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luxS in bacteria isolated from 25- to 40-million-year-old amber

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Abstract

Interspecies bacterial communication is mediated by autoinducer-2, whose synthesis depends on *luxS*. Due to the apparent universality of *luxS* (present in more than 40 bacterial species), it may have an ancient origin; however, no direct evidence is currently available. We amplified *luxS* in bacteria isolated from 25- to 40-million-year-old amber. The phylogenies and molecular clocks of *luxS* and the 16S rRNA gene from ancient and extant bacteria were determined as well. Luminescence assays using *Vibrio harveyi* BB170 aimed to determine the activity of *luxS*. While the phylogeny of *luxS* was very similar to that of extant *Bacillus* spp., amber isolates exhibited unique 16S rRNA gene phylogenies. This suggests that *luxS* may have been acquired by horizontal transfer millions of years ago. Molecular clocks of *luxS* suggest slow evolutionary rates, similar to those of the 16S rRNA gene and consistent with a conserved gene. Dendograms of the 16S rRNA gene and *luxS* show two separate clusters for the extant and ancient bacteria, confirming the uniqueness of the latter group.

Introduction

Interspecies bacterial communication, or quorum sensing (QS), is mediated by autoinducer-2 (AI-2), a furanosyl borate diester (Schauder *et al.*, 2001). Synthesis of AI-2 depends on *luxS*, which is the product of S-ribosylhomocysteine lyase. *luxS* was first identified in *Vibrio harveyi*, *Escherichia coli*, and *Salmonella typhimurium*, and its expression has been associated with virulence in *E. coli* and *Streptococcus pyogenes* (DeLisa *et al.*, 2001; Lyon *et al.*, 2001) and biofilm formation in *Bacillus cereus* (Taga *et al.*, 2001; Xavier & Bassler, 2005a, b; Auger *et al.*, 2006). More than 40 bacterial species harbor *luxS*, and this apparent universality makes it attractive for evolutionary analyses (Bassler, 1999; Surette *et al.*, 1999; Winzer *et al.*, 2003; Rezzonico & Duffy, 2008).

We propose that the evolution of QS mediated by *luxS* can be studied directly given that bacteria have been previously isolated from 25- to 40-million-year-old amber. Amber bacteria differ from present-day bacteria in their enzymatic and biochemical profiles, as well as their 16S rRNA gene phylogenies (Greenblatt *et al.*, 1999). Most amber isolates are *Bacillus* spp., but Gram-positive cocci (Lambert *et al.*, 1998; Greenblatt *et al.*, 2004) and Gram-negative bacteria have been isolated as well, representing an opportunity to study QS in diverse ancient microorganisms (Jones *et al.*, 2005; Auger *et al.*, 2006; Rollins & Schuch, 2010). In this study, we report *luxS* sequences in ancient microorganisms, reconstruct the phylogenies of *luxS* and the 16S rRNA gene from ancient and extant bacteria, and calculated molecular clocks for both *luxS* and the 16S rRNA gene.

Materials and methods

Amber isolates characterization and DNA extraction

All experiments were performed in a laminar flow cabinet, exclusive for amber bacteria. Amber bacteria were previously isolated by the Ambergene Corporation, under Class III aseptic protocols (Cano & Borucki, 1995).

Isolates were grown in nutrient broth, brain–heart infusion broth, or trypticase soy broth supplemented with agar (1.5% w/v) (Difco) and incubated for 24–72 h at 28 or 37 °C. Individual colonies were morphologically characterized by Gram-staining to confirm that the isolates corresponded to those previously reported by the Ambergene Corporation. Isolated colonies were picked and enriched in 1 mL of the broth in which growth was observed. DNA was extracted using the Fermentas GeneJet Genomic DNA Purification Kit following the manufacturer's instructions. Extracted DNA was stained with GelStar Nucleic Acid Gel Stain (20 X) (Lonza, Rockland, ME) and visualized in 0.7% agarose gels. DNA quality and concentration were estimated using a NanoDrop® (ND-1000) spectrophotometer.

luxS and 16S rRNA gene amplification and sequencing

luxS primers were designed using Primer 3 (http://frodo.wi.mit.edu/) and checked for the formation of secondary structures (http://www.premierbiosoft.com/netprimer/index.html) (Supporting Information, Table S1). Primers were designed from consensus sequences to increase the probability of amplification. Primers were designed for *luxS* present in Gram-positive and Gram-negative bacteria, because the phylogeny of *luxS* shows that bacteria cluster by groups (Lerat & Moran, 2004). Primers for the amplification of the 16S rRNA gene were as described elsewhere (Amann *et al.*, 1995; Turner *et al.*, 1999). Amplifications were performed at least three times in 10 μL per reaction as described previously (Patrício *et al.*, 2012) and included reactions without nucleic acids as negative controls. PCR conditions for *luxS* were the following: initial denaturation at 95 °C (2 min), followed by 35 cycles at 94 °C (45 s), annealing at 52 °C (45 s), an extension at 72 °C (45 s), and final extension at 72 °C (7 min). PCR conditions for the 16S rRNA gene were the following: initial denaturation at 95 °C (3 min), followed by 35 cycles at 95 °C (30 s), annealing at 52 °C (30 s), an extension at 72 °C (30 s), and a final extension at 72 °C (10 min). Products were stained as described above, visualized in 1.0% agarose gels, and sequenced using an ABI 3130xl Genetic Analyzer.

Sequence alignments and phylogeny reconstruction

The *luxS* and 16S rRNA gene sequences of 24 present-day bacteria were chosen according to previous studies (Lerat & Moran, 2004), acquired from GenBank (Table S2), and added to a pool of 20 amber isolates that harbor *luxS* and for which the 16S rRNA gene sequences were determined as well. Nucleotide sequences were aligned using CLUSTALW in MEGA, version 4.0 (Tamura *et al.*, 2007), keeping default parameters for multiple DNA alignment. Alignments were screened manually in Mesquite (Maddison & Maddison, 2001) and exported as NEXUS files. The sequence alignment of *luxS* had 567 bp, and the alignment of 16S rRNA gene had 1730 bp. Bayesian Markov chain Monte Carlo (MCMC) inference methods available in BEAST, version 1.7 (Drummond & Rambaut, 2007), were used to reconstruct the phylogenies of the partial gene sequences. MCMC analyses included γ- distributed rate heterogeneity among sites + invariant sites and partition into codon positions (Drummond & Rambaut, 2007; Drummond *et al.*, 2007). Genealogy was estimated with the uncorrelated relaxed lognormal clock (Ho & Larson, 2006) and using the Yule tree prior (Drummond *et al.*, 2007). Two independent MCMC analyses were run for 10 million generations, subsampling every 1000 generations. After a 10% burn-in, the analyses were examined for convergence on Tracer, version 1.5 (Rambaut & Drummond, 2003; Rambaut *et al.*, 2009). Marginal posterior parameter means, the associated 95% highest probability density intervals, and the effective sample size for each parameter were analyzed to assure statistically robust parameter estimates (Drummond *et al.*, 2002). Summary trees were created with TreeAnnotator, version 1.6.0 (Rambaut & Drummond, 2009), and edited in FigTree, version 1.3.1.

Molecular clocks

The evolutionary divergence for chosen sequence pairs (ancient vs. extant) was calculated based on Ochman and Wilson molecular clock for SSU rRNA (0.1 × 10e-9 substitutions/site/year for eubacterial rDNA) (Ochman & Wilson, 1987) and Masatoshi Nei's model of a phylogenetic test of the molecular clock and linearized trees (Ochman & Bergthorsson, 1995). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 5 (Tamura *et al.*, 2011). Trees were built for each ancient isolate against its closest modern ancestor(s). This was performed based on BLAST searches and using a high G+C outgroup (*Streptomyces lavendulae*). Results are similar to those from the Ochman and Wilson model. Molecular clocks for *luxS* were estimated similarly.

Luminescence assays

To evaluate the expression of *luxS* in the amber isolates, luminescence assays were performed using isolates 4_AG11AC10, 10_AG11AC13a, and 16_AG11AC14 and *V. harveyi* BB170 as the reporter strain. Amber isolate 6_AG11AC11 was used as negative control as it lacked *luxS*. The criteria for selection of the isolates for the

assays included differences between the amplified region of the 16S rRNA gene and cell morphology. For these experiments, the growth curves of the amber isolates were determined by $OD_{600\ nm}$ measurements of aliquots collected (in triplicate) every 2 h for up to 8 h. Aliquots were filtered and added to a final concentration of 10% to the reporter strain (final $OD_{600\ nm} = 0.1$). Luminescence emitted by the reporter strain in the presence of the putative AI-2 was measured using a luminometer and is reported as relative light units (RLU). Background luminescence or the luminescence emitted by the reporter strain in the absence of bacterial filtrates was measured as well. Results are reported as plots of the luminescence emitted by the reporter strain in the presence of the supernatant of the amber isolates, and $OD_{600\ nm}$ measurements are shown as well (*y*-axis). The *x*-axis represents the timing of the response of *V. harveyi* BB170 after addition of the putative AI-2.

Statistical analyses

Sequence data matrices were log-transformed, and similarity matrices were used to construct dendograms using Primer E, version 6 (Clarke & Gorley, 2006). For the luminescence data, one-way analysis was performed to test for differences between group means using JMP PRO 10 statistical analysis software (Statistical Discovery™, SAS Institute, Inc.).

Results

Phylogeny and evolution of *luxS*

A total of 20 amber isolates were included in the present study (Table S3). *luxS* was not amplified in most of the Gram-negative isolates, with the exception of isolate 9_AG11AC12a. The tree topology of *luxS* in the present study is comparable to that reported previously (Lerat & Moran, 2004). The amplified region of *luxS* clustered more closely to the *luxS* of *B. megaterium* (Fig. 1a). This was not the case, however, for the 16S rRNA gene phylogeny, where several amber isolates formed distinct branches and clustered with differing bacteria genera (Fig. 1b). The dendogram of the *luxS* clearly showed separate clusters for the extant and ancient taxa (Fig. 2a), while the dendrogram of the 16S rRNA gene sequences showed a similar clustering of the samples by age (extant vs. ancient) (Fig. 2b).

The evolutionary rate or molecular clocks for *luxS* and 16S rRNA gene sequences were calculated. The criteria for selection of the isolates included identification at the species level by BLAST searches of the 16S rRNA gene partial sequences. The evolution rate of the 16S rRNA gene of the amber isolates tested is shown in Table 1 and was estimated to be 14.5–30.3 million years. The results are consistent with the estimated age of the isolates (Table S1). In terms of *luxS*, it exhibited mean evolutionary rates ranging

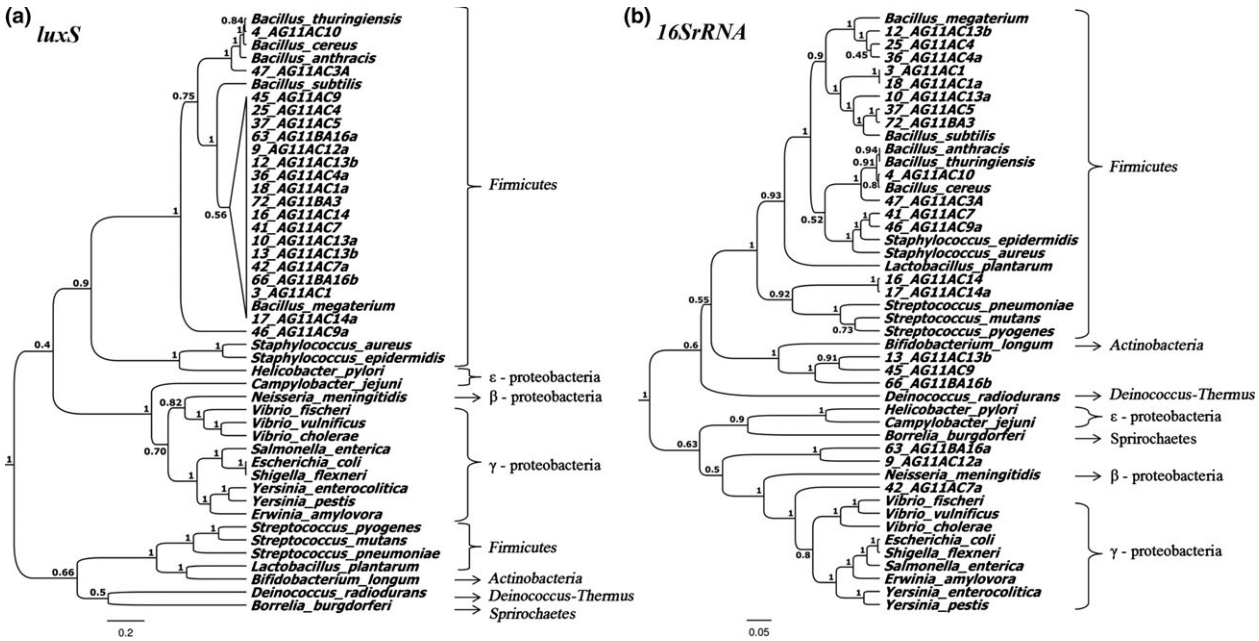

Fig. 1. Phylogeny of *luxS* (a) and the 16S rRNA gene (b) of amber and present-day bacteria.

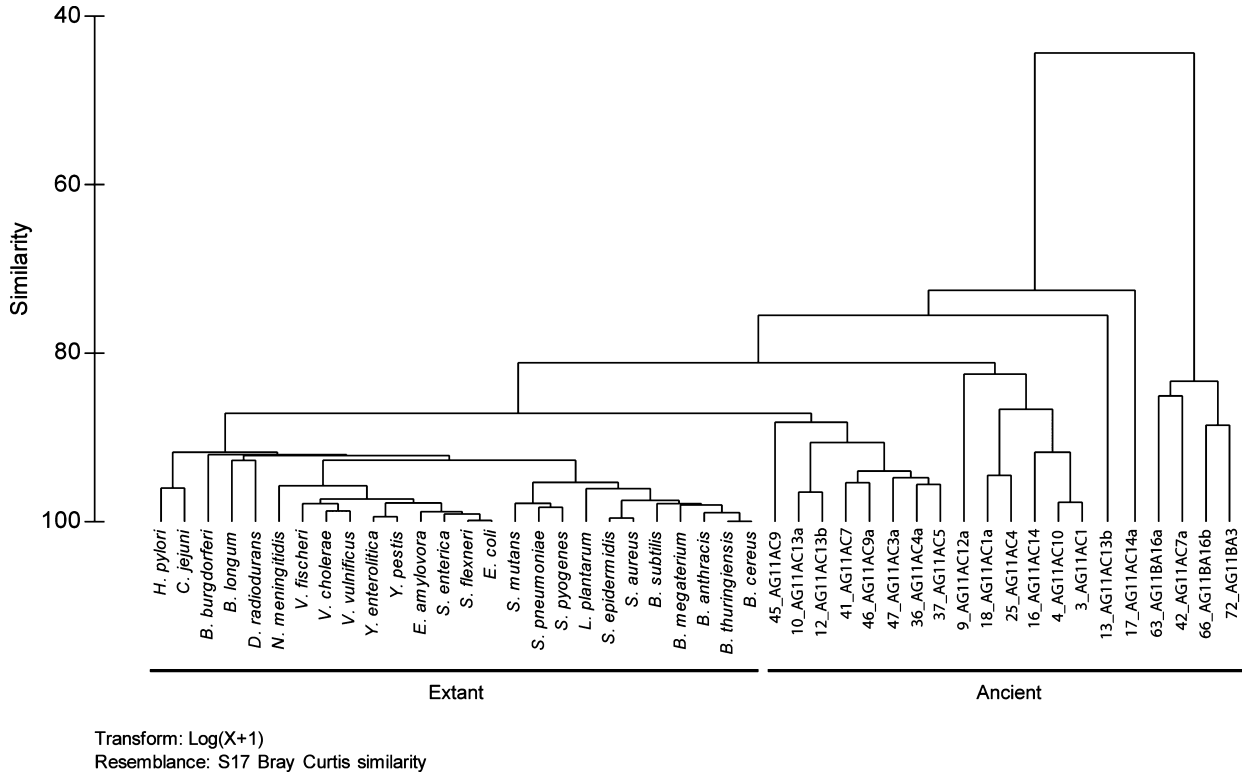

Fig. 2. Dendogram of the *luxS* of ancient and present-day bacteria.

Table 1. Molecular clocks of the 16S rRNA gene for the amber isolates identified at the species level by BLAST searches of the 16S rRNA gene partial sequences

	Molecular clocks (MY)			
Isolate ID	Takezaki *et al.*	Ochman–Wilson	Mean	BLAST closest match
3_AG11AC1	27.5	29.8	28.7	*B. shacklestonii*
4_AG11AC10	17.0	23.6	20.3	*B. cereus*
10_AG11AC13a	18.0	18.5	18.3	*B. safensis*
12_AG11AC13b	18.5	24.3	21.4	*B. megaterium*
16_AG11AC14	23.0	28.2	25.6	*P. alvei*
17_AG11AC14a	19.5	21.5	20.5	*P. alvei*
18_AG11AC1a	26.5	34.0	**30.3**	*B. schakletonii*
25_AG11AC4	13.5	15.5	**14.5**	*B. megaterium*
36_AG11AC4	22.5	27.2	24.9	*B. subtilis*
37_AG11AC5	21.5	25.5	23.5	*B. amyloliquefaciens*
47_AG11AC3a	16.5	19.8	18.2	*B. cereus*
66_AG11BA16b	24.0	23.8	23.9	*Agrococcus jenensis*
72_AG11BA3	20.5	26.8	23.7	*B. amyloliquefaciens*

Time, in millions of years (MY), was calculated using the Takezaki *et al.* and Ochman–Wilson methods.
Bold value indicate lowest and highest means.

from 8.5 to 34.0 million years, which appear to be relatively similar to those values calculated for the 16S rRNA gene (Table 2).

Activity of *luxS*

Luminescence in *V. harveyi* BB170 was induced when exposed to the supernatants of the amber bacteria tested. This was observed at 4 h in all the bacterial isolates tested, which harbored *luxS*, and was not the case for the negative control tested. Luminescence values are shown in Fig. 3, a (isolate 4_AG11AC10), b (isolate 10_AG11 AC13a), and c (isolate 16_AG11AC14). The negative control (6_AG11AC11) did not emit statistically significant luminescence in any of the time points (Fig. 3d). Importantly, the luminescence emitted by the reporter strain in the presence of the putative AI_2 of all amber isolates tested is statistically significant, as shown by the one-way analysis of response (Fig. S1). The overlapping circles for each pair Student's *t* and Best Hsu's MCB also indicate significant difference between the three strains and the control.

Discussion

Our results are the first to report the presence and evolutionary rate of genes involved in QS in ancient bacteria. The amplification of *luxS* in several of the amber isolates tested is neither contamination nor systematic errors of the PCRs. This was highly predicated by the *luxS* and 16S

Table 2. Molecular clocks of *luxS* of chosen amber isolates in this study

Strain ID	No. of substitutions	Total bases	*K*	Time (MYBP)	*r*	BLAST closest match
3_AG11AC1	12	240	0.05	26.3	1.9E−09	*B. megaterium*
4_AG11AC10	4	236	0.02	23.2	7.3E−10	*B. thuringiensis*
10_AG11AC13a	11	238	0.05	**8.5**	5.4E−09	*B. megaterium*
12_AG11AC13b	11	239	0.05	22.1	2.1E−09	*B. megaterium*
16_AG11AC14	11	240	0.05	32.5	1.4E−09	*B. megaterium*
17_AG11AC14a	12	241	0.05	21.5	2.3E−09	*B. megaterium*
18_AG11AC1a	10	170	0.06	**34.0**	1.7E−09	*B. megaterium*
25_AG11AC4	7	144	0.05	18.7	2.6E−09	*B. megaterium*
36_AG11AC4	13	242	0.05	32.0	1.7E−09	*B. megaterium*
37_AG11AC5	7	154	0.05	25.5	1.8E−09	*B. megaterium*
47_AG11AC3A	6	238	0.03	19.3	1.3E−09	*B. cereus*
66_AG11BA16b	8	171	0.05	23.9	1.9E−09	*B. megaterium*
72_AG11BA3	11	239	0.05	23.7	1.9E−09	*B. megaterium*
Mean rate			0.05	23.9	2.1E−09	

r (evolutionary rate) = *K*/years; *K*, no. of substitutions/total bases.

Amber isolates were chosen as these were identified at the species level using the 16S rRNA gene partial sequences. Results show the number of substitutions, total bases used for the molecular clock analyses, *K*, time (MYBP), *r*, and the BLAST search closest match.

Lowest and highest times (MYBP) are shown in bold.

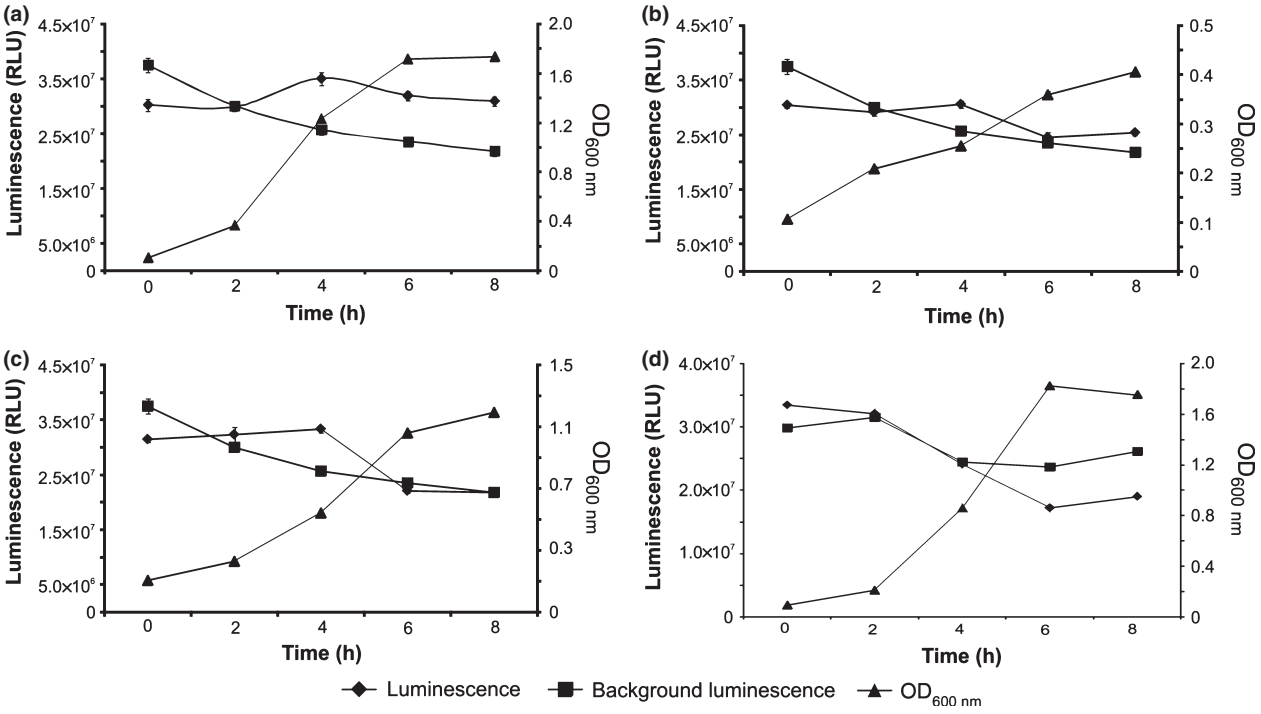

Fig. 3. The y-axis shows the possible expression of *luxS* in bacteria isolated from amber by luminescence assays using *Vibrio harveyi* BB170 as the reporter strain. Optical densities were also measured (in triplicate) every 2 h for up to 8 h, and standard deviations are represented by error bars. Isolates included (a) 4_AG11AC10, (b) 10_AG11AC13a, (c) 16_AG11AC14, and (d) 6_AG-11-AC-11 (control). Luminescence produced by the reporter strain after the addition of the supernatant, and without it (background luminescence), was measured and is presented in RLU. The x-axis represents the timing of the *Vibrio harveyi* BB170 response after addition of the putative AI-2.

rRNA gene dendogram analyses, which clearly show a separation between the extant and ancient bacteria. Cross-contamination can also be discarded due to the differing 16S rRNA gene sequences among the isolates that were positive for *luxS*. Moreover, all three sets of *luxS* primers were tested in *c.* 130 amber isolates, regardless of being a

Gram-positive or Gram-negative. If contamination of the primer sets would have occurred, *luxS* would have been amplified in all or most of the isolates tested. It should be noted that amber possesses preservative properties, representing an opportunity to isolate and extract suitable ancient DNA for analyses such as those performed in the present study (Cano, 1996).

Most *luxS* sequences in the amber isolates were similar to the *luxS* sequences of extant *Bacillus* spp. when performing the BLAST search. This may be due to the unchanged nucleotide sequence of the amplified region of *luxS*. This may not have been the case for most of the Gram-negative bacteria tested (except for isolate 9_AG11AC12a), which were negative for *luxS*. This may suggest that Gram-negative bacteria lacked *luxS* millions of years ago or that these harbored *luxS* sequences different from those of present-day bacteria. The presence of a *luxS* sequence similar to that of *Bacillus* spp. in an ancient Gram-negative isolate (isolate 9_AG11AC12a) is a matter of further research as this could suggest the horizontal transmission of the gene between Gram-positive and Gram-negative bacteria. Cross-contamination is a possibility that can be discarded as this isolate was identified as a *Brevundimonas* sp. by a BLAST search of the 16S rRNA gene sequence. Notably, the presence of a *luxS* sequence similar to that of *Bacillus* spp. in nonsporulating bacteria, such as those identified as *Curtobacterium* sp. (isolate 13_AG11AC13b) and *Brevundimonas* sp. (isolate 9_AG11AC12a), suggests a possible horizontal transmission of the gene as well (Urbanczyk *et al.*, 2012). However, the possibility remains that the data presented here are biased by the type of bacteria able to survive in amber and/or those that are cultivable. The lack of amplification of *luxS* in Gram-negative bacteria isolated from amber still leaves a gap in terms of the status of the gene in this bacterial group.

The *luxS* sequences corresponding to the amber bacteria accounted for the differences in the tree topologies of both genes considered. The reason is that the *luxS* sequences grouped with *Bacillus* spp., whereas the 16S rRNA gene sequences formed distinct clades in the phylogenetic tree. This suggests that *luxS* in the ancient bacteria tested was acquired by horizontal gene transfer from *Bacillus* spp. Our data suggest that the lateral transmission of *luxS* took place at least 40 million years ago. While the exact time of the horizontal transmission of *luxS* is certainly hard to estimate, it is possible that it was acquired over 40 million years ago by certain bacteria. The similarity of the *luxS* tree topology to that corresponding to the 16S rRNA gene suggests that in extant bacteria, *luxS* may have been acquired mainly by vertical transmission (Lerat & Moran, 2004; Sun *et al.*, 2004). The biological reasons and mechanisms of the horizontal transfer of *luxS* are a matter of further research, but this is a rare event in extant bacteria (Schauder *et al.*, 2001).

The relatively low mutation rate of *luxS* (similar to that of the 16S rRNA gene) may suggest that the gene has been conserved for millions of years and may have an important function in ancient microorganisms as well. Although this may be apparent, no data so far have shown directly that *luxS* has been conserved for millions of years. This, in turn, raises new questions about the possible role(s) of *luxS* in QS and metabolic processes in ancient bacteria. It is known that the primary role of LuxS resides in the activated methyl cycle, and this remains to be addressed for ancient bacteria (Winzer *et al.*, 2003; Vendeville *et al.*, 2005; Xavier & Bassler, 2005a, b; Rezzonico & Duffy, 2008). Notably, the luminescence assays confirmed the activity of *luxS* in the amber isolates tested. The high luminescence of the reporter strain at 4 h suggests that AI-2 could be important for processes associated with the mid-log phase, as in the case of biofilm formation (Auger *et al.*, 2006). These data, although preliminary, open the opportunity to further determine the possible role of AI-2 in these unique isolates. It is known that *luxS* has an essential role in metabolic pathways; yet, its role in other biological processes (e.g. virulence), as those shown with extant bacteria, is a matter of further research. While experiments were performed using three amber isolates harboring *luxS*, results are still valuable as they provide insights into the expression of *luxS*. We are in the process of performing the luminescence experiments in more amber isolates.

Conclusions

The present study reported *luxS* sequences in 25- to 40-million-year-old bacteria, such as those identified as *Bacillus schakletonii* and *B. aryabhattai*, two extant bacterial species that had not been previously reported as carrying *luxS*. This opens the opportunity to study possible novel QS mechanisms. The amplified region of *luxS* may be at least 40 million years-old and that it has remained largely unchanged. Our data provide direct evidence of an ancient origin of a possible functional *luxS*. This in turn raises new questions on the specific role(s) of *luxS* in ancient microorganisms and whether it is involved in the regulation of metabolism in amber bacteria.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Oneway analysis of response of the luminescence assays.
Table S1. Primers used in this study.
Table S2. Extant bacteria in included in the phylogenetic and evolutionary analyses in the present study.
Table S3. Amber isolates harboring luxS included in the present study.