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Characterizing the Host and Symbiont Proteomes in the Association between the Bobtail Squid, *Euprymna scolopes*, and the Bacterium, *Vibrio fischeri*

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Abstract

The beneficial symbiosis between the Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium, *Vibrio fischeri*, provides a unique opportunity to study host/microbe interactions within a natural microenvironment. Colonization of the squid light organ by *V. fischeri* begins a lifelong association with a regulated daily rhythm. Each morning the host expels an exudate from the light organ consisting of 95% of the symbiont population in addition to host hemocytes and shed epithelial cells. We analyzed the host and symbiont proteomes of adult squid exudate and surrounding light organ epithelial tissue using 1D- and 2D-polyacrylamide gel electrophoresis and multidimensional protein identification technology (MudPIT) in an effort to understand the contribution of both partners to the maintenance of this association. These proteomic analyses putatively identified 1581 unique proteins, 870 proteins originating from the symbiont and 711 from the host. Identified host proteins indicate a role of the innate immune system and reactive oxygen species (ROS) in regulating the symbiosis. Symbiont proteins detected enhance our understanding of the role of quorum sensing, two-component signaling, motility, and detoxification of ROS and reactive nitrogen species (RNS) inside the light organ. This study offers the first proteomic analysis of the symbiotic microenvironment of the adult light organ and provides the identification of proteins important to the regulation of this beneficial association.

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Introduction

The light organ symbiosis between the Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium, *Vibrio fischeri*, is used as a model association for understanding host/microbe interactions [1–3]. Hours after hatching from its egg case, the host is colonized when environmental *V. fischeri* take up residence in epithelia-lined crypt spaces located within a specialized light organ [1]. *V. fischeri* is the sole bacterium that colonizes the light organ and prior research has focused on understanding the mechanisms for establishing and maintaining the high degree of specificity between the partners [1–4]. While in the light organ, the bacteria are connected directly to the external environment through ciliated ducts and pores (Fig. 1). This conduit is important as it serves as an interface between the host and the environment and is used in a daily venting of the symbionts. The venting behavior is linked to the nocturnal foraging activities of the host. At night the light organ crypt spaces contain the highest densities of bacteria (10^9/adult squid; [5]), and the light provided by these symbionts is used to avoid predation [6]. At dawn the host expels 95% of its symbionts from the light organ, while entering a quiescent state in which it buries in the substrate [5,7]. The remaining bacteria repopulate the crypts ensuring a full complement of symbionts by the following nightfall. This venting mechanism helps regulate the symbiont population in the light organ as well as increases the concentration of *V. fischeri* in the immediate squid habitat, allowing future generations to be colonized [1,8].

The exudate of adult hosts emerges from the light organ pores as a thick paste-like substance that can be easily collected for experimental analyses (Fig. 1). This material represents the immediate microenvironment of the light organ crypts and is comprised of symbiont cells and a mixed population of host cells (macrophage-like hemoctyes and shed epithelial cells), all surrounded by an acellular matrix [5]. In order to understand the host and symbiont contributions to this microenvironment, previous studies have focused on the cellular and biochemical components of the exudate [5,9]. Recent work has focused on changes in host and symbiont gene expression during the daily rhythm within the light organ [10]. Transcriptome analyses at different time points during the day/night revealed dynamic changes both metabolically and physiologically for the host and symbiont, and identified a large number of differentially expressed genes [10]. In addition, microscopy at these time points revealed that the crypt epithelium also undergoes morphological changes whereby apical surfaces are blebbed into the crypt spaces [10]. Many of these gene expression and cellular changes were most dramatic in the hours just before and after dawn, reflecting the dynamic turnover that occurs in the light organ upon venting.

In this study, we employed a number of techniques to characterize the host and symbiont proteomes of the adult light organ microenvironment at dawn when the association undergoes a dramatic reduction in symbiont population. To date, proteomic

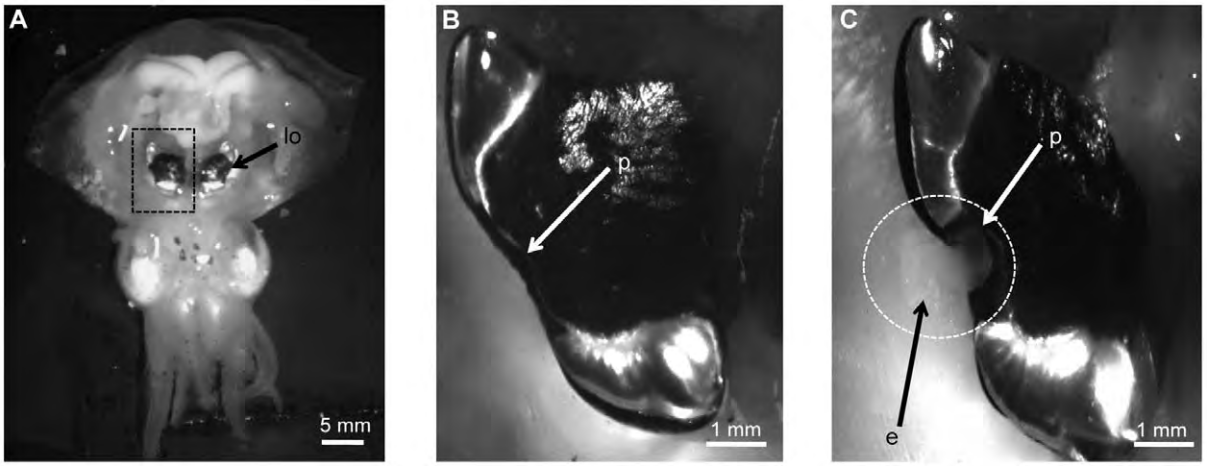

Figure 1. Host and symbiont cells are expelled each morning as a thick exudate. A. A ventrally dissected adult squid reveals the bilobed light organ (lo), which is located in the center of the mantle cavity. (Dashed box highlights the region of light organ in **B** and **C**) **B.** One half of the light organ prior to expelling the light organ contents. **C.** One half of the light organ during the venting process. The exudate (e) emerges from a lateral pore (p) as an opaque paste (dotted circle).
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analyses of the squid/*Vibrio* association are limited. A previous study used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to reveal numerous differences in the soluble proteins present in the light organs of juvenile aposymbiotic (uncolonized) and symbiotic (colonized) squid during the development of the symbiosis, however no proteins were identified [11]. Recent advances in proteomics, including multidimensional protein identification technology (MudPIT), have provided the tools to allow the identification of a large number of host and symbiont proteins in the squid/*Vibrio* association for the first time [12,13]. MudPIT utilizes strong cation exchange chromatography (SCX) to separate peptides by charge prior to liquid chromatography tandem mass spectrometry (LC MS/MS), thus increasing the number of identified peptides. In this study we utilized MudPIT, in addition to 1D- and 2D-PAGE, to describe both the host and symbiont proteomes in the light organ exudate and the surrounding host epithelial tissue. These analyses identified components of the host's innate immune system as well as numerous proteins involved in the detoxification of reactive oxygen species (ROS). Symbiont proteins detected were involved in stress responses, quorum sensing, motility, and two-component signaling pathways. Our data also highlight many proteins that are presently uncharacterized with regard to the squid/*Vibrio* symbiosis. Identifying the host and symbiont proteins present in the light organ represents a first step to understanding key functional aspects of the association's molecular dialogue that is responsible for maintaining this highly specific relationship and complements a number of other molecular and genetic techniques that have been applied to this symbiosis.

Materials and Methods

Ethics statement

Euprymna scolopes is an invertebrate and is not regulated by animal care regulations in the United States. All field collection of research animals was done in accordance with state and federal regulations. The State of Hawaii does not require collection permits for this species outside of marine reserves. None of the animals collected for this study were caught/collected within a marine reserve or regulated area.

General methods

Adult animals were collected in shallow sand flats of Maunalua Bay, Oahu, HI by dip net and were either maintained in the laboratory in re-circulating natural seawater aquaria at the Hawaii Institute of Marine Biology or at the University of Connecticut with artificial seawater (ASW, Instant ocean) at 23°C. All animals were acclimated at least 48 hours under laboratory conditions and kept on an approximate 12 hr light/12 hr dark cycle before sample collection. *V. fischeri* strain ES114 was grown in saltwater tryptone (SWT) at 28°C as previously described [14].

Exudate and central core collection

Exudate was collected as previously described [5]. Briefly, adult squid were anesthetized in a 2% ethanol/seawater solution and ventrally dissected under red light within minutes prior to dawn. A light stimulus (150 W halogen light) was used to induce venting behavior. Within 1 h, the squid had expelled the light organ contents, which were collected with a 10-µl disposable micropipette (Drummond Scientific Company) and stored on ice after the addition of a 1× protease inhibitor cocktail according to the manufacturer's protocol (Sigma Aldrich, P2714). Post-vented central cores were also dissected and removed from the light organ. All samples were flash frozen with liquid nitrogen and stored at −80°C until further analysis. No differences were detected between samples collected from animals maintained at either the Hawaii Institute of Marine Biology or at the University of Connecticut (data not shown).

Gel-based proteomic methods

Exudate sample preparation for 1D- and 2D-polyacrylamide gel electrophoresis. For PAGE applications, symbiont cells from freshly collected light organ exudate were separated from the soluble fraction, a source of host proteins, by centrifugation (Eppendorf 5810 R, 5,000 rpm, 10 minutes, 4°C). The symbiont pellet was washed three times with 0.22 µm filtered ASW to remove additional soluble proteins. Symbiont proteins were extracted by a modified method from Ho and Hsu [15]. Briefly, 10 consecutive liquid nitrogen freeze/thaw cycles were performed in the presence of a 1× protease inhibitor cocktail (Sigma Aldrich, P2714) with 80 mM Tris, pH 8.0 for cell lysis. After separation from

the bacterial pellet, soluble host proteins were quantified (see below) and stored until further analysis. For cultured *V. fischeri*, cells were grown to early stationary phase [14] and proteins were extracted as described for the symbiont exudate pellet. Protein concentrations of separate symbiont exudate and host soluble fractions, as well as culture-grown *V. fischeri*, were determined spectrophotometrically using the method of Whitaker and Granum [16] and/or a Bradford assay (Bio-Rad). Typically, protein extractions of exudate resulted in 10–20 μg of protein combined from the host soluble and symbiont pellet fractions. Comparison of 2D-PAGE gels from soluble proteins of culture-grown *V. fischeri* and the host soluble fraction of the exudate demonstrated that the soluble host fraction was devoid of bacterial proteins (data not shown).

1D-polyacrylamide gel electrophoresis of light organ exudate. Between 10 and 20 μg of exudate protein from either the host (soluble protein separated from bacterial pellet) or symbiont fraction (bacterial pellet) were resolved with 12.5% polyacrylamide gels (Bio-Rad). Electrophoresis was performed with a Hoeffer 250 mini-gel apparatus at 23 mA or a Bio-Rad Mini PROTEAN® Tetra cell at 200 V for 30 minutes. Gels were either stained with Bio-Rad Brilliant Blue Coomassie R-250 or a Bio-Rad Silver Stain Plus Kit (Bio-Rad). 1D-PAGE of both the soluble host fractions and the bacterial pellets were shown to be reproducible (n = 3, separate and fractionated pooled exudate samples for each; data not shown).

2D-polyacrylamide gel electrophoresis of light organ exudate. 2-D PAGE was performed using the Amersham Pharmacia Biotech Multiphor II system as previously described [11]. 40 μg of pooled exudate protein from either the soluble host fraction or the bacterial pellet, originating from 2 or more adult squid or culture-grown *V. fischeri* cells, were denatured 1:4 in 9 M urea, 1% DTT, 2% Pharmalyte 3–10, 0.5% Triton-X-100, 0.14% phenylmethylsulfonyl fluoride, loaded onto a first dimension gel strip with an immobilized pH gradient (4–7) and focused over a 20 hour period. Samples were then separated by molecular weight on pre-cast 12% to 14% polyacrylamide gradient gels (GE Healthcare Life Sciences). Gels were silver stained as previously described [11,17]. 2D-PAGE from the soluble host fractions and the bacterial pellets or culture-grown cells were deemed to be highly reproducible (n = 3, separate and fractionated pooled exudate samples; data not shown). For comparison, 2D gels were visually aligned and similarities and differences of the molecular weights and individual protein species were noted. Five spots of interest from the 2D-PAGE gel of the exudate bacterial pellet were excised and successively washed in 50% acetonitrile, 50% acetonitrile/50 mM NH_4HCO_3, and 50% acetonitrile/10 mM NH_4HCO_3. The five gel spots were then dried by speed vacuum (Eppendorf Concentrator 5301) and resuspended in 10 mM NH_4HCO_3. Digestion was completed with 0.1 μg trypsin (Promega, V5111) per each 15 mm^3 of gel in a final volume of 35 μl of 10 mM NH_4HCO_3 at 37°C for 24 hours. The digested samples were stored at −80°C until submission to the W. M. Keck Biotechnology Resource Laboratory, Yale University, for LC MS/MS (See below, Mass spectrometry proteomics).

Mass spectrometry proteomic methods

Protein preparation for multidimensional protein identification technology and liquid chromatography tandem mass spectrometry. For MudPIT and LC MS/MS, pooled host and symbiont fractions from freshly collected light organ exudate were combined and quantified as described above. Additionally, central cores were homogenized in the presence of a 1× protease inhibitor cocktail (Sigma Aldrich, P2714) with 80 mM Tris, pH 8.0 using a ground-glass homogenizer. Proteins from central cores were collected from the supernatant of the homogenate after centrifugation (Eppendorf 5810 R, 14,000 rpm, 30 minutes, 4°C) and quantified as described above. Extractions of the central core tissue resulted in approximately 20 μg of soluble protein per central core. Total protein from pooled exudate samples (50 μg, n = 7 squid and 100 μg, n = 7 squid) and pooled central core samples (40 μg, n = 3 squid) were precipitated with 10% trichloroacetic acid (Fisher Scientific) at 4°C overnight. The protein precipitates of the exudates and central cores were collected by centrifugation (Eppendorf 5810 R, 11,000× *g*, 30 minutes, 4°C) and washed twice with ice-cold acetone. The protein pellets were briefly air-dried and then solubilized in 25 μl of 8 M urea, 0.4 M ammonium bicarbonate, pH 8.0. Both samples were reduced and alkylated with 5 μl of 45 mM dithiothreitol (DTT; Acros Organics) at 37°C for 20 minutes and 5 μl of 100 mM iodoacetamide (Acros Organics) at room temperature in the dark for 20 additional minutes. Sequencing grade trypsin was added 1:15 (w/w enzyme to protein; Promega, V5111). The solutions were diluted in water to 100 μl (2 M urea final concentration). Both samples were digested at 37°C for 18–24 hours and then stored at −80°C until submission to the W. M. Keck Biotechnology Resource Laboratory, Yale University for LC MS/MS.

For MudPIT, tryptic digests of pooled exudate proteins from *E. scolopes* underwent strong cation exchange (SCX) on an Applied Biosystems Vision Workstation at the W. M. Keck Biotechnology Resource Laboratory at Yale University. During SCX, peptides were separated by charge into fractions, which were then analyzed by LC MS/MS. MudPIT analyses of separate pooled exudate samples were run in duplicate. The first analysis used 10 SCX fractions (50 μg, n = 7 squid) and the second used 20 SCX fractions (100 μg, n = 7 squid), allowing greater coverage of lower abundance peptides. The central core sample (40 μg, n = 3 squid) and symbiont exudate 2D-PAGE spots (n = 5 spots) were analyzed by one-dimensional LC MS/MS (see below).

For SCX, the tryptic digests of pooled exudate proteins were acidified with 2 μl of 1 M phosphoric acid. A 2.1 mm×200 mm PolySULFOETHYL A™ column (PolyLC Inc.) was used to establish a linear gradient for 118 minutes. The gradient was maintained in 10 mM potassium phosphate, 25% acetonitrile (pH 3.0) and the same buffer with the addition of 1 M potassium chloride. Fractions were collected every 2 minutes at a flow rate of 150 μl/min. All fractions were dried, dissolved in 5 μl of 70% formic acid, and diluted to 15 μl in 0.1% trifluoroacetic acid for subsequent LC MS/MS.

Liquid chromatography tandem mass spectrometry. LC MS/MS of each exudate SCX fraction, central core peptides, and 2D gel spot peptides was completed at the W. M. Keck Biotechnology Resource Laboratory at Yale University. A LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a Waters nanoAcquity UPLC system operated with a Waters Symmetry® C18 180 μm×20 mm trap column, and a 1.7 μm, 75 μm×250 mm nanoAcquity™ UPLC™ column (35°C) was used for peptide separation. Trapping was performed at 15 μl/min with Buffer A (100% water, 0.1% formic acid) for 1 minute. Peptide separation was performed at 300 nl/min with Buffer A and Buffer B (100% CH_3CN, 0.075% formic acid); a 51 minute linear gradient was established starting with 5% Buffer B, increasing to 50% B at 50 minutes, and finally 85% B at 51 minutes. MS was acquired in the Orbitrap using 1 microscan followed by four data dependent MS/MS acquisitions. Neutral loss scans (MS^3) were also obtained for 98.0, 49.0, and 32.7 amu.

Data analysis. All MS/MS spectra were analyzed using the Mascot algorithm for uninterpreted MS/MS spectra [18]. The Mascot Distiller program used the MS/MS spectra to generate

Mascot compatible files by combining sequential MS/MS scans from profile data that have the same precursor ion. A charge state of +2 and +3 were preferentially located with a signal to noise ratio of 1.2 or greater and a peak list was created for database searching. The peak list was searched by Mascot using *V. fischeri* amino acid sequence and juvenile *E. scolopes* light organ expressed sequence tag (EST) databases [19]. Search parameters included partial methionine oxidation, carboxamidomethylated cysteine, a peptide tolerance of ±20 ppm, MS/MS fragment tolerance of ±0.6 Daltons (Da), and peptide charges of +2 or +3. Normal and decoy database were also searched. Mascot significance scores are based on a MOlecular Weight SEarch (MOWSE) scores and rely on multiple matches of more than one peptide to the same protein [20]. The MOWSE based ions score is equal to $(-10)*(\mathrm{Log}_{10}\mathrm{P})$, where P is the absolute probability that a match is random. For a match to be significant, the probability of it being a random match should be below 5% (E-value$<$0.05) [21]. The protein threshold score depends on the size of the database being searched, therefore, Mascot determined that scores greater than 68 were significant when searching the juvenile light organ EST database and scores greater than 48 were significant when searching the *V. fischeri* ES114 amino acid database. Proteins were considered identified when 2 or more peptides matched the same protein and if the Mascot score was above the respective significance threshold. Proteins with putative identifications contained two or more peptide matches, but had a Mascot score below the threshold for the respective database (E-value$>$0.05).

Mascot also calculates the exponentially modified protein abundance index (empai) which estimates the abundance of protein species by using the number of peptides detected in the analysis compared to the number of possible peptides for a particular protein [22,23].

Host proteins identified by Mascot using the juvenile light organ EST database were further analyzed using the Bioinformatics Utility for Data Analysis of Proteomics using ESTs (BUDAPEST) which removed any peptides matching to non-coding reading frames [24]. BLASTx (E-value cutoff 10^{-6}) against the NCBI nr database was used to determine the top protein hit for each EST [25]. In addition, BUDAPEST calculated a peptide score for each protein identified. This score was equal to the number of correct reading frame peptides squared divided by the total number of peptides (all reading frames) identified for that EST. BUDAPEST scores greater than 1 can be considered significant, however, in our study scores of 2 or greater were chosen to represent significant protein identifications.

Results

Exudate samples collected from adult *E. scolopes* light organs were analyzed using a number of proteomic techniques. 1D- and 2D-PAGE revealed that the host soluble fraction of the exudate, derived from host hemocytes and apical surfaces of shed light organ crypt epithelial cells, was comprised of a complex mixture of proteins and peptides, the majority of which are represented between the isoelectric points of 4 to 7 and a size of 7 to 100 kilodaltons (kD) (Fig. 2A, B). Similar analyses of the symbiont fraction of the exudate also revealed a complex protein profile (Fig. 3A, B). Comparison of the host and symbiont PAGE gels support previous observations that the exudate appears enriched in bacteria. When comparing proteins expressed by *V. fischeri* in the light organ to proteins expressed by *V. fischeri* in culture, a protein with an isoelectric point of 5.5 and a molecular weight of 10 kD was present in the light organ, but not in solubilized proteins from culture-grown *V. fischeri* (Fig. 3C). The protein spot

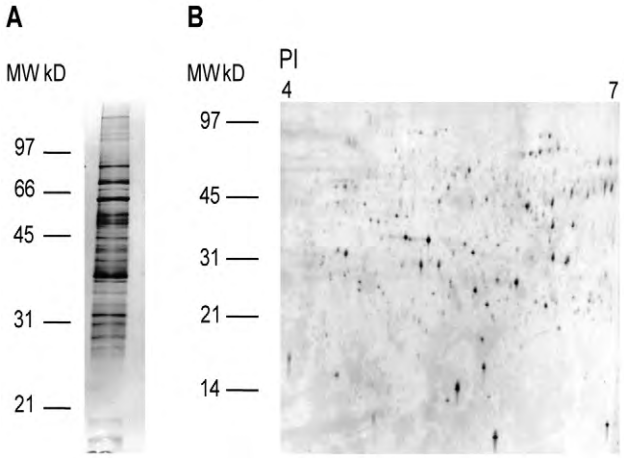

Figure 2. PAGE analysis of the soluble host fraction from light organ exudate. A. 1D-PAGE of the host fraction of the exudate on a 12.5% polyacrylamide gel. **B.** 2D-PAGE of the host fraction of the exudate on a 12–14% polyacrylamide gel.
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of interest (Fig. 3C, spot 2) and four surrounding protein spots (common to both the light organ and culture) were excised and identified by LC MS/MS (Table 1). The unique symbiont light organ protein was determined to be a quorum sensing-regulated protein (QsrP), which has been previously identified as being expressed by *V. fischeri* in the light organ, but remains functionally uncharacterized [26].

In an effort to further characterize the proteins expressed by the host and symbiont we utilized shotgun proteomic techniques (LC MS/MS and MudPIT). These methods allowed us to putatively identify a combined 1581 host and symbiont proteins present in the light organ. For MudPIT, light organ exudate samples of 10 or 20 SCX peptide fractions (see Materials and Methods) were analyzed (Table 2). In addition, to increase our representation of host proteins we analyzed post-vented central cores by single fraction LC MS/MS (Table 2). A total of 870 unique symbiont proteins were putatively identified by Mascot from all the light organ samples (exudates and central cores; Table S1). 516 of these proteins were above the significance threshold set by Mascot (E-value$<$0.05). For the host, we utilized BUDAPEST, a software program developed specifically to identify proteins in the correct open reading frame in cases when only EST databases are available [24]. 676 host proteins with more than 2 peptides matching to the correct reading frame and a BUDAPEST score of greater than or equal to 2 were identified from combining the LC MS/MS and MudPIT data of the exudate and central core samples (Table S2).

All host and symbiont proteins, including putative identifications, were organized functionally according to the Clusters of Orthologus Groups database (COG and KOG; Fig. S1, Table S1, Table S2) [27,28]. In order to achieve a more thorough understanding of the functions represented by the proteins in our data, we first analyzed the relative abundance of each symbiont protein. The 25 most abundant symbiont proteins determined by empai include the protein subunits of luciferase (LuxAB), QsrP, alkyl hydroperoxide reductase C22 (AhpC), and several cold shock proteins (Table 3). Our analyses also identified a number of symbiont proteins related to functions involved in stress responses, quorum sensing, motility, and signaling pathways, all of which have been previously implicated as being important in the squid/*Vibrio* association (Table 4, Table S3; see discussion).

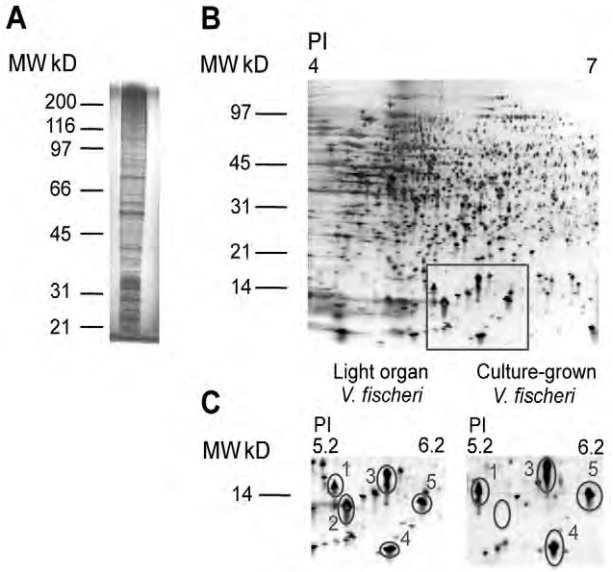

Figure 3. PAGE analysis of the soluble proteins originating from the symbiont fraction of the exudate. A. 1D-PAGE of symbiont fraction of the exudate on a 12.5% polyacrylamide gel. **B.** 2D-PAGE of the symbiont fraction of the exudate on a 12–14% polyacrylamide gel. Black box highlights the region of the gel compared in C. **C.** 2D-PAGE comparison of bacterial soluble proteins from the exudate and culture-grown *V. fischeri*. Numbered protein spots were identified by LC MS/MS (Table 1).
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Several of these identified proteins including AhpC and the cold shock proteins have symbiotic roles yet to be characterized.

Host proteins detected in the light organ highlight the innate immune system, oxidative stress, and signaling pathways (Table 5). Identified proteins include those involved with the NFκB signaling pathway and the recognition of microbial associated molecular patterns (MAMPs) such as peptidoglycan recognition proteins (PGRPs) and a carbohydrate binding protein, galectin (Table 5). Proteins related to oxidative stress consist of superoxide dismutase, peroxiredoxins and numerous peroxidases, including the *E. scolopes* halide peroxidase (EsHPO) (Table 5; see discussion). Additionally, several host proteins involved iron-sequestration were detected in the light organ.

Discussion

The daily expulsion of *V. fischeri* from the light organ of *E. scolopes* provides a unique opportunity to characterize the interactions between the host and symbiont in a natural microenvironment. Previous analyses of this exudate have focused on the cellular and biochemical composition of the expelled matrix [5,9]. In this study we characterized the light organ exudate and surrounding epithelial proteome using MudPIT and PAGE. A total of 1581 unique host and symbiont proteins were putatively identified from the light organ, offering the first proteomic analyses of this symbiotic microenvironment.

Table 2. Number of host and symbiont proteins identified by shotgun proteomics[a].

Sample	*Vibrio fischeri*	*Euprymna scolopes*[b]
Exudate (10 Fractions)	214	163
Exudate (20 Fractions)	708	591
Central Core (1 Fraction)	90	234
Total unique proteins	870	711

[a]Protein identifications required 2 or more peptides per protein. Total unique proteins summarize the combined unique proteins from all samples. Numbers include putative identifications (see Materials and Methods).
[b]Host protein identifications counts are post-BUDAPEST analysis.
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Innate immune system

MAMPs and host pattern recognition receptors (PRRs) are two components underlying host/microbe interactions and are significantly involved in the development of this association [29]. MAMPs including lipopolysaccharide (LPS), and peptidoglycan and its derivatives, function in determining the specificity of the squid/*Vibrio* symbiosis as well as initiating morphogenetic changes to the light organ [30–32]. We identified several host proteins related to pattern recognition in both the exudate and central core tissues (Table 5). *E. scolopes* PGRP2 and 3 (EsPGRP2 and EsPGRP3) are involved in detecting peptidoglycan, a major cell wall component of bacteria [33]. EsPGRP2 is secreted into the crypts of the light organ where it is thought to degrade tracheal cytotoxin (TCT), a monomer of peptidoglycan [34]. The role of EsPGRP3 in the symbiosis is currently under investigation, but has been detected in adult and juvenile hemocytes (unpublished data). Certain carbohydrates, such as beta-galactosides, are another type of MAMP that are recognized by carbohydrate binding proteins known as galectins [35]. A putative galectin was identified in both the exudate and central core tissue (Table 5, Table S2) and may have an uncharacterized role in the squid/*Vibrio* symbiosis.

Aside from PRRs and MAMPs, cellular adhesion is often important for host/microbe cell-to-cell interactions. Outer mem-

Table 1. Exudate proteins identified by LC MS/MS from symbiont 2D-PAGE analysis (Fig. 3).

Spot	Mw (kD)/pI[a]	Top Protein Hit to NCBI nr Database	ORF[b]	gi	Score[c]
1	13.3/5.4	50S ribosomal protein L9	VF_2310	59712917	1045
2	11.0/5.5	Quorum sensing regulated protein QsrP	VF_A1058	59714241	628
3	14.7/5.7	Transcriptional dual regulator H-NS	VF_1631	59712238	712
4	8.8/5.7	Cold shock protein	VF_2561	59713168	473
5	9.3/6.1	30S ribosomal protein S6	VF_2312	59712919	501

[a]Predicted molecular weight (Mw) and isoelectric point (pI) for the proteins identified.
[b]Open reading frame (ORF) locations of the respective genes on the chromosomes of *V. fischeri*.
[c]Scores were assigned by Mascot. Scores greater than 83 were significant (E-value<0.05) for searches of the NCBI nr database.
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Table 3. The 25 most abundant symbiont proteins present in light organ exudates and central cores identified by MudPIT and LC MS/MS in descending empai order (excluding ribosomal proteins).

#	gi	ORF[a]	Gene	Protein Name	empai	Score[b]
1	59714104	VF_A0921	luxA	luciferase alpha chain LuxA	28.74	2840
2	59712269	VF_1662	-	DNA-binding protein	21.59	257
3	59712346	VF_1739	acpP	acyl carrier protein	19.95	290
4	59714241	VF_A1058	qsrP	LuxR-regulated periplasmic protein QsrP	13.61	691
5	59712582	VF_1975	ahpC	alkyl hydroperoxide reductase, C22	10.54	994
6	59711657	VF_1050	-	hypothetical protein	9.35	179
7	59711351	VF_0744	ybeD	hypothetical protein	7.94	194
8	59712374	VF_1767	cspD	DNA replication inhibitor	7.66	309
9	59714005	VF_A0822	-	hypothetical protein	7.55	101
10	59711823	VF_1216	infC	protein chain initiation factor IF-3	6.86	378
11	59711844	VF_1237	ihfA	integration host factor subunit alpha	6.41	193
12	59712727	VF_2120	arcA	two-component response regulator	6.32	799
13	59711232	VF_0625	Ndk	nucleoside diphosphate kinase	5.92	245
14	59712758	VF_2151	-	iron(III) ABC transporter	5.3	1109
15	59711304	VF_0697	-	putative lipoprotein	5.22	72
16	59711049	VF_0442	Pgk	phosphoglycerate kinase	5.08	1747
17	59711497	VF_0890	grxA	glutaredoxin 1	4.88	216
18	59710869	VF_0262	rpoA	DNA-directed RNA polymerase alpha	4.84	647
19	59713778	VF_A0595	-	cold shock protein	4.78	273
20	59712703	VF_2096	-	hypothetical protein	4.77	146
21	59714277	VF_A1094	cspG	DNA-binding transcriptional regulator	4.64	776
22	59712568	VF_1961	Tsf	elongation factor Ts	4.36	794
23	59714103	VF_A0920	luxB	luciferase beta chain LuxB	3.93	1434
24	59710881	VF_0274	-	immunogenic protein	3.87	442
25	59711114	VF_0507	deoD	purine nucleoside phosphorylase	3.86	140

[a]Open reading frame (ORF) locations of the respective genes on the chromosomes of *V. fischeri*.
[b]Scores were assigned by Mascot. Scores greater than 48 were significant for *V. fischeri*.
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brane proteins (OMPs) are localized at the bacterial cell surface and are good candidates for mediating recognition between the partners. OmpU, a symbiont outer membrane protein that we have identified in the light organ (Table S3), was shown to be important in mediating adhesion to adult host hemocytes and during the early stages of colonization [36,37]. Other OMPs identified, such as a hypothetical protein VF_1010, have roles yet to be characterized in binding and adhesion, but may have similar functions (Table S3). Understanding how the symbiont outer membrane proteome varies in the light organ vs. the free-living environment and between symbiosis-competent and incompetent strains may shed light on mechanisms of mediating specificity in this symbiosis.

An immune pathway highlighted by our proteomic data includes NFκB signaling (Table 5). The role of NFκB signaling during the establishment of the squid/*Vibrio* symbiosis is currently under investigation, however, many important members of the pathway have been identified from juvenile light organ ESTs [33]. We detected NFκB repressing factor (see below) and importin alpha 3, a protein involved in shuttling proteins into the nucleus by recognizing nuclear localization signals (Table 5) [38]. *In vitro* and *in vivo* studies using cancer cell lines reveal this protein is a member of the NFκB signaling pathway and aids in the transport of NFκB transcription factors into the nucleus [39].

Recently, *E. scolopes* has been shown to have a complement pathway that in other systems is involved with mediating inflammation and opsonization [29,40,41]. The function of this pathway has yet to be described in the squid/*Vibrio* symbiosis, however, we detected putative components of the complement cascade in both the exudate and the central core (Table 5, Table S2). Although one of these identifications was annotated as a complement component C3 precursor (Table 5, Table S2), closest to the cnidarian *Nematostella*, further analysis of these peptides using *E. scolopes* transcriptomic data revealed that this protein did not align with the previously described *E. scolopes* C3 (data not shown). Instead, this protein, along with two others, were identified as thioester-containing proteins (TEPs). Among invertebrates, TEPs play an important role in innate immune response as members of the complement system or as protease inhibitors [42,43].

Reactive oxygen and nitrogen stress response

The chemical microenvironment of the light organ crypts likely influences the maintenance of the association and helps to ensure specificity. Although oxygen is critical for the bioluminescence reaction, reactive oxygen species (ROS) and toxic oxygen intermediates have been shown to be abundant in the light organ [44]. Host-derived ROS, such as hypohalous acid, are thought to play key roles in initiation and persistence of the squid/*Vibrio*

Table 4. Symbiont proteins detected in light organ exudates and central cores by MudPIT and LC MS/MS[a].

Category	gi	ORF[b]	Protein Name	Score[c]
Quorum Sensing				
	59714104	VF_A0921	luciferase alpha chain LuxA	2840
	59714103	VF_A0920	luciferase beta chain LuxB	1434
	59714241	VF_A1058	LuxR-regulated periplasmic protein QsrP	691
	59711956	VF_1349	subtilisin-like serine protease	491
	59714105	VF_A0922	acyl transferase LuxD	463
	59714077	VF_A0894	putative surface protein	304
	59714106	VF_A0923	acyl-CoA reductase LuxC	225
	59711152	VF_0545	S-ribosylhomocysteinase LuxS	167
	59712332	VF_1725	secretory tripeptidyl aminopeptidase	123
	59712784	VF_2177	LitR	78
	59714102	VF_A0919	long-chain-fatty-acid ligase LuxE	62
	59712585	VF_1978	AcfA-like protein	50
	59711772	VF_1165	macrolide ABC transporter	45
	59714108	VF_A0925	LuxR	21
Oxidative Stress				
	59712582	VF_1975	alkyl hydroperoxide reductase, C22	994
	59711509	VF_0902	thioredoxin reductase	238
	59713192	VF_A0009	hydroperoxidase HPII(III) KatA	184
	59711528	VF_0921	superoxide dismutase, Fe	83
	59712527	VF_1920	thioredoxin-dependent thiol peroxidase	54
	59712923	VF_2316	nitric oxide dioxygenase	46
	59714073	VF_A0890	thioredoxin peroxidase	19
Two-Component Signaling				
	59712727	VF_2120	ArcA	799
	59713744	VF_A0561	two component response regulator	207
	59712949	VF_2342	periplasmic protein CpxP	154
	59712177	VF_1570	TorR	111
	59711061	VF_0454	transcriptional regulator VpsR	83
	59712516	VF_1909	DNA-binding response regulator NarP	63
	59712234	VF_1627	response regulator GacA	59
	59710721	VF_0114	osmolarity response regulator OmpR	54
	59712981	VF_2374	two-component response regulator	46
	59713399	VF_A0216	two component response regulator	36
	59714199	VF_A1016	two component sensory histidine kinase	32
	59711755	VF_1148	response-regulatory protein YehT	23
	59712226	VF_1619	hybrid sensory histidine kinase TorS	20
	59712008	VF_1401	sigma-54 dependent response regulator	16
	59712950	VF_2343	DNA-binding response regulator CpxR	15
Flagellar-related proteins				
	59712463	VF_1856	FlrA	107
	59712488	VF_1881	flagellar anti-sigma-28 factor FlgM	81
	59712471	VF_1864	flagellin	65
	59712473	VF_1866	flagellin	62
	59712478	VF_1871	flagellar basal body L-ring protein	34
	59711322	VF_0715	flagellar motor protein MotB	29
	59712477	VF_1870	flagellar basal body P-ring protein	18
	59712484	VF_1877	flagellar basal body rod protein FlgB	14

Table 4. Cont.

[a]For more complete information on MudPIT and LC MS/MS symbiont protein identifications refer to Table S1 and Table S3.
[b]Open reading frame (ORF) locations of the respective genes on the chromosomes of *V. fischeri*.
[c]Scores were assigned by Mascot. Scores greater than 48 were significant for *V. fischeri*.
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symbiosis [44]. Hypohalous acid, produced by an abundant light organ peroxidase similar to a halide peroxidase, is believed to help to create an oxidative environment that *V. fischeri* must overcome to colonize the host [45,46]. In addition to the previously described EsHPO, a number of other host peroxidases were present, suggesting that additional ROS may be important to this association (Table 5). Peroxiredoxins are antioxidant proteins, which are abundant in the host proteome and have been shown to detoxify reactive molecular species derived from oxygen and nitrogen [47,48]. Therefore, these ROS mediators may indicate a means by which the host protects its own tissues in the oxidative microenvironment of the light organ.

Another role of host ROS may be maintaining specificity by preventing non-symbiotic bacteria and potential pathogens from infecting the host. The light organ crypts are open to the environment via pores on the surface of the light organ, yet *V. fischeri* is thought to be the sole symbiont of this highly specific association [1]. Proteins expressed by the symbiont reveal functions involved with protecting cells from host ROS (Table 4). *V. fischeri* utilizes a periplasmic catalase (*katA*) to sequester hydrogen peroxide from the host, which can be used by EsHPO to generate hypohalous acid [49]. We identified, in addition to KatA, the antioxidant enzymes AhpC and thioredoxin-dependent thiol peroxidase (Bcp) (Table 4). A *V. fischeri katA* mutant showed no additional catalase activity in culture suggesting that KatA is the major scavenger of H_2O_2 [49]. The additional antioxidant proteins identified in this study may indicate a mechanism by which the symbiont can protect itself from other types of ROS or

Table 5. Host proteins detected in light organ exudates and central cores by MudPIT and LC MS/MS[a].

Category	gi	Top protein hit to NCBI nr database	e-value[b]	Score[c]
Immunity				
	225906399	Galectin [*Pinctada fucata*]	4E-69	10
	63033995	Peptidoglycan recognition protein 2 [*E. scolopes*]	1E-121	7
	223670954	C3 precursor [*Nematosella vectensis*][d]	4E-12	6
	144952812	Thioester-containing protein [*Chlamys farreri*]	4E-14	6
	113931358	NFKB repressing factor [*Xenopus tropicalis*]	2E-17	4
	42741753	Importin alpha 3 [*Aplysia californica*]	9E-6	4
	63033997	Peptidoglycan recognition protein 3 [*E. scolopes*]	9E-49	3
	85822201	TEP2 [*Glossina morsitans morsitans*]	3E-16	2
Oxidative stress				
	306451460	Thioredoxin peroxidase [*Cristaria plicata*]	1E-92	13
	110734438	Superoxide dismutase [*Haliotis discus discus*]	7E-61	7
	229366436	Peroxiredoxin-5 [*Anoplopoma fimbria*]	2E-51	5
	67083759	Glutathione-type peroxidase [*Ixodes scapularis*]	8E-50	5
	2239176	Melanogenic peroxidase [*Sepia officinalis*]	3E-59	4.5
	209171295	Peroxiredoxin 4 precursor [*Biomphalaria glabrata*]	1E-101	4
	159008	Halide peroxidase [*Euprymna scolopes*]	1E-141	4
	157136354	Peroxiredoxins, prx-1, prx-2, prx-3 [*Aedes aegypti*]	5E-72	3.2
	77166828	Glutathione peroxidase [*Rhipicephalus microplus*]	4E-62	3
	149688674	Peroxiredoxin [*Chlamys farreri*]	2E-58	2
	126697356	Thioredoxin peroxidase 2 [*Haliotis discus discus*]	1E-54	2
Iron-Binding				
	318067980	Transferrin [*Ictalurus punctatus*]	4E-34	6
	4768842	Ferritin [*Enteroctopus dofleini*]	7E-74	3
	157786780	Melanotransferrin [*Rattus norvegicus*]	6E-21	3

[a]For more complete information on MudPIT and LC MS/MS host protein identifications refer to Table S2.
[b]E-value represents the alignment of the light organ EST with the top protein hit in the NCBI nr database.
[c]Scores were assigned by BUDAPEST and correlate the number of reading frame peptides matched to the light organ EST to the number of overall peptides. Scores greater than 2 were significant.
[d]No alignment with *Euprymna scolopes* C3 (Putatively identified as a thioester-containing protein; see discussion).
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RNS. AhpC, a peroxiredoxin capable of reducing hydrogen peroxide, organic peroxides, and peroxynitrite, is the most abundant antioxidant symbiont protein present in the light organ (Table 3). In *Vibrio vulnificus*, AhpC functions along with another subunit, AhpF, which supplies the reducing equivalents in the form of NADH, to reduce peroxides [50]. However, AhpF is absent from the *V. fischeri* genome, suggesting that another protein is necessary to reduce peroxides by this pathway. Studies involving *Treponema pallidum* show that thioredoxin reductase can substitute for organisms lacking an AhpF homolog [51]. For *V. fischeri*, a thioredoxin reductase FAD/NAD(P)-binding protein (TrxB) was present in our MudPIT data (Table 4) and may have the potential of interacting with AhpC. Along with AhpC, proteins implicating that *V. fischeri* also detoxifies RNS, include nitric oxide dioxygenase (Hmp), and two peptide-methionine (S)-S-oxide reductases (MsrA and VF_A0005; Table S1) [52,53].

Reactive nitrogen species, such as nitric oxide (NO), contribute to signaling and development in the squid/*Vibrio* symbiosis [54]. The role of NO as a toxic product to pathogens has been well studied; however, the function of NO in beneficial associations has only been recently analyzed [54,55]. In juvenile squid the epithelial tissue lining the ducts entering the light organ crypts contain high levels of NO, suggesting that the symbionts must overcome NO in order to colonize the light organ [56]. The detection of nitric oxide dioxygenase (Hmp), recently shown to play a role in NO detoxification, suggests that *V. fischeri* also maintains the ability to manage NO related stress in adult squid (Table 4) [53]. Once *V. fischeri* colonizes the light organ, nitric oxide synthase (NOS) is down-regulated and lower levels of NO likely allow the symbiont to grow in the crypt spaces under reduced RNS stress [56]. We identified NFκB repressing factor, which in addition to other immune functions, has been shown *in vitro* to negatively regulate transcription of NFκB pathway effectors, including NOS, by directly interacting with promoter region sequences (Table 5) [57,58]. The results of this study provide a number of new host and symbiont targets involved in mediating ROS and RNS for further analyses.

The availability of iron has also been shown to be an important factor in squid/*Vibrio* symbioses [53,59,60]. Free iron plays a critical role in host/microbe interactions and under certain circumstances may allow development of pathogenic associations [61,62]. Host proteins involved in sequestering free iron such as ferritin, transferrin, and melanotransferrin were identified (Table 5). These iron-binding proteins provide supporting evidence that iron remains limiting in the light organ and suggest a possible role for these proteins in regulating the growth of *V. fischeri* [59]. In contrast to the host, putative proteins that the symbiont may utilize for acquiring iron include receptors for the siderophores aerobactin and anguibactin (Table S3). Symbiont proteins involved in utilizing heme, another source of iron, are also present, and include HutZ, HutA, HuvX, and HmuT (Table S3). It is likely that *V. fischeri* employs several different strategies to meet its necessary iron requirements.

Quorum Sensing

First described in *V. fischeri*, quorum sensing regulates bioluminescence, the light from which provides the host with an anti-predatory mechanism known as counterillumination [6,63,64]. Lux proteins involved in light production were identified and among the most abundant symbiont proteins (Table 3). Previous PAGE and transcriptomic analyses first revealed additional quorum sensing-regulated proteins, which were also detected by our characterization of the adult light organ proteome [26,65]. QsrP is one of the most abundant proteins present in the symbiont proteome (Table 3), yet this novel protein remains functionally uncharacterized. Another quorum sensing-regulated protein identified in this study is a putative surface protein (VF_A0894) with immunoglobulin-like domains (Table 4). This putative surface protein is similar to the *Leptospira* immunoglobulin-like proteins (LigA, LigB and LigC) of pathogenic *Leptospira spp*., which are thought to mediate adhesion to host cells [66]. These quorum sensing-regulated proteins may be important to a symbiotic lifestyle. We also detected LuxS, AI-2 synthase, which is involved in a second quorum sensing system in *V. fischeri* and has been implicated in regulating motility in *Vibrio alginolyticus* [67–69]. A link between LuxS and motility, may implicate a role for quorum sensing and the onset of motility prior to symbiont expulsion from the light organ (see below).

Symbiont Signaling

Two-component signaling pathways are important mechanisms by which bacteria can sense the environment and have been identified in *V. fischeri* [70–73]. The roles in colonization for some of these regulators, which were present in our proteomic data (Table 4), such as GacA and ArcA, have been studied in detail, and mutagenesis of these genes has demonstrated that they are important in the association [71–73]. Although many regulators have already been characterized with respect to the symbiosis, many proteins involved in two-component signaling have unknown functions in the light organ. For example, CpxP, an abundant symbiont protein (Table 4), is a periplasmic component of *Escherichia coli* and *Vibrio cholerae* and involved in modulating the cell envelope stress response through CpxAR signaling, thus providing an appealing target for future studies [74,75].

Other Related Stresses

Although several were identified in this study, cold shock proteins have yet to be described with respect to the light organ symbiosis. Of the top 25 most abundant symbiont proteins present in the light organ, three were cold shock proteins (CspD, CspG, and VF_A0595; Table 3). Cold shock proteins often bind nucleic acids and function in general stress responses. Furthermore, they have been shown to play a role in regulating bacterial growth at stationary phase and may even serve as MAMPs recognized by hosts [76,77]. One cold shock protein identified in the light organ, CspD, prevents replication from occurring in stationary phase *E. coli* cells by binding to single stranded DNA and blocking replication [78]. Prior to expulsion at dawn, the symbiont population is at its most dense during the day/night cycle. Therefore, cold shock proteins may play a role in either maintaining high cell densities in the light organ and/or assisting during the transition between the symbiotic and free-living state.

Motility

Research involving the role of motility in the squid/*Vibrio* symbiosis has focused on the initiation of colonization. Within the light organ *V. fischeri* cells become differentiated with the loss of their flagella [79]. Upon release from the light organ at dawn, *V. fischeri* cells are believed to fully regenerate their flagella within several hours [79]. Our proteomic data show the presence and putative identification of several proteins related to flagellar structure including filamental proteins (FlaA, FlaC), basal body proteins (FlgB, FlgH, FlgI), and a motor protein (MotB; Table 4). Proteins related to flagellar regulation (FlrA and FlgM) and chemotaxis (CheW and CheZ) were also detected. A recent study indicated an increase in flagellar gene expression by light organ symbionts in the hours preceding dawn and *V. fischeri* mutants of FlaA and FlrA have been shown to be important for symbiotic

competence [10,80,81]. FlrA was also found to be expressed by *V. fischeri* in the light organs of *E. scolopes* and a different squid species, *E. tasmanica*, but not in strains grown in seawater [82]. Together, the data from this present study and others suggests that *V. fischeri* cells are generating flagella prior to expulsion from the light organ and may be preparing for the transition from the symbiotic to the free-living state. Future studies should focus on signals in the changing microenvironment that may initiate this transition.

Symbiont Metabolism

Within the light organ, *V. fischeri* employs a number of metabolic strategies [9,10,83,84]. The daily rhythm of the light organ symbiont population coincides with fluctuations in symbiont metabolism [10]. Transcriptomics revealed a unique pattern in which during the night the symbiont ferments chitin as a means of obtaining energy. After the majority of the symbiont population is expelled from the light organ, the remaining symbionts anaerobically respire glycerol during the hours in which the light organ becomes replenished with a full symbiont population. The results of this study show abundant symbiont chitin binding proteins and chitinases, thus supporting these previous findings (Table S3). The diel shift in metabolism is one piece of evidence that supports the light organ as being a dynamic microenvironment that is under the regulation of both the host and symbiont [10].

Summary

Proteomic studies of symbioses utilizing high-throughput techniques are becoming more common and have been used for analyses of the pea aphid-*Buchnera* symbiosis, nitrogen fixing symbioses of leguminous plants, human gut microbiota, and in characterizing the function of uncultivable symbionts in hydrothermal vent symbioses [85–89]. Characterization of the light organ proteome with high-throughput techniques allowed for the identification of a large number of host and symbiont proteins using little starting material and demonstrates the value of proteomic analyses in an effort to understand the relationship of a symbiotic association. The results of this study complement prior transcriptomic data, but have also identified a number of proteins of previously unknown function in the squid/*Vibrio* symbiosis [10]. The high-throughput techniques used here offer new methods for identification of host and symbiont proteins likely important for the maintenance of this and other host/microbe associations.

Supporting Information

Figure S1 Functional analysis of host and symbiont light organ proteomes. A. COG category counts for all symbiont proteins present in the light organ (including putative identifications). **B.** KOG category counts for all host proteins present in the light organ (including putative identifications) using representative light organ ESTs. (COG/ KOG key: J- translation, ribosomal structure, and biogenesis, A- RNA processing and modification, K- transcription, L- replication, recombination and repair, B- chromatin structure and dynamics, D- cell cycle control, cell division and chromosome partitioning, Y- nuclear structure, V- defense mechanisms, T- signal transduction mechanisms, M- cell wall, membrane and envelope biogenesis, N- cell motility, Z- cytoskeleton, W- extracellular structures, U- intracellular trafficking, secretion and vesicular transport, O- posttranslational modification, protein turnover and chaperones, C- energy production and conversion, G- carbohydrate transport and metabolism, E- amino acid transport and metabolism, F- nucleotide transport and metabolism, H- coenzyme transport and metabolism, I- lipid transport and metabolism, P- inorganic ion transport and metabolism, Q- secondary metabolites biosynthesis, transport and catabolism, R- general function prediction only, S- function unknown).
(DOC)

Table S1 Symbiont proteins detected in light organ exudates and central cores by MudPIT and LC MS/MS.
(XLS)

Table S2 BUDAPEST analysis of host proteins detected in light organ exudates and central core by MudPIT and LC MS/MS.
(XLS)

Table S3 Additional symbiont proteins detected in light organ exudates and central cores by MudPIT and LC MS/MS categorized by functions relevant to survival in the light organ crypts.
(DOC)

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Author Contributions

Conceived and designed the experiments: TRS SVN. Performed the experiments: TRS SVN. Analyzed the data: TRS SVN. Contributed reagents/materials/analysis tools: SVN. Wrote the paper: TRS SVN.

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Video Article

Obtaining Hemocytes from the Hawaiian Bobtail Squid *Euprymna scolopes* and Observing their Adherence to Symbiotic and Non-Symbiotic Bacteria

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Abstract

Studies concerning the role of the immune system in mediating molecular signaling between beneficial bacteria and their hosts have, in recent years, made significant contributions to our understanding of the co-evolution of eukaryotes with their microbiota. The symbiotic association between the Hawaiian bobtail squid, *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* has been utilized as a model system for understanding the effects of beneficial bacteria on animal development. Recent studies have shown that macrophage-like hemocytes, the sole cellular component of the squid host's innate immune system, likely play an important role in mediating the establishment and maintenance of this association. This protocol will demonstrate how to obtain hemocytes from *E. scolopes* and then use these cells in bacterial binding assays. Adult squid are first anesthetized before hemolymph is collected by syringe from the main cephalic blood vessel. The host hemocytes, contained in the extracted hemolymph, are adhered to chambered glass coverslips and then exposed to green fluorescent protein-labeled symbiotic *Vibrio fischeri* and non-symbiotic *Vibrio harveyi*. The hemocytes are counterstained with a fluorescent dye (Cell Tracker Orange, Invitrogen) and then visualized using fluorescent microscopy.

Video Link

The video component of this article can be found at https://www.jove.com/video/1714/

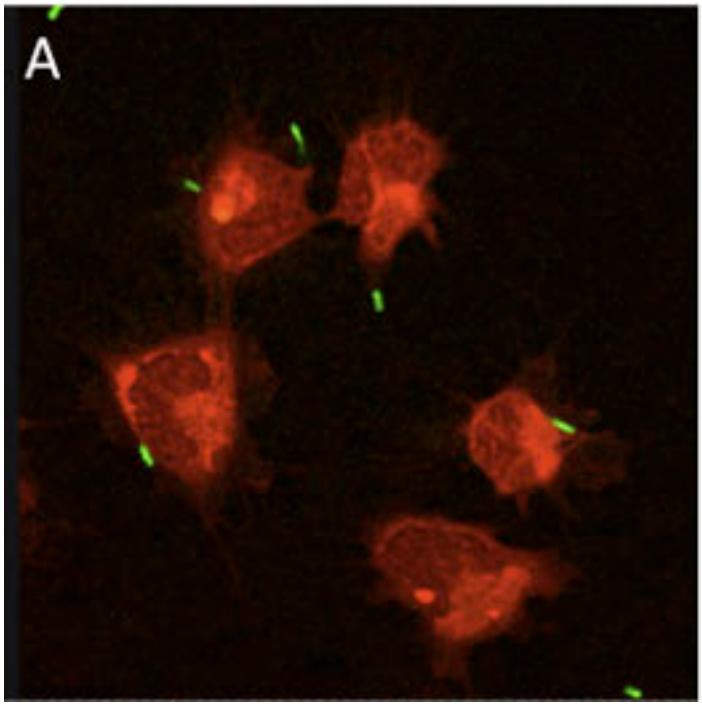
Protocol

1. Prepare 500 mLs of 0.22 µm filter-sterilized artificial seawater (FSW; salinity 35 ppt). Filter artificial or natural seawater through a 0.22 µm micron filter to remove particles and bacteria.
2. Anesthetize one adult Hawaiian bobtail squid (*Euprymna scolopes*) by placing in a 2% solution of ethanol in FSW. Place the animal in anesthetic for approximately 10 minutes. The squid will cease swimming and will not actively respond to touch. Continued respiration, indicated by movement of the mantle, and chromatophore activity should still be observed.
3. Place squid with ventral side facing up on a standard wax dissection tray. Submerge the animal with FSW containing 2% ethanol.
4. Using one standard 200 µl pipette tips, pull back the funnel and mantle to expose the main cephalic blood vessel located between the two eyes.
5. Using a sterile 1 mL syringe with 26.5-gauge needle, puncture the cephalic blood vessel and withdraw between 50-100 µl of hemolymph. Place the hemolymph in a sterile 1.5 mL tube on ice.
   Note: If an animal will serve as a donor multiple times, only withdraw 10-20 µl of hemolymph at any given time. Return the animal to a normal seawater tank. The animal will revive within 30 min.
6. Freshly collected hemocytes are washed and re-suspended in 500 µl of Squid Ringer s solution (S-Ringers; 530 mM NaCl, 10 mM KCl, 25 mM $MgCl_2$, 10 mM $CaCl_2$ and 10 mM HEPES buffer, pH 7.5).
7. Hemocyte concentrations are determined by hemocytometer, and approximately 2,000 cells are added to chambered glass cover slips, and allowed to adhere to the glass for 10 min at room temperature. At this density, the hemocytes form a uniform monolayer on the glass slide surface.
8. To observe bacterial binding to host hemocytes, hemocytes are exposed to a fluorescently labeled bacterial strain such as *Vibrio fischeri* ES114 and/or *Vibrio harveyi* B392, each containing a green fluorescent reporter. *V. fischeri* ES114 and *V. harveyi* B392 are grown to mid-log phase in a sea water tryptone media (SWT) at 28°C in an orbital shaker. The optical density at 600nm is measured spectrophotometrically to determine cell density. The bacteria are pelleted by centrifugation (5,000 rpm for 5 min), the supernatant is discarded, and the pellet is re-suspended in S-Ringers.
9. 100,000 bacterial cells are added to each chamber well so that there are 50 bacteria per hemocyte on average. The hemocyte/bacteria mixtures are incubated in S-Ringer s solution at 25°C for 1 h, a time determined to yield the maximum level of binding.
10. The cytoplasm of the hemocytes are then fluorescently stained with 0.005% CellTracker Orange (Invitrogen) and then washed in S-Ringers to visualize the cells.
11. Stained hemocytes with associated bacteria are viewed by fluorescence using either a Zeiss Discovery V20 fluorescent stereoscope or a Leica SP2 spectral laser confocal microscope, and enumerated over the entire surface of the animal cell.

Representative Results

Because cephalopod hemolymph contains extracellular hemocyanin and not hemoglobin, upon oxygenation, the hemolymph will turn dark blue. An average of ~5000 hemocytes per µl of hemolymph will be obtained using this method. After adherence to the chambered cover slips and fluorescent staining, the hemocytes should appear brightly fluorescently red and amoeboid in shape. For bacterial adhesion, *V. fischeri* will adhere poorly to the hemocytes (1-2 bacterial cells per blood cell) while *V. harveyi* will adhere strongly (10-15 bacterial cells per hemocyte).

Figure 1. Adult Hawaiian bobtail squid *Euprymna scolopes* showing position of cephalic blood vessel.



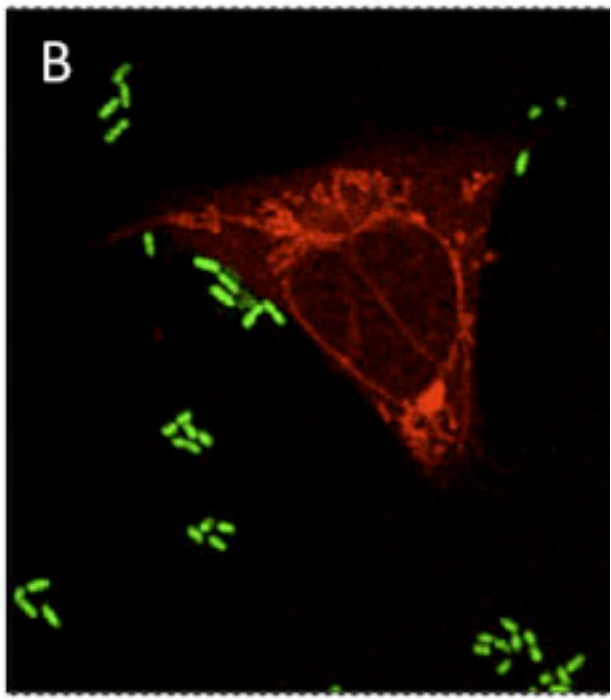


Figure 2. Results of hemocyte exposure to *Vibrio fischeri* (A) and *Vibrio harveyi* (B). Red, Cell Tracker Orange; Green, GFP-labeled bacteria.

Discussion

Studies concerning the role of the immune system in mediating molecular signaling between beneficial bacteria and their hosts have, in recent years, made significant contributions to our understanding of the co-evolution of eukaryotes with their microbiota. The squid/vibrio system has proven itself as a tractable model system to answer fundamental questions in this field[2,3,5,6,8]. The light-organ of the squid *Euprymna scolopes* permits colonization exclusively by the luminous bacterium *Vibrio fischeri*. Because the tissues that house the bacteria remain in contact with seawater, the squid must not only foster the specific symbiosis but also continue to exclude other bacteria. Continued studies have revealed that macrophage-like hemocytes likely play an important role in the establishment and maintenance of this association[1,4,7]. Because the squid host lacks adaptive immunity, the amazing specificity found in this association must be whole or partially mediated through the innate immune system. A recent investigation of these blood cells revealed that hemocytes isolated from *E. scolopes* recognize and phagocytose *V. fischeri* and non-symbiotic bacteria differentially and that colonization likely leads to a type of "immune tolerance" of the symbionts[4]. This protocol will demonstrate how to successfully obtain these blood cells from adult squid and test their ability to bind bacteria.

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GUEST COMMENTARY

Peptidoglycan Monomer Release and *Vibrio fischeri*▽

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Peptidoglycan (PG) is the major component of the bacterial cell wall and is recognized by animals as a signaling molecule indicating the presence of bacteria. PG recycling during cell growth and division is well regulated, but a few gram-negative bacteria also release monomeric forms of PG (2). Until recently, the interaction between these PG monomers and animal host cells was thought to be restricted to pathogenic associations. In this issue, Adin et al. describe a mechanism by which the beneficial bacterium *Vibrio fischeri* releases PG monomers, lending insight into the role that these microbe-associated molecular pattern (MAMP) molecules play in host morphogenesis of the Hawaiian bobtail squid *Euprymna scolopes* (1).

PG monomers. In gram-negative bacteria, PG consists of subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid connected to a short pentapeptide side chain of L-alanyl-D-γ-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (2). During normal recycling of PG, lytic transglycosylases cleave the *N*-acetylmuramic acid-β-1,4-*N*-acetylglucosamine linkage, generating PG monomers (2, 7). In *Escherichia coli*, a permease, AmpG, then aids these PG monomers in entering the cytoplasm, where they are recycled and incorporated into the remodeled cell wall (2, 9). Some gram-negative bacteria, however, release PG monomers that can have a dramatic effect on eukaryotic host epithelial cells. This phenomenon was first described for the pathogens *Bordetella pertussis* and *Neisseria gonorrhoeae*, where release of PG monomers (identical in structure but named tracheal cytotoxin in *B. pertussis* and PG cytotoxin in *N. gonorrhoeae*) causes sloughing of ciliated epithelial cells and contributes to the etiology of whooping cough and gonorrhea, respectively (2, 10, 12).

The squid-vibrio association and PG. The symbiosis between the benthic Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium *Vibrio fischeri* is used as a model system to study the effects of beneficial bacteria on the development of animal host tissues (6, 8, 13). Each generation, the host is colonized by *V. fischeri* cells from the environment. Upon entry, these symbionts are housed in a structure called a light organ, the light of which is used by the host to camouflage itself during its nocturnal feeding behavior. Because the potential symbionts represent only a small fraction of the ambient microbial assemblage found in the surrounding seawater, *E. scolopes* and *V. fischeri* have evolved mechanisms that increase the probability of successful colonization, while discouraging infection of host tissues by nonspecific microorganisms. One such mechanism is a process by which the host harvests *V. fischeri* from seawater by using mucus secretions originating from superficial ciliated epithelia that aggregate environmental bacteria (reviewed in reference 8). The induction of these mucus secretions is initiated by bacterial PG. *V. fischeri* is able to outcompete other environmental bacteria in this mucus and migrate to and colonize epithelium-lined crypt spaces located in the center of the light organ, which is positioned below the superficial ciliated epithelium that is responsible for the mucus secretions (Fig. 1) (8). Following successful colonization, *V. fischeri* induces apoptosis and regression of these superficial ciliated fields via the synergistic action of PG monomers (identical to those released by *B. pertussis* and *N. gonorrhoeae*) and lipopolysaccharide (4). *V. fischeri* PG monomers have also been shown to cause the trafficking of host phagocytic hemocytes into the ciliated fields, presumably a process that aids in the symbiont-induced host cell morphogenesis (5).

Significance of this work. The discovery that a beneficial symbiont employs PG monomers to induce eukaryotic cell morphogenesis as part of the normal developmental program of an animal host broadens the role of MAMPs in microbe-animal interactions beyond that of recognition and removal of pathogens by the innate immune system (4). Adin et al. continue this story by describing a potential mechanism by which *V. fischeri* accumulates extracellular PG monomers. The authors identified and targeted *V. fischeri* homologues to *ampG*, which transports PG into the cytoplasm, and lytic transglycosylase genes (*ltgA*, *ltgD*, and *ltgY*) for mutagenesis. These genes were chosen because *B. pertussis* and *N. gonorrhoeae* employ different mechanisms to make their PG monomers. Whereas in *B. pertussis* the disruption of *ampG* leads to the release of PG monomer into the extracellular environment, *N. gonorrhoeae* uses lytic transglycosylase activity to generate and release PG monomer (reviewed in reference 2). Based on a number of elegant experiments, the major findings of this study include the following: (i) *ampG* mutants have a 100-fold increase in PG monomer release; (ii) mutations of transglycosylase genes in *V. fischeri* led to a decrease in PG monomer release; and (iii) a triple mutant lacking *ltgA*, *ltgD*, and *ltgY* colonized the host but left these squid open to secondary infection.

Because the *V. fischeri ampG* mutant led to a significant increase in PG monomer release while the inactivation of three transglycosylase genes led to the opposite result, the authors conclude that PG monomer release in *V. fischeri* more resembles that of *N. gonorrhoeae*. Interestingly, knocking out all three lytic transglycosylase genes led to susceptibility to a su-

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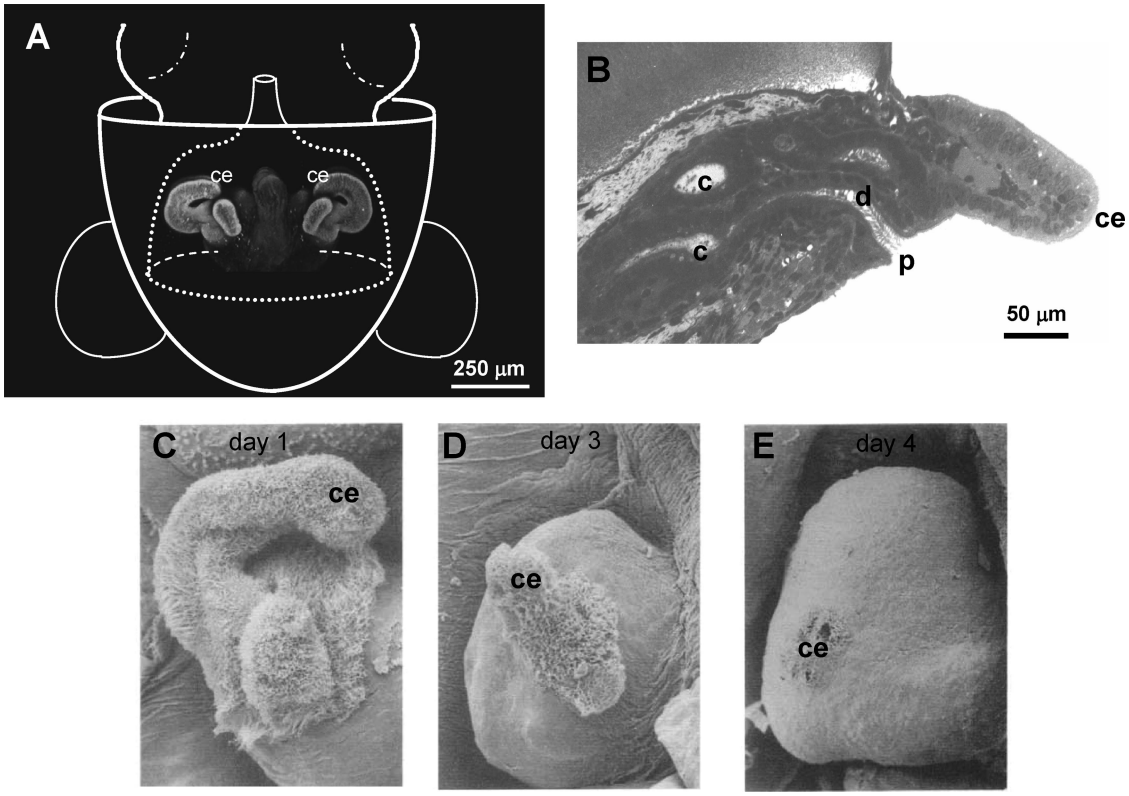

FIG. 1. Effect of *V. fischeri* monomers on host light-organ morphogenesis. (A) At hatching, the juvenile host's light organ is positioned in the center of the mantle cavity, where it is exposed to seawater and a background of environmental bacteria. A ciliated epithelium (ce) located on the surface of the light organ aids *V. fischeri* cells in colonizing the host (7a). (B) During this process, the symbionts migrate through pores (p) on the surface, down a ciliated duct (d), and into epithelium-lined crypt spaces (c), where colonization takes place. (C to E) *V. fischeri* PG monomers along with lipopolysaccharide lead to cell death and regression of the superficial ciliated epithelium (ce) over a 4-day period. (Adapted from reference 2a with the permission of the publisher.)

perinfection of host light organs by another strain of *V. fischeri*, suggesting that the inability to produce PG monomers leads to less regression of the superficial ciliated epithelium and presumably continued colonization of the host light organ. One of the amazing aspects of the squid-vibrio association is the high level of specificity (i.e., only *V. fischeri* has been demonstrated to colonize the light organ, even though the host crypt spaces are physically open to the surrounding seawater, containing on average 10^6 cells/ml of nonsymbiotic bacteria) (8). As the authors so aptly state, PG monomers may help establish this specificity by "closing the door" and preventing future colonization by potential pathogens or interlopers.

Future questions. Besides the results outlined above, this study also generated a number of very useful future tools, *V. fischeri* strains that result in significant increased and decreased PG monomer activity. *V. fischeri* PG monomer and PG derivatives have been implicated in a number of developmental events in the squid host (8, 13). These mutants will no doubt prove to be crucial resources for elucidating the role(s) that PG monomers play in establishing, and perhaps also in maintaining, the squid-vibrio association. Besides the induction of host mucus secretions preceding bacterial aggregation, hemocyte trafficking, and regression of the superficial ciliated epithelium, a number of other developmental events in this association may be mediated by *V. fischeri* PG monomer and/or PG derivatives. For example, by 48 h after colonization, host mucus accumulates in the crypt spaces while mucus secretion from the cells of the superficial epithelium ceases (8). Furthermore, the presence of *V. fischeri* in the crypt spaces is required to prevent continued mucus secretion, as removal of the symbionts from the light organ with the use of antibiotics restores the ability of the host to secrete mucus (8). Does PG or its derivatives play a role in regulating these changes in host mucosecretory behavior?

The host light organ continues to develop after colonization as the hatchling animal grows into an adult. The crypt spaces must accommodate a 1,000-fold increase in *V. fischeri* cells during this period, and a number of changes occur in the overall architecture of the tissues that house the symbionts. Are PG monomers involved with initiating any of these later developmental events, as they are during establishment of the association? The host also has a unique behavior in that every day at dawn, greater than 95% of the symbionts are expelled from the light organ along with a component of host cells comprised of sloughed epithelial cells, epithelial cell fragments, and phagocytic blood cells or hemocytes, the main cellular component of the host's innate immune system (7). This behavior serves two purposes, to seed the environment with symbionts for future host generations and to presumably regulate *V. fischeri* growth in the crypt spaces. The role of the

sloughed epithelial cells in the association is unknown, but this cellular debris may serve as a nutritional source for the symbionts. PG monomers from *B. pertussis* and *N. gonorrhoeae* have been implicated in ciliated cell sloughing in humans (2). The mutants generated in this study may help researchers to understand if *V. fischeri* PG monomers are involved with this daily restructuring of the host epithelium as well as mediation of the host's immune response, leading to the establishment and maintenance of homeostasis in the crypt spaces.

How might *V. fischeri* MAMPs such as PG monomers interact with the host at the molecular level? A number of pattern recognition receptors such as Toll-like receptor and PG recognition proteins along with downstream signaling pathways such as the NF-κB signaling pathway have been implicated in recognizing MAMPs and mediating host responses to microorganisms (2, 11). In *E. scolopes*, homologues to all of these have been found, and now host gene expression of these various receptors and pathways may be studied in response to varying *V. fischeri* PG monomer release in vivo (3).

Research on host-microbe interactions continues to reveal an intricate and conserved repertoire of signals used to mediate molecular cross talk in both pathogenic and beneficial associations. The use of MAMPs such as PG monomers in the initiation and establishment of both types of relationships demonstrates that cell-cell communication between bacteria and eukaryotes is ubiquitous in nature, often serving critical functions in these associations.

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RESEARCH ARTICLE
Host-Microbe Biology

Reproductive System Symbiotic Bacteria Are Conserved between Two Distinct Populations of *Euprymna scolopes* from Oahu, Hawaii

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ABSTRACT Female Hawaiian bobtail squid, *Euprymna scolopes*, harbor a symbiotic bacterial community in a reproductive organ, the accessory nidamental gland (ANG). This community is known to be stable over several generations of wild-caught bobtail squid but has, to date, been examined for only one population in Maunalua Bay, Oahu, HI. This study assessed the ANG and corresponding egg jelly coat (JC) bacterial communities for another genetically isolated host population from Kaneohe Bay, Oahu, HI, using 16S amplicon sequencing. The bacterial communities from the ANGs and JCs of the two populations were found to be similar in richness, evenness, phylogenetic diversity, and overall community composition. However, the Kaneohe Bay samples formed their own subset within the Maunalua Bay ANG/JC community. An *Alteromonadaceae* genus, BD2-13, was significantly higher in relative abundance in the Kaneohe Bay population, and several *Alphaproteobacteria* taxa also shifted in relative abundance between the two groups. This variation could be due to local adaptation to differing environmental challenges, to localized variability, or to functional redundancy among the ANG taxa. The overall stability of the community between the populations further supports a crucial functional role that has been hypothesized for this symbiosis.

IMPORTANCE In this study, we examined the reproductive ANG symbiosis found in two genetically isolated populations of the Hawaiian bobtail squid, *Euprymna scolopes*. The stability of the community reported here provides support for the hypothesis that this symbiosis is under strong selective pressure, while the observed differences suggest that some level of local adaptation may have occurred. These two host populations are frequently used interchangeably as source populations for research. *Euprymna scolopes* is an important model organism and offers the opportunity to examine the interplay between a binary and a consortial symbiosis in a single model host. Understanding the inherent natural variability of this association will aid in our understanding of the conservation, function, transmission, and development of the ANG symbiosis.

KEYWORDS 16S rRNA, *Euprymna*, community analysis, host-microbe interactions, symbiosis

The bobtail squid, *Euprymna scolopes*, is endemic to the Hawaiian archipelago and relies on the well-studied light organ symbiont *Vibrio fischeri* to avoid predation (1). In addition to this symbiosis, adult females also harbor a complex bacterial consortium in their reproductive system (2, 3). The accessory nidamental gland (ANG) is conserved throughout many species of squid, cuttlefish, and bobtail squid (4) and was first described over a century ago (5). This gland consists of a number of epithelium-lined tubules, each of which contains its own dominant bacterial taxon (Fig. 1A) (2). The ANG bacteria are deposited into the jelly coat (JC) of eggs (3), where they are hypothesized

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Euprymna scolopes female ANG reproductive system bacterial symbiosis is conserved across two populations of squid. @AllisonKerwin @spencernyholm

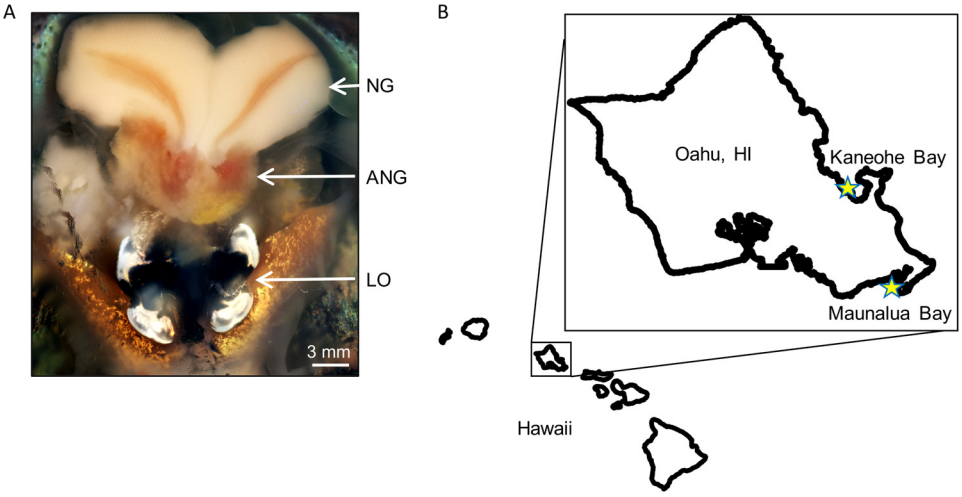

FIG 1 (A) Ventral dissection of adult female squid showing the nidamental glands (NG), accessory nidamental gland (ANG), and light organ (LO). (B) Map of Oahu, HI, showing locations of two squid populations, Kaneohe Bay (21°25′44.0″N, 157°47′32.4″W) and Maunalua Bay (21°16′51.42″N, 157°43′33.07″W).

to be involved in the defense of developing embryos from microbial fouling (2, 6, 7). Bacterial isolates from the ANG have also been demonstrated to inhibit certain marine bacteria via the production of secondary metabolites (7, 8).

Research into these bobtail squid and their symbionts has primarily focused on two populations of *E. scolopes* from the island of Oahu, HI (Fig. 1B). Maunalua Bay (MB), located on the southern coast of Oahu, is a shallow sand flat reaching approximately 600 m from shore to the reef crest. Kaneohe Bay (KB) is located to the north of MB on the eastern coast of Oahu and is the only true barrier reef in the Hawaiian archipelago (9). While bobtail squid are found in several discrete spots throughout KB (10), they are generally collected from a smaller sand flat reaching approximately 120 m from shore to a deeper channel.

These two bobtail squid host populations are located on the same island (Fig. 1B) but exhibit low levels of gene flow and several morphological differences (11). MB females, eggs, and juveniles are significantly larger than those from KB (11). These MB and KB hosts are also known to harbor different strains of *V. fischeri* in their light organs (12), although no evidence for geographic specificity of the strains from these two bobtail squid populations has been found (13), and bacterial lineages from MB and KB hosts show extensive mixing (14). The low levels of gene flow between host populations, along with previously described light organ symbiont strain differences between these two sites, make these populations a good source for examining potential variation in the ANG symbiotic communities.

We hypothesized that the ANGs from these two host populations would contain similar bacterial communities with minor variations, similar to what is seen for the light organ symbiosis. For this study, we collected squid from KB and compared the ANG and egg jelly coat (JC) communities to previously published samples from MB animals (3). We also compared the JC communities to the ANGs from females associated with those eggs, to confirm whether symbionts from mother (ANG) and corresponding egg (JC) communities clustered together, as had previously been demonstrated for the MB population (3).

RESULTS

To examine the natural variability of the *E. scolopes* ANG bacterial community, the V4 region of the 16S rRNA gene from ANG and JC bacterial extracts from Kaneohe Bay was sequenced and compared to previously published samples from Maunalua Bay (3). The bacterial communities from Kaneohe Bay ANGs and JCs clustered together, overlapping with the Maunalua Bay ANGs and JCs but with lower dispersion in a distinct

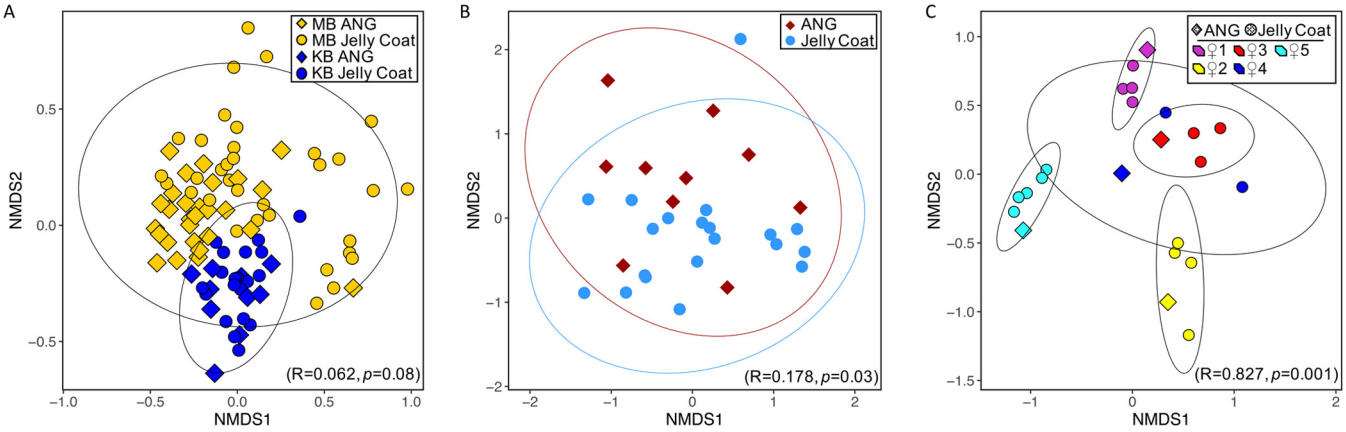

FIG 2 Bray-Curtis beta diversity analysis of Kaneohe Bay (KB) ANG and JC bacterial communities. (A) The overall community composition of KB and Maunalua Bay (MB) ANGs and JCs overlapped, but the KB samples had lower dispersion and clustered apart from the MB samples. (B) ANG and JC bacterial community compositions were not distinct in Kaneohe Bay. (C) KB ANG and JC samples clustered by associated female. Ellipses represent 95% confidence intervals. Results of ANOSIM are presented in parentheses in each plot.

cluster in a Bray-Curtis beta diversity analysis of community composition variation (Fig. 2A). An analysis of similarity (ANOSIM) indicated no dissimilarity between Kaneohe Bay and Maunalua Bay bobtail squid ANG and JC community composition ($R = 0.06$, $P = 0.08$), while a permutational multivariate analysis of variance (PERMANOVA) did find low levels of dissimilarity between the two populations ($F = 6.07$, $P = 0.001$). PERMANOVA is known to be more sensitive to variation in dispersion, and the significance of this test is thus likely due to the lower dispersion of the Kaneohe Bay ANG/JC samples. Kaneohe Bay ANG and JC samples clustered together on a nonmetric multidimensional scaling (NMDS) plot based on the Bray-Curtis metric (Fig. 2B) (ANOSIM: $R = 0.18$, $P = 0.03$; PERMANOVA: $F = 3.06$, $P = 0.02$), in agreement with a previous study, which found a similar pattern for Maunalua Bay ANG and JC samples (3). When compared, the bacterial community composition of Kaneohe Bay JCs reflected that of the ANG of the female that deposited the eggs, with a strong cluster pattern (Fig. 2C) (ANOSIM: $R = 0.83$, $P = 0.001$; PERMANOVA: $F = 7.37$, $P = 0.001$), similar to what was shown previously for the Maunalua Bay population (3).

Alpha diversity was also similar between KB and MB populations, for both ANG and JC bacterial communities (Fig. 3). Three types of alpha diversity were analyzed to give a broad portrait of within-sample diversity. Bacterial richness and evenness (H′), phylogenetic diversity (PD), and richness informed by the number of rare operational taxonomic units (OTUs, Chao1) were all similar between the two populations and two sample types, when analyzed via two-way ANOVA (Fig. 3). The larger spread in alpha diversity of MB JC samples than of other sample types is attributed to including a wider set of JCs from different stages of embryogenesis in the initial study (3), while this study included JCs only from eggs collected during early embryogenesis.

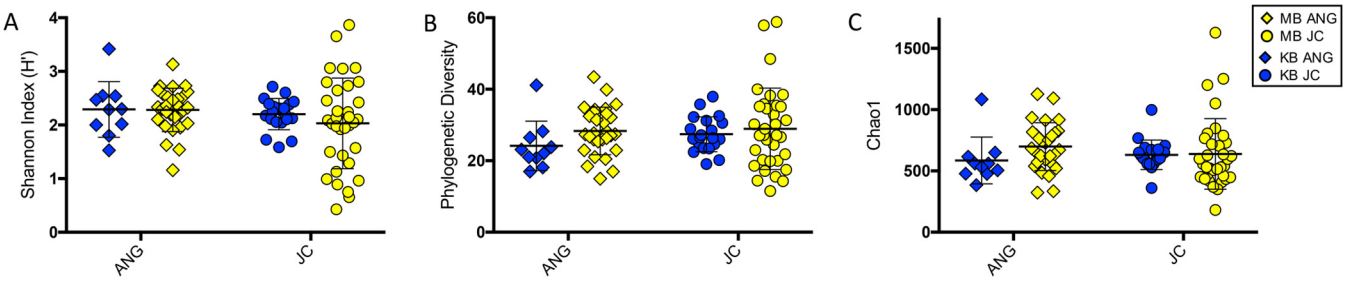

FIG 3 Alpha diversity analysis of Kaneohe Bay ANG and JC bacterial communities. Two-way ANOVA (see Table S1 in the supplemental material) revealed no effect of population or tissue type on bacterial community richness/evenness (A), phylogenetic diversity (B), or richness informed by the number of rare taxa present (C). Thick bars indicate means; thin bars indicate standard deviations.

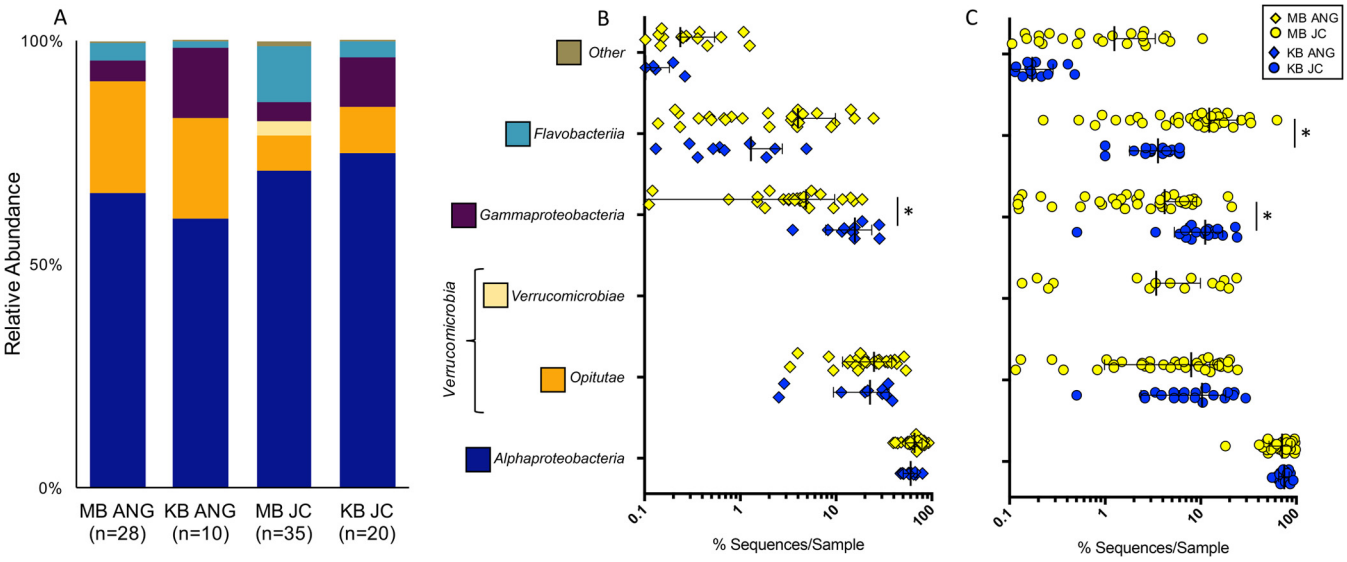

FIG 4 (A) Kaneohe Bay (KB) ANG and JC bacterial communities resembled those observed in Maunalua Bay (MB) at the class level. (B) KB animals had significantly more *Gammaproteobacteria* in their ANGs than MB hosts ($t_{36} = 5.129$, $P < 0.0001$). (C) JCs from KB animals had significantly more *Gammaproteobacteria* than MB JCs ($t_{53} = 4.73$, $P = 0.0001$) and also fewer *Flavobacteriia* ($t_{53} = 3.138$, $P = 0.01$). Taxa are presented at the class level (*Verrucomicrobiae* and *Opitutae* are both classes within the *Verrucomicrobia* phylum); the scatter plot is presented on a log scale to show variation for taxa present at lower average abundances. Thick bars represent means; thin bars represent standard deviations; asterisks represent significant differences between populations (B and C). Error bars that would have extended below 0.1% sequences/sample have been omitted from the graph (B and C). The "other" component included taxa present in more than one sample and at less than 0.3% average abundance.

The cluster patterns found via beta diversity analysis between the Kaneohe and Maunalua Bay ANG and JC bacterial communities can be explained by the observed differences in the relative abundances of certain taxa. The KB and MB ANGs were both dominated by *Alphaproteobacteria* (60.3% KB versus 65.9% MB) and *Verrucomicrobia* (22.6% KB versus 25.0% MB) (Fig. 4A). The JCs from both populations had higher levels of *Alphaproteobacteria* (74.8% KB versus 70.9% MB) and lower levels of *Verrucomicrobia* (10.3% KB versus 11.4% MB) than the ANGs (Fig. 4A). However, *Gammaproteobacteria* in KB ANGs accounted for a significantly higher proportion of the community than in MB ANGs (15.7% KB versus 4.9% MB, $t_{34} = 4.635$, $P = 0.0002$) (Fig. 4B). The higher proportion of *Gammaproteobacteria* in KB was due to an *Alteromonadaceae* genus (BD2-13, 11.9% KB versus 2.0% MB, $t_{34} = 5.023$, $P = 0.0003$) (Fig. 5A). A similar difference was seen in the JC for the same genus (9.4% KB versus 2.0% MB, $t_{53} = 5.588$, $P = 0.00001$) (Fig. 5B).

Two *Alphaproteobacteria* taxa were significantly higher in the JCs of Maunalua Bay than in those of Kaneohe Bay (*Methylocystaceae* [family], 1.8% MB versus 0.5% KB, $t_{53} = 3.639$, $P = 0.01$; *Rhizobiales* [order], 0.6% MB versus 0.3% KB, $t_{53} = 3.107$, $P = 0.04$), while a third (*Leisingera* sp.) was significantly higher in the Kaneohe Bay JCs than in the Maunalua Bay JCs (0.01% MB versus 0.8% KB, $t_{53} = 3.085$, $P = 0.04$) (Fig. 5B). However, the ANGs showed no differences in these specific *Alphaproteobacteria* groups between the two populations. These changes within the JC *Alphaproteobacteria* taxa indicate that this group may also shift slightly between the populations, although none of these individual taxa account for substantial proportions of the communities. The *Leisingera* sp. differences in this study between MB and KB JCs appear to be due in large part to a few outliers within the Kaneohe Bay JCs (Fig. 5B). Previous research demonstrated that a majority of the *Rhodobacteraceae* found in the *E. scolopes* ANG/JC community belonged to the *Leisingera* genus (2, 3, 7, 15); however, the 16S rRNA gene V4 region does not provide enough resolution to consistently resolve *Rhodobacteraceae* genera. The lower reported values of *Leisingera* in the MB ANGs and JCs in this study than those previously published for the same samples (3) are due in part to this lack of resolution in the V4 region of the 16S rRNA gene, as well as to the use of *de novo* clustering instead of reference-based OTU clustering.

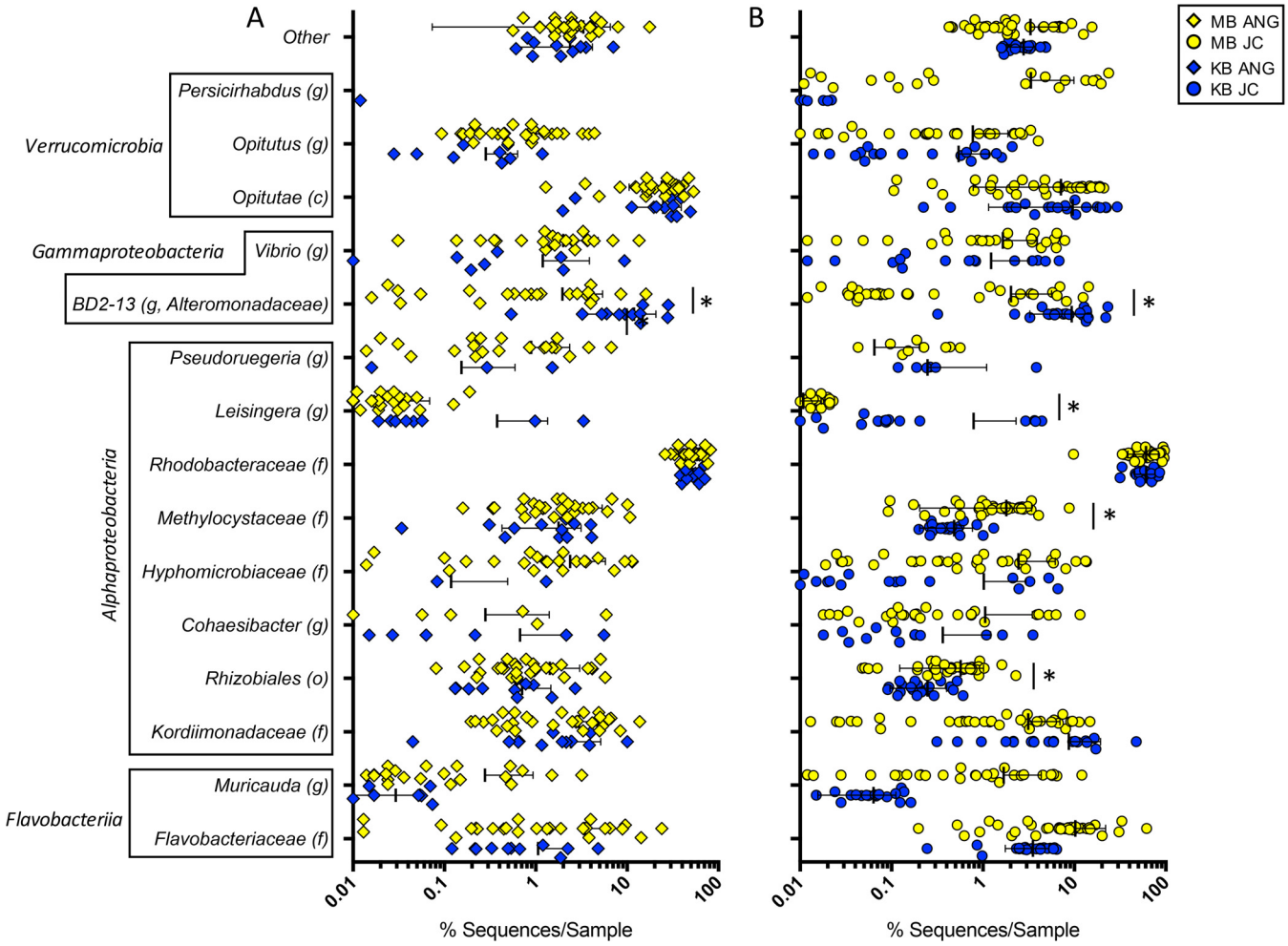

FIG 5 A higher abundance of *Gammaproteobacteria* in the Kaneohe Bay (KB) ANG and JC bacterial communities was due to a shift in BD2-13, a genus from the *Alteromonadaceae* family. BD2-13 ($t_{38} = 5.22$, $P = 0.0001$) was significantly more abundant in KB ANGs (A) and in KB JCs ($t_{53} = 5.612$, $P = 0.00001$) (B). One *Alphaproteobacteria* taxon (*Leisingera*: $t_{53} = 3.085$, $P = 0.04$) was also significantly higher in KB JCs (B), while two others were significantly lower in KB JCs (*Rhizobiales*: $t_{53} = 3.107$, $P = 0.04$; *Methylocystaceae*: $t_{53} = 3.639$, $P = 0.009$). Taxa are presented at the finest level obtained (c, class; o, order; f, family; g, genus); the scatter plot is presented on a log scale to show variation for taxa present at lower average abundances. Thick bars represent means; thin bars represent standard deviations; asterisks represent significant differences between populations. Error bars that would have extended below 0.01% sequences/sample have been omitted from the graph (A and B). The "other" component included taxa present in more than one sample and at less than 0.3% average abundance.

DISCUSSION

In this study, we find that the bobtail squid ANG MB and KB bacterial communities are largely similar, with small yet significant changes between some members. The ANG and JC communities were dominated by *Alphaproteobacteria* from the *Rhodobacteraceae* family and *Verrucomicrobia* from the *Opitutae* class in both locations (Fig. 4). Alpha diversity metrics showed no differences between the populations, demonstrating that the communities are similar in terms of species richness, evenness, and phylogenetic diversity. However, beta diversity analysis revealed that samples from Kaneohe Bay exhibited tighter dispersion, clustering apart from the Maunalua Bay samples but still contained within the larger MB group, indicating a distinct but similar community composition (Fig. 2 and 3). The Kaneohe Bay population contained a significantly higher proportion of *Gammaproteobacteria* from the BD2-13 genus (a member of the *Alteromonadaceae*) (Fig. 5). Altogether, these data suggest that the community is stable between these two host populations, with the slight differences in community composition potentially reflecting local adaptation to differing environmental conditions, localized variability, or functional redundancy between the members.

The main taxonomic difference between the MB and KB ANG communities was due to an increase in relative abundance of *Alteromonadaceae* in the KB population. *Alteromonadaceae* are known to produce many secondary metabolites with antibacterial and anticancer properties (16, 17). Secondary metabolite production by symbiotic bacteria is hypothesized to contribute to cephalopod egg defense from potential fouling and infection during development (2, 6–8), although this has yet to be demonstrated *in vivo*. The potential for functional redundancy between alteromonads and other bacterial groups in the ANG could also provide further insight into how this bacterial consortium contributes to egg defense. The two host populations in MB and KB are known to contain different light organ strains of *V. fischeri* (12). However, colonization efficiency is not affected by the source population of squid or *V. fischeri* symbionts, suggesting a lack of host-symbiont coevolution (13) and functional redundancy between the strains. The higher prevalence of *Alteromonadaceae* in the KB ANG symbiosis should be explored further and may provide a novel source for natural product discovery. While *Actinobacteria* and to a lesser degree *Cyanobacteria* have been responsible for the majority of natural product drug discovery in marine ecosystems, the *Alteromonadaceae* are responsible for a high proportion of natural products derived from *Proteobacteria*, especially compared to the *Alphaproteobacteria* (16–18).

The minor differences found in the ANG community composition between these two populations may be potentially tied to morphological differences already described for these squid. MB day 0 eggs are known to be larger than KB day 0 eggs (3.5-mm diameter versus 3.0-mm diameter, respectively [11]). This size difference could indicate a larger amount of jelly coat in the MB eggs and thus a higher overall abundance of bacteria, or alternatively may be due to a larger yolk sac and/or embryo. Differences in bacterial communities caused by abundance and/or strain differences may influence the defensive potential (i.e., secondary metabolite production) of eggs from one population versus the other. Further research comparing *E. scolopes* ANG/JC populations from other geographic locations and comparisons between cephalopod species may answer some of these questions.

While functional redundancy may explain the variation in communities found between these two populations, differing environmental conditions might also play a role. Kaneohe Bay is a barrier reef, while Maunalua Bay is a fringing reef, and thus, these two sites are subjected to different types of water currents. Both sites contain a high level of nonindigenous and cryptogenic species (KB, 18.8%; MB, 18% of total biota), as well as invasive macroalgae (19, 20). However, the observed species distributions at sites of *E. scolopes* collections are distinct, with the highest number of macrofaunal taxa found in Kaneohe Bay belonging to polychaeta and gastropods, while in Maunalua Bay amphipods and red algae dominate (19, 20). These ecosystems could exert different predation and fouling pressures on bobtail squid eggs, leading to local adaptations of the symbiont populations. Additionally, distinct bacterial communities in the KB seawater and sediment could provide different source populations for the environmental transmission of the community. The developing squid are hypothesized to reacquire their ANG symbionts every generation from the environment (3), and so variation in symbiont availability between KB and MB may influence the ANG community compositions of these two populations.

In many other host-microbe associations, symbionts have been found to diverge between different populations. Gut communities frequently vary due to differences in diet between populations, as is observed in the human gut microbiome (21, 22) or in the juvenile Atlantic salmon, *Salmo salar*, where the gut microbiome varied between populations only in *Mycoplasmataceae* strains (23). The hindgut microbiota of termites, *Reticulitermes flavipes*, from different but nearby populations, showed similar abundance patterns for the core taxa but did exhibit variation hypothesized to allow the termites to distinguish nestmates from invaders (24). Furthermore, obligate nutritional endosymbionts, such as *Symbiodinium* in corals, can vary at the strain level (25).

In a symbiosis that may be functionally similar to that of the ANG, the epithelial bacterial community of *Hydra oligactis* has been shown to provide protection from

fungal fouling (26). The *H. oligactis* epithelial symbiosis and the ANG symbiosis appear to share similar population dynamics. A comparison of *Hydra oligactis* populations from two German lakes found that the populations contained many of the same bacterial taxa and grouped together apart from the community of *Hydra vulgaris* from one of the same lakes (27). However, each population did contain some bacterial taxa not found in the other population (27), similar to what we observed in this study for the two populations of *E. scolopes*.

Despite the population differences, the overall ANG community dynamics within each host population appear to be similar. We found no dissimilarity between the overall ANG and JC community composition, and the JC bacterial community of a given female's eggs clustered with its corresponding ANG (Fig. 2), providing additional evidence for the deposition of the ANG bacterial community into the egg JC. Expanding on the conclusions reached for the MB population (3), comparison of the ANG communities from genetically isolated host populations reinforces the hypothesis that ANG symbionts are taxonomically conserved in this and other cephalopod species. The conserved bacterial taxa between these isolated populations lead us to predict that similar ANG symbiotic communities will be found across populations of *E. scolopes*. Previous studies suggest that similar bacterial taxa are shared between *E. scolopes* and other ANG-containing cephalopods (28–31). *Alphaproteobacteria* appear to make up the majority of the taxa found in these symbioses, along with *Gammaproteobacteria* to a lesser extent (28–31). Future studies should also focus on determining whether functional conservation exists between the different bacterial strains found in various cephalopods.

The selective pressure exerted on a defensive symbiosis will largely depend on the abundance and fitness effects of specific pathogens/foulers in the host's natural environment (32–35). If fouling only rarely impacts *E. scolopes* clutches, or if that fouling does not negatively impact host survival or fitness, then the selective pressure to conserve the symbiosis throughout the species should be low. Distinct environmental conditions between populations could result in different selective pressures. The largely conserved ANG symbiosis between the MB and KB bobtail squid populations may reflect the strong threat of egg fouling or infection by marine microbes. In the future, *in situ* experiments investigating fouling of eggs where the JC community has been altered (e.g., by antibiotic treatment) may lend insight into the occurrence of this threat in the host's natural environment. Examination of bobtail squid populations from other islands in the Hawaiian archipelago will also enhance our understanding of the stability of the ANG community across the species. A previous genome study of roseobacters isolated from the ANG of MB *E. scolopes* suggested that there are differences between closely related strains (15). Future work on specific KB ANG isolates along with metagenomic and transcriptomic studies may lead to a better understanding of these differences. The overall stability of the community between host populations supports a critical functional role for this symbiosis, while the few variable taxa open up potential avenues for understanding how local host-microbe populations adapt to different conditions and for isolating additional drug discovery candidates.

MATERIALS AND METHODS

Ten sexually mature female squid (ranging in mantle length from 19 mm to 30 mm) were collected from Kaneohe Bay (KB, 21°16′51.42″N, 57°43′33.07″W) using dip nets and were immediately transferred to Kewalo Marine Laboratory, Oahu, HI. Squid either were sacrificed within 2 days or were shipped to Connecticut and maintained in our squid facility for up to 4 months. Lab-maintained females were regularly mated and kept in individual tanks to allow clutches to be matched to the individual mothers. Bobtail squid were anesthetized in 2% ethanol in artificial seawater prior to sacrifice. Egg clutches were collected and dissected within 12 h of deposition. All samples were surface sterilized in 99% ethanol and filter-sterilized squid Ringer's solution (FSSR [2]) to remove transient bacterial contaminants.

DNA extraction from ANGs ($n = 10$) and egg JCs ($n = 20$) was completed as previously described (3). Briefly, ANGs were homogenized in FSSR, followed by differential centrifugation to separate the bacterial cells from host tissue. DNA extraction of the bacterial component was completed using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) with bead beating (Mini-Beadbeater-16; BioSpec Products, Bartlesville, OK). Ten JCs were separated from their outer egg capsules and yolk sacs and pooled in a bead-beating tube. The JCs were flash-frozen to −80°C for a minimum of 30 min, and DNA was extracted

using the MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI) with bead beating and an increased concentration of proteinase K (0.833 μg/ml).

Extracted DNA was amplified using bar-coded primers developed for the V4 region of the 16S rRNA gene by Caporaso et al. (36) and sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA) according to established protocols (24, 37). Samples were processed either in the Nyholm lab or at the University of Connecticut Microbial Analysis, Resources and Services facility (MARS). An average of 50,052 ± 12,197 reads/sample was obtained for KB ANG samples (n = 10, minimum 23,976 reads/sample). An average of 66,550 ± 32,128 reads/sample was obtained for KB JCs (n = 20, minimum 17,654 reads/sample). MB samples were previously published and reanalyzed for this study and contained an average of 82,077 ± 31,037 reads/sample for the JC and 74,739 ± 31,370 reads/sample for the ANG (3). Both negative-extraction (no-sample) and PCR (no-template) controls were processed and sequenced simultaneously with all samples. Fewer than 1,000 sequences/control were obtained in all cases, and the majority of sequences in these controls belonged to a single *Escherichia* OTU. Most other OTUs present in the controls were not present in the ANG samples. Three *Rhodobacteraceae* OTUs also associated with the community were obtained in the controls as well but accounted for less than 1% of sequences for the control samples. In addition, the presence of *Rhodobacteraceae* in the ANG has been previously established through the use of fluorescence *in situ* hybridization (2) and culturing techniques (15). No *Verrucomicrobia* OTUs were found in any of the control samples.

Sequencing data were analyzed using QIIME (38). *De novo* methods were used to assign operational taxonomic units (OTUs) at the 97% identity level (24). Samples were rarefied to 10,000 sequences. Alpha diversity was analyzed in QIIME, and the $\log_2$ Shannon index was converted to a natural log Shannon index. Alpha diversity plots were created, and differences in alpha diversity were tested using two-way ANOVA with *post hoc* Tukey tests in Prism. Beta diversity was analyzed using the Bray-Curtis metric, with community composition similarity tested by ANOSIM and PERMANOVA in QIIME and NMDS plots created in R using the Vegan package (39). Differences in relative abundance between various taxa were analyzed by unpaired t test and corrected for multiple comparisons using the Holm-Sidek method in Prism. KB sequences were compared to MB sequences previously published and available under project identifier (ID) PRJEB14655, accession numbers ERS1498392 to ERS1498398, ERS1496666 to ERS1496676, and ERS1496678 to ERS1496722 (3). MB sequences were reanalyzed for this study for consistency.

Accession number(s). KB sequences were deposited in the European Nucleotide Archive (ENA) and are available under project ID PRJEB23264.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00531-17.

TABLE S1, TIF file, 0.2 MB.

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Symbiotic bacteria associated with a bobtail squid reproductive system are detectable in the environment, and stable in the host and developing eggs

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Summary

Female Hawaiian bobtail squid, *Euprymna scolopes*, have an accessory nidamental gland (ANG) housing a bacterial consortium that is hypothesized to be environmentally transmitted and to function in the protection of eggs from fouling and infection. The composition, stability, and variability of the ANG and egg jelly coat (JC) communities were characterized and compared to the bacterial community composition of the surrounding environment using Illumina sequencing and transmission electron microscopy. The ANG bacterial community was conserved throughout hosts collected from the wild and was not affected by maintaining animals in the laboratory. The core symbiotic community was composed of *Alphaproteobacteria* and *Opitutae* (a class of *Verrucomicrobia*). Operational taxonomic units representing 94.5% of the average ANG abundance were found in either the seawater or sediment, which is consistent with the hypothesis of environmental transmission between generations. The bacterial composition of the JC was stable during development and mirrored that of the ANG. Bacterial communities from individual egg clutches also grouped with the ANG of the female that produced them. Collectively, these data suggest a conserved role of the ANG/JC community in host reproduction. Future directions will focus on determining the function of this symbiotic community, and how it may change during ANG development.

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Introduction

A number of host-microbe interactions rely on symbiotic bacteria that are environmentally transmitted each generation. In marine ecosystems, cephalopods (loliginids, sepiids and sepiolids, Buchner, 1965) form symbioses with bacterial consortia that are associated with a specialized organ of the female reproductive system called the ANG. The ANG is made up of epithelium-lined tubules that house bacterial symbionts (Fig. 1, Bloodgood, 1977; Collins *et al.*, 2012). Although this bacterial association has been recognized for a century (Pierantoni, 1918), the function of the ANG remains largely uncharacterized. Published studies from two cephalopod species suggest that bacteria are deposited into the egg cases (Kaufman *et al.*, 1998; Collins *et al.*, 2012), where they are hypothesized to play a role in egg defense (Biggs and Epel, 1991). The dominant bacterial taxa of cephalopod ANGs are generally a combination of *Alphaproteobacteria, Gammaproteobacteria* and *Verrucomicrobia*, depending on the host species analysed (Barbieri *et al.*, 2001; Grigioni *et al.*, 2000; Pichon *et al.*, 2005; Collins *et al.*, 2012).

The bobtail squid, *Euprymna scolopes*, is endemic to the Hawaiian archipelago and lives in symbiosis with the bioluminescent bacterium *Vibrio fischeri*. This light organ symbiosis has served as a model for studying numerous beneficial host-microbe interactions, including quorum sensing, host immune response to beneficial and environmental microbes, and symbiont specificity (Nyholm and McFall-Ngai, 2004; Nyholm and Graf, 2012; Miyashiro and Ruby 2012; McFall-Ngai, 2014). Recent investigations have also focused on the ANG bacterial community of *E. scolopes*, which is unique among the cephalopods in containing a large contingent of *Verrucomicrobia* (Collins *et al.*, 2012). Fluorescence *in situ* hybridization (FISH) demonstrated that some members of the ANG bacterial community are also present in the *E. scolopes* egg JC (Collins *et al.*, 2012). However, the exact composition of the bacterial community in *E. scolopes* eggs and a comparison between the egg and ANG bacterial communities

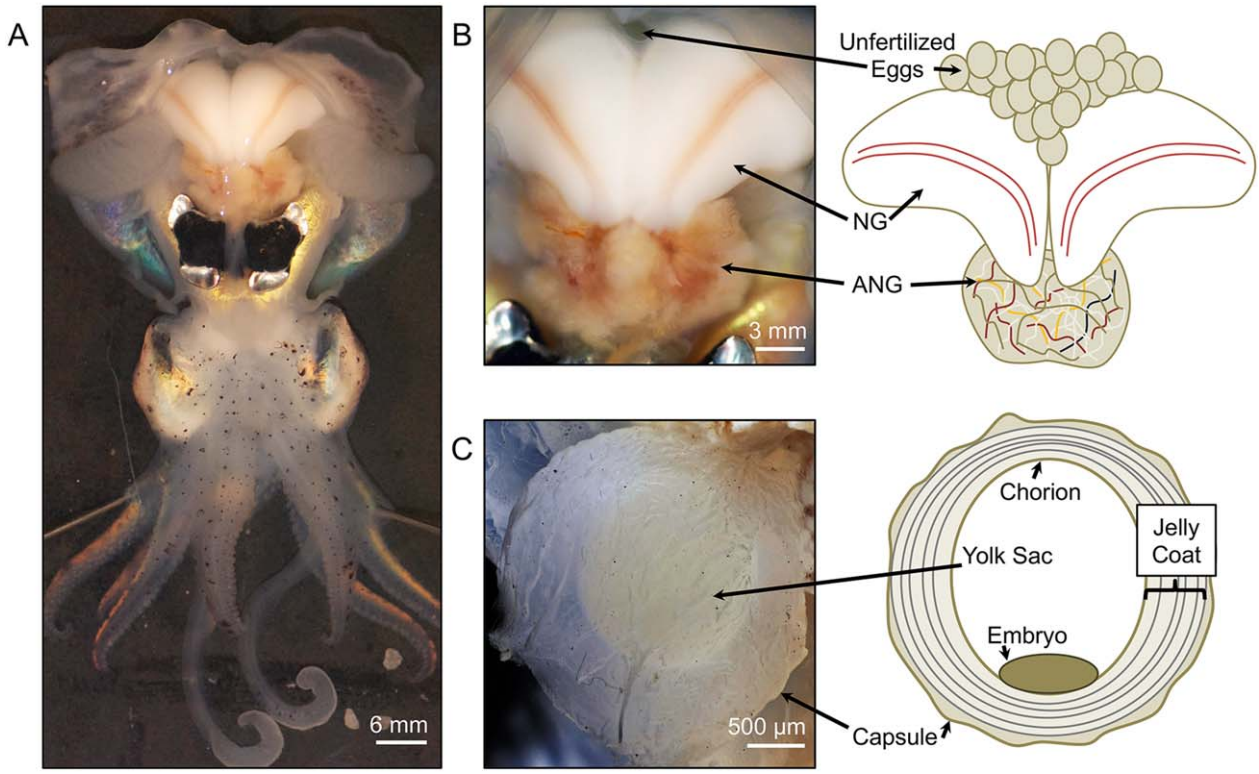


Fig. 1. Ventral dissection of a female *E. scolopes* (A), the female reproductive system (B) and an egg (C). Bacteria from the ANG are deposited into the JC layers that are secreted by the nidamental glands (NG). The embryo develops inside the central yolk sac, bounded by the chorion membrane. The JC surrounds the chorion and is encapsulated by an outer layer called the capsule which cements the egg to the rest of the clutch.

from corresponding individual females has not been reported.

Whether the ANG community is altered by maintaining mature animals in the laboratory is also unknown. Removing wild-caught animals from their native environments, especially when those environments are marine, and maintaining them in the laboratory can lead to changes in an animal's resident microbiota (Ford *et al.*, 1986; Kooperman *et al.*, 2007; Scott *et al.*, 2010; Devine *et al.*, 2012; Pratte *et al.*, 2015). While *E. scolopes* is easily maintained in the laboratory with little evident influence on fecundity, the effect of laboratory conditions on the ANG bacterial community is unclear.

E. scolopes lives in close contact with the microbial communities found in the environment, burrowing in the sediment during the day and actively swimming in the water column while hunting at night. Seawater flows constantly through the host's mantle cavity and this process is important in selecting *V. fischeri* for its light organ association (reviewed in Nyholm and McFall-Ngai, 2004). Microbial symbioses are transferred to the next generation either by direct maternal transfer of symbionts to offspring (vertical transmission), or by reacquiring symbionts from the environment (horizontal/environmental transmission; Bright and Bulgheresi, 2010). Juvenile cephalopods lack an ANG and are hypothesized to acquire their ANG symbionts from the environment during sexual development, despite the presence of those bacteria in the eggs (Kaufman *et al.* 1998, S. Nyholm pers. obs.). However, the microbial communities of the near-shore seawater and sediment in the natural habitat of *E. scolopes* remain poorly described. One goal of this research was to determine the seawater and sediment community composition of the bobtail squid's environment to understand whether bacteria associated with the ANG are present.

This research examines the variability of the ANG bacterial community of *E. scolopes* from Maunalua Bay, Oahu, Hawaii, and determines the core community of the ANG within this population. We analysed differences in bacterial composition of the ANG from wild and laboratory-maintained animals. Because the ANG association is thought to be environmentally transferred between generations, the bacterial communities of the seawater and sediment from Maunalua Bay were also examined. Finally, bacterial communities from the host's eggs were compared to ANGs, and the stability of the community during embryogenesis was characterized.

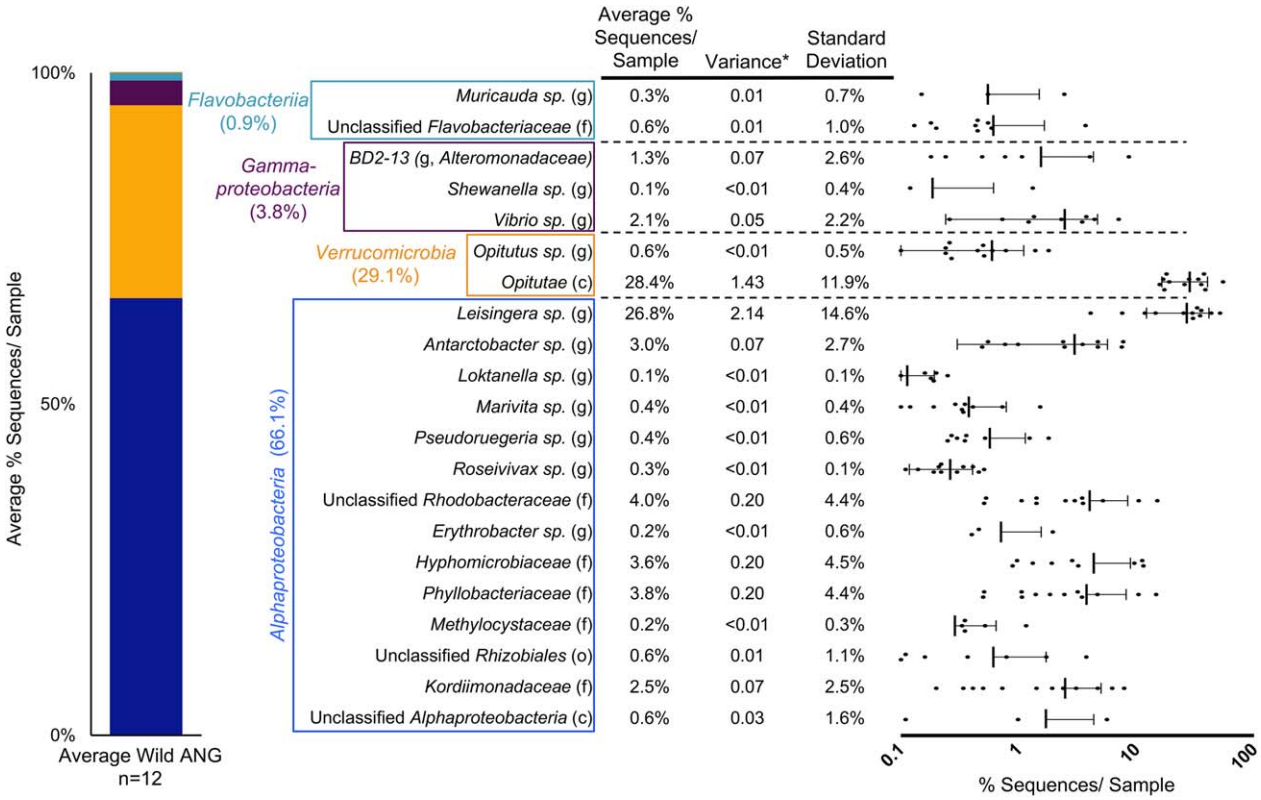


Fig. 2. The average symbiont population in the ANG of mature, wild-caught animals was composed of four bacterial classes. The percentage of sequences per sample for each taxon did not vary widely indicating a consistent community composition throughout the population of *E. scolopes*. Taxa present in more than one sample, and at greater than 0.1% of the average community, were included. Other taxa made up 0.15% of the community and included *Betaproteobacteria*, *Deltaproteobacteria* and *Sphingobacteriia* sequences. Taxa presented at the finest level obtained, c – class; o – order; f – family; g – genus. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. *Variance units are $\%^2$.

Results

High-throughput Illumina sequencing of the 16S rRNA V4 gene region revealed the phylogenetic diversity and variability of the ANG and JC bacterial communities across space and time. An examination of ANGs collected from wild mature bobtail squid ($n = 12$) showed that the average community was composed of four main bacterial groups: 66.1% ± 11.1% *Alphaproteobacteria*, 29.1% ± 11.8% *Verrucomicrobia*, 3.8% ± 3.6% *Gammaproteobacteria* and 0.9% ± 1.4% *Flavobacteriia* (Fig. 2). For all identified taxa the variance was low, indicating little individual differences in bacterial taxa between hosts from this population.

To determine whether bobtail squid maintained in the lab for an extended period had an altered ANG community, we compared lab-maintained animals to wild animals. ANGs from laboratory-maintained hosts ($n = 17$) had a similar composition to those from wild bobtail squid ($n = 12$, Fig. 3A). Operational taxonomic units (OTUs) identified as most closely related to *Betaproteobacteria* were only found in the ANGs of two of the lab-maintained animals, with one outlier at a comparatively higher relative abundance (2.4% ± 9.7%). ANGs from lab-maintained and wild *E. scolopes* clustered together via beta-diversity metrics (Fig. 3B), and one-way ANOSIM did not reveal significant dissimilarity between the groups ($R = -0.04$, $p = 0.79$), indicating that the bacterial consortium was stable when hosts were maintained in aquaria over their lifetime, usually a period of several months. When R is closer to zero in an ANOSIM analysis the similarity of the samples within a group is the same as the similarity between groups. Furthermore, animals collected over a period of seven years and different seasons had similar bacterial taxa, suggesting that the ANG community is stable over time and across generations.

The average JC community ($n = 35$) included fewer OTUs most closely related to the *Opitutae* class (*Verrucomicrobia*) than the average ANG community ($n = 29$, 8.0% vs. 25.0%), and more *Alphaproteobacteria* (71.3% vs. 65.0%, Fig. 4A). However, JC samples clustered with the

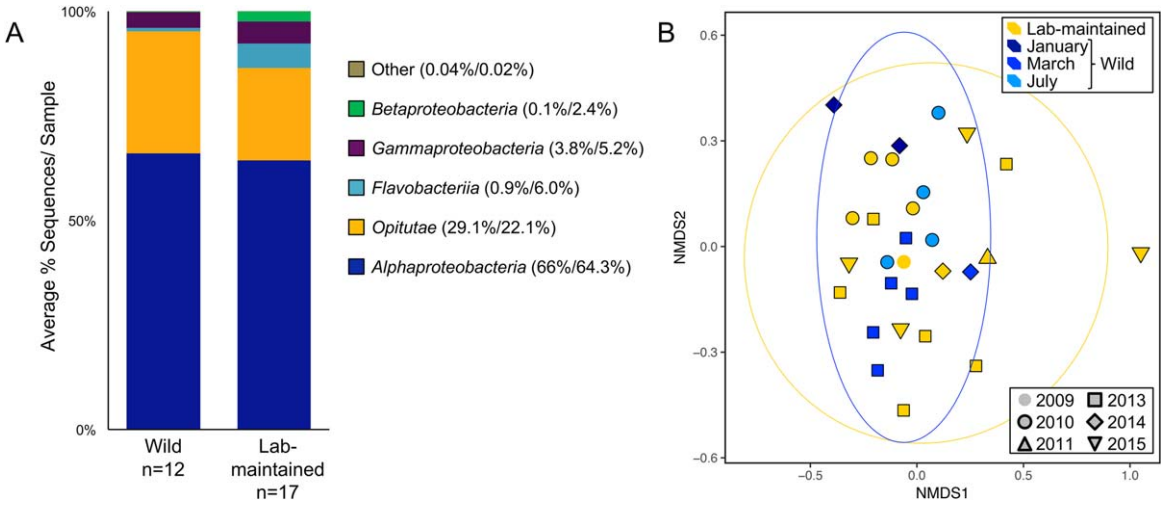


Fig. 3. Wild ($n = 12$) and lab-maintained ($n = 17$) ANGs had similar bacterial community compositions, both in the most abundant bacterial classes (A) and in beta-diversity (B). Bray Curtis NMDS plot of the two types of communities shows overlap of the sample types indicating that the community composition is similar. Shades of blue indicate month of collection for wild *E. scolopes*, while shape indicates year of collection. Community compositions were similar despite collection during different seasons and years. Ellipses indicate 95% confidence intervals of lab-maintained and wild groups. Taxa present in more than one sample, and at greater than 0.1% of the average community, were included (A). Other taxa made up 0.02% of the lab-maintained community and included *Deltaproteobacteria* and *Sphingobacteriia* sequences.

ANGs from wild and lab-maintained animals using beta-diversity metrics and one-way ANOSIM showed only low levels of dissimilarity between the groups ($R = 0.15$, $p = 0.001$), indicating that the bacterial consortium found in the JCs reflected that found in the ANG (Fig. 4B). While the spread of the JC samples was greater than that of the ANG samples which clustered closely together (Fig. 4B), the JCs and ANGs clustered more closely with each other than with environmental samples (Fig. 5A). JC samples generally clustered closer to the ANG of the female that produced those eggs than to other ANGs ($n = 5$), demonstrating that the JC community may reflect low levels of individual ANG variation (Fig. 4C, Supporting Information Fig. S1). JCs taken from various points in embryogenesis showed no clear clustering by embryonic stage. Clustering reflected the female that produced the eggs, but within each cluster the early- (day 0–2) and mid-stage (day 10–12) communities tended to group closer together and apart from the late-stage (day 17–24) community (Fig. 4D). Overall, this pattern indicates that the JC community is stable in terms of relative bacterial community composition throughout much of embryogenesis.

Eggs were also examined using TEM to determine whether the JC changed between early and late embryogenesis, and whether any patterns of bacterial distribution could be detected. During early embryogenesis, single bacterial cells were scattered throughout the JC layers, with no particular pattern in terms of cell morphology or distribution among inner vs. outer layers of the JC (Fig. 6A). By late embryogenesis single cells and small microcolonies of morphologically similar bacteria (typically 3–4 cells) were observed throughout the JC layers. Again, no pattern of distribution was observed (Fig. 6B). At both stages of embryogenesis, cells were observed in the process of cell division (Fig. 6C/D). An electron-dense material appeared to divide the various JC layers from each other (Fig. 6E). The abundance of culturable bacteria (CFUs) in the JC increased from an average of 2.1×10^4 CFU/JC at early embryogenesis (day 0) to an average of 3.0×10^5 CFU/JC by late embryogenesis (day 19–24, $n = 5$ clutches).

The Maunalua Bay *E. scolopes* ANG core bacterial community (OTUs present in 90% of ANG samples) consisted of 52 OTUs from two bacterial phyla, the *Verrucomicrobia* and the *Proteobacteria*. The *Verrucomicrobia* core members were all from the *Opitutae* class (12 OTUs), and in four cases were further identified to the genus *Opitutus* (Table 1). The *Proteobacteria* core members consisted solely of *Alphaproteobacteria* from the *Kordiimonadales* and *Rhizobiales* orders, and, most abundantly, the *Rhodobacteraceae* family. The majority of these core OTUs were classified to the family level of *Rhodobacteraceae* (17 OTUs), but five genera, specifically *Leisingera*, *Loktanella*, *Marivita*, *Roseivivax*, and *Antarctobacter* (14 OTUs) were also identified. The core community represented on average 79.5% of the sequences recovered per ANG sample. However, the abundance of those core OTUs varied from animal to animal, and the remaining 20.5% of sequences present in the average ANG was also variable, although the majority of the remaining OTUs belonged to the same taxonomic groups discussed here.

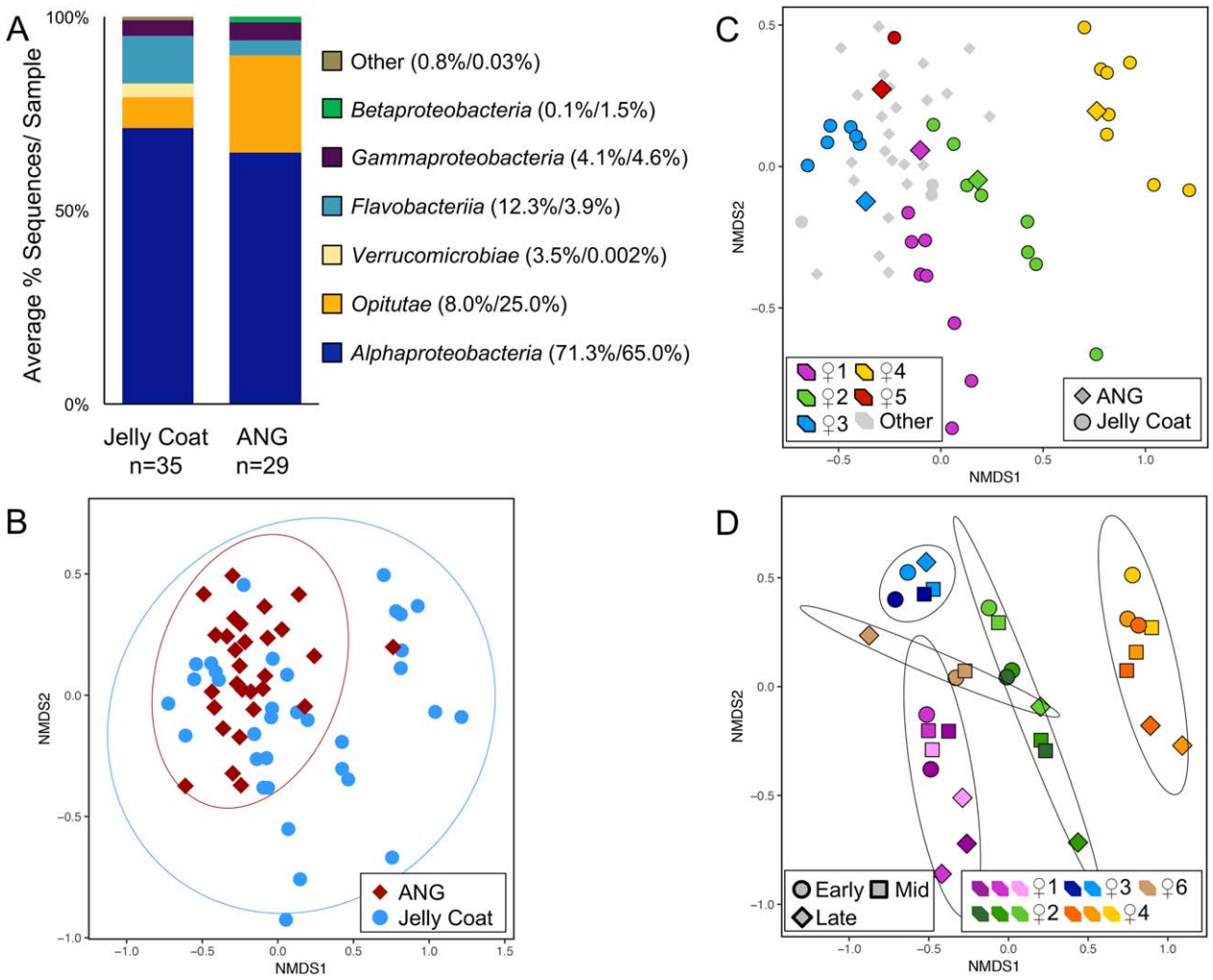
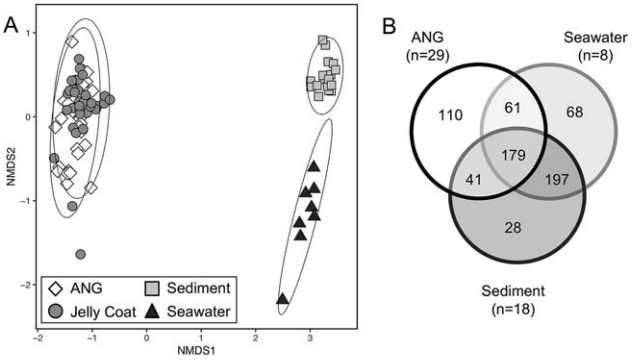


Fig. 4. The egg JC ($n = 35$) and ANG ($n = 29$) had similar bacterial community compositions, consisting of 4 main bacterial taxa (A). The 'other' component included taxa present in more than one sample, and at less than 0.5% of the average community composition. Other taxa made up 0.8% of the JC community and included unclassified *Bacteria* and *Proteobacteria*, *Clostridia*, *Deltaproteobacteria*, *Planctomycetia* and *Acidimicrobiia*. Bray Curtis NMDS analysis showed that the ANG and JC communities overlap (B). The same analysis demonstrated that JCs cluster with the ANG from the female that produced those eggs (C), and that JCs cluster by the individual female (colour), but not by stage of embryogenesis (D; shape, early = day 0–2, mid = day 10–12, late = day 17–24). JCs labelled as 'other' (C) are samples for which no matching ANG was analysed, or ANGs for which no matching JCs were obtained, and are provided for context. Ellipses indicate 95% confidence intervals (B/D).

The sediment community (n = 18) was similar throughout the sites sampled, and contained sequences belonging to 37 classes of bacteria and archaea (Supporting Information Fig. S2, Table S1). The seawater community ($n = 8$) was also similar between samples and contained sequences from 22 classes of bacteria and archaea (Supporting Information Fig. S3, Table S2). Both the sediment and seawater communities were more diverse than the ANG and JC communities, both in richness/evenness metrics (H' and E_H), and phylogenetically (PD, Fig. 5D). Beta-diversity metrics showed distinct clustering of the seawater and sediment samples apart from the ANG/JC samples, and one-way ANOSIM revealed significant dissimilarity between the environmental samples and bobtail squid-associated samples ($R = 1.0$, $p = 0.001$), indicating that the overall community composition of the three sample types was different (Fig. 5A). However, a substantial overlap of the OTUs present in the average ANG with those found in the environment was noted (Fig. 5B and C). Seventy-two percent of the 391 total OTUs recovered from all ANG samples were found in a seawater or sediment sample, or both. The OTUs unique to the ANG represented 5.5% of the average ANG sequences, and only 1.7% of the average core ANG sequences. These results suggest that the majority of the ANG community is also present in the environment.



C

	# OTUs	% of Average ANG Sequences	% of Average ANG Core Sequences
ANG/Sediment/Seawater	179	87.08%	75.11%
ANG/Sediment	41	2.26%	1.47%
ANG/Seawater	61	5.14%	1.24%
Unique to ANG	110	5.51%	1.65%
Total	391	100.00%	79.47%

D

	n	Shannon Index (H')	Shannon Equitability (E_H)	Phylogenetic Diversity (PD)
ANG	29	2.38 ± 0.38	0.51 ± 0.07	11.26 ± 2.32
Jelly coat	35	2.15 ± 0.76	0.47 ± 0.14	10.74 ± 2.63
Seawater	8	2.92 ± 0.36	0.60 ± 0.05	22.09 ± 3.71
Sediment	18	4.41 ± 0.19	0.82 ± 0.03	32.35 ± 2.58

Fig. 5. Comparison of the bacterial communities found in the Maunalua Bay seawater, sediment, JC, and ANG. The overall community composition of the sediment, seawater, and ANG/JC were distinct when compared via Bray Curtis beta-diversity analysis (A). However, the ANG community had substantial overlap with the seawater and sediment communities in terms of which OTUs were present (B) and in the percentage of the average ANG community those OTUs represented (C). Shannon index and equitability metrics indicated that the ANG and JC communities were less diverse than the seawater and sediment communities, and the same was true for phylogenetic diversity (D). Ellipses represent 95% confidence intervals (A).

Sequencing of the laboratory aquaria seawater and substrate revealed a subset of the natural bobtail squid environmental community (Supporting Information Fig. S4). While many of the bacterial classes present in the wild were also present in the lab, the relative abundances of those classes varied widely. The overlap of the ANG community with the lab environment was less than the overlap seen with the natural environment.

Discussion

The cephalopod ANG-bacteria association is widely distributed, but no study to date has examined the bacterial consortia of ANGs and eggs collected from individuals in the same environment. In this study, a core ANG community from *E. scolopes* was determined and found to be consistent and conserved in multiple mature female bobtail squid collected from Maunalua Bay, Oahu, HI. ANG communities were also stable when maintained in the lab over several months, making this symbiosis tractable for study in the laboratory. Furthermore, the OTUs that make up the ANG community were detected in the local environment, supporting the hypothesis of environmental symbiont transmission. Finally, the bacterial community of the egg JC reflected that of the associated ANG, and was stable throughout the duration of embryogenesis.

The ANG bacterial consortium was consistent and stable across individuals from this single population. For all taxa found in the ANG, the relative abundances of the bacterial community members did not vary substantially between individuals, indicating a stable community (Fig. 2). The core bacterial ANG community was dominated by two conserved members, the *Opitutae* (*Verrucomicrobia*) and *Rhodobacteraceae* (*Alphaproteobaceria*, Table 1), consistent with a previous study from *E. scolopes* (Collins *et al*., 2012). Prior research and culturing efforts demonstrated that most of the *Rhodobacteraceae* found in the ANG belong to *Leisingera* sp. (Collins *et al*., 2012, 2015), and while the 16S rRNA gene V4 region does not provide enough resolution to consistently resolve genera within the *Rhodobacteraceae* family, our results confirm that a majority of the ANG *Rhodobacteraceae* OTUs belong to *Leisingera* sp. (Fig. 2, Table 1). Prior research demonstrated that many of the *Rhodobacteraceae* isolates from the ANG are very similar to each other at the genome level (Collins *et al*., 2015). Members of this group, commonly known as roseobacters, can be free-living or symbiotic (Collins *et al*., 2015), and frequently produce pigments in culture, potentially accounting for the bright colouration of the tubules that make up the ANG (Fig. 1, Collins *et al*., 2012; Gromek *et al*., 2016). In the cuttlefish, *Sepia officinalis*, ANG pigmentation has been linked to the bacterial component of the organ (van den Branden *et al*., 1979; 1980; Richard *et al*., 1979). In this study, the *Rhodobacteraceae* accounted for 14.5% ± 5.12% of the average water column community and 3.2% ± 1.6% of the average sediment community in Maunalua Bay and thus are also a significant free-living component of the bobtail squid's natural habitat.

The second most dominant group of bacteria in the ANG was the *Opitutae* class of *Verrucomicrobia*. This group of bacteria is intriguing as it has only recently been shown to be involved in symbiotic associations (Vandekerckhovem *et al*., 2000; Petroni *et al*., 2000; Romero-Perez *et al*., 2011). Few examples of symbiotic *Verrucomicrobia* are well described, but a closely related symbiotic verrucomicrobium has been described in a ciliate (Petroni *et al*., 2000), and other *Verrucomicrobia* have been found in the human and bovine GI tracts (Romero-Perez *et al*., 2011; Lozupone *et al*., 2012). *Opitutae* were also found in both the sediment and seawater samples, but at very low

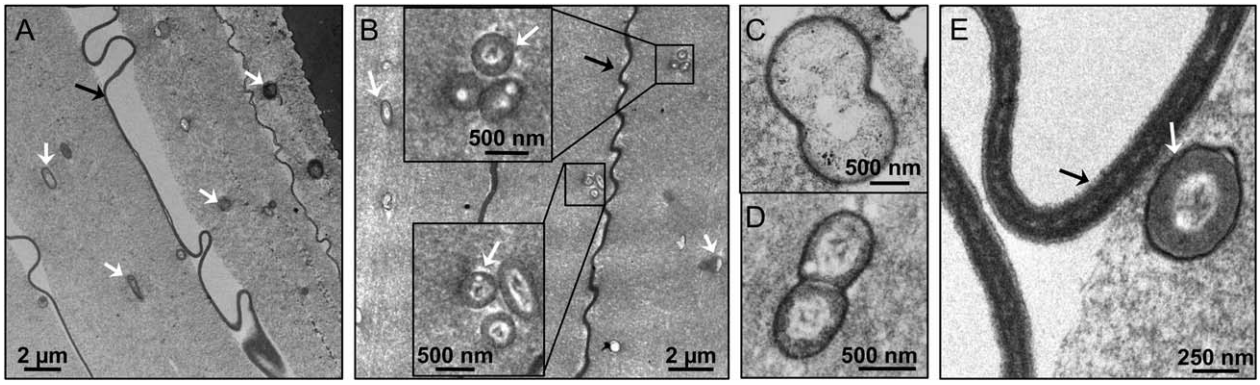


Fig. 6. Transmission electron micrographs of *E. scolopes* egg JCs collected at day 0 (A) or day 21 (B) of embryogenesis. At day 0 bacteria occurred as single cells throughout the various layers of JC (A), while at day 21 small microcolonies of bacteria of similar morphologies were common (B, see insets). Bacterial cells in the process of dividing were observed at both days 0 (C) and 21 (D). Layers of JC are separated by an electron-dense material composed of two layers (E). White arrows indicate bacterial cells and black arrows indicate membrane-like structures (A, B, E).

average relative abundances (0.3% ± 0.3% and 0.02% ± 0.01% respectively).

Both the *Rhodobacteraceae* and *Opitutae* are greatly enriched in the ANG community compared to both the sediment and water column. A similar enrichment can be seen for the *E. scolopes* light organ symbiont, where *V. fischeri* is abundant in the host but present only at low levels in the environment (Lee and Ruby, 1994, 1992). Sponge symbionts are also found at very low abundances in surrounding seawater and sediment environments, leading to the hypothesis that these rare microbes in the environment could serve as a 'seed bank' for colonization (Schmitt *et al*., 2012; Thomas *et al*., 2016). Future research will examine whether ANG bacteria are present at higher levels in local Hawaiian habitats with and without the host.

The 52 OTUs found in the core community represented 80% of the sequences present in the average ANG, providing further evidence of the stability and consistency of the ANG bacterial community. The animals included in this study were collected over the course of seven years, and no seasonal or yearly pattern was noted (Fig. 3). Because the lifespan of *E. scolopes* is predicted to be less than one year, the sampling in this study also represents multiple generations of bobtail squid from the same habitat. Such stability in a complex marine symbiosis appears uncommon: sponges and corals often have widely variable symbiont communities, and both microbiomes can contain thousands of OTUs (Ainsworth *et al*., 2015; Thomas *et al*., 2016). However, host-symbiont stability is a hallmark of other associations, including the binary symbioses of squid-vibrio associations or siboglinid tubeworms and sulfide-oxidizing bacteria (Dubilier *et al*., 2008; McFall-Ngai, 2014).

Bringing animals into captivity and maintaining them in the laboratory often results in changes in their microbiota (Ford *et al*., 1986; Scott *et al*., 2010; Devine *et al*., 2012). For example, the viable number of bacterial cells present on the mantle tissue of lab-maintained western Atlantic

Table 1. Core ANG bacterial community of *Euprymna scolopes* from Maunalua Bay. OTUs present at 97% identity level in 90% of 29 sampled bobtail squid are shown. OTUs in the core represent 79.5% of sequences present per average sample.

Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
Proteobacteria	*Alphaproteobacteria*	*Kordiimonadales*	*Kordiimonadaceae*		7	1.34%
		Rhizobiales	Unclassified *Rhizobiales*		1	0.05%
			Phyllobacteriaceae		1	3.01%
		Rhodobacterales	*Rhodobacteraceae*	Unclassified *Rhodobacteraceae*	17	15.56%
				Leisingera sp.	7	29.30%
				Loktanella sp.	1	0.08%
				Marivita sp.	1	0.34%
				Roseivivax sp.	1	0.14%
				Antarctobacter sp.	4	5.51%
Verrucomicrobia	*Opitutae*	Unclassified *Opitutae*			8	23.18%
		Opitutales	*Opitutaceae*	*Opitutus sp.*	4	0.95%
				Total	52	79.47%

brief squid, *Lolliguncula brevis*, increased ten-fold, a proliferation attributed to *Vibrio* sp. (Ford *et al.*, 1986). Studies examining corals, *Siderastrea siderea* and *Fungia granulosa*, the sea slug, *Elysia chlorotica*, and leaf-cutter ant gardens also found shifts in microbial communities when either aquatic or terrestrial animals were maintained in a laboratory (Kooperman *et al.*, 2007; Scott *et al.*, 2010; Devine *et al.*, 2012; Pratte *et al.*, 2015). To understand whether the ANG bacterial community is altered by laboratory conditions, ANG diversity was examined over the life of the bobtail squid. No shift was found in ANGs from laboratory-maintained animals, which were indistinguishable from those of wild individuals (Fig. 3). Such stability is not unprecedented, the gut microbial community of the Sonoran Desert turtle ant, *Cephalotes rohweri*, was unaffected by laboratory-maintenance (Lanan *et al.*, 2016), while the presence of the endosymbiont of the olive fruit fly, *Bactrocera oleae*, was also undisturbed by captivity under certain conditions (Estes *et al.*, 2012). The epithelia-associated microbiota of two species of *Hydra* maintains the species-specificity found in wild populations despite laboratory culture for over 30 years (Fraune and Bosch, 2007). *E. scolopes* has been used as a model system for studying host-microbe associations in part because animals are easily maintained and bred in the laboratory (Arnold *et al.*, 1972; Hanlon *et al.*, 1997; Nyholm and McFall-Ngai, 2004; Koch *et al.*, 2013). The stability of the ANG bacterial community suggests that this association may be studied intact over the adult life of the host under laboratory conditions, although whether the community changes over multiple reared generations has yet to be tested.

In certain cephalopods, the transfer of bacterial consortia from the ANG to eggs is hypothesized to aid in the protection of developing embryos, possibly from predation, pathogens, and/or biofouling (Biggs and Epel, 1991). Several members of the community have been shown to produce a number of secondary metabolites, some of which are able to inhibit certain marine vibrios (Collins *et al.*, 2015; Gromek *et al.*, 2016). A previous FISH analysis of eggs from *E. scolopes* demonstrated that JCs contain some of the same bacterial groups as those found in the ANG (Collins *et al.*, 2012). Despite differences in the relative abundances of the bacterial community between the JC and ANG of *E. scolopes*, we show that the JC community contains bacteria found in the ANG, and the bacteria in eggs produced by a given female group with that female's ANG (Fig. 4C, Supporting Information Fig. S1), providing evidence that bacteria from the ANG are deposited into the JC. The clustering of JCs by the associated female also accounts for the wider spread of the JC samples, which radiated outward from the ANG samples in the beta diversity analysis (Fig. 4C, Supporting Information Fig. S1). Since the ANG and JC of the squid *Doryteuthis pealeii* also have similar bacterial communities (Barbieri *et al.*, 2001), this deposition of ANG bacteria to eggs is likely to occur with other cephalopods.

If the bacteria in the JC play a defensive role for developing eggs, then the community should be conserved over the course of embryogenesis to maintain any protective effect. Once a female deposits her clutch, the eggs are potentially susceptible to fouling by microorganisms present in the seawater. The community composition of the JC remained stable despite the exposure of eggs to the environment for three weeks under laboratory conditions (Fig. 4D). The JC samples clustered by the female that produced the eggs and not by the stage of embryogenesis, although within those clusters the community seemed more similar at early- and mid-stages of embryogenesis. The differences in the late-stage communities were attributed to an occasional increase in *Flavobacteriia* (data not shown) but additional research is needed to confirm this observation.

While the overall community composition was not affected by embryonic stage, the bacteria in the JC did appear to be metabolically active. Microcolonies were detected during late embryogenesis along with bacterial cells in the process of cell division (Fig. 6). Cell division apparently occurred at a fairly slow rate given the small number of cells present in the microcolonies after 21 days. Culture-dependent estimates of bacterial abundance showed an average increase of an order of magnitude over the course of embryogenesis. These efforts to quantify the bacterial abundance in the JCs are an underestimation due both to an inability to culture the *Verrucomicrobia* contingent of the community, and difficulties in completely homogenizing the JC to ensure a uniform distribution prior to plating. Given these technical challenges, the overall measured abundance of the JC bacteria is a conservative estimate and the actual numbers are likely $>2 \times 10^4$ CFU/JC, and $>3 \times 10^5$ CFU/JC in early- and late-stage eggs respectively.

Transmission electron microscopy of eggs revealed the presence of a previously undescribed component of the JC, an electron-dense dual-layered structure that strongly resembles a membrane (Fig. 6E). This structure may be involved in maintaining the configuration of the egg capsule. Similar structures are visible in published images of other cephalopod eggs, including *D. pealeii*, *Rossia macrosoma*, *Loligo forbesi*, *D. opalescens*, and *Sepia officinalis*, although the structure is rarely commented upon (Biggs and Epel, 1991; Lum-Kong, 1992; Boletzky, 1998; Barbieri *et al.*, 2001; Cornet *et al.*, 2015). No segregation of the bacterial community based on morphological characteristics was observed in individual JC layers.

Despite the presence of ANG bacteria in the eggs, the ANG symbiosis is hypothesized to be environmentally transmitted. *E. scolopes* reaches sexual maturity in the laboratory in approximately 60 days (Hanlon *et al.*, 1997)

and the ANG develops between 1 and 1.5 months post-hatching (S. Nyholm, pers. obs.). While the ANG could be colonized by bacteria deposited in the juvenile at hatching and stored until ANG development begins, studies in other cephalopods suggest that ANG bacteria are likely environmentally transmitted (Kaufman *et al*., 1998; Barbieri *et al*., 2001) and that ANG development is correlated with this transmission (Kaufman *et al*., 1998). Sepiolid (bobtail) squids are inherently benthic, spending time buried in sand and hunting in the water column. Colonization of the ANG may thus occur from bacteria in the seawater, substrate or both. The symbiosis itself could be a source of enrichment for the various symbionts in the environment as well, possibly after the juveniles hatch as the egg casings degrade. While it appears that the ANG does not experience the same daily venting as is seen in the light organ (reviewed in Nyholm and McFall-Ngai, 2004), the deposition of bacteria into the JC could result in the release of bacteria into the environment during egg laying. Comparative analysis of the ANG and environmental communities revealed substantial overlap in shared OTUs, with 94.5% of the average ANG sequences also found in the environment. The remaining OTUs may be rare members of the environment, accounting for the lack of detection to date.

Analysis of the microbial communities found in laboratory aquaria substrate and artificial seawater revealed less of an overlap in shared OTUs with the ANG community compared with the natural environment, (74.2% of the average ANG sequences accounted for, in comparison to 94.5%). While this analysis does not preclude the adult female as a source of enrichment for the ANG bacteria in her environment, it also does not provide strong evidence for the female seeding the environment, especially as no *Verrucomicrobia* were detected in either the laboratory artificial seawater or substrate. However, our laboratory conditions may have prevented the establishment of some ANG bacteria if released by females. Detected environmental bacteria in the laboratory could have been introduced during squid collection and transit.

The high overlap in the OTUs present in both the seawater and surface sediment (Fig. 5B) may have resulted from mixing of the communities during sampling, although the distinct clustering of the seawater community from that of the sediment, which takes into account OTU abundance, provides evidence that the two sampling methods resulted in distinct sample types (Fig. 5A). The seawater community was consistent across the sampling area, but varied from the Hawaii Ocean Time-Series (HOT) that characterized the bacterial and archaeal composition of the seawater at various depths approximately 100 km offshore of Oahu, HI (Supporting Information Fig. S3, Karner *et al*., 2001; DeLong *et al*., 2006; Brown *et al*., 2009). Differences in the near-shore seawater community may be impacted by terrestrial runoff, anthropogenic activities, and the presence of certain algae (Smith *et al*., 1999; Goeke *et al*., 2010; Nogales *et al*., 2011).

The presence of an ANG bacterial community is conserved throughout a diverse group of cephalopods (Buchner, 1965) but has only been examined via next-generation sequencing in *E. scolopes* (Collins *et al*., 2012; this study). The conservation of a common bacterial community across many species, especially the *Alphaproteobacteria* (Grigioni *et al*., 2000; Barbieri *et al*., 2001; Pichon *et al*., 2005), may reflect a conserved function of the ANG bacteria. Ongoing research in our laboratory is examining the putative role of these bacteria in egg protection. Differences in community composition between cephalopod species may be due to functional redundancy of the bacterial groups found, similar to what has been described in the mammalian gut (Ley *et al*., 2006; Dethlefsen *et al*., 2008), or could be a response to differing challenges found in environments where eggs develop. The conservation of the bacterial community in individual females across this population and the stability of the community for the duration of embryogenesis both support the hypothesis of a critical functional role in host development.

E. scolopes has served as a model organism for symbiosis research (McFall-Ngai, 2014). The ease of maintaining the host in the laboratory and the stability of the ANG consortium make this bobtail squid species an ideal candidate for studying cephalopod-ANG associations. Future efforts will also focus on examining the function and putative environmental transmission of this symbiosis. Developing a model for the comprehensive understanding of the establishment and maintenance of ANG bacteria will aid in our understanding of that community's function. Given the wealth of information obtained from decades of research about interactions with the light organ symbiont, studying the ANG symbiosis in *E. scolopes* may also provide insight into conserved and new mechanisms by which animals and symbiotic bacterial partners interact, both in the host and environment.

Experimental procedures

Animal collections

Female *E. scolopes* were collected from Maunalua Bay (21°26'3.36"N, 157°47'20.78"W), a sheltered sandflat on the island of Oahu, Hawaii, between March 2009 and August 2015. Bobtail squid were either sacrificed in Oahu (wild, $n = 12$) or were shipped to the University of Connecticut and maintained in the laboratory (lab-maintained, $n = 17$) for as long as four months (Supporting Information Fig. S5). Lab-maintained, mated females were kept in individual tanks, and egg clutches were moved within twelve hours of deposit to baskets in a separate tank, allowing for the tracking of eggs produced by an individual female. In one case, eggs were from a female that laid a clutch in tanks with flowing Hawaiian seawater (Kewalo Marine Laboratory, University of Hawaii,

Oahu, HI). Dissected tissues were surface-sterilized by washing first in 99% ethanol followed by filter-sterilized squid Ringer's solution (FSSR, Collins *et al*., 2012).

DNA extraction: ANG. All sacrificed females were mature animals with a mantle length ≥20 mm, and had a fully developed reproductive system with eggs present in the mantle cavity. Prior to sacrifice, animals were anesthetized in 2% ethanol in filter-sterilized seawater (FSSW).

ANGs were homogenized in FSSR with a sterile plastic pestle. Differential centrifugation was used to separate bacterial cells from host tissue. The homogenized ANGs were centrifuged for five min at 100 X*g* to pellet the host tissue, then the supernatant containing the bacterial cells was removed and centrifuged for five min at 5000 X*g* to pellet the bacteria. DNA was extracted from samples using the DNeasy Blood and Tissue kit according to the manufacturer's protocol (Qiagen, Valencia, CA). The pelleted bacteria were combined with ATL buffer, Proteinase K, and zirconia beads (0.1 and 0.5 mm) and dissociated using a bead-beater for three min (Mini-Beadbeater-16, BioSpec Products, Bartlesville, OK). The solution was incubated for 30 min at 56°C, followed by bead-beating for an additional three min, and then incubated for 30 min at 56°C. Samples were centrifuged at room temperature for five min at 6000 X*g* to pellet the beads. DNA concentration was determined throughout using the Qubit® dsDNA High Sensitivity assay (ThermoFisher Scientific, Waltham, MA), and averaged 36.9 ng/μl ± 19.7 ng/μl (all samples >4 ng/μl, Supporting Information Fig. S6).

DNA extraction and bacterial quantification: egg JCs. Eggs were removed from a given clutch at various stages of embryogenesis after a clutch was deposited (early, day 0–2; mid, day 10–12; or late, day 17–24). Eggs were dissected using sterile forceps to remove the outer capsule and inner yolk sac, leaving only the JC.

DNA was extracted from 10 JCs/sample using the MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). JCs were first flash-frozen to −80°C for a minimum of 30 min. The provided Tissue and Cell Lysis buffer was prepared with Proteinase K to a final concentration of 0.833 μg/ml, and added to the frozen JCs with 0.1 and 0.5 mm zirconia beads. Samples were then subjected to bead-beating for five min followed by incubation at 65°C, with shaking overnight to allow the viscous JC material to break down, and subsequent bead-beating for five min. The manufacturer's protocol was then followed, repeating the protein precipitation step three times. DNA concentrations averaged 12.7 ng/μl ± 12.8 ng/μl (majority of samples >1 ng/μl, all samples >0.1 ng/μl, Supporting Information Fig. S6).

JC material from five eggs/sample was surface sterilized by washing first in 99% ethanol followed by FSSR. Samples were taken from early and late time points from the same clutches ($n = 5$). JCs were homogenized in FSSR using a sterile plastic pestle and then diluted prior to plating on triplicate seawater-tryptone plates (Lee and Ruby 1992). Colony counts were completed after three days of growth at 28°C.

Environmental DNA isolation. Sediment samples ($n = 18$) were collected via four transects at the site of animal collection, spanning approximately 600 m of the coast and extending 100–500 m from the shore towards the reef crest. Sterile tubes were used to collect the top three centimeters of sediment from four points on each transect. Samples were frozen at −80°C within one hour. Excess water was drained prior to processing to remove as much seawater from samples as possible. DNA extraction was completed on 250 mg of sediment via the DNeasy Blood and Tissue kit with bead-beating. DNA concentrations averaged 4.0 ng/μl ± 2.3 ng/μl (all samples >1 ng/μl, Supporting Information Fig. S6).

Seawater samples ($n = 8$) were collected from the points closest to shore and 250m from shore on each of the four transects described above. Samples were collected in sterile buckets and were transported back to the lab for immediate processing. Five liters of seawater from each collection point were filtered through 0.22 μm Whatman filters (GE Healthcare Life Sciences, Pittsburgh, PA) which were then frozen at −80°C. DNA was extracted using the PowerWater DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). DNA concentrations averaged 19.0 ng/μl ± 5.4 ng/μl (all samples >12 ng/μl, Supporting Information Fig. S6).

Substrate ($n = 3$) and artificial seawater ($n = 3$) samples were also collected from laboratory aquaria using the methods described above. Because aquaria water was circulated through particle and charcoal filters and subjected to UV sterilization, these samples resulted in low yields of total bacterial DNA. Laboratory substrate sample DNA concentrations averaged 0.8 ng/μl ± 0.1 ng/μl, while aquaria artificial seawater sample DNA concentrations averaged 0.3 ng/μl ± 0.2 ng/μl (Supporting Information Fig. S6). While use of low DNA concentration samples may increase the risk of contamination (Salter *et al*., 2014), these samples were included for a point of comparison.

DNA amplification, sequencing, and analysis

Extracted DNA was amplified using barcoded primers developed by Caporaso *et al*., (2012) for the V4 region of the 16S rRNA gene and sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) following established protocols (Nelson *et al*., 2014; Benjamino and Graf, 2016). Some sample processing was performed by the UConn Microbial Analysis, Resources and Services facility.

Sequencing data were analysed following established protocols (Nelson *et al*., 2014; Benjamino and Graf, 2016) using QIIME (Caporaso *et al*., 2010). OTUs were assigned at the 97% identity level using Greengenes (2013-08 release, DeSantis *et al*., 2006) and *de novo* methods. The dataset was rarified to 10,000 sequences per sample. A core community was determined as OTUs present in 90% of ANG samples. Alpha diversity was analysed in QIIME, and the log2 Shannon Index was converted to a natural log Shannon Index. NMDS plots of beta-diversity analyses using Bray-Curtis were created in *R* using the VEGAN package (Oksanen *et al*., 2016), and community composition similarity was tested via ANOSIM in QIIME. Sequences were deposited in the European Nucleotide Archive (ENA) under the project ID PRJEB14655.

Transmission electron microscopy (TEM)

A freshly deposited *E. scolopes* clutch was maintained in aerated FSSW, which was changed daily. At 0 and 21 days post-

deposit, eggs were obtained from the clutch and the outer capsule was removed. Decapsulated eggs were prepared for TEM following established protocols (Collins *et al*., 2012) with the following alterations. Eggs were fixed (2.5% gluteraldehyde/2% paraformaldehyde solution, Collins *et al*., 2012) at room temperature for 1 h, placed at 4°C for 10 min, and then transferred to fresh fixative and stored at 4°C for up to 22 days. Post-fixation protocols were carried out on all treatments at the end of the experiments, but no anomalies were noted in the day 0 eggs, which were stored in fixative the longest. The yolk sac (Fig. 1) was pierced prior to osmication to allow for complete infiltration during the remaining steps. After ethanol dehydration, eggs were transferred to a transition fluid, 100% propylene oxide, for two washes of 15 min. Tissues were embedded in Spurr's resin and sectioned on a Leica UCT Ultramicrotome (Leica Microsystems, Buffalo Grove, IL) into 90 nm ultrathin sections. Samples were imaged on a Tecnai Biotwin transmission electron microscope (7–12 sections/sample, FEI, Hillsboro, OR).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Bray Curtis NMDS analysis showed that JCs (circles) cluster with the ANG (diamond) from the female (colour) that produced those eggs. Ellipses indicate 95% confidence intervals. This figure is another version of that shown in Figure 4C, but includes only the groups of ANGs/JCs. Female 5 from that analysis is left out here as it included only a single JC with the ANG, and a 95% confidence interval requires at least three data points.

Fig. S2. The average sediment sample contained 13 classes from seven bacterial/archaeal phyla. Taxa present at greater than 1% average abundance in more than one sample are included, and presented at the class level. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. The 'other' category includes taxa present at less than 1% average abundance: *Acidobacteria-6*, *Gemm-2*, *Gemm-4*, *Ellin6529*, *Opitutae, Nitrospira, Phyciphaerae, PRR-12*, *OM190*, *SAR202*, *Bacteroidi*a, *C6*, *Chloracidobacteria*, *VHS-B5-50*, *Fusobacteria, Gemm-1*, unclassified *Planctomycetes*, *Actinobacteria*, *Chlamydiia*, *Bacilli*, *Anaerolineae*, *Verrucomicrobiae*, *Synechococcophycideae*, and *Clostridia*. *Probably of eukaryotic macro- or microalgal origin.

Fig. S3. The average seawater sample contained six classes from four bacterial/archaeal phyla. Taxa present at greater than 1% average abundance in more than one sample are included and presented at the class level. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. The 'other' category includes taxa present at less than 1% average abundance: *Thaumarchaeota*, *Acidimicrobiia*, *Planctomycetia*, *Deltaproteobacteria*, *Fusobacteria*, *Oscillatoriophycidae*, *Betaproteobacteria*, *Epsilonproteobacteria*, *Opitutae*, *Ellin6529*, *Acidobacteria-6*, *Sphingobacteriia*, *Gemm-2*, *OM190*, *Gemm-4*, and *Sva-0725*. *Probably of eukaryotic macro- or microalgal origin.

Fig. S4. Laboratory artificial seawater (n=3) and substrate (n=3) contained many of the same taxa as the natural Hawaiian environment, but at different relative abundances (A, B, D). Taxa present at greater than 1% are included (A). The lab environment contained fewer OTUs that overlapped with the ANG community than the natural environment (C). Lab substrate exhibited similar levels of diversity as natural Hawaiian sediment, but the lab artificial seawater was more diverse than that from Hawaii (E). *Probably of eukaryotic macro- or microalgal origin.

Fig. S5. Laboratory-maintained *E. scolopes* were kept in captivity for periods ranging from two weeks to four months.

Fig. S6. DNA extractions yielded a variety of concentrations, but the majority if not all of the replicates for most sample types were $>$1ng/μl (A). The exception to this cutoff were the JC samples, five of which were below this cutoff but which appeared similar in composition to others that were sequenced, and the lab substrate and lab seawater samples, sequenced as controls. All included samples yielded $>$ 10,000 sequences, with the exception of one lab seawater sample, one JC sample, and one Hawaiian sediment sample, all of which yielded 5,000-10,000 sequences (B).

Table S1. Conserved bacterial community of the Maunalua Bay, Oahu, HI sediment. OTUs present at 97% identity level present in 90% of 18 sediment samples are shown. Conserved OTUs represent 87% of sequences present per average sample. *Probably of eukaryotic macro- or microalgal origin.

Table S2. Conserved bacterial community of the Maunalua Bay, Oahu, HI seawater. OTUs present at 97% identity level present in 85% of 8 water samples are shown. Conserved OTUs represent 98.4% of sequences present per average sample. *Probably of eukaryotic macro- or microalgal origin.

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Leisingera sp. JC1, a Bacterial Isolate from Hawaiian Bobtail Squid Eggs, Produces Indigoidine and Differentially Inhibits Vibrios

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Female members of many cephalopod species house a bacterial consortium in the accessory nidamental gland (ANG), part of the reproductive system. These bacteria are deposited into eggs that are then laid in the environment where they must develop unprotected from predation, pathogens, and fouling. In this study, we characterized the genome and secondary metabolite production of *Leisingera* sp. JC1, a member of the roseobacter clade (*Rhodobacteraceae*) of *Alphaproteobacteria* isolated from the jelly coat of eggs from the Hawaiian bobtail squid, *Euprymna scolopes*. Whole genome sequencing and MLSA analysis revealed that *Leisingera* sp. JC1 falls within a group of roseobacters associated with squid ANGs. Genome and biochemical analyses revealed the potential for and production of a number of secondary metabolites, including siderophores and acyl-homoserine lactones involved with quorum sensing. The complete biosynthetic gene cluster for the pigment indigoidine was detected in the genome and mass spectrometry confirmed the production of this compound. Furthermore, we investigated the production of indigoidine under co-culture conditions with *Vibrio fischeri*, the light organ symbiont of *E. scolopes*, and with other vibrios. Finally, both *Leisingera* sp. JC1 and secondary metabolite extracts of this strain had differential antimicrobial activity against a number of marine vibrios, suggesting that *Leisingera* sp. JC1 may play a role in host defense against other marine bacteria either in the eggs and/or ANG. These data also suggest that indigoidine may be partially, but not wholly, responsible for the antimicrobial activity of this squid-associated bacterium.

Keywords: symbiosis, *Euprymna*, roseobacter, *Rhodobacteraceae*, indigoidine, *Leisingera*, DART-MS, secondary metabolite regulation

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INTRODUCTION

It is becoming increasingly evident that many animals and plants use compounds produced by symbiotic bacteria for protection against pathogens and other fouling organisms (reviewed in Flórez et al., 2015). In marine and aquatic environments a number of invertebrates (including sponges, tunicates, bryozoans, and molluscs) host microorganisms that produce compounds used for such protection. These groups have served as an important source for studying defensive symbioses and for the discovery of novel bioactive natural products (see example in Schmidt and Donia, 2010).

Among molluscs, one common yet poorly understood animal–bacterial association occurs between members of squid and cuttlefish species and bacterial consortia that reside within a reproductive gland of female hosts called the accessory nidamental gland (ANG; Kaufman et al., 1998; Grigioni et al., 2000; Barbieri et al., 2001; Pichon et al., 2005; Collins et al., 2012). This organ harbors a dense consortium of bacteria housed in epithelium-lined tubules that are attached to the nidamental gland, the organ that secretes the jelly coat (JC) surrounding fertilized eggs. Bacteria from the ANG are deposited into the JC where they have been hypothesized to help protect developing eggs from fouling microorganisms, pathogens, and/or predation (Barbieri et al., 1997, 2001; Collins et al., 2012, 2015).

The Hawaiian bobtail squid, *Euprymna scolopes,* has been used as a model organism to study bacteria–host interactions, mainly due to the host's relationship with the bioluminescent bacterium *Vibrio fischeri* (McFall-Ngai, 2014). Recent studies have also focused on a second association found within the ANG of this species (Collins and Nyholm, 2011; Collins et al., 2012, 2015). These studies demonstrated that the ANG consortium in *E. scolopes* is dominated by members of the *Rhodobacteraceae* (roseobacters) within the *Alphaproteobacteria*, a common group of marine bacteria. A number of roseobacter-clade organisms are known to produce unique antimicrobial molecules and other secondary metabolites. For example, the antibiotic tropodithietic acid (TDA) and the algicidal roseobacticides are produced by *Phaeobacter* species and the antibacterial compound indigoidine is produced by *Leisingera* (formerly *Phaeobacter*) sp. Y4I (Geng et al., 2008; Seyedsayamdost et al., 2011; Cude et al., 2012). Most of these studies have focused on either free-living or plankton-associated roseobacters and the potential antimicrobial activity of the ANG strains has not been explored. A study that analyzed the genomes of 13 ANG roseobacter strains from *E. scolopes* did reveal the potential for secondary metabolite production (Collins et al., 2015) and *Gammaproteobacteria* from the ANG of another squid species have been shown to inhibit other bacteria (Barbieri et al., 1997).

In this study, we characterized the genome and secondary metabolite production of a new bacterial strain, *Leisingera* sp. JC1, isolated from the JC of *E. scolopes* squid eggs. Whole genome sequencing and biochemical analyses revealed the potential for and production of a number of secondary metabolites, including siderophores and acyl-homoserine lactones involved with quorum sensing. The complete indigoidine biosynthetic gene cluster was detected in the genome and mass spectrometry confirmed the production of this compound. Furthermore, we investigated the regulation of indigoidine under co-culture conditions with *V. fischeri*, the light organ symbiont. Finally, both *Leisingera* sp. JC1 and extracts from this strain exhibited differential antimicrobial activity against a number of marine vibrios, suggesting that indigoidine may be partially, but not wholly, responsible for the antimicrobial activity of this squid-associated bacterium.

MATERIALS AND METHODS

Bacterial Isolation

Hawaiian bobtail squid, *E. scolopes*, were obtained from sand flats in Oahu (Maunalua Bay, 21°16′51.42″ N, 157°43′33.07″ W), Hawaii and maintained in aquaria as previously described (Schleicher and Nyholm, 2011). Eggs laid in captivity from one adult female were collected, flash frozen on the 11th day of development, and stored at −80°C. Ten eggs were thawed for bacterial isolation and their outer capsules and embryos were removed and discarded with sterile forceps. The JCs were isolated, surface sterilized with 70% ethanol, and rinsed with filter-sterilized squid Ringers (FSSR, 530 mM NaCl, 25 mM $MgCl_2$, 10 mM $CaCl_2$, 20 mM HEPES, pH = 7.5). The 10 JCs were pooled and homogenized in FSSR, then serially diluted and plated on seawater tryptone (SWT) medium (5 g/L tryptone, 3 g/L yeast extract, 3 mL/L glycerol, 700 mL/L Instant Ocean sea salts, 15 g/L agar, 300 mL/L DI water). *Leisingera* sp. JC1 colonies appeared dark blue on this medium and were streaked to isolation.

Genomic Sequencing and Analysis

Genomic DNA was extracted using the MasterPure DNA Purification kit (Epicentre, Madison, WI, USA) from an overnight liquid culture of *Leisingera* sp. JC1 grown shaking at 30°C in SWT. DNA was quantified using a Qubit 2.0 fluorometer (Life Technologies, Agawam, MA, USA) and checked for quality on a 1% agarose gel and using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Agawam, MA, USA). A paired end library was prepared from 1 ng of genomic DNA using the Nextera XT DNA library kit (Illumina, Inc., San Diego, CA, USA) and quantified using the Qubit fluorometer and bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The library was sequenced on an Illumina MiSeq sequencer using 2 bp × 250 bp reads at the Microbial Analysis Resources and Services (MARS) facility at the University of Connecticut (Storrs, CT, USA).

Reads were trimmed using the CLC Genomic Workbench (Qiagen, Hilden, Germany) and a draft genome was assembled using the A5 assembler (Tritt et al., 2012). Coverage was determined by mapping trimmed reads to the draft genome assembly using CLC Genomic Workbench. The genome was annotated using the Rapid Annotation using Subsystem Technology (RAST, Aziz et al., 2008)[1] server and analyzed with the Antibiotic and Secondary Metabolite Analysis Shell 3.0 (antiSMASH, Weber et al., 2015)[2] for potential secondary metabolite biosynthesis gene clusters. The draft genome assembly has been deposited in DDBJ/EMBL/GenBank under accession LYUZ00000000. The version described in this paper is version LYUZ01000000.

[1] http://rast.nmpdr.org

[2] http://antismash.secondarymetabolites.org

Taxonomic Analysis and Whole Genome Comparison

Initial 16S identity suggested JC1 belonged to the genus *Leisingera* (data not shown). To validate this conclusion and to evaluate its relationship to the previously sequenced ANG isolates, a further taxonomic analysis was undertaken that used 17 previously described *Leisingera* genomes (Collins et al., 2015). A 33 gene multilocus sequence analysis was carried out following the methodology described in Collins et al. (2015). After generating alignments for each of the 33 genes using MUSCLE (Edgar, 2004), a concatenated alignment was generated using in-house python scripts. An optimal model of evolution was determined using the Akaike information criterion with correction for small sample size as implemented in jModelTest v2.1.4 (Darriba et al., 2012). The best-fitting model reported was GTR + Gamma estimation + Invariable site estimation. A maximum-likelihood (ML) phylogeny was generated from the concatenated multi-sequence alignment using PhyML v3.0_360-500M (Guindon et al., 2010). PhyML parameters consisted of GTR model, estimated p-invar, four substitution rate categories, estimated gamma distribution, sub-tree pruning and regrafting enabled with 100 bootstrap replicates. In addition to the maximum-likelihood analysis, a Bayesian inference analysis was also conducted using MrBayes v3.2.4 x64 (Ronquist et al., 2012). A mixed model with gamma estimation and invariable sites was used. The mixed model settled on a GTR submodel with only one parameter difference from the default GTR model with a posterior probability > 0.8. The standard GTR model accounted for the remainder of the model probability. The analysis used two cold chains with three heated chains each and ran for one million generations. After the run finished, convergence was assessed using average standard deviation of split frequencies of the cold chains, potential scale reduction factors of parameters, and minimum effective sample sizes of parameters. All criteria indicated the runs had converged.

Average nucleotide identity (ANI) was calculated using JSpecies 1.2.1 (Richter and Rosselló-Móra, 2009). The calculations were made using the MUMmer aligner with its default options. Contig files were generated for this analysis using the seqret function of the EMBOSS package (Rice et al., 2000). The reciprocal comparisons were averaged for reporting. Estimates of *in silico* DDH were made using the Genome-to-genome distance calculator 2.1 (Meier-Kolthoff et al., 2013) using the BLAST+ alignment method and the formula 2 algorithm outputs.

Select genomes were compared using the BLAST Ring Generator (BRIG) v1.0 (Alikhan et al., 2011). Default BLAST options were used. A whole genome alignment was generated using the Mauve program v2.3.1 (Darling et al., 2010). The progressiveMauve algorithm was used with default options.

Homoserine Lactone Detection

Homoserine lactone (HSL) production was detected using a well-diffusion assay with the HSL-sensing bacterium *Agrobacterium tumefaciens* NTL4 pZLR4 (Cha et al., 1998) as previously described (Ravn et al., 2001; Collins et al., 2015). In brief, *A. tumefaciens* NTL4 was grown in 3 mL of LB with 30 μg/mL gentamicin for 24 h at 30°C. This culture was used to inoculate 50 mL of AB minimal media with 0.5% casamino acids and 0.5% glucose (Chilton et al., 1974), and allowed to grow for another 24 h at 30°C. This culture was used to inoculate 100 mL of AB minimal media to which 1.2% agar had been added and a final concentration of 0.5% casamino acids, 0.5% glucose, and 75 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added after autoclaving. The inoculated molten agar was allowed to solidify in Petri dishes and wells were cut into the media using a sterile borer.

Leisingera sp. JC1 and *Leisingera* sp. ANG1 were grown overnight at 30°C in 3 mL of SWT broth with 30 μM $FeCl_3$, 0.5% glucose, and 0.5% casamino acids to induce HSL production. Cells were pelleted and the supernatant was collected and filtered through a 0.22 μm filter (Thermo Scientific, Agawam, MA, USA). Cell-free supernatant (60 μl) was added to the wells in the *A. tumefaciens* plates. The *N*-3-oxohexanoyl homoserine lactone standard was serially diluted and added to wells of an *A. tumefaciens* plate as a control and for semi-quantitative comparison. All plates were incubated at 28°C for 24 h before imaging.

Siderophore Detection

To qualitatively detect siderophore production, *Leisingera* sp. JC1 was plated in triplicate on chrome azurol S (CAS) indicator agar, modified for marine bacteria as previously described (Whistler and Ruby, 2003), and incubated at 28°C for 24 h before imaging. Sequestration of iron from CAS causes a color change from blue to orange, indicating siderophore production.

Detection of Indigoidine Biosynthesis Genes in JC1 Genomic DNA

To confirm the presence of indigoidine biosynthesis genes in JC1, genomic DNA was extracted and quantified as described for genomic sequencing above. Primers were designed (Supplemental Table S1) to amplify the *igiCDR* genes based on the draft genome assembly and using Primer3 software (Untergasser et al., 2012). PCR amplification was performed using the standard GoTaq Green Master Mix (Promega, Madison, WI, USA) protocol with 30 cycles and 55°C annealing temperature.

Leisingera sp. JC1 Large Scale Culture

Leisingera sp. JC1 was cultured for extraction using SWT media (as described above except without addition of glycerol, delineated hereafter as SWT_{ng}). A three step culturing process was employed to produce sufficient scale for secondary metabolite extraction, while ensuring that the bacterium was in late stationary phase for optimal production of secondary metabolites (Ruiz et al., 2010). First, small scale cultures were prepared by inoculating a JC1 colony into 5 mL of media in a 24 deep well plate, which was incubated for 3 days at room temperature while shaking at 200 rpm. Then, medium scale cultures were prepared by transferring 1.5 mL of the small scale cultures into 125 mL baffled flasks with 50 mL media, which were incubated for 3 days at room temperature while shaking at 125 rpm. Lastly, large scale cultures were prepared by transferring

15 mL of medium scale cultures into 1 L baffled flasks with 500 mL of media, which were incubated for 3 days at room temperature while shaking at 125 rpm.

Extraction of *Leisingera* sp. JC1

All extraction solvents were ACS grade and purchased from Sigma Aldrich (St. Louis, MO, USA).

Normal Extraction

Diaion HP20 resin (Supelco, Bellefonte, PA, USA) was pre-washed by sequentially rinsing resin with methanol and Millipore water (EMD Millipore, Billerica, MA, USA). Large scale JC1 cultures were sonicated to lyse cells prior to addition of pre-washed Diaion HP20 resin (50 g, 10% w/v), followed by incubation for 24 h at room temperature while shaking at 125 rpm. Bacterial culture and resin were then filtered using a coarse glass frit filter and washed with Millipore water to remove aqueous media components. The resin and bacterial culture were then sequentially extracted with methanol, dichloromethane, and acetone (2 × 150 mL). Organic portions were combined, extracted with ethyl acetate to remove residual aqueous material, and concentrated.

Indigoidine Enriched Extraction

Because indigoidine is poorly soluble in water and most organic solvents, a second extraction protocol was utilized to prepare an indigoidine enriched extract following modified literature procedures (Yu et al., 2013). Briefly, large scale cultures were sonicated to lyse cells and transferred to centrifuge tubes. Cells were then separated from supernatant by low-speed centrifugation (850 *g* × 5 min; Beckman Coulter Avanti J-E Centrifuge, Brea, CA, USA). Supernatant was transferred to new tubes and subjected to high-speed centrifugation (21,000 *g* × 10 min) to obtain an indigoidine enriched pellet. The pellet was washed with methanol, transferred to a microcentrifuge tube, dried under N_2 gas, and dissolved in dimethyl sulfoxide (DMSO).

Detection of Indigoidine Production by *Leisingera* sp. JC1 via LC–MS

All HPLC grade solvents and reagents were purchased from Sigma-Aldrich. LC–MS data were collected on an Agilent ESI single quadrupole mass spectrometer coupled to an Agilent 1260 HPLC system with a G1311 quaternary pump, G1322 degasser, and a G1315 diode array detector (Agilent Technologies, Santa Clara, CA). A gradient elution was used from 10% methanol in H_2O to 90% methanol in H_2O over 25 min using an Agilent Eclipse XDB-C_{18} RP-HPLC column (4.6 mm × 150 mm, 5 μm) and a flow rate of 1 mL/min. Indigoidine enriched extracts were prepared at 5 mg/mL in DMSO. Indigoidine eluted at retention time (t_R) 10.7 min in agreement with literature (Yu et al., 2013).

Zone of Inhibition Assays

To observe inhibition of vibrio strains and ANG isolate strains by *Leisingera* sp. JC1 (Supplementary Table S3), a zone of inhibition (ZOI) assay was used. The vibrio strains *V. anguillarum* 775, *V. parahaemolyticus* KNH1, *V. fischeri* ES114, *V. harveyi* B392, and *Photobacterium leiognathi* KNH6 were grown for 2.5 h (to stationary phase) at 30°C in YTSS (4 g/L tryptone, 2.5 g/L yeast extract, 15 g/L Instant Ocean sea salts) broth and then serially diluted from 10^7 to 10^4 CFU/mL in YTSS broth to observe density dependent inhibition. Each dilution was plated in triplicate on YTSS agar using a sterile swab to form a lawn. All ANG isolates tested were grown overnight (∼4 × 10^8 CFU/mL) in SWT broth at 30°C and plated on SWT agar using a sterile swab to form a lawn. *Leisingera* sp. JC1 was grown overnight to a density of ∼1 × 10^8 CFU/mL in SWT when testing with ANG isolates and in YTSS when testing with vibrio strains. This overnight broth of *Leisingera* sp. JC1 was spotted (10 μL) on the surface of each lawn in quadruplicate. All plates were incubated at 28°C for 24 h before imaging and ZOI measurements around the *Leisingera* sp. JC1 colonies. SWT or YTSS broth (10 μL) were spotted on each lawn as media controls, and 10 μL of the overnight culture of *Leisingera* sp. JC1 was spotted in quadruplicate on SWT or YTSS agar without any bacterial lawns as a growth control.

To quantify inhibition, an average of three ZOI diameters were measured and an average of three diameters of the JC1 colonies were measured using ImageJ (Schneider et al., 2012). Due to slight variations in JC1 colony size across trials, the measurements were normalized by subtracting the average JC1 colony diameter from the average ZOI diameter. To determine if differences in ZOIs across lawn densities per organism were statistically significant, one-way ANOVAs were performed. If the results of the one-way ANOVA indicated statistically significant differences, multiple comparisons *post hoc* Tukey tests were performed to determine which lawn densities were significantly different.

96-Well Liquid Assays

Leisingera sp. JC1 extracts were tested for antibacterial activity against *V. fischeri* ES114, *V. anguillarum* 775, and *V. parahaemolyticus* KNH1. High throughput assays with these bacterial strains were developed based on similar assays with natural product extracts and human pathogens (Zgoda and Porter, 2001), including obtaining CFU counts and growth curves for each of the vibrio strains as well as determining proper incubation times and temperatures and finding appropriate controls. These assays were performed in 96-well plates (Corning Costar, Corning, NY, USA) with SWT media and incubated at 28°C while shaking at 200 rpm. The bacterial inocula were prepared by adding select colonies into 5 mL of media and adjusted to OD_{600} 0.1 (approximately 1–2 × 10^8 CFU/mL as per Clinical and Laboratory Standards Institute, 2012). Colony forming unit (CFU) counts were manually confirmed to ensure accurate approximation for each vibrio strain.

Extracts were screened as previously described (Zgoda and Porter, 2001) with the following modifications. Briefly, master mix was prepared by addition of 1.6 mL adjusted vibrio inoculum, 7.84 mL sterile water, and 6.4 mL of SWT media. To each well, 198 μL of master mix was added with 2 μL of either positive control (chloramphenicol, final testing concentration 2.5 μg/mL), negative control (DMSO), or extract prepared in DMSO (screened at final concentration of 500 μg/mL; MIC performed using serial dilutions). Sterility control wells consisted

of 98 μL sterile water, 100 μL of SWT media, and 2 μL of DMSO. All controls and samples were tested in technical triplicates with experiments repeated a minimum of three times to confirm results. Plates were read at 600 nm every 2 h from 0 to 10 h with a final reading at 24 h using a Synergy H1 Hybrid Reader (Biotek, Winooski, VT, USA). Results are given as percent control activity (PCA) calculated in comparison with DMSO, the negative control.

Localization of Indigoidine Production by *Leisingera* sp. JC1 Using DART-MS

Direct analysis in real time-mass spectrometry (DART-MS) analysis was performed using a JEOL AccuTOF with DART ion source (IonSense, Inc., Saugus, MA, USA). High purity helium 5.0–6.0 grade (greater than 99.999% purity) was heated to 300°C and used for ionization. Five locations were selected on JC1 colonies in the presence or absence of *V. fischeri*, including (A) center of colony, (B) midpoint between center and edge of colony, (C) edge of colony, (D) ZOI (in the absence of *V. fischeri* sample was obtained from a point equidistant from colony edge), and (E) outside ZOI. At each location a sterile single use syringe needle (BD Medical, Franklin Lakes, NJ, USA) was placed in the sample and then placed between the DART ion source and the MS inlet. Positive ion MS data were obtained over a *m/z* range of 60–700 and relative percent abundance was obtained for the indigoidine ion. Standards were run after sampling each colony and mass spectral data were monitored in real time to ensure no residual indigoidine remained after each sample. DART-MS is only semi-quantitative due to the potential for differential ionization, suppression of ions, and/or changes in sample concentration in the DART ion source (Sanchez et al., 2011). Therefore, relative indigoidine ion abundance was used to generate heatmaps representing a gradient from less abundance (black) to more abundance (red).

Measurement of Indigoidine Production by *Leisingera* sp. JC1 in Co-culture

JC1 bacterial inoculum was prepared by adding JC1 colonies into 5 mL of SWT_{ng} media in a 24 deep well plate, incubated for 24 h at room temperature while shaking at 200 rpm. Bacterial inocula for the vibrios were prepared by adding bacterial colonies of each species separately into 5 mL of SWT_{ng} media in 24 deep well plates, incubated for 2 h at 28°C while shaking at 200 rpm. All bacterial inocula (JC1, *V. fischeri*, *V. anguillarum*, *V. parahaemolyticus*) were adjusted to OD_{600} 0.1 prior to use.

Co-cultures of JC1 with individual vibrios were prepared by adding 1 mL of adjusted JC1 inoculum to 10 mL SWT_{ng} media in 125 mL baffled flasks, incubated for 24 h at room temperature while shaking at 125 rpm, followed by addition of 200 μL of *V. fischeri, V. anguillarum*, or *V. parahaemolyticus*. After addition of the vibrio strain, co-cultures were incubated for an additional 24 h at room temperature while shaking at 125 rpm. Monocultures of JC1, *V. fischeri*, *V. anguillarum*, and *V. parahaemolyticus* were prepared by adding 1 mL of adjusted inoculum to 10 mL SWT_{ng} media in 125 mL baffled flasks, incubated for 48 h while shaking at 125 rpm.

All co-cultures and monocultures were extracted using the indigoidine enriched protocol described above. LC–MS data was obtained on the Agilent LC–MS system described above, using an isocratic method to ensure minimal baseline variation (10% acetonitrile in H_2O with 0.1% formic acid over 15 min at a flow rate of 1 mL/min with 20 μL injection volume). Extracts were prepared at 5 mg/mL in DMSO. Indigoidine was detected and quantitated via measurement of area under the curve at UV absorbance 299 nm and confirmed by MS.

RESULTS AND DISCUSSION

Genome Characteristics and General Metabolism

Taxonomic Placement of JC1

Leisingera sp. JC1 has a draft genome size of 5.19 Mb and GC content of 62.3% (**Table 1**), which is average for members of the roseobacter clade and similar to other squid-associated isolates (Collins et al., 2015). This larger genome size reflects the generalist lifestyle and ability to use diverse energy sources common of roseobacters (Newton et al., 2010). The *repABC* genes for plasmid replication are present as well as *tra* genes necessary for conjugative plasmid transfer, indicating the potential presence of extrachromosomal DNA. Further sequencing is necessary to confirm the number, size, and content of these putative plasmids.

Phylogenetic reconstruction methods (Bayesian and maximum-likelihood) used with the 33-gene concatenation returned identical topologies with overall strong statistical supports (**Figure 1**), placing JC1 close to the *Leisingera* taxa previously isolated from the ANG. Average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization estimates (*is*DDH) support this placement. JC1 had higher ANI (90.5–91.7%) (Supplementary Table S2) and *is*DDH (38.8–44.6%) values with the ANG isolates than with any other *Leisingera* sp. These results also show that JC1 does not group with either *Leisingera* sp. ANG-M7 or the ANG1 group, but is still related to both (**Figure 1**; Supplementary Table S3), which is not unusual since other ANG isolates also fall outside the main ANG1 clade (Collins et al., 2015).

There are indications that JC1 may be more similar to *Leisingera* sp. ANG-M7 than to the *Leisingera* sp. ANG1 group. Both the ANI and *is*DDH values between JC1 and ANG-M7 are elevated in comparison to their values with the ANG1 group. There are no support statistics for ANI so it is uncertain if the 1.2% (JC1-M7 ANI versus JC1 compared to the ANG group) and 1.6% (M7-JC1 ANI versus M7 compared to the ANG group) higher values are significantly different. However, *is*DDH values are supported by 95% confidence intervals. The lower interval for JC1-M7 does not overlap with the upper interval for any comparison with a member of the ANG group, suggesting the *is*DDH values are significantly different. Additionally, the Bayesian inference found a small fraction of topologies in which the placements of JC1 and ANG-M7 were reversed, while the maximum-likelihood analysis found this occurrence in 33 of

TABLE 1 | Genome statistics of *Leisingera* sp. JC1.

Genome size (Mb)	Number of contigs	N_{50} (bp)	G + C content (%)	Number of genes	Missing genes* (% of total)	Fold coverage
5.19	168	123,213	62.3	5,074	54 (1.1)	37

**As predicted by the RAST server (Aziz et al., 2008).*

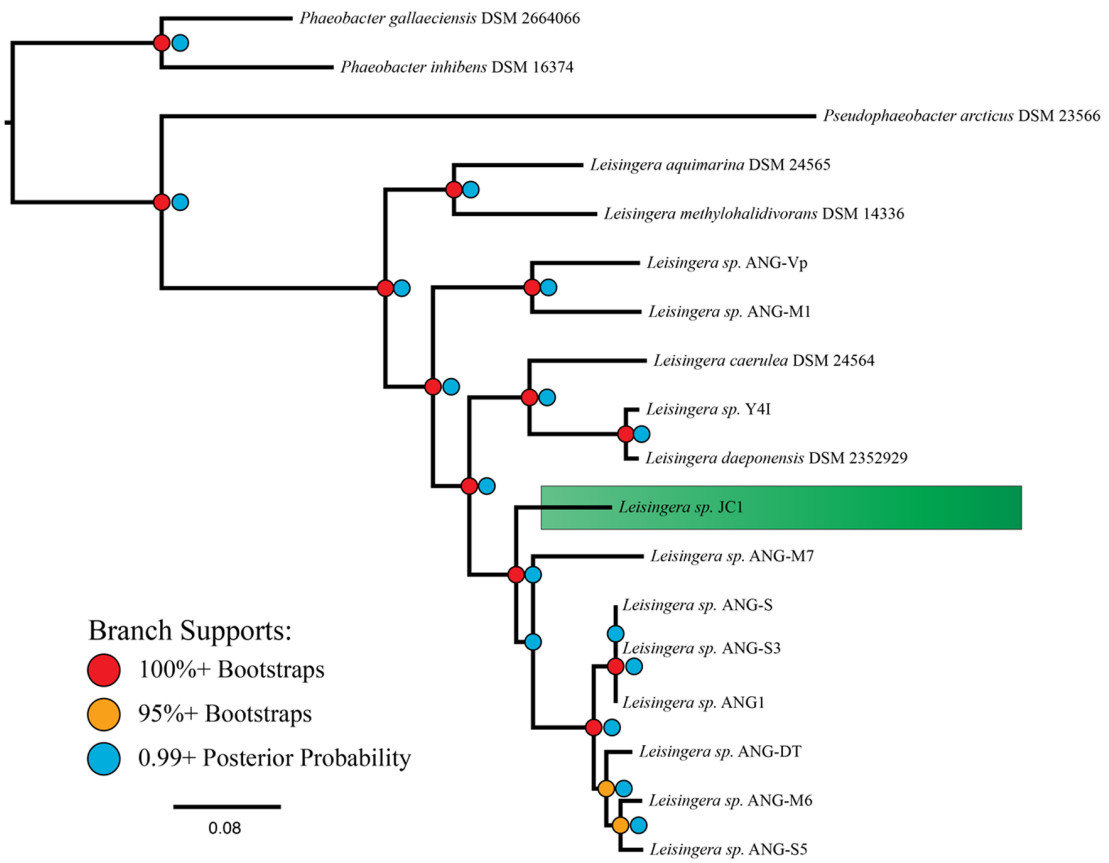

FIGURE 1 | MLSA analysis places *Leisingera* sp. JC1 in a sister group to other previously isolated ANG bacteria. Based on a comparison of 33 single-copy housekeeping genes, the egg jelly coat (JC) isolate JC1 is placed in the previously described roseobacter clade, "Clade 1" (Newton et al., 2010), along with seven previously isolated ANG bacteria (Collins et al., 2015). The phylogenetic tree reflects both Bayesian inference and maximum-likelihood methods with both posterior probability and bootstrap supports displayed.

100 bootstrap replicates. Overall, these analyses suggest that *Leisingera* sp. JC1 is distinct from, but related to the current ANG isolates.

Isolates having similar pigmentation to *Leisingera* sp. JC1 were cultured from other egg clutches, an ANG, and ovary from different females (data not shown). Among these, colonies with a similar dark blue morphology were isolated from the JCs of 1 and 23 day old eggs laid by different females. Similar colonies were isolated from the ANG of one of these females and the ovary of another female. Preliminary 16S sequencing placed two of these isolates in the genus *Leisingera* (data not shown), and further sequencing will reveal if these are the same strain as JC1. In addition, the production of the pigment indigoidine was confirmed by these additional strains (see below). These data suggest that *Leisingera* sp. JC1 and/or other indigoidine-producing strains may be selected for in the ANG/JC symbiosis.

Primary Metabolism

Leisingera sp. JC1 has a complete Entner-Duodoroff pathway and tricarboxylic acid cycle for metabolism of glucose. JC1 lacks any orthologs of phosphofructokinase, a major enzyme of glycolysis, but does contain a glucokinase and two distinct glucose-6-phosphate-1-dehydrogenases (GAPDHs). A glucose-6-phosphate-1-dehydrogenase (GPDH) is present, which catalyzes the first step of the alternative pathways for glucose metabolism, indicating that the Entner-Duodoroff pathway is probably used instead of glycolysis. *Leisingera* sp. JC1 only has the first two enzymes of the oxidative pentose phosphate pathway, but any 6-phosphate-gluconate produced can be further dehydrated by the Entner-Duodoroff pathway. Glycolate is a dissolved organic carbon often excreted by phytoplankton, and can be a carbon source for marine heterotrophic bacteria (Edenborn and Litchfield, 1985). *Leisingera* sp. JC1 is predicted

to oxidize glycolate to glyoxylate by a glycolate oxidase. JC1 has one system for glycerol uptake, the Ugp system, which can transport glycerol-3-phosphate against the concentration gradient. Sulfur oxidation genes are present, as well as a complete denitrification pathway with a copper-containing nitrite reductase. An assimilatory nitrate reductase is also present, which can convert nitrate to nitrite. An ammonia assimilation pathway is present with a ferrodoxin-dependent GOGAT, but no adenylyltransferase gene (GlnE) is present.

Transport

The high-affinity inorganic phosphate transport genes *pstABCS* and their regulatory genes *phoBUR* are present in JC1. The siderophore biosynthesis genes *asbAB* and *siderX456,* which encode high-affinity iron chelators, and the ferric iron ABC transporter, *pitADC,* are also present. JC1 has ABC transporters for dipeptides, oligopeptides, branched-chain amino acids, alkylphosphonate, and tungstate. The tripartite ATP-independent periplasmic (TRAP) transporter genes *dctMPQ* are present for unknown substrates, as well as the twin-arginine translocation (TAT) system genes, *tatABC*.

Leisingera sp. JC1 contains all 13 genes that encode the structural proteins essential for the Type VI Secretion System (T6SS) to function (Cianfanelli et al., 2016). The T6SS is a one-step mechanism for delivery of effectors across the Gram-negative outer membrane and membrane of the target cell, be it bacterial or eukaryotic. Widespread amongst the *Proteobacteria*, some T6SSs have been implicated in eukaryotic virulence (Pukatzki et al., 2006; Sana et al., 2012), but the majority are believed to play a role in bacterial competition (Hood et al., 2010; Schwarz et al., 2010). While it is possible for one T6SS system to affect both bacterial and eukaryotic targets (Jiang et al., 2014) it is believed that the system evolved for interactions with other bacteria, even in the case of intraspecific competition (Unterweger et al., 2014). Little work has been done, however, to investigate the role of T6SSs in beneficial host-symbiont relationships. Eleven of the 12 previously described ANG isolates also possess a T6SS (Collins et al., 2015), and it is possible that this system plays a role in interactions with other ANG or JC bacteria and/or the squid host. In the ANG, bacteria are partitioned into densely packed, epithelium-lined tubules, where each tubule is dominated by a particular taxon (Collins et al., 2012). These ANG/JC isolates may utilize the T6SS to outcompete other bacteria to establish colonization of a single tubule. While *Leisingera* sp. JC1 groups closely with other ANG isolates that also possess a T6SS (**Figure 1**, Collins et al., 2015), intraspecific effectors may facilitate competition between these strains, since ANG tubules are often highly pigmented with a single color (e.g., all dark blue matching the pigmentation of JC1 or all red-orange matching the pigmentation of several ANG isolates). Future studies will investigate the nature of JC1's T6SS effector proteins in the ANG symbiosis. There are numerous classes of evolved effector VgrG proteins, each with their own enzymatic function (reviewed in Durand et al., 2014). Understanding the number and type of effectors that JC1 can produce and deliver may help elucidate any role in the symbiosis.

Secondary Metabolite Biosynthesis

Analysis with the antibiotic and Secondary Metabolite Analysis Shell (antiSMASH, Weber et al., 2015) predicted several potential secondary metabolite biosynthesis gene clusters (Supplementary Table S4). These results included three separate siderophore clusters, one bacteriocin, one HSL, one type 1 polyketide synthase (T1 PKS), one other PKS (not type 1,2,3, or *trans*-AT), and two clusters classified as "other." Of these two "other" clusters, one contains the biosynthesis cluster for the known antimicrobial metabolite, indigoidine (Cude et al., 2012), while the other contains a previously described putative hybrid polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) gene cluster known to be conserved amongst roseobacters (Martens et al., 2007). This PKS/NRPS gene cluster encodes a polyketide synthase, glycosyl transferase, non-ribosomal peptide synthetase, and phosphopantetheinyl transferase, but the product of this cluster has not yet been identified. The top homologous gene cluster of the T1 PKS is 45% similar to a cluster in the ANG isolate, *Leisingera* sp. ANG-M7. While some roseobacters are capable of producing the novel secondary metabolite TDA (Bruhn et al., 2006, 2007; Geng et al., 2008), genes for synthesis of this molecule were not found nor was the molecule detected via LC–MS (data not shown).

Quorum Sensing

AntiSMASH predicted one *luxIR* homolog in *Leisingera* sp. JC1, flanked by an acyltransferase, crotonyl-CoA reductase, helicase, and oxidoreductase, similar to the previously published gene arrangement in bacterial isolates from the ANG (Collins et al., 2015). Production of HSLs by JC1 was confirmed in the *A. tumefaciens* NTL4 reporter assay, in which cell-free supernatant of a JC1 culture did induce β-galactosidase activity, indicating the presence of HSLs (**Figures 2A,B**). When compared to a dilution series of the *N*-3-oxohexanoyl HSL, JC1 produced a halo similar to that seen by 25 nM of HSL standard. The HSL production of JC1 was also slightly less than that of a closely related ANG isolate, *Leisingera* sp. ANG1.

Understanding the gene regulation by quorum sensing will be an important avenue of research for *Leisingera* sp. JC1 and the other *E. scolopes* ANG isolates due to the different habitats these bacteria experience. It is hypothesized that cephalopod ANGs are colonized via horizontal transmission from the environment (Kaufman et al., 1998), and potential symbionts must switch from living at very low cell densities in the seawater to very high cell densities in the ANG tubules (Collins et al., 2012). When ANG bacteria are deposited into the JC layers of eggs, these bacteria again experience a switch from the very high densities of the ANG to a lower density in the eggs. Due to this change in environments and cell densities, quorum sensing may play a role in gene regulation for ANG/egg JC bacteria.

Quorum sensing is also important in host-microbe interactions involving other roseobacters. For example, quorum sensing regulates motility and biofilm formation during host colonization in the sponge symbiont *Ruegeria* sp. KLH11 (Zan et al., 2012) and is necessary for colonization of the alga, *Ulva*

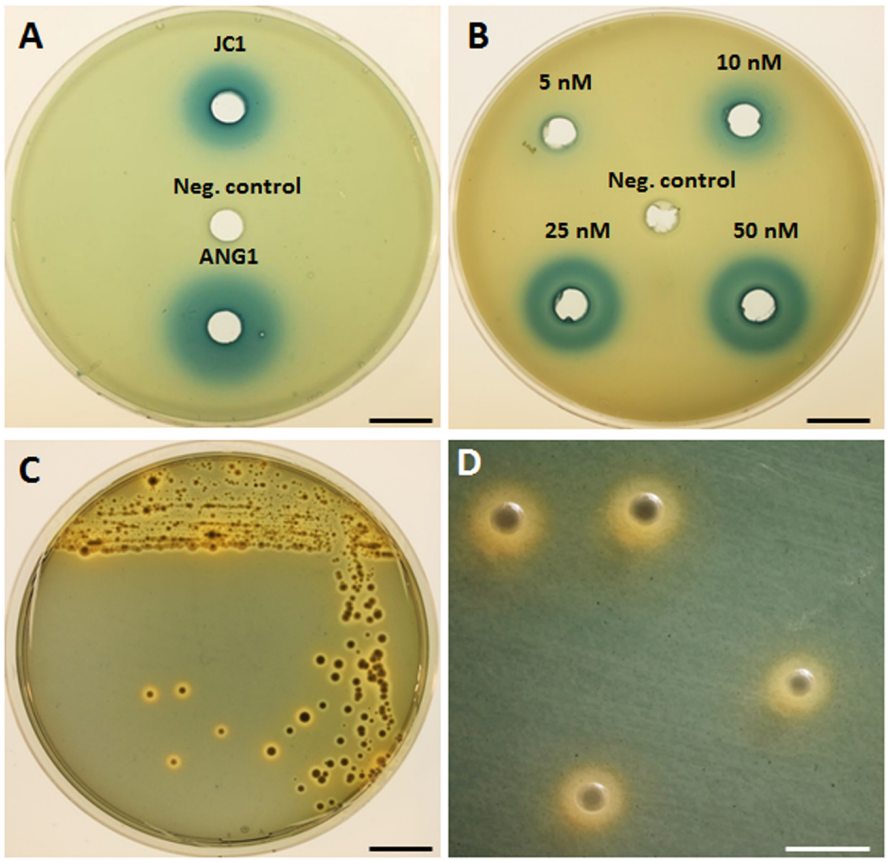

FIGURE 2 | Detection of homoserine lactone production and siderophore production by *Leisingera* sp. JC1. (A) Homoserine lactones were detected by β-galactosidase activity in cell-free supernatant of *Leisingera* sp. JC1 and compared with previously tested *Leisingera* sp. ANG1 (Collins et al., 2015); SWT broth was used as a negative control. **(B)** Dilution of *N*-3-oxohexanoyl homoserine lactone used as a positive control for the HSL assay; DMSO was the HSL standard solvent and was the negative control. **(C)** *Leisingera* sp. JC1 plated on CAS agar. Sequestration of iron changes the media from blue to orange, indicating siderophore production. **(D)** Magnified view of JC1 colonies from the plate in **(C)**, showing the orange halos in the media indicative of siderophore production. Scale bars, 1.5 cm **(A–C)**, 4 mm **(D)**.

australis by *Phaeobacter gallaeciensis* 2.10 (Rao et al., 2007). In other roseobacters, quorum sensing regulates secondary metabolite production, such as TDA in *Phaeobacter gallaeciensis* (Berger et al., 2011). In the indigoidine producing roseobacter, *Leisingera* sp. Y4I, there are two quorum sensing systems that regulate indigoidine production, *pgaIR* and *phaIR* (Cude et al., 2015). The JC1 *luxI* homolog has a 72% amino acid similarity to *pgaI* (RBY4I_1689) in Y4I, and the JC1 *luxR* homolog has an 81% amino acid similarity to *pgaR* (RBY4I_3631) in Y4I. The second set of *luxIR* homologs in Y4I, *phaIR* (RBY4I_3464 and RBY4I_1027), is not present in JC1. PgaI synthesizes the C8-HSL, produced by several proteobacteria, while PhaI synthesizes the $3OHC_{12:1}$-HSL, which may be species specific. JC1 lacking the *phaIR* system may reflect its divergence from *Leisingera* sp. Y4I. Further analyses will be needed to understand if indigoidine production in *Leisingera* sp. JC1 is regulated by quorum sensing.

Siderophore Production

Three separate siderophore biosynthesis gene clusters were detected in the genome, as described above, and production of iron chelators was confirmed by plating on CAS agar (**Figures 2C,D**). Appearance of an orange halo around colonies indicates that iron was sequestered from the chrome-azurol S dye in the media. Siderophores are high-affinity iron chelators, and can provide a growth advantage to cells in iron-limited environments, such as in seawater and in colonization of hosts. Although, the presence of siderophore biosynthesis genes in the genomes of currently sequenced roseobacter clade members is rare, 10 of the 12 previously sequenced *E. scolopes* ANG roseobacter symbionts did have the genes and/or demonstrate production of siderophores (Collins et al., 2015). Similar to the majority of the squid-associated roseobacter clade, the *Leisingera* sp. JC1 genome contains siderophore biosynthesis genes, indicating that siderophore production may play a role in the ANG symbiosis.

Indigoidine Biosynthesis Genes

The indigoidine biosynthesis gene cluster in *Leisingera* sp. JC1 contains all six biosynthesis genes previously described for *Leisingera* sp. Y4I (Cude et al., 2012) and shares a similar genome

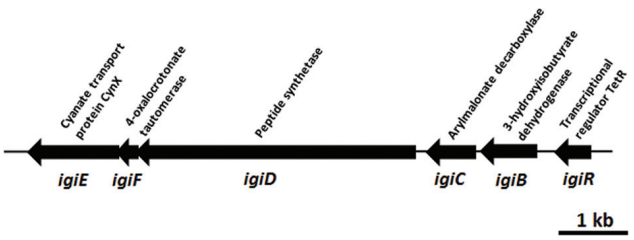

FIGURE 3 | *Leisingera* sp. JC1 indigoidine biosynthesis operon. Operon organization obtained from the published genome sequence (OBY26161.1, OBY26149.1, OBY26162.1, OBY26150.1, OBY26151.1, OBY26152.1). Arrows drawn to scale.

arrangement (**Figure 3**). To confirm the presence of individual members of the indigoidine biosynthesis gene cluster, primers were designed to three different components of the pathway, the non-ribosomal peptide synthetase (*igiD*), the transcriptional regulator (*igiR*), and one of the three indigoidine modification genes (*igiC*). The presence of these genes in JC1 genomic DNA was confirmed by PCR (Supplementary Figure S1).

Indigoidine biosynthesis genes have been detected in a diverse group of bacteria, including the *Actinobacteria* (*Streptomyces),* and *Alpha-*, *Beta-*, and *Gamma-proteobacteria*. The JC1 indigoidine biosynthesis operon shares the closest homology to the operon in *Leisingera* sp. Y4I, with 90–95% amino acid similarity for all gene products (**Table 2**). Other indigoidine biosynthesis operons share the non-ribosomal peptide synthetase, *igiD*, but many lack the same accessory genes required to modify indigoidine. When compared to other indigoidine producing strains, the *igiD* of JC1 is functionally homologous to other NRPS genes, sharing 49–53% amino acid similarity with *Vogesella indigofera*, *Streptomyces lavendulae*, and *Dickeya dadantii* 3937 (**Table 2**). A comparison with the genome of *Leisingera* sp. Y4I also confirmed that the indigoidine gene cluster is shared between these strains although absent from related ANG isolate *Leisingera* sp. M7 (Supplementary Figures S4 and S5).

Detection of Indigoidine Production by *Leisingera* sp. JC1

Because of the distinctive morphology and the genetic evidence for indigoidine biosynthesis, *Leisingera* sp. JC1 was cultured and extracted to obtain chemical evidence of indigoidine production. Using a three-step culture process, a deep blue liquid culture was obtained. However, upon extraction using a typical resin-based organic extraction protocol, most of the blue color was insoluble in organic solvents and little evidence of indigoidine production was observed via liquid chromatography-mass spectrometry (LC–MS, see **Figures 4D,E**), integrating to only 0.8% of the JC1 normal extract and indicative of negligible indigoidine extraction using this method. Therefore, an indigoidine enriched extraction protocol was utilized to pellet the insoluble indigoidine away from other media and cellular components followed by dissolving the sample in DMSO (Yu et al., 2013), resulting in an indigoidine enriched extract with 91.1% indigoidine. Analysis via LC–MS confirmed the presence of indigoidine (**Figure 4A**) in the indigoidine enriched extract (**Figures 4B,C**) with a peak eluting at 10.7 min with an $[M\text{-}H]^-$ of 247.0, consistent with the molecular weight and fragmentation pattern of indigoidine (248.2 g/mol) and in agreement with literature precedent (Yu et al., 2013). In addition, indigoidine was detected in two other JC and ANG isolates that exhibited a similar dark blue coloration in culture (data not shown).

Antibacterial Activity of *Leisingera* sp. JC1

Zone of Inhibition

Zone of inhibition assays were performed to test the ability of *Leisingera* sp. JC1 to inhibit other marine bacteria, both free-living and symbiotic (Supplementary Table S3). JC1 was tested against the *E. scolopes* light organ symbiont, *V. fischeri* ES114; another bioluminescent member of the *Vibrionaceae*, *P. leiognathi* KNH6, isolated from Hawaiian seawater; *V. harveyi* B392; *V. parahaemolyticus* KNH1 and *V. anguillarum* 775. These bacteria were plated at lawn densities from 10^4 to 10^7 CFU/mL to test the efficacy of possible inhibition at varying densities

TABLE 2 | Comparison of indigoidine biosynthesis operon in *Leisingera* sp. JC1 to other indigoidine producing strains.

Gene	Annotation	% Amino acid identity to *Leisingera* sp. Y4I operon	% Amino acid identity to *Vogesella indigofera* operon	% Amino acid identity to *Streptomyces lavendulae* operon	% Amino acid identity to *Dickeya dadantii* 3937 operon
igiE	Cyanate transport protein, CynX	95	58	NA	NA
igiF	4-oxalocrotonate tautomerase	91	NA*	NA	NA
igiD	Peptide synthetase	91	53	50	49
igiC	Arylmalonate decarboxylase	95	56	NA	NA
igiB	Hydroxyisobutyrate dehydrogenase	93	51	NA	NA
igiR	Transcriptional regulator, TetR	90	42	NA	NA

**NA, not applicable, no homolog of the gene present in that organism.*

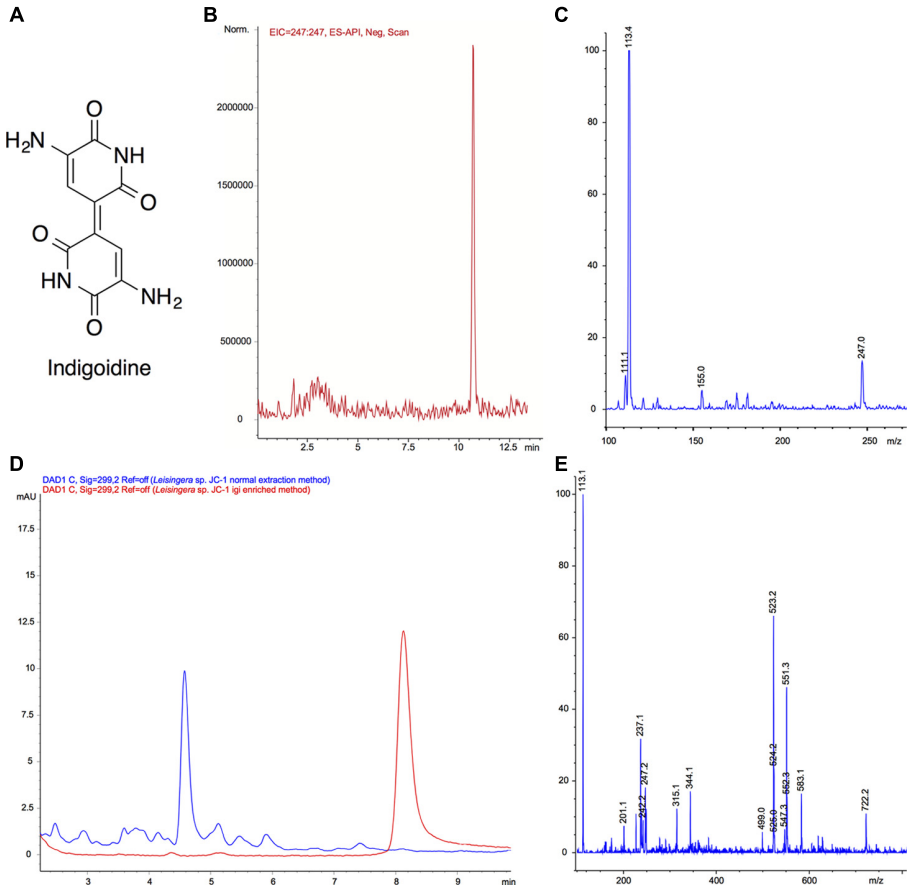

FIGURE 4 | Mass spectral confirmation of indigoidine production by *Leisingera* sp. JC1 and comparison of normal and igi enriched extracts. **(A)** Structure of indigoidine. **(B)** Extracted ion chromatogram (EIC) of indigoidine to confirm presence in extract ($[M\text{-}H]^-$ 247.0) using a previously reported LC–MS solvent gradient (Yu et al., 2013). **(C)** Negative ionization mass spectrum of peak at 10.7 min. The $[M\text{-}H]^-$ peak of 247.0 is consistent with a molecular formula of $C_{10}H_7N_4O_4$, confirming the presence of indigoidine (248.19 g/mol). **(D)** LC–MS UV chromatogram overlays (299 nm) of normal extract (blue) with indigoidine (igi) enriched extract (red). The peak eluting at t_R 8.2 min in the igi enriched extract was confirmed to be indigoidine with a purity of 91.1% (t_R different due to use of isocratic solvent system to minimize baseline variation). Although, a small peak was present at t_R 8.2 min in the normal extract, this integrates to only 0.8% indicating that the *Leisingera* sp. JC1 normal extract contains negligible amounts of indigoidine. **(E)** Negative ionization mass spectrum of peak at t_R 8.2 min for normal extraction method. Although there was an ion corresponding to $[M\text{-}H]^-$ of 247.2, this was not the most dominant ion and the more quantitative UV analysis in D shows only a very small peak, indicative of negligible indigoidine in the JC1 normal extract.

which more closely reflect biologically relevant concentrations. Overall, *Leisingera* sp. JC1 differentially inhibited the five vibrios tested (**Figure 5**). For two of the strains tested, *V. fischeri* ($F_{3,76} = 12.63$, $P < 0.0001$) and *P. leiognathi* ($F_{3,60} = 137.5$, $P < 0.0001$), JC1 showed significantly greater inhibition at lower lawn densities (**Figure 5A**; Supplementary Figure S2). When measured ZOIs were normalized for variations in JC1 colony diameter, there was an average 4.2 mm ZOI at 10^4 CFU/mL of *V. fischeri*, while at the 10^7 CFU/mL density, there was a 2.3 mm ZOI (Supplementary Figures S2I–L). A multiple comparisons *post hoc* Tukey test determined that the ZOI for the 10^4–10^6 CFU/mL lawn densities of *V. fischeri* were significantly greater than the ZOI at the 10^7 CFU/mL density. The change in ZOI with test strain lawn density was most apparent for *P. leiognathi*, where the average ZOI at the 10^4–10^5 CFU/mL lawn densities ranged from 2.4 to 2.9 mm, and then dropped to 0 mm at the 10^6–10^7 CFU/mL densities (**Figures 5A,D**; Supplementary Figures S2A–D). A multiple comparisons *post hoc* Tukey test showed that the ZOI at 10^4 CFU/mL of *P. leiognathi* was significantly different from the ZOI at 10^5 CFU/mL, and that both ZOIs at 10^4 and 10^5 CFU/mL were significantly different from the 10^6–10^7 CFU/mL results. *Leisingera* sp. JC1 showed a trend toward inhibition of *V. anguillarum* with ZOIs ranging from 2.7 to 3.3 mm (**Figures 5A,E**; Supplementary Figures S2E–H) although, a one-way ANOVA determined that the ZOIs were not statistically different ($F_{3,60} = 2.553$, $P = 0.0639$). No inhibition was observed when JC1 was tested against *V. parahaemolyticus* or *V. harveyi* at any lawn density (**Figures 5A,F,G**; Supplementary Figures S2M–P).

Leisingera sp. JC1 was also tested in a ZOI assay against the 12 previously described ANG isolates (Collins et al., 2015) and one additional ANG isolate, *Muricauda* sp. ANG21. All ANG isolates were only tested at a lawn density of approximately 10^8 CFU/mL. Inhibition was observed against *Ruegeria* sp. ANG-S4,

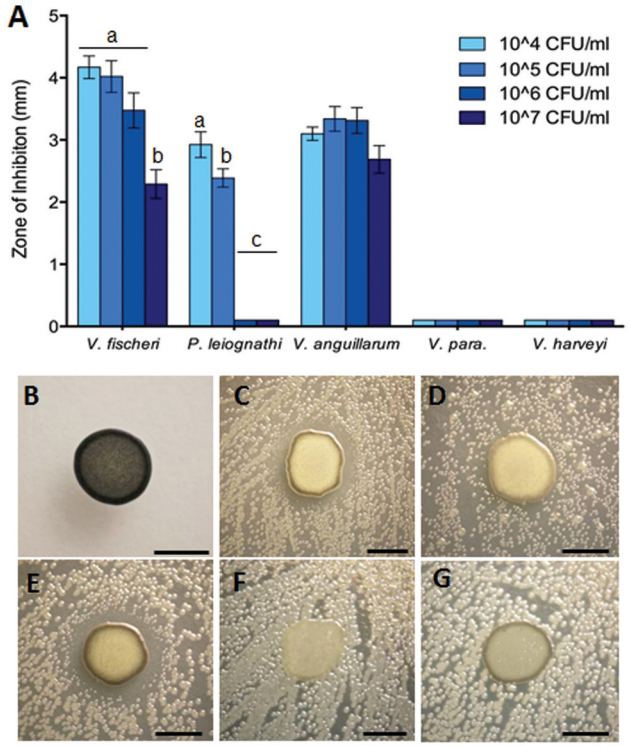

FIGURE 5 | *Leisingera* sp. JC1 differentially inhibits pathogenic and non-pathogenic vibrio species. (A) Zones of inhibition around JC1 colonies were measured with increasing lawn densities of each vibrio species. *Leisingera* sp. JC1 differentially inhibited five vibrio strains. Significantly greater inhibition of *Vibrio fischeri* was seen between the 10^4–10^6 CFU/ml and the 10^7 CFU/ml lawn density. Significantly greater inhibition of *Photobacterium leiognathi* was seen between the 10^4 and 10^5 CFU/ml lawn densities, as well as between the 10^4–10^5 and the 10^6–10^7 CFU/ml densities. JC1 inhibited *Vibrio anguillarum* uniformly across all lawn densities and did not inhibit *Vibrio parahaemolyticus* or *Vibrio harveyi*. Letters indicate significantly different groups based on results from *post hoc* Tukey tests (see Results and Discussion). Representative 24 h images of **(B)** JC1 plated alone on YTSS agar and with 10^6 CFU/ml of **(C)** *V. fischeri*, **(D)** *P. leiognathi*, **(E)** *V. anguillarum*, **(F)** *V. parahaemolyticus*, and **(G)** *V. harveyi*. Scale bars: 5 mm **(B–G)**.

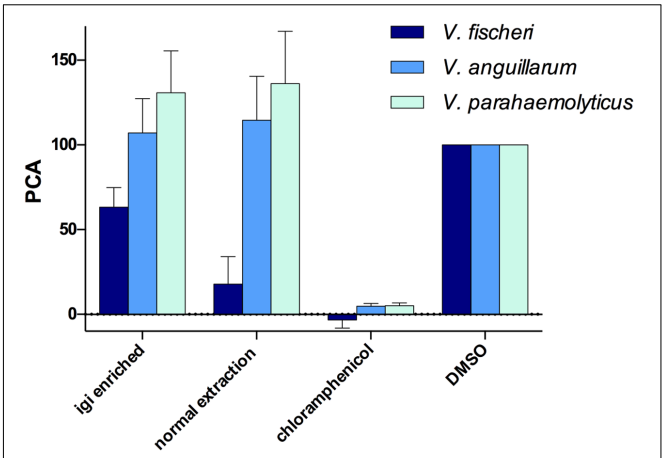

FIGURE 6 | *Leisingera* sp. JC1 extracts inhibit *V. fischeri* in 96-well liquid assays. JC1 cultures were grown at large scale and extracts were prepared using either an indigoidine enriched procedure (igi enriched) or a normal extraction method. All extracts were screened at 500 μg/mL against *V. fischeri*, *V. anguillarum,* and *V. parahaemolyticus*. The JC1 normal extract strongly inhibited growth of *V. fischeri* (PCA 17.9 ± 9.3) with less activity of the JC1 igi enriched extract against *V. fischeri* (PCA 63.3 ± 6.7). No inhibition of *V. anguillarum* or *V. parahaemolyticus* was observed. Data shown are average PCA values from three experiments (PCA, percent control activity, as compared with DMSO negative control).

with an average ZOI of 6.3 mm (±0.7) and against *Muricauda* sp. ANG21, with an average ZOI of 5.9 mm (±0.6; Supplementary Figure S3). *Leisingera* sp. JC1 was not able to inhibit any of the other *Leisingera* spp. previously isolated from ANGs. Since partitioning between bacterial taxa is observed in the ANG tubules some activity against other ANG isolates may contribute to competition between strains during colonization (Collins et al., 2012).

96-Well Liquid Assay

Both the normal and indigoidine enriched JC1 extracts were screened for activity using 96-well plate liquid assays with several of the vibrios tested above, including *V. fischeri* ES114, *V. anguillarum* 775, and *V. parahaemolyticus* KNH1 (**Figure 6**). Both extracts were initially tested at 500 μg/mL with minimum inhibitory concentrations (MICs) determined for active samples. The JC1 normal extract was found to strongly inhibit growth of *V. fischeri* with a PCA value of 17.9 ± 9.3 during screening and was determined to have a MIC of 250 μg/mL. The JC1 indigoidine enriched extract also exhibited moderate inhibition of *V. fischeri* with a PCA of 63.3 ± 6.7.

In contrast to the ZOI data above, no inhibition was observed for either extract when tested against *V. anguillarum*, potentially due to differences between the activity of indigoidine in agar versus liquid assays, as seen with *Leisingera* sp. Y41 and hypothesized to result from changes in the redox state of indigoidine (Cude et al., 2012). These results may also be attributed to differences in the chemical composition between extracts and the bacteria *in situ* (e.g., aqueous soluble metabolites are generally excluded from the extraction protocols used in this study). Neither JC1 extract inhibited *V. parahaemolyticus*, in agreement with the ZOI data above.

Previous studies with a mutant of *Leisingera* sp. Y4I that did not produce indigoidine suggested that production of the compound is required for inhibition of *V. fischeri* (Cude et al., 2012). However, with the more potent inhibition of *V. fischeri* seen in the JC1 normal extract versus the indigoidine enriched extract in this study (**Figure 6**), indigoidine production does not seem to be the only mechanism of inhibition for *Leisingera* sp. JC1. Given that the JC1 normal extract contains only minimal amounts of indigoidine (0.8% as discussed above), the bacterium may be utilizing other secondary metabolites in conjunction with indigoidine for chemical defense. The JC1 genome includes several other secondary metabolite biosynthetic gene clusters for HSL, siderophore, bacteriocin, PKS, and PKS/NRPS production and thus *Leisingera* sp. JC1 likely utilizes one or more of the

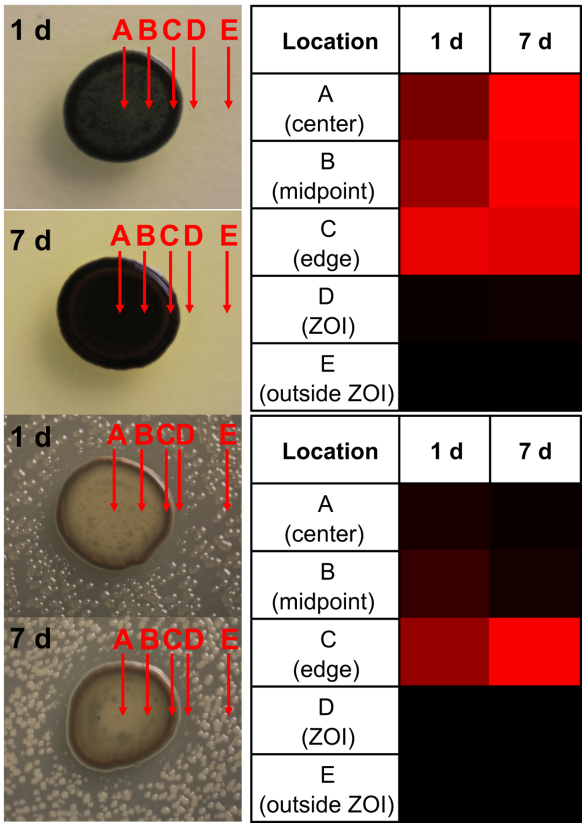

FIGURE 7 | *Leisingera* sp. JC1 localizes indigoidine production to outer edge of colony in the presence of *V. fischeri*. Five locations (A–E) were analyzed for the presence of indigoidine via direct analysis in real time-mass spectrometry (DART-MS) over 7 days. (A) In the absence of *V. fischeri*, indigoidine production was more uniformly distributed across the colony. (B) In the presence of *V. fischeri*, indigoidine production was primarily localized to outer edge of colony (location C) whereas minimal indigoidine was detected in the center of the colony (location A). Very little indigoidine was detected in the ZOI or outside ZOI in the presence or absence of *V. fischeri*. Heatmaps represent a gradient of indigoidine ion abundance from less abundance (black, 0%) to more abundance (red, 100%).

compounds encoded by these pathways for chemical defense, in addition to the defensive capabilities attributed to indigoidine. Creating an indigoidine mutant of *Leisingera* sp. JC1 will help test this hypothesis, in conjunction with identification of additional metabolite(s) responsible for JC1 antimicrobial activity.

Localization of Indigoidine Production by *Leisingera* sp. JC1

While performing ZOI assays, there was a dramatic change in colony pigmentation of *Leisingera* sp. JC1 when grown alone (**Figure 5B**) as compared to growth under challenge with various vibrio strains (**Figures 5C–G**). Deep blue pigment production was observed uniformly when JC1 was grown in monoculture and appeared to localize to the outer edges of the colonies when presented with vibrio strains. Direct analysis in real time-mass spectrometry (DART-MS) is an ambient ionization technique in which samples can be analyzed without sample preparation or extraction (Sanchez et al., 2011). DART-MS was utilized to chemically confirm the visual observations of localization of indigoidine production of JC1 in monoculture and co-culture with *V. fischeri* over the course of 7 days (**Figure 7**). Five locations were selected on each colony including center (A), midpoint (B), edge (C), ZOI (D), and outside ZOI (E). In the absence of *V. fischeri*, indigoidine was uniformly produced throughout JC1 colonies (locations A–C). However, in the presence of *V. fischeri* there was little to no indigoidine production in the center or midpoints of JC1 colonies, but intense indigoidine detected around colony edges (**Figure 7**, location C only). Indigoidine was only minimally detected in the ZOI or outside the ZOI for either monoculture or co-culture. Trends in the localization of indigoidine production were even more apparent upon measurement after 7 days.

There are several examples of pigment production being induced when in the presence of other bacteria, such as in *Staphylococcus aureus* when co-cultured with *Pseudomonas aeruginosa* (Antonic et al., 2013) or production of a red pigment by *Streptomyces lividans* TK23 when co-cultured with *Tsukamurella pulmonis* TP-B0596 (Onaka et al., 2011). Pigment production can also be induced under other stress response conditions, such as protection from UV radiation (Tong and Lighthart, 1997). Pigment production has also been tied to photosynthesis (Orf and Blankenship, 2013), however, *Leisingera* sp. JC1 lacks genes associated with photosynthesis or carbon fixation (data not shown).

When grown alone, *Leisingera* sp. JC1 exhibited a uniform blue–black pigmentation across the colony which was confirmed by mass spectrometry to be essentially uniform production of indigoidine. Secondary metabolite biosynthesis is an energy intensive endeavor and production of antimicrobial compounds would typically be thought to be reserved for defensive situations. Since indigoidine is produced throughout the colony when in monoculture, and given its relatively moderate antibacterial activity as suggested by assays with the indigoidine enriched extract, it is also possible that indigoidine serves multiple functions for *Leisingera* sp. JC1. However, *Leisingera* sp. JC1 localized indigoidine production to the outer edges of the colony when co-cultured with *V. fischeri* and other vibrios. If utilized as a defensive compound, indigoidine may be localized to points of direct interaction with other microorganisms. Secondary metabolite production can be localized to susceptible locations such as in plants, sponges, and other sessile terrestrial and marine organisms (Amsler et al., 2001; Furrow et al., 2003; Van Dyck et al., 2010). The role of *Leisingera* sp. JC1 has yet to be examined directly in the ANG symbiosis but localized production of indigoidine or other secondary metabolites may play a role in egg defense or inhibition of other bacteria from colonizing the ANG (see conclusions below).

Regulation of Indigoidine Production by *Leisingera* sp. JC1

After observing localized production of indigoidine when grown on solid media with *V. fischeri*, additional co-culture experiments were undertaken in liquid media using several

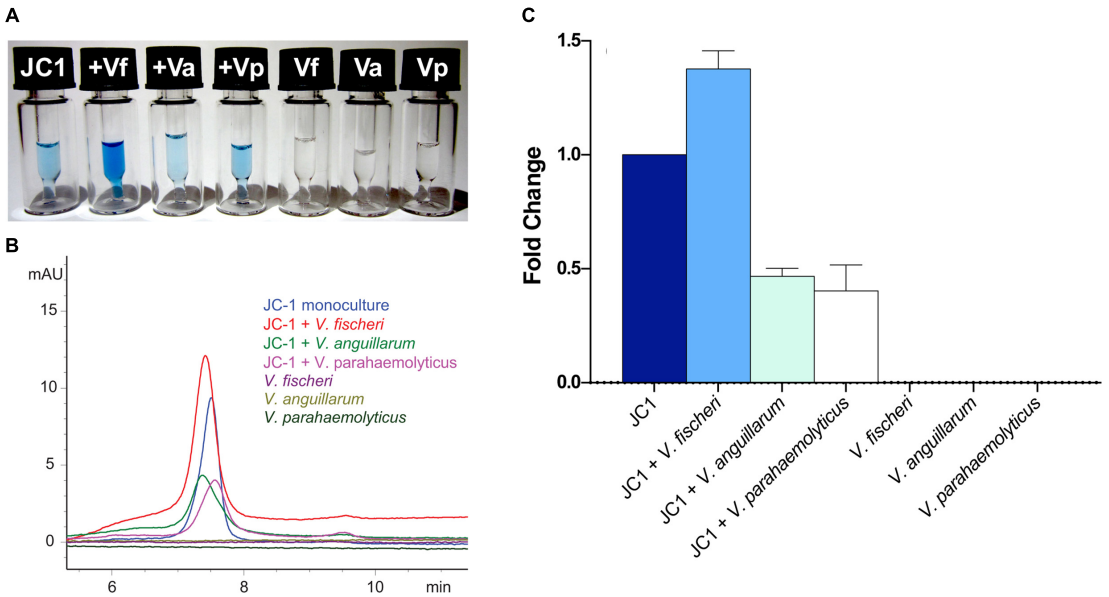

FIGURE 8 | *Leisingera* sp. JC1 upregulates indigoidine production in the presence of *V. fischeri*. JC1 was cultured alone and in the presence of *V. fischeri*, *V. anguillarum*, and *V. parahaemolyticus* (each vibrio was also monocultured). **(A)** Extracts were prepared in DMSO at 5 mg/mL for LC–MS (from left to right): JC1 monoculture, JC1 + *V. fischeri*, JC1 + *V. anguillarum*, JC1 + *V. parahaemolyticus*, *V. fischeri* monoculture, *V. anguillarum* monoculture, and *V. parahaemolyticus* monoculture. As shown, the highest pigment production was observed when JC1 was co-cultured with *V. fischeri*. **(B)** LC–MS UV chromatograms (299 nm) for all extracts. JC1 indigoidine production was upregulated when co-cultured with *V. fischeri* (red). Down regulation was observed when co-cultured with *V. anguillarum* or *V. parahaemolyticus* (green and pink, respectively). **(C)** Following extraction, indigoidine production was measured using HPLC and quantitated via measurement of the area under the curve for UV absorption at 299 nm. Fold change was calculated as compared with JC1 monoculture.

of the vibrios from the antimicrobial assays above. *Leisingera* sp. JC1 was grown in monoculture and in the presence of *V. fischeri*, *V. anguillarum*, and *V. parahaemolyticus*, followed by extraction and measurement of indigoidine production (**Figure 8**). Monocultures of all three vibrios were also grown and extracted as controls. Addition of *V. fischeri* to established cultures of *Leisingera* sp. JC1 resulted in a 1.38 fold increase in indigoidine. Co-cultures with *V. anguillarum* and *V. parahaemolyticus* resulted in a decrease in indigoidine production of approximately 0.5 fold for both organisms. Vibrio monocultures confirmed that these species do not produce indigoidine. Changes in indigoidine production were also visually evident with darker, more intense blue observed for extracts cultured with *V. fischeri* in comparison with JC1 monoculture, as well as lighter blue extracts observed for *V. anguillarum* and *V. parahaemolyticus* co-cultures (**Figure 8A**).

The increase in indigoidine production of JC1 with *V. fischeri* is consistent with the antibacterial activity observed for *Leisingera* sp. JC1 on both solid and liquid media (**Figures 5** and **6**), strengthening the hypothesis that indigoidine may play a protective role in association with *E. scolopes*. In addition, the downregulation of production with *V. anguillarum* and *V. parahaemolyticus* also supports the liquid culture bioassay data (**Figure 6**). The differential antimicrobial activity and indigoidine production between the three vibrios may be due to the purported role of the ANG and JC bacteria in the host. The ability of *Leisingera* sp. JC1 to inhibit *V. fischeri* may be related to the fact that the ANG is located directly posterior to the light organ, which harbors high densities of the sole symbiont, *V. fischeri* (McFall-Ngai, 2014). Each day 95% of viable *V. fischeri* cells in the light organ are expelled directly into the mantle cavity of the host as part of the regulatory mechanisms of that association (Boettcher et al., 1996; Nyholm and McFall-Ngai, 1998). A study from another squid, *Doryteuthis pealeii* (Kaufman et al., 1998) suggests that ANG bacteria are environmentally transmitted during development. Given that *V. fischeri* is not detected in the ANG (Collins et al., 2012), the inhibitory effect of *Leisingera* sp. JC1 and other ANG isolates may prevent *V. fischeri* and other vibrios from colonizing the ANG and thus help shape the consortium during development. Alternatively, inhibition against vibrios may play a role in egg defense since eggs are exposed to seawater for approximately three weeks and vibrios are known to be common members of the bacterioplankton.

CONCLUSIONS

Genome analyses confirm that *Leisingera* sp. JC1 is part of the squid-associated roseobacter clade. Both *in silico* and *in vitro* analyses confirmed the secondary metabolite potential and production of siderophores, acyl-homoserine lactones associated with quorum sensing, and the pigment indigoidine. *Leisingera* sp. JC1 and its extracts had inhibitory activity against a variety of marine bacteria including the light organ symbiont *V. fischeri*. Furthermore, JC1 challenged with *V. fischeri* led to increased localized

production of indigoidine as well as an increased production of indigoidine when co-cultured in liquid media. Taken together these results suggest that *Leisingera* sp. JC1 may play a protective role in egg defense and/or in shaping the microbial community of the ANG. The importance of defensive symbioses in nature is becoming increasingly more evident (Flórez et al., 2015). A number of both terrestrial and marine organisms use novel secondary metabolites produced by bacteria toward defense from potential pathogens and fouling microorganisms. Since roseobacters have been found in the ANGs of a number of cephalopods from diverse marine environments (Kaufman et al., 1998; Grigioni et al., 2000; Barbieri et al., 2001; Pichon et al., 2005; Collins et al., 2012) there may be a conserved function of this group in this symbiosis. Further studies from this group may reveal novel compounds that are important for the biology of these associations and that exhibit antimicrobial activity.

AUTHOR CONTRIBUTIONS

MB, SN, SG, and AS conceptualized and designed research; SG, AS, MF, and JLG conducted experiments; MB, SN, SG, AS, MF, and JPG analyzed data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01342

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Comparative genomics of *Roseobacter* clade bacteria isolated from the accessory nidamental gland of *Euprymna scolopes*

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The accessory nidamental gland (ANG) of the female Hawaiian bobtail squid, *Euprymna scolopes*, houses a consortium of bacteria including members of the *Flavobacteriales*, *Rhizobiales*, and *Verrucomicrobia* but is dominated by members of the *Roseobacter* clade (Rhodobacterales) within the *Alphaproteobacteria*. These bacteria are deposited into the jelly coat of the squid's eggs, however, the function of the ANG and its bacterial symbionts has yet to be elucidated. In order to gain insight into this consortium and its potential role in host reproduction, we cultured 12 Rhodobacterales isolates from ANGs of sexually mature female squid and sequenced their genomes with Illumina sequencing technology. For taxonomic analyses, the ribosomal proteins of 79 genomes representing both roseobacters and non-roseobacters along with a separate MLSA analysis of 33 housekeeping genes from *Roseobacter* organisms placed all 12 isolates from the ANG within two groups of a single *Roseobacter* clade. Average nucelotide identity analysis suggests the ANG isolates represent three genera (*Leisingera*, *Ruegeria*, and *Tateyamaria*) comprised of seven putative species groups. All but one of the isolates contains a predicted Type VI secretion system, which has been shown to be important in secreting signaling and/or effector molecules in host–microbe associations and in bacteria–bacteria interactions. All sequenced genomes also show potential for secondary metabolite production, and are predicted to be involved with the production of acyl homoserine lactones (AHLs) and/or siderophores. An AHL bioassay confirmed AHL production in three tested isolates and from whole ANG homogenates. The dominant symbiont, *Leisingera* sp. ANG1, showed greater viability in iron-limiting conditions compared to other roseobacters, possibly due to higher levels of siderophore production. Future comparisons will try to elucidate novel metabolic pathways of the ANG symbionts to understand their putative role in host development.

Keywords: symbiosis, *Euprymna scolopes*, *Roseobacter* clade, genomics, Cephalopoda, *Alphaproteobacteria*

INTRODUCTION

The *Roseobacter* clade is a pervasive and diverse group of marine *Alphaproteobacteria*. This group is estimated to account for 10% of all marine bacteria, with higher percentages in coastal seawater (Wagner-Döbler and Biebl, 2006). These organisms have usually been investigated from an ecological perspective due to their abundance in seawater. The combined metabolic potential of such a large bacterial population may contribute to both sulfur cycling, primarily through metabolism of dimethylsulfoniopropionate (DMSP), and carbon cycling, as roseobacters oxidize a variety of carbon sources to CO_2 (González et al., 2000).

Many of the characterized *Roseobacter* isolates can be described as free-living, having been isolated from seawater or inert marine surfaces. However, some roseobacters also associate with other organisms, including oysters (Ruiz-Ponte et al., 1998), sponges (Zan et al., 2014), algae (Rao et al., 2007; Case et al., 2011), and cephalopods (Grigioni et al., 2000; Pichon et al., 2005; Collins et al., 2012). Among many squid and cuttlefish, roseobacters have been found associated with the accessory nidamental gland (ANG), part of the female reproductive system and comprised of many epithelium-lined tubules that house dense populations of bacterial symbionts (**Figure 1**, Bloodgood, 1977; Collins et al., 2012). Evidence suggests that these bacteria are embedded in the jelly coat of the squid's eggs that are then deposited in masses on the ocean floor where they resist fouling and degradation over ~3 weeks of development (Barbieri et al., 2001; Collins et al., 2012).

Studies that have investigated the ANG consortium have found members of the *Roseobacter* clade among many cephalopods, including *Doryteuthis pealeii, Sepia officinalis,* and *Euprymna scolopes* (Grigioni et al., 2000; Barbieri et al., 2001; Pichon et al., 2005; Collins et al., 2012). In the Hawaiian bobtail squid, *E. scolopes*, roseobacters comprise ~50% of the microbial population according to 16S rDNA surveys, predominantly from the genus *Leisingera* (formerly *Phaeobacter*; Collins et al., 2012). Other members of the consortium include *Flavobacteria* and *Verrucomicrobia*

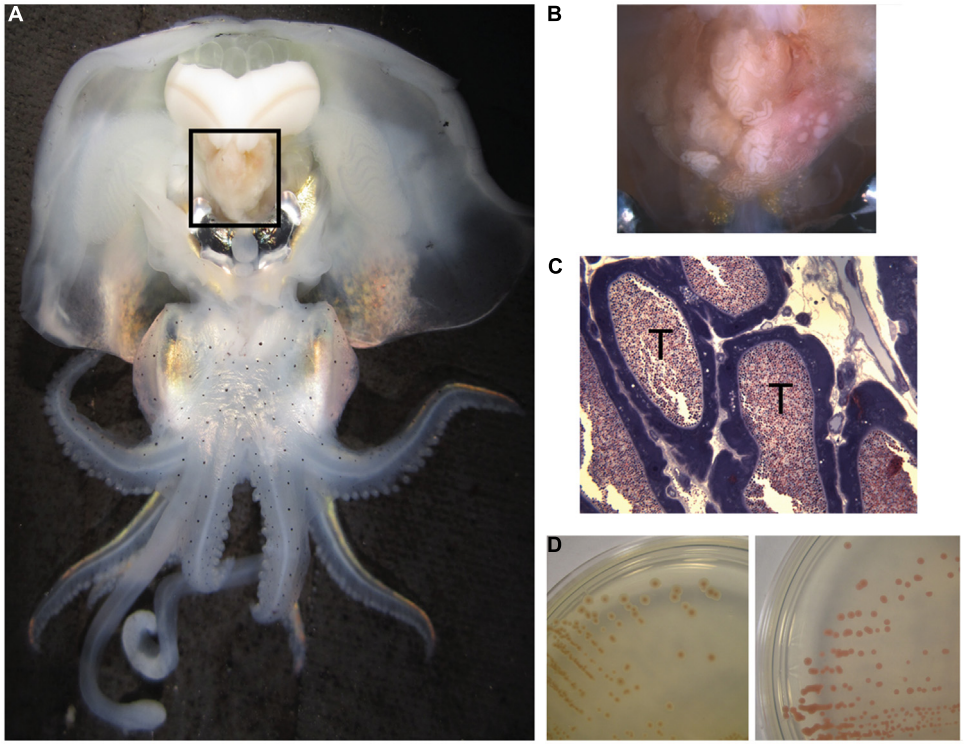


FIGURE 1 | Anatomy of *Euprymna scolopes*, the accessory nidamental gland (ANG) and associated bacteria. (A) The ANG (black box) is located on the ventral side of the squid, posterior to the ink sac and light organ. **(B)** The ANG is comprised of many pigmented convoluted epithelium-lined tubules that may appear white, pink, or brown. **(C)** Microscopy of fixed and stained sections of the ANG reveal dense populations of bacteria within tubules (T). **(D)** Bacteria from the ANG that were isolated on complex media also had a variety of pigments, from brown (*Leisingera* sp.ANG-Vp, left) to red (*Tateyamaria* sp. ANG-S1, right).

and each of these groups are partitioned such that only one taxon dominates any given tubule (Collins et al., 2012).

Roseobacter clade bacteria are known to produce several antimicrobial compounds, including tropodithietic acid (TDA), which has antimicrobial and anti-algal properties (Brinkhoff et al., 2004). Under certain conditions, likely when associated with dying algae, *Phaeobacter inhibens* can also produce anti-algal compounds known as roseobacticides derived from *p*-coumaric acid, a product of lignin degradation (Seyedsayamdost et al., 2011). *Leisingera* sp. Y4I and *Leisingera daeponensis* produce indigoidine, an antimicrobial blue pigment that is synthesized from a unique polyketide/non-ribosomal peptide synthase gene cluster and has been shown to inhibit marine bacteria, including *Vibrio fischeri* (Cude et al., 2012; Dogs et al., 2013).

The function of the ANG and its associated bacterial population remains unknown although protective roles against predation and/or fouling have been suggested (Biggs and Epel, 1991). The distribution of roseobacters among cephalopod ANGs suggests that they have a conserved function in these animals. Furthermore, they must contain traits that allow them to survive in multiple habitats such as seawater, a specialized organ such as the ANG, and within squid egg jelly coats. To shed light on the metabolic capabilities of these bacteria and investigate possible adaptations to living in these different habitats, we examined the genomes of 12 isolates from the ANG of *E. scolopes* and compared them to others from the *Roseobacter* lineage. Here, we describe the genetic content from this select group of roseobacters that exist in conserved symbioses with cephalopods worldwide.

MATERIALS AND METHODS

CULTURING BACTERIA FROM THE ANG

Animals were collected in sand shallows on Oahu, Hawaii and maintained in artificial aquaria as previously described Schleicher and Nyholm (2011). To obtain ANGs, five mature females were anesthetized in Instant Ocean with 2% ethanol. Organs were removed and surface sterilized with 70% ethanol before being homogenized in filter-sterilized squid Ringer's solution (530 mM NaCl, 25 mM $MgCl_2$, 10 mM $CaCl_2$, 20 mM HEPES, pH = 7.5). Tissue homogenate was serially diluted and plated on either salt water tryptone (SWT) or Reasoner's 2A medium (R2A) supplemented with a 70:30 mixture of Instant Ocean and distilled water (Reasoner and Geldreich, 1985; Nyholm et al., 2009). Plates were incubated aerobically at 28°C for 2–7 days. For each animal, colonies with different morphology and/or color were isolated for further analysis.

GENOME SEQUENCING AND ANNOTATION

Genomic DNA was isolated using the MasterPure DNA Extraction kit (Epicentre) from liquid cultures of ANG bacteria grown

overnight at 28°C in either SWT or R2A. DNA was quantified using a Qubit fluorescence assay (Invitrogen). Illumina sequencing libraries were created from 1 ng of genomic DNA using the Nextera XT library kit and the libraries were quantified by a HS DNA Bioanalyzer assay (Agilent). Libraries were sequenced on an Illumina MiSeq sequencer using 2 × 250 bp reads. Draft genomes were assembled using the CLC Genomic Workbench (CLC) using default parameters. For *Leisingera* sp. ANG1 (formerly *Phaeobacter gallaeciensis* ANG1), additional sequencing data was added from a previous sequencing effort using an Illumina mated-pair library (Collins and Nyholm, 2011). Assemblies were annotated using the Rapid Annotation using Subsytem Technology (Aziz et al., 2008, RAST, rast.nmpdr.org) server. To search for Type IV secretion systems (T4SS), the VirB4 protein from *P. inhibens* DSM17395 was used to query the ANG isolate genomes using tblastn. Genomes were also analyzed with Anti-SMASH (Blin et al., 2013, Antibiotic and Secondary Metabolite Analysis Shell, antismash.secondarymetabolites.org) and BAGEL3 (van Heel et al., 2013, BActeriocin Genome mining tool, bagel.molgenrug.nl) for secondary metabolite and bacteriocin biosynthesis gene clusters. Draft genome assemblies have been deposited in DDBJ/EMBL/GenBank under accession numbers AFCF00000000 and JWLC00000000-JWLM00000000. The versions described in this manuscript are AFCF02000000 and JWLC01000000-JWLM01000000.

TAXONOMIC ANALYSIS

A total of 79 genomes were used for analyses in this study. Fifty-seven *Roseobacter* genomes and 10 non-*Roseobacter* genomes were obtained from the NCBI ftp site (ftp://ftp.ncbi.nih.gov/genomes/, listed in Supplementary Figure 1). Twelve *Roseobacter* genomes are new to this study, including an improved assembly of the previously published *Leisingera* sp. ANG1 (**Table 1**). To ensure equal gene calling across the genomes, all genomes, including the 67 draft and completed genomes obtained from the NCBI ftp, were re-annotated using the RAST server (Aziz et al., 2008). Assembled contigs were reconstructed from the RAST-generated GenBank files for all genomes using the seqret application of the EMBOSS package (Rice et al., 2000).

An initial survey of the *Roseobacter* clade was made using 51 ribosomal proteins. Queries were obtained from the BioCyc database (Caspi et al., 2010) for *Roseobacter denitrificans* OCh 114, excluding methyltransferases and putative proteins. Unlike many previous studies (Soucy et al., 2014) nucleotide sequences were used to potentially allow finer resolution of relationships. The top hits for each gene were aligned separately using MUSCLE (Edgar, 2004) and evaluated by hand to verify that the sequences were homologs. In-house python scripts created a concatenated alignment from all 51 genes. An optimal model of evolution was determined using the akaike information criterion with correction for small sample size (AICc). The program jModelTest 2.1.4. was used to compute likelihoods from the nucleotide alignment and to perform the AICc (Guindon et al., 2010; Darriba et al., 2012). The best-fitting model reported was GTR + Gamma estimation + Invariable site estimation. A maximum likelihood (ML) phylogeny was generated from the concatenated multi-sequence alignment using PhyML v3.0_360-500M (Guindon et al., 2010). PhyML parameters consisted of GTR model, estimated p-invar, 4 substitution rate categories, estimated gamma distribution, subtree pruning and regrafting enabled with 100 bootstrap replicates. This tree (Supplementary Figure 1) placed all of the new ANG isolates from this study into a single clade, corresponding to three groups (Clades 1, 2, and 4) previously described by Newton et al. (2010). Clade 4's placement sister to clade 2 is discussed in Section "Results and Discussion."

To further explore the relationships within these three clades a new scheme was devised. Forty-four genomes were selected from the clade, including all members corresponding to Newton's Clade 1, for inclusion in this step. As most ribosomal proteins are quite short, only 18 ribosomal genes were used and 15 single-copy housekeeping genes were added. This offered the advantage of adding a net of ∼8,300 positions to the alignment, most of which are likely under less stringent selection than those of a ribosomal protein. An added advantage is that all 33 genes are shared with the Newton set. This creates a direct relationship facilitating comparison with that previous work. The top Blast hits for the 44 genomes were processed as described above for the ribosomal tree. The AICc test reported the same model for evolution as above. The tree was also generated using SPR and 100 bootstrap replicates. The resulting tree was rooted based on the ribosomal tree's placement of the clades. This corresponded to the root being placed where Newton's clades 1 and 2/4 diverge.

AVERAGE NUCLEOTIDE IDENTITY

JSpecies1.2.1 (Richter and Rosselló-Móra, 2009) was used as described previously (Fullmer et al., 2014) to analyze the genomes for average nucleotide identity (ANI) and tetramer frequency patterns.

SIDEROPHORE BIOCHEMICAL ASSAYS

To reduce contaminating iron, all glassware was washed and all solutions were prepared using water treated with a Nanopure Diamond filtration system (Barnstead, Lake Balboa, CA, USA). Siderophore production was confirmed using chrome azurol S (CAS) agar, modified for marine bacteria as previously described Whistler and Ruby (2003).

To test viability of ANG bacteria in iron-limiting conditions, several isolates were grown in the presence of the iron chelator ethylenediamine-N,N'-bis (2-hydroxyphenylacetic acid) (EDDHA) as described previously (McMillan et al., 2010). Cultures were grown for 24 h at 26°C in SWT then washed 3x in minimal sea salts solution (MSS, 50 mM $MgSO_4$, 10 mM $CaCl_2$, 350 mM NaCl, 10 mM KCl, 18.5 mM NH_4Cl, 333 μM K_2PO_4, $FeCl_3$ 10 μM, 100 mM PIPES, pH = 7.2) with no added iron or EDDHA. Cultures were inoculated to an OD_{600} of 0.05 in MSS with 10 μM $FeCl_3$. Glucose and casamino acids were added as carbon sources at 0.2 and 0.3% respectively and cultures were grown for 24 h at 26°C with shaking. To create iron-limiting conditions, EDDHA was added to the growth media at 10–30 μM. To test viability in iron-limiting conditions, cultures were grown for 24 h at 26°C, and the OD_{600} of each culture was measured and compared to control cultures without EDDHA. Siderophore

Table 1 | Genome assembly statistics for *Roseobacter* clade ANG isolates.

Isolate	Genome size (Mb)	# of genes	Missing genes* (% of total)	% GC	N50 (kb)	Contigs	Fold- coverage	Female ID
ANG-Vp	5.150	4,941	51 (1.0)	62.3	70	165	69.2	1
ANG-M1	5.375	5,097	63 (1.2)	62.0	211	180	132.3	3
ANG1	4.587	4,484	26 (0.6)	62.8	450	36	1,455†	1
ANG-DT	4.596	4,467	23 (0.5)	62.6	189	116	115.4	5
ANG-S	4.572	4,458	19 (0.4)	62.8	196	83	65.5	4
ANG-S3	4.597	4,468	18 (0.4)	62.7	300	84	129.0	2
ANG-M6	4.542	4,429	26 (0.6)	62.7	157	65	118.0	3
ANG-S5	4.660	4,534	33 (0.7)	62.5	233	54	123.5	2
ANG-M7	4.582	4,498	46 (1.0)	62.5	263	61	148.7	3
ANG-R	4.685	4,755	43 (0.9)	57.4	390	47	98.1	4
ANG-S4	4.538	4,619	9 (0.2)	57.2	978	20	71.9	2
ANG-S1	4.425	4,478	33 (0.7)	60.6	229	33	110.7	2

**As predicted by the RAST server (Aziz et al., 2008).*
† *Leisingera* sp. ANG1 was previously sequenced with an Illumina mate-pair library and is therefore and a much higher fold coverage than other genomes (Collins and Nyholm, 2011).

production was measured from supernatants using the CAS liquid assay as described previously (Schwyn and Neilands, 1987). Further chemical characterization of siderophores was done using the Arnow (1937) and Csáky (1948) assays.

HOMOSERINE LACTONE DETECTION

Homoserine lactone (HSL) production was detected using the HSL-sensing bacterium *Agrobacterium tumefaciens* NTL4 (pZLR4; Cha et al., 1998). To determine acyl homoserine lactone (AHL) production, we used a well-diffusion assay as previously described Ravn et al. (2001). Briefly, a 3-mL culture of *A. tumefaciens* NTL4 was grown for 24 h in LB with gentamicin 30 μg/mL at 28°C. One milliliter of this culture was used to inoculate 50 mL of AB minimal media containing 0.5% glucose and 0.5% casamino acids (Chilton et al., 1974). After a 24-h incubation, 100 mL of AB minimal media containing 1.2% agar was autoclaved. Once the molten agar had cooled sufficiently, glucose and casamino acids were added to 0.5% each and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to a final concentration of 75 μg/mL. The molten agar was then combined with the 24-h culture of *A. tumefaciens*, distributed into petri dishes and allowed to solidify.

To induce HSL production by ANG isolates, cultures were grown overnight at 26°C in either SWT or MSS with 30 μM $FeCl_3$ and 0.5% of both glucose and casamino acids. To prevent the degradation of HSLs in alkaline conditions, the growth medium was buffered to pH 6.8 and never rose above 7.5 for any experiments. After a 24-h incubation, the cells were pelleted by centrifugation and the supernatant was filtered through a 0.22-μm filter. Wells were created in the *A. tumefaciens* agar plates using a sterile borer and 60 μL of cell-free supernatant was deposited into each well.

Accessory nidamental gland tissue was tested for the presence of AHLs by dissecting three separate ANGs from mature females as described above. Each ANG was homogenized in 300 μL of squid Ringer's solution and the homogenate was centrifuged at 1,000 × *g* for 10 min to pellet the ANG tissue. The supernatant containing bacterial cells was removed and centrifuged again at 10,000 × *g* for 10 min and 60 μL of the resulting clarified homogenate was deposited in a well of the AHL detection plates. All AHL detection plates were incubated at 28°C and photographed after 48 h.

RESULTS AND DISCUSSION

The genomes sequenced in this study were of a typical size for roseobacters, ranging from 4.4 to 5.4 Mb (**Table 1**). These large genomes are typical of the many cultured and sequenced organisms of the *Roseobacter* clade and reflect the diverse metabolisms reported in these bacteria (Newton et al., 2010). These data suggest that there has been little gene loss (or genome decay) as a result of close association with a host. However, several uncultivated roseobacters have streamlined genomes and may have a different lifestyle than most cultured members of this group (Luo et al., 2012, 2014). Many combinations of gene clusters for plasmid replication and partitioning were detected, particularly *repABC* genes. These data suggest that the ANG isolates have several extrachromosomal elements that may be resolved pending further sequencing efforts.

TAXONOMIC ANALYSIS

Of the ANG isolates identified, there were nine *Leisingera* (ANG1, ANG-DT, ANG-S, ANG-S3, ANG-S5, ANG-M6, ANG-M7, ANG-Vp, and ANG-M1), two *Ruegeria* (ANG-R and ANG-S4), and one *Tateyamaria* (ANG-S1) isolates. The 33 gene phylogenetic reconstruction placed these ANG isolates in five well-supported clades (**Figure 2**). The *Leisingera* isolates all grouped together in a single strongly supported clade, sister to four other described *Leisingera* taxa. This placement supports their recent designation as members of the *Leisingera* genus (Breider et al., 2014). The two

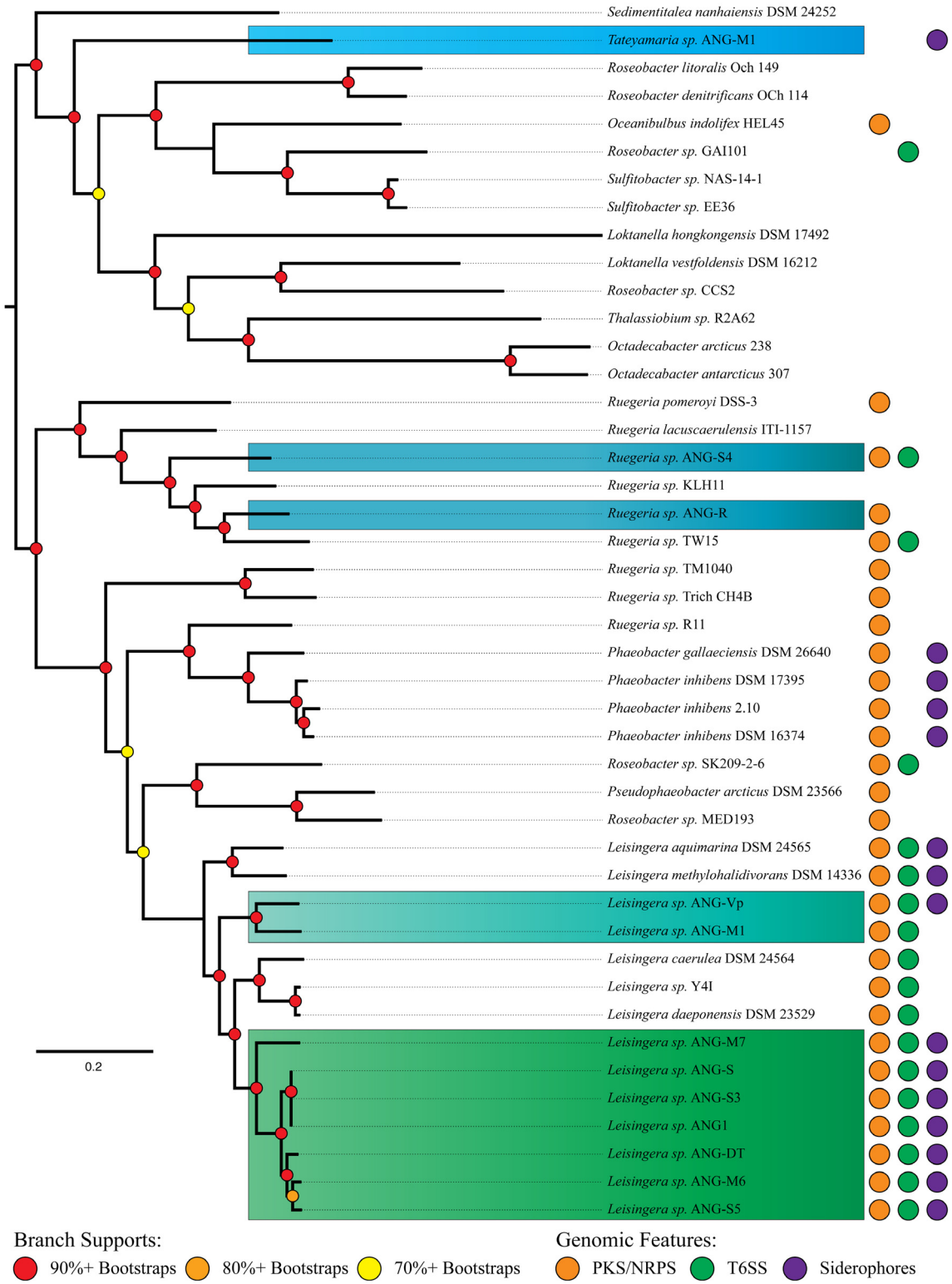


FIGURE 2 | MLSA analysis of *Roseobacter* clade isolates from the ANG with closely related organisms and distribution of significant gene clusters. Phylogenetic analysis of 33 single-copy housekeeping genes places most ANG isolates in the previously described "Clade 1" of the *Roseobacter* clade (Newton et al., 2010). A polyketide/non-ribosomal peptide synthase gene cluster is distributed throughout most of the *Roseobacter* clade, while Type VI secretion systems and siderophores are limited to fewer members. Isolates are color-coded to indicate genus. Blue, *Ruegeria*; Light blue, *Tateyamaria*; Teal and green, *Leisingera*.

Ruegeria ANG taxa did not place together, however, they are part of a clade composed of only *Ruegeria* taxa, affirming the putative genus designation. *Tateyamaria* placed on a basal branch long enough to suggest it is not closely associated with any of the taxa analyzed for this study.

The structure of the ribosomal tree (Supplmentary Figure 1) shares similarities with Newton et al.'s (2010) phylogeny. However, there are notable differences. First, the taxa of Newton's clade 3 are split into two separate clusters. Second, all but one member of Newton's clade 4 groups sister to clade 2. Finally, the two Rhodobacterales bacteria (HTCCs 2255 and 54623) fall among clades 2 and 4 rather than as part of the outgroups. The placement of clades 3 and 4 may be explained by the nature of gene concatenation. Concatenations can yield trees with high support values on topologies for which none of the constituents' gene phylogenies match (Salichos and Rokas, 2013; Colston et al., 2014). Gene choice can result in significantly different well-supported topologies. Thus, the averaged history of the ribosome may have been "outvoted" by the average history of the balance of Newton's seventy single-copy genes. The topology of the ribosomal tree was used to assign the root in the 33 gene tree (**Figure 2**) on the assumption that the ribosomal phylogeny was accurate in clade 4's placement. The clade 4 taxa could be used as outgroups to clades 1 and 2 instead with no significant change to the further analyses of the ANG isolates.

The structure of the 33 gene tree (**Figure 2**) compares well with Newton's phylogeny. Taxa previously identified as *Phaeobacter, Ruegeria, and Leisingera* formed polyphyletic clades. This occurrence was not unanticipated as the Newton et al. (2010) study showed a 70-gene tree with the same structure, albeit with fewer taxa. The genes analyzed in this study represent a subset of those analyzed in Newton et al. (2010) and therefore were expected to recapitulate this result. Our tree also aligns well with the recent reclassification by Breider et al. (2014). *Sedimentitalea nanhaiensis*, formerly *Leisingera nanhaiensis*, placed at the base of Newton's clade 2, which is separated from the balance of the *Leisingera* genus. *Pseudophaeobacter arcticus*, formerly *Phaeobacter arcticus*, fell in a clade sister to the *Leisingera*, also isolated from the newly redefined *Phaeobacter* genus. Thus, its reclassification resolves a polyphyly observed in our tree. Likewise, *L. caerulea* and *L. daeponensis*, also reclassified from the *Phaeobacter* genus, resolve a separate polyphyly. As these two taxa are sister to established *Leisingera*, we find reassigning them to this genus in line with our results. The only remaining question of polyphyly in our 33 gene phylogeny is *Ruegeria* sp. R11, which groups with the *Phaeobacter/Pseudophaeobacter/Leisingera* clade. This isolate has been proposed as *Nautella* based on 16S rDNA similarity to the *Nautella* type strain and may not be a member of the *Ruegeria* genus (Fernandes et al., 2011).

The phylogenetic analyses identified apparent relationships at approximately the genus level. In order to attempt to refine these results and provide species-level putative designations, ANI was employed using the accepted ANI cutoff of 95% (**Figure 3**, Konstantinidis et al., 2006; Richter and Rosselló-Móra, 2009). The ANG isolates fall into seven putative species groups. Six *Leisingera* isolates (ANG1, ANG-DT, ANG-S, ANG-S3, ANG-S5, and ANG-M6) formed one group, and three other *Leisingera* isolates (ANG-M7, ANG-M1, ANG-Vp) and the two *Ruegeria* isolates each formed its own singleton group. The *Leisingera* isolates are of particular interest as previous research has shown that the most common symbionts within the ANG belong to the genus *Leisingera,* though they were previously classified within the genus *Phaeobacter* (Collins and Nyholm, 2011; Collins et al., 2012). One putative species of *Leisingera* was consistently isolated from the five individual ANGs used in this study. This cluster of isolates likely represents the dominant culturable symbiont present in the ANG and includes the previously sequenced isolate, *Leisingera* sp. ANG1. Notably, the ANI values of the ANG isolates all fell short of even 90% identity with any of the previously described species. These data suggest that each of these putative ANG species is, indeed, a novel taxon. Providing comprehensive polyphasic species descriptions is beyond the scope of this work, so we propose these taxa as sp. of their various assigned genera.

RECLASSIFICATION OF *Phaeobacter gallaeciensis* ANG1

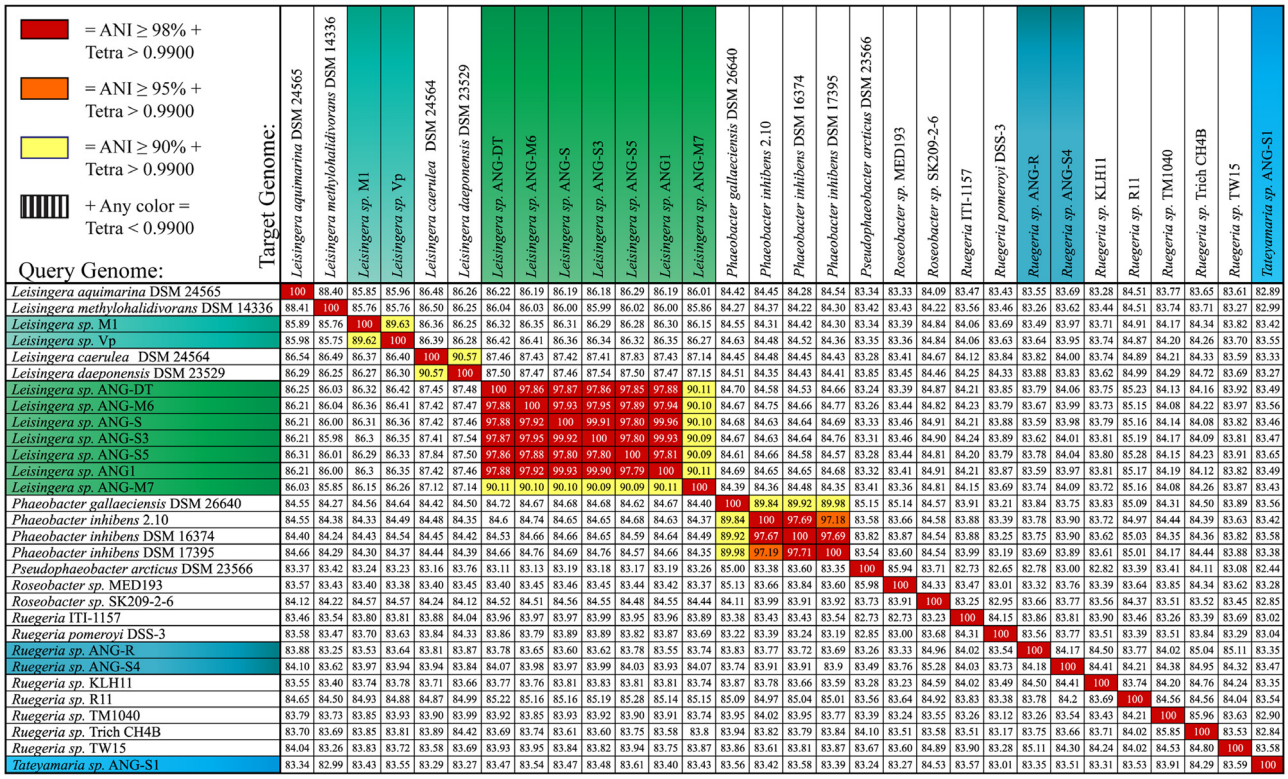
Consistent with previous research, our results suggest the isolate we had previously identified as *P. gallaeciensis* is phylogenetically distinct from the type species, *P. gallaeciensis* DSM 26640 (Thole et al., 2012; Breider et al., 2014). We therefore reclassify the isolate *P. gallaeciensis* ANG1 as *Leisingera* sp. ANG1 pending further phenotypic analyses.

GENOME CHARACTERISTICS AND GENERAL METABOLISM

Of the 12 ANG symbionts examined in this study, all have genes encoding a complete Entner–Doudoroff pathway for metabolizing glucose. Furthermore, all of them lack the gene for phosphofructokinase, a key enzyme from the Embden–Meyerhof–Parnas pathway. This is typical of many previously sequenced and complete genomes from the *Roseobacter* lineage (Moran et al., 2004; Newton et al., 2010; Wagner-Döbler et al., 2010). Two organisms (*Tateyamaria* sp. ANG-S1 and *Ruegeria* sp. ANG-S4) contain all genes for a complete pentose-phosphate pathway. The others contain most genes for the pathway, with the exception of a gene encoding 6-phosphogluconate dehydrogenase. As an alternative metabolic pathway, 6-phosphogluconate produced by the first two enzymes of the pentose phosphate pathway could feed into the Entner–Doudoroff pathway for further carbohydrate metabolism (Fuchs, 1999; Berger et al., 2014).

While the *Roseobacter* clade was first described as a group of obligate aerobic organisms, recently it has been shown that some members contain enzymes needed for anaerobic respiration of nitrate (Dogs et al., 2013). All of the isolates from the ANG contain the gene for nitrate reductase that could be used for anaerobic respiration of nitrogen. Most isolates, with the exception of *Tateyamaria* sp. ANG-S1, also contain genes for other denitrifying enzymes to further reduce nitrogenous oxyanions. These data suggest that the ANG isolates may be able to survive and thrive in anaerobic environments by respiring nitrogenous oxyanions.

Although genes associated with phototrophy were detected in *Tateyamaria* ANG-S1, including bacteriochlorophyll a, these genes were not detected in the other ANG isolates. These data are consistent with previous observations of Clade-1 roseobacters which



Query Genome:	Leisingera aquimarina DSM 24565	Leisingera methylohalidivorans DSM 14336	Leisingera sp. M1	Leisingera sp. Vp	Leisingera caerulea DSM 24564	Leisingera daeponensis DSM 23529	Leisingera sp. ANG-DT	Leisingera sp. ANG-M6	Leisingera sp. ANG-S	Leisingera sp. ANG-S3	Leisingera sp. ANG-S5	Leisingera sp. ANG1	Leisingera sp. ANG-M7	Phaeobacter gallaeciensis DSM 26640	Phaeobacter inhibens 2.10	Phaeobacter inhibens DSM 16374	Phaeobacter inhibens DSM 17395	Pseudophaeobacter arcticus DSM 23566	Roseobacter sp. MED193	Roseobacter sp. SK209-2-6	Ruegeria ITI-1157	Ruegeria pomeroyi DSS-3	Ruegeria sp. ANG-R	Ruegeria sp. ANG-S4	Ruegeria sp. KLH11	Ruegeria sp. R11	Ruegeria sp. TM1040	Ruegeria sp. Trich CH4B	Ruegeria sp. TW15	Tateyamaria sp. ANG-S1
Leisingera aquimarina DSM 24565	100	88.40	85.85	85.96	86.48	86.26	86.22	86.19	86.19	86.18	86.29	86.19	86.01	84.42	84.45	84.28	84.54	83.34	83.33	84.09	83.47	83.43	83.55	83.69	83.28	84.51	83.77	83.65	83.61	82.89
Leisingera methylohalidivorans DSM 14336	88.41	100	85.76	85.76	86.50	86.25	86.04	86.03	86.00	85.99	86.02	86.00	85.86	84.27	84.37	84.22	84.30	83.42	83.43	84.22	83.56	83.46	83.26	83.62	83.44	84.51	83.74	83.71	83.27	82.99
Leisingera sp. M1	85.89	85.76	100	89.63	86.36	86.25	86.32	86.35	86.31	86.29	86.28	86.30	86.15	84.55	84.31	84.42	84.30	83.34	83.39	84.84	84.06	83.69	83.49	83.97	83.71	84.91	84.17	84.34	83.82	83.42
Leisingera sp. Vp	85.98	85.75	89.62	100	86.39	86.28	86.42	86.41	86.36	86.34	86.32	86.35	86.27	84.63	84.48	84.52	84.36	83.35	83.36	84.84	84.06	83.63	83.64	83.95	83.74	84.87	84.20	84.26	83.70	83.55
Leisingera caerulea DSM 24564	86.54	86.49	86.37	86.40	100	90.57	87.46	87.43	87.42	87.41	87.83	87.43	87.14	84.45	84.48	84.45	84.43	83.28	83.41	84.67	84.12	83.84	83.82	84.00	83.74	84.89	84.21	84.33	83.59	83.33
Leisingera daeponensis DSM 23529	86.29	86.25	86.27	86.30	90.57	100	87.50	87.47	87.46	87.54	87.50	87.47	87.15	84.51	84.35	84.43	84.41	83.85	83.45	84.46	84.25	84.33	83.88	83.83	83.62	84.99	84.29	84.72	83.69	83.27
Leisingera sp. ANG-DT	86.25	86.03	86.32	86.42	87.45	87.48	100	97.86	97.87	97.86	97.85	97.88	90.11	84.70	84.58	84.53	84.66	83.24	83.39	84.87	84.21	83.85	83.79	84.06	83.75	85.23	84.13	84.16	83.92	83.49
Leisingera sp. ANG-M6	86.21	86.04	86.36	86.41	87.42	87.47	97.88	100	97.93	97.95	97.89	97.94	90.10	84.67	84.75	84.66	84.77	83.26	83.44	84.82	84.23	83.79	83.67	83.99	83.73	85.15	84.08	84.22	83.97	83.56
Leisingera sp. ANG-S	86.21	86.00	86.31	86.36	87.42	87.46	97.88	97.92	100	99.91	97.80	99.96	90.10	84.68	84.63	84.64	84.69	83.33	83.46	84.91	84.21	83.88	83.59	83.98	83.79	85.16	84.14	84.08	83.82	83.46
Leisingera sp. ANG-S3	86.21	85.98	86.3	86.35	87.41	87.54	97.87	97.95	99.92	100	97.80	99.93	90.09	84.67	84.63	84.64	84.76	83.31	83.46	84.90	84.24	83.89	83.62	84.01	83.81	85.19	84.17	84.09	83.81	83.47
Leisingera sp. ANG-S5	86.31	86.01	86.29	86.33	87.84	87.50	97.86	97.88	97.80	97.80	100	97.81	90.09	84.61	84.66	84.58	84.57	83.28	83.44	84.81	84.20	83.79	83.78	84.04	83.80	85.28	84.15	84.23	83.91	83.65
Leisingera sp. ANG1	86.21	86.00	86.3	86.35	87.42	87.46	97.88	97.92	99.93	99.90	97.79	100	90.11	84.69	84.65	84.65	84.68	83.32	83.41	84.91	84.21	83.87	83.59	83.97	83.81	85.17	84.19	84.12	83.82	83.49
Leisingera sp. ANG-M7	86.03	85.85	86.15	86.26	87.12	87.14	90.11	90.10	90.10	90.09	90.09	90.11	100	84.39	84.36	84.48	84.35	83.41	83.36	84.81	84.15	83.69	83.74	84.09	83.72	85.16	84.08	84.26	83.87	83.43
Phaeobacter gallaeciensis DSM 26640	84.55	84.27	84.56	84.64	84.42	84.50	84.72	84.67	84.68	84.68	84.62	84.67	84.40	100	89.84	89.92	89.98	85.15	85.14	84.57	83.91	83.21	83.84	83.75	83.83	85.09	84.31	84.50	83.89	83.56
Phaeobacter inhibens 2.10	84.55	84.38	84.33	84.49	84.48	84.35	84.6	84.74	84.65	84.65	84.68	84.63	84.37	89.84	100	97.69	97.18	83.58	83.66	84.58	83.88	83.39	83.78	83.90	83.72	84.97	84.44	84.39	83.63	83.42
Phaeobacter inhibens DSM 16374	84.40	84.24	84.43	84.54	84.45	84.42	84.53	84.66	84.66	84.65	84.59	84.64	84.49	89.92	97.67	100	97.69	83.82	83.87	84.54	83.88	83.25	83.75	83.90	83.62	85.03	84.35	84.36	83.82	83.58
Phaeobacter inhibens DSM 17395	84.66	84.29	84.30	84.37	84.44	84.39	84.66	84.76	84.69	84.76	84.57	84.66	84.35	89.98	97.19	97.71	100	83.54	83.60	84.54	83.99	83.19	83.89	83.89	83.61	85.01	84.17	84.44	83.88	83.38
Pseudophaeobacter arcticus DSM 23566	83.37	83.42	83.24	83.23	83.16	83.76	83.11	83.13	83.19	83.18	83.17	83.19	83.26	85.00	83.38	83.60	83.35	100	85.94	83.71	82.73	82.65	82.78	83.00	82.82	83.39	83.41	84.11	83.08	82.44
Roseobacter sp. MED193	83.57	83.43	83.40	83.38	83.40	83.45	83.40	83.45	83.46	83.45	83.44	83.42	83.37	85.13	83.66	83.84	83.60	85.98	100	84.33	83.47	83.01	83.32	83.76	83.39	83.64	83.85	84.34	83.62	83.28
Roseobacter sp. SK209-2-6	84.12	84.22	84.57	84.57	84.24	84.12	84.52	84.51	84.56	84.55	84.48	84.55	84.44	84.11	83.99	83.91	83.92	83.73	83.91	100	83.25	82.95	83.66	83.77	83.56	84.37	83.51	83.52	83.45	82.85
Ruegeria ITI-1157	83.46	83.54	83.80	83.81	83.88	84.04	83.96	83.97	83.97	83.99	83.95	83.96	83.89	83.38	83.43	83.43	83.54	82.73	82.73	83.23	100	84.15	83.86	83.81	83.90	83.46	83.26	83.39	83.69	83.02
Ruegeria pomeroyi DSS-3	83.58	83.47	83.70	83.63	83.84	84.33	83.86	83.79	83.89	83.89	83.82	83.87	83.69	83.22	83.39	83.24	83.19	82.85	83.00	83.68	84.31	100	83.56	83.77	83.51	83.39	83.51	83.84	83.29	83.04
Ruegeria sp. ANG-R	83.88	83.25	83.53	83.64	83.81	83.87	83.78	83.65	83.60	83.62	83.78	83.55	83.74	83.83	83.77	83.72	83.69	83.26	83.33	84.96	84.02	83.54	100	84.17	84.50	83.77	84.02	85.04	85.11	83.35
Ruegeria sp. ANG-S4	84.10	83.62	83.97	83.94	83.94	83.84	84.07	83.98	83.97	83.99	84.03	83.93	84.07	83.74	83.91	83.91	83.9	83.49	83.76	85.28	84.03	83.73	84.18	100	84.41	84.21	84.38	84.95	84.32	83.47
Ruegeria sp. KLH11	83.55	83.40	83.74	83.78	83.71	83.66	83.77	83.76	83.81	83.83	83.81	83.81	83.74	83.87	83.78	83.66	83.59	83.28	83.23	84.59	84.02	83.49	84.50	84.41	100	83.74	84.20	84.76	84.24	83.35
Ruegeria sp. R11	84.65	84.50	84.93	84.88	84.87	84.99	85.22	85.16	85.16	85.19	85.28	85.14	85.15	85.09	84.97	85.04	85.01	83.56	83.64	84.92	83.83	83.38	83.78	84.2	83.69	100	84.56	84.56	84.04	83.54
Ruegeria sp. TM1040	83.79	83.73	83.85	83.93	83.90	83.99	83.92	83.85	83.93	83.92	83.90	83.91	83.74	83.95	84.02	83.95	83.77	83.39	83.24	83.55	83.26	83.12	83.26	83.54	83.43	84.21	100	85.96	83.63	82.90
Ruegeria sp. Trich CH4B	83.70	83.69	83.85	83.81	83.89	84.42	83.69	83.74	83.61	83.60	83.75	83.58	83.8	83.94	83.82	83.79	83.84	84.10	83.51	83.58	83.51	83.17	83.75	83.66	83.71	84.02	85.85	100	83.53	82.84
Ruegeria sp. TW15	84.04	83.26	83.83	83.72	83.58	83.69	83.93	83.95	83.84	83.82	83.94	83.75	83.87	83.86	83.61	83.81	83.87	83.67	83.60	84.89	83.90	83.28	85.11	84.30	84.24	84.02	84.53	84.80	100	83.58
Tateyamaria sp. ANG-S1	83.34	82.99	83.43	83.55	83.29	83.27	83.47	83.54	83.47	83.48	83.61	83.40	83.43	83.56	83.42	83.58	83.39	83.24	83.27	84.53	83.57	83.01	83.35	83.51	83.31	83.53	83.91	84.29	83.59	100

FIGURE 3 | Average nucleotide identity (ANI) comparison shows seven unique isolates from the ANG. Six of the isolates cultured from the ANG are highly similar, sharing >98% ANI (ANG-DT, ANG-M6, ANG-S, ANG-S3, ANG-S5, and ANG1). These isolates dominate the culturable isolates of the ANG and have been consistently isolated from five different animals. These isolates appear to be a novel species while isolate ANG-M7 may be a second novel *Leisingera* taxon. Isolates ANG-Vp and ANG-M1 share 90% ANI but each appear to represent a novel taxon. The other isolates (ANG-R, ANG-S4, and ANG-S1) are unique from other sequenced roseobacters. Isolates are color-coded to indicate genus. Blue, *Ruegeria*; Light blue, *Tateyamaria*; Teal and green, *Leisingera*.

were not found to be phototrophic (Newton et al., 2010; Luo and Moran, 2014).

PROTEIN SECRETION SYSTEMS

While a Type IV secretion system is present in many roseobacters, we detected *virB* in only two of the genomes examined here (ANG-M1 and ANG-R). Previous literature has suggested these systems are used for communication between bacteria and eukaryotic cells (Luo and Moran, 2014). However, given that a large proportion of isolates from the ANG appear to lack this system, the T4SS may not be a critical means of communication between the consortium and its host.

An interesting feature of the *Leisingera* genus is that all sequenced genomes contain genes for a Type VI secretion system (T6SS, **Figure 2**). In *L. daeponensis* and *L. caerulea* it has been shown that this T6SS exists on a plasmid (Beyersmann et al., 2013; Dogs et al., 2013). In *Leisingera* sp. ANG1 the T6SS is located on a large contig (>500 kb) containing *repAB* plasmid partitioning genes, suggesting that the T6SS in this species is also located on a plasmid. Similar conclusions were reached with the genomes of *L. caerulea, L. daeponensis, L. methylohalidivorans,* and *L. aquimarina.* Each of these organisms has genes for a T6SS on plasmids that vary in size (from 109 kB in *L. caerulea* to 526 kB in *Leisingera* sp. ANG1); however, all have a DnaA 1-like replicase (Beyersmann et al., 2013; Buddruhs et al., 2013; Dogs et al., 2013; Riedel et al., 2013). While other roseobacters contain a T6SS, the conservation of the T6SS on similar plasmids could be characteristic of this genus.

Several functions of the T6SS have been proposed, including antimicrobial roles, as evidenced by direct cell-contact mediated killing (Murdoch et al., 2011; Russell et al., 2011). The T6SS has also been shown to be involved with host–microbe interactions, particularly in the *Rhizobiales. A. tumefaciens* shows attenuated ability to create crown gall tumors when the T6SS is deleted (Wu et al., 2008). Similarly, the nitrogen-fixing plant symbiont *Rhizobium leguminosarum* lacking a T6SS will successfully colonize its host, however, it will fail to fix nitrogen (Bladergroen et al., 2003). The T6SS has also been implied in many other general associations between microorganisms, including predator evasion (Pukatzki et al., 2006) and self/non-self recognition (Gibbs et al., 2008).

It is interesting that all of the isolates, with one exception (*Ruegeria* sp. ANG-R), have genes for a T6SS, including isolates outside of the *Leisingera* genus. This suggests that the T6SS in these bacteria may be important for communication with the host and/or with other bacteria. In the ANG of *E. scolopes,* bacteria are housed in high densities within the epithelium-lined tubules

of the organ (Collins et al., 2012). Such high densities of bacterial cells foster close contact with other bacteria and many host cells, including the ANG epithelium and hemocytes, the principle cellular innate immunity component of the host. Given that the T6SS functions by direct cell-to-cell contact, it would be an ideal mechanism for the delivery of effectors directly to other symbionts and/or host tissues. The T6SS may play a role in mediating how these organisms are selected from the environment and explain how some species are able to dominate the bacterial populations within a given tubule (Collins et al., 2012).

SECONDARY METABOLITES

Members of the *Roseobacter* clade have been shown to produce several unique secondary metabolites. Some of the most notable ones include antibacterials such as TDA, produced by organisms such as *P. inhibens* and *Ruegeria* sp. TM1040, and the blue pigment indigoidine, produced by organisms such as *Leisingera* sp. Y4I and *L. daeponensis* (Geng et al., 2008; Cude et al., 2012). None of the biosynthetic genes for either of these compounds were found in any of the genomes sequenced. Furthermore, no classical antibiotic synthesis pathways (e.g., tetracycline, carbapenems, etc.) were found.

However, analysis with the Antibiotic and Secondary Metabolite Analysis Shell (AntiSMASH, Blin et al., 2013) revealed several gene clusters encoding potential secondary metabolism (**Table 2**). These included gene clusters for siderophore synthesis, autoinducer synthases (*luxI* homologs), polyketide/non-ribosomal peptide synthases (PKS/NRPS) and production of volatile compounds such as terpenes. The BActeriocin GEnome mining tooL (BAGEL, van Heel et al., 2013), was used to screen genomes for possible bacteriocin producing gene clusters, which were found in the *Ruegeria* isolates (ANG-R and ANG-S4) as well as *Tateyamaria* sp. ANG-S1 (**Table 2**). Bacteriocins are a broad group of proteins that can be used to kill other bacteria but have also been shown to act as inducers of invertebrate metamorphosis and thus may serve a number of functions (Cotter et al., 2013; Shikuma et al., 2014).

All isolates have a conserved non-ribosomal peptide/polyketide synthase gene cluster characterized previously (**Table 2**, Martens et al., 2006). This gene cluster is conserved in the *Roseobacter* lineage, being found in 28 of 57 previously sequenced genomes, and is comprised of four genes: a non-ribosomal polypeptide synthase, a polyketide synthase, a glycosyltransferase and a phosphopantetheinyl transferase. However, the product of this gene cluster has not yet been characterized. Given that this gene cluster is well-conserved throughout the *Roseobacter* lineage, its product and function should be elucidated through future experiments.

QUORUM SENSING

Homoserine lactones produced by LuxI homologs have been widely studied as quorum sensing molecules in bacteria, including the *luxIR* system of *V. fischeri*, the light organ symbiont of *E. scolopes* (Antunes et al., 2007; Miyashiro and Ruby, 2012). AntiSMASH detected 2 separate pairs of *luxIR* homologs in the ANG isolates that were most similar to the *ssaIR* and *ssbIR* previously described in *Ruegeria* sp. KLH11 (Zan et al., 2012). However, only the *Ruegeria* isolates, ANG-S4 and ANG-R, have both pairs of *luxIR* homologs. Most of the ANG bacteria only have homologs of *ssbIR*. In *Ruegeria* sp. KLH11, these two systems work together to control biofilm formation and motility (Zan et al., 2012). The genes *ssaI* and *ssaR*, are shown to regulate the change between adherent and planktonic lifestyles. Increased levels of HSLs promote flagellar growth and motility, while lower levels foster biofilm development. The actions of these genes can be indirectly repressed by *ssbIR*. The fact that so many ANG isolates have only the *ssbIR* homologs suggest that there may be a unique function for these quorum sensing genes independent of the *ssaIR* quorum sensing system. In addition to *ssbIR*, the ubiquitous *luxIR* homologs in the *Roseobacter* genomes from the ANG are also similar to the *raiIR* genes described in *Rhizobium etli* (Rosemeyer et al., 1998). Both SsbIR and RaiIR are known to produce 3-hydroxyl-HSL compounds, but *raiIR* has been shown to control growth and nitrogen fixation, not motility. This raises the possibility that the *luxIR* genes in ANG roseobacters may regulate growth of bacteria within the ANG.

Table 2 | Secondary metabolite gene clusters detected with AntiSMASH and BAGEL.

	PKS/NRPS	LuxRI	Bacteriocin	Siderophore	Terpene	Ectoine
Leisingera sp. ANG-Vp	1	1	0	1	0	1
Leisingera sp. ANG-M1	1	1	0	0	0	1
Leisingera sp. ANG1	1	1	0	1	0	0
Leisingera sp. ANG-DT	1	1	0	1	0	0
Leisingera sp. ANG-S	1	1	0	1	0	0
Leisingera sp. ANG-S3	1	1	0	1	0	0
Leisingera sp. ANG-M6	1	1	0	1	0	0
Leisingera sp. ANG-S5	1	1	0	1	0	0
Leisingera sp. ANG-M7	1	1	0	1	0	0
Ruegeria sp. ANG-R	1	2	3	0	0	1
Ruegeria sp. ANG-S4	2	2	3	0	0	0
Tateyamaria sp. ANG-S1	0	1	2	1	1	0

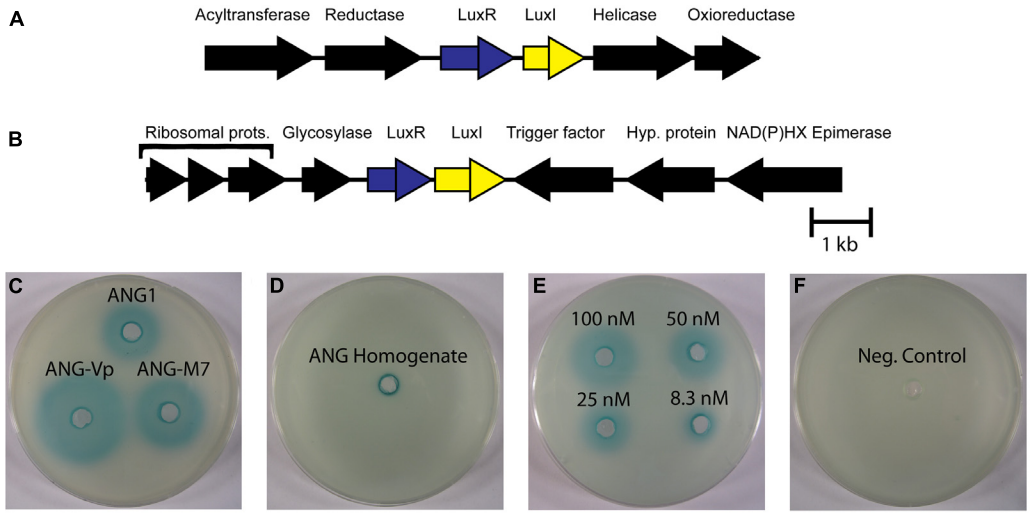


FIGURE 4 | LuxIR homologs in *Roseobacter* clade organisms from the ANG and associated homoserine lactone production. (A) All ANG isolates have a pair of *luxIR* homologs flanked by potential anabolic genes (crontonyl CoA reductase and acetyltransferase) and a helicase and oxioreductase. **(B)** Another pair of *luxIR* homologs is present only in the *Ruegeria* species (isolates ANG-S4 and ANG-R) flanked by ribosomal proteins, a cell division trigger factor and a glycosylase. **(C)** Representative picture of β-galactosidase activity induced by homoserine lactone in supernatants from cultures of *Leisingera* sp. ANG1, *Leisingera* sp. ANG-M7, and *Leisingera* sp. ANG-Vp. **(D)** Homoserine lactones were also detected in ANG homogenates (representative image, $n = 3$ separate ANGs). **(E)** Semi-quantitative dilution of N-3-oxo-hexanoyl-homoserine lactone. **(F)** 60 μL growth medium (Negative control).

To determine if HSLs are present in the ANG and are produced by the bacterial symbionts, we tested for the presence of AHLs using a semi-quantitative biosensor assay. All isolates that could grow to high density in liquid medium produced detectable HSLs (**Figure 4**). Species like *Tateyamaria* sp. S1 did not grow to a very high density and failed to produce enough HSL to be detected by the assay (not shown). The homogenates of three ANGs were also tested and resulted in small zones of β-galactosidase activity around the assay wells, suggesting that HSLs are produced in the ANG and could contribute to the symbiosis by influencing gene expression of the bacterial consortium. As a negative control, host gill tissue was also homogenized in a similar manner to ensure that compounds from squid tissue were not inducing expression of β-galactosidase in the *A. tumefaciens* biosensor. No enzymatic activity was observed in this control (not shown), confirming the specificity of the assay.

While HSLs were detected in both pure culture and in ANG homogenate, gene regulation by HSL quorum sensing may be different than what has been described for their nearest homologs in *Ruegeria* sp. KLH11. Most ANG isolates, including the dominant *Leisingera* species, lack the *ssaIR* homologs directly responsible for the increase of motility described in *Ruegeria* sp. KLH11. This suggests there is a yet undescribed role for the *ssbIR* homologs in the *Roseobacter* clade isolates from the ANG.

Future research should investigate the chemical nature of the HSL produced by the autoinducer synthases in individual ANG isolates. The nearest characterized homologs, both RaiI in *R. etli* and SsbI in *Ruegeria* sp. KLH11 produce 3-hydroxyl-HSL compounds (Rosemeyer et al., 1998; Zan et al., 2012). Future studies should confirm that the members of the ANG consortium also produce this type of HSL. Genetics have not yet been developed in any of the cultured ANG isolates, however, creating a non-functioning mutant of the autoinducer synthase could reveal phenotypes controlled by quorum sensing. Comparing transcriptomes between HSL^- and wild type strains may also reveal genes that are controlled by quorum sensing. Moreover, previous research has shown that these symbionts are likely environmentally transmitted (Kaufman et al., 1998). Thus, the symbionts encounter three environments of varying cell density, from ambient seawater with a low density of symbionts, to the tubules of the ANG where the cells are highly concentrated, to the egg jelly coat with a lower density. Given the profound differences in cell density between free-living symbionts in seawater and the tubules of an ANG, quorum sensing may be an ideal mechanism for gene regulation between the different environments experienced by ANG bacteria (host/ANG, egg, free-living). Further studies should also examine how gene expression changes from the high-cell density environment of the ANG to the egg jelly coat, where cell densities will be lower, but where any anti-fouling compounds may be produced.

SIDEROPHORES

Another group of secondary metabolite biosynthesis genes that was detected in the genomes of ANG isolates were siderophores. Siderophores are small molecules with high affinities for iron and can be used by bacteria for iron scavenging. Iron is needed for many cellular functions, including respiration, detoxification of reactive oxygen species (e.g., catalases, super-oxidase dismutase), and metabolism (e.g., aconitase of the TCA cycle). Very few organisms are known to survive without iron (Andrews et al., 2003). One way that bacteria can acquire iron in environments where it is a limiting resource is by producing siderophores to sequester iron from other sources.

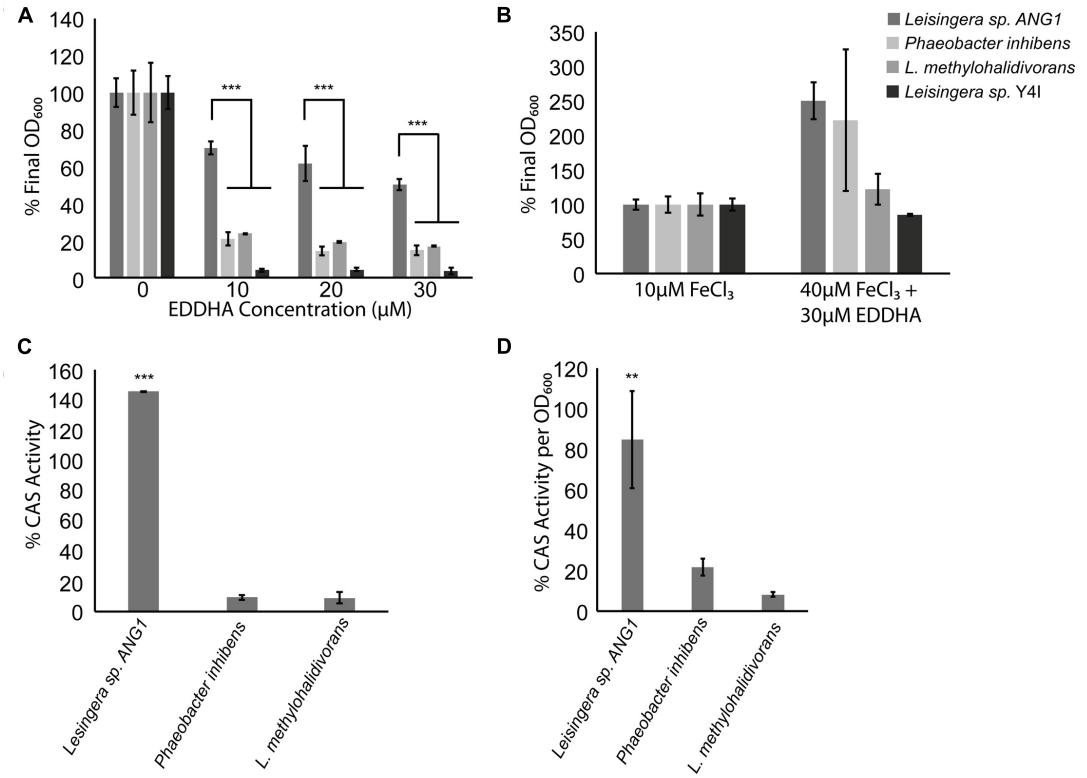


FIGURE 5 | *Roseobacter* clade symbionts from the ANG have a growth advantage in iron-limiting conditions, possibly due to siderophore production. (A) While other *Roseobacter* clade organisms were inhibited by the presence of an iron-chelator, *Leisingera* sp. ANG1 grew to more than 50% of its optical density even if EDDHA was at three times the concentration of available iron. **(B)** EDDHA is not toxic to *Roseobacter* clade organisms, as adding enough iron to overwhelm the chelator restored the growth defect to all *Roseobacter* clade organisms. **(C)** *Leisingera* sp. ANG1 produced more siderophore than non-ANG isolates in the presence of EDDHA. **(D)** Even if no iron chelator were present, siderophores were more abundant in the supernatants from cultures of *Leisingera* sp. ANG1. ** $p < 0.01$, *** $p < 0.001$.

Siderophore synthesis genes in the *Roseobacter* clade are rare. Of previously sequenced *Roseobacter* genomes, only six genomes from four species (*L. aquimarina*, *L. methylohalidivorans*, *P. inhibens,* and *P. gallaeciensis*) are predicted to have siderophore synthesis genes (**Figure 2**). However, all roseobacters isolated from the ANG of *E. scolopes*, with the exception of *Ruegeria* sp. ANG-R and ANG-S4, have either siderophore biosynthesis genes or showed siderophore activity in biochemical assays (**Table 2** and **Figure 5**). For example, *Leisingera* sp. ANG-M1 had no predicted siderophore synthesis genes, but siderophore activity was detected when grown on CAS agar and in CAS liquid assays, suggesting that these biosynthetic genes may not be annotated, perhaps due to the fragmented state of the assembled genome for this isolate. Conversely, *Tateyamaria* sp. ANG-S1 has siderophore biosynthetic genes, but failed to show siderophore activity (not shown). Taken together, these data suggest induction of siderophore synthesis genes may be controlled very differently in *Tateyamaria* sp. ANG-S1 and may be induced only under specific conditions.

We compared growth and siderophore production in iron-limiting conditions of *Leisingera* sp. ANG1, a representative of the dominant ANG symbionts, to three other species from the *Roseobacter* lineage. Siderophore-producing strains *P. inhibens* DSMZ 17395 and *L. methylohalidivorans* DSM 14336 were tested along with the non-siderophore producing strain *Leisingera* sp. Y4I. When grown in the presence of the iron chelator EDDHA, most roseobacters had a growth defect, growing to only 20% of the control density (**Figure 5A**). However, *Leisingera* sp. ANG1 had a much smaller growth defect ($p < 0.001$), growing to greater than 50% of the control OD when concentrations of EDDHA were three times the concentration of available iron in the media (**Figure 5A**). To show this was not due to a toxic effect of EDDHA, $FeCl_3$ was added to higher concentrations (40 μM) to overwhelm the iron chelator, which restored the growth of all organisms (**Figure 5B**).

The survival of *Leisingera* sp. ANG1 under iron-limiting conditions could be due to the higher levels of siderophores produced by these organisms. Supernatants from cultures of strains that failed to grow (*P. inhibens* and *L. methylohalidivorans*) showed very little CAS activity while supernatants from cultures of ANG1 had very high levels of CAS activity, indicative of a high concentration of siderophores ($p < 0.001$, **Figure 5C**). To determine if this increase was a consequence of the increased growth of *Leisingera* ANG1, CAS activity was measured in supernatants from cultures without any iron chelator added. This allowed the bacteria to grow and deplete the iron available in the media, leading to induction of

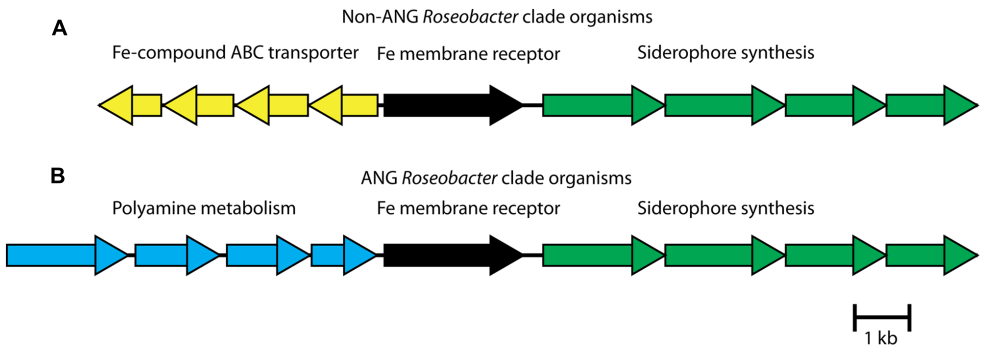


FIGURE 6 | *Roseobacter* isolates from the ANG have a unique genome rearrangement upstream of siderophore biosynthesis group. (A) In previously sequenced *Leisingera* and *Phaeobacter* species, an ABC transporter, predicted to transport iron compounds like iron dictate, lies upstream of a membrane receptor protein and the siderophore synthesis gene cluster. **(B)** In ANG bacteria, polyamine metabolism genes replace the ABC transporter genes. In addition to modifying regulatory elements, the four polyamine genes could synthesize a polyamine backbone of the siderophores, such as putrescine.

siderophore synthesis. Supernatants from cultures of *Leisingera* sp. ANG1 had more CAS activity than either *P. inhibens* DSM17395 or *L. methylohalidivorans* DSM14336 per unit OD_{600} ($p < 0.01$, **Figure 5D**). These data suggest that the abundance of siderophores produced by *Leisingera* sp. ANG1 is not just due to an increase in cell number, but instead to increased siderophore production at the cellular level.

Examining the siderophore biosynthesis genes in roseobacters isolated from the ANG, revealed a unique genome rearrangement (**Figure 6**). In all other siderophore-producing roseobacters, siderophore synthesis genes are located downstream of an iron membrane receptor and an iron-compound ABC transporter. In roseobacters isolated from the ANG, four genes related to polyamine metabolism are inserted upstream of the iron membrane receptor (**Figure 6**). The polyamine genes upstream of the siderophore synthesis cluster are sufficient to synthesize putrescine, a backbone of certain catechol siderophores such as photobactin from *Photorhabdus luminescens* (Ciche et al., 2003). Testing the supernatant of *Leisingera* sp. ANG1 with the Arnow assay showed that catechol siderophores were being produced. This genome rearrangement may be responsible for the higher production of siderophores in *Leisingera* sp. ANG1, perhaps by altering the regulatory elements upstream of the siderophore biosynthesis genes or perhaps by coupling the production of putrescine and the catecholate siderophore. Future research may determine if putrescine is a structural component of the catechol siderophores produced by the ANG symbionts such as the dominant *Leisingera* symbionts.

Producing siderophores can be beneficial to bacteria that colonize animal tissues. Iron-chelating proteins produced by hosts can effectively deplete freely available iron to the associated microbiota (Ong et al., 2006). Furthermore, a host infected with a pathogen will sometimes increase production of iron-chelating proteins as a way to starve infectious bacteria of a critical resource (Jurado, 1997). One of the most-widely studied models is the siderophore enterobactin which is produced by several species of enteric bacteria, including *Salmonella* and *Escherichia* species (Raymond et al., 2003). This iron-chelating molecule acquires iron from serum proteins carrying iron, such as transferrin, and the siderophore-iron complex is taken up by the infecting bacteria to keep them supplied with iron. To combat this, the innate immune system produces proteins to bind siderophores in order to prevent the iron-scavenging molecules from fulfilling their purpose (Goetz et al., 2002; Abergel et al., 2008).

In invertebrates, iron sequestration can be performed by two ubiquitous proteins, ferritin, and transferrin. Ferritin is present in the hemolymph of invertebrates where it can function as an iron transporter or iron scavenger (Ong et al., 2005) and transferrin is up-regulated in insect epithelia during bacterial infection (Buchon et al., 2009; Wang et al., 2009). Both of these proteins have been found in transcriptomic and proteomic data from both hemocytes and light organ tissues of *E. scolopes* (Schleicher and Nyholm, 2011, Collins, unpublished data). These iron chelators, if present in the ANG, could provide a selective pressure that other roseobacters would have to overcome. In such a case, siderophore-producing organisms such as *Leisingera* sp. ANG1 may have an advantage over other bacteria and this may contribute to its dominance in the consortium. Colonization of cephalopod ANGs is likely via environmental transmission (Kaufman et al., 1998) and overcoming iron-limitation may be one part of what is likely a complex process for establishment and development of the association.

The function of the ANG and its bacterial consortium remains unknown even though it was hypothesized that the bacteria deposited in the jelly coats of squid eggs may play a role in protecting the egg masses from fouling, possibly through the production of antimicrobial compound(s) (Biggs and Epel, 1991). Previous research in the eggs of the shrimp (*Palaemon macrodactylus*) have shown that, once the eggs are brooded, *Alteromonas* sp. bacteria colonize the surface of the egg and produce the antimicrobial compound 2, 3-indolinedione that protects the eggs from fungal infection (Gil-Turnes et al., 1989). However, shrimp eggs acquire these epibionts from seawater which is an important distinction from squid eggs, where the bacterial symbionts from the ANG are actively deposited into jelly coat layers. Future research will attempt to understand the role of

these bacteria within the eggs of developing embryos and try to discern what contribution they may make to deter fouling organisms.

This study sets the foundation for future research on the ANG symbionts by characterizing the genomes of several isolates from the *Roseobacter* lineage. We have identified many features of these genomes that may be important in the ANG association including Type VI secretion systems, siderophore production and putative quorum sensing systems using HSLs. The ANG and associated roseobacters are found worldwide in many different cephalopod species. This trend suggests that the consortium may play a similar and conserved role in squid and cuttlefish. Future research will hopefully elucidate the contribution of these bacteria to the development and survival of cephalopods and their embryos. Genome analyses of the *Roseobacter* clade bacteria that dominate the ANG, along with future genomic and transcriptomic studies of other ANG symbionts and the entire consortium will provide a number of exciting avenues of research to help elucidate the nature of this widely distributed association.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00123/abstract

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Diversity and Partitioning of Bacterial Populations within the Accessory Nidamental Gland of the Squid Euprymna scolopes

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Diversity and Partitioning of Bacterial Populations within the Accessory Nidamental Gland of the Squid *Euprymna scolopes*

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Microbial consortia confer important benefits to animal and plant hosts, and model associations are necessary to examine these types of host/microbe interactions. The accessory nidamental gland (ANG) is a female reproductive organ found among cephalopod mollusks that contains a consortium of bacteria, the exact function of which is unknown. To begin to understand the role of this organ, the bacterial consortium was characterized in the Hawaiian bobtail squid, *Euprymna scolopes*, a well-studied model organism for symbiosis research. Transmission electron microscopy (TEM) analysis of the ANG revealed dense bacterial assemblages of rod- and coccus-shaped cells segregated by morphology into separate, epithelium-lined tubules. The host epithelium was morphologically heterogeneous, containing ciliated and nonciliated cells with various brush border thicknesses. Hemocytes of the host's innate immune system were also found in close proximity to the bacteria within the tubules. A census of 16S rRNA genes suggested that *Rhodobacterales*, *Rhizobiales*, and *Verrucomicrobia* bacteria were prevalent, with members of the genus *Phaeobacter* dominating the consortium. Analysis of 454-shotgun sequencing data confirmed the presence of members of these taxa and revealed members of a fourth, *Flavobacteria* of the *Bacteroidetes* phylum. 16S rRNA fluorescent *in situ* hybridization (FISH) revealed that many ANG tubules were dominated by members of specific taxa, namely, *Rhodobacterales*, *Verrucomicrobia*, or *Cytophaga-Flavobacteria-Bacteroidetes*, suggesting symbiont partitioning to specific host tubules. In addition, FISH revealed that bacteria, including *Phaeobacter* species from the ANG, are likely deposited into the jelly coat of freshly laid eggs. This report establishes the ANG of the invertebrate *E. scolopes* as a model to examine interactions between a bacterial consortium and its host.

Many aquatic and marine invertebrates, including some cephalopods (squid, octopuses, and cuttlefish), lay their eggs in clutches or masses on benthic substrates, where they take weeks or even months to develop before hatching (2, 5, 12, 33). During this time, the developing embryos are unprotected, and prior observations suggest these egg clutches resist predation and/or fouling by microorganisms, although clear mechanisms for this resistance have yet to be described. Sexually mature females of some species have an accessory nidamental gland (ANG), a reproductive organ that houses a dense consortium of bacteria in pigmented epithelium-lined tubules and is attached to the nidamental gland (NG), the organ that secretes the jelly coat surrounding fertilized eggs (6). Culture-dependent and -independent methods have identified the dominant members of these microbial communities for some squid species (3, 6, 25, 39). All squid ANGs examined to date are dominated by alphaproteobacteria, usually members of the *Roseobacter* clade within the *Rhodobacterales* (6, 16, 39) with additional members belonging to the *Gammaproteobacteria* (vibrios, pseudoalteromonads, and pseudomonads) and the *Bacteroidetes*. Similar taxonomic groups were also found in the egg casings of the squid *Loligo pealei*, suggesting that the ANG serves to inoculate the egg clutches with a bacterial population (6). Although the exact role of these consortia has not been determined, those past studies suggest a symbiotic relationship between these bacteria and their hosts that should be investigated further.

In this study, we examined the accessory nidamental gland of the Hawaiian bobtail squid, *Euprymna scolopes* (Fig. 1). The symbiosis between *E. scolopes* and the bioluminescent bacterium *Vibrio fischeri* is used as a model system to study the effects of beneficial bacteria on the development of animal host tissues (26, 29, 30, 35). Adult *E. scolopes* squid can easily be collected and bred in the laboratory and are readily accessible to use as experimental animals to research host/microbe interactions. In addition, its responses, i.e., biochemical, cellular, genetic, and developmental, to bacterial colonization are the best characterized for any cephalopod species.

In order to better understand the role of the ANG consortium in the biology of *E. scolopes*, the host and bacterial cell morphologies as well as the microbial diversity were characterized using transmission electron microscopy (TEM), 16S ribosomal sequence analysis, restriction fragment length polymorphism (RFLP) analysis, fluorescent *in situ* hybridization (FISH), and high-throughput 454 metagenomic sequencing. Here we report the initial characterization of the ANG microbiota for the model host, *E. scolopes*. This study is the first to use high-throughput sequencing to characterize the bacteria in any accessory nidamental gland. More importantly, it sets the foundation for exploration of a bacterial consortium in the same host as has already been used to research a well-studied monospecific symbiosis.

MATERIALS AND METHODS

Animal maintenance. Adult animals were collected from shallow sand flats off Oahu, HI, by dip net and maintained in 42-liter recirculating

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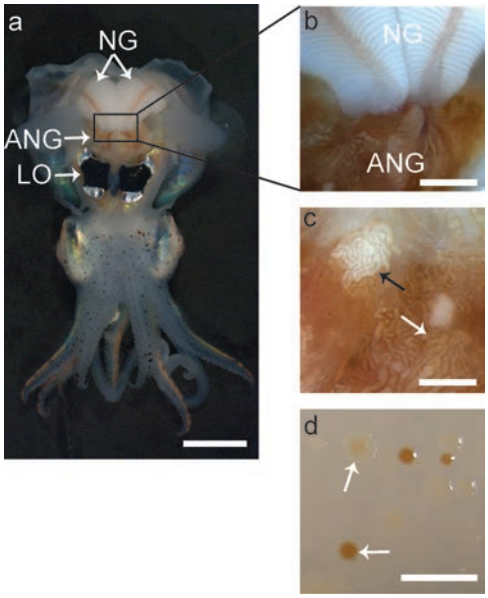

FIG 1 Anatomy of a female *Euprymna scolopes* and morphology of ANG isolates. (a) Ventral dissection of *E. scolopes*, showing the accessory nidamental gland (ANG) located posterior to the light organ (LO) and in close proximity to the nidamental gland (NG). (b) Pigmented ducts in the NG converge at the ANG. (c) Magnification of the ANG reveals convoluted tubules, most of which are dark orange in pigmentation (white arrow), but others appear white (black arrow) or yellow (not shown). (d) Culturing of ANG symbionts results in many colonies with pigmentation similar to that of the ANG tubules (white arrows). Bars, 1 cm for panels a and d, 2.5 mm for panel b, and 1 mm for panel c.

aquaria at the University of Connecticut with artificial seawater (ASW; Instant Ocean) at 23°C and kept on an approximately 12 h light/12 h dark cycle (33).

Dissection and DNA extraction. Female squid that had been maintained in the laboratory for between 24 h and 4 months were anesthetized in 2% ethanol in filter-sterilized ASW and ventrally dissected to remove the accessory nidamental gland. Once removed, the ANGs were flash frozen in liquid nitrogen and stored at −80°C until use. For 16S clone libraries, ANGs were homogenized in lysis buffer and total DNA was isolated using a DNeasy Tissue Prep kit (Qiagen, Hilden, Germany). To obtain DNA for 454 library construction, frozen ANGs were first thawed and then homogenized in squid Ringer's solution (530 mM NaCl, 25 mM $MgCl_2$, 10 mM $CaCl_2$, 20 mM HEPES, pH 7.5) using a ground glass homogenizer. The homogenate was spun for 10 min at 5,000 × *g* at 4°C. To remove solubilized host tissues, the supernatant was removed and the pellet was repeatedly washed (at least three times) with squid Ringer's solution until the protein concentration of the supernatant was sufficiently low (<0.5 mg/ml), as measured spectrophotometrically by A_{280} analysis. For 454 sequencing, total genomic DNA was extracted from the resulting pellet by the use of a DNA MasterPure kit (Epicentre, Madison, WI).

Culturing bacteria from the ANG. Frozen ANGs from three sexually mature ANGs were homogenized in squid Ringer's solution, and the homogenate was serially diluted 10-fold and plated in triplicate onto R2A media (42) supplemented with 27 g of marine salts (Instant Ocean). Plates were incubated aerobically at 28°C for 3 days, and the resulting colonies were observed for pigmentation.

Microscopy. Immediately after dissection, ANGs were cut in half and fixed at room temperature in 2.0% paraformaldehyde–2.5% glutaraldehyde in buffer A (0.1 M sodium cacodylate, 0.375 M NaCl, 1.5 mM $CaCl_2$, and 1.5 mM $MgCl_2$, pH 7.4). After an initial 15-min fixation period, the tissue samples were cut into smaller pieces (~0.25 cm thick) and placed in fresh fixative for an additional 5 h at 4°C. Following fixation, tissue pieces were washed several times in cold buffer A and left at 4°C overnight. The following day, tissues were postfixed in a solution of 1% osmium tetroxide–0.8% potassium ferricyanide–0.1 M sodium cacodylate–0.375 M NaCl for 1.5 h at 4°C and then washed in distilled water, dehydrated through an ascending ethanol series, cleared in 100% acetone, and embedded in an epoxy mixture of Embed 812 (Electron Microscopy Sciences, Hatfield, PA) and Araldite 506 (Ernest Fulham Inc., Albany, NY). Semithin (2-μm) sections were obtained with a glass knife using an LKB Ultramicrotome V and stained with methylene blue and azure II followed by counterstaining with basic fuchsin. Stained sections were viewed on an Axiovert 200 M (Zeiss, Oberkochen, Germany) microscope. Thin (80-nm) sections were obtained using a diamond knife on a LKB Ultramicrotome V followed by staining with 2% uranyl acetate and Reynold's lead citrate (43) and viewed with an FEI Tecnai Biotwin G2 Spirit electron microscope (Hillsboro, OR) operated at 80 kV.

16S clone library construction and RFLP and sequencing analyses. To examine the bacterial diversity in ANGs, total genomic DNA from the ANGs of five sexually mature females were used to make five separate 16S clone libraries. ANGI, ANGII, ANGIII, and ANGIV came from each of four females that were kept in our squid facility for 9, 14, 12, and 17 weeks, respectively. ANGV came from an individual that had been field caught and was maintained in our facility for 24 h. 16S genes were amplified using 25-μl GoTaq reaction mixtures (Promega, Madison, WI) with the 27F and 1406R eubacterial 16S primers (Table 1). PCR conditions were as follows: 95°C for 3 min, then 35 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 90 s, followed by a final elongation at 72°C for 10 min. PCR

TABLE 1 Primers and FISH probes used in this study

Primer, probe, or target organism category	Name	Sequence	Hybridization buffer % formamide (probes only)	Reference or source
Primer				
Eubacterial	27F	AGAGTTTGATCCTGGCTCAG		Lane (23)
	1406R	ACGGGCGGTGTGTRCAA		Lane (23)
FISH probes				
Eubacterial (universal)	Eub338I	GCTGCCTCCCGTAGGAGT	30	Amann (1)
	Eub338III	GCTGCCACCCGTAGGTGT	30	Daims et al. (9)
Roseobacter	G Rb	GTCAGTATCGAGCCAGTGAG	30	Giuliano et al. (13)
Bacteroidetes (*Cytophaga-Flavobacteria*)	CF319	TGGTCCGTGTCTCAGTAT	30	Manz et al. (28)
Verrucomicrobia	Verruco_193	CGCCATTACAAGCTTTAGTA	20	This study
Phaeobacter	Phaeo_126	TGGCTATTTTAGAGAAGGGCA	20	This study
Alphaproteobacteria	Alph_968	GGTAAGGTTCTGCGCGTT	30	Neef (34)
Eubacteria (negative control)	NonEub338	ACTCCTACGGGAGGCAGC	30	Wallner et al. (49)

products were ligated and cloned using a PGEM-T Easy kit with JM109 cells (Promega, Madison WI). A total of 417 colonies were selected for restriction fragment length polymorphism (RFLP) analysis by incubating cloned genes with 10 U of MspI restriction enzyme (New England BioLabs, Ipswich, MA) at 37°C for 15 min. The resulting fragments were visualized on 1.5% agarose gels, and clones were grouped according to unique RFLP patterns. Representative clones from each group were sequenced using BigDye version 1.1 (Applied Biosystems, Carlsbad, CA) according to the manufacturer's specifications. Any clone that could not be grouped with an RFLP pattern was also sequenced. The 16S rRNA genes from 25, 45, and 27 clones from ANGI, ANGII, and ANGIII, respectively, were fully sequenced to confirm the accuracy of the restriction digest grouping. Sequences were analyzed with the Bellerophon chimera server (18), and 25 chimeric sequences were discarded, leaving 392 clones that were included in the analysis. The full-length sequences were used to search the Greengenes 16S rRNA gene database of named isolates by the use of BLAST (11). Operational taxonomic units (OTUs) were assigned to each sequence based on highest percent identity. Sequences from the *Verrucomicrobia* isolates had few quality alignments and were therefore characterized as representing a phylum.

454 metagenomic sequencing. To identify other bacterial members isolated from the ANG that might not have been detected with 16S clone libraries and to increase our sequencing depth, we analyzed bacterial diversity using 454-metagenomic analyses. Bacterial DNA was extracted from 3 ANGs as described above. The samples were pooled, and 500 ng was used to construct a 454-shotgun metagenomic library using a Rapid Library kit (Roche Applied Science, Basel Switzerland). After the small-volume (SV) emulsion PCR (emPCR) titration was performed, the library was used in two 454 sequencing runs with FLX Titanium chemistry (Roche Applied Science, Basel, Switzerland). After removing 454 artifacts by the use of a 454 replicate filter (15), 622,987 sequences with an average length of 389.68 bases (total = 242.77 Mb) were analyzed. Roughly 1% of the reads (6,350) were eukaryotic in origin and not used in our analyses.

For 16S analysis of 454 data, reads were annotated using the MG-RAST server (32). Using the algorithm available from the Ribosomal Database Project (RDP), reads with at least a 200-bp alignment to a known 16S gene were extracted and used to search the NCBI nucleotide database with BLAST. OTUs were assigned as described above.

FISH. To localize bacteria to the ANG, organs were dissected from six sexually mature female squid and prepared for fluorescent *in situ* hybridization (FISH). Two were freshly collected and dissected in Hawaii; the other four were kept in our animal facility for 8 to 14 weeks prior to dissection. Time in captivity did not affect results (data not shown). Three ANGs were fixed with Carnoy's solution (ethanol:chloroform:acetic acid [6:3:1]) overnight, and three were fixed in 1× PBS–4% paraformaldehyde for 4 h. Tissues were embedded in paraffin, and hybridization was performed as previously described (21). Three egg capsules were removed from freshly laid egg clutches, fixed in squid Ringer's solution–4% paraformaldehyde for 4 h, and embedded in paraffin as described above.

Based on our 16S data, several ribosomal probes were used at 50 pmol/ml each for hybridization (Table 1). Probes that corresponded to species of *Eubacteria*, *Alphaproteobacteria*, the *Roseobacter* clade, or *Cytophaga-Flavobacteria-Bacteroidetes* (CFB) were designed on the basis of published data. Novel 16S probes for *Verrucomicrobia* and *Phaeobacter* species were designed based on 16S sequence data, and specificity was confirmed with ProbeCheck (Table 1) (24) and fixed cultures of closely related members of genera of *Rhodobacterales* (e.g., *Phaeobacter*, *Ruegeria*, *Tateyamaria*, and *Nautella* for the *Phaeobacter*-specific probes; data not shown). All probes were synthesized by Eurofins MWG Operon (Huntsville, AL) and conjugated to fluorescein isothiocyanate (FITC), Cy3, or Cy5. After an overnight hybridization at room temperature in formamide hybridization buffer (0.9 M NaCl, 20 mM Tris [pH 8], 0.01% sodium dodecyl sulfate [SDS]) (Table 1), the tissue was washed in hybridization buffer and then counterstained with 300 nM DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA) in 1× PBS for 5 min. The following negative controls were performed: no probe, a nonsense probe (complementary to the eubacterial probe Eub338), and competition with nonlabeled probes. Tissue sections were imaged on a Leica SP2 confocal microscope (Wetzlar, Germany) or a Zeiss Axiovert 200 M epifluorescence microscope (Carl Zeiss, Germany) using DAPI, FITC, Cy3, and Cy5 filter sets.

Accession numbers. 16S clone library sequences were deposited in the European Nucleotide Archive (ENA) with accession numbers HE574851 to HE574928. Metagenomic reads were deposited in the NCBI Short Read Archive with accession numbers SRR329677.8 and SRR329678.5.

RESULTS

Morphological and EM observations. The ANG of *E. scolopes* (Fig. 1a) contains many convoluted tubules that are highly pigmented (Fig. 1b). While most tubules have a dark orange pigmentation, some appear white (Fig. 1c) or, more rarely, yellow (not shown). As with other ANGs, the bacteria within the tubules likely synthesize these pigments, as colonies isolated from the organ also appeared similarly pigmented when grown in culture (Fig. 1c) (6).

Light and electron microscopy of fixed sections of the ANG revealed that the organ is highly vascularized, with many blood vessels among tubules lined with ciliated epithelial cells and containing populations of bacteria. In some tubules, however, bacteria were not observed (Fig. 2A). Two morphologically distinct and segregated cell types were observed (Fig. 2B): a large coccoid bacterium (LCB) and a smaller bacillus bacterium (SBB). These two bacterial morphotypes appeared in separate tubules with strikingly different epithelia. One type of epithelium, associated with the SBB, appeared vacuole-rich (Fig. 2B and C), while the other associated with the LCB, had an electron-dense staining pattern lacking vacuoles (Fig. 2B and F). Within the tubules housing the bacteria were microvillar brush borders 1 to 5 μm in thickness along with membrane-bound vesicles that may be secreted or blebbed by the host (Fig. 2D). Some of the vacuole-rich epithelial cells had the distinct appearance of being secretory in nature, containing numerous large electron-light vacuoles and smaller electron-dense granules located at the apical surfaces of the epithelium (Fig. 2C). Hemocytes, the primary innate immune cells of *E. scolopes*, were observed in the lumina of the tubules; however, phagocytosed bacteria were not observed within these cells (Fig. 2E). Each tubule was dominated by one of the two morphologies: either the SBB (Fig. 2C and D) or the LCB (Fig. 2F). Mixtures of both LCB and SBB morphotypes were also observed outside the tubules within the connective tissue (Fig. 2G and H). The epithelial membranes appeared well-preserved, suggesting that these observations were not from a fixation artifact and that the bacteria can travel outside the ANG lumina. Hemocytes were also observed within the connective tissue (Fig. 2H), but as in the lumina of the tubules, no intracellular or phagocytosed bacteria were noted. Under higher magnification, the LCB cells appeared to be filled with many granules (Fig. 2I). This was in stark contrast to SBB, which were either mostly electron dense (Fig. 2J) or contained large electron-light storage vacuoles which resembled polyhydroxybutarate (PHB) (19, 27) (Fig. 2K).

16S diversity. In order to identify members of the microbial community of the *E. scolopes* ANG, we constructed five 16S clone libraries from five sexually mature adult female squid. Sequences of 417 clones were binned by RFLP analysis. Of these, 96 full-length 16S sequences were analyzed and 25 identified chimeric sequences were removed. Analysis of these data showed that most (302/392; Table 1) clones belonged to the *Alphaproteobacteria* and

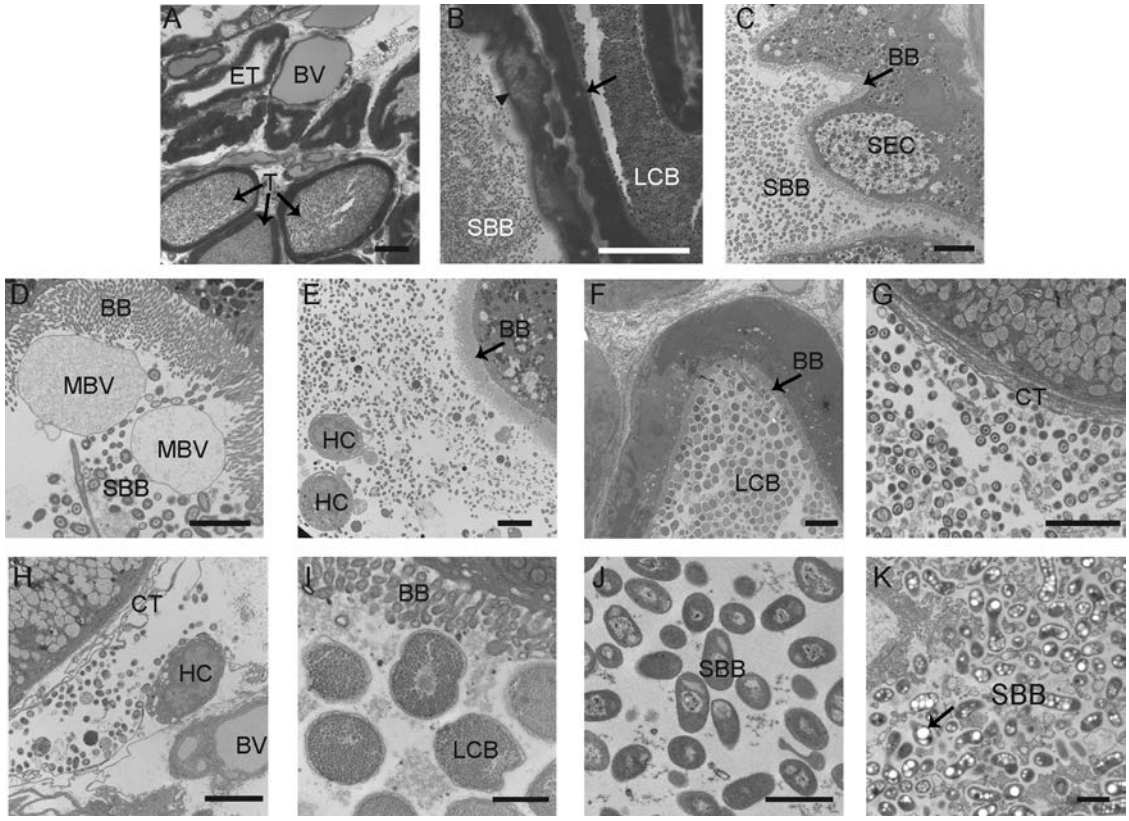

FIG 2 Light microscopy and TEM of fixed sections from the *E. scolopes* ANG. (A) Cross-section of ANG tissue, showing many tubules interspersed between blood vessels (BV). Most tubules contained dense bacterial populations (T); however, some were empty (ET). (B) Closer inspection revealed that tubules have two distinct morphotypes, comprising a large coccoid bacterium (LCB) and a smaller bacillus-shaped bacterium (SBB) that were observed in tubules with differing epithelial morphologies, either a vacuole-rich epithelium (arrowhead) or dense epithelium (black arrow). (C) Tubule with brush border (BB) dominated by SBB with an epithelial morphology suggesting secretory cells (SEC). (D) SBB inhabiting tubules with a thick microvillar brush border (BB) and membrane-bound vesicles (MBV). (E) Hemocytes (HC) in the lumen of a tubule with a population of bacteria. (F) Tubule dominated by LCB. (G) Bacteria were also seen in the connective tissue (CT) outside the tubules; note the absence of a brush border. (H) Hemocytes were also seen with bacteria among the connective tissue. (I) Closer inspection of the LCB revealed many storage granules. (J and K) One SBB morphotype showed a nucleoid structure, while the other (K) showed many polyhydroxybutyrate-like granules (black arrow). Bars, 30 μm (A and B), 5 μm (C to H), and 1 μm (I to K).

that *Phaeobacter* was the most commonly observed genus (221/392). Other *Alphaproteobacteria* species belonged to genera within the *Rhodobacterales*, primarily of the *Roseobacter* clade (for example, *Ruegeria*, *Labrenzia*, and *Pseudoruegeria*). Twelve were from the genus *Kordiimonas*, and eight sequences were from the *Rhizobiales*. The next most common group of sequences (89/392) had greatest similarity to the sequences corresponding to members of the phylum *Verrucomicrobia*. These *Verrucomicrobia* sequences displayed only ~90% identity to those from the Greengenes database, most likely due to the lack of characterized verrucomicrobial isolates. Only one sequence belonged to the *Gammaproteobacteria* and corresponded to the genus *Shewanella*.

The overall bacterial populations of ANGs from separate animals were similar, with two OTUs conserved across all five clone libraries, *Phaeobacter* and *Verrucomicrobia* (Table 2). Members of four other genera of the alphaproteobacteria (*Ruegeria*, *Kordiimonas*, *Cohaesibacter*, and *Nautella*) were conserved among the same four ANG libraries. Length of time spent in the mariculture facility did not seem to influence the microbial communities found in the ANG, as animals maintained for either 1 day (library ANG5) or 4 months (library ANG4) were found to have similar bacterial taxa (Table 2).

In addition to the RFLP and sequence data from the five clone libraries, 16S gene fragments from the 454 metagenome were also analyzed. A total of 532 genomic fragments with at least a 200-bp alignment to a reference 16S sequence in the RDP database were used for this analysis. The taxonomies of these 16S sequences were similar to those identified in the 16S libraries; species of *Rhodobacterales*, *Rhizobiales*, and *Verrucomicrobia* were dominant (Fig. 3). 72.55% of the 16S sequences belonged to the *Alphaproteobacteria*, and the most common genus was *Phaeobacter* (177/532). Members of the order *Rhodobacterales* was the most common, with *Verrucomicrobia* being the second largest taxonomic contingent overall (89/532). Members of the *Rhizobiales* and the phylum *Bacteroidetes*, which were not seen in our 16S library, accounted for less than 20% of the 16S sequences. Only 2 of the 532 16S sequences were from the *Gammaproteobacteria*.

FISH. Observations from electron microscopy suggested that different morphotypes (SBB or LCB) dominated individual tubules within the ANG (Fig. 2). To test whether this could have been due to different phylogenetic groups occupying separate tubules, fluorescent *in situ* hybridization (FISH) was used to visualize dominant bacterial taxa within the ANG. Ribosomal FISH revealed that most tubules within the ANG contained a specific

TABLE 2 Operational taxonomic units within five ANG 16S clone libraries[a]

Phylotype	No. of OTUs				
	ANG1	ANG2	ANG3	ANG4	ANG5
Alphaproteobacteria					
Rhodobacterales					
Phaeobacter	20	49	78	40	26
Ruegeria	9	6	0	6	4
Nautella	2	4	0	5	1
Labrenzia	0	6	0	1	0
Pseudoruegeria	0	1	0	0	0
Oceanicola	0	1	0	0	0
Marinovum	0	0	2	0	0
Salipiger	0	0	4	0	0
Rhizobiales					
Cohaesibacter	0	1	0	2	3
Mesorhizobium	0	0	2	0	0
Kordiimonadales					
Kordiimonas	1	4	0	3	4
Verrucomicrobia	37	12	3	25	29
Gammaproteobacteria					
Shewanella	0	1	0	0	0
Total no. of clones	69	85	89	82	67

[a] A total of 392 clones were binned into taxonomic groups by RFLP analysis. The full-length 16S rRNA gene of 71 clones from ANG1, ANG2, and ANG3 was analyzed using the Greengenes database (11) after chimeric sequence removal (Materials and Methods).

bacterial group (Fig. 4). Staining with an FITC-labeled *Roseobacter* clade-specific probe and a cocktail of the eubacterial probes Eub338 and Eub338III (Table 1) showed that the *Roseobacter* clade probe hybridized to the majority of the bacteria of one tubule (Fig. 4a). Similarly, using both the *Verrucomicrobia*- and *Alphaproteobacteria*-specific probes, tubules were dominated by only one of the two fluorescent signals (Fig. 4b), suggesting bacterial partitioning among the ANG tubules. The presence of members of the *Cytophaga-Flavobacteria-Bacteroidetes* (CFB) that were identified in the 454 metagenomic sequencing analysis was also confirmed with this technique (Fig. 4c).

Applying FISH to the jelly capsule of freshly laid squid eggs also revealed a mixture of bacteria within the capsule, with an abundance of *Alphaproteobacteria* present, including *Phaeobacter* sp. (Fig. 4d and e). No bacterial cells were observed in direct contact with the developing embryo (not shown). These data suggest that bacteria from the ANG are deposited directly into host egg capsules.

DISCUSSION

We used a variety of microscopy and molecular methods to characterize the bacterial population of the ANG of *E. scolopes*, ultimately to understand its role in host reproduction. These analyses show that *Alphaproteobacteria* species from the *Roseobacter* clade within the *Rhodobacterales* are prevalent and that members of the genus *Phaeobacter* dominate the consortium, while other major constituents are members of the *Rhizobiales*, *Verrucomicrobia*, and *Flavobacteria* (Table 2 and Fig. 3). This bacterial consortium is contained within heteromorphic epithelium-lined tubules that are infiltrated by host hemocytes. Moreover, FISH analyses confirmed that many tubules of the ANG are dominated by single taxonomic groups, suggesting niche specificity in this association (Fig. 4).

The dominance of *Roseobacter* clade members in the ANG of *Euprymna scolopes* is similar to what has been described for other cephalopod ANGs, including those of other squid (3, 39) and cuttlefish (16), by the use of 16S clone sequencing. Like that of *Loligo pealei*, the ANG of *Euprymna* has a large *Alphaproteobacteria* contingent, comprising *Roseobacter* clade members as well as members of the marine *Rhizobiales*. The *E. scolopes* ANG also has a *Flavobacteria* contingent, similar to observations made using the egg casings of *L. pealei* (3).

The presence of *Verrucomicrobia* and the lack of *Gammaproteobacteria* make the consortium in *E. scolopes* strikingly different from the ANG consortia previously described for other cephalopods. Members of the *Verrucomicrobia* have been detected in relatively few host/microbe associations (38, 44, 47, 51), and the major presence of this group in the squid ANG represents a potentially novel symbiotic role for this phylum. Less than 1% of 16S genes from our clone libraries and from the 454 metagenome belonged to the *Gammaproteobacteria*. This is surprising for a number of reasons. In *L. peali*, it has been estimated that 5% of the bacterial population of the ANG is made up of this group (3). Furthermore, *E. scolopes* has a binary association with the bioluminescent *Gammaproteobacterium Vibrio fischeri* (29, 30, 35). Given that the host expels 10^6 to 10^9 symbionts from its light organ as part of a daily rhythm (7, 36), the close proximity of the two organs, and that the ANG consortium is likely environmentally transmitted (see below), it is surprising that *V. fischeri* was not detected in our analyses.

Previous work has shown that the bacterial consortia within cephalopod ANGs are likely established by horizontal/environmental transmission (20). In that work, Kaufman et al. examined development of the ANG in *Loligo opalescens* and found that the organ develops 11 weeks after hatching and that colonization is likely due to horizontal/environmental transmission. This conclusion is also supported by the observation that the nearest relatives of ANG isolates from *L. pealei* are environmental strains (3). The ANG of *E. scolopes* is absent at hatching, and females tend to reach sexual maturity within 60 days (17). Therefore, horizontal transmission of the *E. scolopes* ANG consortium is also probable. Field-caught animals at different stages of development of the ANG symbiosis will be used for future analyses of both the organ and the microbial community. Current efforts are also under way to rear animals to sexual maturity in our laboratory.

The data presented here suggest that establishment and maintenance of the bacterial consortium may be an intricate process, as both electron microscopy and FISH analyses showed bacterial partitioning among the ANG tubules (Fig. 2 and 4). Electron microscopy revealed distinct morphotypes (LCB and SBB) prevalent in each tubule, and FISH revealed that members of the *Rhodobacterales*, *Cytophaga-Flavobacteria-Bacteroidetes*, and *Verrucomicrobia* dominated separate tubules (Fig. 2 and 4). Other studies have noted dominant morphotypes (coccus and bacillus) within the lumina of cephalopod ANGs (3, 6). Electron micrographs from the cuttlefish *Sepia officinalis* show a coccoid bacterium with a morphology very similar to that of the granular, coccoid cells ob-

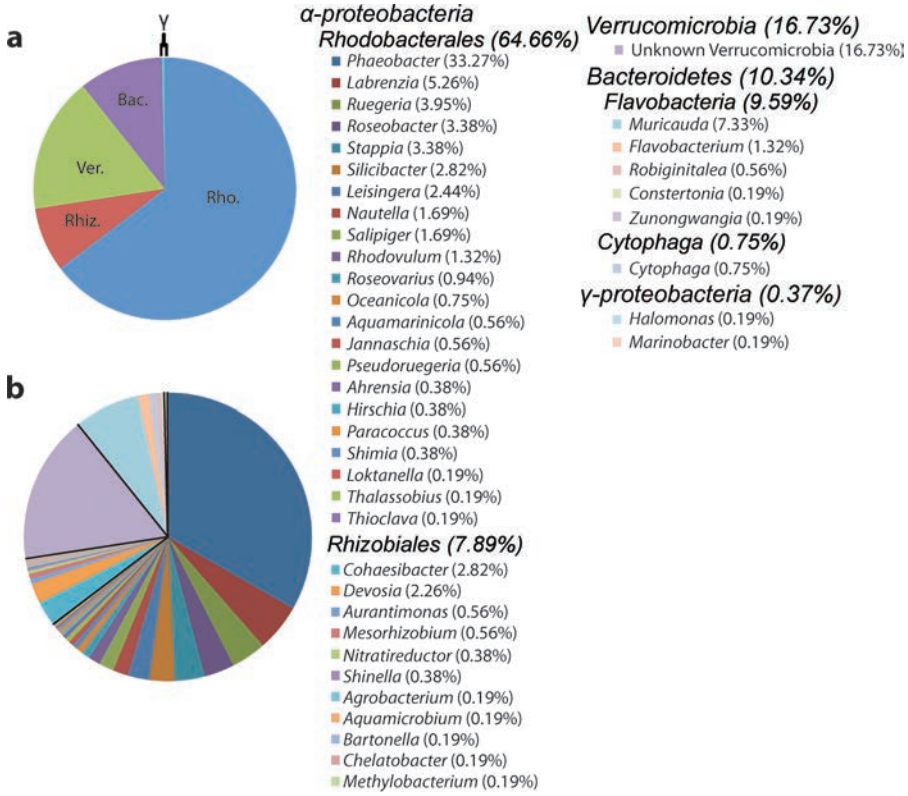

FIG 3 Taxonomic analysis of 454 metagenomic data. Higher (a) and lower (b) taxonomy of 532 16S gene fragments found within the ANG metagenome. Members of the *Alphaproteobacteria*, comprising mostly *Rhodobacterales* (Rho.), accounted for the vast majority of sequences (72.55%). Members of the *Verrucomicrobia* (Ver.) were the next largest contingent (16.73%), and members of the *Bacteroidetes* (Bac.), mostly from the *Flavobacteria*, made up a smaller fraction (10.34%). Less than 1% of 16S fragments belonged to the *Gammaproteobacteria*. The presence of a single 16S sequence is represented by "0.19%." Rhiz., *Rhizobiales*.

served in the *E. scolopes* ANG (48). The data from this study suggest that the different bacterial morphotypes are different taxa occupying separate tubules. Bacterial morphology by itself is not a reliable taxonomic identifier; however, studies of the marine verrucomicrobium *Coraliomargarita akajimensis* revealed a morphology similar to the LCB morphotype observed in this study (52). The two epithelial morphologies of the ANG tubules are very distinct from one another, suggesting that each tubule fosters a unique microenvironment optimized to contain a specific bacterial taxon or that specific bacteria influence development of different epithelia. The mechanism(s) for establishing and maintaining bacterial tubule dominance is not yet known, but these bacterial groups may be adapted to specific niches or microenvironments within the ANG. Alternatively different taxa may dominate specific tubules during colonization due to a founder effect.

Just as carbon and energy sources influence bacterial diversity in digestive tracts, nutrition may play a role in the segregation of the bacteria within the ANG. While members of the *Verrucomicrobia* have not been thoroughly described, many have been shown to degrade polysaccharides such as mucin in the human gut (10) or fucoidan in the gut of a sea cucumber (45). The genome of *Phaeobacter gallaeciensis* ANG1, a dominant member of the ANG consortium in *E. scolopes*, reveals that it has many pathways for energy and carbon assimilation; however, it lacks enzymes to degrade polysaccharides, including chitinases, amylases, agarases, and α-L-fucosidase (8). Therefore, the host may provide different nutrients in different tubules, thereby enriching for certain bacteria. The presence of PHB-like granules in some cells (Fig. 2K) could be explained by nutrient restriction, as PHB improves survivability during starvation and/or stress tolerance in other systems (27, 41).

There are other clues that can be gathered from the host as to how a microenvironment can be created to foster dominance of specific bacteria. The light organ symbiosis between *E. scolopes* and *V. fischeri* has been studied in detail for more than 20 years, and previous studies have shown that the host and symbiont work in concert to create a microenvironment that selects for *V. fischeri* to the exclusion of nonsymbiotic bacteria (30, 35, 50). The stark differences in epithelial tissues in the ANG suggest that unique microenvironments exist between tubules. In the light organ, hemocytes, representing the sole cellular component of the host's innate immune system, have been implicated in establishing and maintaining specificity (22, 31, 36, 37). Hemocytes were also observed to infiltrate the lumina of the ANG tubules and were found to come in direct contact with the bacterial consortium (Fig. 2). Whether these hemocytes contribute to specificity in the ANG association remains to be determined, but we have isolated several ANG bacterial strains that are available to use in adhesion and phagocytosis assays. Future research should examine how components of the innate immune system as well as other host and symbiont factors may influence the development and maintenance of this association.

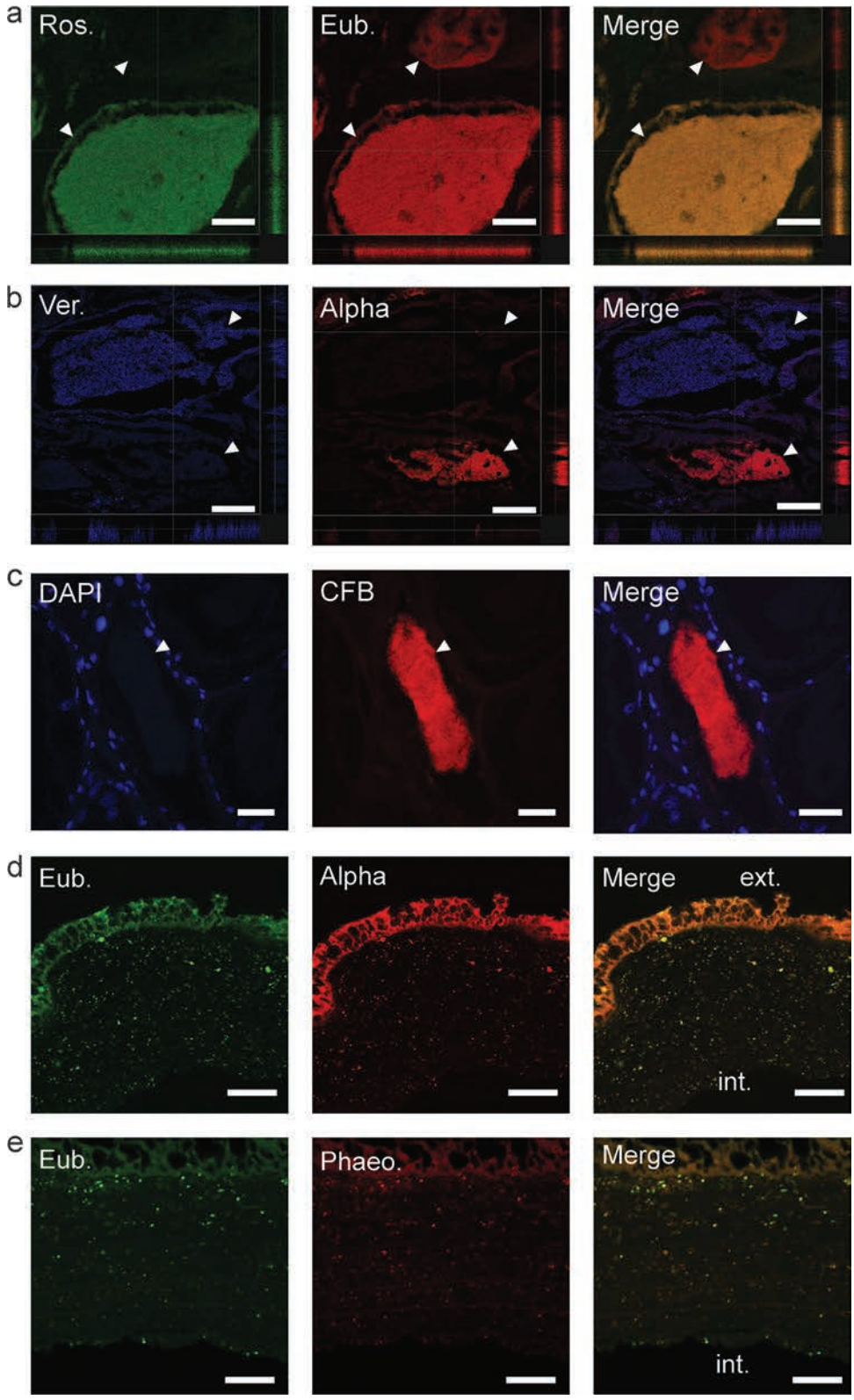

FIG 4 Fluorescent *in situ* hybridization of fixed ANG paraffin-embedded sections. (a) 16S FISH with FITC-conjugated *Roseobacter* (Ros.) probe (green) and CY3-conjugated eubacterial (Eub.) cocktail (red). (b) Cy3-conjugated *Alphaproteobacteria* (Alpha; red) and Cy5-conjugated *Verrucomicrobia* (Ver.; blue) probes were observed dominating separate tubules, suggesting specificity and/or segregation of the bacterial populations. Axes denote positions of "slices" through confocal sectioning. (c) 16S FISH with Cy3-conjugated *Cytophaga-Flavobacteria-Bacteroidetes* probe (CFB; red) and DAPI staining (blue). (d) Hybridization with an FITC-conjugated eubacterial cocktail (green) and a Cy3-conjugated *Alphaproteobacteria* probe (red) revealed a population of bacteria within the jelly capsule of a freshly laid egg. (e) Hybridization with a Cy3-conjugated *Phaeobacter* (Phaeo.) probe confirmed that many of the bacteria in the jelly capsule were *Phaeobacter* species. The exterior (ext.) and interior (int.) of the capsule are labeled. White arrowheads indicate tubules. Bars, 30 μm (a), 50 μm (b), and 20 μm (d and e).

Despite numerous studies that have characterized the bacterial communities within cephalopod ANGs, its function is still unknown. The ANG may provide antimicrobial or antifouling compounds that protect the squid's eggs throughout their development (4, 14). The genome of *Phaeobacter gallaeciensis* ANG1 revealed no classical antibiotic synthesis pathways (8), but future analyses should take into account the genomes of the other ANG members and possible uncharacterized pathways of novel antimicrobial compounds. Two other *P. gallaeciensis* strains have shown the ability to inhibit fungal, bacterial, and/or algal growth (40, 46), and future studies should test whether removing the ANG consortium from female squid and/or their egg clutches influences fecundity and egg development. Currently, we have 14 other *E. scolopes* ANG rhodobacterial isolates in culture, and characterizing these strains in greater detail should shed light on the function of the ANG.

The female members of many squid species found worldwide harbor a consortium of bacteria within their ANG. Surprisingly, much of the composition of the microbial communities is the same (e.g., the dominance of *Rhodobacterales*), even though these hosts are found in very different environments with different physical and biological parameters (e.g., salinity, temperature, predators, and life histories). This trend suggests that these microbial consortia play similar roles in their squid hosts. Future experiments can utilize high-throughput sequencing techniques to reveal gene expression of the bacteria in the ANG and within the egg capsule. The results of this study lay the foundation for the development of *E. scolopes* as a model for studying a consortial symbiosis (ANG) and a binary symbiosis (light organ) in the same host.

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Draft Genome of Phaeobacter gallaeciensis ANG1, a Dominant Member of the Accessory Nidamental Gland of Euprymna scolopes

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Draft Genome of *Phaeobacter gallaeciensis* ANG1, a Dominant Member of the Accessory Nidamental Gland of *Euprymna scolopes*▽

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***Phaeobacter gallaeciensis* strain ANG1 represents the dominant member of the bacterial consortium within the reproductive accessory nidamental gland (ANG) of the squid *Euprymna scolopes*. We present a 4.59-Mb assembly of its genome, which may provide clues as to how it benefits its host.**

The accessory nidamental glands (ANGs) of female cephalopods are reproductive organs containing dense consortia of bacteria dominated by *Alphaproteobacteria* from the *Roseobacter* clade (5, 11, 13). Although the function of this organ has not been demonstrated, the production of antimicrobial and/or antifouling compounds from ANG bacterial isolates has been proposed (4, 5). Here, we describe the draft genome of *Phaeobacter gallaeciensis* ANG1, a member of the *Rhodobacteraceae*, isolated from the ANG of a sexually mature Hawaiian bobtail squid, *Euprymna scolopes*.

The *Roseobacter* clade is a ubiquitous, diverse group that can comprise up to 20% of bacterial populations in marine environments (7). Besides being implicated in a number of large-scale ecological roles, i.e., carbon cycling (12) and sulfur metabolism (10), roseobacters are also found as members of many eukaryotic-bacterial symbioses. For example, they form obligate associations with marine algae (1), are major colonizers of corals (2), and are commonly found as dominant members in the ANGs of several cephalopods—including loliginid squid (5), cuttlefish (11), and the Hawaiian bobtail squid *Euprymna scolopes* (unpublished data).

We cultured three isolates from the ANG of *E. scolopes* and identified them by sequencing the full-length 16S rRNA gene. The isolate that was sequenced was Gram negative and positive for oxidase and catalase. The 16S rRNA gene was 99.7% identical to 16S gene sequences found in three separate *E. scolopes* ANG clone libraries and 99.2% identical to a coastal isolate, *Phaeobacter gallaeciensis* SCH0407, in GenBank (accession no. AY881240). The genome of this isolate, *P. gallaeciensis* ANG1, was sequenced using Illumina mated paired-end technology. A total of 1.73×10^8 36-bp reads were assembled using the CLC Genomic Workbench assembler (CLC Bio, Denmark), resulting in 1,370-fold coverage of a 4.59-Mb genome contained in 135 contigs. Glimmer (8), GeneMark (6), and the RAST server (3) were used to predict open reading frames (ORFs). A total of 4,389 protein-coding genes, 35 tRNAs, and one ribosomal operon were identified.

Phaeobacter gallaeciensis ANG1 has complete Embden-Myerhoff-Parnas, Entner-Doudoroff, and pentose phosphate pathways and a complete tricarboxylic acid (TCA) cycle. The genome contains an abundance of predicted ABC transporters, particularly for peptides, amino acids, and polyamines (i.e., putrescine, spermidine). Other transport systems include a twin-arginine transport system, the Sec pathway, and a type IV and a type VI secretion system. In addition, all cobalamin synthesis genes are present, suggesting that *P. gallaeciensis* ANG1 may provide this nutrient for its host. While there is high similarity to two other *Phaeobacter gallaeciensis* genomes available (strains BS107 and 2.10), strain ANG1 lacks the genes needed to synthesize the antibiotic tropodithietic acid (TDA) (9), and classical pathways for antibiotic production were not found.

This genome shows the metabolic and transport potential of a major bacterial constituent of the accessory nidamental gland of *E. scolopes*. Further analyses of this genome may provide significant clues to understanding the role of *P. gallaeciensis* in this symbiotic organ.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited in DDBJ/EMBL/GenBank under accession no. AFCF00000000. The version described in this paper is the first version, AFCF01000000.

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Cephalopod genomics: A plan of strategies and organization

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The Cephalopod Sequencing Consortium (CephSeq Consortium) was established at a NESCent Catalysis Group Meeting, "Paths to Cephalopod Genomics- Strategies, Choices, Organization," held in Durham, North Carolina, USA on May 24-27, 2012. Twenty-eight participants representing nine countries (Austria, Australia, China, Denmark, France, Italy, Japan, Spain and the USA) met to address the pressing need for genome sequencing of cephalopod mollusks. This group, drawn from cephalopod biologists, neuroscientists, developmental and evolutionary biologists, materials scientists, bioinformaticians and researchers active in sequencing, assembling and annotating genomes, agreed on a set of cephalopod species of particular importance for initial sequencing and developed strategies and an organization (CephSeq Consortium) to promote this sequencing. The conclusions and recommendations of this meeting are described in this white paper.

Cephalopods

Cephalopods (octopus, squid, cuttlefish, *Nautilus*) have captured the imagination of scientists and the general public since Aristotle. These predatory creatures are an ancient group, known from at least the Late Cambrian and today comprising more than 700 species [1,2]. Cephalopods range in size from the pygmy squids (thumbnail-sized adults) to the colossal and giant squids (18 meters in total length), which are the largest known invertebrates. Cephalopods are believed to be among the most "advanced" invertebrates, having evolved large, highly differentiated brains, a sophisticated set of sensory organs that includes vertebrate-like eyes, and fast jet-propelled locomotion [3]. The neuroendocrine and heart-blood vascular systems of cephalopods have long been recognized for their complexity and similarity to those found in vertebrates [4-6]. A particularly striking trait of cephalopods is that they are masters of rapid adaptive coloration, having the ability to change quickly the texture, pattern, color and brightness of their skin. Dynamic camouflage helps the animals evade detection by predators and approach prey with stealth; the same systems produce signals for communication with conspecifics [3]. The remarkable morphological and physiological innovations of cephalopods provide the scientific community with a tremendous opportunity for insight into mechanisms of evolutionary convergence and innovation in structure and function.

Cephalopods have diversified to inhabit all oceans of the world, from benthic to pelagic zones, from intertidal areas to the deep sea, and from the polar regions to the tropics. They share the "behavioral space" in their many marine habitats with teleost fishes and marine mammals [7], placing them in some of the most competitive ecohabitats on Earth. Cephalopods are ecologically important for the central position they play in trophic predator-prey relationships; they are a primary food source for marine mammals and for many harvested fish species. Their importance in the food web is often underestimated, but they constitute a crucial element in coastal ecosystem equilibrium. Moreover, cephalopods themselves are the target of large commercial fisheries worldwide, with an annual harvest of two million metric tons of squid alone [8].

Cephalopod biological research has a long history involving a wide range of experimental paradigms, the best known of which is the work on squid giant axon physiology that led to Nobel Prize awards for Alan Hodgkin and Andrew Huxley. Also prominent are the extensive investigations by J.Z. Young, Brian Boycott, Martin Wells and colleagues into cephalopod brain and behavior, with a particular focus on the sophisticated learning and memory systems of the octopus [9]. Cephalopod biology has recently become relevant to the field of biomimetic research, particularly for robotics and materials science [10,11]. There are likely to be many new areas of cephalopod-based research. For example, cephalopods immobilize prey organisms withtoxins, some of which are very poisonous to humans [1]. Study of such toxins may serve to identify new biomedically valuable reagents [12].

Cephalopods are mollusks, which show a greater variety of forms than do any other extant animal phylum. Even within the Mollusca, cephalopods display a remarkable level of modification in body plan organization. Particularly notable among the soft-bodied (coleoid) cephalopods are the reduction or loss of the shell, the adaptation of the mantle for locomotion and respiration, and the modification of the ventral molluscan foot into arms [2]. These innovations are undoubtedly tightly linked to the selective pressures from the loss of the shell and the development of a "high-performance" nervous system. The cephalopod lineage, and its origins from a monoplacophoran-like molluscan ancestor [2,13], thus represents a deeply attractive model for understanding the acquisition of novelty through evolutionary time.

All of these areas of cephalopod biology, from neuronal function at the cellular and systems levels to cephalopod population dynamics to the evolution of gene regulatory elements mediating body plan variation, would benefit greatly from the molecular insight that high-quality cephalopod genomics would provide. Indeed, it is astonishing that, in 2012, with the explosion of genome resources for so many life forms, there is not yet available a single assembled cephalopod genome. The goal of the NESCent meeting and this white paper is to provide organizational mechanisms for cephalopod biology to move from the pre-genomic to the post-genomic age.

Genomics

Genomic and transcriptomic sequencing will greatly aid the biological study of cephalopods. A sequenced genome produces a comprehensive list of genes, and contains the regulatory blueprint dictating their expression [14]. Sequenced transcriptomes reveal the expression levels of gene sets for different cells, tissues and organs at different developmental stages and under different physiological states [15,16]. Resequencing individuals of a genome-enabled species offers unprecedented datasets that can be applied to long-standing questions in population genetics, disease, and the characterization of species of commercial importance where there may be little *a priori* genetic knowledge [17,18]. Comparative genomics has revolutionized and stabilized our understanding of the evolutionary relationships among organisms throughout the Tree of Life, both living and recently extinct [19,20]. Sequence data have also advanced novel areas of research, such as nanotechnology, biomaterials and synthetic biology [21-23].

The most obvious benefit of cephalopod genomics will be to individual laboratories already studying cephalopod biology. With a full inventory and complete sequences for known genes of interest, laboratories can study gene function much more rapidly and thoroughly. In addition, with a near-complete inventory of protein-coding and non-coding RNA genes, these researchers can assess a much larger set of candidate genes for function in their biological processes of interest.

The greater benefits may come, however, to biological researchers outside the existing cephalopod field. Until very recently, genome-scale analyses of biological processes have favored the sequencing of two out of the three major divisions of bilateral animals [24]: deuterostomes (primarily vertebrates, with an expanding study of other chordates and selected non-chordates such as sea urchins and hemichordates) and ecdysozoans (from which the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* both come). In contrast, there has been far less genomic analysis of lophotrochozoans, with genomes published for only a handful of organisms, including three trematode parasitic worms and one oyster [25-29]. The genes and gene networks regulating the independent evolution of the host of highly derived features displayed in cephalopods are unknown, making comparative analyses of these phenomena at the level of gene function and regulation impossible. Sequencing of cephalopods would do more than expand our knowledge of genome organization within lophotrochozoans. With genomic data, researchers currently studying molecular evolution of complex metazoans would be able to investigate cephalopods as a new, independent instance of such evolution.

The genomes of cephalopods are known to be larger and more repeat-rich than many previously sequenced metazoan genomes [30]. With newly developed methods for sequencing and assembly [31,32], these genomes are now more tractable than they would have been even a few years ago. Indeed, the likely challenges of cephalopod genomics will prove an important test of these emerging technologies.

Genomic data will allow analyses of cephalopod molecular biology that have, until now, not been considered by the cephalopod community. Detailed studies of the genomes of mammals, flies, and nematodes have revealed unanticipated mechanisms of gene regulation: microRNAs-first characterized through nematode genetics and then shown to be ubiquitous [33]; epigenetic modification of the genome-first documented through the genetics of *Drosophila* position-effect variegation and then mechanistically clarified by studies in many species, including mammals [34,35]; and long non-coding RNAs-initially identified in mammals (*Xist*, *H19)* and flies (BX-C) and subsequently found to be pervasive [36,37]. The extent to which gene and protein expression in mollusks is regulated by the mechanisms identified in mouse, fruit fly, and nematode is unknown, but one striking example is provided by RNA editing. This regulatory process for protein diversification was initially described in mammals, but now appears to be much more widely employed in cephalopods than in vertebrates [38,39]. It is possible that deeper genomic studies of mollusks, and in particular cephalopods, will reveal additional, as yet undiscovered mechanisms of animal gene regulation.

Another promising arena of research that may benefit from cephalopod genomics is the global analysis of protein-coding gene families [40], which has to date been strongly biased towards deuterostomes and ecdysozoans. Proteins in these two groups feature extremely well characterized domains as well as domains that remain completely obscure and are typically described as "Domain

of Unknown Function" [41]. Cephalopod genomics can be expected to enrich our knowledge of such protein domain modules. Moreover, study of cephalopods will also almost undoubtedly expand the pool of protein domains, as it has already done in the identification of the reflectin protein family [11].

Choices of cephalopod species for genomic sequencing

Within the Mollusca, cephalopods diverged from a monoplacophoran-like ancestor over 500 million years ago, later branching into the extant clades Nautiloidea (*Nautilus* and *Allonautilus*) and Coleoidea (squid, cuttlefish and octopus) [2,42-44]. The CephSeq Consortium has come together with the intention of using strategic genomic and transcriptomic sequencing of key cephalopod species to address previously unanswerable questions about this group. Taking into account the challenges of cephalopod genome sequencing, as well as the necessity to address nodal taxa, we have identified a set of species on which to focus our initial efforts. Selected species have been chosen based on the curiosity of their biological features as well as the possible advantages of their practical use. These species also cover ecologically diverse life histories, representing benthic, nectobenthic and nectonic animals.

Cephalopods are animals with advanced cognitive skills and a complex repertoire of behavioral abilities [3,45]. Their brains are comparable both in size and complexity with those of vertebrates, and have been the focus of a number of studies on the neurobiology of behavior [46]. In particular, they have served as models for the cellular and systems circuitry of learning and memory [4,9]. Historically, *Octopus vulgaris* has been a key species for this work through studies of anatomy [9], behavior following lesions and brain stimulation [3,4,47] and cellular neurophysiology [48,49]. *O. vulgaris* has also served as an attractive model for neuroendocrine studies in invertebrates [5,50].

Recently, *Octopus bimaculoides* (California Two-spot Octopus) has emerged as a model system for cephalopod biology. The large size of *O. bimaculoides* eggs grants unique access to early embryonic stages, making this species a prime candidate for future genetic and developmental studies. The hardiness, ready availability in the United States and easy husbandry of adult *O. bimaculoides* [51] add to the appeal of this model species.

The deadly venom of blue-ringed octopus *Hapalochlaena maculosa* makes this species of interest for study of the evolution and regulation of toxicity within octopods [1].

Comparative studies of these octopus species would illuminate the bases of both their shared characteristics as well as those of their divergent features. Additionally, these species have essentially non-overlapping geographic distributions, providing animal accessibility to cephalopod researchers globally.

Within the decapodiforms, *Sepia* and *Loligo* are the most studied genera. Historically, *Sepia officinalis* has been a key cephalopod for neurobiological research, and is a critical species in global fisheries. *S. officinalis* possesses a complex chromatophore network for countershading, camouflage and communication [3,52,53]. Its internal calcified shell supplies buoyancy and the effect of global climate changes on this structure has become a focus of recent study [54,55]. *S. officinalis* is emerging as a particularly versatile model organism in eco-evo-devo studies [56]. As a practical matter, *S. officinalis* eggs are voluminous, and easily collected, maintained and reared in the laboratory [57]. The morphological events in *S. officinalis* embryogenesis are well described in the literature [58-61].

Loligo, and particularly its giant fiber system, has served as the fundamental basis for our understanding of nerve impulse conduction. The giant synapse system has recently been employed as a biomedical model of neurological disease [62]. *Loligo* is one of the most important groups for cephalopod fisheries in the North Atlantic [8]. *Loligo pealeii* is the premier experimental species of the loliginids, with not only an extensive publication base [63], but also annual availability at the Marine Biological Laboratory (Woods Hole, MA).

Euprymna scolopes is a unique cephalopod model organism because of its well-described symbiotic relationship with the luminescent bacterium *Vibrio fischeri*. This important biomedical model has been employed to study the mechanisms of host colonization and symbiont specificity, host/microbe cell-cell signaling, and innate immunity [64-67]. *Euprymna scolopes*' short life cycle and small egg size also make it an attractive choice for developmental studies in culture [68,69]. In 2005, the *V. fischeri* genome was sequenced [70]; having access to the host genome would allow this field to advance rapidly.

Pygmy squids (*Idiosepius*) have one of the smallest genomes among cephalopods (2.1 Gb), making them strong candidates for assembly and annotation [30]. Their small body size and exceptionally short life cycle also distinguish these cephalopods as possible model organisms [71].

The giant squid *Architeuthis dux* serves to represent deep-sea cephalopods. Little is known about the species of *Architeuthis*. *Architeuthis* is globally distributed and a recent analysis of the complete mitogenomes of multiple giant squid worldwide showed no detectable phylogenetic structure on the mitochondrial level and an exceptionally low level of nucleotide diversity, suggesting that there is only one global species of giant squid [72]. A nuclear reference genome for *Architeuthis* would clarify the population genetics of this species and provide critical information for comparative studies across cephalopods.

Nautilus, the cephalopod "living fossil", is a representative of a phylogenetically unique branch of the cephalopods, the nautiloids. *Nautilus* possesses many presumably ancestral anatomical features not shared with other cephalopods, including pinhole eyes, rhinophores for odor detection, an external shell, and numerous tentacles, all without suckers [73]. Comparative genomic studies employing *Nautilus* would highlight the genetic bases of these divergent features.

Sequencing strategy

Cephalopod genomes are large, complex and full of repeats. Sequencing and assembly may be technically very challenging. Below we recommend what, with the current state of hardware and software, would be excellent approaches to tackling cephalopod genomes. Researchers in the CephSeq Consortium will undoubtedly choose varying combinations of approaches for their specific projects. In any event, with rapid changes in the underlying technologies for sequencing, assembly and annotation, this series of technical recommendations will need to be revisited on a regular basis, and should be viewed as the snapshot it is of a particular moment (May 2012) in a rapidly advancing field.

Our recommendation for the initial approach to genome sequencing of cephalopods is to use a proven low-cost short-read sequencing approach (Illumina HiSeq with long-insert mate pairs). The current best practices for initial assembly of complex (≥1 Gb) eukaryotic genomes involve a mixture of high read coverage derived from short insert libraries (300-2000 bp) and high clone-coverage of longer insert (5-10 kb) and fosmid jump libraries (or mate-pair libraries). In this approach, approximately 45× coverage from the smaller insert libraries and 45× coverage from a 5-kb insert library would be produced for each taxon. In addition, 5× read coverage would be generated for 10-kb insert size libraries. For increasing genomic contiguity and long-range scaffolding, 40-kb fosmid jump libraries at 1× genomic coverage should be added for the ten pioneer cephalopod genomes (see Table 1). These methods have been tested and were successful in the sequencing of the 2.4 Gb giant panda [74] and the *de novo* assembly of the 3.2 Gb human genome with ALLPATHS-LG [75]. Additional approaches, such as sequence-based genetic mapping to bridge the gap between scaffolds and chromosomes and emerging long-read single molecule technologies (PacBio RS), could also be employed.

Initial efforts in cephalopod genomics, as well as more mature efforts in other molluscan genomes (*Aplysia, Biomphalaria, Lottia*), have identified many challenges in generating useful genomic assemblies. Many specific taxa were discussed at the NESCent meeting, and several collaborative projects have been initiated. For example, two species of *Octopus* will soon have genomic sequence generated, and two groups plan to sequence the smallest known cephalopod genomes, those of the genus *Idiosepius* (2.1 Gb). There was broad support at the meeting for sequencing *Sepia*, *Loligo*, and *Euprymna*, based on biological significance, research community size and phylogenetic position. Limited genome sequence data from *Sepia officinalis*, *Euprymna scolopes*, *Hapalochlaena maculosa*, *Architeuthis dux* and *Nautilus pompilius* are or will soon be available. Integration of these sequence data will assist with annotation and gene detection by sampling broadly across the phylogeny of cephalopods, with *Nautilus* providing an important outgroup for the coleoid cephalopods. Interpretation of cephalopod-specific genetic novelty and the innovations involved in nervous system specialization would be further assisted by the sequencing of an outgroup such as one from the Monoplacophora. While contiguous and annotated genomes are our ultimate goal, the strong sense of the community is that intermediate assemblies and transcriptome sequencing would be immensely helpful, and ideally would be exchanged prior to publication.

Table 1. Cephalopod species proposed for initial sequencing efforts.

Species	Estimated genome size (Gb)	Current sequencing coverage	Geographic distribution	Lifestyle juvenile/adult	Research importance
O. vulgaris	2.5-5	46×	world-wide	planktonic/ benthic	classic model for brain and behavior, fisheries science
O. bimaculoides	3.2	50×	California, Mexico	benthic	emerging model for development and behavior, fisheries science
H. maculosa	4.5	10×	Indo-Pacific	benthic	Toxicity
S. officinalis	4.5	-	East Atlantic-Mediterranean	nectobenthic	classic model for behavior and development, fisheries science
L. pealeii	2.7	-	Northwest Atlantic	nectonic	cellular neurobiology, fisheries science
E. scolopes	3.7	-	Hawaii	nectobenthic	animal-bacterial symbiosis, model for development
I. paradoxus	2.1	80×	Japan	nectobenthic	model for development, small genome size
I. notoides	-	50×	Australia	nectobenthic	model for development, small genome size
A. dux	4.5	60×	world-wide	nectonic	largest body size
N. pompilius	2.8-4.2	10×	Indo-Pacific	nectonic	"living fossil", outgroup to coleoid cephalopods

It must be emphasized that all the projects described above are in their infancy and are expected to benefit from the formation of the CephSeq Consortium. Indeed, representatives from each of these cephalopod sequencing efforts participated in the NESCent meeting and agreed to the formation of the Consortium.

Annotation of novel genomes is a complex problem [76]. Efforts at automated annotation of molluscan genomic sequences have demonstrated the challenge facing the future annotation of cephalopod genomes. Long branch lengths within the phylum, the taxonomic distances to well annotated animal genomes, and the relatively low quantity of previous molecular and genetic work in the Mollusca will demand the generation of additional resources to assist and train automated gene detection programs. Of primary importance will be the generation of transcript inventories to identify genes, refine gene models, detect start points and intron-exon boundaries, and train automated gene identification algorithms. Transcriptome data such as those from RNAseq are quick and relatively inexpensive to generate, and will be immensely useful. Systematic sequencing of nervous system tissues and embryonic stages can be combined with relatively early-stage assemblies to generate gene models and exon structures. In addition, pairs of *Octopus* species (*O. vulgaris* and *O. bimaculoides*) and *Idiosepius* species (*I. notoides* and *I. paradoxus*), through comparative sequence analysis, may be critical for annotation.

Annotation efforts are labor-intensive but also offer an opportunity to grow the cephalopod research community and attract outside expertise. For example, domain experts of particular gene families or pathways can be recruited to assist in the description of likely protein function. Bioinformatics researchers interested in the problems of annotation across long phylogenetic distances, the assessment of unique gene families and the evolution of biochemical novelty, and the likely challenges of extensively RNA-edited transcriptomes, will also be enlisted. Finally, annotation provides an outreach opportunity to involve young scientists and K-12 classrooms in cutting-edge scientific discovery on these fascinating organisms.

Data sharing plan

An important goal of the CephSeq Consortium is to share data rapidly and effectively both within and beyond the Consortium. Data sharing is necessary to foster the broadest possible impact of our sequencing and annotation efforts. This sharing will prove critically important for the cephalopod community. We expect sequence homology within the taxon to be an important foundation for collaboration within the field because cephalopods have evolved many new and unique character features. Sharing data prior to publication could significantly accelerate cephalopod research. However, data sharing policies must also recognize that there is significant publication, funding, and career recognition risks involved in making data available before publication: often the first to publish a particular observation garners the most recognition.

Broad data-sharing agreements such as the Ft. Lauderdale agreement [77] have already been adopted by the international genomics community, and, most significantly, by many large sequencing centers. However, as the sequencing capacity of small collaborations has increased, this type of agreement is an increasingly poor fit for the data being generated. Moreover, for a federated community such as the CephSeq Consortium, with significant international participation by many small groups, enforcement of any agreement is challenging. We believe that an explicit policy should be adopted to protect data generators while creating incentives for the earliest possible sharing of data. An effective policy should also encourage use of cephalopod sequence data beyond the currently defined cephalopod community, while protecting the interests of those generating the data.

We therefore propose to adopt a liberal opt-in data sharing policy, modeled in part on the JGI data usage policy [78], which will support the rapid sharing of sequence data, subject to significant restrictions on certain types of usage. Community members will be encouraged to submit their data, but not required to do so. We plan to provide incentives for this private data sharing by (1) developing a community data and analysis site with a simple set of automated analyses such as contig assembly and RNAseq transcript assembly; (2) offering pre-computed analyses such as homology search across the entire database; and (3) supporting simple investigative analyses such as BLAST and HMMER. We also plan to provide bulk download services in support of analysis and reanalysis of the entire dataset upon mutual agreement between the requesting scientist and the CephSeq Consortium Steering Committee (see below), who will represent the depositing scientists. Collectively, these policies would provide for community engagement and participation with the CephSeq Consortium while protecting the interests of individual contributors, both scientifically and with respect to the Convention on Biological Diversity [79]. Policy details will need to be specified and implementation is subject to funding. Our intent is to build an international community by putting the fewest barriers between the data and potential researchers, while still protecting the data generators.

The CephSeq Consortium: Mission statement and organization

Mission Statement: The vision of the Cephalopod Sequencing Consortium is rapid advancement of cephalopod science into the genomics era, one employing the most modern and efficient methods available and engaging broad international participation by the entire cephalopod scientific community. This vision entails communication and active promotion of sequencing technologies and findings to researchers across a great diversity of fields. Bioinformatics experts initially outside of cephalopod biology will participate with cephalopod researchers in this effort. The Consortium will help facilitate funding endeavors by individuals and groups by providing basic summary documents (*e.g.*, white papers, letters of support) that describe the current state and consensus goals of

cephalopod genomics efforts worldwide. In addition to promoting and accelerating scientific progress, the CephSeq Consortium aims to translate the contributions of cephalopod science to society at large by encouraging applied science in fields as diverse as fisheries science, materials science and biomedical research. Education and outreach will be emphasized for broad dissemination of progress in cephalopod genomics at multiple levels, including K-12, undergraduate and graduate students, and the public at large.

Organizational Structure: Establishment of a Steering Committee was agreed upon at the May 2012 NESCent Catalysis Group Meeting. The composition of the committee was initially set at seven members, with broad international representation of cephalopod biologists, genomicists and bioinformaticians. The Committee will initially meet every 4 months, either in person, or remotely, or both. The Steering Committee is charged with providing international oversight of the community's activities, fostering the free-flow of information among CephSeq Consortium members (see Data Sharing Plan), promoting collaborations, and ensuring that the CephSeq Consortium remains focused on the Mission Statement objectives set forth above. The Steering Committee will also work to facilitate community-wide efforts to annotate assembled genomes.

The tenure of the Committee will initially be two years, and any and all cephalopod researchers are encouraged to contact the Committee about the changing needs of the community. The inaugural members are: Laure Bonnaud (Univ. Paris, France), C. Titus Brown (Michigan State Univ., USA), Roger Hanlon (Marine Biological Laboratory, USA), Atsushi Ogura (Ochanomizu Univ., Japan), Clifton Ragsdale/Chair (Univ. Chicago, USA), Jan Strugnell (La Trobe Univ., Australia) and Guojie Zhang (BGI, China).

A web site [80] will serve as a point of contact for the worldwide community. An auxiliary site for sharing cephalopod genomic and transcriptomic data is to be established within the next six months (see Data Sharing Plan). The CephSeq Consortium will coordinate internationally with the Cephalopod International Advisory Council (CIAC) [81] and with the newly established CephRes-Associazione Cephalopod Research-ONLUS [82], which is based in Europe.

Workshops will be organized annually to ensure coordinated and cooperative progress in genomics on an international scale. One likely venue for such workshops would be society meetings, such as the annual meeting of the Society for Integrative and Comparative Biology (SICB).

The Steering Committee urges scientists who support the goals of this white paper to join the consortium by signing the white paper and participating in the activities of the consortium.

Broader impacts

A specific recommendation of this white paper is to compete for a Research Coordination Network (RCN) grant from the NSF. A Cephalopod RCN would facilitate annotation of the cephalopod genomes being produced worldwide, mediate the exchange of emerging technologies that will benefit from genomic resources and accelerate the advent of new areas of research made possible by cephalopod genomics. It would also serve to expand the next generation of cephalopod researchers. Consequently, a central element of a Cephalopod RCN would be short-term laboratory exchanges for undergraduate and graduate students to aid in genome annotation and analysis, to promote education in bioinformatics and cephalopod biology and to foster new collaborations across the cephalopod community.

Cephalopods are important to science, including the fields of cellular neurobiology, learning and memory, neuroethology, biomaterial engineering, animal-microbe interactions, developmental biology, and fundamental molecular biology such as RNA editing. Access to genomic information will greatly facilitate this ongoing research, particularly through gene discovery. Cephalopod genomics will also drive the creation of new areas of investigation, including such biomedically important topics as regeneration and aging [83,84]. Other examples of promising post-genomic cephalopod research include study of the unknown chemosensory systems by which cephalopods monitor their marine environments, and the isolation of cephalopod neurotoxins, which could lead to novel reagents for research and drug-based therapies [12]. Cephalopod genomics will also be important for evolutionary biology, particularly for understanding the great diversity and genomic complexity of the whole molluscan phylum and for probing the emergence of the evolutionary innovations that are represented by cephalopod eyes, large brains and prehensile arms.

Cephalopods are a critical component of marine ecology, are important commercially to the fisheries industry and are an emerging aquaculture taxon. The effects of global warming and marine acidification and hypoxification on cephalopod health and viability are unknown and can only be fully assessed with improved species delineation and a deeper understanding of population dynamics. Specifically, cephalopod genomics will aid our ability to track population migrations and monitor demographic expansions and contractions. This information will in turn directly inform efforts to assess the effects of climate change on cephalopod stocks [85]. Cephalopods are a critical food source and genomic resources can also be expected to help monitor cephalopod overfishing and improve cephalopod aquaculture.

People are fascinated by cephalopods, from *Nautilus* to the octopus to the giant squid. The coupling of genomics to cephalopod biology represents a fusion of two areas of great interest and excitement for the public. This fusion presents a tremendous educational platform, particularly for K-12 students, who can be engaged in the classroom and through the public media. Public outreach about cephalopod genomics will help build support for basic scientific research, including study of marine fauna and ecology, and will add to the public's understanding of global changes in the biosphere.

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Christon J. Hurst *Editor*

The Mechanistic Benefits of Microbial Symbionts

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Hawaiian bobtail squid, *Euprymna scolopes*. Courtesy of Margaret McFall-Ngai

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Christon J. Hurst
Editor

The Mechanistic Benefits of Microbial Symbionts

Contents

Chapter 11
Fiat Lux: The Squid–Vibrio Association as a Model for Understanding Host–Microbe Associations

Spencer V. Nyholm

Abstract The symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* offers an experimentally tractable model for understanding the role of beneficial bacteria on animal development and the mechanisms by which host and symbionts establish and maintain highly specific associations. The symbiont is transmitted from the environment each generation, and mechanisms must be in place to ensure specificity. Research over the years has revealed some of the "molecular dialogue" that occurs between the partners during and after colonization. Many of these interactions involve microbe-associated molecular patterns (MAMPs) and host pattern recognition receptors (PRRs) as well as components of the host's innate immune system. The role of light production by the symbiont and light detection by the host is also critical to the association and has likely served as a driving force during the evolution of this symbiosis. Finally, the host harbors a second symbiosis, housing a consortium of bacteria in the female reproductive system. *Euprymna scolopes* therefore offers the unique opportunity to study both a binary and consortial symbiosis in the same host.

11.1 Importance of Model Associations in Symbiosis Research

All animals and plants form beneficial associations with microorganisms, and such associations have had a profound effect on the evolution of these groups (McFall-Ngai et al. 2013; Oldroyd 2013). In recent years it has become evident that symbionts play a critical role in the development and health of not only individual hosts, but entire ecosystems [e.g., coral reefs; see Chap. 10 by V. Weis and hydrothermal vent and other chemoautotrophic ecosystems (Dubilier et al.

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2008)]. Understanding the molecular mechanisms by which these associations are established and maintained can be difficult because often the symbionts occur as complex consortia where delineating the role of any one member is challenging. Therefore, employing the use of model systems with fewer partners is often advantageous. The binary association between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* is one such system that has been used to understand how beneficial host–microbe associations are formed (McFall-Ngai 2014). This chapter will review how this highly specific association is established and maintained, highlighting the molecular mechanisms by which the partners communicate to achieve this goal and how the host's innate immune system contributes to the specificity of the symbiosis.

11.2 Bioluminescent Symbioses

Bioluminescence (the production of light by living organisms) is a common biological phenomenon in many environments but is especially common in marine ecosystems (Widder 2010). The light produced by organisms is used for a number of behaviors including finding prey, camouflage to avoid predation, and attracting mates. The vast majority of fauna use autogenic bioluminescence, meaning they produce the chemicals (substrate luciferin and enzyme luciferase) necessary for light generation. A few groups, mainly found among fishes and squid, rely on a symbiotic relationship with bacteria for light production.

The majority of bioluminescent bacteria in the marine environment belong to members of the *Gammaproteobacteria* group *Vibrionaceae* and primarily within the genera *Vibrio* and *Photobacterium* (Guerrero-Ferreira et al. 2013; Urbanczyk et al. 2011). Members of these groups have formed associations with a number of different species of fish and squid (Guerrero-Ferreira and Nishiguchi 2007; Urbanczyk et al. 2011). Perhaps the best studied of this group is *Vibrio fischeri* (also referred to as *Aliivibrio fischeri*) which produces light through a process known as quorum sensing. This phenomenon, which was first discovered in *V. fischeri*, regulates light production based on density-dependent cell–cell communication [reviewed in Verma and Miyashiro (2013) and Miyashiro and Ruby (2012)]. *Vibrio fischeri* produces a freely diffusible chemical autoinducer known as an N-acyl homoserine lactone (3-oxo-C6-HSL) that initiates gene expression when a quorum or critical density of bacterial cells is present (e.g., as found in culture or contained within the light organ of a host). The chemistry of bacterial bioluminescence in *V. fischeri* is based on production of an enzyme (luciferase) that oxidizes substrates [bacterial luciferin; reduced flavin mononucleotide (FMNH2)] and a long-chain fatty acid (RCHO) into FMN and aliphatic acid (RCOOH). The genes for all of these factors are encoded by the *lux* operon (Lux ICDABEG) (Gray and Greenberg 1992) and are transcriptionally activated when 3-oxo-C6-HSL binds the LuxR activator. A positive feedback loop allows for the production of more autoinducer (LuxI) and thus increases luminescence output. Luminescence in *V. fischeri* is also regulated by

two other quorum sensing systems AinS–AinR and LuxS–LuxP/Q [reviewed in Verma and Miyashiro (2013)]. Unlike many nutritional symbioses discussed in other chapters of this book, luminescence (light production) is the main selective force in bioluminescent symbioses.

11.3 The Association Between *Euprymna scolopes* and *Vibrio fischeri*

The association between the model cephalopod *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* has been used for over 25 years now to understand interactions of animal hosts with beneficial bacteria (McFall-Ngai 2014). This association has many advantages that lend themselves to the study of host–microbe interactions. For example, each partner can be raised independently in the laboratory and is readily available for molecular, biochemical, and genetic analyses. The female host lays clutches of hundreds of eggs that hatch after an approximate 20-day embryogenesis, allowing a high sample number of squid for any given experiment. Numerous researchers are currently maintaining squid rearing facilities, and a small cohort of animals (typically 10–20 breeding pairs) will yield approximately 20,000–60,000 juvenile squid per year that may be used for experimentation. The establishment of the symbiosis takes place over a short time frame, i.e., colonization occurs within hours after the host hatches from egg cases and therefore experiments can often quickly be resolved.

Euprymna scolopes is a relatively small squid (average adult length = 30–40-mm) that is an active nocturnal predator endemic to the Hawaiian archipelago. It belongs to a family of squid known as the Sepiolidae whose members are found in the Indo-Pacific and Mediterranean Sea and often form associations with bioluminescent bacteria. As with other bioluminescent hosts, a defining feature of *E. scolopes* is the presence of a bilobed light organ that is located in the center of the mantle cavity and is part of the hindgut-ink sac complex (McFall-Ngai and Montgomery 1990). The light organ itself is made up of a number of complex tissues including a lens, reflective tissue, and epithelium-lined crypt spaces that house the extracellular symbionts (to densities of 10^9 *V. fischeri* cells per adult squid). The crypt spaces are connected to the environment via a ciliated duct that terminates at a pore on either side of the light organ (Fig. 11.1). Like other squid, *E. scolopes* is relatively short lived (9 months to 1 year). Juvenile squid hatch from externally laid egg cases that are deposited in the environment among coral reefs and shallow sand flats. Embryogenesis is approximately 3 weeks, and juvenile squid hatch without their symbionts and thus must be colonized by *V. fischeri* from the environment each generation. *Euprymna scolopes* is a nocturnal predator that hunts for small crustaceans near coral reefs. The light produced by the bacteria is used to camouflage the host in a behavior known as counterillumination (Jones and Nishiguchi 2004). The host is able to match down-welling moonlight and

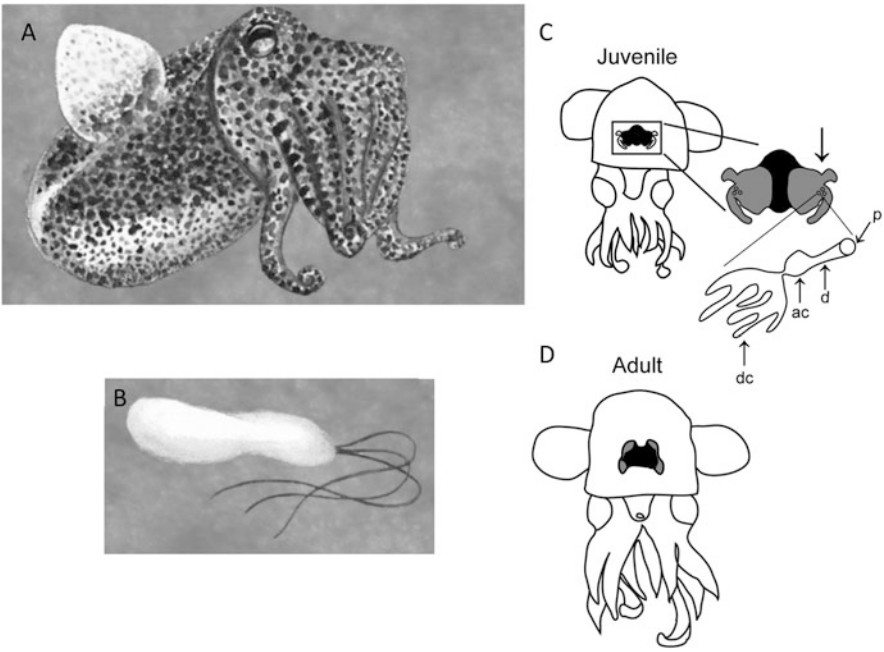

Fig. 11.1 The *Euprymna scolopes–Vibrio fischeri* symbiosis. The Hawaiian bobtail squid (**a**) forms a highly specific association with the bioluminescent bacterium *Vibrio fischeri*. (**b**) The *V. fischeri* bacteria are acquired from the environment and colonize the nascent host light organ for each new generation of squid. *Vibrio fischeri* is flagellated and its motility is crucial during the early stages of colonization. (**c**) Ventral surface of a juvenile squid. The nascent light organ is located in the center of the mantle cavity and includes superficial ciliated epithelial fields (*gray*). The interior of one juvenile light organ crypt system, including the pores (p), ducts (d), antechambers (ac), and the deep crypts (dc). (**d**) Ventral surface of an adult squid showing the mature light organ (**a** and **b** drawn by Andrea Suria; **c** and **d** are adapted from Rader and Nyholm (2012))

starlight such that the silhouette that would normally be viewed by predators is obscured. In exchange, the symbionts are housed in the light organ where they receive all of their nutrition from the host.

Vibrio fischeri is a cosmopolitan bacterium that is found throughout the world's oceans. In Kaneohe Bay on the island of Oahu, Hawaii, *V. fischeri* populations are as much as 24–30 times higher in areas that also harbor the Hawaiian bobtail squid compared to surrounding waters that lack *E. scolopes* (Lee and Ruby 1994; Jones et al. 2007). This increase in the abundance of bacteria in the squid's habitat is likely due to a unique diel rhythm that occurs in this association (Fig. 11.2). While the host is hunting at night, it has a full complement of *V. fischeri* such that the symbionts are at a high cell density and bioluminescence is induced (see above). At dawn, the host undergoes a quiescent period where it buries in the substrate. At this time and in response to a light stimulus (sunlight), the musculature of the light organ contracts and expels the contents of the crypt spaces into the surrounding seawater (Boettcher et al. 1996; Nyholm and McFall-Ngai 1998). Approximately 95 % of the symbionts are expelled, and the remaining 5 % of *V. fischeri* cells divide

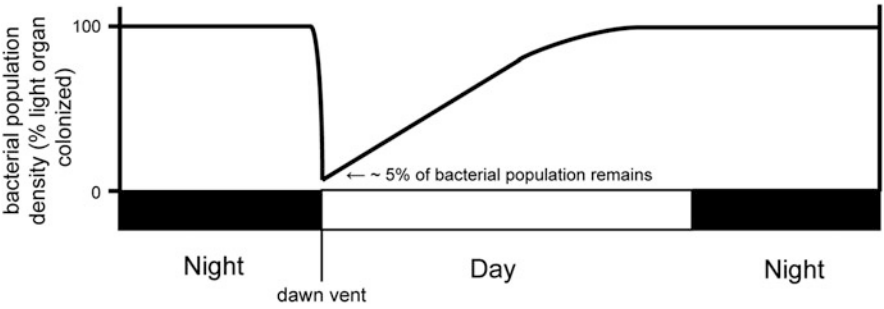

Fig. 11.2 Host-symbiont daily rhythm. The squid–vibrio association undergoes a diel rhythm whereby the light organ contains a full symbiont complement when the host is hunting at night. Because *V. fischeri* cells are at a high density during this time, the genes for bioluminescence are activated, and light is produced which the host in turn uses as a means of camouflage known as counterillumination. At dawn each day, 95 % of the symbionts are expelled, and the remaining symbionts divide during the day to repopulate the organ. This rhythm serves to regulate the symbiont population and to seed the environment with viable *V. fischeri* [Adapted from Rader and Nyholm (2012)]

during the day so that there is once again a full population when the host emerges at dusk to hunt. The squid's light organ can therefore be thought of as a chemostat whereby this "bioreactor" leads to a daily growth and expulsion of large numbers of *V. fischeri*. The expelled bacteria are viable and able to transition to a free-living state. These active and motile cells are then capable of colonizing the next generation of squid. Dawn therefore represents a dramatic transition in the association. Not only are the symbionts expelled, but the exudate also contains shed apical surfaces of host epithelial cells and blood cells (hemocytes) all embedded in a thick matrix (Nyholm and McFall-Ngai 1998). The host's crypt epithelium undergoes a restructuring whereby the apical surfaces are effaced and blebbed into the crypt spaces in the hours preceding dawn (Wier et al. 2010). There is also an upregulation of greater than 50 cytoskeleton-related genes during this time reflecting dynamic changes to the crypt epithelium. After dawn, the epithelium reestablishes polarity, and there is a concomitant downregulation of the same cytoskeleton-related genes. The symbionts also undergo cyclic transcriptional changes that are notably tied to metabolism. Genes associated with anaerobic respiration of glycerol are upregulated during the day and transition to a metabolism of chitin fermentation in the hours before dawn. Lipid analysis also suggests that host-derived fatty acids are incorporated into the symbionts' membranes as a result of effacement of the crypt epithelium (Wier et al. 2010).

11.4 Steps to Colonization

One of the hallmarks of the squid–vibrio association is the high degree of specificity. *Vibrio fischeri* is the only bacterium that is capable of colonizing the light organ. Work over many years has focused on the intricate communication that occurs between the host and symbiont and the molecular and cellular dialogue that is involved. *V. fischeri* from the environment must colonize the host among a background of a number of nonsymbiotic microorganisms. In areas with large squid populations, *V. fischeri* can be found at concentrations of 100–1500 cells per ml of seawater among a background of a million other nonsymbiotic bacteria (Lee and Ruby 1994). The light organ of the juvenile host is also quite different from the adult and is poised to interact with bacteria from the environment. Notably, the juvenile light organ has elaborate ciliated epithelial fields on its lateroventral surfaces. The fields are comprised of two appendages that form a ring surrounding three pores on either side of the light organ. These cilia begin to beat within seconds after the squid emerge from their egg case and help entrain seawater near the vicinity of the pores. *Vibrio fischeri* must enter these pores and travel down a ciliated duct before entering an antechamber that leads to crypt spaces where colonization occurs (Fig. 11.1). One of the challenges for *V. fischeri* is that the mantle volume of the squid is quite small (1.3 μl) such that only a few *V. fischeri* cells are present in the mantle cavity and any given time. Because the squid is constantly ventilating, *V. fischeri* must find one of the three pores on either side of the light organ before being expelled back into the environment. How does *V. fischeri* overcome this challenge, and what prevents nonsymbiotic bacteria from entering and colonizing the light organ? There are a series of physical, chemical, and cellular barriers that assist the host in selecting the correct symbiont from seawater, and in turn, *V. fischeri* has mechanisms to ensure that it can overcome each of these potential barriers and successfully colonize the light organ.

One way in which the host helps entrain environmental bacteria is the secretion of mucus from the ciliated fields soon after hatching. Gram-negative bacteria from the environment, including *V. fischeri*, are then capable of aggregating in this mucus forming a type of biofilm (Nyholm et al. 2000). In addition to the host mucus, *V. fischeri* is capable of generating a biofilm using an 18-gene *syp* (symbiosis polysaccharide) locus that is important for this purpose (Yip et al. 2005, 2006; Norsworthy and Visick 2013). Association with the biofilm also represents the first site of specificity as *V. fischeri* outcompetes other Gram-negative bacteria in these aggregations (Nyholm and McFall-Ngai 2003). Recent work has shown that only a few cells (three to five) are necessary to aggregate and initiate colonization (Altura et al. 2013). In addition to association with the mucus, these cells also appear to directly bind cilia associated with the surface epithelium before migrating to and entering the light organ pores (Altura et al. 2013). Amazingly, interactions with these few initial cells lead to widespread transcriptional changes in the host that appear to prime the organ for colonization (Kremer et al. 2013). Specifically, the

host upregulates expression of an endochitinase that hydrolyzes polymeric chitin found in the host mucus secretions to chitobiose. Chitobiose, in turn, acts as a chemoattractant for cells of *V. fischeri* that migrate to the pores (Mandel et al. 2012).

The ability to migrate to the pores and away from the initial aggregate is dependent on motility. The mantle cavity of a juvenile squid is a very dynamic microenvironment. The ciliated fields create microcurrents that help to move particles toward the pores, but then outward-beating cilia in the ducts present a challenge for bacteria moving down these ducts to the antechamber within the light organ tissue. *Vibrio fischeri* has a tuft of polar flagella that it uses to move and chemotax toward the ducts (Ruby and Asato 1993). Knocking out various genes involved with motility has demonstrated that both non-motile or hyper-motile mutants are deficient in colonization (Graf et al. 1994; Nyholm et al. 2000; Millikan and Ruby 2002, 2004; Wolfe et al. 2004; Brennan et al. 2013). The symbiont also undergoes morphological changes during its transition from a free-living bacterium to a symbiont. For example, after colonization, *V. fischeri* loses its flagella while it divides and populates the light organ (Ruby and Asato 1993). A proteomics study of the crypt contents showed that a number of symbiont proteins involved with bacterial flagellar production were present during venting, suggesting that *V. fischeri* may be capable of anticipating the transition from the squid host to the environment since the symbiont loses its flagella after colonization (Schleicher and Nyholm 2011). Symbionts that are vented from the light organ each morning are still viable and regain their flagella-based motility.

In addition to motility, chemotaxis is also important in the trek that *V. fischeri* must make to the host. This chemotaxis often relies on two-component regulatory systems consisting of a response regulator and sensor kinase, along with methyl-accepting chemotaxis proteins [MCPs; reviewed in Norsworthy and Visick (2013)]. These MCPs are coupled to a sensor kinase (CheA) and two response regulators (CheY and CheB). Methylation of MCPs allows bacteria to respond rapidly to environmental cues and is regulated by a methyltransferase (CheR) and methylesterase (CheB). *Vibrio fischeri* has a number of putative MCPs (43 predicted from the genome) suggesting that the symbiont can respond to many environmental cues (Brennan et al. 2013). Experimental evidence showed that deletions to either CheY or CheR lead to a deficiency in colonization (Hussa et al. 2007; Deloney-Marino and Visick 2012). *Vibrio fischeri* is also capable of chemotaxing toward *N*-acetylneuraminic acid (NANA) (DeLoney-Marino et al. 2003) and chitin derivatives (Mandel et al. 2012) (GlcNAc and GlcNAc2) which are found in the shed mucus and light organ crypts, respectively (Nyholm et al. 2000; Heath-Heckman and McFall-Ngai 2011). Furthermore, disruption of a chitin gradient prevents *V. fischeri* from entering the light organ pores (Mandel et al. 2012).

11.5 Molecular Dialogue Between the Partners

Much of the molecular "conversation" between the partners is mediated by what are known as microbe-associated molecular patterns (MAMPs) (Koropatnick et al. 2004) (Table 11.1). These are molecules unique to microbes and include, for example, the cell wall component peptidoglycan, outer-membrane proteins, or other compounds like lipopolysaccharide (LPS). In turn, these MAMPs are often detected by host pattern recognition receptors (PRRs), the binding of which results in downstream signaling cascades that influence host transcription, often related to the immune system (Nyholm and Graf 2012). One of the first MAMPs that *E. scolopes* encounters is bacterial peptidoglycan (PGN). The host hatches from its egg case into seawater containing numerous environmental bacteria (up to 10^6 cells/ml). Since both Gram-positive and Gram-negative bacteria have PGN, it is quite abundant in seawater. Exposing hatchling squid to bacteria or exogenous PGN leads to mucus secretion from the ciliated epithelium of the nascent light organ (Nyholm et al. 2002). One area of intensive research in the squid–vibrio association is the influence of the symbiont on the development of the light organ. Early observations showed that colonization by *V. fischeri* leads to morphogenesis of the light organ whereby ciliated epithelial tissues that assist in colonization undergo apoptosis and regress over the first days of the host-symbiont association (McFall-Ngai and Ruby 1991; Montgomery and McFall-Ngai 1994; Doino and McFall-Ngai 1995; Foster and McFall-Ngai 1998). Two MAMPs produced by *V. fischeri*, LPS and a PGN derivative called tracheal cytotoxin (TCT), work synergistically to induce this morphogenesis (Foster and McFall-Ngai 1998; Koropatnick et al. 2004). These findings are significant as these MAMPs are often associated with inducing virulence in pathogenic associations. For example, TCT had been only known in the human pathogens *Neisseria gonorrhoeae* and *Bordetella*

Table 11.1 Host and symbiont mediators of specificity and persistence in the squid–vibrio association

Host mediators of specificity and persistence	Symbiont mediators of specificity and persistence
Innate immune system – Hemocytes (trafficking to light organ, tolerance to *V. fischeri*, delivery of chitin to symbionts) – Pattern recognition receptors (Toll-like receptors, TLRs; peptidoglycan recognition proteins; PGRPs, galectins, lipid-binding proteins, LBPs – Reactive oxygen and nitrogen species – Galaxins – Cryptochromes – Hemocyanin Alkaline phosphatases Specialized ciliated epithelium Host mucus secretions Endochitinase	Microbe-associated molecular patterns (MAMPs) – Lipopolysaccharide (LPS) – Peptidoglycan (PGN) – Tracheal cytotoxin (TCT) Light production (dark mutants do not persist in light organ) Motility Biofilm formation (Syp locus) Chemotaxis (CheR)

pertussis where it promotes virulence by damaging epithelial cells. The structure of TCT in *V. fischeri* is identical yet it does not cause virulence in the squid. These data suggest that what would normally be classified as a toxin may also serve as a signaling molecule during normal development.

Euprymna scolopes has a number of pattern recognition receptors (PRRs) that have the potential to respond to MAMPs. These include five peptidoglycan recognition proteins (PGRPs), a Toll-like receptor (TLR), and LPS-binding proteins (LBPs) (Goodson et al. 2005; Troll et al. 2009, 2010; Krasity et al. 2011; Collins et al. 2012b). Host PGRPs are expressed in specific tissues and cell types, and some appear to change upon colonization. For example, EsPGRP1 is present in the nuclei of host epithelial cells but is later absent in cells that undergo apoptosis during light organ morphogenesis (Troll et al. 2009). Mutants of *V. fischeri* that are defective in TCT release do not induce loss of EsPGRP1. The EsPGRP2 protein has a secreted form that has been found in the light organ crypt spaces as well as in mucus produced during the onset of colonization (Troll et al. 2010). This same protein, EsPGRP2, also has an amidase activity enabling it to degrade TCT. In addition, the host has two alkaline phosphatases (APs) that are present in the light organ and have the ability to dephosphorylate and inactivate the lipid A portion of *V. fischeri* LPS (Rader et al. 2012). Twelve hours postcolonization, a time point when light organ morphogenesis is irreversible (Doino and McFall-Ngai 1995), there is an increase of AP expression in the light organ that mirrors the daily rhythm of the symbiosis, higher in the evening and lower during the day. Taken together these data suggest that the host has mechanisms to respond to *V. fischeri* MAMPs in order to promote colonization and then to maintain the association.

The production of light by *V. fischeri* serves as the basis of the squid–vibrio association. The effectiveness of counterillumination is difficult to demonstrate outside a controlled experiment in the presence of a predator, but the importance of light production by the symbiont and the ability of the host to detect that light are strongly supported. One question that is often posed is how the host prevents a "cheater," signifying in this case bacteria that consume resources but do not provide illumination, from colonizing the light organ. Is it possible for a "dark" strain of *V. fischeri* to colonize the light organ, effectively parasitizing the host without having to produce light? An experimental study showed that a strain of *V. fischeri* that is deficient in producing light was unable to persist in the light organ (Visick et al. 2000). Furthermore, mutants defective for light production are impaired in terms of inducing developmental phenotypes normally associated with colonization (Chun et al. 2008; McFall-Ngai et al. 2012). Light produced by the symbiont also appears to lead to an increase in expression of PRRs and innate immunity factors like EsPGRP1, LPS-binding protein, and galaxins (Chun et al. 2008). These data suggest that the host has the ability to detect and respond to light produced by the symbiont. How is this achieved? In some ways the light organ very much resembles an eye. There are a lens and reflective tissue that help to transmit the light produced by the symbionts. In addition, the light organ tissues of the host express the genes necessary for phototransduction including opsin, rhodopsin kinase, and arrestin (Tong et al. 2009), and colonization by *V. fischeri* subsequently influences the

expression of host genes associated with eye specification and development (Peyer et al. 2014). The host also has two cryptochromes, proteins that in other systems help regulate circadian rhythms (Heath-Heckman et al. 2013). Expression of one of these, *escry1*, had an expression pattern that mirrored the daily rhythm of bacterial bioluminescence, being highest when luminescence was also at its peak. Colonization by a bacterial mutant defective in light production was noted to disrupt the rhythm of *escry1* expression, and *escry1* expression subsequently was capable of being rescued by exposure of the squid to blue light. Interestingly, exposure to just blue light and the MAMP TCT and lipid A also led to rhythmic *escry1* expression. Therefore in the squid–vibrio association, light acts as a signal and morphogen similar to the MAMPs described above. How does the host sanction "dark" cheaters? This question remains to be answered but given the importance of light production to the host's survival, having the ability to regulate and respond to the symbiont's light is paramount.

11.6 Interactions with the Innate Immune System

All organisms have mechanisms to defend themselves against pathogenic microorganisms (usually bacteria, viruses, or eukaryotic parasites). The immune system often mediates these interactions and much research has been focused on understanding how hosts overcome these challenges. However, a more recent view of the immune system suggests that it also has a significant role in mediating the establishment and maintenance of beneficial associations. Having a need to interact and "communicate" with environmental microorganisms was likely a major driving force during the evolution of immune systems, and studies have shown that the immune system plays a critical role in mediating symbioses with microbes (Nyholm and Graf 2012).

Invertebrates lack canonical adaptive immunity meaning they don't have the capability to produce antibodies to specific antigens (this trait appeared with the jawed vertebrates). Alternate mechanisms that generate highly variable immune proteins have been described for some specific organisms [e.g., fibrinogen-related proteins in the snail *Biomphalaria glabrata* (Zhang et al. 2004) or Down's syndrome cell adhesion molecules in *Drosophila melanogaster* (Watson et al. 2005)]. However, most invertebrates are thought to rely on the innate immune system, primarily comprised of phagocytes and the production of antimicrobial compounds or reactive oxygen and nitrogen species. How is it that invertebrates can form highly specific associations with microorganisms in the absence of antibody-based immunological memory? Many studies have shown that the innate immune system of animals can interact specifically with microorganisms to form these associations [reviewed in Nyholm and Graf (2012)].

The cellular-based immune system of cephalopods (squid, octopuses, and cuttlefish) largely consists of macrophage-like hemocytes (Fig. 11.3). Hematopoiesis of these cells occurs in a specialized organ called the white body from which mature

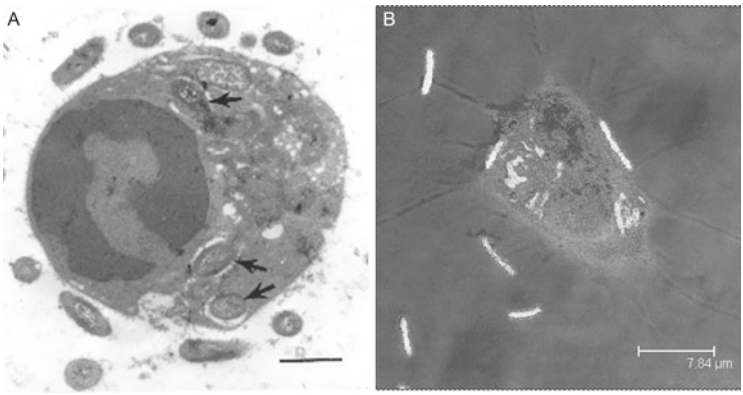

Fig. 11.3 Interactions with host hemocytes. Specificity is partially mediated by the main innate immune cell type of the host, the macrophage-like hemocyte. (**a**) Hemocytes are sometimes observed with phagocytosed bacteria (*arrows*) within the light organ crypt spaces. (**b**) The ability for hemocytes to differentially bind symbiotic and nonsymbiotic bacteria is influenced by colonization of the light organ. Removing the symbiont by curing the host with antibiotics leads to increase binding of *V. fischeri* (Nyholm et al 2009) [(**a**) Adapted from Nyholm and McFall-Ngai (1998)]

hemocytes enter the circulatory system. Unlike other molluscs, cephalopods have a very complex and enclosed circulatory system with a systemic and two branchial hearts that distribute hemolymph containing the main oxygen-binding protein hemocyanin, other extracellular proteins, and hemocytes to all of the tissues and organs. The light organ is also highly vascularized, and hemocytes are capable of migrating to and entering the crypt spaces that house *V. fischeri*, and thus the hemocytes are expelled with the daily venting of the symbionts each morning (Nyholm and McFall-Ngai 1998). Why do hemocytes migrate into the light organ? Are they involved with regulating the symbiosis, perhaps preventing overgrowth of the symbiont or sanctioning strains of *V. fischeri* that are inefficient or underperforming in light production? Because the crypt spaces are directly linked to the environment via the pores and ciliated ducts, the potential for other nonsymbiotic or pathogenic microorganisms to enter the crypts is always present. Observations of symbiotic juveniles have shown that hemocytes in the crypt spaces occasionally have engulfed or phagocytosed bacteria (Nyholm and McFall-Ngai 1998). This observation begs the question: are these phagocytosed bacteria *V. fischeri* or nonsymbiotic bacteria that have made their way into the light organ and are in the process of being removed?

To understand the dynamics of this process, in vitro binding assays showed that host hemocytes that were isolated from colonized adult animals recognized and bound the cells of four marine bacterial species to different degrees (Nyholm et al. 2009). Specifically, hemocytes bound significantly more cells of *Vibrio harveyi* and *Photobacterium leiognathi* than those of *V. fischeri* and *Vibrio parahaemolyticus*. To examine whether hemocyte binding behavior was influenced by colonization, the symbiont population was eliminated from a subset of animals

by antibiotic treatment prior to the collection of the hemocytes. A cohort of animals was maintained in the cured state for an additional 4 days, during which time bacteria-binding efficiency was determined for hemocytes isolated either from cured (naïve) or symbiotic animals. No change was detected in the ability of the hemocytes from symbiotic hosts to bind any of the three bacterial strains over the 5-day experiment; however, by day 4, the ability of the naïve hemocytes to bind *V. fischeri* cells had become significantly greater, increasing to fivefold by 5 days. This increased binding activity was specific toward *V. fischeri* as binding to *V. parahaemolyticus* or *V. harveyi* was unchanged.

These results show that the ability to avoid adherence to hemocytes of *E. scolopes* varied among related bacterial species, and the hemocytes' response was specifically altered by the colonization state of the light organ. These data suggest that colonization can lead to a type of host immune tolerance to *V. fischeri*. The mechanisms by which *V. fischeri* avoids adherence and induces this tolerance remain to be characterized, but experimental data show that a *V. fischeri* outer-membrane protein (OmpU) may be involved as deletion of OmpU leads to a significant increase in binding to hemocytes. Ultimately we'd like to understand the molecular mechanisms by which the hemocytes can distinguish between symbiotic and nonsymbiotic bacteria. Research in the Nyholm lab is currently focused on understanding changes in hemocyte gene expression and protein abundance in response to colonization. A number of genes and proteins from hemocytes have been identified that are predicted to be involved with immune response, including the detection of MAMPs and downstream signaling. Furthermore, colonization state has been found to influence hemocyte gene expression of the PRR EsPGRP5, nitric oxide synthase (NOS), and a squid orthologue of the complement component C3 (Collins et al. 2012b) as well as protein abundance of a number of factors involved with the cell skeleton, lysosomal function, and other components of innate immunity (Schleicher et al. 2014).

In addition to their traditional role in immunity, hemocytes appear to play other functions in the light organ. As mentioned earlier, there is a pronounced diel rhythm whereby 95 % of the symbionts are expelled each morning at dawn. Hemocytes and shed epithelial cells are also expelled with this exudate. An analysis of both host and symbiont transcription over the diel cycle suggests that *V. fischeri* switches between oxidative phosphorylation during the day to chitin fermentation at night (Wier et al. 2010). Where does this chitin come from? A survey of light organ tissues revealed that only hemocytes contain chitin and it is often in cytoplasmic granules (Heath-Heckman and McFall-Ngai 2011). Therefore hemocyte migration into the crypt spaces may also serve the purpose of providing a source of chitin to *V. fischeri*. Hemocyte trafficking is also influenced by early exposure to the symbiont and may be involved with early morphogenesis. After hatchling squid are exposed to *V. fischeri* for 2 h; hemocytes traffic into the ciliated epithelial fields that will then undergo apoptosis and regression 4 days after colonization (Koropatnick et al. 2007). One area of future research is to understand the extent to which hemocytes traffic into the light organ and whether they then migrate out into the circulatory system. Could *V. fischeri* or bacterial MAMPs have a more

systemic effect on the host (e.g., by influencing other organs as has been shown for gut bacteria in mammals), and do hemocytes act as a potential mediator for signaling to other tissues? These questions remain to be answered.

In addition to cellular-based immunity, the host also has a number of other classical components of the innate immune system including reactive oxygen and nitrogen species (Tomarev et al. 1993; Weis et al. 1996; Small and McFall-Ngai 1999; Schleicher and Nyholm 2011). A squid halide peroxidase (sHPO) is present in the ducts and crypt spaces of the light organ (Weis et al. 1996). This enzyme converts hydrogen peroxide (H_2O_2) into hypohalous acid (HOCl) against which bacteria are not known to have an effective defense. To overcome this challenge, *V. fischeri* has a periplasmic catalase that effectively converts hydrogen peroxide to water and oxygen thus preventing production of hypohalous acid (Visick and Ruby 1998). A mutation in the gene that encodes this catalase (katA) was associated with both sensitivity to H_2O_2 and defective colonization when competed against wild-type *V. fischeri* (Visick and Ruby 1998). A proteomics study of the light organ exudate also detected the antioxidant enzymes alkyl hydroperoxide reductase (AhpC) and thioredoxin-dependent thiol peroxidase (Bcp) in addition to KatA (Schleicher and Nyholm 2011). So far, it is unclear whether these additional antioxidant enzymes play a role in the symbiosis.

In addition to ROS, the host also has a nitric oxide synthase (NOS) that is capable of producing nitric oxide (NO). NO along with other antimicrobial factors like PGRP2, chitinases, lysozyme, and proteases are secreted in the mucus of the host (Kremer et al. 2014; Troll et al. 2010). Colonization induces an attenuation of NOS and NO, and the *V. fischeri* MAMPs LPS and TCT can together induce this effect (Davidson et al. 2004; Altura et al. 2011). *Vibrio fischeri* has a homologue to a heme NO/oxygen-binding (H-NOX) protein that may help mediate responding to NO but is also linked to iron uptake for the symbiont (Wang et al. 2010; Wang and Ruby 2011). Other compounds including a flavohemoglobin, an alternative oxidase (AOX), and NO-responsive regulatory protein (NsrR) also likely play a role in protecting *V. fischeri* from the effects of NO (Wang et al. 2010; Dunn et al. 2010).

The host harbors a number of other potentially antimicrobial compounds. For example, the oxygen-binding protein hemocyanin is found in the crypt spaces of the light organ. In addition to likely serving as a means to deliver oxygen to the symbionts for bioluminescence, hemocyanin has antimicrobial activity and is present in the mucus secreted by ciliated fields during colonization and may play a role in recruitment of *V. fischeri* (Kremer et al. 2014). Analyses of the transcriptome and proteome of light organs and hemocytes also revealed a number of putative members of the complement cascade often associated with the innate immune system of other animals (Castillo et al. 2009; Collins et al. 2012b). An orthologue of the complement component C3 was localized to the light organ crypt epithelium and in hemocytes, and that orthologue of C3 was differentially expressed in blood cells from both symbiotic and cured (antibiotic-treated) hosts. The hemocyte proteome also revealed a number of thioester-containing proteins (TEPs) that may be involved in a complement-like response.

Recently, the role of a protein known as galaxin was characterized in the squid–vibrio association (Heath-Heckman et al. 2014). Galaxins are a group of proteins that have been described in other hosts (corals and hydrothermal vent tubeworms), yet their role in symbiosis is poorly understood. A gene expression study showed that in *E. scolopes*, galaxin is upregulated after colonization of the light organ (Chun et al. 2008) and follows the daily rhythm in adult light organs (Wier et al. 2010). The protein EsGal1 is the dominant galaxin in the light organ and expression of this protein can be partially induced by the MAMPs TCT and Lipid A (Heath-Heckman et al. 2014). The EsGal1 protein is also found on the surface of the light organ epithelium and in mucus secreted during initial aggregation. In vitro assays have shown that EsGal1 inhibited the growth of Gram-positive bacteria and therefore may be involved with the initial selection of the symbiont since Gram-positive bacteria are excluded from aggregations that form outside the light organ during colonization (Heath-Heckman et al. 2014; Nyholm et al. 2000; Nyholm and McFall-Ngai 2004).

11.7 Developing *E. scolopes* as a Model for Both Binary and Consortial Symbioses (Accessory Nidamental Gland Association)

A common yet poorly understood animal–bacterial association occurs between members of squid and cuttlefish species and bacterial consortia that reside within a reproductive gland of female hosts called the accessory nidamental gland (ANG) (Bloodgood 1977). This highly pigmented organ of the reproductive tract of sexually mature females harbors a dense consortium of bacteria (Fig. 11.4). Research in several squid species shows that these bacteria are housed in epithelium-lined tubules that are attached to the nidamental gland, the organ that secretes the jelly coat surrounding fertilized eggs, and these bacteria are deposited into the egg cases (Barbieri et al. 2001; Collins et al. 2012a). Many squid lay their eggs in clutches or masses on the seafloor bottom where they must develop over a period of approximately 1 month. During this time, the developing embryos are exposed and unprotected. Although the role of these bacteria is unclear, they may help prevent both unwanted fouling from other microorganisms and predation. Culture-dependent and independent methods have identified the dominant members of bacteria that are housed in cephalopod ANGs. In *E. scolopes*, as with other cephalopod species, the ANG is dominated by *Alphaproteobacteria*, usually members of the *Roseobacter* clade within the *Rhodobacterales*, commonly found in marine environments (Collins et al. 2012a). Other contributing taxa include members of the *Bacteroidetes*, *Gammaproteobacteria,* and *Verrucomicrobia* (Fig. 11.4). Efforts are underway to understand the role that this consortium plays in the reproduction of *E. scolopes* but there are some common themes that have also been observed in the light organ association, such as housing a dense bacterial

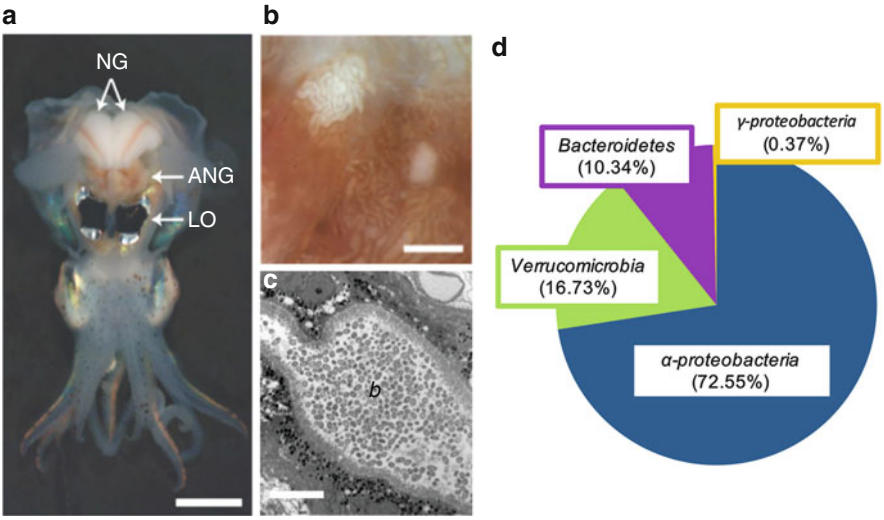

Fig. 11.4 The accessory nidamental gland symbiosis of *E. scolopes*. (**a**) Ventral dissection showing the position of the nidamental gland (NG) and accessory nidamental gland (ANG) in relation to the light organ (LO). Scale, 5 mm. (**b**) Close-up of ANG showing pigmented tubules containing bacteria. Scale, 0.5 mm. (**c**) Electron micrograph showing bacteria (b) within host tubules. Scale, 5 μm. (**d**) Results of sequencing of the 16S ribosomal gene from the ANG of *E. scolopes*. The communities had a number of members of the *Rhodobacterales*, similar to findings for other squid species. Members of the *Verrucomicrobia* and the *Bacteroidetes* phyla were also prominent members. The ANG and light organ association in *E. scolopes* will allow researchers to explore both a binary and consortial symbiosis in the same host [Adapted from Collins et al. (2012a); Copyright © American Society for Microbiology]

population in epithelium-lined crypt spaces and the trafficking of hemocytes into the crypts (Collins et al. 2012a). The same sHPO that was found in the light organ is also present in the ANG (Small and McFall-Ngai 1999), and a second galaxin (EsGal2) has higher expression in the ANG when compared to the light organ (Heath-Heckman et al. 2014).

11.8 Conclusions and Future Directions

The squid–vibrio association has provided a wealth of information on how beneficial bacteria interact with animal hosts. Because of the binary nature of the association and the fact that each partner can be maintained independently, a broad number of experimental approaches have been applied successfully to study the squid host and its symbionts. However, no model system is ideal and each faces its own set of challenges. One thing that may further contribute to the use of the squid–vibrio association among researchers will be the development of more tools on the host side. For example, unlike other model hosts such as *Drosophila*

melanogaster, zebrafish, and mice, genetic knockout and knockdown techniques have not yet been developed in the squid. Also, while there is a growing body of transcriptome information available for *E. scolopes*, the genome itself has not been sequenced although efforts are underway. The present lack of a full genomic sequence for this squid species has made identifying its genes and proteins from proteomic studies to be an unnecessarily difficult task. Cephalopod genomes have posed significant challenges for sequencing as they tend to be quite large (*E. scolopes* is 3.7 Gb) and have abundant repetitive sequences (Albertin et al. 2012). Most of the colonization experiments in the squid–vibrio association are carried out over the first 5 days after the squid hatch. Although methods for raising the squid to maturity have been described, it is often a labor intensive and time-consuming process (Hanlon et al. 1997; Lee et al. 2009). Recent efforts with aquaculture of the host, however, are opening up new and exciting avenues of research (Koch et al. 2014). By reliably raising the squid, the research community can analyze the long-term developmental effects of colonization. On the symbiont side, the ability to mutagenize *V. fischeri* has always been a powerful tool for understanding the effects of specific genes on the symbiosis (Ruby 2008). Comparative genomics between native and nonnative *V. fischeri* strains has also proven valuable for understanding the genes as well as the metabolic and physiologic processes that are important for host-symbiont specificity (Mandel et al. 2009). The diel rhythm of the squid–vibrio association and the ability to passage bacteria from one host to another are also proving to be an important asset that has allowed researchers to apply experimental evolution techniques to understand traits that are important for colonization (Schuster et al. 2010; Soto et al. 2012).

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