, Ma L, Bouchier C, Etienne J, Hartland EL, Buchrieser

C. 2010. Analysis of the *Legionella longbeachae* genome and transcriptome uncovers

unique strategies to cause Legionnaires' disease. PLoS Genetics 6:e1000851.

50. Allombert J, Lazzaroni JC, Bailo N, Gilbert C, Charpentier X, Doublet P, Vianney A.

2014. Three antagonistic cyclic di-GMP-catabolizing enzymes promote differential

Dot/Icm effector delivery and intracellular survival at the early steps of *Legionella*

pneumophila infection. Infect Immun 82:1222-33.

51. Levi A, Folcher M, Jenal U, Shuman HA. 2011. Cyclic diguanylate signaling proteins

control intracellular growth of *Legionella pneumophila*. MBio 2:e00316-10.

52. Trigui H, Dudyk P, Oh J, Hong JI, Faucher SP. 2015. A regulatory feedback loop

between RpoS and SpoT supports the survival of *Legionella pneumophila* in water. Appl

Environ Microbiol 81:918-28.

53. Mendis N, McBride P, Saoud J, Mani T, Faucher SP. 2018. The LetA/S two-component

system regulates transcriptomic changes that are essential for the culturability of

Legionella pneumophila in water. Sci Rep 8:6764.

54. Hammer BK, Swanson MS. 1999. Co-ordination of *Legionella pneumophila* virulence

with entry into stationary phase by ppGpp. Mol Microbiol 33:721-731.

55. Zusman T, Gal-Mor O, Segal G. 2002. Characterization of a *Legionella pneumophila*

relA insertion mutant and toles of RelA and RpoS in virulence gene expression. J

Bacteriol 184:67-75.

56. Dalebroux ZD, Edwards RL, Swanson MS. 2009. SpoT governs *Legionella pneumophila*

differentiation in host macrophages. Mol Microbiol 71:640-58.

57. Byrne B, Swanson MS. 1998. Expression of *Legionella pneumophila* virulence traits in

response to growth conditions. Infect Immun 66:3029-3034.

58. Hales LM, Shuman HA. 1999. The *Legionella pneumophila rpoS* gene is required for

growth within *Acanthamoeba castellanii*. J Bacteriol 181:4879-4889.

59. Hammer BK, Tateda ES, Swanson MS. 2002. A two-component regulator induces the

transmission phenotype of stationary-phase *Legionella pneumophila*. Mol Microbiol

44:107-18.

60. Gal-Mor O, Segal G. 2003. The *Legionella pneumophila* GacA homolog (LetA) is

involved in the regulation of icm virulence genes and is required for intracellular

multiplication in *Acanthamoeba castellanii*. Microb Pathog 34:187-94.

61. Lynch D, Fieser N, Gloggler K, Forsbach-Birk V, Marre R. 2003. The response regulator

LetA regulates the stationary-phase stress response in *Legionella pneumophila* and is

required for efficient infection of *Acanthamoeba castellanii*. FEMS Microbiol Lett

219:241-8.

62. Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB.

1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*.

J Clin Microbiol 10:437-441.

63. Horwitz MA. 1983. Formation of a novel phagosome by the Legionnaires' disease

bacterium (*Legionella pneumophila*) in human monocytes. J Exp Med 158:1319-1331.

64.   Sadosky AB, Wiater LA, Shuman HA. 1993. Identification of *Legionella pneumophila*

genes required for growth within and killing of human macrophages. Infect Immun

61:5361-5373.

65.   Mampel J, Spirig T, Weber SS, Haagensen JAJ, Molin S, Hilbi H. 2006. Planktonic

replication is essential for biofilm formation by *Legionella pneumophila* in a complex

medium under static and dynamic flow conditions. Appl Environ Microbiol 72:2885-95.

  Figure Legends

FIG 1. The *Legionella* quorum sensing (Lqs)-LvbR-cyclic di-GMP regulatory network.

The environmental bacterium *L. pneumophila* employs a complex regulatory network to

integrate and respond to extra- and intracellular signals. The *Legionella* quorum sensing (Lqs)

system produces, detects and responds to the endogenous signaling molecule LAI-1

(*Legionella* autoinducer-1; 3-hydroxypentadecane-4-one). The Lqs system comprises the

autoinducer synthase LqsA, the homologous sensor kinases LqsS and LqsT, as well as the

cognate response regulator LqsR. Lpg1057 is a diguanylate cyclase, which produces the

intracellular second messenger cyclic di-GMP (c-di-GMP). Hnox1 is a receptor of nitric oxide

(NO) and an inhibitor of the diguanylate cyclase Lpg1057. The transcription factor LvbR

negatively regulates *hnox1*expression, and *lvbR* expression is negatively regulated by LqsS.

Thus LvbR links the Lqs system and c-di-GMP signaling. The Lqs-LvbR regulatory network

controls bacterial virulence, motility, natural competence, expression of a genomic island,

planktonic growth and biofilm architecture.

FIG 2. The Lqs-LvbR network regulates *L. pneumophila* growth and density in AYE

medium and MDM. (A-C) *L. pneumophila* JR32, $\Delta lvbR$, $\Delta lqsR$, $\Delta lqsA$, $\Delta lqsS$, $\Delta lqsT$ or

$\Delta lqsS$-$\Delta lqsT$ mutant strains were grown in AYE medium for 23-24 h at 37°C and inoculated

in AYE medium at an initial OD_{600} of 0.1. Growth of strains at (A) 30°C, (B) 40°C or (C)

45°C was monitored over time by measuring OD_{600} with a microplate reader. (D-F) *L.*

pneumophila JR32, $\Delta lvbR$, $\Delta lqsR$, $\Delta lqsA$, $\Delta lqsS$, $\Delta lqsT$ or $\Delta lqsS$-$\Delta lqsT$ mutant strains were

grown in MDM for 28-29 h at 37°C and inoculated in MDM medium at an initial OD_{600} of

0.1. Growth of strains at (D) 30°C, (E) 40°C or (F) 45°C was monitored over time by

measuring OD_{600} with a microplate reader. Growth curves shown are means and standard

deviations of biological triplicates and representative of at least four independent experiments.

FIG 3. Temperature-dependent growth of environmental and clinical *Legionella* isolates.

L. pneumophila strains JR32, 500, 509, 525, 529 and *L. longbeachae* strain NSW150 were

diluted in (A) AYE medium or (B) MDM to an initial OD_{600} of 0.2 and grown at the

temperatures indicated (18-55°C). Bacterial growth was determined over time by measuring

OD_{600} using a microplate reader. From the resulting growth curves, the growth rates were

calculated using a nonlinear regression model, only considering exponential growth phases

defined in semi-logarithmic plots. Data shown are means and standard deviation of growth

rates and doubling times from three independent experiments performed in biological

triplicates each.

FIG 4. Growth onset and cell density of environmental and clinical *Legionella* strains in

AYE medium and MDM. (A-C) *L. pneumophila* strains JR32, 500, 509, 525, 529 and *L.*

longbeachae strain NSW150 were grown in AYE medium for 23-24 h at 37°C and inoculated

in AYE medium at an initial OD_{600} of 0.2. Growth of *Legionella* strains at (A) 30°C, (B) 40°C

or (C) 45°C was monitored over time by measuring OD_{600} with a microplate reader. (D-F) *L.*

pneumophila strains JR32, 500, 509, 525, 529 and *L. longbeachae* strain NSW150 were

grown in minimal defined medium (MDM) for 28-29 h at 37°C and inoculated in MDM at an

initial OD_{600} of 0.2. Growth of strains at (D) 30°C, (E) 40°C or (F) 45°C was monitored over

time by measuring OD_{600} with a microplate reader. Growth curves shown are means and

standard deviations of biological triplicates and representative of six independent

experiments.

FIG 5. Presence of *lqsR, lqsA, lqsS, lvbR* and *hnox1* gene regions in environmental and

clinical *Legionella* isolates. The presence of (A) P_{lqsR}-*lqsR*, P_{lqsA}-*lqsA*, P_{lqsS}-*lqsS* or (B) P_{lvbR}-

lvbR, *lvbR*_1-201, LvbR_BS or P_{hnox1}-*hnox1* in the genomes of *L. pneumophila* strains JR32

(positive control), 500, 509, 525, 529 or *L. longbeachae* strain NSW150 (negative control)

was assessed by PCR amplification of the corresponding genes (and putative promoters)

and/or intergenic regions indicated. PCR products were analyzed by agarose gel

electrophoresis and visualized with MaestroSafe staining: P_{lqsR}-$lqsR$ (1727 bp), P_{lqsA}-$lqsA$

(1936 bp), P_{lqsS}-$lqsS$ (1984 bp), and P_{lvbR}-$lvbR$ (948 bp), $lvbR$ (264 bp), $lvbR$_1-201 (201 bp),

LvbR_BS (347 bp), P_{hnox1}-$hnox1$ (1344 bp) and LvbR_BS-$hnox1$ (969 bp). Schemes depicting

the lqs or the $lvbR$-$hnox1$ genomic region and the PCR fragments amplified are shown below

the gels.

FIG 6. Regulation of *lqsR, lqsA*, *lqsS*, *lvbR* and *hnox1* promoters in environmental and

clinical *Legionella* isolates. *L. pneumophila* strains JR32, 500, 509, 525 and 529 harboring

(A) P_{lqsR}-*gfp* (pRH037), (B) P_{lqsA}-*gfp* (pRH038), (C) P_{lqsS}-*gfp* (pRH051), (D) P_{lvbR}-*gfp*

(pRH023) or (E) P_{hnox1}-*gfp* (pRH026) reporter constructs (promoters of strain JR32) were

grown in AYE medium for 23-24 h at 37°C and inoculated in AYE medium at an initial

OD_{600} of 0.2. Strains were incubated at 30°C, and GFP fluorescence was measured over time

(relative fluorescence units, RFU) using a microplate reader. Data shown are means and

standard deviations of biological triplicates and representative of three independent

experiments.

FIG. 7. Growth of environmental and clinical *L. pneumophila* isolates harboring

promoter reporters. *L. pneumophila* strains JR32, 500, 509, 525 and 529 containing (A)

P_{lqsR}-*gfp* (pRH037), (B) P_{lqsA}-*gfp* (pRH038), (C) P_{lqsS}-*gfp* (pRH051), (D) P_{lvbR}-*gfp* (pRH023)

or (E) P_{hnox1}-*gfp* (pRH026) reporter constructs (promoters of strain JR32) were grown in AYE

medium for 23-24 h at 37°C and inoculated in AYE medium at an initial OD_{600} of 0.2.

Bacterial growth at 30°C was followed over time by measuring OD_{600} with a microplate

reader. Data shown are means and standard deviations of biological triplicates and

representative of three independent experiments.

Table 1. Doubling times of environmental and clinical *Legionella* isolates in AYE medium or
MDM.

Strain	t_d 30°C [a]	t_d 40°C [a]	t_d 45°C [a]
AYE medium			
JR32	4.06 ±0.03	2.22 ±0.02	2.51 ±0.04
500	3.99 ±0.03	2.72 ±0.08	3.05 ±0.10
509	4.28 ±0.02	2.41 ±0.01	3.73 ±0.37
525	4.58 ±0.05	2.44 ±0.03	2.62 ±0.01
529	4.45 ±0.03	2.33 ±0.01	2.00 ±0.02
NSW150	2.86 ±0.06	2.16 ±0.09	-
MDM			
JR32	7.93 ±0.09	5.00 ±0.04	7.32 ±0.21
500	10.29 ±0.08	8.61 ±0.12	15.57 ±0.70
509	9.08 ±0.16	7.31 ±0.02	19.40 ±0.73
525	31.61 ±0.44	25.08 ±0.31	-
529	8.86 ±0.10	7.27 ±0.02	9.70 ±0.39
NSW150	-	-	-

769   [a] Doubling times (t_d) are indicated in [h]. Data shown are means and (±) standard deviation
770   from doubling times derived from three independent cultures, (-) no growth observed.

772   **Table 2.** *Legionella* strains and plasmids used in this study.

Strain or plasmid [a]	Relevant properties [b]	References/Source [b]
L. pneumophila		
JR32 [a]	*L. pneumophila* Philadelphia-1, serogroup (sg) 1, salt-sensitive isolate of AM511, clinical isolate, reference strain	(64)
AK01 (ΔlqsT)	JR32 *lqsT*::Km	(33)
AK02 (ΔlqsS-ΔlqsT)	JR32 *lqsS*::Km *lqsT*::Gm	(33)
AK03 (ΔlvbR)	JR32 *lvbR*::Km	(39)
NT02 (ΔlqsA)	JR32 *lqsA*::Km	(35)
NT03 (ΔlqsR)	JR32 *lqsR*::Km	(36)
NT05 (ΔlqsS)	JR32 *lqsS*::Km	(35)
478	*L. pneumophila*, sg 6, clinical isolate	NRCL (#80970); this study

480	*L. pneumophila*, sg 5, clinical isolate	NRCL (#7980); this study
481	*L. pneumophila*, sg 1, clinical isolate	NRCL (#10891); this study
485	*L. pneumophila*, sg 1, clinical isolate	NRCL (#10943); this study
488	*L. pneumophila*, sg 5, environ. isolate	NRCL (#7797); this study
489	*L. pneumophila*, sg 6, environ. isolate	NRCL (#7775); this study
493	*L. pneumophila*, sg 1, environ. isolate	NRCL (#11045); this study
500 [a]	*L. pneumophila*, sg 1, environ. isolate	NRCL (#10909); this study
509 [a]	*L. pneumophila*, sg 1, clinical isolate	NRCL (#760); (29, 65)
514	*L. pneumophila*, sg 1, clinical isolate	NRCL (#883); (29, 65)
525 [a]	*L. pneumophila*, sg 6, environ. isolate	NRCL (#1365); (65)
526	*L. pneumophila*, sg 6, clinical isolate	NRCL (#1374); (65)
529 [a]	*L. pneumophila*, sg 1, environ. isolate	NRCL (#1488); (29, 65)
534	*L. pneumophila*, sg 1, environ. isolate	NRCL (#1563); (29, 65)
L. longbeachae		
NSW150 [a]	*L. longbeachae*, clinical isolate, reference strain	(48)
496	*L. longbeachae*, clinical isolate	NRCL (#8565); this study
L. micdadei		
501	*L. micdadei*, clinical isolate	NRCL (#634); this study
503	*L. micdadei*, clinical isolate	NRCL (#668); this study
Plasmids		
pAK18	pMMB207C-*gfp* (const.)-P$_{lvbR}$-*lvbR*, Cm	(39)
pCM009	pMMB207C-P$_{flaA}$-*gfp* (ASV), Cm	(30)
pNT28	pMMB207C-*gfp* (const.), Cm	(36)
pRH023	pMMB207C-P$_{lvbR}$-*gfp* (ASV), Cm	(39)
pRH026	pMMB207C-P$_{hnox1}$-*gfp* (ASV), Cm	(39)
pRH037	pMMB207C-P$_{lqsR}$-*gfp* (ASV), Cm	This study
pRH038	pMMB207C-P$_{lqsA}$-*gfp* (ASV), Cm	This study
pRH051	pMMB207C-P$_{lqsS}$-*gfp* (ASV), Cm	This study
pTS02	pMMB207C-RBS, P$_{tac}$-*lqsA*, Cm	(29)
pTS04	pMMB207C-RBS, P$_{tac}$-*lqsR*, Cm	(36)
pTS10	pMMB207C-RBS, Cm	(36)

773   [a] Environmental and clinical *Legionella* isolates analyzed in detail in this study.

774   [b] Abbreviations: NRCL, Swiss National Reference Center for *Legionella*; Km, kanamycin

775   resistance; Gm, gentamycin resistance; Cm, chloramphenicol resistance.

776 **Table 3.** Oligonucleotides used in this study.

Oligonucleotide	Sequence 5' - 3' [a]	Comments
oRH051	TCAAAAAGCATATGTGCTATGC	LvbR_BS (fo) (39)
oRH068	CTCATTTGGTGTGACTTCTAATCC	*lvbR* 1-201 (re), sequencing
oRH069	CTATGGTCTTCAGCATAAACAAATC	LvbR_BS (re) (39), sequencing
oRH104	CCACATGAATTGTTCCATCAATGC	sequencing
oRH178	CAAAACAATGCATCCCGCTGC	P$_{lqsR}$-*lqsR* (fo)
oRH179	GAGTGCCTGGTCAGACTTGC	P$_{lqsR}$-*lqsR* (re)
oRH188	<u>GGAAACAGAATTCGAGCT</u>CACTGCAATAAGCGCCTGCC	P$_{lqsR}$ (fo) (into *Sac*I and *Xba*I site)
oRH189	<u>CATATGTATATCTCCTTCTTAAAT</u>CTAGAGGCTCCTCCTGAGCAAAACG	P$_{lqsR}$ (re) (into *Sac*I and *Xba*I site)
oRH190	<u>GGAAACAGAATTCGAGCT</u>CCCCCTTGATTGCAGAAGC	P$_{lqsA}$ (fo) (into *Sac*I and *Xba*I site)
oRH191	<u>CATATGTATATCTCCTTCTTAAAT</u>CTAGACCATAACTCAGATTCTTTTCCC	P$_{lqsA}$ (re) (into *Sac*I and *Xba*I site)
oRH199	ATACAAGTACCTTTTGCTGGC	P$_{lqsA}$-*lqsA* (fo)
oRH200	CAATACCTGGAAGAGTTAGATGC	P$_{lqsA}$-*lqsA* (re)
oRH201	GAAACATGTATTCACCAAAGGC	P$_{lvbR}$-*lvbR* (fo)
oRH202	ATTAATTTTCCTTATCCTGCAAGC	P$_{lvbR}$-*lvbR* (re)
oRH208	ATGAAAAAACAAACCGACTTAATTG	*lvbR* ORF (fo)
oRH209	TTATGAGGCTGAGTTTTTAGTTATTTTC	*lvbR* ORF (re)
oRH219	AATAACCCTTTCCCTCCTGC	P$_{lqsS}$-*lqsS* (fo)
oRH220	CGCTATCTCAGTAAAAACGGC	P$_{lqsS}$-*lqsS* (re)
oRH221	ATTTTCCAGCAATAATTCGGC	P$_{hnox1}$-*hnox1* (fo)
oRH223	<u>GGAAACAGAATTCGAGCT</u>CATCGCCAGAGGTTATCATGG	P$_{lqsS}$ (fo) (into *Sac*I and *Xba*I site)
oRH224	<u>CATATGTATATCTCCTTCTTAAAT</u>CTAGATTCTTATCCTTAAATCCAGCTAAAAAACG	P$_{lqsS}$ (re) (into *Sac*I and *Xba*I site)

777 [a] Regions overlapping with destination vector are underlined.