The ISME Journal (2011) 5, 1505–1516

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ORIGINAL ARTICLE

Diversity and functional analysis of *luxS* genes in Vibrios from marine sponges *Mycale laxissima* and *Ircinia strobilina*

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Sponges harbor highly diverse and dense microbial communities, providing an environment in which bacterial signaling may be important. Quorum sensing (QS) is a cell density-dependent signaling process that bacteria employ to coordinate and regulate their gene expression. Previous studies have found that bacteria isolated from sponges are able to produce acyl-homoserine lactones (AHLs), an important class of QS molecules found in proteobacteria. Autoinducer-2 (AI-2) is a second class of QS molecule, and is considered to be an interspecies signal. However, AI-2 signaling has not been reported in sponge bacterial symbionts. In this study, degenerate primers were designed based on known *Vibrio luxS* sequences to amplify the *luxS* genes encoding AI-2 synthases of several *Vibrio* isolates from marine sponges *Mycale laxissima* and *Ircinia strobilina*. All the vibrios isolated from these two sponges had *luxS* genes and were able to produce signals with AI-2 activity as detected using a biological reporter. A novel group of *luxS* sequences was found, thus extending the known diversity of *luxS* genes. One isolate was chosen for further analysis of its *luxS* gene by expression of the gene in *Escherichia coli* DH5α and by characterization of the profile of AI-2 activity. This work provides the first information about *luxS* genes and AI-2 activity in sponge-associated bacterial communities.
The ISME Journal (2011) **5,** 1505–1516; doi:10.1038/ismej.2011.31; published online 31 March 2011
Subject Category: microbe–microbe and microbe–host interactions
Keywords: sponges; symbionts; quorum sensing; acyl-homoserine lactone; autoinducer-2; *Vibrio*

Introduction

Sponges (phylum Porifera) are one of the most primitive multicellular animals and are sessile, filter-feeders. Sponges can form close associations with microbes that in some cases comprise up to 60% of the biomass of the sponges (Vacelet, 1975; Vacelet and Donadey, 1977; Wilkinson, 1978; Hentschel *et al.*, 2006). Molecular techniques have revealed that these bacterial communities are remarkably diverse and include many novel bacteria (Taylor *et al.*, 2007; Mohamed *et al.*, 2008c; Webster and Blackall, 2009). Symbiotic relationships between sponges and microorganisms are considered to contribute to the health and nutrition of sponges (Mohamed *et al.*, 2010). Many studies have shown the metabolic phenotypes of sponge-associated microbes (Taylor *et al.*, 2007; Mohamed *et al.*, 2008d, 2010), although little evidence exists confirming the contribution of symbiotic microbes to sponge well-being or survival (Webster and Blackall, 2009). Exceptions are the translocation of photosynthate from cyanobacteria to the host sponge (Wilkinson, 1979) and a decrease in health status of sponges associated with loss of cyanobacteria (Thacker, 2005).

Bacteria are known to effectively communicate with each other (Hooshangi and Bentley, 2008). One of the mechanisms for communication is termed quorum sensing (QS), in which bacteria monitor and respond to their own population density. In this process, diffusible signal molecules, sometimes called autoinducers, are produced at basal levels. The signals can accumulate proportionally to population density, and at critical threshold concentration, the signal binds to receptor proteins, which trigger expression of a specific spectrum of genes and phenotypes (Fuqua *et al.*, 1994). Bacterial processes such as biofilm formation, bioluminescence, motility, virulence factor secretion, antibiotic production, sporulation and competence for DNA uptake can be controlled by QS systems (Ng and Bassler, 2009).

In Proteobacteria, acyl-homoserine lactones (AHLs) are a major type of molecular cue for QS, and were originally described as autoinducers. Bacteria that are able to produce AHLs have been

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Received 5 July 2010; revised 1 February 2011; accepted 20 February 2011; published online 31 March 2011

isolated from marine sponges (Taylor *et al.*, 2004; Mohamed *et al.*, 2008a). Sponges harbor a higher proportion of AHL producers in their culturable bacterial communities compared with that of surrounding seawater (Mohamed *et al.*, 2008a). AHL regulatory systems among these bacteria may have roles during bacterial colonization of the sponges or in controlling activities within the sponge symbiont community (Cicirelli, Hill and Fuqua, in preparation). They may also provide one means by which sponges could interact or interfere with bacteria (Taylor *et al.*, 2007).

Autoinducer-2 (AI-2) is another well-known molecular cue and has been extensively analyzed in *Vibrio harveyi* and *Vibrio cholerae*, where it is involved in regulation of bioluminescence and virulence-associated traits (Miller *et al.*, 2002; Lenz *et al.*, 2004; Henke and Bassler, 2004a, b). The activated methyl cycle is a crucial metabolic pathway to recycle homocysteine from the major methyl donor *S*-adenosyl methionine. LuxS, an *S*-ribosylhomocysteinase, catalyzes part of the cycle and functions to convert *S*-ribosylhomocysteine to homocysteine; meanwhile, it can also, as a side reaction, synthesize 4,5-dihydroxy-2,3-pentanedione, the precursor of AI-2. 4,5-Dihydroxy-2,3-pentanedione can spontaneously give rise to several furanone derivatives, collectively referred to as AI-2. The dual roles of *luxS* in metabolism and in AI-2 formation have led to controversy regarding its function in QS (Doherty *et al.*, 2006; Rezzonico and Duffy, 2008). *V. harveyi* produces a third type of autoinducer CAI-1, an alpha-hydroxyketone (Tiaden *et al.*, 2010), in addition to the AHL autoinducer (3-OH-C4-HSL) and AI-2. The three different types of autoinducers in *V. harveyi* bind to their respective membrane-bound receptors and converge on a central signal transduction pathway to regulate gene expressions (Ng and Bassler, 2009).

Thus far, no studies have been performed on the AI-2 systems among sponge-associated bacteria. During the process of isolating bacteria from the sponges *Mycale laxissima* and *Ircinia strobilina*, we obtained many *Vibrio* strains. We therefore focused on this investigation of the presence of *luxS* genes in sponge-associated bacteria on *Vibrio* spp. because of their high incidence in the sponges. Degenerate primers of *luxS* based on known *Vibrio luxS* sequences were designed and applied to all the *Vibrio* isolates. The AI-2 activity of all the *Vibrio* isolates was tested and a novel *luxS* sequence was cloned and analyzed further. The same set of primers was used to PCR-amplify the *luxS* genes directly from sponge bacterial communities.

Materials and methods

Sample collection

Three individuals of each of *M. laxissima* and *I. strobilina* were collected by SCUBA at Conch Reef, Key Largo, FL, USA in late October 2008 at a depth of *ca* 20 m. The water salinity was measured to be 36 p.p.t. using a portable refractometer (Fisher Scientific, Pittsburgh, PA, USA) at a water temperature *ca* 28 °C. Sponge samples were transported to the laboratory within 1 h of collection and sponges were rinsed three times with sterile artificial seawater for bacterial isolation and frozen at −20 °C for later DNA extraction. Water samples were collected within 1 m of the sponges at a depth of *ca* 20 m in a sterile 20-l container. Approximately 6 l of water was filtered through each of three 0.22 um pore-size Sterivex filters (Millipore, Billerica, MA, USA). The filters for extraction of DNA from bacteria in the water samples were flushed with 1X SET buffer (0.1 M NaCl, 10 mM Tris-Hcl, pH 8.0 1 mM EDTA) as described by Somerville *et al.* (1989) and stored at −20 °C.

Bacterial isolation and enumeration

Sponges were rinsed with sterile artificial seawater to remove transiently associated bacteria and then processed for isolation of culturable bacteria. Sponge tissue (1 cm^3) was ground in artificial seawater using a sterile mortal and pestle and 10-fold serial dilutions were plated on Difco Marine Agar 2216 (BD Biosciences, Franklin Lakes, NJ, USA). Plates were incubated at 30 °C for 1 week at which time bacterial colonies were counted on plates with suitable dilutions (30–300 colonies). Approximately 50 colonies were selected for identification by 16S ribosomal RNA (rRNA) gene sequencing (below) to determine the proportion of vibrios in the culturable community. Serial dilutions of water samples were processed similarly for bacterial isolation. Total bacterial counts were determined using methods described by Mohamed *et al.* (2008d).

Identification of bacterial isolates by 16S rRNA gene sequence analysis

A single colony of each isolate was transferred to 15 ml of Marine Broth 2216 and incubated at 30 °C for 24–48 h. Bacterial genomic DNA was extracted from isolates using the UltraClean microbial kit (MoBio Laboratories, Carlsbad, CA, USA). Representatives of each morphotype based on careful observation of colony appearance were subcultured and cryopreserved at −80 °C in Marine Broth 2216 supplemented with 30% glycerol. Almost full-length 16S rRNA gene fragments were PCR-amplified using universal primers 27F and1492R as described by Enticknap *et al.* (2006). PCR products were sequenced using an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) and primers 27F and1492R. Sequences were assembled using online software CAP3 (http://pbil.univ-lyon1.fr/cap3.php) with a manual check. Chimeric sequences were identified by using the

CHECK_CHIMERA program of the Ribosomal Database Project (Maidak *et al.*, 2001) and the sequences were analyzed initially by using the BLASTn tool at the National Center for Biotechnology Information website (NCBI).

Extraction of genomic DNA from sponges and surrounding water samples

Freeze-dried sponge tissue (1 cm^3) was ground using a sterile mortar and pestle. Total genomic DNA was extracted by a published method (Pitcher *et al.*, 1989), modified for sponge tissues (Enticknap *et al.*, 2006; Mohamed *et al.*, 2008d). DNA was extracted from the filters obtained from surrounding seawater samples as described by Somerville *et al.* (1989).

Amplification of partial luxS gene sequences from Vibrio isolates

On the basis of sequences of known *Vibrio luxS* genes downloaded from Genbank, degenerate primers were designed for amplification of *luxS* gene fragments using online primer design software consensus-degenerate hybrid oligonucleotide primers (Rose *et al.*, 2003). The primers were designated VluxsF (5′-TGCTGGACTCCTTCACCGTNGAYCAYAC-3′) and VluxsR (5′-TGCATGGCGGCGGTNCCRCAYTGTT-3′). These primers were designed based on an alignment of all available *Vibrio luxS* gene sequences. PCR mixtures consisted of 50 μl containing two units Platinum Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), 1 × PCR Buffer, 2 mM $MgCl_2$, 200 mM dNTPS (Fermentas, Glen Burnie, MD, USA), 0.2 μM each primer and 10–20 ng of genomic DNA or distilled water as a negative control. PCR cycling conditions for *Vibrio luxS* gene amplification consisted of 94 °C for 5 min followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C. A final 10 min extension step was done at 72 °C. PCR reactions were performed in a PTC-200 cycling system (Bio-Rad, Hercules, CA, USA). The PCR products with expected size ~400 bp were purified using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and sequenced as described above for 16S rRNA gene fragments using primer VluxsF.

Measurement of AI-2 activity

AI-2 activity was detected by using the reporter strain *V. harveyi* TL-26, in which all three signaling pathways are disrupted. Receptor genes for AHL and CAI-1 are mutated as well as the *luxS* gene. Thus, TL-26 can respond only to exogenously added AI-2 (not AHLs or CAI) and cannot produce AI-2 (Long and Bassler; personal communication). All the *Vibrio* isolates and *V. harveyi* TL-26 were grown overnight in Luria–Bertani medium with 2% NaCl. Culture supernatants of test strains were filter sterilized (0.22 μm) and 60 μl of sterile supernatant was added to 140 μl AB medium (Greenberg *et al.*, 1979) containing *V. harveyi* TL-26 (inoculated 1:5000 from an overnight culture). *Agrobacterium tumefaciens* KYC55, which does not produce AI-2 (Zhu *et al.*, 2003), was used as a control to test if this assay is specific. *Vibrio mimicus* ATCC 33653 was used as a positive control. The culture was incubated at 30 °C with shaking at 180 r.p.m. for 16 h and light production was measured by using a *FLUOstar* OPTIMA fluorescence microplate reader (BMGlabtech, Cary, NC, USA). The induction of luminescence by each tested supernatant was expressed relative to that in a negative control comprising sterile Luria–Bertani medium instead of culture supernatant.

AI-2 activity of JZ08IS72, one *Vibrio* isolate from the sponge *I. strobilina*, at different growth phases was measured. Briefly, a culture of JZ08IS72 grown in Luria–Bertani medium with 2% NaCl, incubated at 30 °C and shaken at 200 r.p.m. was sampled at several different points over a continuous period of 30 h. AI-2 activity was measured as described above.

Full-length cloning of luxS gene from isolate JZ08IS72

The full-length *luxS* gene (hereafter referred to here as $luxS_{72}$) of isolate JZ08IS72 from the sponge *I. strobilina* was cloned using a GenomeWalker Universal Kit according to the manufacturer's instructions (Clontech Laboratories, Inc., Mountain View, CA, USA). Briefly, genomic DNA of JZ08IS72 was extracted using a CTAB method (Murray and Thompson, 1980). The DNA was digested with restriction enzymes provided by the kit and ligated to Genome Walker adaptors to construct a genomic library. The library was screened by $luxS_{72}$ specific primers using a nested PCR method. The primer used for the first round PCR amplification of the 5′ end of the gene was luxsR1 (5′-GGTATGGATACCCT TTTCAGAAAGCAG-3′) and for the second round was luxsR2 (5′-CTGAGTAAAGCGAAGGTCAAACA CAGT-3′). The primer used for the first round PCR amplification for the 3′ end was luxF1 (5′-CGTTGAG ATCATCGACATCTCTCCTAT-3′) and for second round was luxF2 (5′- TACATGAGTCTGATCGGTAC ACCTACT-3′). The primers targeting the adaptor sequences were provided by the kit. All the PCR conditions were the same as recommended by the manufacturer. The PCR products were gel purified and sequenced. The sequences were aligned using Clustal X 2.0.12 (http://www.clustal.org/) and assembled using online software CAP3 (http://pbil.univ-lyon1.fr/cap3.php) and also checked manually. The assembled sequences were further analyzed by BLAST.

Heterologous expression of $luxS_{72}$ in Escherichia coli *DH5α*

The coding sequence of $luxS_{72}$ was PCR amplified using forward primer 5′-GCCCTGCAGCTGAAACAG GAAACAGCTATGCCTTTACTAGATAGC-3′ (the *Pst*I

recognition site is underlined and the Shine–Dalgarno sequence from *lacZ* is both underlined and in italics) and reverse primer 5′-GGCCTCGAGT TACACCTTGAGGG-3′ (the *Xho*I recognition site is underlined). The PCR product was cloned into pCR4-TOPO vector (Invitrogen Life Technologies) and then subcloned into expression vector pSRKGm (Khan *et al.*, 2008), named pSRKGm $luxS_{72}$ and subsequently introduced into *E. coli* DH5α using standard techniques in Sambrook *et al.* (1989) and induced for expression using 1 mM isopropyl β-D-1-thiogalactopyranoside. At each cloning step, five clones were sequenced to identify clones with correct inserts. The activity of expressed $luxS_{72}$ in *E. coli* DH5α was measured using the TL-26 AI-2 reporter as described above; and *E. coli* M4100, which can produce AI-2 (Sperandio *et al.*, 1999), was used as positive control.

Clone library construction of luxS *genes amplified from sponge-associated bacterial communities*

Degenerate primers VluxsF and VluxsR were used to PCR-amplify putative *Vibrio luxS* genes from total genomic DNA extracted from sponges *M. laxissima* and *I. strobilina*, and also from surrounding seawater using the same PCR conditions used to amplify *luxS* genes from bacterial isolates. The PCR products were gel purified using a QIAquick gel extraction kit (Qiagen). Corresponding areas from the negative control sample in which the PCR was performed with no added template DNA were excised and taken through the same cloning procedure to provide strict negative controls. Purified PCR products were ligated into the pCR-XL-TOPO vector and transformed into One Shot TOP 10 chemically competent *E. coli* cells using a TOPO XL PCR cloning kit (Invitrogen Life Technologies). Plasmid DNA was isolated from individual clones and purified using a SprintPrep 384 kit (Agencourt Bioscience, Beverly, MA, USA). Sequencing was performed as described using M13 forward sequencing primer.

Phylogenetic analysis of 16S rRNA gene sequences and luxS *gene sequences*

16S rRNA gene sequences from isolates were edited using PreGap3 and Gap4 from the Staden package and analyzed using BLASTn at the NCBI website. Isolates were presumptively identified according to the identity of the closest cultured relative. Partial 16S rRNA gene sequences of all the *Vibrio* isolates were aligned using Clustal × 2.0.12 (http://www.clustal.org/) and a phylogenetic tree was constructed using software MEGA 4.0 (http://www.megasoftware.net/). *luxS* gene sequences from all the *Vibrio* isolates were converted to amino acid (aa) sequence using open reading frame finder and BLASTx at the NCBI website. *luxS* gene sequences from clone libraries were edited and the vector sequences were removed using the VecScreen tool at the NCBI website and were converted into aa sequences as described for isolates. The *luxS* gene sequences from isolates and clones were aligned and the phylogenetic tree was constructed as described for the 16S rRNA gene. To examine the novelty of the new cluster of isolates with *luxS* genes obtained in this study, the novel *luxS* genes were analyzed using BLASTn and the top 100 hits in Genbank were obtained. In addition, *luxS* genes in *Vibrio* spp. were searched for in Genbank using the key words 'Vibrio AND luxS'. These two data sets were pooled and aligned together with all the *luxS* sequences obtained from this study. In order to present a comprehensive but legible tree, the following approach was used: if the reference sequences from GenBank shared higher than 99% similarity with each other, then only one representative was chosen for inclusion in the tree and the total number of sequences that shared over 99% similarity to that representative was given in brackets. The name and accession number of these sequences is presented in Supplementary Table.

Rarefaction analyses of the *luxS* genes clone libraries were performed using the mothur program (Schloss *et al.*, 2009).

Nucleotide sequence accession numbers

16S rRNA gene sequences from isolates were submitted to GenBank under accession no. HM117107-HM117132. *luxS* gene sequences from isolates were submitted to GenBank under accession no. HM117133-HM117158 and *luxS* gene sequences from clone libraries were submitted to GenBank under accession no HM117021-HM117106.

Results

Partial luxS sequence and detection of AI-2 activity of Vibrio isolates from sponges

In total, 14 out of 47 isolates from *M. laxissima*, 10 out of 40 isolates from *I. strobilina* and 2 out 36 isolates from surrounding seawater were identified as *Vibrio* spp. by 16S rRNA gene sequences analysis (>1300 bp). For *M. laxissima*, the plate count was $1.2 \pm 0.2 \times 10^7$ colony-forming units (c.f.u.) cm^{-3} of sponge tissue (mean ± s.e.), for *I. strobilina*, the plate count was $2.6 \pm 0.8 \times 10^7$ c.f.u. cm^{-3} of sponge tissue and for surrounding seawater, the plate count $2.0 \pm 0.5 \times 10^5$ c.f.u. ml^{-1}. Total *Vibrio* isolates in 1 cm^3 sponge tissue were calculated at *ca* 3.5×10^6 c.f.u. and 6.5×10^6 c.f.u. for *M. laxissima* and *I. strobilina*, respectively. For surrounding seawater, the culturable *Vibrio* count was *ca* 1.1×10^4 c.f.u. ml^{-1}. Total bacterial counts obtained by microscopic enumeration of 4,6-diamidino-2-phenylindole-stained cells was $1.1 \pm 4.4 \times 10^9$ cells cm^{-3} and $2.8 \pm 0.4 \times 10^9$ cells cm^{-3} for *M. laxissima* and *I. strobilina*, respectively (Mohamed, 2007; Mohamed *et al.*, 2008d).

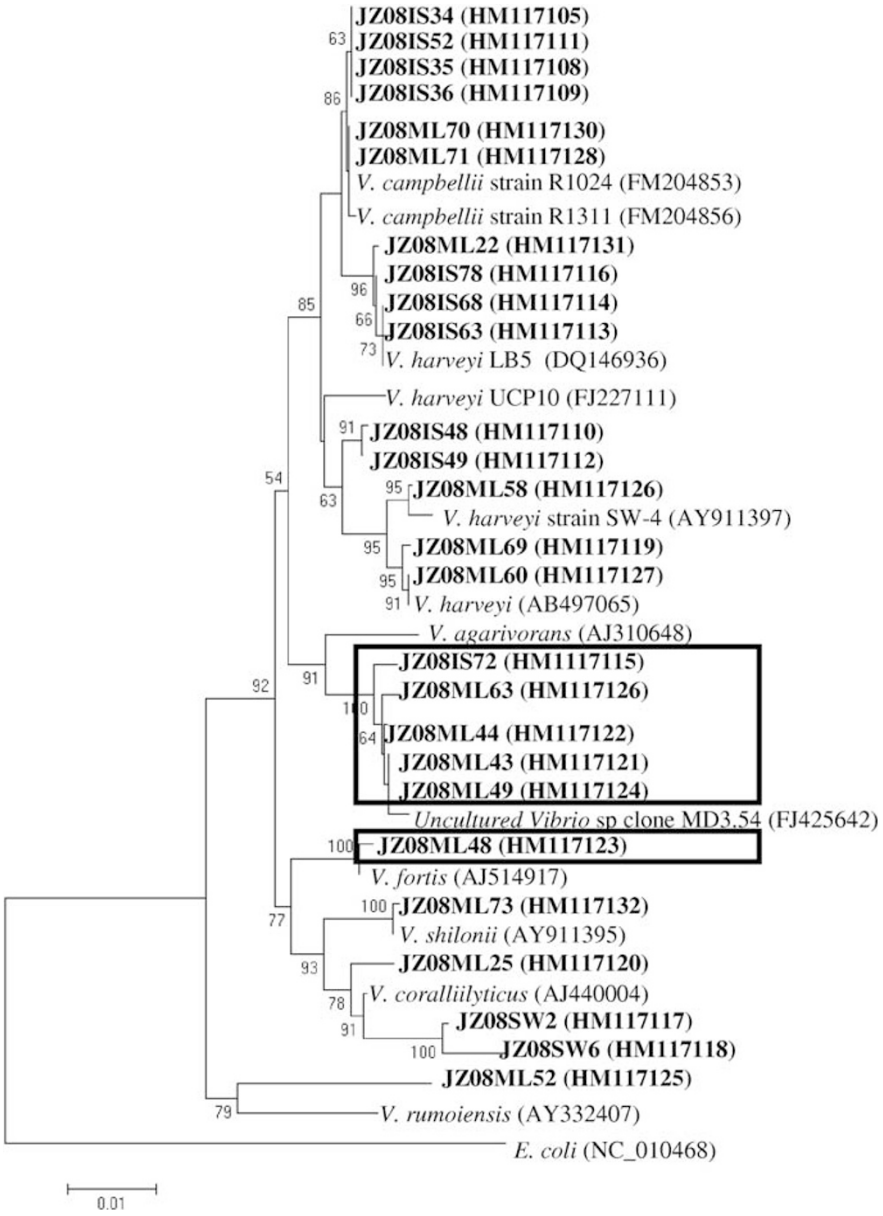

Figure 1 Rooted neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences (>1300 bp) of *Vibrio* strains that were recovered from *M. laxissima* (JZ08ML), *I. strobilina* (JZ08IS) and seawater (JZ08SW). All the sequences retrieved in this study are in bold and accession numbers are listed in parentheses for each entry. Isolates in box represent the *Vibrio* isolates that carry novel *luxS* gene sequences shown in Figure 2. Reference sequences are shown with GenBank accession numbers listed after each sequence name. Bootstrap values >50% are shown at nodes. The scale bar indicates 0.10 substitutions per nucleotide position.

As shown in the phylogenetic tree (Figure 1), a variety of different *Vibrio* species were retrieved. Six and nine isolates from the two sponges were closely related to strains of *V. campbelli* and *V. harveyi*, respectively. The other isolates from sponges were closely related to various *Vibrio* species, such as *Vibrio agarivorans*, *Vibrio shilonii* and *Vibrio fortis*. JZ08ML52 shared only 96% identity to its closest relative *Vibrio rumoiensis* (AY332407), indicating this strain might be a new species. 16S rRNA genes of two isolates from seawater (JZ08SW2 and JZ08SW6) shared 96–97% identity to that of *Vibrio coralliilyticus (*DJ440004), indicating that these isolates may also be new *Vibrio* spp.

Degenerate primers VluxsF and VluxR based on *Vibrio luxS* sequences were able to amplify partial *luxS* gene sequences from all the *Vibrio* isolates. A phylogenetic tree of the *luxS* gene sequences from these vibrios is shown in Figure 2. Using the approach described above to examine the novelty of the *luxS* cluster MI, about 130 sequences were analyzed. The tree based on *Vibrio luxS* sequences only (Figure 2) was very similar to that obtained by including all *luxS* sequences (Supplementary

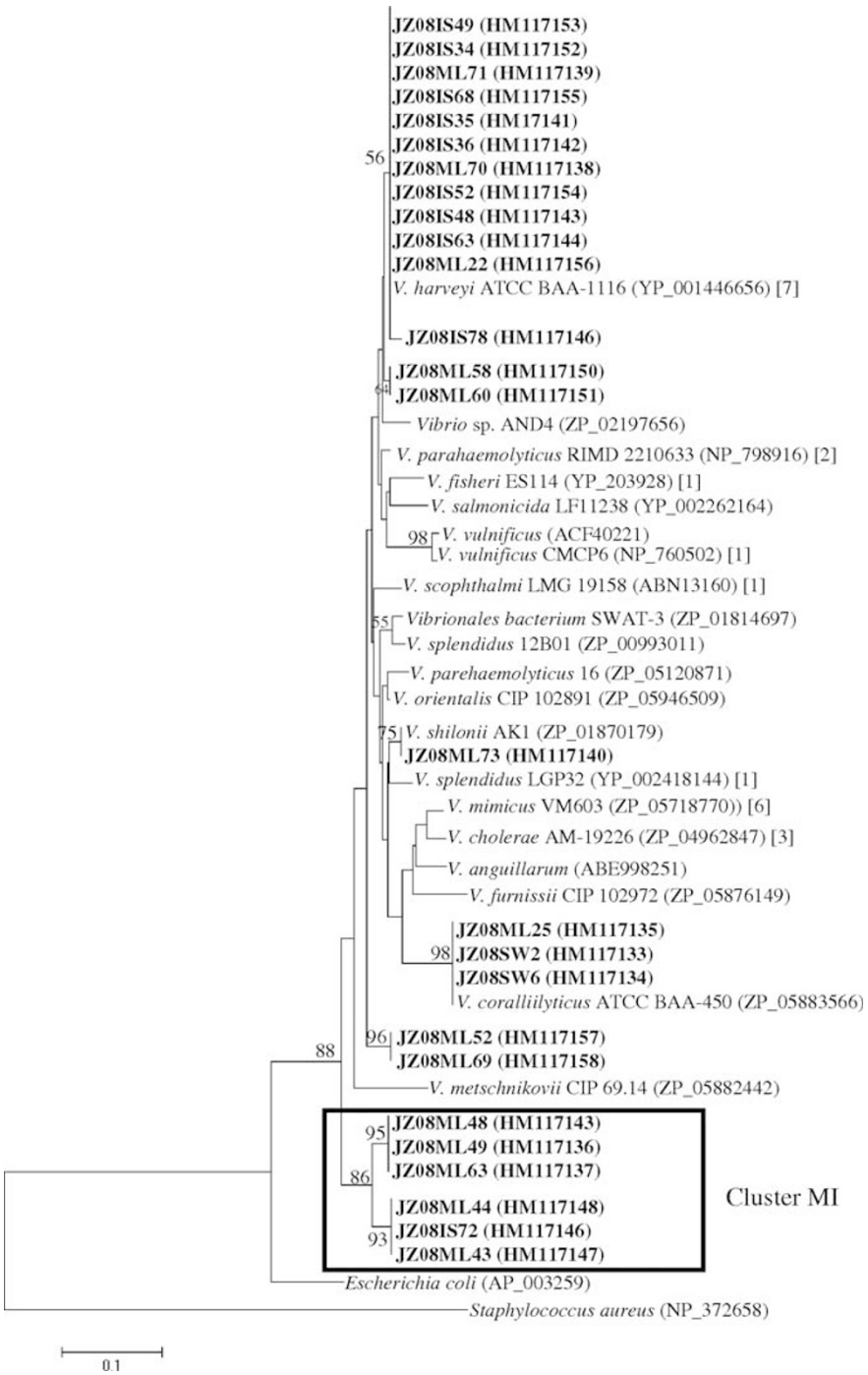

Figure 2 Phylogenetic tree using neighbor-joining method based on the predicted 96 aa residues encoded by *luxS* genes from *Vibrio* isolates. Sequences isolated from *M. laxissima* (JZ08ML), *I. strobilina* (JZ08IS) and seawater (JZ08SW) in this study are in bold and the accession number of each of these *luxS* sequences is listed after the isolate name. The numbers in [] indicates the numbers of sequences in Genbank that shared over 99% identity to the sequence on the tree and these sequences are listed in Supplementary Material Table 1. The novel *luxS* cluster is named Cluster MI. Bootstrap values >50% is shown at nodes. The scale indicates the number of aa substitutions per site.

Figure 1). The majority of *luxS* genes from these sponge-derived *Vibrio* isolates are closely related to that of *V. harveyi,* which is consistent with the close relationship between these isolates and *V. harveyi* shown by 16S rRNA phylogeney. However, in several cases the phylogeny of *luxS* genes does not match that indicated by the corresponding 16S rRNA genes. For example, the 16S rRNA gene sequences of isolates JZ08ML69 and JZ08ML52 are closely related to those of *V. harveyi* (AB497065) and *V. rumoiensis* (AY332407), respectively; but the *luxS* gene sequences of these two isolated shared over

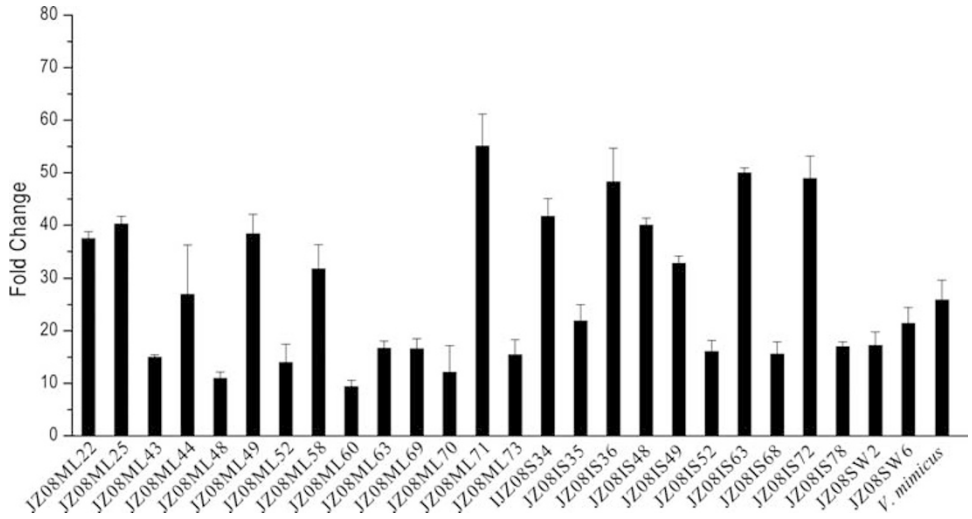

Figure 3 AI-2 activities of all *Vibrio* isolates measured by reporter strain *V. harveyi* TL-26. Fold changes were calculated by comparing the light production induced by supernatant of individual log phase cultures to that using Luria–Bertani (LB) medium instead of culture supernatant. *V. mimicus* ATCC 33653 was used as positive control. Supernatant from *A. tumefaciens* KYC55 failed to induce light production. Bars are means from triplicate experiments; error bars represented the s.e.m.

99% identity to each other. Similarly, the 16S rRNA genes of isolate JZ08ML48 grouped with *V. fortis* (AJ514917), but its *luxS* gene sequence clustered with the *luxS* genes from isolates JZ08ML43, JZ08ML44, JZ08ML49, JZ08ML63 and JZ08IS73.

A novel cluster (named cluster MI) of *luxS* gene sequences, comprising those from isolates JZ08ML43, JZ08ML44, JZ08ML48, JZ08ML49, JZ08ML63, JZ08IS73, shared only 88–90% identity to their closest relative in Genbank (boxed in Figure 2). JZ08IS72 was chosen as a representative of this group for further analysis to confirm the novelty of these *luxS* genes.

All the isolates are evaluated for AI-2 activity using reporter strain *V. harveyi* TL-26. All isolates were able to induce light production (Figure 3) whereas *A. tumefaciens* KYC55 did not induce light production (data not shown), showing that all the *Vibrio* isolates, all of which have *luxS* genes, are able to synthesize AI-2-type molecules.

JZ08IS72 was grown in shaken culture in Luria–Bertani medium with 2% NaCl at 30 °C. AI-2 activity in the culture supernatant was detected from early exponential phase (2 h after inoculation) and onward. It reached the maximal level during transition to stationary phase (6 h after inoculation) and decreased afterward with some fluctuations as shown in Figure 4.

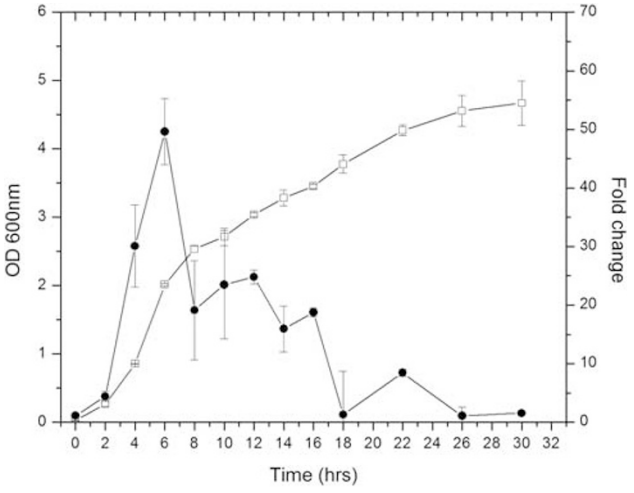

Figure 4 AI-2 production profile during growth of strain JZ08IS72. Strain JZ08IS72 was grown in Luria–Bertani (LB) with 2% NaCl. The growth (open squares) and AI-2 activity (closed circles) were monitored at the indicated time intervals. The error bars represent s.e. of triplicate samples.

Characterization of luxS *sequence of JZ08IS72 and expression in* E. coli *DH5α*

Using a genome walking method, the full length of $luxS_{72}$ was cloned. Results showed that an open reading frame encoding a putative *luxS* gene designated $luxS_{72}$ had 516 nucleotides and encoded 171 aa. It shared 88% identity with its closest *luxS* relative from *Vibrio parahaemolyticus* 16 (accession number ZP_05120871) at the aa level, although no significant similarity was found between $luxS_{72}$ and that of *V. parahaemolyticus* 16 at the nucleotide level, indicating high divergence of DNA sequence of $luxS_{72}$. Alignment of $luxS_{72}$ to that of several other *Vibrio* species indicated that the variance of aa largely occurred in the *C*-terminus of the protein and $luxS_{72}$ has all the conserved aa identified in Hilgers and Ludwig (2001) (see Supplementary Material Figure S2).

By walking upstream and downstream of $luxS_{72}$ gene, partial sequence of the glutamate-cysteine ligase gene (closely related to that of *V. harveyi*

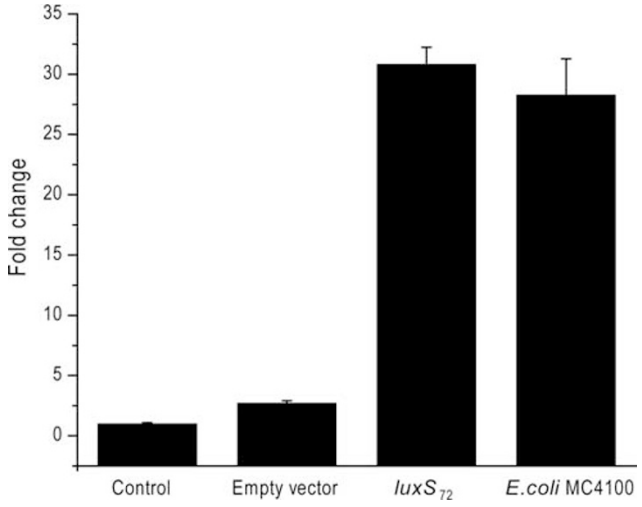

Figure 5 Heterologous expression of $luxS_{72}$ in *E. coli* DH5α. $luxS_{72}$ was cloned into expression vector pSRK-Gm and chemically transformed into *E. coli* DH5α. The transformant was grown in Luria–Bertani (LB) at 37 °C up to an OD_{600} of *ca*1.2 and induced by isopropyl *β*-D-1-thiogalactopyranoside (1 mM) for 3 h. *E. coli* MC4100 was used as a positive control. All the strains were grown up to an OD_{600} of 1.80 ± 0.02. The AI-2 activity was measured as described in Materials and methods section. The bars (from the left) represent the result of AI-2 activity from *E. coli* DH5α, the empty vector control, *E. coli* DH5α expressing $luxS_{72}$, and the positive control *E. coli* MC4100. Experiments were performed in triplicate and error bars indicate the s.e.m.

HY01 (ZP_01988480)) was found upstream of $luxS_{72}$ and partial sequence of a putative Mg^{2+} and Co^{2+} transporter *corB* gene was found downstream of $luxS_{72}$ (data not shown).

In order to confirm the function of the novel $luxS_{72}$, it was cloned into the pSRKGm expression vector, placing it under control of the isopropyl *β*-D-1-thiogalactopyranoside-inducible *lacZ* promoter (P_{Lac}) and expressed in *E. coli* DH5α, which does not produce AI-2 because of a mutation in the *luxS* gene (Surette and Bassler, 1998). Results shown in Figure 5 demonstrate that $luxS_{72}$ was successfully expressed in the heterologous host. Significant AI-2 activity was detected compared with the empty vector and control ($P<0.05$) and the heterologous production of AI-2 because of expression of $luxS_{72}$ was comparable to a *luxS*-proficient strain *E. coli* MC4100 ($P>0.05$).

Sequence analysis of luxS *genes from clone libraries*

luxS genes were successfully PCR-amplified from marine sponges *M. laxissima* and *I. strobilina* and also from surrounding seawater from Key Largo, using degenerate primers VluxSF and VluxSR. Phylogenetic relationships of the *luxS*-deduced aa sequences from these two marine sponges and surrounding seawater samples were analyzed. Phylogenetic relationships based on nucleotide sequences were also determined. Overall, these two trees based on aa sequences and nucleotide sequences were generally congruent with each other (data not shown). In total, 33, 30 and 23 clones of *luxS* genes were recovered and sequenced from the sponges *M. laxissima*, *I. strobilina* and seawater, respectively. To determine the relative richness and coverage of *luxS* genes sequenced from *M. laxissima*, *I. strobilina* and surrounding seawater, rarefaction analyses were performed. The rarefaction curves for *luxS* genes from *M. laxissima* and *I. strobilina* approached a plateau, and nearly plateaued for surrounding seawater at the distance of 0.01 (see Supplementary Material Figure S3). At the distance of 0.03, all the three rarefaction curves reached a plateau, indicating sufficient sampling of these clone libraries (data not shown). The phylogenetic analysis of these sequences is shown in Figure 6. In all, 31 out of 33 clones from *M. laxissima*, referred to as ML group 1 shared 97–99% identity to *luxS* gene of *V. mimicus* VM603 (ZP_05718770), and the other two clones, referred to as ML group 4 shared 100% identity to the *luxS* gene sequence of *V. harveyi* ATCC BAA-1116 (YP_001446656). In total, 28 out of 30 clones from *I. strobilina* and nine clones from seawater, referred to as IS and SW group 5, were closely related to the *luxS* gene of V. *harveyi* 1DA3 (ZP_06175640), sharing 96–99% identity and the other two clones from *I. strobilina* (IS group 3) shared 94% identity to the *luxS* gene of *V. parahaemolyticus* RIMD 2210633 (NP_798916). In all, 14 out of 23 clones from seawater, referred to as the SW group 2, were closely related to the *luxS* gene of *V. mimicus* VM603 (ZP_05718770), sharing 98–99% identity.

Discussion

The marine sponges *M. laxissima* and *I. strobilina* contained several different *Vibrio* strains, identified by 16S rRNA gene sequence analysis. Associations of vibrios with other marine organisms range from pathogenic to mutualistic. Close relatives of the *Vibrio* isolates described in this study include *V. harveyi*, a major pathogen of marine animals (Zhang *et al.*, 2001) and *V. shiloli* (AF007115), well established as a causative agent of coral bleaching (Kushmaro *et al.*, 1996, 1997). Pathogenicity is often strain-specific rather than species-specific so the close relationship between the sponge-associated *Vibrio* species and known pathogens does not necessarily indicate that our *Vibrio* isolates are potential pathogens of their host sponges. Known mutualist relationships between *Vibrio* spp. and marine invertebrates include *Vibrio fischeri*-bobtail squid (McFall-Ngai, 2008). Sponge-associated vibrios might offer the host sponge or the associated bacterial communities some benefits. One possibility is that nitrogen-fixing vibrios may supply the holobionts with fixed nitrogen. Shieh and Lin (1994) described vibrios as nitrogen-fixing bacteria associated with sponge *Halichondria*. However, none of the vibrios isolated in this study are closely related

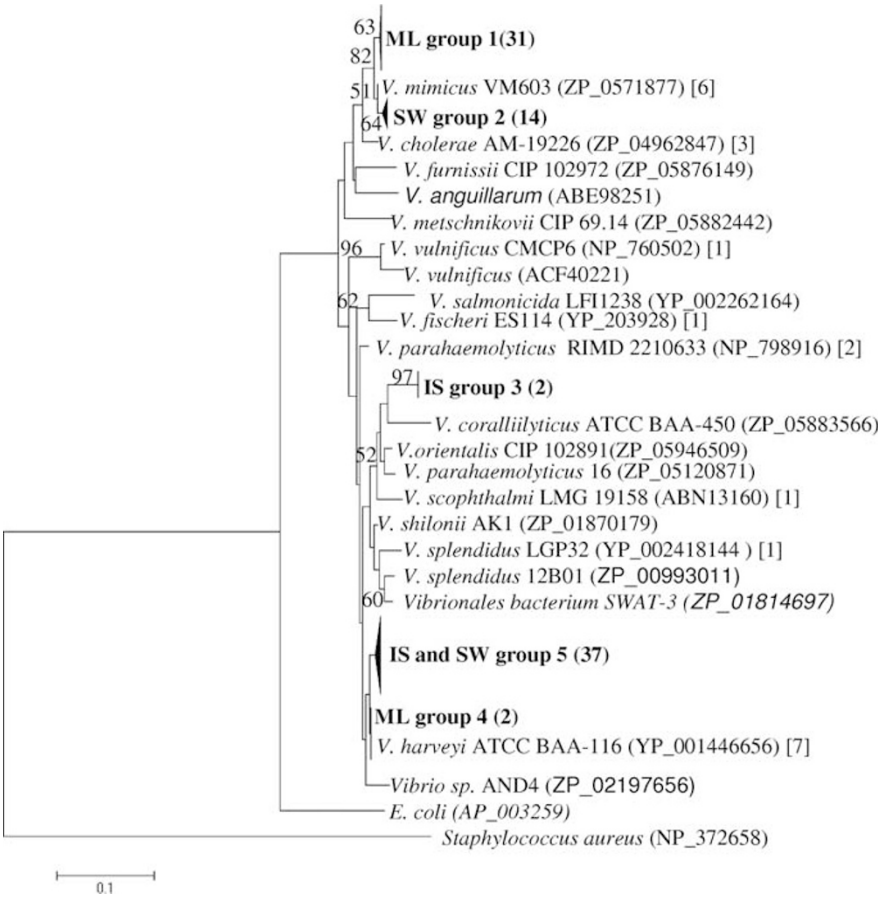

Figure 6 Phylogenetic tree using the neighbor-joining method based on the predicted 117 aa residues encoded by the *luxS* gene amplified from the bacterial communities of *M. laxissima* (ML), *I. strobilina* (IS) and surrounding seawater (SW). Sequences obtained in this study are in bold. Bootstrap values > 50% are shown at each node. The scale bar indicates the number of aa substitutions per site. Numbers in () after each group name indicate the number of clones in each group. See Supplementary Material Table 1 for accession numbers of each clone. Numbers in [] after reference sequences are the same as in Figure 2 legend. See Supplementary Material Table 2 for accession numbers of each clone.

to previously described nitrogen-fixing *Vibrio* spp. and none of the *nifH* gene sequences retrieved from the microbial communities of *M. laxissima* and *I. strobilina* is closely related to the *nifH* genes of vibrios (Mohamed *et al*., 2008b). The role of the *Vibrio* spp. associated with *M. laxissima* and *I. strobilina* therefore remains speculative.

Using degenerate *luxS* primers based on *Vibrio luxS* gene sequences, partial *luxS* gene sequences were detected in all *Vibrio* isolates from the two marine sponges and surrounding seawater. A novel cluster containing *luxS* genes from six isolates shared only low identity (88–90%) to known *luxS* gene sequences. Our results thus extend the known diversity of this QS gene. *luxS* genes are highly divergent among gram-negative and gram-positive bacteria but might have similar functions. For example, the *luxS* gene of *Bacillus subtilis* shares only *ca* 40% identity to that of *V. cholerae* and *V. harveyi*; however, AI-2 produced in *B. subtilis* can induce the bioluminescence of *V. harveyi* BB170 and LuxS-dependent QS is required to form a differentiated biofilm and also for swarming on solid surfaces (Lombardia *et al*., 2006). Further, even among the vibrios, the *luxS* gene of *V. cholerae* shares only 87% identity to that of *V. harveyi* yet the *lux* operon of *V. harveyi* can function well in *V. cholerae* (Lenz *et al*., 2004). This suggests that the novel *luxS* cluster obtained in this study might be involved in the same pathway as that of *V. harveyi* or *V. cholerae*. The $LuxS_{72}$ aa sequence has all of the uniformly conserved residues for other *S*-ribosylhomocysteinases, including those implicated in metal binding (Hilgers and Ludwig, 2001). It is therefore not surprising that this protein can function to drive synthesis of AI-2 when expressed in *E. coli* DH5α.

A comparison of the plate counts obtained on marine agar and the total cell counts present in the sponges revealed that *ca* 1% of sponge-associated bacteria were culturable from both sponges. Vibrios comprised a major group of bacteria in the culturable assemblage from both sponges (*ca* 30% and 25% for *M. laxissima* and *I. strobilina*, respectively). We therefore focused on *Vibrio* spp. for this investigation of the presence of *luxS* genes in

sponge-associated bacteria because of the high incidence of *Vibrio* spp. in the total bacterial assemblage and because there is a precedent for *luxS* signaling to be important in colonization of a marine invertebrate by a *Vibrio* sp., in the *V. fischeri*/*Euprymna scolopes* symbiosis (Lupp and Ruby, 2004). We acknowledge that the proportion of *Vibrio* spp. in the total sponge-associated bacterial community may be low (our data establish a lower limit of *ca* 3 *Vibrio* cells/10 000 prokaryote cells in these two sponges) and that the ecological significance of *luxS* signaling in these communities remains to be established. A recent study obtained 25 *Vibrio* isolates from healthy and diseased corals and showed that all the *Vibrio* isolates were able to activate an AI-2 reporter, although no *luxS* genes were amplified in this study (Tait *et al*., 2010). The investigators speculated that vibrios might use several different types of QS signal molecules to regulate processes that are used to colonize corals during adverse environmental conditions. Studies by Bansal *et al*. (2008) and Englert *et al*. (2009) showed that *E. coli* cells are attracted by AI-2 and AI-2 can be as effective an attractant as L-aspartate. On the basis of these studies, one hypothesis is that *E. coli* might use AI-2 to join groups of bacteria that produce AI-2 (Defoirdt, 2010). It is also possible that chemotaxis to AI-2 might serve to attract free-living bacteria to biofilms (Hegde *et al*., 2011). AI-2 is better understood in Vibrios than in other bacteria. AI-2 has been well characterized in *V. harveyi* and *V. cholerae* as a signaling molecule. It controls bioluminescence, metalloprotease production and type III secretion in *V. harveyi* and represses biofilm and virulence and activates protease activity in *V. cholerae* (Ng and Bassler, 2009). In light of these observations, it is possible that similar functions are controlled by AI-2 signaling in sponge-associated Vibrios.

Interestingly, in some cases there was a large difference in the levels of AI-2 activity between closely related isolates (for example, isolates JZ08ML70 and JZ08ML71 show around fivefold difference in AI-2 activity. The *luxS* gene has been showed to be regulated both at the transcriptional and translational levels (Zhang *et al*., 2008; Udekwu, 2010). The difference in the levels of AI-2 activity between closely related isolates, (for example, JZ08ML70 and JZ08ML71 show around fivefold difference in AI-2 activity) could be due to the difference in the gene regulation.

AI-2/*luxS* QS systems among vibrios can regulate genes involved in biofilm formation, virulence factors, bioluminescence and colonization (Ng and Bassler, 2009). In the well-characterized *V. fischeri*—*E. scolopes* colonization system, double mutants of *luxS* and *ainS* (an AHL synthase gene) reduces the already diminished level of colonization of *ainS* mutants by 50% (Lupp and Ruby, 2004). Almost all the *Vibrio* isolates obtained in this study (Zan, Fuqua and Hill, in preparation) and in previous report by Mohamed *et al*. (2008a) also produce AHL. The role of the AI-2/*luxS* system in the colonization of sponges by vibrios provides an interesting area for future work.

The AI-2 activity of JZ08IS72 peaked 6h after inoculation, decreased during the transition to stationary phase and there was almost no AI-2 activity detectable after 18h following inoculation. In contrast, the AI-2 activity of *V. mimicus* ATCC 33653 detected in parallel with that of JZ08IS72 did not show this pattern. In *V. mimicus* ATCC 33653, AI-2 activity peaked between 8 and 10h after inoculation and remained relatively constant with some fluctuations thereafter (data not shown). There are no other reports of AI-2 systems from *Vibrio* species showing a peak in production followed by a decline, not even in *V. harveyi* and *V. cholerae*, in which the AI-2 QS pathway is well studied. However, in *Salmonella enterica* serovar Typhimurium and *E. coli* K-12 strain MG1655, AI-2 production peaks in mid-to-late exponential phase and quickly declines either in the transition to stationary phase or in stationary phase (Xavier and Bassler, 2005; Doherty *et al*., 2006). This is because of the internalization of AI-2 by the Lsr transporter in *S. enterica* serovar Typhimurium and by the b1513 transporter (a homolog of the Lsr transporter) in *E. coli* K-12 strain MG1655 (Xavier and Bassler, 2005). No homolog of the Lsr transporter was found in the genomes of *V. harveyi* and *V. cholerae* (data not shown). This suggests that a potential new pathway involving in internalization of AI-2 or perhaps degradation may exist in JZ08IS72, which warrants further investigation. It is also possible that other environmental factors might affect the AI-2 activity of JZ08IS72 during stationary phase, such as the lack of a preferred carbon source, neutral pH, and low osmolarity (Surette *et al*., 1999). The ecological relevance of the peak-shaped pattern is not clear.

In the *luxS* gene clone library from *M. laxissima*, ML group 1 was clearly distinct from SW group 2 with a high bootstrap value of 82% and did not cluster with any other known *luxS* gene sequences, implying that ML group 1 might be a sponge-specific *luxS* gene group. IS group 3 is a relatively small group that is distantly related to any known *Vibrio luxS* sequence, suggesting that this group comprises a sponge-specific *luxS* gene group. Clones from *I. strobilina* and seawater in IS and SW group 5 were closely related to each other, suggesting that strains carrying these genes may be derived from the surrounding seawater.

Conclusions

All the *Vibrio* spp isolated from two marine sponges were shown to have *luxS* gene and were demonstrated to induce light production in a reporter strain. A novel cluster of *luxS* gene (cluster MI) sequences was obtained. The relationship between

AI-2/*luxS* QS and the colonization on sponge hosts by vibrios deserves further investigation. This work provides the first information about *luxS* genes and AI-2 activity in sponge-associated bacterial communities.

Acknowledgements

We acknowledge the National Undersea Research Center (NURC), University of North Carolina at Wilmington for providing sampling opportunities in Key Largo, FL, USA. Funding for this research was provided by the National Science Foundation Directorate for Biological Sciences Molecular and Cellular Biosciences (MCB-0238515) and Integrative Organismal Systems (IOS-0919728). We thank Tao Long and Bonnie Bassler for the gift of *V. harveyi* TL-26. Ryan Powell is thanked for assistance in preparation of figures and Leah Blasiak is thanked for comments on the paper. We thank two anonymous reviewers for thoughtful comments.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)