ORIGINAL ARTICLE

From deep-sea volcanoes to human pathogens: a conserved quorum-sensing signal in *Epsilonproteobacteria*

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Chemosynthetic *Epsilonproteobacteria* from deep-sea hydrothermal vents colonize substrates exposed to steep thermal and redox gradients. In many bacteria, substrate attachment, biofilm formation, expression of virulence genes and host colonization are partly controlled via a cell density-dependent mechanism involving signal molecules, known as quorum sensing. Within the *Epsilonproteobacteria*, quorum sensing has been investigated only in human pathogens that use the *luxS*/autoinducer-2 (AI-2) mechanism to control the expression of some of these functions. In this study we showed that *luxS* is conserved in *Epsilonproteobacteria* and that pathogenic and mesophilic members of this class inherited this gene from a thermophilic ancestor. Furthermore, we provide evidence that the *luxS* gene is expressed—and a quorum-sensing signal is produced—during growth of *Sulfurovum lithotrophicum* and *Caminibacter mediatlanticus*, two *Epsilonproteobacteria* from deep-sea hydrothermal vents. Finally, we detected *luxS* transcripts in *Epsilonproteobacteria*-dominated biofilm communities collected from deep-sea hydrothermal vents. Taken together, our findings indicate that the epsilonproteobacterial lineage of the LuxS enzyme originated in high-temperature geothermal environments and that, in vent *Epsilonproteobacteria*, *luxS* expression is linked to the production of AI-2 signals, which are likely produced *in situ* at deep-sea vents. We conclude that the *luxS* gene is part of the ancestral epsilonproteobacterial genome and represents an evolutionary link that connects thermophiles to human pathogens.

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Introduction

Epsilonproteobacteria have been recognized as an ecologically significant group of bacteria in deep-sea geothermal environments as they are highly represented in moderately warm (25–35 °C) and oxidized as well as in relatively hot (40–70 °C) and anoxic vents (Longnecker and Reysenbach, 2001; Nakagawa *et al.*, 2005; Campbell *et al.*, 2006; Moussard *et al.*, 2006; Huber *et al.*, 2007; Sievert and Vetriani, 2012). At deep-sea hydrothermal vents, chemosynthetic *Epsilonproteobacteria* mediate the transfer of carbon and energy from the geothermal source to higher trophic levels, functioning as primary producers.

The metabolic versatility observed in pure cultures of vent *Epsilonproteobacteria* (Campbell *et al.*, 2006; Sievert and Vetriani, 2012), along with their high growth rates (Campbell *et al.*, 2006) and ability to attach to substrates and form biofilms (Alain *et al.*, 2004), is likely to contribute to the ecological success of these bacteria in environments characterized by high turbulence and steep thermal and redox gradients. *Epsilonproteobacteria* also include human pathogens (for example, *Helicobacter* and *Campylobacter* spp., Supplementary Figure S1) (Nakagawa *et al.*, 2007), whose ability to colonize their hosts is related to high growth rates, attachment and biofilm formation (Stark *et al.*, 1999; Joshua *et al.*, 2006).

Quorum sensing (QS), a cell-to-cell communication mechanism that relies on cell density and the production of extracellular signaling molecules, is used by bacteria to control coordinated gene expression, including virulence genes and functions associated with biofilm formation and host colonization (Bassler *et al.*, 1994; Keller and Surette, 2006). One QS system that appears to be widespread across the bacterial domain (Figure 1a), and that has been proposed to function as the universal language for

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interspecies communication, is based on a furanone derivative, known as autoinducer-2 (AI-2), synthesized by the LuxS enzyme (Kaper *et al.*, 1999; Rader *et al.*, 2007). The LuxS enzyme is an integral component of the activated methyl cycle (AMC), the major methyl donor in the cell (Vendeville *et al.*, 2005; Turovskiy *et al.*, 2007). In addition to its role as AI-2 synthase, the main function of the LuxS enzyme in the AMC is the conversion of S-ribosylhomocysteine to homocysteine, with the production of 4,5-dihydroxyl-2,3-pentanedione (DPD) as a by-product of this reaction (Chen *et al.*, 2002; Winzer *et al.*, 2003). Through processes of dehydration and cyclization, DPD is rearranged into a mixture of chemically related molecules that function as AI-2 chemical signals in QS. These molecules include 4-hydroxy-5-methyl-3 (2H) furanone (MHF), (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxy-tetrahydrofuran (R-THMF) and furanosyl borate diester (S-THMF borate; (Schauder *et al.*, 2001; Winzer *et al.*, 2002; Chen *et al.*, 2002; Winzer *et al.*, 2003; Vendeville *et al.*, 2005; Rader *et al.*, 2007; Shen *et al.*, 2010).

The importance of *luxS*/AI-2 for the coordinated expression of virulence factors, biofilm formation and bioluminescence has been confirmed in a variety of microorganisms (Bassler *et al.*, 1994; Forsyth and Cover, 2000; Wright *et al.*, 2000; Elvers and Park, 2002; Shimizu *et al.*, 2002; Stevenson and Babb, 2002; Winzer *et al.*, 2002; McNab *et al.*, 2003; Rhee *et al.*, 2003). In *Epsilonproteobacteria*, the role of AI-2-mediated QS has been investigated exclusively in the human pathogens, *Helicobacter* and *Campylobacter* spp., where AI-2 secretion into the extracellular environment has been shown to occur by a LuxS-dependent mechanism (Forsyth and Cover, 2000; Joyce *et al.*, 2000; Elvers and Park, 2002; Rader *et al.*, 2007). In *Helicobacter pylori*, AI-2 modulates flagellar morphogenesis and gene expression in a dose-dependent manner (Rader *et al.*, 2007; Shen *et al.*, 2010; Rader *et al.*, 2011). Similarly, in *Campylobacter jejuni*, *luxS* mutants showed reduced motility and attachment during host colonization (Plummer *et al.*, 2012). LuxS-dependent motility/positioning patterns have been shown to be important virulence strategies required for colonization and dispersal of both *Helicobacter* and *Campylobacter* spp. in several animal models (Eaton *et al.*, 1996; O'Toole *et al.*, 2000; Ottemann and Lowenthal, 2002; Blaser and Atherton, 2004; Rader *et al.*, 2007; Plummer *et al.*, 2012).

As LuxS has been linked to the regulation of motility/colonization functions in pathogenic *Epsilonproteobacteria* (Eaton *et al.*, 1996; Forsyth and Cover, 2000; Joyce *et al.*, 2000; O'Toole *et al.*, 2000; Wright *et al.*, 2000; Elvers and Park, 2002; Rader *et al.*, 2007; Shen *et al.*, 2010; Plummer *et al.*, 2012), we investigated the distribution of the *luxS* gene in *Epsilonproteobacteria* and we reconstructed the evolutionary history of the epsilonproteobacterial LuxS lineage. Furthermore, we used *Sulfurovum*

lithotrophicum and *Caminibacter mediatlanticus* as model strains for mesophilic and thermophilic vent *Epsilonproteobacteria*, respectively, to investigate the expression of the *luxS* gene and the production of a QS signal during growth. Finally, we identified *luxS* transcripts in *Epsilonproteobacteria*-dominated chemosynthetic biofilms collected from active deep-sea hydrothermal vents.

Materials and methods

Organisms and growth conditions

S. lithotrophicum (DSM 23290^T; Inagaki *et al.*, 2004), a microaerobic, facultative anaerobic thiosulfate-oxidizing mesophile, was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Caminibacter mediatlanticus* strain TB-2 (DSM 16658^T), a strictly anaerobic, hydrogen-oxidizing thermophile was isolated and described previously in our laboratory (Voordeckers *et al.*, 2005). The two reporter strains, *Vibrio harveyi* strains BB170 and BB120 (Bassler *et al.*, 1993), were kindly provided to us by Dr Chikindas (Rutgers University, New Brunswick, NJ, USA) and Dr Bassler (Princeton University, Princeton, NJ, USA). *S. lithotrophicum* was grown anaerobically (N₂/CO₂, 80%:20%) at 30 °C in 356 MJ basal medium (Inagaki *et al.*, 2004) supplemented with 10 mM Na₂S₂O₃ • 5H₂O and 14 mM KNO₃ as electron donor and acceptor, respectively. *C. mediatlanticus* was grown at 55 °C in modified SME medium (Stetter *et al.*, 1983) supplemented with 14 mM KNO₃ under a gas phase of H₂/CO₂ (20%:80%). Strains of *V. harveyi* were grown in modified Autoinducer Bioassay (AB) medium (Turovskiy and Chikindas, 2006). Time course experiments for *S. lithotrophicum* and *C. mediatlanticus* were performed in 1 liter of culture medium in triplicate.

Autoinducer-2 bioassay

The AI-2 detection assay utilizes the *luxN*-null mutant, *V. harveyi* BB170, as a reporter strain that produces bioluminescence only in response to AI-2 QS signals (Bassler *et al.*, 1994). The assay was initiated by growing *V. harveyi* BB170 cultures for 16 h in modified Autoinducer-2 Bioassay (AB) medium (Turovskiy and Chikindas, 2006) with aeration at 30 °C. The cultures were diluted 1:5000 in fresh AB medium to obtain ~10⁵ CFUs per ml. Cell-free supernatant (CFS) preparations of *S. lithotrophicum* and *C. mediatlanticus* were added to the diluted *V. harveyi* to a 10% v/v final concentration, and these cultures were shaken (140 r.p.m.) at 30 °C for 5 and 6 h, respectively. Bioluminescence measurements were taken every 30 min with a Luminoscan TL plus luminometer (ThermoFisher Scientific, Waltham, MA, USA). Uninoculated *S. lithotrophicum* and *C. mediatlanticus* media were used as negative controls, whereas the CFS from the wild-type *V. harveyi* strain BB120 was used as a

positive control. All bioluminescence induction values obtained from the three independent growth experiments for each bacterium are averages of duplicate AI-2 bioassays.

The activity of AI-2 is expressed in relative bioluminescence units. The relative bioluminescence units were normalized by subtracting the light intensity values of the negative control from the light

intensity values of all samples (including the positive controls) in the assays. The relative bioluminescence unit values were expressed for both *S. lithotrophicum* and *C. mediatlanticus* as percentages of bioluminescence induction, where each measured time point was divided by the maximum bioluminescence induction value obtained from each bacterium, including the positive controls.

The CFSs of *S. lithotrophicum* and *C. mediatlanticus* were prepared from 10 ml of cell suspensions at different stages during the time course growth experiments. The CFS of *V. harveyi* BB120 (positive control) was prepared from cell suspensions of approximately OD₆₀₀ of 0.5–0.6. All cell suspensions were filtered through a 0.2 µm-pore-size filter, centrifuged at 6000 × g for 30 min (Nalgene Nunc International, Rochester, NY, USA) and transferred into new tubes. Samples were stored at –20 °C until further processing.

Microscopy and cell counts

Cells were routinely stained with 0.1% acridine orange and visualized with an Olympus BX 60 microscope (Center Valley, PA, USA) with an oil immersion objective (UPlanF1 100/1.3). Samples for direct cell counts were collected to monitor cell growth in the time course experiments and at different time points during the AI-2 bioassay to determine the number of *V. harveyi* BB170 cells per ml (Supplementary Table S2).

RNA extraction and quantitative reverse transcription PCR (qRT-PCR) analyses of *luxS* transcripts in model organisms

Total RNA was isolated from *S. lithotrophicum* and *C. mediatlanticus* cultures at different phases during growth. Then, 75 ml of *S. lithotrophicum* cultures and 50 ml of *C. mediatlanticus* cultures were preserved in a 1:1 volume of RNeasy lysis buffer (Qiagen, Valencia, CA, USA) and stored at –80 °C until further processing. RNA was extracted from cell pellets using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and treated with TURBO DNase (Ambion), as recommended by the manufacturer. The quantity and quality of the RNA obtained was evaluated both spectrophotometrically on a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific) and by agarose gel electrophoresis.

Absolute quantification of *luxS* and 16S rRNA transcripts was carried out by qRT-PCR using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA, USA) and the real-time detection system iCycler iQ (Bio-Rad). Reactions,

each in triplicate, were carried out according to the manufacturer's instructions, using 0.15 µg of total RNA for *S. lithotrophicum* and 0.3 µg of total RNA for *C. mediatlanticus* in 15 µl reactions. Primers were manually designed and analyzed using the Oligo Analyzer 3.1 from IDT (Integrated DNA Technologies, Coralville, IA, USA) to have a composition suitable for use in qRT-PCR (Supplementary Table S3). Specificity of each pair of primers was confirmed by PCR amplification using the genomic DNA of the model organisms followed by sequencing. The efficiency of the PCR amplifications was determined from the slopes of the dilution curves of the target RNA.

Standard curves for the *luxS* and 16S rRNA genes were constructed using dilutions (range of standards: 10³–10⁷ copies per ml for *S. lithotrophicum* and *C. mediatlanticus* in a 15 µl reaction) of the corresponding PCR amplicon (Chini *et al.*, 2007; Cuebas *et al.*, 2011) using the iQ SYBR Green Supermix (Bio-Rad). The cycle threshold (C_t) values obtained were used to calculate the absolute number of transcript copies per ng of RNA for both strains (Supplementary Tables S4 and S5). The number of 16S rRNA transcripts was monitored as the internal standard to ensure that equal amounts of RNA were used in each reaction and to normalize the levels of *luxS* transcripts across samples (Supplementary Figure S2). Values for 16S rRNA and *luxS* transcript copies per ng presented here are an average of triplicate qRT-PCR experiments for the three independent growth experiments carried out for each model organism. Negative controls omitting the templates were included.

Sampling of natural biofilms from deep-sea hydrothermal vents

Samples of microbial biofilms were collected during R/V *Atlantis* cruises AT 15-15 (January–February 2007) and AT 15-28 (December 2007–January 2008) using the Deep-Submergence Vehicle *Alvin*. Both biofilms used in this study were collected using experimental microbial colonization devices made of stainless steel mesh. The first biofilm (designated as low T biofilm) was collected from a diffuse flow vent with no associated megafauna ('Mk 33', fluid temperature: 10–28 °C, depth 2506 m, East Pacific Rise, 9° 50' N, 104° 18' W). The second biofilm (designated as high T biofilm) was collected from the wall of a sulfide structure colonized by *Alvinella pompejana* tubeworms venting 20–50 °C fluids

Figure 1 (a) Distribution of the *luxS* gene among phyla of the domain Bacteria. The tree was constructed using the neighbor-joining method from the 16S ribosomal RNA genes of 469 representative bacterial species. The *luxS* homologs were identified in 167 *Beta*-, *Delta*-, *Gamma*- and *Epsilonproteobacteria*, *Firmicutes*, *Fusobacteria*, *Actinobacteria*, *Deinococcus/Thermus*, *Spirochaetes* and *Bacteroidetes/Chlorobi* (branches shown in bold face). The number of *luxS* genes identified in each phylum is indicated in parenthesis, followed by the total number of genomes surveyed. The bar represents 0.10 base substitutions per nucleotide. The asterisks indicate branches that include *Firmicutes*. (b) Phylogenetic tree of *LuxS* amino acid sequences of *Epsilonproteobacteria*. The tree was constructed with Bayesian inference and maximum likelihood methods. The genera of the organisms within each collapsed branch are shown. Supplementary Table S6 lists all the organisms used in this analysis. The ancestral nodes A, B and C are shown as solid circles. Posterior probability values that support each topological element are shown at the branch nodes. Bar, 20% estimated substitutions.

(‘Jumeaux’ site, depth 2621 m, East Pacific Rise, 12° 48 N, 103° 56 W).

Vent natural biofilms: RNA extraction, RT-PCR

detection of luxS transcripts and library construction

Samples of the low T and high T biofilms were resuspended in RNAlater (Ambion) and stored at –80 °C. Total RNA was extracted from the biofilm samples using the RNeasy kit (Qiagen) and the DNase-treated RNA (0.5 µg) was reversed transcribed in 25 µl reactions using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Two sets of primers, the first based on the *luxS* genes of *S. lithotrophicum* and *Sulfurimonas denitrificans* (SL/SD *luxS* primers; Supplementary Table S3) and the second based on the *luxS* genes of *C. mediatlanticus* and *Nautilia profundicola* (CM/NP *luxS* primers; Supplementary Table S3), were designed and used in RT-PCR reactions to amplify the *luxS* gene transcripts from the low T and high T biofilms. Both primer sets were used to amplify *luxS* transcripts in the biofilm samples.

Touchdown PCR conditions for all *luxS* amplifications were as follow: an initial denaturation for 5 min at 94 °C, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 44 °C for 1 min, extension at 72 °C for 1 min and 30 s, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 1 min, extension at 72 °C for 1 min and 30 s and a final extension of 5 min at 72 °C.

The amplified *luxS* transcripts were gel-purified using the MinElute Gel Extraction Kit (Qiagen), cloned into the pCR4-TOPO plasmid vector and the ligation products were transformed into *Escherichia coli* One Shot TOP10 Competent Cell using the TOPO TA Cloning Kit for Sequencing (Invitrogen). In all, 52 and 46 randomly chosen clones from the two *luxS* environmental libraries were analyzed, respectively, for insert-containing plasmids by direct PCR followed by gel electrophoresis of the amplified products. The resulting PCR fragments were screened by restriction fragment length polymorphism analysis using the *RsaI* restriction endonuclease (Boehringer Mannheim, Mannheim, Germany). The restriction fragment length polymorphism reactions were analyzed on a 3% MetaPhor Agarose (Lonza, Rockland, ME, USA) gel. Representative *luxS* genes with unique restriction fragment length polymorphism patterns were purified using the MiniPrep Plasmid Purification Kit (Qiagen) and sequenced.

PCR amplification of the luxS gene from reference strains

Genomic DNA extracted from 10 ml cultures of *Caminibacter* sp. strain TB1 (Voordeckers *et al.*, 2005), *Nautilia* spp. strains MT3 and MT4 (Voordeckers *et al.*, 2008), *Nautilia nitritireducens* strain MB-1 (Perez-Rodriguez *et al.*, 2010) and *S. lithotrophicum* (Inagaki *et al.*, 2004) was used for

the amplification of partial *luxS* genes using the *luxS* primers CM/NP and SL/SD (*Caminibacter/Nautilia* and *Sulfurovum/Sulfurimonas* spp., respectively; Supplementary Table S3) and the touchdown PCR conditions described above. Long-term stocks of these reference strains, prepared by adding 50 µl of dimethyl sulfoxide to 1 ml of liquid cultures, are stored at –80 °C. Live cultures are available upon request.

Phylogenetic analyses

The distribution of the *luxS* gene within the *Bacteria* was investigated in 465 representative taxa within the bacterial domain by genome search or PCR amplification in reference strains. A phylogenetic tree based on the 16S ribosomal RNA genes of the taxa surveyed was then constructed using the ARB neighbor-joining method (<http://www.arb-home.de/home.html>; Figure 1a).

The phylogenetic tree in Figure 1b was constructed by aligning trimmed LuxS amino acid sequences from GenBank, using Muscle (Edgar, 2004) and Gblocks (Castresana, 2000). Trees were reconstructed with Bayesian inference and maximum likelihood methods. The maximum likelihood phylogeny was inferred from the alignment using PHYML (Guindon and Gascuel, 2003) with the CpREV substitution model and 1000 bootstrap resamplings. The substitution model was selected based on AIC values using ProTest3 (Darriba *et al.*, 2011). Bayesian phylogeny was inferred using MrBayes (Ronquist and Huelsenbeck, 2003) performing 500 000 generations with two parallel searches with the CpREV amino acid matrix model (selected using forward selection among all possible substitution models) and a burn-in of 125 000 generations. The tree was rooted using the mid-point convergence method and manually using LuxS from Gram-positive bacteria as the outgroup. Both rooting methodologies gave the same results. The organisms used in the phylogenetic tree reconstruction shown in Figure 1b are listed in Supplementary Table S6. Ancestral state reconstruction of the LuxS sequence was performed using the MEGA package (Tamura *et al.*, 2013) using the maximum likelihood option with the CpREV amino acid matrix model of substitution.

For the construction of the phylogenetic tree shown in Figure 3, the *luxS* sequences obtained by PCR from reference strains and natural vent biofilm communities were translated into amino acid sequences using EMBOSS Transeq (<http://www.ebi.ac.uk/emboss/transeq/>), aligned using ClustalX v 1.8 (Thompson *et al.*, 1997) and manually adjusted using Seaview (Galtier *et al.*, 1996). Phylogenetic distances were calculated using the CpREV substitution model and the maximum likelihood method to evaluate tree topologies and their robustness was tested by bootstrap analysis with 100 resamplings.

Nucleotide sequence accession numbers

The *luxS* sequences from this study are available through GenBank under the following accession numbers: KF876791-KF876794 for *luxS* genes in *Caminibacter* sp. strain TB1, *Nautilia* sp. strain MT3, *Nautilia* sp. strain MT4 and *N. nitratireducens* strain MB1; KF876760-KF876777 for *luxS* transcripts obtained from the low T biofilm; and KF876778-KF876790 for *luxS* transcripts obtained from high T biofilm.

Results

Distribution of the LuxS enzyme in the bacterial domain and reconstruction of the evolutionary history of the epsilonproteobacterial LuxS lineage

In this study we investigated the distribution of the *luxS* gene by searching representative genomes within the bacterial domain and we identified *luxS* gene homologs in 167 out of 469 bacterial taxa (Supplementary Tables S7 and S8). Representative species coding for *luxS* were found within the Beta- (5%), Delta- (9%), Gamma- (55%) and *Epsilonproteobacteria* (100%), *Firmicutes* (63%), *Fusobacteria* (60%), *Actinobacteria* (24%), *Deinococcus/Thermus* (100%), *Spirochaetes* (40%) and *Bacteroidetes/Chlorobi* (13%) (Figure 1a and Supplementary Table S7).

Bayesian phylogenetic analyses of the LuxS enzyme of *Epsilonproteobacteria* indicated that the lineage found in pathogenic, commensal and mesophilic *Epsilonproteobacteria* shared a common ancestor with members of the order *Nautiliales* (node A in Figure 1b; 100% posterior probability) and with *Nitratiruptor* sp. (node B in Figure 1b). All members of the *Nautiliales* and *Nitratiruptor* sp. are deep-sea vent thermophilic chemolithoautotrophs (Campbell et al., 2006; Sievert and Vetriani, 2012) (Figure 1b and Supplementary Table S6). A reconstruction of the amino acid sequence of the LuxS ancestral state indicated that the sequence corresponding to node A (Figure 1b) was 83.1% and 72.3% identical to the LuxS of *C. mediatlanticus* and *Campylobacter fetus*, respectively.

All the mesophilic *Epsilonproteobacteria*, which include environmental species of the genera *Sulfurovum*, *Sulfurimonas*, *Arcobacter* and *Sulfurospirillum*, the commensal organism *Wolinella succinogenes* and the pathogenic species, *Campylobacter* and *Helicobacter*, shared a common ancestor (node C in Figure 1b) nested within the thermophilic lineage. However, the topology of this section of the LuxS tree showed a polytomy; hence the evolutionary relationships between mesophilic, commensal and pathogenic *Epsilonproteobacteria* could not be resolved. Interestingly, a group of LuxS sequences found in members of the *Gammaproteobacteria* (including species of the genera *Vibrio*, *Photobacterium*, *Salmonella*, *Escherichia* and *Enterobacter*) clustered with the *Arcobacter* lineage and

nested within a group of *Campylobacter* sequences (*Campylobacter* group 1 in Figure 1b). Finally, our analysis showed that the LuxS enzyme of *H. pylori* was related to the Gram+ lineage (Figure 1b).

Quantitative detection of luxS transcripts and AI-2 activity in S. lithotrophicum and C. mediatlanticus

We carried out a time course experiment and quantified *luxS* transcripts at different phases during the growth cycle of these bacteria (Figures 2a and d). We found that in both *S. lithotrophicum* and *C. mediatlanticus*, the expression of the *luxS* gene was highest during the mid-exponential phase of growth and gradually dropped to baseline levels during the stationary phase (Figures 2b and e). The drop in *luxS* transcripts was ~336-fold in *S. lithotrophicum* from 30–100 h of growth and ~59-fold in *C. mediatlanticus* from 8 to 40 h of growth (Figures 2b and e).

To establish whether *S. lithotrophicum* and *C. mediatlanticus* produced an AI-2-like QS signal during the time course experiment, we carried out an AI-2 detection assay using *V. harveyi* strain BB170, a reporter strain that produces bioluminescence in response to AI-2 QS signals (Bassler et al., 1994; Turovskiy et al., 2007). Indeed, supplementing cultures of *V. harveyi* BB170 with CFSs of *S. lithotrophicum* and *C. mediatlanticus* induced a bioluminescence response (Figures 2c and f). In both microorganisms, the observed bioluminescence induction increased with cell densities during growth (Figures 2c and f). Maximum bioluminescence induction (expressed in relative bioluminescence units) was observed during the stationary phase of growth, at 68.7 ± 8.1 h for *S. lithotrophicum* and at 26 ± 3.5 h for *C. mediatlanticus* (Supplementary Table S1). In fact, bioluminescence induction in *V. harveyi* BB170 was significantly correlated to cell density in both *S. lithotrophicum* ($r=0.60$, $P<0.01$, $n=25$) and *C. mediatlanticus* ($r=0.63$, $P<0.01$, $n=33$). Bioluminescence induction using the CFSs of *V. harveyi* strain BB120 (wild type) as a positive control was 2.5- and 2.2-fold higher than that measured when the CFSs from *S. lithotrophicum* and *C. mediatlanticus* were used, respectively (Supplementary Table S2). The net positive bioluminescence induction measured in *V. harveyi* BB170 in response to the CFSs of *S. lithotrophicum* and *C. mediatlanticus* indicated that growth of these organisms was necessary for the synthesis of AI-2-like molecules and to elicit a bioluminescence response in the reporter strain.

When changes in *luxS* transcription and bioluminescence induction at different times during growth were compared, a negative correlation was established between the two variables. Expression of the *luxS* gene (Figures 2b and e) in both *S. lithotrophicum* ($r(24) = -0.68$, $P<0.01$) and *C. mediatlanticus* ($r(31) = -0.54$, $P<0.01$) is inversely correlated to bioluminescence induction in *V. harveyi* (Figures 2c and f).

Figure 2 Growth curves for *S. lithotrophicum* (a) and *C. mediatlanticus* (d) cultured under nitrate-reducing conditions. Expression of the *luxS* gene (filled symbols) and induction of bioluminescence (open symbols) in *S. lithotrophicum* (b, c) and *C. mediatlanticus* (e, f). Exponential (E), stationary (S) and death (D) growth phases are labeled within each graph. All experiments were carried out in triplicates.

Detection of *luxS* transcripts in biofilm communities from deep-sea hydrothermal vents

Following our finding that laboratory strains of vent *Epsilonproteobacteria* express the *luxS* gene during growth and elicit a QS response in a *V. harveyi* reporter strain, we hypothesized that the *luxS* gene was expressed *in situ* at deep-sea vents during the formation of microbial biofilms. To this end, we collected recently established biofilms (3 to 7 days

old) from two vents located on the East Pacific Rise at 9° and 13° North, respectively, that were characterized by different thermal and biological regimes. Both the low T and high T biofilms were dominated by *Epsilonproteobacteria* (93.1% and 86.6%, respectively; data not shown). The *luxS* transcripts related to *Epsilonproteobacteria* were amplified by RT-PCR from RNA extracted from the biofilm communities. However, *luxS* transcripts

from the low T biofilm were detected only when the SL/SD primers were used, whereas *luxS* transcripts from the high T biofilm were obtained only with the CM/NP primers. Phylogenetic analysis of the amino acid sequences of the LuxS enzyme deduced from RT-PCR-amplified transcripts showed that the high T biofilm expressed enzymes related to *C. mediatlanticus*, *Caminibacter* sp. strain TB-1 and *N. nitratireducens* (Figure 3; 98–99% amino acid identity to LuxS of *C. mediatlanticus*). In contrast, the LuxS retrieved from the low T biofilm was distributed in three clusters. The majority of the sequences (cluster 1 in Figure 3) were grouped in three subclusters with 82–83% amino acid identity to the LuxS of *S. lithotrophicum*. Two sequences were related to the enzyme of *S. lithotrophicum* and *Sulfurovum* sp. strain NBC37-1 (low T biofilm A6 and B8; 88% and 87% identity to the enzyme of *S. lithotrophicum*, respectively), whereas a single sequence (low T biofilm C12) was placed in a discrete cluster (83% identity to the LuxS of *S. lithotrophicum*).

Discussion

Thermophilic ancestry of the LuxS lineage and luxS gene flow in Epsilonproteobacteria

Bayesian reconstruction of the LuxS evolutionary history indicated a thermophilic origin of the epsilonproteobacterial lineage (Figure 1b). This finding is supported by the observation that the LuxS sequences from mesophilic, commensal and pathogenic *Epsilonproteobacteria* are nested within the thermophilic clades and that the ancestral state reconstruction of the LuxS sequence (corresponding to node A in Figure 1b) showed higher amino acid identity to the enzymes found in modern thermophiles than those found in modern mesophiles. As all members of the *Nautiliales* and *Nitratiruptor* sp. are thermophiles, the most parsimonious interpretation of the data is that the ancestral LuxS proteins represented by nodes A and B were in thermophilic *Epsilonproteobacteria*, and that a more recent ancestor was transferred to mesophilic *Epsilonproteobacteria* between nodes B and C (Figure 1b). This interpretation is in line with phylogenomic and 16S rRNA gene-based reconstructions of the epsilonproteobacterial tree that showed the *Nautiliales* as the first line of divergence (Zhang and Sievert, 2014). Overall, our data suggest that the epsilonproteobacterial *luxS* lineage originated in geothermal environments. However, the direction of the *luxS* gene flow among mesophilic *Epsilonproteobacteria* (for example, from environmental strains to commensals/pathogens of warm-blooded animals or vice versa) was less defined and remains unresolved (Figure 1b).

Our phylogenetic analysis revealed two events of *luxS* horizontal gene transfer within the *Epsilonproteobacteria*. *H. pylori* appears to have acquired the gene from *Enterococcus faecium*, a Gram-positive bacterium that, like *H. pylori*, inhabits the human

gastrointestinal tract (Figure 1b). This finding is in line with previous studies that showed that the LuxS from *H. pylori* was related to the enzyme found in members of the *Firmicutes* (Lerat and Moran, 2004; Doherty et al., 2010). Moreover, marine *Gammaproteobacteria* of the genera *Photobacterium* and *Vibrio*, along with *E. coli* and other human-associated bacteria, appear to share a common *luxS* ancestor with *Arcobacter nitrofigilis* and *A. butzleri*, a marine organism and a human pathogen, respectively (Figure 1b and Supplementary Table S6). These findings indicate that bacteria inhabiting similar ecological niches, regardless of their taxonomic distance, share closely related forms of the *luxS* gene. Because habitat and niche play an important role in horizontal gene transfer (Polz et al., 2013), we suggest that inter-species QS within the same habitat is an important driver in the evolution of *luxS*.

luxS expression and AI-2 activity in laboratory cultures of vent Epsilonproteobacteria

Here we present one of the few studies on how the expression of the *luxS* gene relates to AI-2 activity. In *Salmonella typhimurium*, the constitutive expression of *luxS* suggests that AI-2 production is regulated by the availability of the LuxS substrate, S-ribosylhomocysteine (Beeston and Surette, 2002). In contrast, the expression of *luxS* in *E. coli* and *Edwardsiella* spp. is under the control of catabolite repression, and it is correlated with growth and with AI-2 activity levels (Wang et al., 2005; Zhang et al., 2008; Zhang and Sun, 2012). In comparison, the number of *luxS* transcripts and AI-2 extracellular activity levels in *S. lithotrophicum* and *C. mediatlanticus* showed highest *luxS* expression during the mid and late exponential phases of growth, whereas maximum AI-2 activity levels occurred during the stationary phase (Figures 2b–f). This temporal uncoupling between *luxS* expression and AI-2 activity levels is conserved in both vent strains and may be indicative of a mechanism of feedback inhibition of *luxS* expression, perhaps controlled by the concentration of AI-2.

LuxS function in vent Epsilonproteobacteria: QS vs AMC

The dual function of LuxS as AI-2 synthase and in recycling methionine via the homocysteine precursor in the AMC cannot be ignored when investigating its role in new species. However, in geothermal environments, where reduced sulfur is not limiting, the *de novo* synthesis of methionine is thought to be energetically inexpensive; hence, the function of LuxS in recycling methionine may not be as critical as in other habitats (Winzer et al., 2003). This consideration suggests that *luxS* expression in vent *Epsilonproteobacteria* may be more directly involved in AI-2 production rather than in recycling

Figure 3 Maximum likelihood phylogenetic tree inferred from the amino acid sequence deduced from a fragment of the *luxS* gene transcripts obtained from the high and low T biofilm communities collected from deep-sea hydrothermal vents on the East Pacific Rise. The tree shows the position of the biofilm-derived *LuxS* sequences relative to that of pure cultures of *Epsilonproteobacteria*. The model organisms, *Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum*, are shown in bold face. Percentages > 50% of 100 bootstrap resampling that support each topological element are shown at the branch nodes. Bar, 10% estimated substitutions.

methionine. Moreover, the ability of *H. pylori* to synthesize cysteine under an incomplete AMC via the reverse transsulfuration pathway further suggests the importance of *LuxS* in AI-2 production in *Epsilonproteobacteria* (Doherty *et al.*, 2010; Shen *et al.*, 2010).

Although we have not identified the specific furanone derivative responsible for the signaling activity that we measured in our experiments,

previous studies showed that the precursor of AI-2, DPD, spontaneously cyclizes into different isomers, including S-THMF borate with AI-2 activity in *V. harveyi*, R-THMF with AI-2 activity in *S. typhimurium* and the less active metabolic by-product, MHF (Feather, 1981; Nedvidek *et al.*, 1992). In particular, MHF has been shown to exhibit at least a 10-fold reduction in activity compared with S-THMF borate when tested as a QS-inducing

molecule in *V. harveyi* reporter strains (Feather, 1981; Nedvidek *et al.*, 1992; Schauder *et al.*, 2001; Winzer *et al.*, 2003). The bioluminescence induced by *S. lithotrophicum* and *C. mediatlanticus* was very similar (2.5- and 2.2-fold lower, respectively) to that induced by the CFSs of the wild-type *V. harveyi* strain BB120, suggesting that the CFSs of the vent strains contained an active form of AI-2-like molecule with a much higher activity than MHF. Furthermore, as bioluminescence induction by *S. lithotrophicum* and *C. mediatlanticus* was measured using similar concentration of *V. harveyi* cells in all the assays (Supplementary Table S2), our results indicate that the gradual increase in bioluminescence induction during growth (Figures 2a and d) reflected the increasing production of AI-2-like molecules in the CFS of the two strains (Figures 2c and f). We argue that the significant correlation between AI-2 activity levels and cell density in our model organisms could, in principle, provide a measure of population density.

As no AI-2 receptors have yet been identified in *Epsilonproteobacteria*, the complete *luxS*/AI-2 QS circuit remains unclear. Two main classes of AI-2 receptors have been characterized: the LuxP family receptors, found in *Vibrio* spp. (Chen *et al.*, 2002), and the LsrB family, found in *S. typhimurium* and other enteric bacteria (Miller *et al.*, 2004). In addition, the AI-2-binding capability has been demonstrated in RbsB-like receptors of the ribose binding protein family (Armbruster *et al.*, 2011). However, no homologs to these receptors have been found in *Epsilonproteobacteria*. Hence, other receptors must exist, as responses to AI-2 have been reported in bacteria lacking LuxP, LsrB and RbsB receptors. For instance, the established role of *luxS*/AI-2 in cell–cell signaling in *H. pylori* in the absence of LuxP, LsrB or RbsB homologs suggests the existence of yet unidentified receptors (Doherty *et al.*, 2010). This is further supported by the small sequence similarity between LuxP, LsrB and RbsB (Pereira *et al.*, 2009) that likely evolved independently while converging toward the same function.

Vent *Epsilonproteobacteria* express the *luxS* gene *in situ* during the formation of biofilms

We demonstrated that the *luxS* gene was expressed *in situ* in two biofilms from active vents characterized by different thermal regimes. The *luxS* gene transcripts that we identified in these biofilms reflected the distribution of specific taxa of *Epsilonproteobacteria* along the thermal gradient. Transcripts related to *Sulfurovum* and *Sulfurimonas* spp., two sulfur-oxidizing mesophiles, were detected in the low T biofilm, whereas transcripts related to *Caminibacter* and *Nautilia* spp., two hydrogen-oxidizing thermophiles, were identified in the high T biofilm (Figure 3). In light of our finding that in *S. lithotrophicum* and *C. mediatlanticus* the expression the *luxS* gene is correlated to AI-2-like activity and

to a QS response in *V. harveyi*, the detection of epsilonproteobacterial *luxS* transcripts in vent microbial biofilms implies that AI-2 signals are produced *in situ*. It is reasonable to hypothesize that, similar to what has been reported in *H. pylori* and *C. jejuni*, AI-2 activity in vent *Epsilonproteobacteria* might be involved in controlling the genes involved in flagellar motility, adhesion and other functions associated with the formation of biofilms.

Concluding remarks

Epsilonproteobacteria are a versatile group of bacteria that include chemolithoautotrophic thermophiles and mesophiles inhabiting marine geothermal environments, symbionts of invertebrates, commensals of mammals and human pathogens (Dubilier *et al.*, 2008; Ruehl and Dubilier, 2010). Identifying the genes encoded by the core—or ancestral—genome shared by *Epsilonproteobacteria* may reveal how these microorganisms evolved to colonize very diverse habitats. The *luxS* gene is part of the epsilonproteobacterial core genome and represents an evolutionary link that connects vent thermophiles to human pathogens. Why is *luxS*/AI-2, rather than other QS mechanisms, conserved in *Epsilonproteobacteria*? These bacteria live in environments, such as the deep-sea vents, the rumen of cows and the human stomach, characterized by harsh physicochemical conditions. DPD, the precursor of AI-2, is very stable over a broad pH range, whereas other QS signaling molecules, such as acylhomoserine lactones and autoinducing peptides, appear to have inherent susceptibility to hydrolytic degradation (Globisch *et al.*, 2012). In line with this hypothesis, a study where the vent hyperthermophiles *Thermotoga maritima* and *Pyrococcus furiosus* were found to produce AI-2 via a *luxS*-independent biotic/abiotic reaction pathway also evidenced the stability of AI-2 at elevated temperature (Nichols *et al.*, 2009). Hence, it is possible that the chemical stability of DPD has played a role in the selection of AI-2 as a chemical signal in *Epsilonproteobacteria*.

Although it is known that *luxS*/AI-2 regulates functions associated with biofilm formation in pathogenic *Epsilonproteobacteria* (Eaton *et al.*, 1996; O'Toole *et al.*, 2000; Ottemann and Lowenthal, 2002; Blaser and Atherton, 2004; Rader *et al.*, 2007; Plummer *et al.*, 2012), its involvement in attachment and colonization of substrates in geothermal environments remains unknown. Future efforts leading to the development of a genetic system in vent *Epsilonproteobacteria* will provide the necessary tools to further understand the *luxS*/AI-2 QS system in geothermal environments.

Conflict of Interest

The authors declare no conflict of interest.

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