**Legionella spp. All Ears? The Broad Occurrence of Quorum Sensing Elements outside Legionella pneumophila**

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**Abstract**

Legionella spp. are ubiquitous bacteria principally found in water networks and ~20 species are implicated in Legionnaire’s disease. Among them, Legionella pneumophila is an intracellular pathogen of environmental protozoa, responsible for ~90% of cases in the world. Legionella pneumophila regulates in part its virulence by a quorum sensing system named “Legionella quorum sensing,” composed of a signal synthase LqsA, two histidine kinase membrane receptors LqsS and LqsT and a cytoplasmic receptor LqsR. To date, this communication system was only found in L. pneumophila. Here, we investigated 58 Legionella genomes to determine the presence of a lqs cluster or homologous receptors using TBlastN. This analysis revealed three categories of species: 19 harbored a complete lqs cluster, 20 did not possess lqsA but maintained the receptor lqsR and/or lqsS, and 19 did not have any of the lqs genes. No correlation was observed between pathogenicity and the presence of a quorum sensing system. We determined by RT-qPCR that the lqsA gene was expressed at least in four strains among different species available in our laboratory. Furthermore, we showed that the lqs genomic region was conserved even in species possessing only the receptors of the quorum sensing system, indicating an ancestral acquisition and various loss dynamics during evolution. This system could therefore function in interspecific communication as well.

Key words: Legionella spp, quorum sensing, comparative genomics, phylogeny.

**Significance**

Legionella pneumophila has a quorum sensing communication system composed of three genes organized as a cluster that allows it to finely regulate its virulence and its biphasic lifestyle. We investigated the presence of such a system on the scale of the whole Legionella genus, thanks to the availability of genomes for 58 Legionella species. We found that this cluster is not exclusive to L. pneumophila, and that many other species actually possess homologs for all the necessary genetics to carry out this communication. In addition, some species lack homologs of the signal molecule, whereas displaying homologs of the receptors. This indicates that they may be equipped to hear this signal, which would be implicated in an interspecies communication.

**Introduction**

The Legionella genus includes all the pathogens involved in the Legionnaire’s disease. Only two species (Legionella pneumophila and Legionella longbeachae) are responsible for 95% of the world’s cases but the genus is composed of over 60 species and for most of them the pathogenicity could not be demonstrated. To this day, 20 species in addition to L. pneumophila have been documented to be human pathogens, like Legionella feli, Legionella micdadei, Legionella dumoffii, and Legionella anisa (Muder and Yu 2002; Kümpers et al. 2008; Hubber et al. 2017). These species have different ecological niches, such as water networks, cooling towers, river water or soil (even potting soil in the case of L. longbeachae, Steele et al. 1990), but one thing they have in common is that they infect protozoans, such...
as amoebas as part of their life-cycle (Gomez-Valero and Buchrieser 2019). In addition, some Legionellae exhibit proliferation capacities in mammalian host cells, such as macrophages and epithelial cells (Newton et al. 2010). The regulation of infection mechanisms remains less studied in non-pneumophila strains (Muder and Yu 2002), and therefore the regulation of virulence in these bacteria is still very poorly known. In view of the prevalence of legionellosis cases due to L. pneumophila, most epidemiological and pathogenic studies focus on this species and on L. longbeachae, the other prevalent epidemiologic strain, to a lesser extent (Bacigalupo et al. 2017).

Legionella pneumophila is a facultative intracellular bacterium with a biphasic lifestyle, alternating transmissive and replicative phases (Mondino et al. 2020). The transition between these two states is tightly regulated by a quorum sensing (QS) communication system called Legionella quorum sensing (lqs), mediated through the production of a signal molecule, an α-hydroxyketone called “Legionella autoinducer-1” (LAI-1). This communication system regulates virulence, mobility, competence, biofilm formation (Hochstrasser and Hilbi 2017) and, as demonstrated very recently, lqs is implicated in the formation of virulent Legionella persisters in amoeba and macrophage models (Personnic et al. 2019). A quorum sensing system is defined by the presence of a synthase producing a communication signal and a receiver allowing the transduction of the signal. They are generally organized in a genetic cluster or lie in close proximity in the genome (Waters and Bassler 2005; Ryan and Dow 2008; Patankar and González 2009; Waters and Bassler 2005; Ryan and Dow 2008; Patankar and González 2009; Sakurooka et al. 2019). The lqs system is organized as a genomic cluster lqsA-lqsR-hdeD-lqsS (Tiaden et al. 2007) which corresponds to genes encoding the signal synthase, a cytoplasmic receptor, a protein of unknown function and a histidine kinase membrane receptor, respectively. Moreover, a second histidine kinase membrane receptor is encoded by an orphan gene lqsT (Kessler et al. 2013). However, LqsT itself is a homologue of LqsS, although functional studies indicate that they are not equivalent transducers of the LAI-1 signal (Kessler et al. 2013). Communication using α-hydroxyketonones was first described in Vibrio cholerae through the products of the cqs cluster (CqsAV/CqsS) (Miller et al. 2002) but was also found later in several other environmental bacteria. The genes they carry share 30–45% identity with the lqs cluster of L. pneumophila (Tiaden, Spirig, Hilbi, et al. 2010). However, the cqs system lacks lqsR and hdeD. A further specificity of the lqs system of L. pneumophila compared with V. cholerae is the presence of a cytoplasmic receptor LqsR. Indeed, in V. cholerae, signal transduction is carried out directly via the CAI-1 signal produced by CqsA and the CqsS histidine kinase membrane receptor. In the case of L. pneumophila, the binding of LAI-1 by LqsS and LqsT will allow the phosphorylation of LqsR which induces its dimerization and the transduction of the signal to the target genes (Tiaden et al. 2007; Hochstrasser and Hilbi 2017; Hochstrasser et al. 2020). Therefore, the lqs system, as it is currently described, apparently retains the same gross functionality (Quorum Sensing), gene homology (lqsA, lqsS), but with an extra step in the transduction cascade (lqsR), a second branch of signaling (through lqsT) and a gene of unknown function (hdeD).

Only L. pneumophila strains and three other species Legionella bozemanii, Legionella rubrilucens, and Legionella taunensis have been investigated for a lqs system (Spirig et al. 2008). To date, the presence of this cluster has been demonstrated only in L. pneumophila strains (Spirig et al. 2008). However, even in the absence of the lqs cluster, a homologue of LqsT, called BaeS, was found in L. longbeachae or L. dumoffi (71% homology) and another in Legionella drancourtii (58% homology) (Kessler et al. 2013). This suggests that these strains could perceive and react to the LAI-1 signal, as it was described for some N-acyl homoserine lactone signal (Al-1) nonproducer strains which can respond to exogenous Al-1 through an orphan LuxR receptor (Patankar and González 2009). This makes LqsT a receptor of interest under the scope of cross-species communication.

In this study, we explored in silico the presence of the lqs communication system among Legionella species. To do so, we recovered the protein sequences of the lqs cluster from L. pneumophila Paris then studied their distribution among Legionella spp. and their organization in the genome. To confirm the presence and the expression of lqsA when it is present, we performed RT-qPCR in eight Legionella species.

Materials and Methods

Bacterial Culture

The strains used in this study are described in table 1. Legionella strains were cultured in buffered charcoal yeast extract (BCYE) broth (1% ACES, 1% yeast extract, 0.2% charcoal, 1.5% agar, 0.025% iron (III) pyrophosphate, 0.04% L-cysteine, pH 6.9) for 3 days at 37°C before experiment. Then, Legionella spp. were inoculated at OD₆₀₀ of 0.05 in buffered yeast extract (BYE) at 37°C for 24 h to 30 h at 37°C with constant shaking.

DNA, RNA Extraction, and RT-qPCR Analysis

The presence of lqsA in Legionella spp. was assessed by PCR. Total genomic DNA was extracted from the overnight culture in BYE at 37°C using a DNeasy Blood and Tissue Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer’s protocol. The lqsA and 16S genes were amplified at 45°C with the degenerated primer pairs lqsAF402 5’-CAATCCAGTTTGGTCGCCAA-3’ and lqsAR776 5’-GCTTTAGCAAGACTTGCW-3’ and at 55°C with the universal primers 27F and 786R for 16S rRNA, respectively. lqsA expression in replicative phase culture (OD₆₀₀: 1) and early stationary phase (OD₆₀₀: 2.5) cultures was determined by
RT-qPCR was performed using LightCycler 480 instrument (Roche Applied Science, Germany) according to the manufacturer’s instructions. The qPCR reactions were performed using the TakyonTM No Rox Sybr MasterMixdTTP Blue (Eurogentec) on the LightCycler 480 instrument (Roche Applied Science, Germany) according to the manufacturer’s instructions. The qPCR program was: 10 min at 95 °C and 45 cycles of the amplification step (10 s at 95 °C, 10 s at 58 °C, and 10 s at 72 °C for extension time in a single acquisition mode), the melting curves step for 1 min at 65 °C for annealing and the cooling 30 s at 40 °C. Expression level was normalized to the housekeeping gene gyrB (Mou and Leung 2018). The relative levels of genes expression were evaluated using 2−ΔΔCt method (Livak and Schmittgen 2001) and standardized to the expression level at an OD600 of 0.4. All results are averages of three independent experiments. All the data were presented as mean ± standard error (± SEM). Statistical analysis was performed using the ordinary one-way ANOVA followed by Dunnett’s post hoc test (GraphPad Prism 6). Significant differences are indicated by asterisks as follows: * P < 0.05; ** P < 0.01.

Phylogeny Reconstruction and Evolutionary Analyses

We selected 58 Legionella species for which all the coding sequences deduced from their genomes are freely available in the NCBI database (accession numbers in Supp. T1). The completeness of these genomes was checked using Busco 4.0.5 with legionellales_odb10 database (April 24, 2019) (Seppey et al. 2019). Each genome was over 95% complete except for L. oakridgensis (92.0%), L. norlandica (91.9%), and L. tunisiensis (68.7%). The coding sequences of HdeD, LqsA, LqsR, LqsS, and LqsT were searched by TBLASTN, using the already annotated sequences of L. pneumophila as queries (E-value < 10−6 and qcov_hsp_perc > 65%). For LqsA, we also fixed a minimum of 50% of identity (pident). The ID of each coding sequence of the synthase and receptors are listed in supplementary table S1, Supplementary Material online. To generate a phylogenetic tree of the Legionella species, we also searched by TBLASTN the 78 markers used in Burstein et al. (2016) (E-value < 10−6 and one maximum target sequence). We retrieved 55 of them, found in each of the 58 species of our data set (supplementary F1, Supplementary Material online). All the alignments were performed individually with Muscle (Edgar et al. 2004), implemented in Seaview (Gascuel et al. 2010). Occasionally, a few sequences were manually edited by adding a N base to correct a shift in the reading frame. The final sets of sites were computed with Gblocks (Castresana 2000). For the species phylogeny, the sequences of the 55 markers were concatenated with Seaview, leading to a 54,786 bp alignment. Phylogenetic trees were generated by the Maximum Likelihood method, using RAxML 8.2.12 (Stamatakis 2014) with the GTRGAMMAI model of evolution and 100 rapid bootstrap replicates.

Syntheny Reconstruction

The length of the fragments and the direction of the ORF were obtained through the software genepalette (a java script program source code can be obtained through the www.genepalette.org website) from geneflat file generated from NCBI’s graphics genome visualization tool. The figures were reworked and mounted with Inkscape software.

Results

The lqs Cluster Is Present in Numerous Legionella Species

This study was performed on genomes from Legionella species published in the NCBI database with fully sequenced and annotated genomes (supplementary table T1, Supplementary Material online). To investigate the unexpected presence of a LAI-1-based QS system or that of a homologous receptor in other species, we performed a TBLASTN search using all the genes composing the lqs cluster present in L. pneumophila. The protein sequences of L. pneumophila Paris were used: LqsA (Lpp2787), LqsR (Lpp2788), HdeD (Lpp2789), LqsS (Lpp2790), and LqsT (Lpp2574). All protein candidates

| Strains                      | Description                        | References               |
|------------------------------|------------------------------------|--------------------------|
| Legionella longbeachae      | Clinical isolate                   | Cazalet et al. (2010)    |
| Legionella pneumophila str. | Environmental isolate              | Cazalet et al. (2004)    |
| Legionella bozemanaea       | Clinical isolate                   | Brenner et al. (1980)    |
| Legionella micdadei         | Clinical isolate                   | Garrity et al. (1980)    |
| Legionella dumoffii         | Clinical isolate                   | Brenner et al. (1980)    |
| Legionella feeleii          | Environmental isolate              | Hervaldt (1984)          |
| Legionella oakridgensis     | Environmental isolate              | Orrison et al. (1983)    |
| Legionella hackelia         | Human lung aspirate                | Wilkinson et al. (1985)  |

**Table 1**

Bacterial Strains Used in This Study
obtained from the BLAST analysis are listed in the supplementary table T1, Supplementary Material online, and corresponding phylogenetic trees are represented in the supplementary figure S1, Supplementary Material online.

In order to map the distribution of the elements of the *L. pneumophila* QS system in the *Legionella* genus, the homologue genes were plotted on a tree reflecting the phylogeny of the *Legionella* species, constructed de novo according to Burstein et al. (Burstein et al. 2016) (fig. 1). As compared with Burstein et al., 20 species were added and a similar topology was found, *Legionella geestiana* representing the most basal species. First, we found that *hdeD* was present in all species. As regards QS elements, the species fell into three categories. The first category displayed homologs for all the genes from the *lqs* cluster found in *L. pneumophila* (red) and is composed of 19 species (fig. 1). This category is further divided into three groups of strains named group I, II, III with *L. pneumophila* alone representing the group II. Although most clustered as affiliated species, they did not form discrete monophyletic groups (fig. 1). The second category was composed of 20 species that possessed a homologue for *lqsR* or *lqsS* or both (blue) but did not have a homologue of the *lqsA* synthase. It has to be noticed that a homologue of *lqsS* was never found alone in the absence of homologue of *lqsR*, irrespective of the presence of the other *lqs* homologs genes. Sixteen species out of 20 were found in two monophyletic groups in a basal position near the root, whereas the rest fell at various positions on the tree. A third category lacking the entire gene cluster (green) was composed of 19 species.

A specificity of the communication by α-hydroxyketones in *L. pneumophila* is the presence of the cytoplasmic receptor *LqsR*. Thirty-nine species harbored a homologue of *lqsR* from which 34 possessed also a *lqsS* homologue. The phylogenetic tree of the sequences similar to *LqsR* (supplementary file S1B, Supplementary Material online) displayed two branches. The upper branch contained the homologs of the *LqsR* present in the *lqs* cluster. In the lower branch, the sequences were more similar to a *che*-Y-like gene; in addition, they were not found in preserved genetic regions. In some cases (black frame), the candidate gene (TBlasN ca. 70% identity) was carried by a plasmid: pB3526CHC and pB1445CHC of *L. longbeachae* str. B3526CHC and str. B1445CHC, respectively, plasmid pLA01-117 of *L. sainthelenensis* and plasmid pLELO of *L. pneumophila* str. Lorraine. Moreover, except for *L. maceachernii*, the species present in this subgroup did not have the *lqs* cluster. *Legionella anisa* was the only species not possessing the *lqs* cluster to have two *lqsR* homologs.

As the *L. pneumophila lqsT* is an orphan gene, we investigated separately the prevalence of its homologs, such as BaeS in *L. longbeachae* (71% homology with *LqsT*), in the
Legionella genus. As a BLAST query, we used the LqsT sequence also from L. pneumophila Paris. In total, 28 species possessed a LqsT homologue (fig. 1). Homologs for LqsT were not preferentially found in any of the three groups possessing an equivalent of the lqs cluster but were instead spread quite haphazardly in the tree. As for these groups, eight had a homologue of the orphan LqsT receptor in addition to an equivalent of the lqs cluster, that is seven out of 13 in group I, plus L. pneumophila that also makes group II (fig. 1). Homologs were especially absent in group III.

To confirm the presence or the absence of lqsA homologs in Legionella spp. other than L. pneumophila, we amplified by PCR the corresponding locus with degenerated primers using 16S rDNA as a control, on eight strains available in our laboratory: That is four predicted to harbor lqsA and four others predicted not to (fig. 2A). The PCR results confirmed the presence of a lqsA homologue in four strains, L. pneumophila, L. micdadei, L. feelei, and Legionella hackelia. Conversely, it was not detected in L. longbeachae, Legionella oakridgensis, Legionella bozemanae, and L. dumoffii, which was consistent with the absence of detection in silico. Moreover, the expression of lqsA in Legionella non-pneumophila strains was monitored using RT-qPCR in replicative growth phase and in early stationary growth phase cultures of each species in BYE at 37°C. All expression has been normalized to housekeeping gene gyrB. Results are averages of three independent experiments and Error bars show the SEM (standard error of the mean). Statistical analysis was performed using the ordinary one-way ANOVA followed by Dunnett’s post hoc test (* P < 0.05, ** P < 0.01) with comparison to the control condition at OD600: 0.4.

In order to show whether the presence of a QS system is correlated with legionellosis, a Venn diagram was made that combines the presence of homologs of the lqs cluster (lqsA, lqsR, and lqsS) or lqsT and literature reports on the involvement of strains in the disease (fig. 3). From the 22 species reported to be involved in the disease, only four possessed homologs of all four lqs genes, three had the lqs cluster but no lqsT homologue and eight contained only a homologue of lqsT. Interestingly, seven species involved in the disease had neither lqsT nor lqs cluster homologs, whereas 12 species that possessed the lqs cluster have never been described as pathogenic to humans. On the whole, no correlation could be drawn between the presence of the lqs cluster and the disease, or between the disease and phylogenetically related species (fig. 1).

The Genetic Environment of the lqs Cluster Is Conserved in All Legionella Species

Complete genome availability made it possible to obtain the genomic organization and explore the direct environment of the lqs genes. For the species containing the lqs cluster (first category described above: groups I, II, III), the order and the orientation of the homologs of the lqsA, lqsR, and lqsS genes were strongly conserved (fig. 4). The complete cluster was localized in the same place between a “cytochrome c5 family
protein” and the “hydroxymethylbilane synthase.” The direct environment was also relatively conserved ranging from the “peptidyl-prolyl cis-trans isomerase” and the “small ribosomal subunit biogenesis GTPase RsgA.” Beyond these limits, the environment was less conserved.

Although homologs of hdeD were detected in all species (fig. 1), they mostly occurred at various positions in the genome: They resided within the cluster in only six species, in which case they were always located at the same position as in L. pneumophila, between lqsR and lqsS (fig. 4). The insertion of one sequence, with different length, coding for hypothetical proteins of unknown function was observed between the homologs of hdeD and lqsS (Legionella moravica and L. quateriensis) or lqsR and lqsS (Legionella shakespearei). In the immediate vicinity of the QS cluster, L. feeliei, Legionella donaldsonii, and L. tunisiensis displayed an insertion of the pur operon between lqsA and the “cytochrome c5 family protein.” Legionella worlensensis presented a highly peculiar
organization, as the \textit{lqsA} homologue was isolated from the \textit{lqsR-hdeD-lqsS} sequences which were transferred to the negative strand, upstream in the genome.

In species displaying a partial \textit{lqs} cluster (second category described above: Lacking \textit{lqsA}, and \textit{lqsS} as well in five cases, blue area in fig. 1), \textit{lqsR} was systematically observed, the surrounding genetic environment being also preserved (fig. 5). For species missing the \textit{lqsA} gene alone, \textit{Legionella noorlandica} was the only one to display an extensive disorganization of the cluster so that it could not be aligned with any other species (therefore not presented in fig. 5). In species lacking the \textit{lqs} cluster (green area in fig. 1), the genetic environment of \textit{hdeD} was the same and the area of insertion was present in all genomes. In these cases, \textit{hdeD} was always located between “cytochrome c5 family protein” and “hydroxymethylbilane synthase” except for \textit{Legionella tusconensis}, \textit{L. bozemaniae}, \textit{Legionella parisiensis}, and \textit{L. geestiana} (fig. 6). For the other groups of species, no preferential insertion sites of \textit{hdeD} were observed.

**Discussion**

Communication in the environment is an essential process in many ecosystems and regulates many biological processes. The study of quorum sensing in \textit{Legionella} spp. has been carried out only in \textit{L. pneumophila}, the only species in which this system was detected so far (Personnic et al. 2019). In this study, we established instead that genes displaying significant homologies (high protein identities) with elements of the \textit{lqs} cluster were also present in \textit{Legionella non-pneumophila} strains. Most prominently, a complete cluster similar to the \textit{lqs} cluster was found within 18 other species, with conservation of the genomic organization and of the orientation of its genes. The only differences were the absence of a homologue of \textit{hdeD} in 12 species, an additional insert of unknown function in three species, and a disrupted cluster in a single species (\textit{L. worsenlensis}). The organization was similarly conserved for species that had an incomplete cluster, that is, displaying only homologs of the cytoplasmic \textit{lqsR} and/or the membrane \textit{lqsS}, with the exception of \textit{L. noorlandica}. The cluster was inserted in the same genetic environment in all species with very minor variations, and the absence of \textit{lqsA} in some species did not alter the neighboring genes.

All these results suggest that the elements of the \textit{L. pneumophila} QS system are not exclusive to this species, and could result from a deeper recruitment history. The \textit{lqsA} possessing species fell into three groups that did not display a direct phylogenetic affiliation: Bar the possibility of multiple horizontal transfer events, this rather suggests an ancient acquisition of this gene, followed by several episodes of loss. The same could be said of the receptors \textit{lqsR} and \textit{lqsS}, which appeared early in the Legionellae filiation, that is, after divergence from the basal \textit{L. geestiana}, unless their absence in this species relates to loss. From these patterns and from the conservation of the surrounding genomic area, we hypothesize that the \textit{lqs} cluster was acquired as a unit by a common ancestor and that several episodes of successive losses have constructed the distribution of this communication system. An unexpected trait is that in the loss dynamics, the genes did not behave as a unit but as independent elements, resulting in species having the receptors and not the signal molecule. A homologue of the \textit{lqsA} gene was never found alone. In such species, the receptors could well function in interspecies communication based on LAI-1, following the broad occurrence of $\alpha$-hydroxyketone signaling in the environment and among different \textit{Legionella} species.

Indeed, communication based on $\alpha$-hydroxyketone is found in many other environmental species, such as \textit{V. cholerae}, \textit{Nitrooccus mobilis}, \textit{Burkholderia xenovoran}, or \textit{Palaromonas sp.}, with recognized homologies between the components of the systems (Tiaden, Spirig, Hilbi, et al. 2010). Moreover, an expression of \textit{lqsA} partially complements a \textit{V. cholerae cqsA} mutant strain (Spirig et al. 2008), further highlighting the molecular similarities of the pathways. However, at this scale, the diversity of these species and the sparseness of the data precludes conclusion on their recruitment histories, that is, through vertical or horizontal transmission. In any case, the \textit{lqs} system of \textit{L. pneumophila} differs notoriously from the \textit{cqs} system of \textit{V. cholerae} (\textit{CqsA/CqsS}) through the insertion of the cytoplasmic receptor \textit{lqsR} in the mediation of QS (Tiaden, Spirig, Hilbi, et al. 2010). The \textit{lqsR} homologue was highly conserved in the \textit{Legionella} genus (present in 39 species out of 58), emphasizing the importance of this protein in signal transduction in a \textit{lqs}-like system. Interestingly, we found in 19 species a second \textit{lqsR} homologue, phylogenetically more distant and displaying homologies with \textit{che-Y}. This gene is described as a response regulator receiver, involved in the transduction cascade of a chemotaxis pathway widespread among Prokaryotes and well described in \textit{Escherichia coli} (Bai and Sourjik 2018). It is possible that \textit{lqsR} originated from a duplication of this gene and was recruited in a new pathway. In fact, \textit{N. mobilis}, \textit{B. xenovoran}, \textit{Palaromonas sp.}, also possess a \textit{lqsR} homologue in addition to \textit{lqsA} and \textit{lqsS}-like genes (Tiaden, Spirig, Hilbi, et al. 2010).

As these homologs further function in QS in these species, there is ground to propose that those we found in the \textit{Legionellae} beyond \textit{L. pneumophila} likewise mediate QS. In \textit{L. pneumophila}, the regulation of the biphasic lifestyle in particular by QS is important for virulence (Personnic et al. 2018), and is expected to participate in the mediation of two physiologically different states, the replicative and the transmissive phases. This is reflected by a differential expression of \textit{lqsA} in the biphasic lifestyle, with a preferential expression at the end of the replicative phase with a continued expression in the transmissive phase (Kessler et al. 2013). Here, in three species belonging to what we designated as group I, the expression of \textit{lqsA} displayed a similar profile. Thus, the expression of LAI-1 seems to be growth phase dependent whatever the species. However, the manifestation of
QS, that is the genes that are regulated downstream of signaling could differ: The QS system could have different roles or a different level of involvement in the virulence of the various Legionella species. In this regard, even though it lacks lqsA and therefore, the ability to perform QS on its own (considering interspecies communication as a possibility), L. longbeachae

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**Fig. 5.** Synthesis alignment of the genomic region of Legionella species that have lost lqsA. Homologue genes were represented with the same color except for unique genes or genes not found in two or three species (black with white point). The comparison shows that the regions upstream and downstream of lqs elements are relatively conserved.

**Fig. 6.** Schematic representation of the genomic region of hdeD in species that do not have any genes from the lqs cluster.
nevertheless displays a biphasic regulation of its genes, although it is less pronounced (Cazalet et al. 2010).

In *L. pneumophila*, it was shown that LqsS and LqsR are sufficient to transduce the quorum sensing signal because in ΔlqsA mutants the native phenotype can be restored by the addition of supernatant from wild strains or by synthetic LAI-1 (Tiaden, Spiring, Sahr, et al. 2010; Schell et al. 2016). LqsR and LqsS seem to be enough to drive the signal. Nevertheless, a second receptor exists, LqsT, which is homologous to LqsS (Kessler et al. 2013). It is described as acting as a second branch of signaling through LqsR, whereas it was demonstrated that their respective deletion mutants display nonequivalent phenotypes (Kessler et al. 2013). The LqsT homologue discovered in *L. longbeachae*, *L. dumoffii*, and *L. drancourtii* actually displays a further homology with BaeS (Kessler et al. 2013): This gene is in fact the histidine kinase receptor component of the widespread two-components system Bae, described in *E. coli* as involved in envelope stress response (Delhaye et al. 2019). Interestingly, in *E. coli* BaeS is able to trans-phosphorylate a noncognate response regulator, namely Che-Y (Yamamoto et al. 2005). Of note, however, as an orphan receptor, BaeS phosphorylates its response regulator to induce gene transcription, whereas in the described lqs model, LqsS or LqsT-mediated phosphorylation of LqsR occurs under low LAI-1 concentrations and represses target genes transcription (Personnic et al. 2018). Among the *Legionella*, it was present in 28 species, of which 12 displayed only this receptor. Despite the absence of the synthase and of the LqsR receptor, we cannot exclude that LqsT homologs alone are able to recognize and respond to an exogenous LAI-1 signal, or even an entirely different, analogue signal. They may even convey the signaling through a transduction pathway not involving LqsR. This broadens the scope for interspecies communication to noncognate ligands, and possibly cross-talks with other pathways.

As regards the mysterious HdeD, it appeared as an intruder of the lqs cluster: It was present at a conserved position in only six closely related species. Even if it was not present within the lqs cluster, all the *Legionella* species had a hdeD counterpart. Moreover, the ubiquitous gene hdeD is found in several other environmental species but never located inside the QS cluster (Tiaden, Spiring, Hilbi, et al. 2010). To our knowledge, its function in the cluster is still unknown. A homologue of hdeD is also found in *E. coli* (which has no gene homologous to the lqs cluster) and codes for a protein involved in acidity resistance (Masuda and Church 2003). Studies to understand the involvement of HdeD in *L. pneumophila* do not seem conclusive. Indeed, no phenotype or physiological response seems to be associated with the hdeD mutant. The loss of HdeD in *L. pneumophila* does not affect its virulence, phagocytosis efficacy, or acid resistance (Tiaden et al. 2008). Nevertheless, in the *Legionella* genus, hdeD is very well preserved. These results suggest that HdeD has no link with the lqs cluster and is not involved in the communication: Its presence in the lqs cluster would then be coincidental.

Involvement in legionellosis is characterized solely from the isolation of the species from affected patients, and with the exception of *L. longbeachae*, cases related to *Legionella non-pneumophila* are rare (Muder and Yu 2002). They occur mainly in severely immunocompromised patients, indicating a lower capacity for infection of pulmonary macrophages (Khodr et al. 2016). Moreover, some cause Pontiac fever rather than pneumonia, the severe form of the disease (Muder and Yu 2002). Rarity, infection of more susceptible hosts and lesser pathogenicity suggest a lesser virulence. In our analysis, the presence of a lqs QS system was not correlated with involvement in disease. Indeed, species lacking the lqs cluster, such as *Legionella cincinnatiensis* (Thacker et al. 1988) or *L. longbeachae* (responsible for at least half of the cases in Australia, Wood et al. 2015) are associated to disease. In contrast, some species with a complete lqs cluster, such as *L. moravica*, *Legionella brunensis*, *Legionella naaturn*, or *Legionella quaterinensis* have never been described as involved in human pneumonia (Wilkinson et al. 1988; Dennis et al. 1993). These results suggest that the strength of virulence is not controlled by the lqs system in itself, even though it could participate in its triggering. Even in *L. pneumophila*, the biphasic lifestyle and thus virulence triggering are only partly regulated by QS, the major part of regulation being devoted to stringency response and metabolism (Oliva et al. 2018). Virulence levels should rather reflect a distinctive panel of effectors that allow growth in human cells; Gomez-Valero et al (2019) highlight that there are in fact several panels that emerged independently, as they did not find a core set common to disease-inducing *Legionella* spp.

As a conclusion, LAI-1 appeared to be a communication system nonexclusive to *L. pneumophila* and could be an interspecies QS molecule. Many questions remain to be answered. What is the role of the communication system in these other species? Why was this communication system partially or completely lost in these different species and why does this system seem to be so important in *L. pneumophila* but not in *L. longbeachae*? Is the presence of only one membrane receptor (LqsR or LqsS or LqsT) enough to induce interspecies communication?

**Supplementary Material**

Supplementary data are available at Genome Biology and Evolution online.

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Data Availability

Raw sequencing data are available from the National Center for Biotechnology Information database and all accession number were grouped together in the supplementary table T1, Supplementary Material online.

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