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 pathogeny could not be demonstrated. To this day, 20 species in addition to *L. pneumophila* have been documented to be human pathogens, like *Legionella feeleii*, *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 (Bacigalupe 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; Sakuraoka 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 transductors of the LAI-1 signal (Kessler et al. 2013). Communication using α-hydroxyketones was first described in *Vibrio cholerae* through the products of the *cqs* cluster (CqsA/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 rubriluscens*, and *Legionella taurinensis* 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 (AI-1) nonproducer strains which can respond to exogen AI-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 OD_{600} 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′-CAATCAGGTTGGTGCGCKAA-3′ and lqsAR776 5′-GCTTTAGCAAGACTTGCWGT-3′ and at 55 °C with the universal primers 27F and 786R for 16 S rRNA, respectively. *lqsA* expression in replicative phase culture (OD_{600}: 1) and early stationary phase (OD_{600}: 2,5) cultures was determined by

Table 1
Bacterial Strains Used in This Study

Strains	Description	References
Legionella longbeachae NSW150	Clinical isolate	Cazalet et al. (2010)
Legionella pneumophila str. Paris	Environmental isolate	Cazalet et al. (2004)
Legionella bozemanea ATCC 33217	Clinical isolate	Brenner et al. (1980)
Legionella micdadei ATCC 33218	Clinical isolate	Garrity et al. (1980)
Legionella dumoffii ATCC 33279	Environmental isolate	Brenner et al. (1980)
Legionella feeleii ATCC 35072	Clinical isolate	Herwaldt (1984)
Legionella oakridgensis ATCC 33761	Environmental isolate	Orrison et al. (1983)
Legionella hackelia ATCC 35999	Human lung aspirate	Wilkinson et al. (1985)

RT-qPCR using "High Pure RNA isolation Kit" (Roche) for RNA extraction and "GoscriptTM Reverse Transcription System" (Promega) for reverse transcription according to the manufacturer's recommendations. Primers used for RT-qPCR in this study are listed in the supplementary table T2, Supplementary Material online. All cDNA were normalized (200 ng/µL) before proceeding to qRT-PCR reaction. All 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 $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and standardized to the expression level at an OD_{600} 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. norrlandica* (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

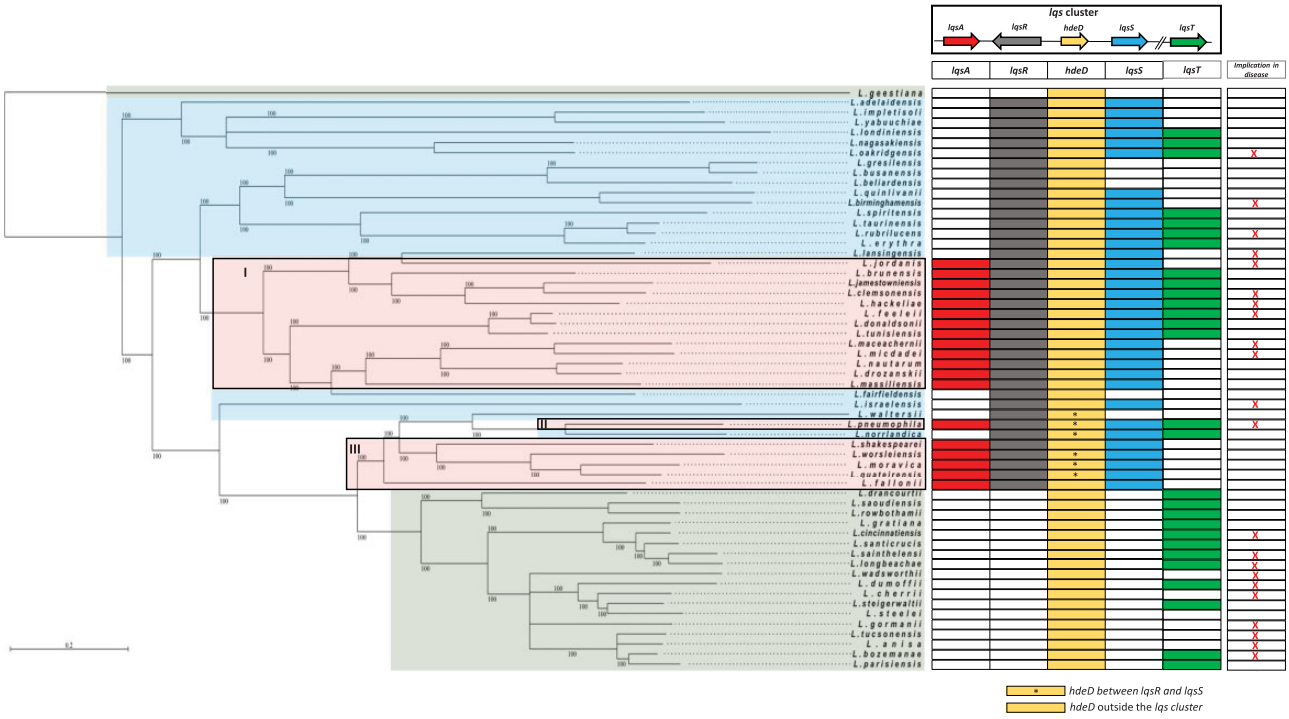

Fig. 1.—Phylogenetic tree of *Legionella* spp. showing genomic presence of *lqs* genes. Alignments were performed with Muscle using concatenated sequences of the 55 *Legionella* spp. markers with Seaview. Phylogenetic trees were generated by the Maximum Likelihood method, using RAxML 8.2.12 (Stamatakis 2014) with the GTRGAMMAI model of evolution and 100 rapid bootstrap replicates. Presence of *lqsA*, *lqsR*, *hdeD*, *lqsS*, and *lqsT* were reported on the phylogenetic tree manually. The bar at the bottom represents the estimated evolutionary distance. Human infection: https://www.specialpathogenslab.com/legionella-species.php and Connell et al. (1996).

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 S1*B*, 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 (TBlastN ca. 70% identity) was carried by a plasmid: pB3526CHC and pB1445CHC of *L. longbeachae* str. B3526CHC and str. B1445CHC, respectively, plasmid pLAI01-117 of *L. sainthelensi* 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

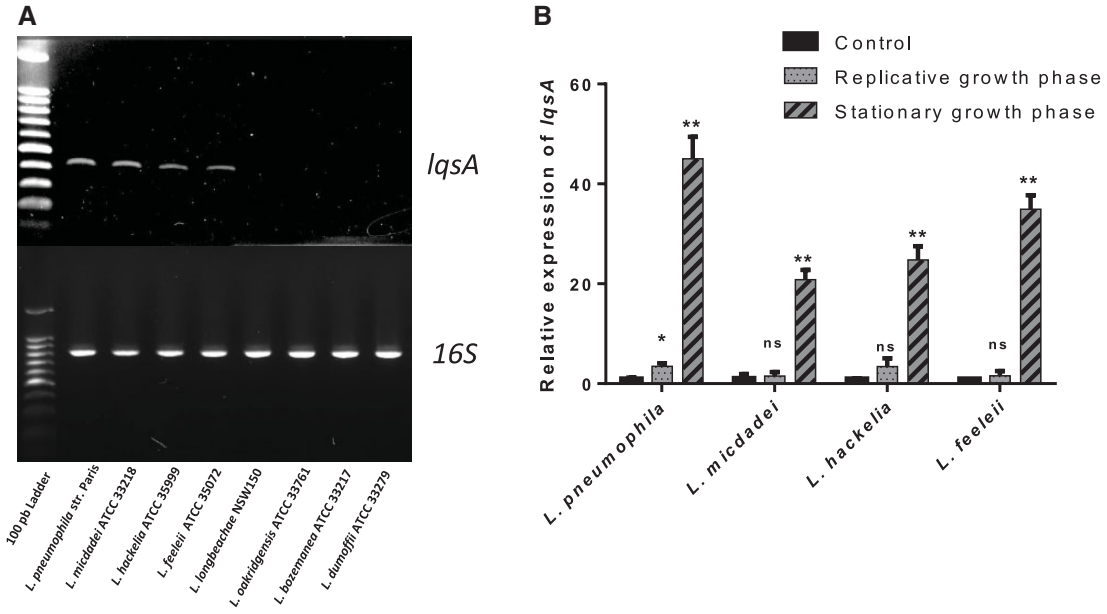

Fig. 2.—Laboratory *Legionella* spp. harboring a *lqsA* gene. (*A*) the presence of the *lqsA* gene was determined by PCR amplification from genomic DNA using *lqsA* degenerated primers on *Legionella pneumophila*, *Legionella feeleii*, *Legionella micdadei*, *Legionella hackelia*, *Legionella oakridgensis*, *Legionella bozemanea*, and *Legionella dumoffii*. As a positive control, the 16S rRNA gene was amplified. (*B*) The expression of *lqsA* was determined by RT-qPCR after RNA extraction from replicative growth phase and 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 OD_{600}: 0.4.

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. 2*A*). The PCR results confirmed the presence of a *lqsA* homologue in four strains, *L. pneumophila*, *L. micdadei*, *L. feeleii*, and *Legionella hackelia*. Conversely, it was not detected in *L. longbeachae*, *Legionella oakridgensis*, *Legionella bozemanea*, 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 (fig. 2*B*). Upon culturing in BYE growth medium, the species wherein *lqsA* had been detected by PCR were found to express *lqsA*. For all species, the expression profiles appeared very similar (fig. 2*B*) with a higher expression in the early stationary phase of growth.

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

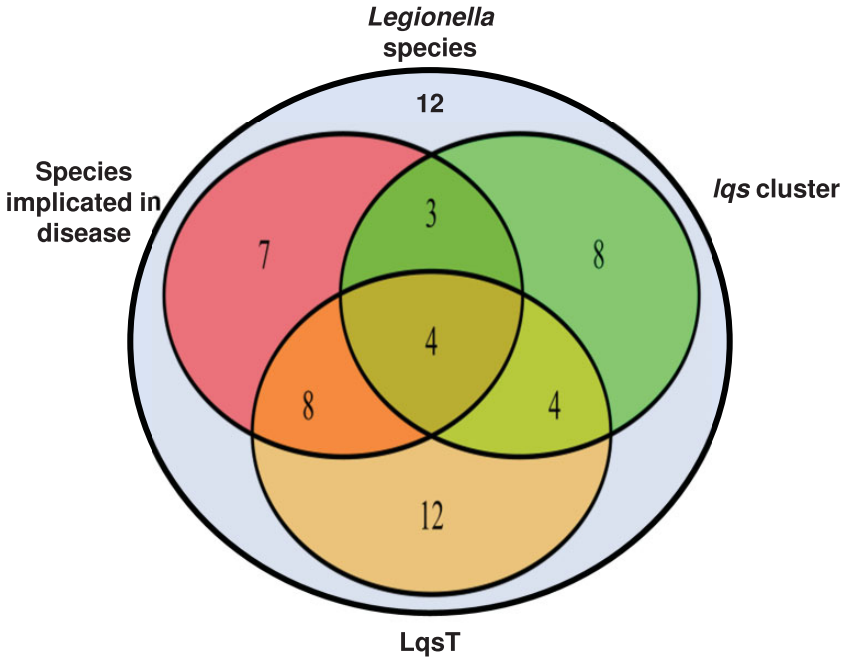

Fig. 3.—Venn diagram depicting strains harboring complete *lqs* cluster (*lqsA*, *lqsR*, and *lqsS* at least) and *lqsT* in relation with implication in disease. (Disease information was collected from https://specialpathogenslab.com/legionella).

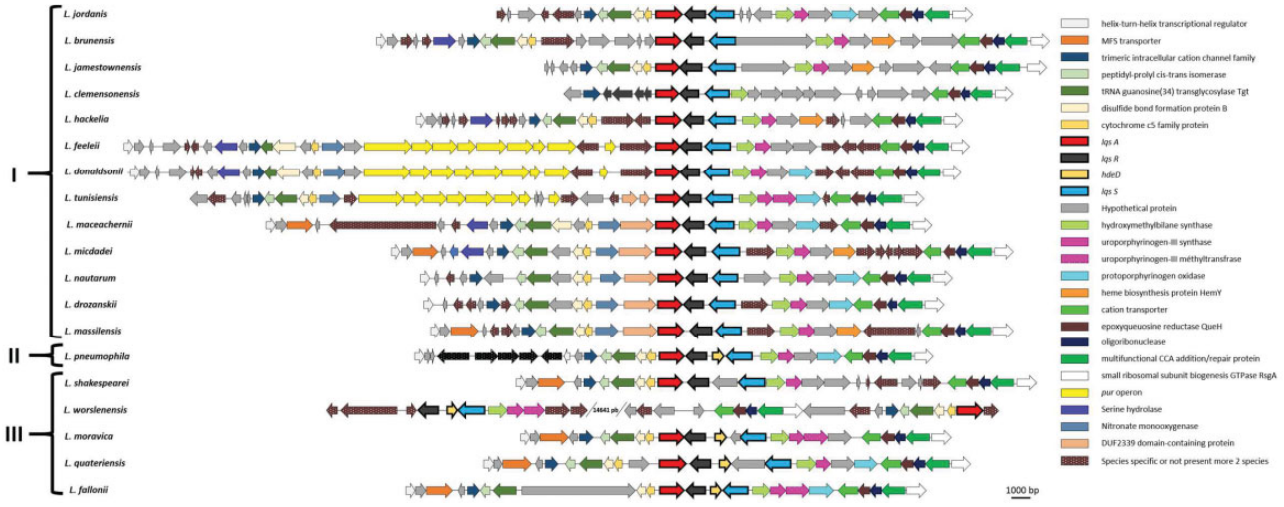

Fig. 4.—Syntheny alignment of the genomic region of *lqs* gene cluster of *Legionella* species containing the quorum sensing autoinducer synthase gene *lqsA*. The *lqs* cluster was found in 19 species. Homologue genes were represented with the same color except for unique genes or genes not found in two or three species (black with white point). The comparison shows that the regions upstream and downstream of *lqs* elements are relatively conserved.

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. feeleii*, *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 *lqsA* homologue was isolated from the *lqsR-hdeD-lqsS* sequences which were transferred to the negative strand, upstream in the genome.

In species displaying a partial *lqs* cluster (second category described above: Lacking *lqsA*, and *LqsS* as well in five cases, blue area in fig. 1), *lqsR* was systematically observed, the surrounding genetic environment being also preserved (fig. 5). For species missing the *lqsA* gene alone, *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 *lqs* cluster (green area in fig. 1), the genetic environment of *hdeD* was the same and the area of insertion was present in all genomes. In these cases, *hdeD* was always located between "cytochrome c5 family protein" and "hydroxymethylbilane synthase" except for *Legionella tusconensis*, *L. bozemanae*, *Legionella parisiensis*, and *L. geestiana* (fig. 6). For the other groups of species, no preferential insertion sites of *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 *Legionella* spp. has been carried out only in *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 *lqs* cluster were also present in *Legionella* non-*pneumophila* strains. Most prominently, a complete cluster similar to the *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 *hdeD* in 12 species, an additional insert of unknown function in three species, and a disrupted cluster in a single species (*L. worsenlensis*). The organization was similarly conserved for species that had an incomplete cluster, that is, displaying only homologs of the cytoplasmic *lqsR* and/or the membrane *lqsS*, with the exception of *L. noorlandica*. The cluster was inserted in the same genetic environment in all species with very minor variations, and the absence of *lqsA* in some species did not alter the neighboring genes.

All these results suggest that the elements of the *L. pneumophila* QS system are not exclusive to this species, and could result from a deeper recruitment history. The *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 *lqsR* and *lqsS*, which appeared early in the Legionellae filiation, that is, after divergence from the basal *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 *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 *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 α-hydroxyketone signaling in the environment and among different *Legionella* species.

Indeed, communication based on α-hydroxyketone is found in many other environmental species, such as *V. cholerae*, *Nitrococcus mobilis*, *Burklhoderia xenovorans*, or *Palaromonas* sp., with recognized homologies between the components of the systems (Tiaden, Spirig, Hilbi, et al. 2010). Moreover, an expression of *lqsA* partially complements a *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 *lqs* system of *L. pneumophila* differs notoriously from the *cqs* system of *V. cholerae* (CqsA/CqsS) through the insertion of the cytoplasmic receptor *lqsR* in the mediation of QS (Tiaden, Spirig, Hilbi, et al. 2010). The *lqsR* homologue was highly conserved in the *Legionella* genus (present in 39 species out of 58), emphasizing the importance of this protein in signal transduction in a *lqs*-like system. Interestingly, we found in 19 species a second *lqsR* homologue, phylogenetically more distant and displaying homologies with *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 *Escherichia coli* (Bi and Sourjik 2018). It is possible that *lqsR* originated from a duplication of this gene and was recruited in a new pathway. In fact, *N. mobilis*, *B. xenovorans, Palaromonas* sp. also possess a *lqsR* homologue in addition to *lqsA* and *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 Legionellae beyond *L. pneumophila* likewise mediate QS. In *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 *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 *lqsA* displayed a similar profile. Thus, the expression of LAI-1 seems to be growth phase dependent whatever the species. However, the manifestation of

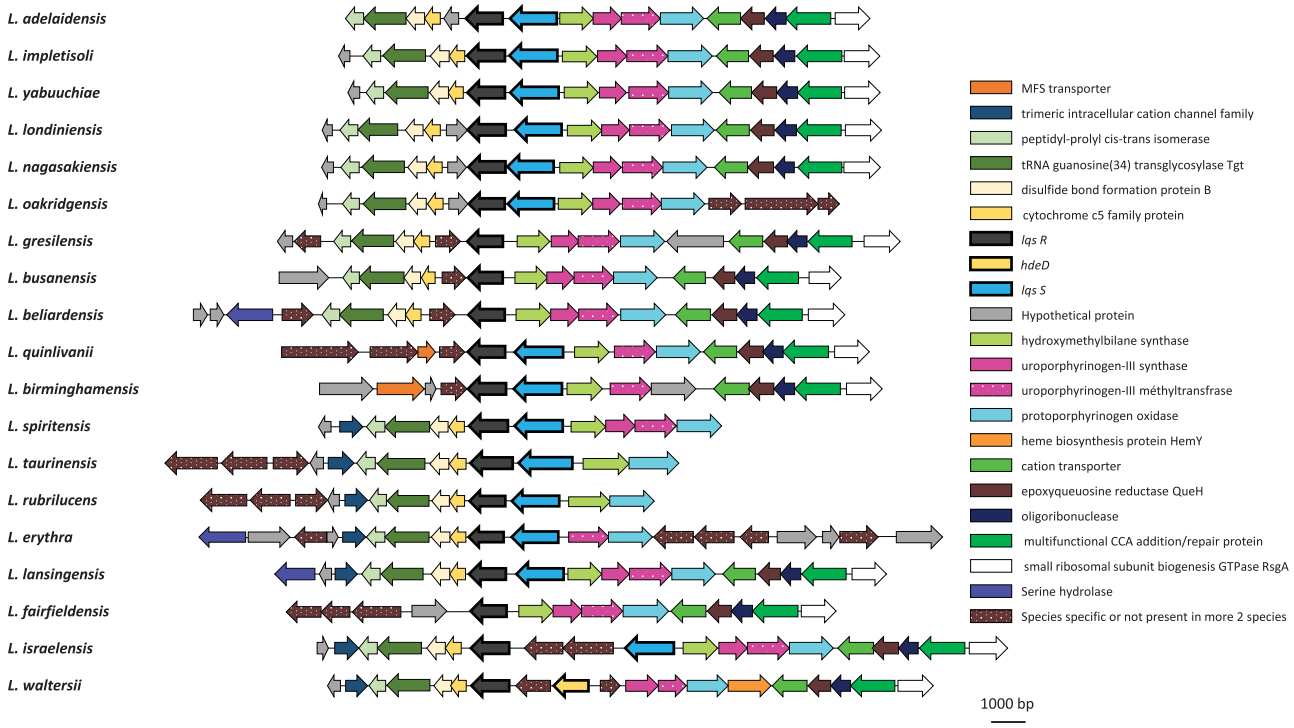

Fig. 5.—Syntheny alignment of the genomic region of *Legionella* species that have lost *lqsA*. Homologue genes were represented with the same color except for unique genes or genes not found in two or three species (black with white point). The comparison shows that the regions upstream and downstream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

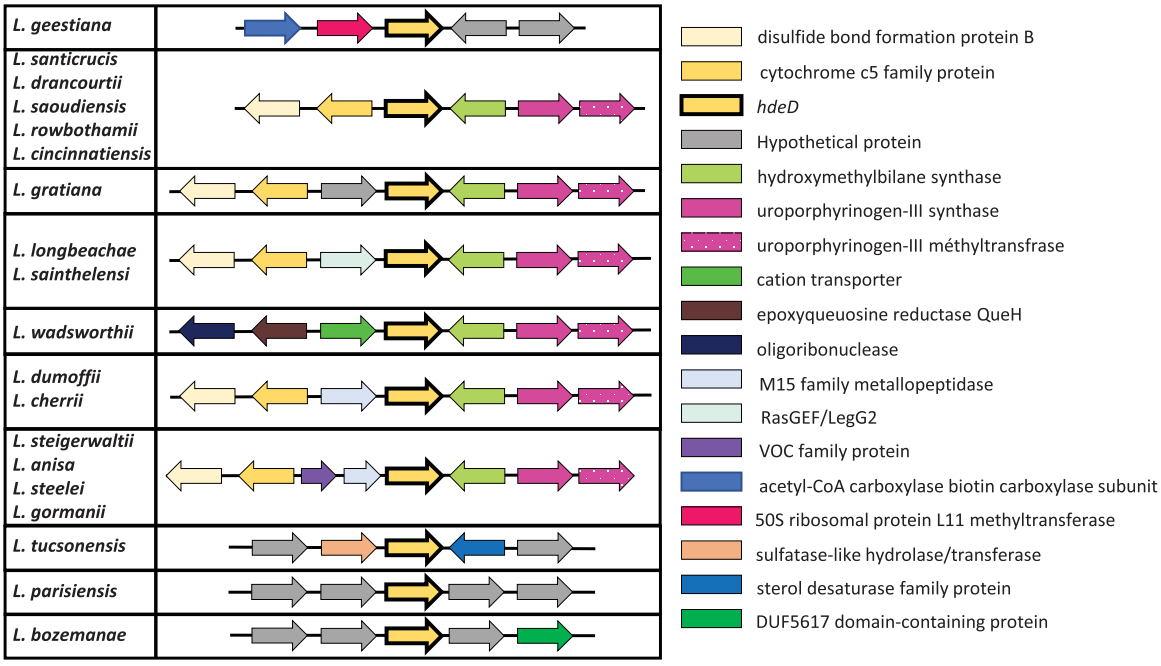

Fig. 6.—Schematic representation of the genomic region of *hdeD* in species that do not have any genes from the *lqs* cluster.

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*

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 Δ*lqsA* mutants the native phenotype can be restored by the addition of supernatant from wild strains or by synthetic LAI-1 (Tiaden, Spirig, 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. dumoffi*, 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 *Legionellae*, 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 possible 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, Spirig, 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 nautarum*, 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 devolved 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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