A New *N*-Acyl Homoserine Lactone Synthase in an Uncultured Symbiont of the Red Sea Sponge *Theonella swinhoei*

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Sponges harbor a remarkable diversity of microbial symbionts in which signal molecules can accumulate and enable cell-cell communication, such as quorum sensing (QS). Bacteria capable of QS were isolated from marine sponges; however, an extremely small fraction of the sponge microbiome is amenable to cultivation. We took advantage of community genome assembly and binning to investigate the uncultured majority of sponge symbionts. We identified a complete *N*-acyl-homoserine lactone (AHL)-QS system (designated TswIR) and seven partial *luxI* homologues in the microbiome of *Theonella swinhoei*. The TswIR system was novel and shown to be associated with an alphaproteobacterium of the order *Rhodobacterales*, here termed *Rhodobacterales* bacterium TS309. The *tswI* gene, when expressed in *Escherichia coli*, produced three AHLs, two of which were also identified in a *T. swinhoei* sponge extract. The taxonomic affiliation of the 16S rRNA of *Rhodobacterales* bacterium TS309 to a sponge-coral specific clade, its enrichment in sponge versus seawater and marine sediment samples, and the presence of sponge-specific features, such as ankyrin-like domains and tetratricopeptide repeats, indicate a likely symbiotic nature of this bacterium.

Symbiosis between metazoans and microbes exists in virtually every environment, including in marine sponges (phylum *Porifera*) (1). Marine sponges are important members of shallow and deep reef communities and are a rich source of secondary metabolites with pharmaceutical potential, and these metabolites are often produced by the sponges' microbial symbionts (2, 3). These host-associated bacteria are sponge specific and can comprise up to 35% of the sponge volume (4), spanning at least 47 bacterial phyla (5).

In recent years, symbioses between microorganisms and eukaryotic hosts have received growing attention. The host, together with all its symbionts, is now seen as a single entity designated the holobiont, defined as the sum of the genetic information of the host and its microbiota (6). Sponge holobionts provide an enclosed niche in which extensive interactions among dense and diverse microbial populations are accomplished (7). The mechanisms underlying cell-cell interactions among bacteria within the sponge and the genetic regulation systems controlling gene expression in sponge symbionts are largely unknown. One of the best understood microbial quorum-sensing (QS) systems is the one that uses *N*-acyl-homoserine lactones (AHLs) as signals (e.g., 8–10). QS enables bacteria to regulate their gene expression in a population density-dependent manner. At a low cell density, low levels of AHLs, which diffuse in and out of the bacterial cell, are present. As the bacterial density increases, especially for bacteria enclosed in niches, AHLs accumulate, and once they reach a critical level (quorum), they interact with LuxR cognate receptor proteins, which then affect target gene expression (11). A canonical AHL-QS system produces AHLs via the LuxI family AHL synthase protein and responds to them via the LuxR cognate receptor protein (12). The regulation of target genes is mediated by the formation of LuxR-AHL complexes that directly bind to specific promoter sequences (*lux* boxes) upstream of QS-regulated genes

(11). Not all AHL synthases belong to the LuxI family proteins; AHLs can also be synthesized by LuxM- and AinS-type proteins (11). QS systems can regulate a variety of cellular processes that are beneficial to a bacterial community, such as motility, sporulation, production of virulence factors and secondary metabolites, plasmid transfer, and biofilm formation (11), and they can facilitate the colonization of desirable niches (13).

Bacteria with AHL-QS systems have been isolated from marine sponges (for examples, see references 7 and 14), and signaling molecules able to activate AHL biosensors have been reported in sponge extracts (7, 14, 15); the chemical nature of most of these signaling compounds is currently unknown. Gardères et al. (16) identified AHLs produced *in vivo* by symbionts of the Celtic Sea sponge *Suberites domuncula*; more recently, it was determined that the signal molecule produced, *N*-3-oxododecanoyl-L-homoserine lactone, affects the expression of immune and apoptotic genes of the host sponge *S. domuncula*, possibly enabling the

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sponge to monitor and regulate bacterial populations (17). Furthermore, Zan et al. (18) studied QS in the AHL-producing *Ruegeria* sp. strain KLH11 isolated from the marine sponge *Mycale laxissima* (18). This bacterium possesses a complex and interconnected QS system composed of two pairs of *luxIR* homologues and one orphan *luxI* (a LuxI gene that does not possess a cognate LuxR [14, 19]). QS regulates the expression of flagellar motility and biofilm formation in KLH11 and may dictate the transition between free-living and sessile modes of growth, possibly limiting aggregation within the sponge (14). These studies provided evidence of AHL-QS regulation in sponge-associated bacteria. However, considering that a large fraction of sponge-associated microbes are not yet amenable to cultivation, culture-independent studies are required to obtain a general insight into the extent to which AHL-QS is present within a sponge microbiome.

In this study, we used culture-independent techniques to determine which AHL-QS systems are found within the microbiome of *Theonella swinhoei* (class *Demospongiae*) and their relative abundances. This sponge species was chosen because it is of wide chemical interest (20), harbors a dense microbial community (21), and is ubiquitous in the Western Indo-Pacific Ocean.

MATERIALS AND METHODS

AHL detection in sponge tissue using *Agrobacterium tumefaciens* reporter. A few cubic centimeters of sponge tissue was collected from 26 different *T. swinhoei* (22) specimens from Eilat, Israel (29°29'45.28"N, 34°54'43.02"E), during 2013 to 2015. The sponge samples were immediately frozen in liquid nitrogen after being brought to the surface from the dives. Frozen tissues were freeze-dried using a lyophilizer (VirTis). Two grams of dried tissue was ground into powder in liquid nitrogen, and AHLs were extracted using a modified Bligh-Dyer procedure (23) and via solid-phase extraction (SPE), as described in reference 14, in which 10 ml of methanol was added to the sponge powder and incubated overnight with shaking. Five milliliters of chloroform then was added prior to three rounds of sonication. Afterwards, 7.5 ml of water and 5 ml of chloroform were added. The chloroform layer was separated and evaporated to dryness. The residues were then suspended in 6 ml of 1:1 isooctane-ethyl ether and subjected to SPE purification on a Sep-Pak silica cartridge (Waters). The samples were washed with three loads of 1:1 isooctane-ethyl ether and eluted using 6 ml of ethyl acetate. The eluted samples were evaporated using SpeedVac, and AHLs were detected using a thin-layer chromatography (TLC) overlay technique, as previously described (24). Briefly, the organic extract of each individual sponge was dissolved in 40 μ l of ethyl acetate acidified with acetic acid (0.1%). Thirty microliters of these extracts was loaded onto C₁₈ reversed-phase (RP)-TLC plates (Mallipore) and developed in 70% methanol in water. The plates were dried and overlaid with 100 ml of soft AB agar (0.7%) supplemented with 0.5% glucose and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) and then seeded with 10-ml volumes of *A. tumefaciens* NTL4(pZLR4) overnight cultures (25). C₆-homoserine lactone (HSL) and C₈-HSL standards (Sigma-Aldrich, St. Louis, MO, USA) were also loaded on the TLC.

Identification of AHLs from *T. swinhoei* extract. To determine the AHL types present in *T. swinhoei*, we extracted 9 g of a *T. swinhoei* specimen that had showed a positive AHL result, according to the TLC assay with an *A. tumefaciens* reporter. The ethyl acetate extract obtained after SPE purification (see details above) was subjected to silica gel column chromatography with a gradient elution from *n*-hexane/ethyl acetate (EtOAc) (100:0 to 0:100) to EtOAc/methanol (MeOH) (100:0 to 0:100), yielding 12 fractions. Each fraction was evaluated for the presence of AHL using a TLC overlay biosensor assay, followed by high-resolution electrospray ionization-liquid chromatography-mass spectrometry (ESI-LC-MS) and ESI-LC-tandem MS (ESI-LC-MS/MS). We used a Thermo Sci-

entific Dionex UltiMate 3000 rapid separation LC (RSLC) system coupled with a Thermo LTQ Orbitrap XL mass spectrometer. The LC-MS data were recorded and analyzed using the software Thermo Xcalibur 2.07. Five microliters of the extracts was injected (dissolved in MeOH at 1 mg/ml), and AHL standard mixtures and blanks were applied onto an analytical reversed-phase column (Kinetex C₁₈; 100 by 2.1 mm; particle size, 5 μ m; Phenomenex), which was eluted at 200 μ l min⁻¹. The elution procedure consisted of an isocratic profile of acetonitrile-water (10:90 [vol/vol]) for 3 min, followed by a linear gradient from 10 to 90% acetonitrile in water over 30 min, and an isocratic profile over 2 min. Five microliters of commercially available synthetic AHLs (C₄-HSL, C₆-HSL, 3-oxo-C₆-HSL, C₈-HSL, 3-oxo-C₈-HSL, C₁₀-HSL, C₁₂-HSL, C₁₄-HSL, and 3-OH-C₁₈-HSL) was used as the standard (20 μ g/ml). Compounds were detected by high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) under positive-ion conditions. To identify each AHL, extracted ion chromatograms were generated at m/z 172.0968 $\pm$ 0.0010 (C₄-HSL), m/z 200.1281 $\pm$ 0.0010 (C₆-HSL), m/z 214.1074 $\pm$ 0.0010 (3-oxo-C₆-HSL), m/z 228.1594 $\pm$ 0.0010 (C₈-HSL), m/z 242.1387 $\pm$ 0.0010 (3-oxo-C₈-HSL), m/z 256.1907 $\pm$ 0.0010 (C₁₀-HSL), m/z 284.2220 $\pm$ 0.0010 (C₁₂-HSL), m/z 312.2533 $\pm$ 0.0010 (C₁₄-HSL), and m/z 384.3108 $\pm$ 0.0010 (3-OH-C₁₈-HSL). The retention times of the extracted ions were compared with the respective chromatograms of the standards. The identification of AHLs was confirmed by their MS/MS fragmentation pattern, which was identical to that of the corresponding standard, and, for all the AHLs, this included the characteristic product ion at m/z 102.0550, corresponding to the deacylated homoserine lactone (Fig. 1).

Cell separation, microbiome enrichment, and metagenome DNA extraction. An individual *T. swinhoei* sponge was collected by scuba divers from the Gulf of Aqaba, Red Sea, near Eilat, on 31 July 2012. The sponge was transported on ice to the laboratory for processing (within 20 min). Bacteria were enriched from the sponge using a series of filtration and centrifugation steps, according to the method described by Thomas et al. (26). Separated cells were kept at -80°C until DNA was extracted. Genomic DNA was purified by sodium dodecyl sulfate (SDS)-proteinase K lysis. Selective precipitation of cell debris and polysaccharides was performed with cetyltrimethylammonium bromide (CTAB) and isopropanol precipitation, as previously described (27). Information on the presence of AHL for this specific specimen is not available.

Shotgun sequencing. The DNA of the *T. swinhoei* microbiome was sheared using the Covaris sonicator (200- to 400-bp size). Libraries were generated using the TruSeq DNA standard protocol and pooled for sequencing on one lane of the Illumina HiSeq 2000 platform, generating 7 Gb of sequence as 2 $\times$ 100-bp reads (with a short insert size of ~170 bp), and FASTQ files were generated using CASAVA 1.8. Sequence quality was assessed using the FASTX-Toolkit 0.0.13.2 (http://hannonlab.cshl.edu/fastx_toolkit/).

Metagenome assembly and binning. The metagenome was assembled using two different tools, MetaVelvet and IDBA-UD, to better ensure the recovery of QS genes. Both approaches resulted in the assembly of an identical single contig with a complete QS system. Overall, the IDBA-UD assembly recovered more taxonomically binnable contigs and was therefore used for genome recovery (see Table S1 in the supplemental material). For MetaVelvet, merged reads (see below) were assembled using version 1.2.02 (28), with a 61-bp *k*-mer size. Unmerged paired-end reads were then also assembled *de novo* using IDBA-UD 1.1.0 (29), with minimum and maximum *k*-mers of 50 and 70, respectively, and a step size of 5. Contigs were binned taxonomically using multiparameter methods, as described previously (reference 30 and references therein). Briefly, binning was based on concordance among the following phylogenetic and compositional methods: coverage, G+C content, taxonomic affiliation of predicted protein sequences, and emergent self-organizing (ESOM) maps of contig fragment tetranucleotide frequencies (maps were built using 5-kb contig fragments, followed by projections based on 2-kb fragments). To aid binning, protein sequences were predicted using Prodigal version 2.60 in metagenomics mode (31), and sequences were searched against

FIG 1 Mass spectrometry analysis. (A) Extracted ion chromatograms at m/z 228.1549 $\pm$ 0.0010 (C_8 -HSL, denoted by 1), m/z 256.1907 $\pm$ 0.0010 (C_{10} -HSL, denoted by 2), and m/z 384.3108 $\pm$ 0.0010 (3OH- C_{18} -HSL, denoted by 3), derived from fractions of extract of *T. swinhoei*. (B) Extracted ion chromatograms at m/z 228.1594 $\pm$ 0.0010 (C_8 -HSL, denoted by 1), m/z 256.1907 $\pm$ 0.0010 (C_{10} -HSL, denoted by 2), and m/z 284.2220 $\pm$ 0.0010 (C_{12} -HSL, denoted by 4), derived from fractions of extract of *E. coli* DH5 α harboring *tswI*-pBBR1MCS. The characteristic product ion at m/z 102.05, corresponding to the deacylated homoserine lactone, was detected.

the UniRef90 database (32) using ublast (usearch64 [33]). The coverage of each contig was determined by mapping reads back to contigs using Bowtie version 1.0.0, with default parameters (34).

Further recovery and quantification of QS genes. To detect any additional unmapped/unassembled reads of *luxI* origin, a reciprocal BLASTX search was performed as follows: (i) read pairs were merged into single ~105- to 185-bp segments using PANDaseq 2.5 (35) and queried against a target protein database of ~50 LuxI representatives (see Table S2 in the supplemental material), obtained from a study by Nasuno et al. (36); (ii) query sequences with hit E values of $<10^{-2}$ were retrieved and then compared to the NCBI nonredundant (nr) protein database using a cloud server (<http://www.diagcomputing.org>). The investigation of AHL produced by other AHL synthases (e.g., LuxM) was beyond the scope of this study.

To quantify the approximate relative abundances of assembled and unassembled *luxI* homolog genes in the microbiome, we used DNA gyrase subunit B (*gyrB*) as the single-copy normalizing gene and first retrieved read sequences that mapped to the *luxI* and *gyrB* regions on the contigs. To reconcile the effects of gene size on hit retrieval, the number of hits for each *luxI* gene was normalized to the average length (L; in base pairs) of *luxI* (615 bp [37]) and compared to the length of *gyrB* (2,415 bp in *Escherichia coli*) using the following equation: relative abundance of *luxI* = (no. of *luxI* hits/no. of *gyrB* hits) $\times$ (*gyrB* L/*luxI* L) (38).

Heterologous expression of the LuxI homologue from *Rhodobacteriales* bacterium TS309. The *tswI* gene sequence amended with a strong upstream ribosomal signal (AAAGAGGAGAAA) was synthesized as a HindIII-BamHI fragment (GenScript USA, Inc.) and cloned in pUC57. The gene was excised from pUC57 with Hind and BamHI (NEB, United Kingdom) and cloned into the corresponding sites in the broad-host-range cloning vector pBBR1MCS-5 under the expression of the P_{lac} promoter. The plasmid was transformed in *E. coli* DH5 α , as previously described (39). *E. coli* harboring the plasmid carrying *tswI* or the pBBR1MCS-5 empty vector was grown overnight in 100 ml of LB supplemented with 10 μ g/ml gentamicin (Gm). The cultures were centrifuged at 6,000 rpm for 15 min, and the supernatants were filtered (0.45- μ m pore size; Millipore) and extracted twice with an equal vol-

ume of ethyl acetate supplemented with 0.1% acetic acid. The organic fractions were then dried to completeness. AHLs were identified using mass spectrometry, as described above (see "Identification of AHLs from *T. swinhoei* extract").

PCR screen for *tswI* in *T. swinhoei*. For all sponge specimens collected in this study, we also collected a voucher sample (kept at the University of Haifa) with approximately 1 cm³ of tissue preserved in 10 ml of 100% ethanol (Sigma-Aldrich, Germany). DNA was extracted from 15 subsamples of these voucher samples to test if *tswI* could be amplified by PCR. Primers were designed to amplify part of the *tswI* gene (the *luxI* homologue) with IDT OligoAnalyzer 3.1. *tswI* was amplified using ~4 ng/ μ l whole sponge tissue DNA, MyTaq Red mix (Bioline), and the primers *tswI*-qPCR1-F (TCGTCCTGGGGCAACTATAC) and *tswI*-qPCR1-R (ACAAACCCCTCGACAATCAG) (Sigma-Aldrich, Germany), at a final concentration of 0.2 μ M. The PCR temperature profile was as follows: 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 61°C, and 1 min at 72°C, and a final extension step of 3 min at 72°C. The PCR products (size, 194 bp) were visualized by agarose gel electrophoresis. Five random amplicons were sequenced (Macrogen, Republic of Korea) to confirm their identity as *tswI*.

Verification of *tswR*-t truncation. To verify that the truncation of the *luxR* homologue *tswR*-t is not an artifact of the sequence assembly, primers were designed to amplify regions upstream (490 bp) and downstream (60 bp) of the *tswR*-t gene using Primer3Plus (40). *tswR*-t was amplified using DNA extracted from six *T. swinhoei* specimens as the template. PCR amplification was performed using ~4 ng/ μ l template DNA, MyTaq Red mix (Bioline), and the primers *tswR*-t-F (GTATCTCGGC GTCGTCAAAT) and *tswR*-t-R (ATAAGGCCCCGCCCCAAAC) (Sigma-Aldrich, Germany), at a final concentration of 0.2 μ M. The PCR temperature profile was as follows: 3 min at 95°C, followed by 35 cycles of 15 s at 95°C, 30 s at 65.7°C, and 45 s at 72°C, and a final extension step of 1 min at 72°C. The PCR products were visualized by agarose gel electrophoresis, and the product size of 1,080 bp was purified using the Wizard SV gel and PCR Clean-Up system (Promega). PCR products were cloned into pGEM-T Easy (Promega) and chemically transformed into *E. coli* JM109, according to standard protocols (39). Plasmids

TABLE 1 Plasmids used in this study

Plasmid	Relevant feature ^a	Reference or source
pGEM-T Easy	Cloning vector; Amp ^r	Promega
pMP220	Promoter probe vector; IncP; Tc ^r	42
pBBRmcs5	Broad-host-range vector; Gm ^r	41
pUC57TswI	pUC57 containing <i>tswI</i> gene	GenScript USA, Inc.; this work
pUC57TswR	pUC57 containing <i>tswR</i> gene	GenScript USA, Inc.; this work
pUC57TswR-t	pUC57 containing <i>tswR</i> -t gene	GenScript USA, Inc.; this work
pBBTswI	pBBRmcs5 containing <i>tswI</i> gene	This work
pBBTswR	pBBRmcs5 containing <i>tswR</i> gene	This work
pBBTswR-t	pBBRmcs5 containing <i>tswR</i> -t gene	This work
pMPTswIP	pMP220 containing <i>tswI</i> promoter	This work

^a Amp^r, ampicillin resistance; Tc^r, tetracycline resistance; Gm^r, gentamicin resistance.

were purified using the PureYield plasmid miniprep system (Promega) and sequenced (Macrogen, Republic of Korea).

Functionality of the TswIR-QS system based on promoter activity assays. To determine the activity of the LuxR homologues TswR and TswR-t, each of the genes was amended with a strong upstream ribosomal sequence and was synthesized as BamHI-XbaI and XbaI-SacI fragments, respectively (GenScript USA, Inc.). The fragments were cloned into pUC57. To express the LuxR homologues (*tswR* and *tswR*-t) under the control of the *lacZ* promoter, each of the genes was cut from pUC57 and cloned into the corresponding sites in the cloning vector pBBR1MCS-5 (Gm) (41) to generate the plasmids pBTswR and pBTswRt. The intergenic region between TswR and TswI, predicted to contain the promoter region of the TswI AHL synthase (based on Predictions of Bacterial Promoters [BPROM]; Softberry), and the intergenic region between TswR-t and TswR, predicted to contain the promoter region of TswR, were both synthesized as KpnI-XbaI fragments (GenScript USA, Inc.) and cloned in the promoter probe vector pMP220 (42), generating pMPTswIP and pMPTswR, respectively. The restriction enzymes and T4 DNA ligase were purchased from New England BioLabs (NEB, United Kingdom). The plasmid was transformed in *E. coli* DH5 α , as previously described (39). The plasmid pMPTswI in combination with pBTswR or pBTswRt was introduced in the heterologous host AHL-negative (AHL⁻) *A. tumefaciens* NTL4 (43) by triparental conjugation, using HB101 (pRK2013) as a helper strain, as previously described (44). AHL⁻ *A. tumefaciens* NTL4 was chosen for reporter studies because it is an alphaproteobacterium, like *Rhodobacterales* bacterium TS309, and because this strain lacks the pTiC58 plasmid, which carries the QS genes *traI* and *traR*, and for this reason cannot interfere with *tswR* functions. All plasmids that were used in this study are listed in Table 1.

E. coli DH5 α , JM109, and HB101 (pRK2013), the helper strain (42), were grown in LB medium at 37°C. AHL⁻ *A. tumefaciens* NTL4 (43) was grown in yeast extract-Bacto peptone (YEP) medium at 28°C. The following antibiotic concentrations were used: ampicillin (Amp), 100 μ g/ml; kanamycin (Km), 50 μ g/ml; tetracycline (Tc), 15 μ g/ml (*E. coli*) and 30 μ g/ml (*A. tumefaciens*); and gentamicin (Gm), 15 μ g/ml (*E. coli*) and 30 μ g/ml (*A. tumefaciens*).

β -Galactosidase assays were performed as previously described (45). *A. tumefaciens* transconjugants were grown overnight in YEP medium. When required, the cultures were supplemented with the AHLs extracted from the *E. coli* strain expressing the *tswI* gene (pBBTswI). Average Miller

unit values and standard deviations were calculated from the results from three independent experiments.

Genome completeness and annotation. The genome completeness of *Rhodobacterales* bacterium TS309 was determined on the basis of 35 single-copy orthologous groups (OGs) (46). The OGs were obtained from eggNOG version 3.0 (47). Sequences were considered orthologous on the basis of reciprocal BLASTX searches, with a minimum bit score of 60 and at least 50% shared identity. The genomes were annotated using RAST (rast.nmpdr.org). Predicted proteins were also functionally annotated using the RPS-BLAST program on the COG database using WebMGA, a COG annotation tool (48).

Candidate CRISPR analysis. Candidate clustered regularly interspaced short palindromic repeat (CRISPR) arrays were predicted from the DNA sequences of the genomes analyzed in this study by CRISPRFinder, using the default setting (49). CRISPRs containing two spacers with a difference in length exceeding one nucleotide were discarded. CRISPRs containing one spacer were also discarded, except for one CRISPR that was found at the beginning of a contig.

Phylogenetic analysis with concatenated protein tree. A tree using concatenated protein sequences that represent the distinctive characteristics of all alphaproteobacteria was constructed, as previously described (50), with minor changes. Six conserved proteins (arginyl-tRNA synthetase, DNA-directed RNA polymerase β -subunit, gyrase A, Hsp70, phenylalanyl-tRNA synthetase, and RecA) were chosen out of the 12 that are characteristic of all *Alphaproteobacteria* (50). Only 6 out of 12 proteins were chosen based on their shared presence in the two novel binned *Alphaproteobacteria* genomes assembled in this study. The amino acid sequences of these proteins were downloaded from the IMG database for 66 different *Alphaproteobacteria* species (see Table S3 in the supplemental material) and aligned using Clustal W (51). *Helicobacter pylori* was included as an outgroup. The sequence alignments for all 6 proteins were concatenated into a single sequence. Poorly aligned regions were removed using gBlocks (52). The final sequence alignment used for phylogenetic analysis contained 3,203 aligned positions. Maximum likelihood was computed using the WAG+F model plus a gamma distribution using MEGA 6.06 (53). Positions with gaps were deleted. The trees were bootstrapped 500 times.

Phylogenetic analysis with 16S rRNA gene tree. Near-full-length 16S rRNA gene sequences of 6 *Alphaproteobacteria* from the microbiome of *T. swinhoei* were reconstructed from the metagenomic data using a reference-guided method, Expectation Maximization Iterative Reconstruction of Genes from the Environment (EMIRGE) (54). Reconstruction was performed over 100 iterations with clustering of sequences $\geq 97\%$ similar. The 16S rRNA gene sequences were classified with the SINA aligner version 1.2.11 (55), and all obtained *Alphaproteobacteria* sequences were used for further phylogenetic analysis. The sequence data set used for 16S rRNA phylogenetic comparisons consisted of (i) 16S rRNA sequences from the type strain of each *Alphaproteobacteria* genus present in the All-Species Living Tree release LTP 115 (56), (ii) 16S rRNA sequences from IMG derived from genomes used in the concatenated protein tree, and (iii) the closest BLAST hits in GenBank to each of the six EMIRGE-reconstructed *Alphaproteobacteria* sequences. In addition to the *H. pylori* 16S rRNA sequence, we used the same sequences employed by Ferla et al. (57) as the outgroup. The sequences were aligned with the SINA aligner version 1.2.11 using the bacterial mask and default settings. Ambiguously aligned regions present after manual improvement were detected and eliminated using gBlocks version 0.9 (52). The final alignment contained 410 sequences and 1,308 positions and was used for tree reconstruction in MEGA 6.06 (53) using the neighbor-joining algorithm based on Kimura 2-parameter distances, for which ambiguous data in sequence pairs were removed. Support for the phylogenetic relationships was based on 500 bootstrap replicates.

Distribution of *Rhodobacterales* bacterium TS309 among sponge species, seawater, and marine sediment samples. Samples were taken and processed according to standard operating procedures to ensure max-

imum comparability. Each sponge species was sampled at least three times, and samples were collected using sterile equipment. Sample processing, sequencing, and core amplicon data analysis were performed by the Earth Microbiome Project (www.earthmicrobiome.org), and all amplicon data and metadata have been made public through the data portal (www.microbio.me/emp) (58). The V4 region of the 16S rRNA gene was amplified using the primer pair 515f-806rB and sequenced using the HiSeq 2500 platform (Illumina) (59). The sequencing data are publicly available through the Qiita website (<http://qiita.ucsd.edu/>) under project ID 1740.

The Illumina reads were processed in mothur version 1.31.2 (60). First, quality-filtered demultiplexed FASTQ sequences were trimmed according to quality (using the *trim.seqs* command: parameters, qwindow-average = 30, qwindow-size = 5, maxambig = 0, maxhomop = 8, and minlength = 100). To minimize computational effort, the files were reduced to nonidentical sequences (*unique.seqs* and *count.seqs*). Nonredundant sequences were aligned (*align.seqs*: flip = t) to a trimmed reference SILVA 102 (61) bacterial database (*pcr.seqs*: start = 11894, end = 25319, keepdots = F), which was provided by mothur (61). Only sequences that aligned to the expected position were kept (*screen.seqs*: start = 1968, end = 4411; *filter.seqs*: vertical = T, trump = .). The aligned reads were reduced to nonredundant sequences (*unique.seqs*). Chimeric sequences were detected using Uchime (*chimera.uchime*: dereplicate = t) (62) and filtered out (*remove.seqs*). Pairwise distances between the aligned sequences were calculated (*dist.seqs*: cutoff = 0.05) and were used for clustering. Prior to clustering, the aligned sequences were phylogenetically classified based on the trimmed SILVA database (*classify.seqs*) (63). Sequences were clustered (*cluster.split*: fasta =, count =, taxonomy =, split-method = classify, taxlevel = 4, cutoff = 0.03, hard = t, method = furthest) and converted to .shared file format (*make.shared*: list =, count =, label = 0.03). Finally, operational taxonomic unit (OTU) representative sequences were retrieved based on the distances among the cluster sequences (*get.oturep*: list =, label = 0.03, fasta =, count =) and were further classified based on SILVA, Greengenes, and RDP taxonomies (*classify.seqs*: fasta =, template =, taxonomy =, cutoff = 60) (61, 64, 65). FASTQ sequences from additional samples ($n = 340$) that were generated at a later time point were processed with the same pipeline. These sequences were integrated into the shared file using QIIME 1.8 (66), based on their similarity to the OTU representative sequences (*parallel_pick_otus_uclust_ref.py*: -similarity 0.985 -optimal_uclust). Sequences that were not similar to the OTU representative sequences were separately clustered with mothur and integrated into the previous files (.shared and taxonomy files). The integrated OTU table (.shared file) was filtered to remove low-abundance sequences (sequences <0.001% across the whole data set) and chloroplasts (according to SILVA or Greengenes). Additionally, counts from seawater-like OTUs (>0.01% across all seawater samples) were removed from the sponge samples. File manipulation and processing were carried out with Python scripts (<http://www.python.org>).

A subset of this data set was used for downstream analyses and included only samples with a triplicate minimum, with the exception of *T. swinhoei*, for which only a duplicate sample was available (see Table S4 in the supplemental material for more information on the samples used in this analysis).

The 16S rRNA gene sequence that was assembled by EMERGE was used for a MegaBLAST (67, 68) against the subset of the EMP-Sponge Microbiome data set described above. OTUs with $\geq 99\%$ identity to the 16S rRNA of *Rhodobacterales* bacterium TS309 were used to determine the relative abundance of this bacterium among 98 sponge species and 143 seawater, 34 marine sediment, and 4 freshwater samples. Relative abundance was calculated as the sum of reads of relevant OTUs in each sample divided by the total number of reads in the same sample. A box plot was generated using the graphic capabilities of R and the ggplot2 package in R (69).

Nucleotide sequence accession numbers. The sequences of the complete *tswI*-*tswR*-*tswR-t* QS system have been deposited in GenBank under

accession numbers KP092521 to KP092523. The accession numbers of the six (EMERGE-) reconstructed 16S rRNA sequences of *Alphaproteobacteria* from the metagenome are KP092524 to KP092529. The accession numbers of the 6 gene/protein sequences of TS309 and Alpha B (see below) are KP192247 to KP192258. The draft genome of *Rhodobacterales* bacterium TS309 was deposited in NCBI under the accession no. JPPB00000000. The raw sequencing data were submitted to the Sequence Read Archive (SRA) under accession no. SAMN03984007. OTUs with 99% identity to the 16S rRNA gene sequence of *Rhodobacterales* bacterium TS309 can be found at the Qiita website (<http://qiita.ucsd.edu/>) under project ID 1740.

RESULTS

AHLs in sponge extracts. To determine whether AHL or AHL-like molecules are found within the sponge tissue of *T. swinhoei*, 26 individual sponges were collected and screened by a modified Bligh-Dyer procedure and SPE purification (14). The extracts were then analyzed by a further step of TLC purification, which was finally overlaid with a wide-spectrum AHL biosensor. It was observed that in 6 (23%) individuals, the biosensor positively responded to the extract, indicating the possible presence of AHLs or AHL-mimicking molecules in the extracts (data not shown). Chemical analysis by LC-MS of a sponge specimen showing a positive result according to the TLC analysis revealed the presence of C₈-HSL, C₁₀-HSL, and OH-C₁₈-HSL in the sponge extract (Fig. 1A).

Identification of AHL-QS genes in the microbiome of *T. swinhoei*. Based on the presence of AHL signal molecules in sponge extracts, we searched for AHL-QS gene homologues in the microbiome of *T. swinhoei* through metagenomic analysis and assembly. The IDBA-UD assembly generated 881 contigs of >10 kb long, with a total length for all contigs ≥ 2 kb of 34 Mbp, and 36,478 gene candidates (see Table S1 and Fig. S1 in the supplemental material). We identified a single complete *luxI*-*luxR* system, including a *luxI* putative gene and two adjacent *luxR* putative genes: one complete, composed of the two canonical domains, and one that was truncated. These three loci were all contained within a single 2,224-bp contig (Fig. 2A) with 30 $\times$ read coverage. We designated these three putative genes *tswI*, *tswR*, and *tswR-t* (*T. swinhoei* I, R, and R-truncated, respectively). The most similar homologs of *TswI*, *TswR*, and *TswR-t*, identified using BLASTX, were *N*-acyl-L-homoserine lactone synthetase of *Rubellimicrobium thermophilum*, with 61% identity (GenBank accession no. WP_021096725.1); the LuxR family transcriptional regulator of *Halocynthiaibacter namhaensis*, with 55% identity (GenBank accession no. WP_039019741.1); and the transcriptional regulator of *Loktanella* sp. strain S4079, with 45% identity (GenBank accession no. WP_045996373.1). A multiple-sequence alignment with the two predicted LuxR protein sequences (*TswR* and *TswR-t*) and additional selected known LuxR homologues is shown in Fig. 2B. *TswR* (202 amino acids) was found to contain all nine conserved amino acids typical of AHL-LuxR proteins (Fig. 2B, stars), while *TswR-t* (162 amino acids) was missing the last of three conserved amino acids (G) commonly found in the DNA-binding domain of QS-LuxR proteins (Fig. 2B, red star). The truncation of *tswR-t* was verified using PCR amplification and sequencing.

The *TswIR* QS system was also recovered by reciprocal BLASTX search, with 102 distinct read pairs mapped to the assembled *tswI* region (608 bp long). Partial *luxI* homologues that did not assemble were also recovered by reciprocal BLASTX searches, which identified 23 additional read pairs as having best hits to

FIG 2 (A) *tswI*–*tswR*–*tswR*-t gene layout. (B) Alignment of LuxR-type transcriptional regulators. The alignment starts from position 60 of TswR (accession no.): *Rhodobacteriales* bacterium TS309 (KP092522), *Actinobacterium mucosum* KCTC 23349 (KAJ55423), *Oceanicola granulosis* (WP_007253988.1), *Octadecabacter arcticus* (WP_015494454.1), *Loktanella vestfoldensis* (WP_007205120.1), *Roseobacter* sp. CCS2 (WP_008235250.1), *Pseudomonas* (WP_003119559), *Burkholderia xenovorans* (WP_011492631), *Aliivibrio fischeri* (WP_005423460), *Rhizobium etli* (WP_004672081), *Ruegeria* sp. KLH11 (WP_008756899), *Rhodobacter* (WP_002720271), *Ruegeria* sp. KLH11 (WP_008757266), *A. tumefaciens* (WP_010892389), *Rhodobacteriales* bacterium TS309 (KP092523), *L. vestfoldensis* (WP_026352205.1), *Roseobacter* sp. CCS2 (WP_008235255.1), and *A. tumefaciens* (AAC18654.1). The highlighted regions indicate high similarity between all the proteins presented. The nine highly conserved amino acids are indicated with stars. The red star indicates the missing amino acid in the truncated LuxR homologues.

LuxI in the NCBI nr database. The reads were clustered according to the common best BLAST hit and enabled us to detect seven potential additional rare *luxI* homologues in the sponge microbiome metagenome (Table 2).

The gene *tswI* was present at approximately 2.7% relative abundance (in relation to *gyrB*) in the sponge microbiome. The total relative abundance of all eight *luxI* homologues among the sponge-associated bacteria was 3.3% (Table 2). These quantifications should be considered approximate, given the potential lim-

itations related to the identification of unknown *luxI* homologues based on gene similarity alone (70).

Presence of *tswIR* among replicates of *T. swinhoei*. Part of the newly identified *tswI* gene was amplified by PCR from the DNA of 15 *T. swinhoei* sponges. For five of these samples, we sequenced the amplicon and confirmed *tswI* identity. This shows that *tswI* is also present in specimens that did not show AHLs.

AHL in sponge extracts compared to heterologous expression of *tswI* in *E. coli*. To determine if the *tswI* gene was functional

TABLE 2 Relative abundances of *luxI* homologues in *T. swinhoei* microbiome

Name	Best BLAST hit ^a	Accession no.	E value	Identity (%) ^b	No. of reads	Relative abundance (%) ^c
<i>tswI</i>	<i>Rubellimicrobium thermophilum</i>	WP_021096725.1	3.00E-75	61	102	2.7
Partial <i>luxI</i> homologue 1	<i>Acidithiobacillus thiooxidans</i>	WP_024895088.1	6E-17 to 5E-27	72-77	10	0.26
Partial <i>luxI</i> homologue 2	<i>Methylobacter tundripaludum</i>	WP_027148164.1	2E-19 to 1E-9	57-82	6	0.16
Partial <i>luxI</i> homologue 3	<i>Acidovorax radices</i>	WP_010458661.1	6E-20	71	2	0.05
Partial <i>luxI</i> homologue 4	<i>Rhodopseudomonas palustris</i>	WP_011502592.1	0.62 to 0.51	48	2	0.05
Partial <i>luxI</i> homologue 5	<i>Burkholderia ambifaria</i>	WP_006749594.1	0.57	47	1	0.03
Partial <i>luxI</i> homologue 6	<i>Oxalobacteraceae</i> bacterium	WP_026334900.1	4.00E-21	67	1	0.03
Partial <i>luxI</i> homologue 7	<i>Chitinimonas koreensis</i>	WP_028447479.1	8E-10	61	1	0.03

^a Reads from the reciprocal BLASTX analysis were clustered according to common best BLAST hit.

^b An identity range was given when different reads that were clustered together showed different percent identities to the best BLAST hit.

^c Abundance relative to *gyrB* abundance.

and whether it produced the same AHL signal molecules identified in the extract of *T. swinhoei* tissue, the gene was cloned and constitutively expressed in *E. coli*. This led *E. coli* to produce C₈-HSL, C₁₀-HSL, and C₁₂-HSL, the first two being found also in the sponge extract (Fig. 1).

TswIR gene promoter studies. In many AHL-QS systems, *luxI* family AHL synthase genes are positively regulated by the LuxR family protein in the presence of quorum levels of AHL (11). However, when tested in a heterologous system, the *tswI* promoter was constitutively expressed independently of the expression of TswR and TswR-t and of the presence of added AHL signal molecules, suggesting a lack of a positive feedback loop (see Fig. S2A in the supplemental material). The *tswR* promoter was also constitutively expressed (see Fig. S2B in the supplemental material).

Genome reconstruction of the bacterium harboring the newly identified QS system. The assembly of Illumina reads, followed by taxonomic binning, provided 12 partial genomes of likely sponge symbionts, which had a genome completeness of >70% and derived from different phylogenetic groups (data not presented here). The contig bearing *tswI*, *tswR*, and *tswR-t* was binned into a reconstructed alphaproteobacterial partial genome, preliminarily designated Alpha A. Another alphaproteobacterial partial genome was reconstructed and preliminarily designated Alpha B. We estimated that these two genomes were 88.5% and 85.7% complete, respectively. These draft genomes based on *de novo* assembly did not include 16S rRNA genes. Therefore, we used a phylogenetic approach based on 6 concatenated protein sequences to determine their taxonomic affiliation. Alpha A was found to be positioned in the order *Rhodobacterales* (and thus termed *Rhodobacterales* bacterium TS309), while Alpha B was positioned in the order *Rhodospirillales* (Fig. 3). The genome of Alpha A presented several genomic features that support a symbiotic nature: small genome size (see Tables S5 and S6 and Fig. S3A in the supplemental material), high abundance of eukaryotic-like proteins (e.g., ankyrin repeats and tetratricopeptide repeats [see Fig. S3B in the supplemental material]), and the presence of CRISPRs (see Table S5 in the supplemental material).

Phylogenetic affiliation of *Rhodobacterales* bacterium TS309. In addition, 16S rRNA genes were reconstructed independently using a reference-guided method. Thus far, >100 bacterial operational taxonomic units (OTUs) have been identified within *T. swinhoei*, utilizing 16S rRNA amplicon sequencing, including mainly the phyla *Proteobacteria*, *Chloroflexi*, *Poribacteria*, *Acidobacteria*, *Actinobacteria*, and *Cyanobacteria* (21, 71). Similarly, in

this study, based on analysis of reconstructed 16S rRNA genes, the most common phyla detected in the microbiome of *T. swinhoei* were *Proteobacteria* (29%), *Chloroflexi* (24%), *Actinobacteria* (10.5%), and *Acidobacteria* (10%) (Fig. 4A). No reconstructed 16S rRNA gene was obtained for *Poribacteria*, a candidate phylum so far barely detected outside sponges (72, 73). Within the *Proteobacteria*, the most abundant class was *Gammaproteobacteria* (52%), followed by *Alphaproteobacteria* (26%) and *Deltaproteobacteria* (22%) (Fig. 4B). Six different 16S rRNA genes belonging to the class *Alphaproteobacteria* were reconstructed (Table 3). Five belonged to the order *Rhodospirillales* (see Fig. S4 in the supplemental material), and only one was phylogenetically positioned in the order *Rhodobacterales* (see Fig. S4). As previously mentioned, Alpha A, the genome harboring the TswIR QS system, belonged to *Rhodobacterales*, suggesting that the only reconstructed 16S rRNA that was affiliated with a *Rhodobacterales* organism likely belongs to the assembled genome of *Rhodobacterales* bacterium TS309. According to the 16S sequence, TS309 was found within a sponge-associated-enriched clade (73). This affiliation suggests that *Rhodobacterales* bacterium TS309 was the second most common alphaproteobacterium in the microbiome of *T. swinhoei*, whereby the 16S rRNA gene comprised about 1.56% of the genes reconstructed from the sponge microbiome (estimated gene sequence coverage, 31×; Table 3). These results are on the same order of magnitude as the calculated abundance of the TS309 genome, which is 2.7% based on the relative abundance of the TswIR-QS system it harbors (or around 25× genome sequence coverage). We could not associate a 16S rRNA sequence with the Alpha B genome, since five 16S rRNA sequences were all positioned in the order *Rhodospirillales* (Table 3; see also Fig. S4 in the supplemental material).

Distribution of *Rhodobacterales* bacterium TS309 among sponge species, seawater, and marine sediment samples. The EMIRGE-assembled sequence, which likely belongs to *Rhodobacterales* bacterium TS309, was used in a MegaBLAST against the sponge EMP data set. This resulted in 23,083 reads deriving from 15 OTUs with 99% identity to the query sequence (see Table S7 in the supplemental material). Figure 5 and Fig. S5 in the supplemental material show the relative abundances of these 15 OTUs in 26 sponge, 143 seawater, 34 marine sediment, and 4 freshwater samples to provide an overview of the distribution of *Rhodobacterales* bacterium TS309. The results show that these OTUs are almost absent in marine seawater, marine sediment, and freshwater, but they are more abundant in many sponge species tested, including *T. swinhoei* (Fig. 5; see also Fig. S5 in the supplemental material).

FIG 3 Maximum-likelihood distance tree based on concatenated sequences for 6 conserved proteins. The numbers on the nodes indicate the bootstrap percentages out of 500. The bacteria published in this study are shown in bold.

DISCUSSION

Little information is available on the molecular mechanism(s) underlying intraspecies and interspecies bacterial communication that is part of the sponge holobiont. Previous studies have demonstrated QS in isolated bacteria (7, 14–16) and the production of signal molecules in the sponge tissue (16). Through the applica-

tion of culture-independent metagenomic assembly techniques, we reveal the presence of one complete *luxI* gene (*tswI*) and seven partial *luxI* homologues, the most abundant being the novel *tswI* gene, which is part of the TswIR system here assigned to *Rhodobacteriales* bacterium TS309. Recent studies suggest that other members of the abundant marine *Roseobacter* clade, including

FIG 4 (A) Phylogenetic distribution of EMIRGE 16S rRNA OTUs (cutoff, 97% similarity) at the phylum level; (B) phylogenetic distribution of EMIRGE 16S rRNA OTUs at the *Proteobacteria* class level.

another sponge symbiont (14), also possess AHL-based QS systems that likely contribute to their ecological success (74).

Rhodobacterales bacterium TS309 has thus far not been obtained in culture (75; L. Steindler, unpublished data); therefore, to determine whether the AHLs observed in the sponge extracts may be produced by the *tswI* system, we heterologously expressed *tswI* in *E. coli*. C₈-HSL and C₁₀-HSL were identified in *T. swinhoei* extract and produced by *E. coli* expressing *tswI*, suggesting that *Rhodobacterales* bacterium TS309 may be responsible for their production in sponges. OH-C₁₈-HSL may be produced by a different symbiont in this sponge, possibly harboring one of the other seven partial *luxI* homologues identified in this study. Alternatively, *tswI* expressed heterologously in *E. coli* may not produce the same AHLs as in its endogenous host; therefore, we cannot exclude that it may be produced in *Rhodobacterales* bacterium TS309. In fact, previous studies have shown that different bacterial hosts can alter the AHLs produced by the same AHL synthase gene, depending on the acyl-(acyl carrier protein) pool present in the bacterium (14, 76).

Two *luxR* homologues were identified in the *Rhodobacterales* bacterium TS309 genome. From the amino acid alignment, it was determined that one *LuxR* homologue, designated here TswR, was a typical *LuxR* transcriptional regulator, whereas the second one, designated TswR-t, lacked the last one of three conserved amino acids commonly found in the DNA-binding motif of *LuxR* proteins (77). TswR and TswR-t share only 32% identity and are transcribed in opposite directions (Fig. 2A). The function of the truncated *LuxR* homologue is currently unknown; the presence of an AHL-binding domain and the absence of a complete DNA-binding domain lead us to speculate that TswR-t may function as an inhibitor of QS by competing with TswR for AHL binding. Truncated *LuxR* homologs are present also in other bacteria; for example, *A. tumefaciens* possesses the truncated TrlR. In *A. tumefaciens*, the TraI-TraR QS system regulates the genes required for conjugal transfer of the tumor-inducing (Ti) plasmid. In this system, the truncated TrlR inhibits the activity of TraR by protein-protein interaction. TraR and TrlR closely resemble each other on positions 1 to 181, sharing 88% identity; however, as a result of a frameshift at position 182, TrlR is truncated (78). The presence of a putative inhibitor (TswR-t) may explain why only 23% of the *T. swinhoei* specimens tested showed the presence of AHL molecules in sponge extracts, even though the AHL synthase gene (*tswI*) was amplified from 100% of the *T. swinhoei* specimens tested (*n* = 15). We tested the regulation of the promoter of *tswI* in *A. tumefaciens* as a heterologous system. The expression of TswR with or without the concomitant expression of TswR-t and with or without the addition of AHLs did not alter the activity of the *tswI* promoter (see Fig. S2 in the supplemental material). This experiment indicated that the expression of *tswI* is constitutive and independent of TswR, and it did not shed any light on the possible role of TswR-t as an inhibitor of TswR.

According to our calculations, only approximately 3% of the sponge microbiome analyzed harbored AHL synthase genes. The recent discovery of noncanonical AHL-QS systems, in which bacteria may harbor only a *luxR* homologue and no *luxI* (these have been called *luxR* solos [79]), implies that AHL produced by a minority of the sponge microbiome may also affect the genetic regulation of other bacteria that do not synthesize the signal molecules. Moreover, a recent study showed that AHL molecules could also affect the gene expression of the host sponge (17), but

TABLE 3 Alphaproteobacterial 16S rRNA gene sequences reconstructed by EMIRGE

16S rRNA ID	% specific 16 rRNA	Sequence length (bp)	Accession no. of closest 16S rRNA	ARC (×) ^a	16S ID ^b	Related organism taxa ^c
TS-67	2.02	1,419	GU118526.1	35.9	98.94	<i>Rhodospirillales</i> , <i>Rhodospirillaceae</i> , uncultured, uncultured bacterium
TS-309	1.56	1,337	GU118636.1	31.2	95.81	<i>Rhodobacterales</i> , <i>Rhodobacteraceae</i> , uncultured, uncultured bacterium
TS-14	0.62	1,422	GU118606.1	11	98.95	<i>Rhodospirillales</i> , <i>Rhodospirillaceae</i> , <i>Defluviicoccus</i> , uncultured bacterium
TS-168	0.6	1,275	GU118617.1	13.2	97.8	<i>Rhodospirillales</i> , <i>Rhodospirillaceae</i> , <i>Defluviicoccus</i> , uncultured bacterium
TS-145	0.59	1,326	GU118690.1	11.9	96.37	<i>Rhodospirillales</i> , <i>Rhodospirillaceae</i> , <i>Defluviicoccus</i> , uncultured bacterium
TS-105	0.45	1,377	JN655469.1	8.5	98.54	<i>Rickettsiales</i> , TK34, uncultured bacterium

^a ARC, average read coverage (calculated from *k*-mer coverage).
^b 16S ID, percent identity to GenBank 16S rRNA sequences.
^c Related organism taxa according to EMIRGE.

FIG 5 Relative abundances of OTUs with 99% identity to the 16S rRNA of *Rhodobacteriales* bacterium TS309 in the Sponge Microbiome Project. Presented in the figure are all sponge species with median values greater than 0 and three control habitats (blue, seawater and freshwater; brown, sediment). Sponge species with a median equal to zero are shown in Fig. S5 in the supplemental material. The vertical bars represent the medians, the boxes represent the first to third quartiles, and the whiskers show the lowest or highest datum within 1.5 times the interquartile range of the lowest and upper quartile, respectively. The data points beyond the ends of the whiskers are outliers. Information on samples (e.g., geographic location, sample identification [ID], etc.) is provided in Table S4 in the supplemental material.

whether this is true for the signal molecules produced by *Rhodobacteriales* bacterium TS309 remains to be investigated.

With sponges filtering and concentrating bacteria from their surrounding water, the identification of bacteria as true symbionts rather than bacteria consumed as food is often a subject of debate. The results from the three approaches used in this study support the symbiotic nature of *Rhodobacteriales* bacterium TS309: (i) its phylogenetic affiliation to a sponge-coral-enriched cluster (see Fig. S4 in the supplemental material), (ii) its enriched distribution of closely related OTUs in sponges versus seawater and marine sediment samples (Fig. 5), and (iii) the genomic characteristics that are typical of sponge symbionts, such as eukaryotic-like proteins and CRISPRs in its genome (see Fig. S4 and Table S5 in the supplemental material). High abundances of eukaryotic-like proteins (e.g., ankyrin repeats) are hypothesized to shield sponge symbionts from host digestion (26), and ankyrin repeats were also found to be abundant in the genomes of other uncultured sponge-symbiotic bacteria (80–83). CRISPRs are defense mechanisms against the introduction of foreign DNA, including that of phages, and were previously found to be a common feature for many sponge microbiomes and some sponge symbionts (26, 83–85); similarly, they were found also in the genome of *Rhodobacteriales* bacterium TS309 (see Table S5 in the supplemental material).

Recent studies suggest a key role for microbial symbiosis in the

evolutionary history and ecological success of sponges; thus, an understanding of the molecular interactions between microbial symbionts is likely to provide insights into the evolution and maintenance of animal-microbe symbiosis (86). This study determined the relative abundances of eight QS systems in the microbiome of *T. swinhoei*, identified AHLs found in the sponge tissue, and described a novel QS system of an uncultured sponge-associated bacterium belonging to the order *Rhodobacterales* which is likely symbiotic in nature. Future investigations will focus on unraveling the role of such AHL-QS systems in this highly complex symbiosis.

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