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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 over 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. Phylogenies and Principal Component Analyses (PCA) of *luxS* and the 16S rRNA gene from ancient and extant bacteria were constructed. Amber isolates exhibited unique 16S rRNA gene phylogenies, while the *luxS* phylogeny was very similar to that of extant *Bacillus* spp. 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.

Keywords

Ancient bacteria; autoinducer-2; bacterial communication; *luxS*; quorum-sensing

INTRODUCTION

Interspecies bacterial communication, or quorum-sensing (QS), is mediated by autoinducer-2 (AI-2), a furanosyl borate diester (Schauder, Shokat et al. 2001). Synthesis of AI-2 depends on *luxS*, which product is 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, Wu et al. 2001; Lyon, Madden et al. 2001), and biofilm formation in *Bacillus cereus* (Taga, Semmelhack et al. 2001; Xavier and Bassler 2005; Auger, Krin et al. 2006). More than 40 bacterial species harbor *luxS* and this apparent universality makes it attractive for

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evolutionary analyses (Bassler 1999; Surette, Miller et al. 1999; Winzer, Hardie et al. 2003; Rezzonico and 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 isolates differ from present-day bacteria in their enzymatic and biochemical profiles, as well as their 16S rRNA gene phylogenies (Greenblatt, Davis et al. 1999). Most amber isolates are *Bacillus* spp., but Gram-positive cocci (Lambert, Cox et al. 1998; Greenblatt, Baum et al. 2004) and Gram-negative bacteria have been isolated as well, representing an opportunity to study QS in diverse ancient microorganisms (Jones, Jani et al. 2005; Auger, Krin et al. 2006; Rollins and 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 and 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 to 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, USA) 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) (Table 1). 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, since the phylogeny of *luxS* shows that bacteria cluster by groups (Lerat and Moran 2004). Primers for the amplification of the 16S rRNA gene were as described elsewhere (Amann, Ludwig et al. 1995; Turner, Pryer et al. 1999). Amplifications were performed at least three times in 10 μL per reaction as described previously (Patricio, Herbst et al. 2012), and included reactions without nucleic acids as negative controls. PCR conditions for *luxS* were: initial denaturation at 95 °C (2 min), followed by 35 cycles at 94 °C (45 s), annealing at 52 °C for (45 s), an extension at 72°C (45 s) and final extension at 72 °C (7 min). PCR conditions for the 16S rRNA gene consisted of an 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, phylogeny reconstruction and PCA analyses

The *luxS* and 16S rRNA gene sequences of 24 present-day bacteria were chosen according to previous studies (Lerat and Moran 2004), acquired from GenBank (Table 2) and added to a pool of 20 amber isolates that harbor *luxS* and for which the16S rRNA gene sequences were determined as well. Nucleotide sequences were aligned using ClustalW in MEGAv4.0 (Tamura, Dudley et al. 2007), keeping default parameters for multiple DNA alignment. Alignments were screened manually in Mesquite (Maddison and Maddison 2001) and exported as NEXUS files. The sequence alignment of *luxS* had 567 bp and the alignment of 16SrRNA had 1730 bp. Bayesian Markov chain Monte Carlo (MCMC) inference methods available in BEASTv1.7 (Drummond and Rambaut 2007; Drummond and 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, Ho et al. 2007; Drummond and Rambaut 2007). Genealogy was estimated with the uncorrelated relaxed lognormal clock (Ho and Larson 2006) and using the Yule tree prior (Drummond, Ho et al. 2007). Two independent MCMC analyses were run for 10 million generations, sub-sampling every 1,000 generations. After a 10 % burn-in, the analyses were examined for convergence on Tracerv1.5 (Rambaut and Drummond 2003; Rambaut, Ho 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, Nicholls et al. 2002). Summary trees were created with TreeAnnotator v1.6.0 (Rambaut and Drummond 2009) and edited in FigTree v1.3.1.

Partial gene sequences were transformed to numbers (A=1; C=2; G=3; T=4; gaps=0) and were visualized as Principal Component Analysis (PCA) plots. PCA ordinations were calculated in Primer E Software v. 6 (Clarke and Gorley 2006).

Molecular clocks

The evolutionary divergence for chosen sequence pairs (ancient vs. extant) were calculated based on Ochman and Wilson molecular clock for SSU rRNA ($0.1 \times 10e{-9}$ substitutions/site/year for eubacterial rDNA) (Ochman and Wilson 1987). Based on Masatoshi Nei's model of a phylogenetic test of the molecular clock and linearized trees (Ochman and Bergthorsson 1995). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura, Peterson 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

In order 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_AG-11-AC-11 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} 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}=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} 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

Oneway analysis of luminescence data was performed to test for difference between group means using jmp Pro 10 statistical analysis software (Statistical Discovery™, SAS Institute, Inc.).

RESULTS

Evolution and phylogeny of *luxS*

A total of 20 amber isolates were included in the present study (Table 3). *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 and Moran 2004). The amplified region of *luxS* clustered more closely to the *luxS* of *Bacillus megaterium* (Figure 1A). This was not the case, however, for the 16S rRNA phylogeny, where several amber isolates formed distinct branches and clustered with differing bacteria genera (Figure 1B). In the PCA plots, the *luxS* sequences of ancient and extant bacteria exhibited similarities of 60 to 80 % (Figure 2A). The 16S rRNA gene of ancient and extant bacteria exhibited similarities of 80 % (Figure 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 the16S rRNA gene of the amber isolates tested is shown in Table 4 and was estimated to be 14.5 to 30.3 million years. The results are consistent with the estimated age of the isolates (Table 1). In terms of *luxS*, it exhibited mean evolutionary rates ranging from 8.5 to 34.0 million years, which appear to be relatively similar to those values calculated for the 16S rRNA gene (Table 5).

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 Figure 3, **Panel A** (isolate 4_AG11AC10), **Panel B** (isolate 10_AG11AC13a and **Panel C** (isolate 16_AG11AC14). Importantly, these values are statistically significant, as shown by the Oneway analysis of response (Figure 4). 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. Notably, the control did not emitted luminescence in any of the time points.

DISCUSSION

Our results are the first to report the presence and evolutionary rate for 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 PCR reactions. This was predicated by the differing 16S rRNA gene sequences among the isolates that were positive for *luxS*. Contamination would have been detected by looking at the similarities/differences in the16S rRNA sequences amplified from the amber isolates. Moreover, all three sets of *luxS* primers were tested in approximately 130 amber isolates, despite being 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 region of amplified *luxS* region. 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 non-sporulating 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, Furukawa et al. 2012). However, the possibility remains that the data presented 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 sequences formed distinct clades in the phylogenetic tree. This suggests that *luxS* in the ancient bacteria tested may have been acquired by horizontal gene transfer mainly from *Bacillus* spp. Our data suggest that the lateral transmission of *luxS* took place at least 40 million years ago. Due to 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 and Moran

2004; Sun, Daniel 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, Shokat 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 obvious, no data so far have directly shown 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 (AMC) and this remains to be addressed for ancient bacteria (Winzer, Hardie et al. 2003; Vendeville, Winzer et al. 2005; Xavier and Bassler 2005; Rezzonico and Duffy 2008).

luxS is a functional gene, as shown by the luminescence assays using the amber isolates tested. These data, although preliminary, open the opportunity to further determine the possible role of AI-2 in these unique isolates. Although it is known that *luxS* has an essential role in metabolic pathways, its role in other biological processes (e.g. virulence), as those shown with extant bacteria, warrant further studies. While experiments were performed using three amber isolates harboring *luxS*, results are still valuable as they provide insights of 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 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 microbes and if it is involved in the regulation of metabolism in amber bacteria.

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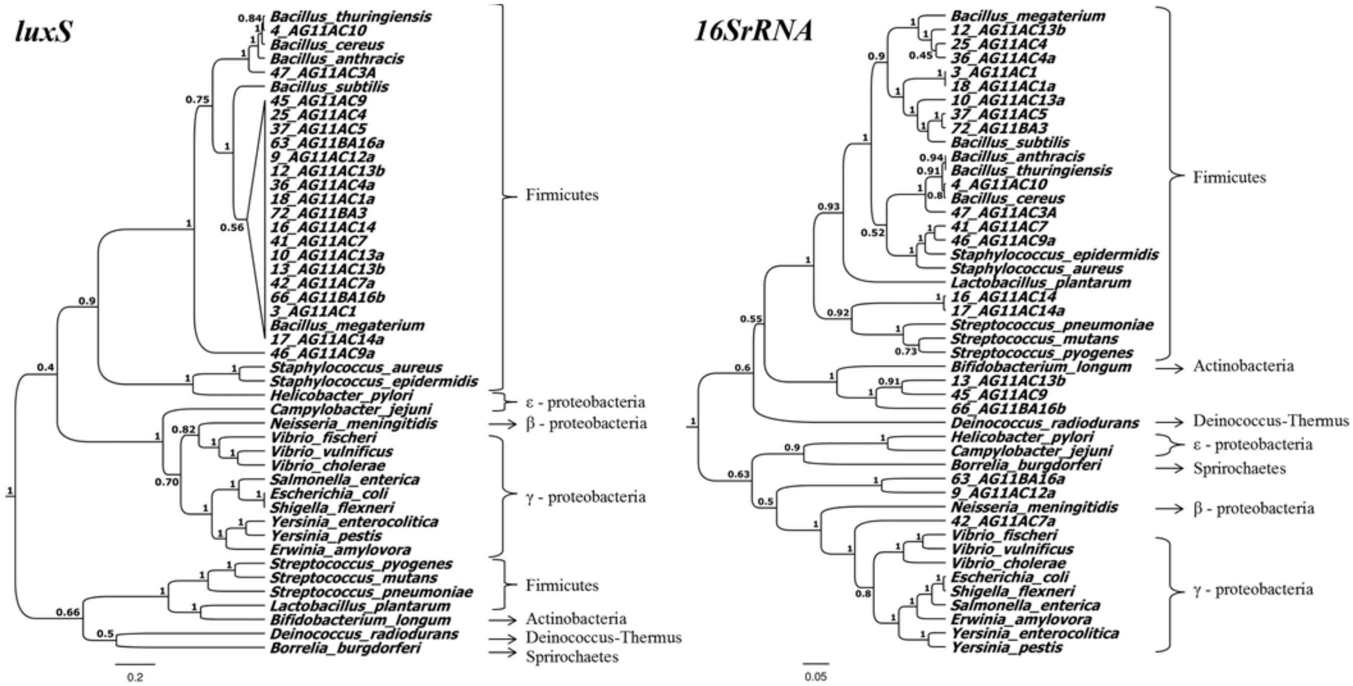

Figure 1.

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 2h for up to 8h 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 Relative Light Units (RLU). The x-axis represents the timing of the *Vibrio harveyi* BB170 response after addition of the putative AI-2.

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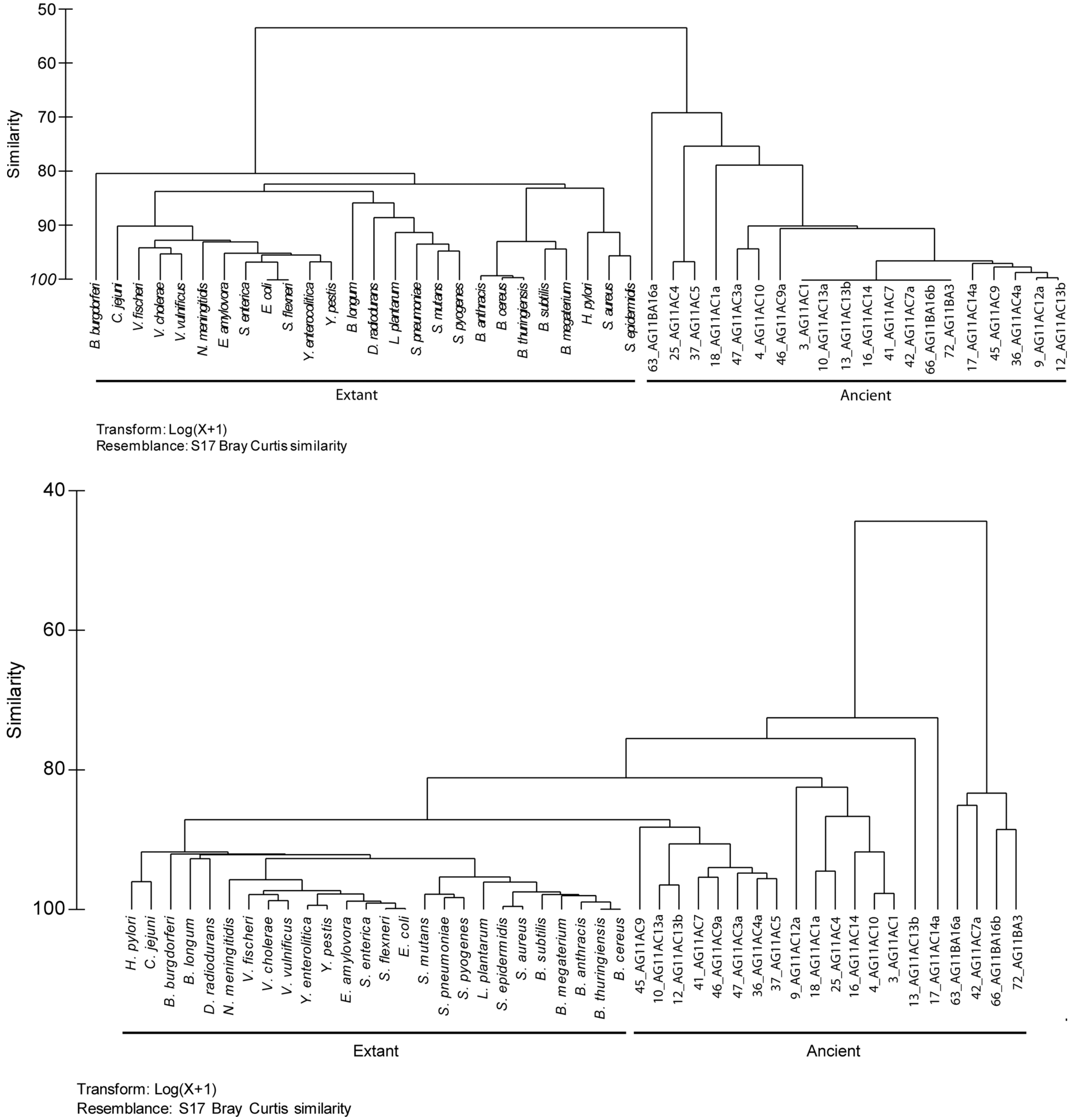

Figure 2.
Phylogeny of *luxS* (A) and the 16S rRNA gene (B) of amber and present-day bacteria.

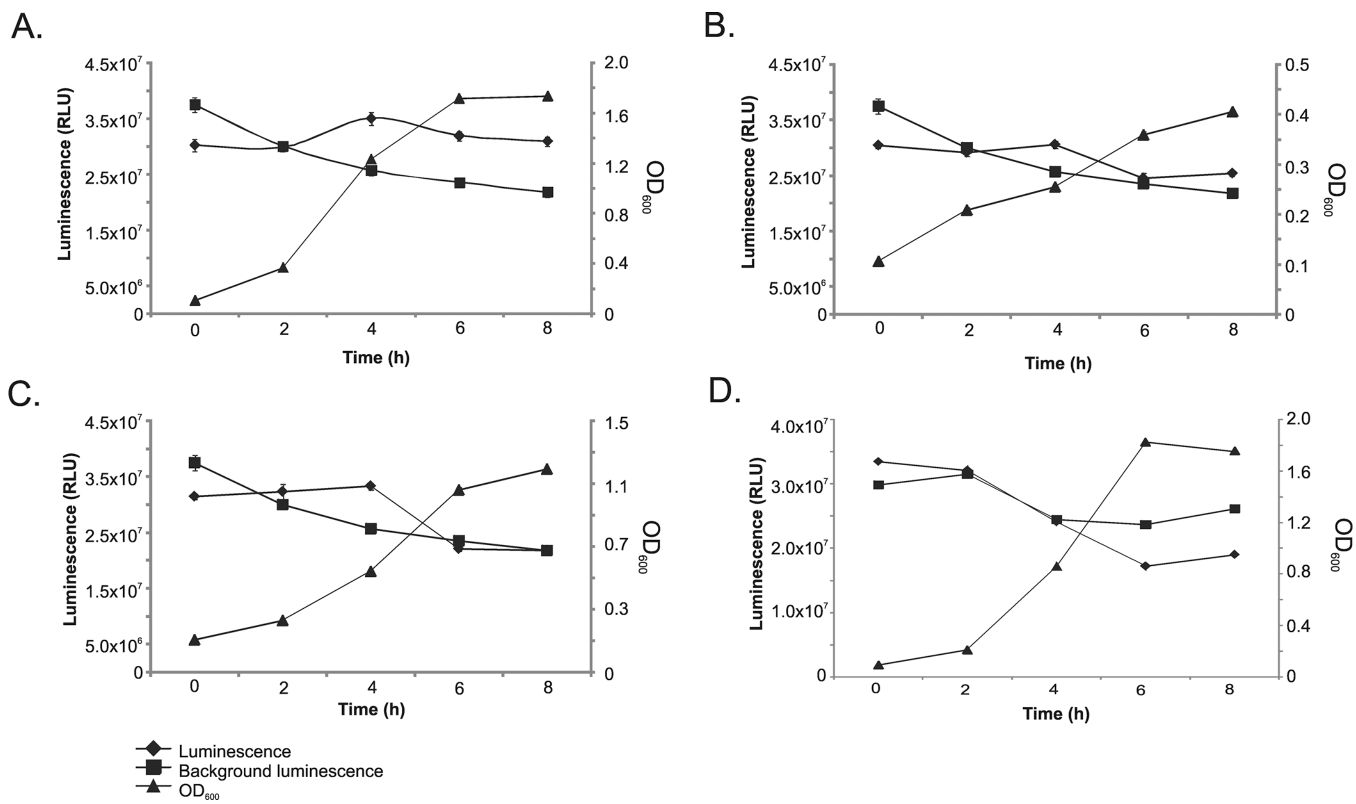

Figure 3.

Dendograms of the *luxS* (A) and the 16S rRNA gene (B) in ancient and present-day bacteria.

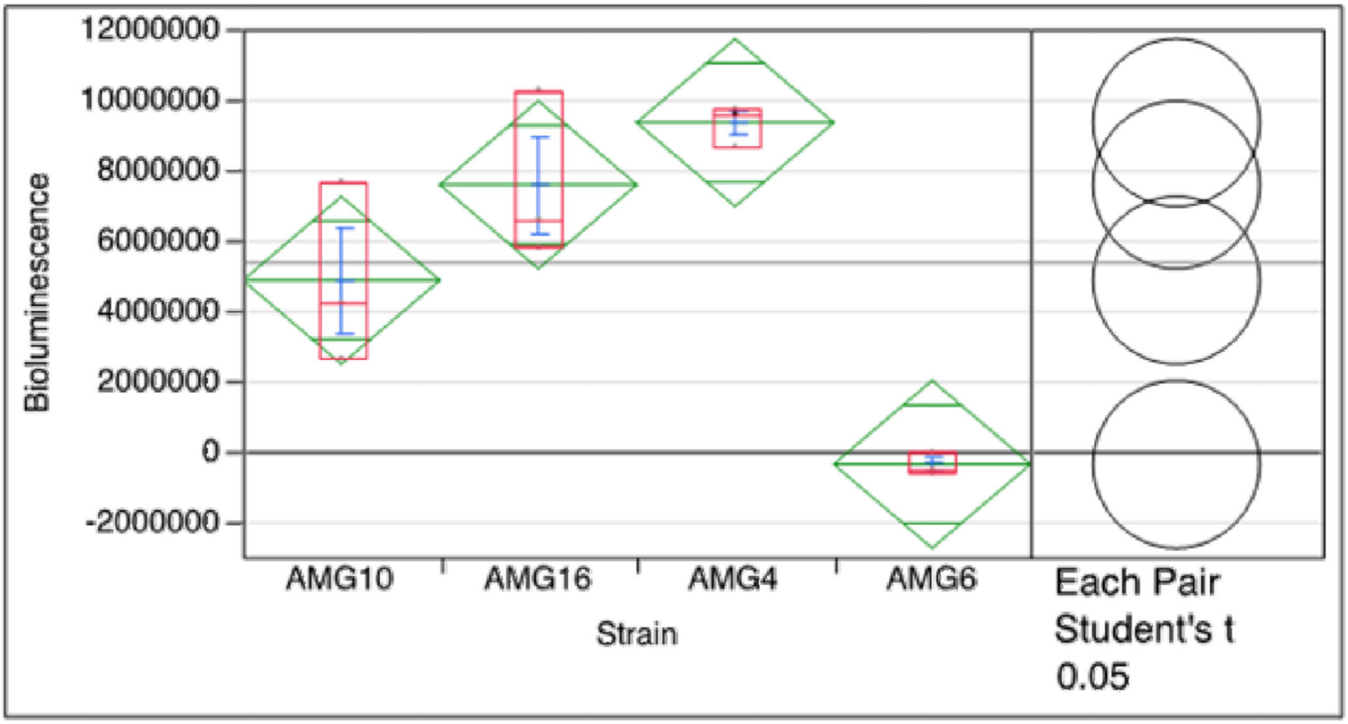

Figure 4.

Oneway analysis of response of the luminescence assays. All three strains (4, 10, and 16) show significantly greater luminescence response than the controls. 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.

Table 1

Primers used in this study. Direction of the primer is represented by F-(Forward) or R-(Reverse). Primers were designed to amplify the *luxS* sequences of Gram-positive and Gram-negative bacteria. Accession Numbers for primer design are specified in the following column.

Primer	Amplicon size (bp)	Target	Reference	Accession numbers
F-GCCAAATAAACAAGCAATGA	239	*luxS* Gram-positive *Bacillus* spp.	This study	NC_014019.1
R-TTGCAGCTGGAATTTCTGTA				NC_012472.1
				NC_000964.3
F-GGATTCATACGCTTGAGCA	184	*luxS* Gram-negative bacteria	This study	NC_000913.2
R-TTCAACACATCTTCCATTGC				NC_003197.1
				NC_008800.1
				NC_013971.1
				NC_010554.1
F-CATATGATTATGTGGGGTCA	180	*luxS* Gram-positive cocci	This study	NC_008533.1
R-TAAGATGAGTTTTGCCCATT				NC_004350.2
				NC_004668.1
F-AGAGTTTGATCCTGGCTCAG	1398	Universal 16S rRNA	Amann et. al., 1995	
R-ACGGGCGGTGTGTRC				
R-GWATTACCGCGGCKGCTG	511	Universal 16S rRNA	Turner et. al., 1999	

Table 2

Extant bacteria in included in the phylogenetic and evolutionary analyses in the present study. Complete *luxS* and 16SrRNA gene sequences were acquired from GenBank. Accession Numbers are shown in the following column.

Extant bacteria	Accession number
Deinococcus radiodurans R1	AE000513.1
Bifidobacterium longum NCC2705	AE014295.3
Neisseria meningitidis 8013	NC_017501.1
Campylobacter jejuni subsp. *jejuni* NCTC 11168	AL111168.1
Bacillus anthracis str. Ames	AE016879.1
Bacillus cereus ATCC 14579	AE016877.1
Bacillus subtilis subsp. *subtilis* str. 168	AL009126.3
Bacillus megaterium QM B1551	NC_014019.1
Lactobacillus plantarum WCFS1	AL935263.2
Staphylococcus aureus subsp. *aureus* N315	NC_002745.2
Helicobacter pylori B8	NC_014256.1
Staphylococcus epidermidis ATCC 12228	NC_004461.1
Streptococcus mutans UA159	NC_004350.2
Streptococcus pneumoniae R6	NC_003098.1
Streptococcus pyogenes SSI-1	NC_004606.1
Enterococcus faecalis V583	NC_004668.1
Escherichia coli O26:H11 str. 11368	NC_013361.1
Salmonella enterica subsp. *enterica* serovar Typhi Ty2	AE014613.1
Vibrio cholerae O1 str. 2010EL-1786	NC_016445.1
Shigella flexneri 2002017	NC_017328.1
Vibrio fischeri ES114	NC_006840.2
Vibrio vulnificus MO6-24/O	CP002469.1
Yersinia enterocolitica subsp. *enterocolitica* 8081	NC_008800.1
Yersinia pestis CO92	NC_003143.1
Erwinia amylovora CFBP1430	NC_013961.1
Borrelia burgdorferi B31	NC_001318.1

Table 3

Amber isolates harboring luxS included in the present study. The negative control, or that lacked *luxS* is shown as well. The following columns show the possible corresponding present-day bacteria as determined by a BLAST search of the 16SrRNA gene, maximum identities (%) and e-values.

Isolate	Amber	Age (My)	16S rRNA gene BLAST hit	Max Identity (%)	e-value
3_AG11AC1	Dominican	25–30	*Bacillus schakletonii*	99	0
4_AG11AC10	Dominican	25–30	*Bacillus cereus*	99	0
9_AG11AC12a	Dominican	25–30	*Brevundimonas* sp.	99	0
10_AG11AC13a	Dominican	25–30	*Bacillus safensis*	99	0
12_AG11AC13b	Dominican	25–30	*Bacillus megaterium*	99	0
13_AG11AC13b	Dominican	25–30	*Curtobacterium* sp.	100	0
16_AG11AC14	Dominican	25–30	*Paenibacillus alvei*	99	0
17_AG11AC14a	Dominican	25–30	*Paenibacillus alvei*	99	0
18_AG11AC1a	Dominican	25–30	*Bacillus schakletonii*	99	0
25_AG11AC4	Dominican	25–30	*Bacillus megaterium*	99	0
36_AG11AC4a	Dominican	25–30	*Bacillus subtilis*	97	0
37_AG11AC5	Dominican	25–30	*Bacillus amyloliquefaciens*	98	0
41_AG11AC7	Dominican	25–30	*Staphylococcus* sp.	95	0
42_AG11AC7a	Dominican	25–30	Uncultured *Pseudomonas* sp.	95	0
45_AG11AC9	Dominican	25–30	*Streptomyces* sp.	97	0
46_AG11AC9a	Dominican	25–30	*Staphylococcus* sp.	94	0
47_AG11AC3a	Dominican	25–30	*Bacillus cereus*	98	0
63_AG11BA16a	Baltic	40	Uncultured *Brevudimonas* sp.	99	0
66_AG11BA16b	Baltic	30	*Agrococcus jenensis*	99	0
72_AG11BA3	Baltic	30	*Bacillus amyloliquefaciens*	99	0
Control					
6_AG-11-AC-11	Dominican	25–30	*Bacillus thuringiensis*	99	0

Table 4

Molecular clocks of the 16SrRNA gene for the amber isolates identified at the species level by BLAST searches of the 16S rRNA gene partial sequences. Time, in millions of years (MY), was calculated using the Takezaki et al. and Ochman-Wilson methods.

Isolate ID	Molecular Clocks (MY)			BLAST Closest Match
	Takezaki et al	Ochman-Wilson	Mean	
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. shacklestonii*
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. amyloliquifaciens*
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. amyloliquefasciens*

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Table 5

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

Strain ID	No. 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. Substitutions/Total Bases