THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 26, pp. 18113–18123, June 27, 2008

The *Legionella* Autoinducer Synthase LqsA Produces an α-Hydroxyketone Signaling Molecule*[S]

Received for publication, March 10, 2008, and in revised form, April 10, 2008 Published, JBC Papers in Press, April 14, 2008, DOI 10.1074/jbc.M801929200

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The opportunistic pathogen *Legionella pneumophila* replicates in human lung macrophages and in free-living amoebae. To accommodate the transfer between host cells, *L. pneumophila* switches from a replicative to a transmissive phase. *L. pneumophila* harbors a gene cluster homologous to the *Vibrio cholerae cqsAS* quorum sensing system, encoding a putative autoinducer synthase (*lqsA*) and a sensor kinase (*lqsS*), which flank a response regulator (*lqsR*). LqsR is an element of the *L. pneumophila* virulence regulatory network, which promotes pathogen-host cell interactions and inhibits entry into the replicative growth phase. Here, we show that *lqsA* functionally complements a *V. cholerae cqsA* autoinducer synthase deletion mutant and, upon expression in *L. pneumophila* or *Escherichia coli*, produces the diffusible signaling molecule LAI-1 (*Legionella* autoinducer-1). LAI-1 is distinct from CAI-1 (*Cholerae* autoinducer-1) and was identified as 3-hydroxypentadecan-4-one using liquid chromatography coupled to high resolution tandem mass spectrometry. The activity of both LqsA and CqsA was abolished upon mutation of a conserved lysine, and covalent binding of the cofactor pyridoxal 5′-phosphate to this lysine was confirmed by mass spectrometry. Thus, LqsA and CqsA belong to a family of pyridoxal 5′-phosphate-dependent autoinducer synthases, which produce the α-hydroxyketone signaling molecules LAI-1 and CAI-1.

Legionella pneumophila is a ubiquitous bacterium that persists in biofilms and replicates within environmental predators including amoebae (1, 2). Upon inhalation of aerosols from contaminated water sources, the Gram-negative bacteria replicate within macrophages and may cause the severe pneumonia Legionnaires disease, which was first recognized 30 years ago (3). The Icm/Dot type IV secretion system (T4SS) governs interactions between *L. pneumophila* and phagocytes (4–10), ultimately leading to a replication-permissive *Legionella*-containing vacuole, which does not communicate with the endosomal pathway but, rather, intercepts the early secretory pathways and possibly other trafficking routes (11, 12). At current count more than 40 "effector" proteins secreted by the Icm/Dot type IV secretion system have been identified, some of which interfere with host cell trafficking by modulating small host cell GTPases or phosphoinositide metabolism (13–15).

L. pneumophila is a facultative intracellular bacterium and, thus, needs to control gene regulation in response to a variety of different environments. The transition of *L. pneumophila* from an intracellular to an extracellular environment coincides with a transition from a replicative growth phase to a transmissive (virulent) phase (16). Upon entry of *L. pneumophila* into stationary growth phase, motility and virulence genes required for transmission are expressed. This transition is also reflected in the gene expression pattern observed in the course of growth in broth or within *Acanthamoeba castellanii* (17). Although in the replicative phase constituents of aerobic amino acid and carbohydrate catabolism are up-regulated, in the stationary phase genes required for transmission and host cell infection are expressed. The latter include genes encoding the flagellar apparatus, type IV pili, as well as Icm/Dot-dependent and -independent virulence factors.

Alternative σ factors are crucial regulators of *L. pneumophila* virulence. In addition to the expression of the flagellar regulon, the flagellar σ factor FliA (σ^{28}) regulates contact-dependent cytotoxicity, infectivity, and lysosome avoidance in macrophages (18, 19) and intracellular replication in *Dictyostelium discoideum* (20) as well as biofilm formation (21). The stationary phase σ factor RpoS (σ^{S}/σ^{38}) promotes growth within *A. castellanii* (22) and in primary macrophages (23) by up-regulating the expression of transmission genes in stationary phase while inhibiting these genes in the replicative growth phase (24).

L. pneumophila response regulators such as LetA (GacA) (18, 25, 26), CpxR (27), and PmrA (28) have also been implicated in the regulation of transmissive traits including virulence. Recently, we characterized the putative response regulator LqsR as a novel element of the *L. pneumophila* virulence regulatory network controlled by RpoS and LetA (29). LqsR promotes pathogen-host cell interactions such as phagocytosis, formation of the *Legionella*-containing vacuole, intracellular replication and toxicity while inhibiting the entry of *L. pneumophila* into the replicative growth phase. The *lqsR* gene is flanked by *lqsA* and *lqsS*, encoding a putative autoinducer synthase and

* This work was supported, in whole or in part, by National Institutes of Health Grant AI044212 (to C. B.). This work was also supported by grants from the Swiss National Science Foundation (631-065952; PP00A-112592), the ETH Zürich (TH 17/02-3), the Commission for Technology and Innovation (6629.2 BTS-LS), and the Swiss Federal Agency for Energy (to H. H.), and by funding from the Agence Française de Sécurité Sanitaire de l'Environment et du Travail (ARCL-2005-002) (to C. B.). The group of H. H. participates in the NEMO (Non-mammalian Experimental Models for the Study of Bacterial Infections) Network supported by the Swiss 3R foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Tables S1 and S2.

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a sensor kinase (see Fig. 1). LqsA and LqsS are homologous to the CqsAS "quorum sensing" system identified in *Vibrio cholerae* and other marine *Vibrio* spp., which is involved in the regulation of virulence, biofilm formation, and bioluminescence (30, 31).

Quorum sensing designates bacterial cell-cell communication via endogenously produced and secreted small molecules termed autoinducers. These signaling molecules regulate gene expression by directly binding to cytoplasmic transcription factors or indirectly by binding to sensor kinases that transmit the signal via phospho-relays (32–34). Autoinducers belong to distinct chemical classes, including *N*-acyl-L-homoserine lactones (AHLs),[2] linear and cyclic peptides, quinolones, and the furanosyl borate diester AI-2. Recently, the signaling molecule CAI-1 (*Cholerae* autoinducer-1) produced by the autoinducer synthase CqsA has been identified as (*S*)-3-hydroxytridecan-4-one (35). Specific AHLs, peptides, or CAI-1 are synthesized by individual bacterial species and, thus, are proposed to promote intraspecies communication. In contrast, AI-2 is produced by LuxS-type synthases and detected by a wide variety of Gram-negative and Gram-positive bacteria. Therefore, this autoinducer might serve as an interspecies signal. AHLs as well as AI-2 are derived from *S*-adenosylmethionine, thus intimately linking bacterial metabolism with the production of a diffusible signal.

L. pneumophila apparently lacks an AI-2 signaling system and AHL-based quorum sensing circuits. Here, we report that the expression of *lqsA* partially complements a *V. cholerae cqsA* mutant strain and produces the diffusible signaling molecule 3-hydroxypentadecan-4-one as the major product (*Legionella* autoinducer, LAI-1). Furthermore, the *L. pneumophila* autoinducer synthase LqsA was found to be a pyridoxal 5′-phosphate (PLP)-dependent enzyme.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—The bacterial strains used in this study are listed in supplemental Table S1. *L. pneumophila* and other *Legionella* spp. were grown on charcoal yeast extract agar plates (36) in AYE broth supplemented with chloramphenicol (5 μg/ml) or kanamycin (50 μg/ml) if necessary. *V. cholerae* strains harboring plasmid pBB1 were cultured in LB containing tetracycline (5 μg/ml). *Escherichia coli* was grown in LB medium supplemented with chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), or ampicillin (100 μg/ml) if required. All reagents were from Sigma unless specified otherwise.

Cloning and Reverse Transcription-PCR—The BLASTP algorithm (37) was used to identify orthologues of *V. cholerae* O1 biovar El Tor CqsA (NP_232914) and CqsS (NP_232913), encoded in the genomes of the *L. pneumophila* strains Philadelphia-1 (38), Paris, Lens (39), and Corby (40). DNA manipulations were performed according to standard protocols, and plasmids were isolated using commercially available kits (Qiagen, Macherey-Nagel). Point mutations were introduced by using the QuikChange kit (Stratagene). The oligonucleotides listed in supplemental Table S2 were used for cloning.

Broad host range expression vectors containing *lqsA* (pTS-2) or *cqsA* (pTS-6) under the control of P_{tac} were constructed by PCR amplification of the putative open reading frame of *lqsA* or *cqsA* from plasmid pNT-1 or genomic DNA of *V. cholerae* strain El Tor VC2740, respectively. The PCR products were ligated into plasmid pGEM-T-Easy, liberated by digestion with NdeI and BamHI, and cloned into pMMB207C-RBS-*lcsC* cut with the same restriction enzymes.

To express a His-tagged LqsA fusion protein in *E. coli* strain BL21(DE3), plasmid pTS-21 was constructed by amplifying *lqsA* by PCR using the primers TS-21-fo/TS-21-re and pNT-1 as a template. The 1250-bp PCR fragment was cut with EcoRI and NotI and ligated into the same sites of plasmid pET-28a(+). Plasmid pTS-22 expressing a His-tagged CqsA fusion protein was constructed by releasing *cqsA* from pTS-6 using BamHI and NdeI and cloning the 1170-bp fragment into pET-28a(+). The conserved lysine residues representing the putative PLP binding sites in LqsA (Lys-258) and CqsA (Lys-236) were replaced by alanine (serine) by site-directed mutagenesis of plasmid pTS-2 or pTS-6, yielding the plasmids pTS-25 (pTS-24) and pTS-26, respectively.

The presence of *lqsA* in *Legionella* spp. was assessed by low stringency PCR. DNA from resuspended bacteria (*L. pneumophila* strains AA100, Corby, 502, 509, 514) or prepared by a kit (remaining strains; GenElute, Sigma) was used as template, and the genes of interest were amplified at 45 °C with the primer pairs LqsA-fo/LqsA-re and oUA64/oUA65 for *lqsA* and 16 S rRNA, respectively. *LqsA* gene expression was determined by reverse transcription-PCR in replicative phase cultures (OD_{600} 0.6) and stationary phase cultures (OD_{600} 3.5) grown in AYE broth (41). To quantify RNA from bacteria grown intracellularly in amoebae, *A. castellanii* were harvested 2 or 17 h post-infection with *L. pneumophila* JR32.

Determination and Characterization of LqsA Activity—*L. pneumophila lqsA* or *V. cholerae cqsA* were expressed under control of the P_{tac} promoter in the *V. cholerae* CAI-1 reporter strain MM920 (30). The emission of light (relative units) was quantified by a luminometer (Victor3 reader; Wallac 1420, PerkinElmer Life Sciences). Production of diffusible autoinducer signals was assessed with *E. coli* BL21(DE3) harboring pTS-2 (*lqsA*), pTS-6 (*cqsA*), or pTS-10 (control) streaked out on charcoal yeast extract agar in 24-well plates. *V. cholerae* MM920 was streaked out in a parallel line after 1 day, and the autoinducer-producing strain was impregnated with 10 μl of a 100 μM isopropyl 1-thio-β-D-galactopyranoside solution. After another day, bioluminescence was determined with the FluorChem 8900 reader (Alpha Innotech Corp.).

The activity of LAI-1 or CAI-1 released into the supernatant of *E. coli* BL21(DE3) producing either His-LqsA (pTS-21) or His-CqsA (pTS-22) under the control of the T7 promoter after 4 h of induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside was determined by bioluminescence in 96-well plates (B&W Isoplate, Wallac) using a Victor3 plate reader. To this end *E. coli* was removed by centrifugation, and 100 μl of (sterile fil-

[2] The abbreviations used are: AHL, *N*-acyl-L-homoserine lactone; AYE, *N*-(2-acetamido)-2-aminoethanesulfonic acid yeast extract; APCI, atmospheric pressure chemical ionization; CAI-1, *Cholerae* autoinducer-1; *cqs*, *cholerae* quorum sensing; Icm/Dot, intracellular multiplication/defective organelle trafficking; LAI-1, *Legionella* autoinducer-1; LC, liquid chromatography; *lqs*, *Legionella* quorum sensing; MS, mass spectrometry; PLP, pyridoxal 5′-phosphate; O-PFB, O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride.

tered) supernatant were added to 100 μl of *V. cholerae* MM920 (overnight culture diluted to an OD_{600} of 0.5) and incubated for 4 h. Supernatant containing LAI-1 or CAI-1 activity was also passed over a PD-10 size exclusion column (Amersham Biosciences) before testing autoinducer activity in 1-ml fractions.

To determine whether LAI-1 or CAI-1 are volatile, the four central wells of a 96-well plate were inoculated with *E. coli* BL21(DE3) harboring plasmid pTS-21 (His-LqsA), pTS-22 (His-CqsA), pTS-25 (His-$LqsA_{K258A}$), pTS-26 (His-$CqsA_{K236A}$), or the corresponding vector (pET-28a(+)). The surrounding wells contained *V. cholerae* MM920, and bioluminescence was determined after 4 h of incubation at room temperature. As a control, the wells were covered with adhesive plastic foil (PVC foil MP30A, MaProline GmbH, Starrkirch-Wil, Switzerland).

Mass Spectrometry—Liquid chromatography-mass spectrometry (LC-MS) analyses were carried out with a Rheos 2200 high performance liquid chromatography (HPLC) system (Flux Instruments, Basel, Switzerland) coupled to an LTQ-Orbitrap (Thermo Fisher Scientific, Waltham), equipped with an atmospheric pressure chemical ionization (APCI) probe. The compounds were separated with acetonitrile using a Gemini C18 analytical column (150 × 2.0 mm, particle size 3; Phenomenex, Torrance, CA) at a flow rate of 200 μl min^{-1} and an injection volume of 10 μl. MS analysis was done in the positive FTMS mode at a resolution of 60,000. The analytes were identified by the exact masses of the corresponding $[M+H]^+$ ions. Fragmentation was performed in the linear ion trap by collision-induced dissociation at a normalized collision energy of 30 using helium as collision gas. Fragment ions were subsequently detected in the positive FTMS mode at a resolution of 30,000. MS/MS spectra of CAI-1 and LAI-1 were analyzed and interpreted using MassFrontier 5.0 software (HighChem, Slovak Republik).

To prepare samples for mass spectrometry, *L. pneumophila* containing pTS-2 (*lqsA*) or no plasmid and *E. coli* containing pTS-21 (*lqsA*), pTS-22 (*cqsA*), or pET-28a (vector) were grown at 37 °C under vigorous shaking in 1 liter of AYE/chloramphenicol or M9/kanamycin, respectively. At an OD_{600} of 2.0 the cultures were induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h. *V. cholerae* MM227 was grown at 30 °C in LB medium to an optical density of 2.9. Cell-free supernatants were prepared by centrifugation (3800 × *g*, 30 min) and extracted with dichloromethane (supernatant:dichloromethane 2:3) in a separation funnel. The organic phase was evaporated to dryness, reconstituted with 200 μl of acetonitrile, and stored at −80 °C.

For oximation, 40 μl of supernatant extract were mixed with 10 μl of saturated *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (O-PFB; ≥98%, Sigma-Aldrich) in acetonitrile. The mixture was incubated for 10 min at room temperature before analysis. Partition coefficients were predicted by ChemBioDraw Ultra 11.0. To test the activity of signals, 100-μl fractions eluting from the LC column were collected. 2 μl of each fraction was incubated with *V. cholerae* MM920 in duplicates as described above.

Identification of Pyridoxal 5′-Phosphate as the Cofactor of LqsA and CqsA—Supernatants of *E. coli* BL21(DE3) harboring plasmid pTS-21 (His-LqsA), pTS-22 (His-CqsA), pTS-25 (His-$LqsA_{K258A}$), pTS-26 (His-$CqsA_{K236A}$), or an empty plasmid (pET-28a(+)) were assayed for autoinducer activity by bioluminescence using *V. cholerae* MM920 as described above. The PLP precursor pyridoxine was added to growing bacteria where indicated.

Covalent binding of PLP to purified His-LqsA, His-$LqsA_{K258A}$, His-CqsA, and His-$CqsA_{K236A}$ was determined by mass spectroscopy using the above *E. coli* strains. To purify the His-tagged proteins, the strains were grown at 30 °C (LB, 50 mg ml^{-1} kanamycin, 0.2 mM pyridoxine) to an OD_{600} of 0.7, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h, centrifuged (8000 × *g*), and resuspended in 50 mM NaP_i, pH 8.0, 15 mM imidazole. After lysis of the bacteria by a French press, cell debris were removed by centrifugation (180,000 × *g*), and the supernatant was loaded onto a nickel-nitrilotriacetic acid-agarose column (Qiagen). After washing with 50 mM NaP_i, pH 8.0, 60 mM imidazole, 300 mM NaCl, the protein was eluted with 4 ml of the same buffer containing 250 mM imidazole and immediately diluted with the same amount of distilled water. The samples were concentrated (Amicon Ultrafiltration Cell, 10 kDa cutoff), purified on C_4-ZipTip (Millipore) using the standard protocol, and eluted with 50% acetonitrile, 0.1% formic acid. The purified samples were injected through a fused silica capillary (inner diameter 75 um) at a flow rate of 0.3 μl/min and analyzed by nanoelectrospray ionization-MS on a quadrupole-time of flight Ultima API mass spectrometer (Micromass). Mass spectra were acquired by scanning an *m*/*z* range from 50 to 1500 with a scan duration of 1 s and an interscan delay of 0.1 s. Spray voltage was set to 2.1 kV, cone voltage was 35 V, and radio frequency lens 1 energy was 50 V. Mass spectra were deconvolved using the MaxEnt 1 software (Micromass) with an accuracy of ±1 Da.

RESULTS

Prevalence of lqsA in L. pneumophila Strains and Gene Expression Analysis—The *lqs* gene cluster (Fig. 1*A*) is present in all four *L. pneumophila* genomes sequenced to date: strain Philadelphia (*lpg2731-2734*), Paris (*lpp2787-2790*), and Lens (*lpl2656-2659*) as well as Corby (*lpc0402-0401-0399-0396*) (38–40). However, the putative autoinducer synthase and sensor kinase genes *lqsA* and *lqsS* genes are distributed more widely than the other genes of the cluster and are also prevalent among marine *Vibrio* spp. (*cqsA*, *cqsS*), where they originally were discovered (30, 31). To further analyze the *L. pneumophila lqs* putative quorum sensing system, we focused on *lqsA*. The *lqsA* gene was found by PCR amplification to be present in laboratory wild-type *L. pneumophila* strains (JR32, AA100, Corby) as well as in all clinical and environmental *L. pneumophila* isolates tested (Fig. 2*A*). In contrast, the *lqsA* gene was not amplified using as a PCR template the genomic DNA of other *Legionella* spp., such as *L. bozemanii*, *L. rubrilucens*, and *L. taurinensis*, indicating that these species either do not contain the *lqsA* gene or harbor a gene too diverse to be amplified by PCR under the low stringency conditions used. The expression of *lqsA* in *L. pneumophila* strain JR32 was determined by semi-quantitative reverse transcription-PCR (Fig. 2*B*). In AYE broth, *L. pneumophila* expressed *lqsA* preferentially in the replicative (OD_{600} = 0.6) rather than in the stationary growth phase

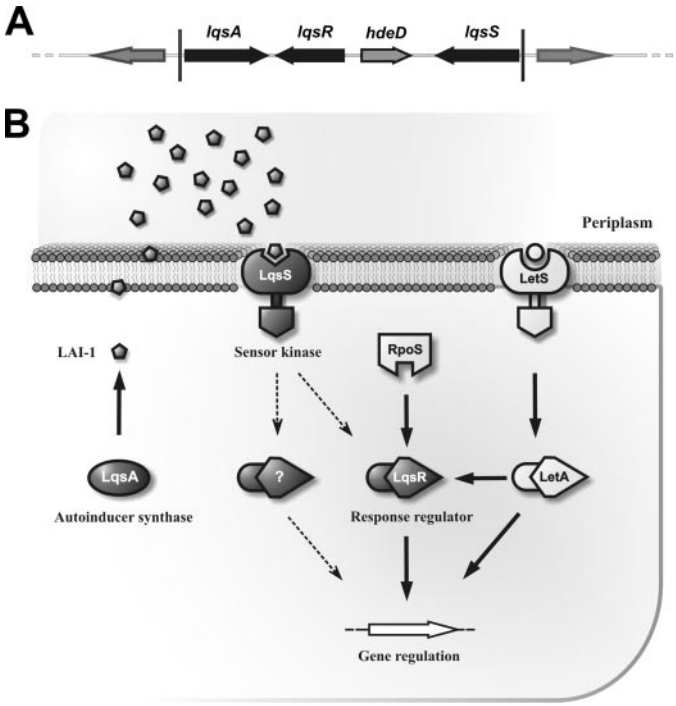

FIGURE 1. **The *lqs* gene cluster and model of the *L. pneumophila* quorum sensing circuit.** *A*, chromosomal map of the *L. pneumophila lqs* gene cluster. The putative autoinducer *lqsA* and sensor kinase *lqsS* are 45 and 29% identical to *V. cholerae cqsA* and *cqsS*, respectively. LqsR encodes a putative response regulator (29), and the *E. coli* homologue of *hdeD* is possibly involved in acid resistance (55). *B*, model of the *L. pneumophila* autoinducer circuit, including LqsA, the low molecular weight diffusible signaling molecule LAI-1, the cognate sensor kinase LqsS, and the response regulator LqsR. The expression of LqsR is controlled by the alternative σ factor RpoS and the two-component system LetA/LetS. *Dashed lines* indicate putative pathways and links.

(OD_{600} = 3.5). Similarly, the bacteria displayed highly elevated levels of *lqsA* RNA upon replication within in *A. castellanii* (17 h post-infection).

LqsA Partially Complements Cell Density-dependent Gene Expression of a V. cholerae cqsA Mutant—The LqsA proteins from the four *L. pneumophila* strains sequenced are 97–99% identical. The *V. cholerae* CqsA autoinducer synthase still shares 42% identity with LqsA over the entire protein length (supplemental Fig. S1), suggesting that the two proteins may perform similar functions. To assay whether *lqsA* complements a *cqsA* deletion mutant, we used the *V. cholerae* CAI-1 reporter strain MM920 (30). This strain harbors plasmid pBB1, encoding a luciferase system induced upon activation of the CqsS sensor kinase. *V. cholerae* MM920 lacks the *cqsA* and *luxQ* genes, encoding the CAI-1 autoinducer synthase ("system 1") and the LuxQ sensor kinase ("system 2"). Thus, via CqsS of system 1, strain MM920 responds to CAI-1 and also to smaller homologues of the CAI-1 hydroxyketone (35), but not to AI-2.

Upon complementation of strain MM920 with the *V. cholerae cqsA* gene, CAI-1 triggered the induction of luciferase and the concomitant emission of light (Fig. 3*A* (30)). Interestingly, the expression of *L. pneumophila lqsA* in *V. cholerae* MM920 also triggered the induction of luciferase and light emission, indicating that *lqsA* and *cqsA* are functionally similar, and a signal produced by LqsA is recognized by the CqsS sensor kinase. The signal intensity produced by the expression of *lqsA* was 50 times above background level (empty plasmid) but was still 2 orders of magnitude smaller than the signal produced by the expression of *V. cholerae cqsA*. The difference in signal intensity was not due to altered growth characteristics of the strains, as expression of *cqsA* or *lqsA* had no effect on the growth of the reporter strain (data not shown). These results suggest that autoinducer molecules produced by CqsA and LqsA are either structurally similar, yet not identical, or produced more efficiently upon expression of *cqsA* by *V. cholerae* compared with the heterologous expression of *lqsA*.

LqsA and CqsA Produce Low Molecular Weight, Volatile Signaling Molecules—Based on the results of the complementation experiment, we tested whether the expression of *lqsA* would produce a diffusible signal that may trigger gene expression intercellularly among different bacterial strains. To this end, *E. coli* containing plasmids with either *lqsA* or *cqsA* was streaked out on agar plates 1 day before *V. cholerae* MM920 was streaked out in a parallel line without contacting the signal-producing strain. Expression of *lqsA* or *cqsA* in *E. coli* resulted in light emission from strain MM920 (data not shown). *E. coli* harboring a control plasmid did not produce any detectable signal. These results are in agreement with a diffusible autoinducer signal being secreted upon production of LqsA or CqsA.

To further study LAI-1 activity, LqsA, or as a control, CqsA, was produced in *E. coli*, and autoinducer activity released into the supernatant was determined using *V. cholerae* MM920. Under these conditions the light intensity produced by LqsA was 4 orders of magnitude higher than the negative control yet still 1–2 orders of magnitude lower than CqsA (Fig. 3*B*). Upon passage of supernatants containing LAI-1 or CAI-1 over a PD-10 size exclusion column, the active molecules did not elute in the void volume but were, rather, recovered in the same fractions (9–12 ml; data not shown), indicating that the LAI-1 and CAI-1 signaling molecules have a molecular mass smaller than the exclusion limit of the column (1 kDa). The signals produced by both enzymes were found to be volatile at room temperature, as *V. cholerae* MM920 emitted light, even if the *E. coli* strains producing the signal molecules were spatially separated in discrete wells of a 96-well plate (Fig. 3*C*). The emission of light by the reporter strain was abolished by covering the wells with a plastic foil.

LqsA and CqsA Produce Distinct Patterns of Hydroxyketone Molecules—We used high resolution mass spectrometry to identify the structure of LAI-1 in cell-free supernatants of *E. coli*, *L. pneumophila*, or *V. cholerae* producing LqsA or CqsA. To this end, the samples were extracted with dichloromethane and analyzed by LC-MS for the presence of hydroxyketones with a chain length of C_8-C_{20}. Compared with control samples, candidate molecules were detected with an elemental composition corresponding to hydroxyketones in this range. However, the bacterial extracts are expected to also contain considerable amounts of fatty acids, which are structurally very similar mass isomers of hydroxyketones and, thus, difficult to distinguish from the latter.

The treatment with O-PFB allows discriminating hydroxyketones from carboxylic acids, as only the former are derivatized by the hydroxylamine. Moreover, the specific pentafluorobenzyl oxime derivatives produced by the carbonyl-reactive

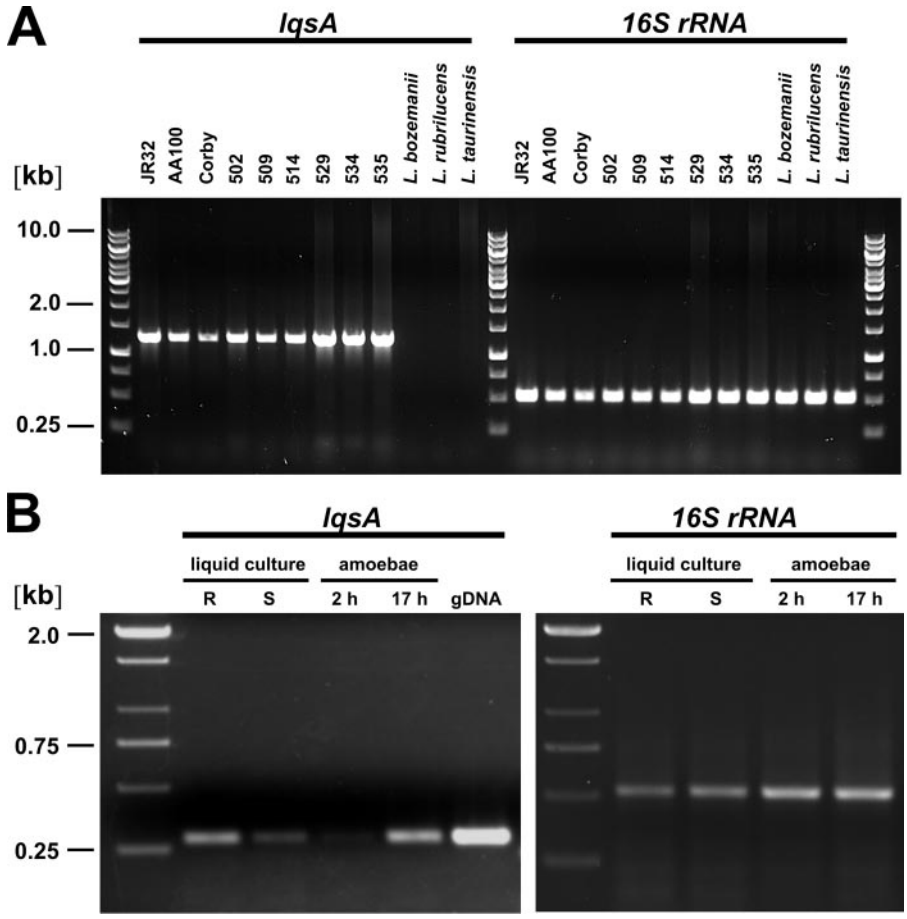

FIGURE 2. **Prevalence of *lqsA* in *L. pneumophila* strains and gene expression analysis.** *A*, the presence of *lqsA* was assessed by PCR amplification of the genes from genomic DNA of laboratory wild-type *L. pneumophila* strains (JR32, AA100, Corby) and clinical (502, 509, 514) and environmental (529, 534, 535) *L. pneumophila* isolates as well as *L. bozemanii*, *L. rubrilucens*, and *L. taurinensis*. As a positive control, the 16 S rRNA gene was amplified. *B*, the expression of *lqsA* in *L. pneumophila* JR32 was determined by semiquantitative reverse transcription-PCR. In AYE broth *lqsA* was expressed preferentially in the replicative (*R*) rather than in the stationary (*S*) growth phase, and in *A. castellanii lqsA* was expressed during replication (17 h). *gDNA*, genomic DNA. Similar results were obtained in at least two independent experiments. *kb*, kilobase.

agent *O*-PFB enhance the detection sensitivity of carbonyl-compounds (42, 43). LC-MS/MS analysis of the different bacterial supernatant extracts identified hydroxyketones with a chain length from 10 to 17 carbon units (Fig. 4). The molecules were not detected in supernatants of *E. coli* or *L. pneumophila* in the absence of plasmids encoding the autoinducer synthases CqsA or LqsA (data not shown).

An estimation of the relative abundance of the hydroxyketones produced by *E. coli* expressing *lqsA* or *cqsA*, *L. pneumophila* expressing *lqsA*, or *V. cholerae* is shown in Table 1. Upon expression of CqsA in *E. coli*, the masses for both CAI-1 (C_{13}) and its C_{11} homologue were detected in the supernatant, and a similar hydroxyketone product pattern was determined in supernatants of wild-type *V. cholerae* MM227. In the supernatants of *E. coli*- or *L. pneumophila*-producing LqsA, several signals with masses exactly matching the corresponding C_{13}-C_{17} hydroxyketones were detected. Interestingly, however, the signal intensity in the pattern was shifted toward longer chain lengths compared with supernatants of bacteria producing CqsA. The major product of LqsA, designated as LAI-1, eluted later from the LC column than the CqsA product (data not shown) and was identified by high resolution MS as the C_{15} hydroxyketone homologue of CAI-1.

Identification of LAI-1 as 3-Hydroxypentadecan-4-one—The characteristic fragment ions of O-PFB oximes greatly facilitate the structural assignment of carbonyl compounds (44). Therefore, the formation of O-PFB derivatives is expected to also specifically shift the mass of LqsA- or CqsA-produced hydroxyketones, thus allowing the detection of structure-determining fragment ions. Using LC-MS, the C_{13} hydroxyketone compounds produced by either CqsA or LqsA were found to elute with the same retention time (data not shown). Subsequent MS/MS analysis yielded identical spectra, including the specific fragment ions at *m*/*z* 152.143, 194.190, and 350.154 (Fig. 5). The strong signals at *m*/*z* 152.143 and 350.154 can be attributed to the cleavage of the C_3-C_4 bond upon a favored collision-induced dissociation between the adjacent hydroxyl and oxime moieties. In summary, the fragmentation patterns of the C_{13} hydroxyketone produced by CqsA and LqsA were indistinguishable and indicated that LqsA synthesizes the molecule 3-hydroxytridecan-4-one (CAI-1) as a minor product (Table 1).

For the major signal present in the LqsA sample (LAI-1), the fragmentation pattern of the selected $[M+H]^+$ ion correlated well with the theoretically predicted fragments for a C_{15} α-hydroxyketone homologue. In agreement with this notion, the loss of water from the C_{15} compound yielded the dominating ion at *m*/*z* 420.233 (Fig. 5). The fragmentation spectrum with the specific ions at *m*/*z* 180.174, 222.222, 378.185, and 420.233 as well as their relative intensities are analogous to the fragmentation products seen for CAI-1. This pattern indicates that an ethyl-α-hydroxyketone moiety is present in LAI-1 as well as CAI-1. Furthermore, the fragment ions *m*/*z* 180.174 and 378.185 allow determining the position of the ketone moiety relative to the C_{15} backbone in LAI-1. The mass of both fragment ions was shifted by 28.03 atomic mass units, relative to the corresponding fragment ions *m*/*z* 152.143 and *m*/*z* 350.154 of CAI-1, respectively. Thus, the ethyl moiety additionally present in LAI-1 compared with CAI-1 is located at the far end of the molecule with regard to the ketone group. Taken together, these fragmentation characteristics strongly support the classification of LAI-1 as an α-hydroxyketone and its identification as 3-hydroxypentadecan-4-one.

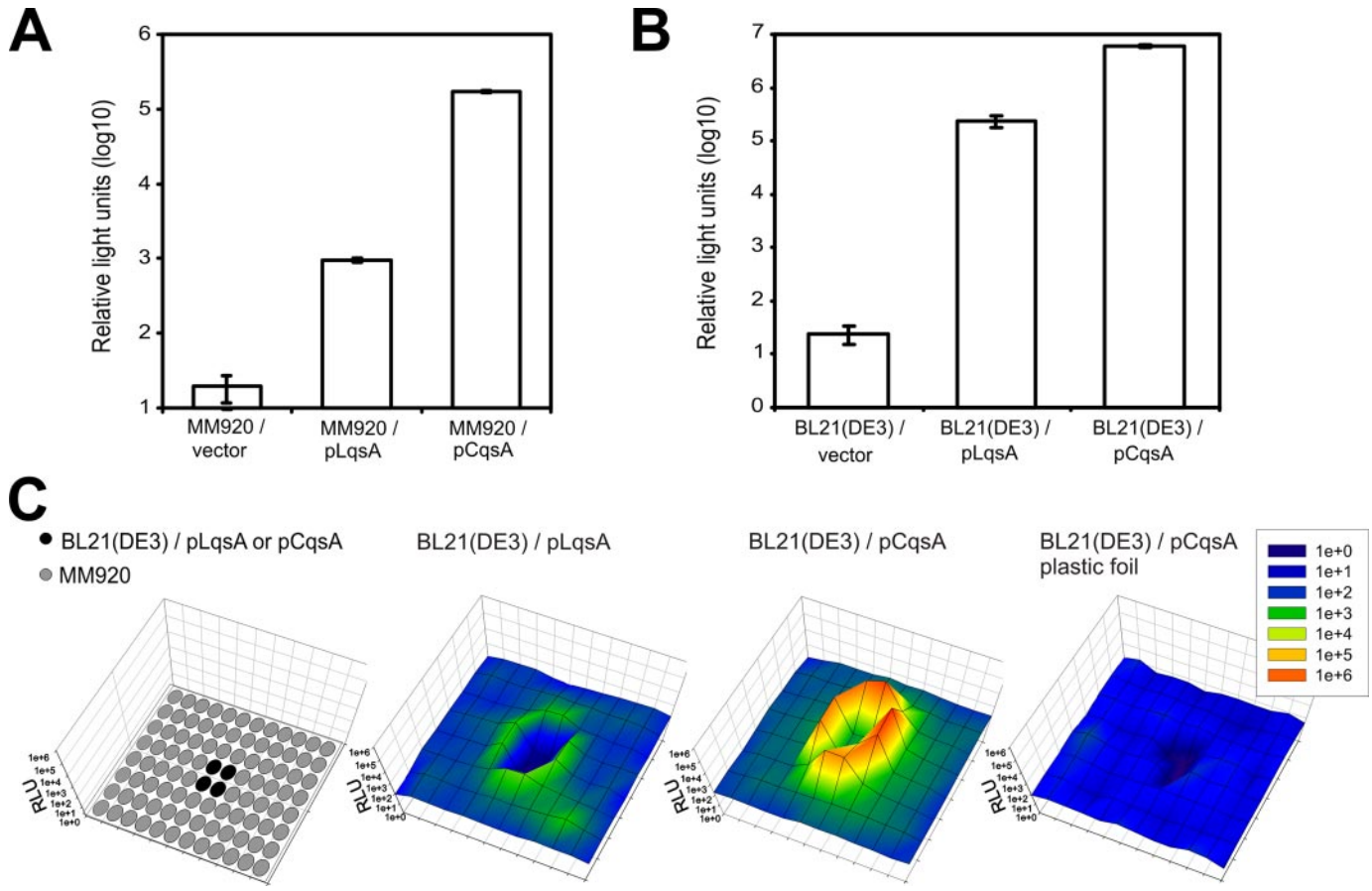

FIGURE 3. **Production of diffusible, volatile signaling molecules by LqsA and CqsA.** *A*, *LqsA* partially complements cell density-dependent gene expression of a *V. cholerae cqsA* mutant. *L. pneumophila lqsA* or *V. cholerae cqsA* was expressed in the *V. cholerae* CAI-1 reporter strain MM920 by introducing pTS-2 (pLqsA) or pTS-6 (pCqsA), respectively. The emission of light (relative units) was quantified by luminescence. *B*, signal activity is produced in *E. coli* upon heterologous expression of LqsA or CqsA. His-LqsA or His-CqsA were produced under the control of the P_{T7} promoter in *E. coli* BL21(DE3) harboring plasmid pTS-21 (pLqsA) or pTS-22 (pCqsA), and the bacterial supernatants were assayed for autoinducer activity by bioluminescence using *V. cholerae* MM920. *C*, the signaling molecules produced by LqsA and CqsA are partially volatile at room temperature. *E. coli* BL21(DE3) harboring plasmid pTS-21 (pLqsA) or pTS-22 (pCqsA) was placed in the 4 central wells of a 96-well plate surrounded by *V. cholerae* MM920 and incubated for 4 h before the determination of bioluminescence. As a control the wells were covered with plastic foil. Means and S.D. of triplicates are shown (*A* and *B*). Similar results were obtained in at least three independent experiments.

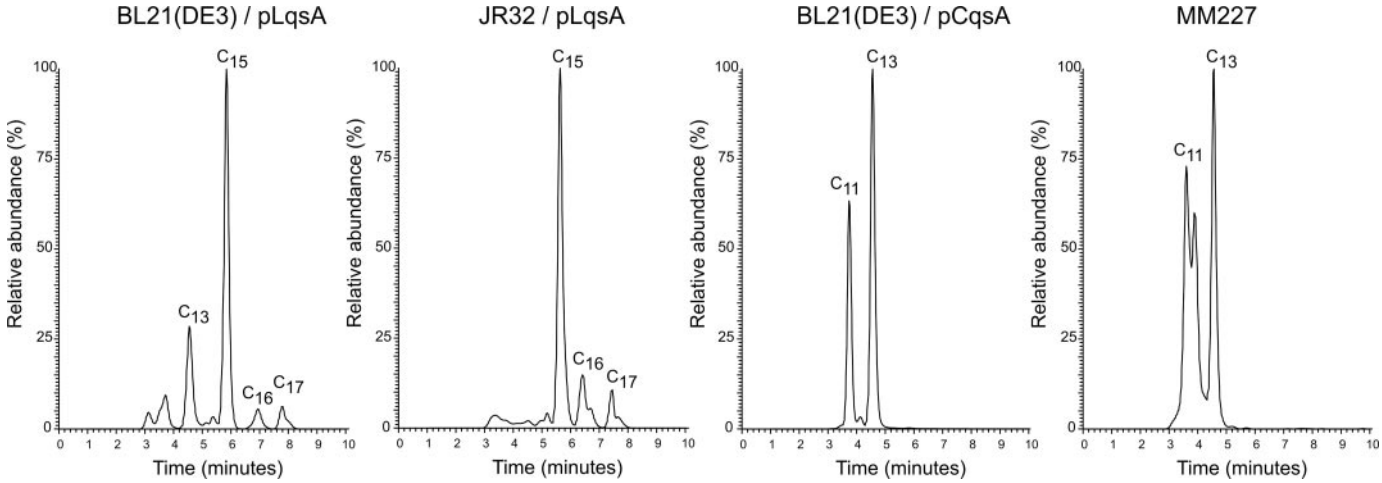

FIGURE 4. **LqsA and CqsA produce a distinct pattern of hydroxyketone molecules.** Selected-ion mass spectrometry chromatograms display the patterns of hydroxyketone molecules produced by *E. coli* BL21(DE3) harboring pTS-21 (pLqsA) or pTS-22 (pCqsA), *L. pneumophila* containing pTS-2 (*pLqsA*), or wild-type *V. cholerae* MM227. Extracted supernatants were treated with O-PFB and analyzed by LC-APCI-MS. In supernatants of *E. coli* or *L. pneumophila* producing LqsA, the C_{15} compound was detected as the major product and termed LAI-1. Supernatants of *E. coli* producing CqsA or supernatants of *V. cholerae* contained high levels of CAI-1 (C_{13}) and its C_{11} homologue.

V. cholerae Does Not Respond to LAI-1—Next, we wanted to correlate specific hydroxyketone homologues produced by LqsA to their autoinducer activity. To this end cell-free supernatants of *E. coli* expressing CqsA or LqsA were extracted with dichloromethane and analyzed by LC-MS. Treatment with O-PBF was omitted to retain the biological activity of the sam-

TABLE 1

Estimation of the relative amounts of α-hydroxyketone signal molecules

The peak areas of the corresponding mass peaks were determined from LC-MS chromatograms and normalized to the sum of peak areas detected in a chromatogram. Ion suppression and concurrence effects cannot be excluded.

	C_{10}	C_{11}	C_{12}	C_{13} CAI-1	C_{14}	C_{15} LAI-1	C_{16}	C_{17}
	%	%	%	%	%	%	%	%
E. coli/pCqsA[a]	1	36	2	61	–	–	–	–
V. cholerae	–[b]	43	4	53	–	–	–	–
E. coli/pLqsA	–	–	–	22	–	**66**	6	6
L. pneumophila/pLqsA	–	–	–	3	4	**68**	16	9

[a] The strains used are *E. coli* BL21(DE3) containing pTS-21 (*lqsA*) or pTS-22 (*cqsA*), *L. pneumophila* JR32 containing pTS-2 (*lqsA*), and *V. cholerae* MM227.

[b] –, not detectable.

ples. The retention times of CAI-1 and LAI-1 were determined from the chromatograms of the corresponding $[M+H]^+$ mass peaks at *m/z* 215.201 and 243.241, respectively (Fig. 6*A*). Identities were confirmed by MS/MS analysis (data not shown).

In parallel, LC fractions of the same samples were collected and tested using the *V. cholerae* CAI-1 reporter strain MM920. The activity eluted as a single peak around 3.3 min for both the LqsA product as well as the CqsA product and correlated well with the elution time of the CAI-1 molecule (Fig. 6*B*). For the fraction containing LAI-1, no reporter activity was found. These data indicate that CAI-1 produced by either CqsA or

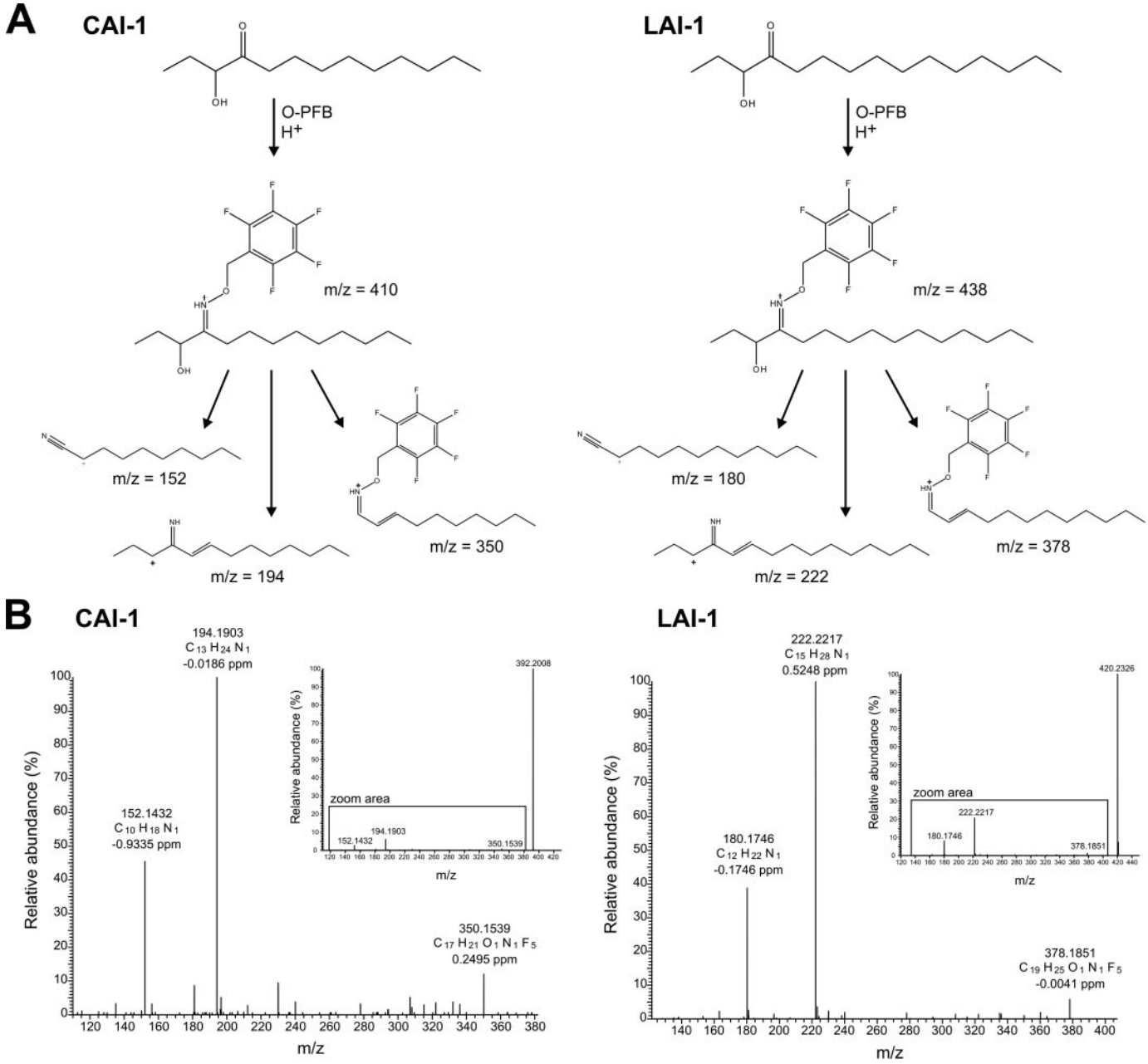

FIGURE 5. **Identification of LAI-1 as 3-hydroxypentadecan-4-one.** *A*, the chemical structures of CAI-1 and LAI-1 are shown. Treatment with O-PFB leads to the oxime adducts depicted, which will yield the proposed fragment ions upon collision-induced dissociation. *B*, the MS/MS spectra for the CAI-1 and LAI-1 oxime derivatives are depicted. The complete fragmentation spectra including the dominating but unspecific $[M-18+H]^+$ fragment ion are shown in the *insets*. The major peaks of the LqsA sample correspond well to the predicted fragmentation products and are analogous to the ions produced by fragmentation of CAI-1, confirming the identity of LAI-1. Each mass peak is labeled with the exact measured *m/z* value, the predicted elemental composition, and the difference between the measured and calculated *m/z* values in ppm, respectively.

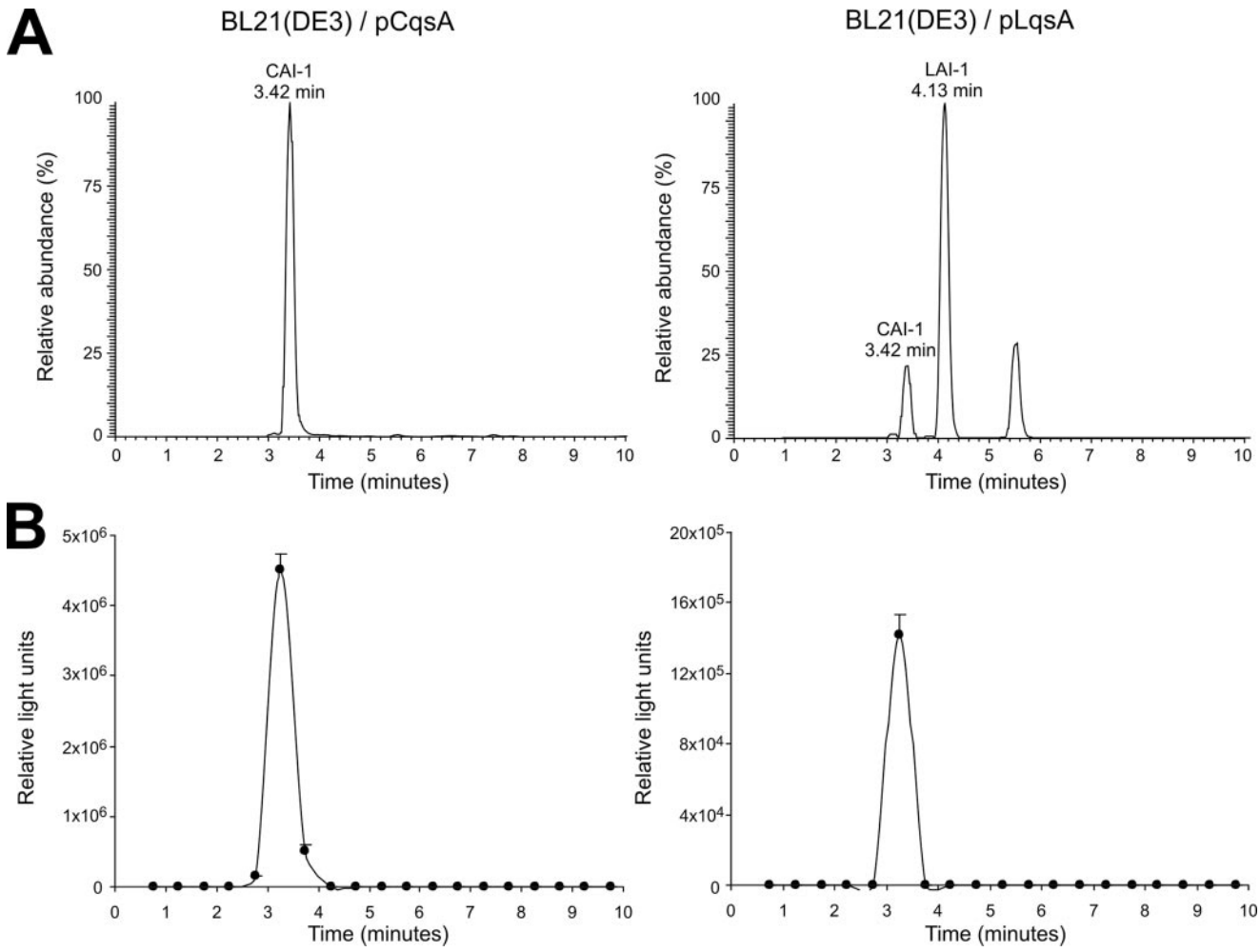

FIGURE 6. **Synthesis of active CAI-1 by CqsA and LqsA.** *A*, selected-ion chromatograms for CAI-1 and LAI-1 display the main products of CqsA and LqsA upon expression in *E. coli* BL21(DE3). The peaks representing the two main autoinducer molecules are labeled, and their elution times are indicated. The identity of CAI-1 and LAI-1 as hydroxyketones was confirmed by MS/MS (data not shown). *B*, activity of the eluted fractions was determined with the *V. cholerae* CAI-1 reporter strain MM920. In both samples the activity was exclusively retained in the fraction containing CAI-1, which indicates that LAI-1 does not contribute to the activation of the reporter. The *error bars* represent S.D. for duplicates.

LqsA triggers the reporter strain MM920, whereas the C_{15} α-hydroxyketone LAI-1 does not. Thus, the activity of LqsA samples on the *V. cholerae* reporter strain results from the synthesis of CAI-1 as a byproduct.

LqsA and CqsA Are Pyridoxal 5′-Phosphate-dependent Enzymes—LqsA is 27 or 23% homologous to the PLP-containing *E. coli* enzymes Kbl (2-amino-3-ketobutyrate-CoA ligase (45)) or BioF (8-amino-7-oxononanoate synthase (46, 47)), respectively. Moreover, LqsA shares a number of conserved amino acids forming the active site of *E. coli* BioF, including the PLP-binding site Lys-236 (supplemental Fig. S1 (46, 47)). To initially test whether PLP might be a cofactor required for the production of signaling molecules, we added the PLP precursor pyridoxine to growing cultures of *E. coli* expressing *lqsA*. Reporter activity increased 3.5-fold upon the addition of 10 μM pyridoxine, suggesting that PLP might be indeed a cofactor of LqsA (Fig. 7*A*).

Next, we addressed the question genetically by replacing with alanine the putative PLP binding amino acid, a conserved lysine residue. Neither the $LqsA_{K258A}$ mutant enzyme (or $LqsA_{K258S}$; data not shown) nor the $CqsA_{K236A}$ mutant produced any activity detectable by the *V. cholerae* reporter strain MM920 (Fig. 7*B*). The observed lack of signal activity was not due to an impaired production or stability of the mutant autoinducer synthases, as the proteins were produced at similar levels as the corresponding wild-type enzymes (supplemental Fig. S2). Moreover, in the 96-well plate setting described above (Fig. 3*C*), the emission of light by *V. cholerae* MM920 was abolished upon expression of either an $LqsA_{K258A}$ or $CqsA_{K236A}$ mutant enzyme by *E. coli* in the central wells (data not shown).

Finally, we confirmed a covalent binding of PLP to the conserved lysine residues of LqsA or CqsA by electrospray ionization-time of flight MS (Fig. 7*C*). Peaks corresponding to purified His-tagged LqsA or CqsA covalently binding PLP were identified for the wild-type enzymes but not for the mutant enzymes, where the conserved lysine was replaced by alanine. Compared with His-CqsA, lower amounts of His-LqsA were produced by *E. coli* (supplemental Fig. S2), and affinity-purified His-LqsA was unstable. The low amounts of His-LqsA-PLP available for MS yielded signals close to the detection limit, and therefore, additional peaks likely originating from contaminant proteins were detected. In contrast, purified His-CqsA was sta-

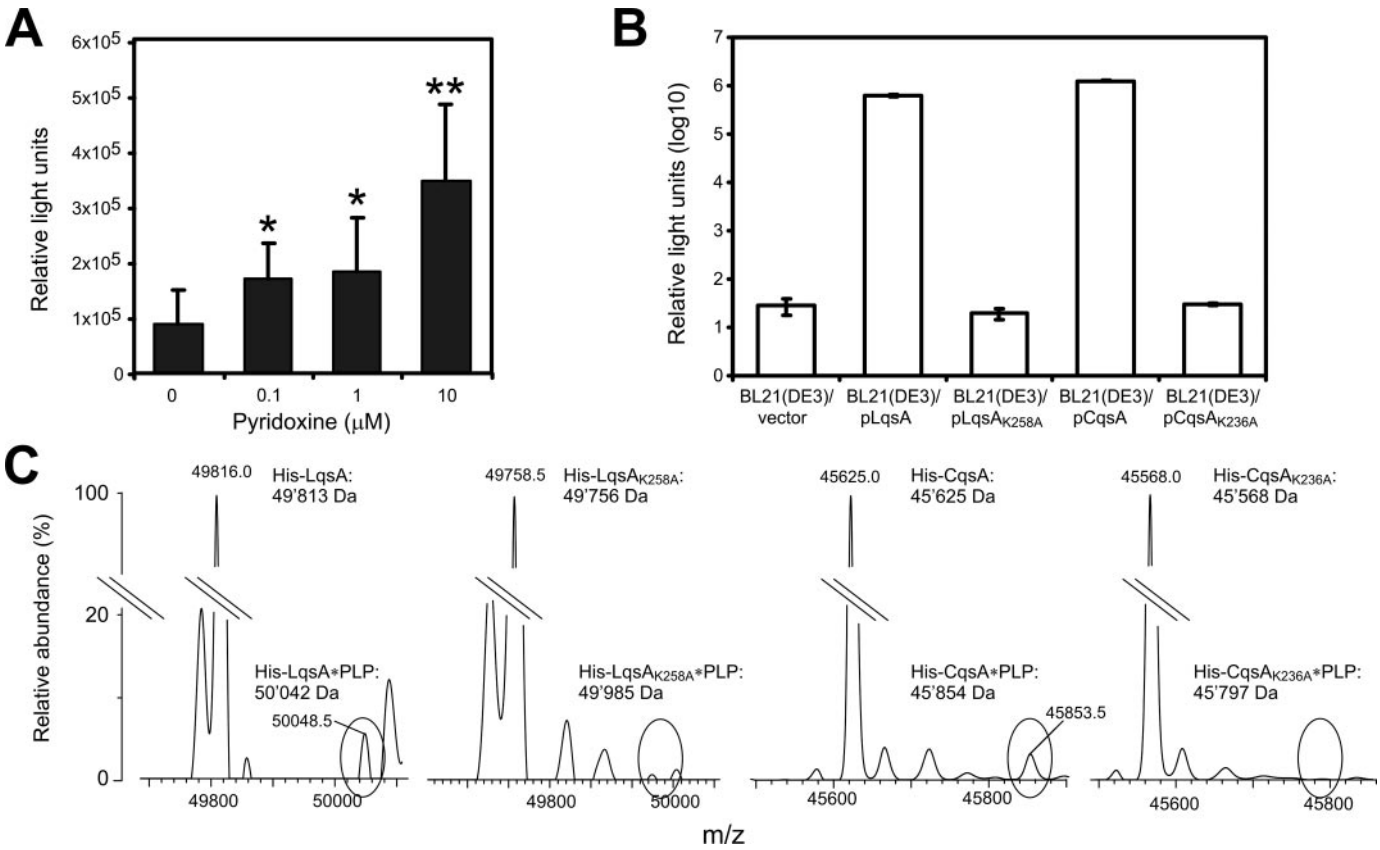

FIGURE 7. **LqsA and CqsA are pyridoxal 5′-phosphate-dependent enzymes.** *A*, the PLP precursor pyridoxine was added at the concentrations indicated to *E. coli* BL21(DE3) harboring plasmid pTS-21 (His-LqsA), and autoinducer activity in cell supernatants was determined by bioluminescence using the *V. cholerae* CAI-1 reporter strain MM920. The data shown are the means and S.D. of 10 samples. *Asterisks* denote the significance of differences relative to the untreated sample (*, $p < 0.01$; **, $p < 0.001$; two-tailed Student's *t* test). Similar results were obtained in three independent experiments. *B*, supernatants of *E. coli* strain BL21(DE3) harboring plasmid pTS-21 (pLqsA), pTS-22 (pCqsA), pTS-25 (pLqsA$_{K258A}$), pTS-26 (pCqsA$_{K236A}$), or plasmid pET-28a(+) (vector) were assayed by bioluminescence using *V. cholerae* MM920. The data shown are the means and S.D. of triplicates and are representative of three independent experiments. *C*, covalent binding of PLP to purified His-LqsA, His-LqsA$_{K258A}$, His-CqsA, or His-CqsA$_{K236A}$ was determined by electrospray ionization-time of flight-MS.

ble, yielding a strong and specific signal with the exact mass calculated for His-CqsA-PLP.

LqsA and CqsA might not only contain PLP and share sequence homologies with the PLP-dependent aminotransferases Kbl and BioF but also display functional similarities. Both Kbl and BioF catalyze the condensation of a small amino acid (Kbl, L-glycine; BioF, L-alanine) with an acyl-CoA moiety (Kbl, acetyl-CoA; BioF, pimeloyl-CoA), thereby liberating CoA-SH. In an attempt to identify the substrates and products of LqsA and CqsA, we assayed in lysates of *E. coli* producing His-LqsA or His-CqsA the release of CoA-SH upon incubation with small amino acids (L-glycine, L-alanine) and acyl-CoA moieties (acetyl-CoA, succinyl-CoA). However, none of the substrate combinations used led to the release of the putative product of the condensation reaction, CoA-SH (data not shown). Attempts using purified His-CqsA or His-LqsA were also not successful.

DISCUSSION

In this study we demonstrate that *L. pneumophila lqsA* functionally complements a *V. cholerae cqsA* mutant strain and produces a diffusible low molecular weight molecule, which is detected by a *V. cholerae* CAI-1 reporter strain (Figs. 3 and 4). These findings suggest that LqsA together with the putative cognate sensor kinase LqsS constitutes the first autoinducer system identified in *L. pneumophila*. Bioinformatic data base searches failed to identify in *L. pneumophila* several autoinducer systems present in other bacteria. These include systems signaling via AHLs, such as the homologues LuxR-LuxI (*Vibrio fisheri*), LasR-LasI (*Pseudomonas aeruginosa*), RhlR-RhlI (*P. aeruginosa*) or TraR-TraI (*Agrobacterium tumefaciens*) (32–34). In the simplest systems, the AHL autoinducer is directly bound by a LuxR-type response regulator containing a DNA binding helix-turn-helix motive. More complex quorum sensing systems that are also missing in *L. pneumophila* include the AHL-based LuxR-LuxN-LuxM system (*Vibrio harveyi*), consisting of a response regulator, a sensor kinase, and an autoinducer synthase, respectively. Finally, the widespread AI-2 system signaling via a furanosyl borate diester is apparently also absent from *L. pneumophila*. Components of the latter system include the autoinducer synthase LuxS, the periplasmic binding protein LuxP, and the sensor kinase LuxQ (32–34).

Only recently the *V. cholerae* CAI-1 activity was identified as (*S*)-3-hydroxytridecan-4-one, and the molecule was synthesized, thus proving to be the biologically most active form of the autoinducer (35). To identify LAI-1, we used LC coupled to high resolution MS. LAI-1 was identified as 3-hydroxypentadecan-4-one based on its retention time on a C_{18} column, specific ion fragmentation patterns, and comparison to data obtained

for CAI-1 (Fig. 5). Supernatants of *E. coli* or *L. pneumophila* producing LqsA triggered signaling by a *V. cholerae* CAI-1 reporter strain, and therefore, it was likely that molecules related or identical to CAI-1 are present in these samples. Reporter strain assays are highly sensitive and widely used tools for the detection of autoinducer molecules. However, due to their intrinsic specificity, these bioassays are often very selective and can neither provide a comprehensive profile nor an accurate quantification of signaling molecules (48, 49). To trigger signaling of the CAI-1 reporter strain, both backbone length and stereochemistry of α-hydroxyketones were found to be important, yet in addition to CAI-1 (C_{13}) smaller homologues (C_{12} and C_{11}), activated the *V. cholerae* quorum sensing circuit as well (35). In contrast, the C_{15} homologue of these hydroxyketones, LAI-1, did not trigger signaling by the CAI-1 reporter strain, and the active compound in samples containing LqsA products was CAI-1 (C_{13}) (Fig. 6). Therefore, *V. cholerae* CqsS apparently recognizes α-hydroxyketones with shorter but not longer linear hydrocarbon backbones than CAI-1.

LC-APCI-MS analysis of extracted supernatants allowed an estimation of the relative amounts of different α-hydroxyketones synthesized by LqsA and CqsA (Fig. 4, Table 1). We detected significant levels of the C_{11} homologue of CAI-1 (C_{13}) not only upon heterologous expression of *cqsA* in *E. coli* but also in the supernatant of wild-type *V. cholerae*. Thus, CqsA apparently shows a broad specificity and yields byproducts of different hydrocarbon length not only upon heterologous production in *E. coli* as reported previously (35) but also in the endogenous *V. cholerae* background. In supernatants of bacterial cultures expressing LqsA, LAI-1 (C_{15}) was the predominant molecule identified (66–68%). However, minor amounts of the C_{13}, C_{14}, C_{16}, and C_{17} homologues were also detected. Noteworthy, although upon expression of *lqsA* in *L. pneumophila* the C_{16} α-hydroxyketone was the second most abundant species (16%), upon expression of *lqsA* in *E. coli* the C_{13} compound (CAI-1) was the second most abundant signal molecule (22%). Taken together, both CqsA and LqsA do not show an exquisite specificity concerning the chain length of their products.

To our knowledge, 3-hydroxypentadecan-4-one has previously not been described in biology. Together with *V. cholerae* CAI-1, *L. pneumophila* LAI-1 forms a family of α-hydroxyketone autoinducer signaling molecules. Like the homoserine lactones, these α-hydroxyketones might represent two examples of an extended autoinducer family, and the substituents as well as the length of the side chain might define the signal specificity. Based on bioassay analysis and data base searches, CAI-1-mediated cell-cell communication was found to be predominant among the genus *Vibrio* (31). Yet in addition to *L. pneumophila* (Fig. 2) and *Vibrio* spp., *lqsA* and *lqsS* homologues are found in a number of environmental bacteria, including *Nitrococcus mobilis*, *Burkholderia xenovorans*, and *Polaromonas* spp. (29). Accordingly, intercellular signaling involving α-hydroxyketones might be common among different bacterial species and genera. *L. pneumophila* persists in biofilms in the environment (1, 50), where interspecies communication with any of these or other bacteria might be relevant. In this context the extended product spectrum of autoinducer synthases of the LqsA/CqsA family might allow interspecies communication with a more diverse group of bacteria.

Although α-hydroxyketone signaling molecules were readily detected by reporter assays and LC-MS in supernatants of *E. coli* and *L. pneumophila* expressing LqsA, these signals were not identified in wild-type *L. pneumophila* grown on plate or in liquid broth. Possible explanations for this observation include that (i) the amount of LAI-1 secreted by *L. pneumophila* is generally very low, (ii) LAI-1 secretion is tightly regulated and low only under the experimental conditions used, or (iii) LAI-1 is not secreted at all.

Perhaps, the threshold for LAI-1 signaling via LqsS is low, and thus, small amounts of LAI-1 are sufficient to efficiently promote cell-cell communication in a confined, possibly signal-impermeable environment such as the intracellular *Legionella*-containing vacuole. At the same time, the concentration of bacteria within *Legionella*-containing vacuoles is high, and accordingly, a considerable concentration of small signaling molecules might be achieved by a relatively small number of bacteria. Moreover, the production of LAI-1 and/or LqsA might be tightly regulated. It has been postulated that *Vibrio* spp. repress the production of CqsA (and consequently, CAI-1), as the expression of the *cqsA* gene alone yielded a stronger autoinducer signal than expression of a 25-kilobase chromosomal region including *cqsA* (31). Along this line, many genes were found to be poorly expressed in *L. pneumophila* broth cultures but are up-regulated upon infection of host cells (17). This is also true for *lqsA*, which is strongly expressed upon intracellular replication of *L. pneumophila* in amoebae (Fig. 2). Finally, under physiological conditions LAI-1 might represent an intracellular metabolite rather than, or in addition to, a secreted signaling molecule. A dual role in cell-cell signaling and metabolism has been proposed for the LuxS autoinducer synthase and its furanosyl borate product AI-2 (51, 52). In any case the characterization of metabolic, signaling and virulence phenotypes of an *L. pneumophila lqsA* mutant strain will shed light on the physiological functions of the corresponding enzyme.

LqsA is 27 or 23% identical to the PLP-containing *E. coli* enzymes Kbl (2-amino-3-ketobutyrate CoA ligase) and BioF (8-amino-7-oxononanoate synthase), respectively, and the autoinducer synthase shares a number of conserved amino acids forming the active site of *E. coli* BioF (supplemental Fig. S1). Based on biochemical and genetic approaches, both LqsA and CqsA were shown to also contain PLP as a cofactor (Fig. 7). The efficient synthesis of signaling molecules by *E. coli* expressing LqsA suggests that common metabolic intermediates are used as substrates for LAI-1 or that *E. coli* harbors additional enzymes required in a putative multistep enzymatic pathway leading to the production of the signaling molecule. Similar observations have been made for *V. cholerae* CqsA (31). However, using different small amino acids (L-glycine, L-alanine) and acyl-CoA moieties (acetyl-CoA, succinyl-CoA), we failed to identify substrates or products of a condensation reaction catalyzed by LqsA *in vitro*. PLP-dependent enzymes perform a vast repertoire of reactions, which is expected to also include novel catalytic activities (53, 54). Further *in vitro* studies are required to gain more insight into the biosynthetic mechanism

of α-hydroxyketone production by bacterial autoinducer synthases of the LqsA/CqsA family.

Acknowledgments—We thank Dr. Bonnie Bassler and Doug Higgins (Princeton University) for providing V. cholerae strains and advice on isolation of hydroxyketones as well as Dr. Christoph von Ballmoos (ETH Zürich) for help with extractions and Serge Chesnov (Functional Genomics Center Zürich) for electrospray ionization-time of flight-MS measurements of PLP-protein samples.

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