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# Evidence for Vertical Transmission of Bacterial Symbionts from Adult to Embryo in the Caribbean Sponge *Svenzea zeai*▽

On On Lee,[1] Pui Yi Chui,[1] Yue Him Wong,[1] Joseph R. Pawlik,[2] and Pei-Yuan Qian[1]*

*Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong,[1] and Department of Biology and Marine Biology and Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington, North Carolina 28409[2]*

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**The Caribbean reef sponge *Svenzea zeai* was previously found to contain substantial quantities of unicellular photosynthetic and autotrophic microbes in its tissues, but the identities of these symbionts and their method of transfer from adult to progeny are largely unknown. In this study, both a 16S rRNA gene-based fingerprinting technique (denaturing gradient gel electrophoresis [DGGE]) and clone library analysis were applied to compare the bacterial communities associated with adults and embryos of *S. zeai* to test the hypothesis of vertical transfer across generations. In addition, the same techniques were applied to the bacterial community from the seawater adjacent to adult sponges to test the hypothesis that water column bacteria could be transferred horizontally as sponge symbionts. Results of both DGGE and clone library analysis support the vertical transfer hypothesis in that the bacterial communities associated with sponge adults and embryos were highly similar to each other but completely different from those in the surrounding seawater. Sequencing of prominent DGGE bands and of clones from the libraries revealed that the bacterial communities associated with the sponge, whether adult or embryo, consisted of a large proportion of bacteria in the phyla *Chloroflexi* and *Acidobacteria*, while most of the sequences recovered from the community in the adjacent water column belonged to the class *Alphaproteobacteria*. Altogether, 21 monophyletic sequence clusters, comprising sequences from both sponge adults and embryos but not from the seawater, were identified. More than half of the sponge-derived sequences fell into these clusters. Comparison of sequences recovered in this study with those deposited in GenBank revealed that more than 75% of *S. zeai*-derived sequences were closely related to sequences derived from other sponge species, but none of the sequences recovered from the seawater column overlapped with those from adults or embryos of *S. zeai*. In conclusion, there is strong evidence that a dominant proportion of sponge-specific bacteria present in the tissues of *S. zeai* are maintained through vertical transfer during embryogenesis rather than through acquisition from the environment (horizontal transfer).**

Besides being the oldest metazoans, sponges are the simplest multicellular animals and possess a low degree of tissue differentiation and coordination (54). Sponges are sessile, filter-feeding organisms that may harbor within their tissues a remarkable array of microorganisms, including bacteria (19, 59, 64), archaea (41), zooxanthellae (22), diatoms (63), and fungi (35). In some cases, microbial consortia can make up to 40 to 60% of the sponge tissue volume (21, 61) and exceed a density of $10^9$ microbial cells per ml of sponge tissue (62), which is several orders of magnitude higher than that found in seawater. Apart from being a source of food (43), bacterial symbionts may participate in the acquisition and transfer of nutrients inside sponges (67, 68), the recycling of insoluble protein (69), the stabilization of the sponge skeleton (44), and the processing of metabolic waste (4, 65). Many antimicrobial compounds have been isolated from sponge bacterial symbionts (24, 47, 53), suggesting the involvement of symbiotic bacteria in sponge chemical defenses. In some cases, bacterial symbionts have been found to be the source of bioactive compounds that were isolated from sponges, which has opened up new research directions in marine natural product chemistry, biotechnology, and pharmaceutical development (18, 23, 40).

Based on immunological evidence from the 1980s (66), sponge-bacterium symbioses are thought to have originated in the Precambrian, when bacteria evolved to form a single clade of sponge-specific bacteria that were distinct from isolates found in the surrounding seawater. Since then, many studies have similarly documented a high level of consistency and specificity in sponge-bacterium associations (20, 27, 59). Nevertheless, questions remain about the acquisition and maintenance of symbionts in host sponges. In general, the following two hypotheses have been proposed: (i) a recently metamorphosed sponge selectively retains specific groups of bacteria from the diverse pool of bacteria present in the water column as it begins filter feeding (horizontal transfer) or (ii) specific bacterial strains are transmitted by the maternal sponge to developing embryos and are already present in the metamorphosing sponge (vertical transfer) (58). The first hypothesis requires some recognition of specific microbes by the sponge, perhaps through an innate immune system (36) or other means to distinguish symbiont strains from food bacteria (70).

Vertical transfer of bacterial symbionts in sponges was first proposed by Lévi and Porte (29), who demonstrated the presence of bacteria inside the larvae of the sponge *Oscarella lobularis*. Later, in 1976, Lévi and Lévi (30) studied the transmission of bacteria in the sponge *Chondrosia reniformis* via sponge

* Corresponding author. Mailing address: Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong. Phone: (852) 2358-7331. Fax: (852) 2358-1559. E-mail: boqianpy@ust.hk.

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oocytes. Since then, vertical transmission of bacterial symbionts via eggs or larvae has been documented for several sponge species, including *Tethya citrina* (15), *Geodia cydonium* (50), *Stelletta grubii* (49), *Hippospongia* sp. (25), *Spongia* sp. (25), *Halisarca dujardini* (10), and *Corticium candelabrum* (8). However, all of these studies employed transmission and scanning electron microscopy and could only examine the presence of bacteria in maternal sponges, oocytes, or larvae at the morphological level, with no determination of microbial identity. With advances in molecular techniques, Enticknap et al. (9) were the first to report the successful isolation of an alphaproteobacterial symbiont, strain NW001, from both the adult sponge *Mycale laxissima* and its larvae. They also did a preliminary denaturing gradient gel electrophoresis (DGGE) analysis of the bacterial community in seawater and compared that with the community in the sponge larval sample. However, such a comparison was not extended to the sponge adult, and no solid conclusion can be drawn for the horizontal transfer mechanism of sponge symbionts. More recently, Sharp et al. (52) used fluorescence in situ hybridization (FISH) and clone library techniques to demonstrate the presence of proteobacteria, actinobacteria, and a clade of sponge-associated bacteria in the embryos and mesohyl of the tropical sponge *Corticium* sp. By clone library and DGGE analyses, Schmitt et al. (48a) identified 28 vertical-transmission clusters in five different Caribbean sponge species and demonstrated that the complex sponge adult microbial community was collectively transmitted through reproductive stages. While these recent studies support the vertical transfer hypothesis, they did not fully address the identities of microbes in the water column surrounding the sponges, which is key to determining whether horizontal transfer may also take place.

The Caribbean reef sponge *Pseudaxinella zeai* was reclassified into a new genus, *Svenzea* (Demospongiae, Halichondria, Dictyonellidae), in 2002 because it has an unusual skeleton arrangement consisting mainly of short stout styles that are arranged in an isodictyal reticulation (2). It is a viviparous sponge that produces the largest embryos (>1 mm in diameter) and larvae (6 mm long) recorded for the phylum Porifera (45). *Svenzea zeai* has also been classified as a bacteriosponge because it contains substantial amounts of unicellular photosynthetic and autotrophic microbial symbionts in its tissues (2, 45). Although bacteria were observed in the embryos and larvae of this sponge based on transmission electron microscopy studies (45), neither the direct linkage between the maternal sponge and the propagules nor the identity of the microbial symbionts had been established.

In this study, our objective was to examine vertical versus horizontal transfer of bacterial symbionts in *Svenzea zeai*. This was achieved by comparing the bacterial community profiles of the adults and embryos of the sponge by use of a combination of molecular techniques, including DGGE and clone library analysis. More than one technique was employed to compensate for deficiencies of each technique in revealing bacterial community structure. Additionally, we used the same techniques to examine the bacterial community in the seawater that surrounded the sponge to determine whether horizontal transfer was evident.

## MATERIALS AND METHODS

**Sample collection and extraction of DNA.** Tissue of the adult sponge *Svenzea zeai* was obtained at a depth of 12 m from San Salvador Island, Bahama Islands (24°03′N, 74°32′W), in June 2007. Three sponge individuals were carefully brought to the water surface by scuba divers and flushed with autoclaved 0.22-μm-filtered seawater to remove loosely attached bacteria. The adult sponges were dissected, and embryos in the adult tissues were carefully removed. Adult sponge tissue (0.5 ml) without any embryos was cut into small pieces and frozen in 0.8 ml of extraction buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 100 mM $Na_2HPO_4$, 1.5 M NaCl, 1% cetyltrimethylammonium bromide, pH 8). The embryos removed from the adult tissue were first briefly washed with 70% ethanol and then thoroughly rinsed twice with autoclaved filtered seawater. Embryo mass (0.5 ml) removed from each of the three individual sponge adults was homogenized and frozen in 0.8 ml of extraction buffer.

Bacterial communities in the surrounding seawater were collected in triplicate by filtering 1 liter of seawater onto 0.22-μm polycarbonate membranes (Osmonics). The membranes were then frozen in extraction buffer. The extraction and purification of total bacterial DNA from the samples were performed following the sodium dodecyl sulfate-based method described by Liu et al. (31). Purified DNA was dissolved in 50 μl of double-distilled water ($ddH_2O$) and kept at −20°C until use.

**DNA fingerprinting analysis of bacterial communities.** Bacterial community structure was revealed by DGGE (39). The 16S rRNA genes in the crude DNA extracts were amplified by PCR, using the universal primers 341F-GC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G CCT ACG GGA GGC AGC AG) and 907R (5′-CCG TCA ATT CMT TTG AGT TT) (39). Each PCR mixture contained 2 μl of DNA template, 1.25 U of *Taq* polymerase (Amersham Biosciences), a 0.25 mM concentration of each deoxynucleoside triphosphate, 0.1 μM of each primer, and 1× PCR buffer in a total volume of 50 μl. PCR was performed in a thermal cycler (MJ Research) under the following thermal conditions: initial denaturation at 95°C for 2 min; 10 touchdown cycles of denaturation at 95°C for 1 min, annealing at 65°C (reduced to 55°C in increments of 1°C $cycle^{-1}$) for 1 min, and extension at 72°C for 1 min; an additional 15 cycles with a constant annealing temperature of 55°C; and a final extension at 72°C for 5 min. PCR products were mixed with loading buffer and loaded onto a 6% acrylamide gel with a denaturing gradient of 35 to 70% (100% denaturant = 7 M urea, 40% [vol/vol] formamide). Electrophoresis was performed using a D-Code system (Bio-Rad) with 1× TAE (20 mM Tris base, 10 mM sodium acetate, and 0.5 mM EDTA) at a constant temperature of 60°C and a voltage of 125 V for 18 h. The gel was stained with 1× SYBR gold (Molecular Probes) for 15 min and photographed with an Alpha Imager 2200 gel documentation system (Alpha Innotech).

**Sequencing analysis of DGGE bands.** Major bands from the DGGE gel were selected and excised from the gel for sequence analysis. Excised gel cubes were first washed with $ddH_2O$ and then immersed in 50 μl of $ddH_2O$ at 4°C overnight. Two microliters of DNA from each excised band was used as the template for the same PCR-DGGE analysis to check for the band position and purity. PCR products were then purified, cloned into the pCR2.1-TOPO vector, and transformed into *Escherichia coli* competent cells by use of a Topo TA cloning kit (Invitrogen) according to the manufacturer's manual. Transformants were screened by blue-white selection on agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)–isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 μg $ml^{-1}$ of ampicillin. White colonies were then transferred to fresh plates and reincubated overnight. DNA was extracted from each positive clone by picking a single white colony from the plate into 100 μl of $ddH_2O$ and lysing the cells by heating at 99°C for 10 min. The lysates were used as DNA templates for subsequent PCR amplification, using the external vector primers M13F and M13R. Purified PCR products were then used as templates for cycle sequencing PCR, using either M13F or M13R primer and a DYEnamic ET dye terminator kit (Amersham Biosciences). Cycle sequencing products were separated using a MegaBACE 500 genetic analyzer (Amersham Biosciences). The nucleotide sequences obtained with the two primers were assembled using Sequencher 4.2 (Gene Codes Corporation), and the assembled sequences were compared with sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/), using BLAST, to obtain their closest phylogenetic affiliations.

**Clone library construction.** Since the bacterial community structures for replicated samples were highly similar, as indicated by DGGE analysis, crude DNA extracts from the three replicates were pooled as templates for the construction of clone libraries. The 16S rRNA genes in the crude DNA extracts were PCR amplified with the universal primers 8F (5′-AGA GTT TGA TCC TGG CTC AG) (3) and 1492R (5′-GGT TAC CTT GTT ACG ACT T) (28), using the following PCR conditions: initial denaturation at 95°C for 5 min; 30 cycles of

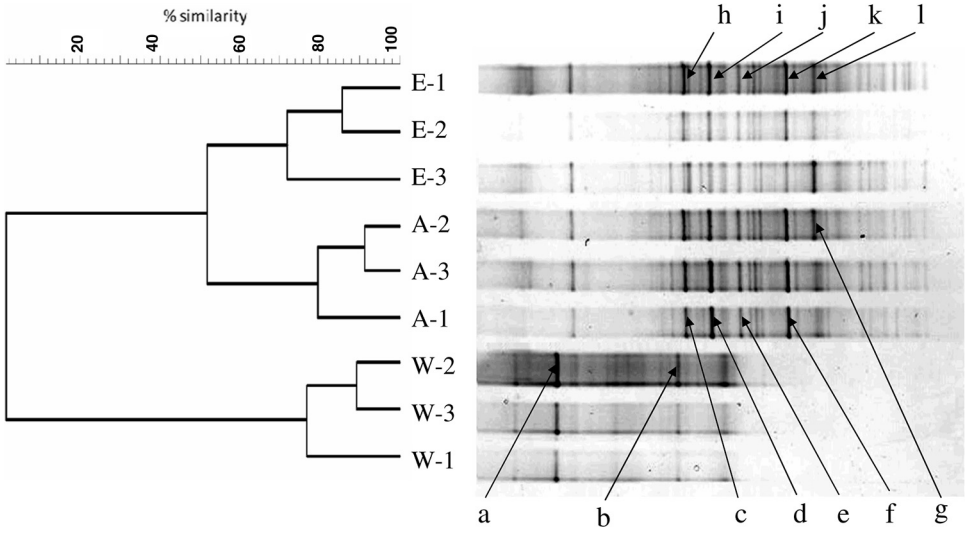



FIG. 1. DGGE band patterns (right) and dendrogram (left) showing the similarities of bacterial communities associated with the adults (A) and embryos (E) of the sponge *Svenzea zeai* and the planktonic bacterial community (W) in San Salvador, Bahama Islands. Labeled bands were excised and sequenced. Details of the excised bands are given in Table 1.

denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Successful PCR amplicons were purified using a PCR purification mini kit (Watson Biotechnologies Inc., Shanghai, China). The quantities of purified PCR products were determined by GeneQuant (Amersham Biosciences). The same amounts of purified DNA from different samples were cloned into the pCR2.1-TOPO vector and then transformed into *E. coli* competent cells by use of a Topo TA cloning kit (Invitrogen) according to the manufacturer's manual. The same procedures as those mentioned above were used for screening of positive clones, extraction of DNA, and subsequent PCR amplification. Aliquots (20 μl) of successful PCR amplicons were digested individually with two restriction enzymes (MspI and HaeIII) according to the manufacturer's instructions (Invitrogen). Restriction fragment length polymorphism (RFLP) patterns of each clone upon two different restriction enzyme digestions were obtained by electrophoresis on 3% agarose gels. Clones that showed the same RFLP patterns for the two enzymes were classified into the same operational taxonomic units (OTUs). One clone from each OTU was randomly selected and subjected to sequencing using vector primers M13F and M13R and internal primers 8F and 1492R. Sequencing analysis was performed as mentioned above. Nearly the full lengths of bacterial 16S rRNA gene sequences were obtained by assembling each fragment sequence using Sequencher 4.2 (Gene Codes Corporation). Chimera Check was used to exclude chimeras (34). The assembled sequences were then compared to the GenBank entries by using BLAST to obtain the closest phylogenetic affiliation for each OTU. Phylogenetic analysis was performed with sequences retrieved for each OTU in comparison with their closest affiliations, using ARB software (33). Phylogenetic trees were then constructed based on the neighbor-joining method (46), maximum parsimony (12), and the unweighted-pair group method using average linkages (UPGMA) (56), using MEGA software (26).

**Statistical analysis.** Bacterial community structures, as revealed by DGGE band patterns, were compared among samples. Previous studies have suggested that major bands on DGGE gels represent the dominant bacterial species present in the respective samples and that band intensity correlates with the relative abundance of the corresponding bacterial species within the sample (13, 38). Therefore, each band in the DGGE gel was described by its position and relative intensity in the profile, using GelCompar II software (Applied Maths). Band matching was performed with 1.00% position tolerance and 1.00% optimization. Similarity matrices were calculated based on the band position and intensity of each sample. Cluster analysis was performed based on the Pearson similarity correlation, and dendrograms were constructed based on the Ward method, using GelCompar II software (Applied Maths).

For each clone library, the Chao estimator (7), the Shannon index (51), and the coverage (37) were calculated. LIBSHUFF analysis was used to compare libraries to determine if they were significantly different from each other (55). A LIBSHUFF comparison of three libraries yielded an experiment-wise critical *P* value of 0.0085 according to the Bonferroni correction (http://libshuff.mib.uga.edu). For each pairwise comparison, if the lower of the two *P* values calculated by LIBSHUFF was less than or equal to the critical *P* value, then there was a significant difference, with a confidence of 95%, in the composition of the communities sampled by each library.

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequences obtained in this study were deposited in GenBank under the following accession numbers: FJ529257 to FJ529375.

## RESULTS

**DGGE analysis of bacterial communities.** DGGE analysis of the bacterial communities associated with the adults and embryos of the sponge *Svenzea zeai* resulted in completely different band patterns in the DGGE gel than those associated with the bacterial communities present in the water adjacent to the adult sponges (Fig. 1, right panel). The water column bacterial communities had an average of 17 bands that concentrated at the upper area of the gel (where the denaturing concentration of the gel was low). In contrast, the average numbers of bands observed for the adult- and embryo-associated bacterial communities were as high as 29 and 30, respectively, and the bands were found mostly in the middle and lower parts of the gel (where the denaturant concentration was high). Cluster analysis using a similarity matrix based on the band position and intensity confirmed the distinctiveness of the water column bacterial community (Fig. 1, left panel). A distinct cluster was formed with replicated samples from the water column, which shared 0% similarity to the cluster formed from replicated samples from the sponge adults and embryos. In contrast, the sponge adult- and embryo-associated bacterial communities shared more than 50% similarity and formed a large cluster which consisted of two smaller clusters comprising the replicated samples from either the sponge adults or embryos.

**Sequence analysis of excised DGGE bands.** Altogether,12 bands from the DGGE gel (2 from the water column samples and 5 each from the sponge adults and embryos) were selected, excised, and sequenced. These bands were selected because of their prominence and uniqueness. Approximately 550 bp of

TABLE 1. Closest phylogenetic affiliations of sequences retrieved from selected bands excised from DGGE gel[a]

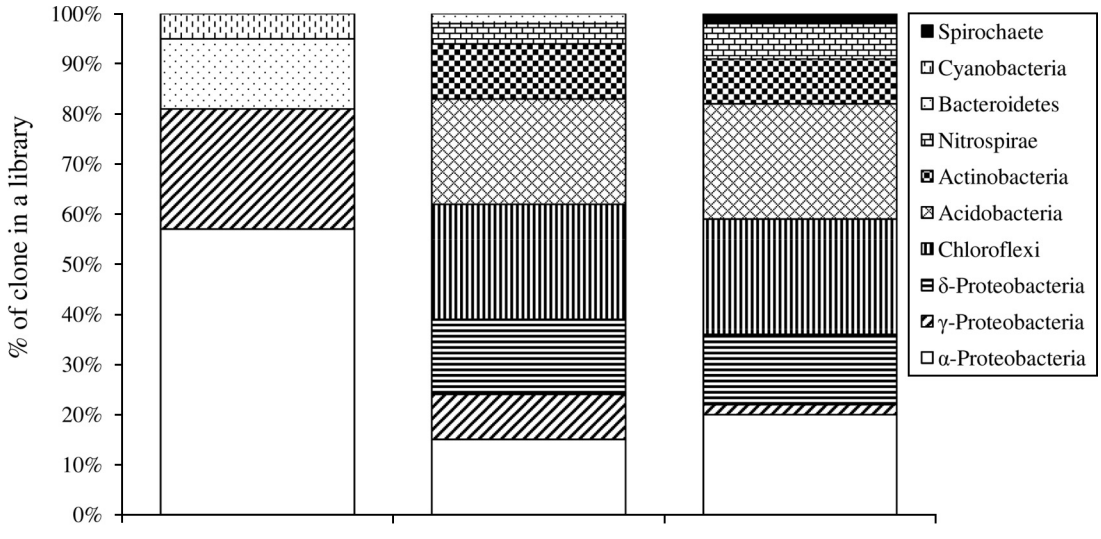
| Band | Source | Closest phylogenetic affiliation | | | | |
|---|---|---|---|---|---|---|
| | | Strain | Source | Phylum | GenBank accession no. | Similarity (%) |
| a | Water | Uncultured *Roseobacter* sp. clone 2_C6 | Coastal seawater from Ria de Vigo, Spain | *Alphaproteobacteria* | EU600651 | 94 |
| b | Water | Uncultured *Rhodobacteriales* bacterium clone HF70_26K06 | Seawater from Hawaii Ocean, North Pacific Subtropical Gyre | *Alphaproteobacteria* | EU361404 | 94 |
| c | Adult | Uncultured sponge symbiont PAUC37f | Marine sponge *Theonella swinhoei* from the Western Caroline Islands in the Republic of Palau | *Acidobacteria* | AF186413 | 97 |
| d | Adult | Uncultured *Chloroflexi* bacterium clone PK067 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076103 | 96 |
| e | Adult | Uncultured *Chloroflexi* bacterium clone PK053 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076110 | 95 |
| f | Adult | Uncultured *Chloroflexi* bacterium clone PK017 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076100 | 99 |
| g | Adult | Uncultured *Chloroflexi* bacterium clone PK064 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076111 | 96 |
| h | Embryo | Uncultured sponge symbiont PAUC37f | Marine sponge *Theonella swinhoei* from the Western Caroline Islands in the Republic of Palau | *Acidobacteria* | AF186413 | 99 |
| i | Embryo | Uncultured *Chloroflexi* bacterium clone PK067 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076103 | 99 |
| j | Embryo | Uncultured *Chloroflexi* bacterium clone PK053 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076110 | 99 |
| k | Embryo | Uncultured *Chloroflexi* bacterium clone PK017 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076100 | 99 |
| l | Embryo | Uncultured *Chloroflexi* bacterium clone PK064 | Marine sponge *Plakortis* sp. from Little San Salvador Island, Bahamas | *Chloroflexi* | EF076111 | 98 |

[a] The sequences of excised bands were compared to nucleotide sequences deposited in GenBank. The closest phylogenetic affiliation for the sequence from each band is indicated by the strain name, phylum, accession number, and similarity. Refer to Fig. 1 for band positions.

sequence was retrieved from each band and compared with nucleotide sequences deposited in GenBank. The closest phylogenetic affiliation of each band sequence is listed in Table 1. All 12 sequences were affiliated with uncultured strains from clones. The 2 sequences from the water samples (bands a and b) were closely related to the phylum *Alphaproteobacteria*, while the other 10 sequences, from the sponge adults (bands c to g) and embryos (bands h to l), belonged to the phyla *Acidobacteria* and *Chloroflexi*. Bands excised from the same portions of the denaturing gradient (i.e., bands c versus h, d versus i, e versus j, f versus k, and g versus l) shared the same closest matches. These results indicate that strains belonging to the phylum *Chloroflexi* contributed large proportions of the bacterial communities associated with the adults and embryos of the sponge.

**Clone library analysis.** Altogether, 228 clones were retrieved from the three clone libraries, 43 of which were from the seawater library, while 90 and 95 were from the sponge adult and embryo libraries, respectively. After RFLP screening, 21, 53, and 44 OTUs were identified from the libraries of seawater and sponge adults and embryos, respectively (Fig. 2). The Chao-1 estimator (7) and the Shannon index ($H$) were used to estimate the species richness and to calculate the diversity of the libraries, respectively. The higher the values, the richer and more diverse was the community. The results indicated that the sponge adults had the highest species richness (Chao-1 = 125 ± 25), followed by the sponge embryos (Chao-1 = 87 ± 19), whereas the lowest species richness was found in the seawater (Chao-1 = 54 ± 18; $H$ = 2.71).

Nearly-full-length 16S rRNA gene sequences were obtained for each OTU and compared with sequences deposited in GenBank. Phylogenetic analysis of the 118 OTUs showed that only 3 of them (2.5%) were affiliated with sequences from isolates, while the remaining 115 OTUs (97.5%) were closely related to uncultured clones (Fig. 3). All clones from the seawater library, except for one (W10), were affiliated with uncultured clones from different marine environments, including seawater from Cocos Island, Monterey Bay, the Mediterranean Sea, and the Red Sea (Fig. 3). In contrast, among the 53 and 44 OTUs retrieved from the sponge adult and embryo libraries, respectively, 42 and 33 of them (a total of 77%), respectively, were closely related to sequences retrieved from other sponge species, including *Plakortis* sp., *Corticium* sp., *Theonella swinhoei*, *Agelas dilatata*, and *Aplysina aerophoba*




| | Water | Adult | Embryo |
|---|---|---|---|
| Clones | 43 | 90 | 95 |
| OTU | 21 | 53 | 44 |
| Chao-1 | 54 ± 18 | 125 ± 25 | 87 ± 19 |
| Shannon index (H) | 2.71 | 3.73 | 3.44 |
| Coverage (%) | 67.4 | 60.0 | 73.7 |
| Evenness | 0.89 | 0.94 | 0.91 |

FIG. 2. 16S rRNA gene phylotype distribution and comparison of clone libraries from water samples and adults and embryos of the sponge *Svenzea zeai*. Species richness was estimated using the nonparametric Chao estimator (7). The Shannon index was calculated based on the number of unique phylotypes detected by RFLP screening. Coverage of the library was expressed as a percentage and calculated from the equation $C = 1 - (n1/N)$, where $n1$ was the number of clones occurring only once in the library and $N$ was the total number of clones examined (34).

(Fig. 3). Only the remaining 23% were related to sequences from other habitats, for instance, mangrove soil and seawater.

Of the 21 OTUs retrieved from the seawater library, 57% belonged to the phylum *Alphaproteobacteria*, 24% to *Gammaproteobacteria*, 14% to *Bacteroidetes*, and 5% to *Cyanobacteria* (Fig. 2). The sponge adult and embryo libraries comprised a wider range of phyla than the seawater library did. Apart from the clones belonging to the phyla *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes*, clones closely related to six other phyla were found (*Deltaproteobacteria*, *Spirochaetes*, *Nitrospirae*, *Actinobacteria*, *Acidobacteria*, and *Chloroflexi*) (Fig. 2). For both the sponge adult and embryo libraries, the largest portion of the clones (>20% each) were affiliated with the phyla *Chloroflexi* and *Acidobacteria*, which were absent in the seawater library.

Figure 3 presents phylogenetic trees reflecting the genetic distances among clones from different libraries with reference to their closest relatives. For all phyla recovered in this study, except for *Spirochaetes*, *Cyanobacteria*, and *Bacteroidetes*, a large proportion of clones from the sponge adults were clustered with those from the sponge embryos, showing extremely high similarity in their 16S rRNA gene sequences. For instance, there was high similarity for clones A86 versus E140 and A76 versus E70 in the *Alphaproteobacteria* (Fig. 3a), clones A16 versus E25 in the *Gammaproteobacteria* (Fig. 3b), clones A51 versus E9 in the *Deltaproteobacteria* (Fig. 3c), clones A29 versus E146 and A124 versus E148 in the *Chloroflexi* (Fig. 3d), and clones A58 versus E38 and A74 versus E115 in the *Acidobacteria* (Fig. 3e). These highly similar clones were defined as vertically transmitted phylotypes and formed 21 monophyletic sequence clusters (clusters of two or more sequences retrieved from both the adult sponges and embryos but not from the seawater). More than half of the sequences derived from the sponge adults and embryos fell into these clusters. On the other hand, clones from the seawater library normally formed lineages distinct from those of the sponge libraries, as shown in the alphaproteobacterial (clones W2, W3, W12, W16, W19, W25, and W26) (Fig. 3a), gammaproteobacterial (clones W5, W11, and W22) (Fig. 3b), and *Bacteroidetes* (clones W8, W10, and W13) (Fig. 3f) trees.

To determine the significance of the differences between the clone libraries based on available sequence data, LIBSHUFF analysis was applied. Employing the Bonferroni correction, a LIBSHUFF comparison of three libraries yielded a critical $P$ value of 0.0085. LIBSHUFF analysis indicated that the sponge adult and embryo libraries were not significantly different from each other, as the lower of the two $P$ values calculated from LIBSHUFF analysis ($P = 0.009$) was larger than the critical $P$ value. In contrast, the seawater library was significantly different from the libraries of the sponge adults (lower $P = 0.001$) and the libraries of the sponge embryos (lower $P = 0.001$).

## DISCUSSION

The Caribbean reef sponge *Svenzea zeai* was categorized as a bacteriosponge on the basis of transmission electron microscopy observations because it contained substantial quantities

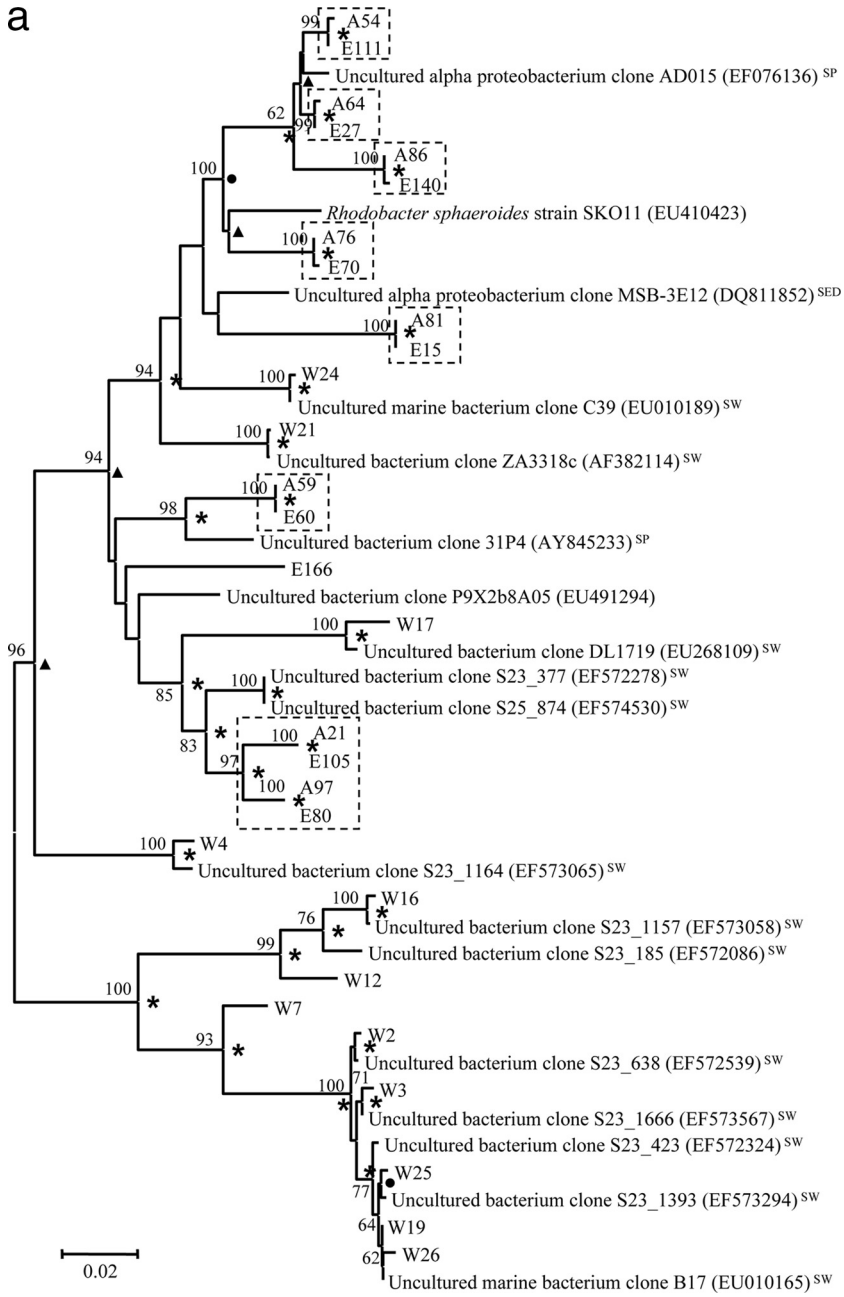



FIG. 3. Phylogenetic trees showing genetic distances among clones retrieved from the libraries for seawater (prefixed with "W"), *S. zeai* adults (prefixed with "A"), and *S. zeai* embryos (prefixed with "E") in reference to members of the *Alphaproteobacteria* (a), *Gammaproteobacteria* (b), *Deltaproteobacteria* (c), *Chloroflexi* (d), *Acidobacteria* (e), and *Actinobacteria*, *Nitrospirae*, *Spirochaetes*, *Cyanobacteria*, and *Bacteroidetes* (f). The trees were constructed based on the neighbor-joining method. Nodes which are observed in both maximum parsimony and UPGMA trees are marked with asterisks, while those observed in either the maximum parsimony or UPGMA tree are marked with filled circles or triangles, respectively. Reference sequences originating from seawater, sediment, and sponges are indicated with the superscripts "SW," "SED," and "SP," respectively. Nucleotide accession numbers of the reference sequences are given in parentheses. Dotted boxes show monophyletic clusters consisting of sequences retrieved from both adult and embryo sponges but not from water. The scale bar represents percent substitutions per nucleotide position. Bootstrap values of >50% based on 1,000 resamplings are indicated by the numbers at the nodes.

of unicellular photosynthetic and autotrophic bacteria (45). The method by which the very large embryos of *Svenzea zeai* acquire their bacterial symbionts from the adult sponge remained unknown, as did the phylogenetic identities of the symbionts. This study represents the only study on the associated bacterial community in the sponge family *Dictyonellidae* and is one of a few detailed studies combining different molecular approaches to investigate vertical transmission as well as horizontal transfer of sponge symbionts in the sponge by incorporating both the sponge embryo-associated and the indigenous bacterioplankton communities for comparison. The results of our study corroborate the most commonly observed

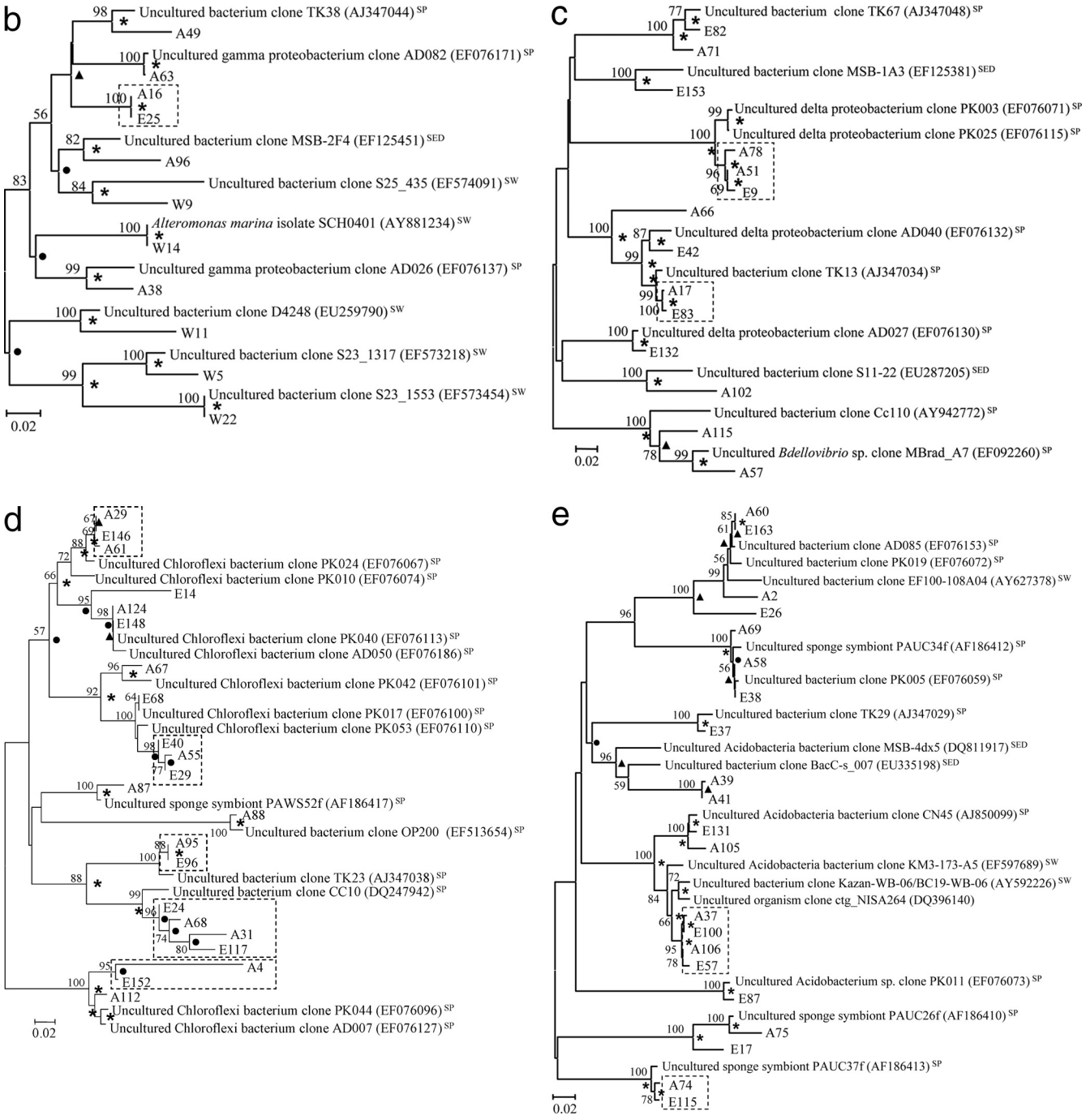



FIG. 3—*Continued.*

method of transmission, which is vertical transfer of symbionts from adult tissue to developing propagules (8, 9, 10, 48a, 52, 58, 60). Moreover, bacterial symbionts associated with both the adult sponge and sponge embryos are primarily bacteria in the phyla *Chloroflexi* and *Acidobacteria*.

The 16S rRNA gene-based DGGE fingerprinting method provided general insights into the compositions of bacterial communities associated with the adults and embryos of *S. zeai* and in the surrounding seawater, while the clone libraries presented a more in-depth analysis of the community composition to reveal the identities of bacterial associates likely involved in vertical transfer. Both methods yielded the same conclusion, that the bacterial communities associated with adult *S. zeai* sponges were highly similar to those associated with embryos but drastically different from those in the surrounding seawater. Twenty-one monophyletic sequence clusters comprising two or more sequences retrieved from the clone libraries of *S. zeai* adults and embryos, but none from the seawater library, were identified upon phylogenetic analysis of clones. More than half of the sponge-derived sequences fell into these clus-

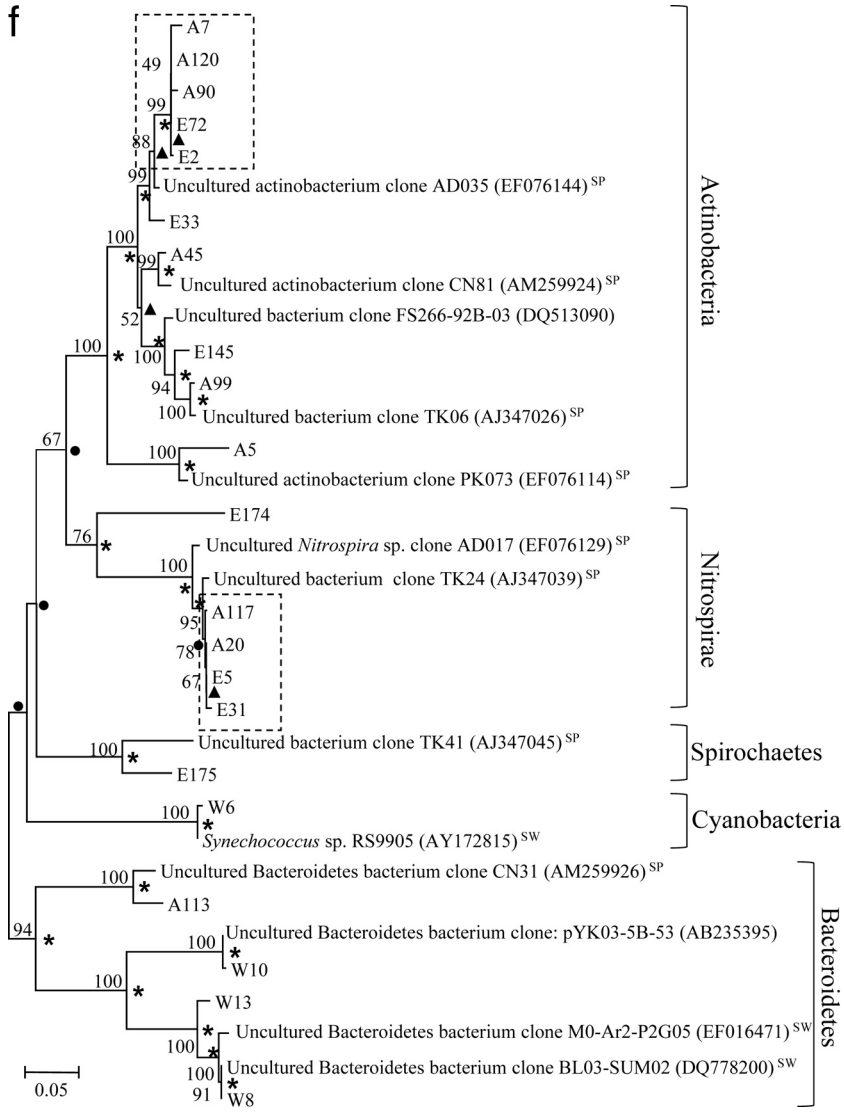



FIG. 3—*Continued.*

ters. Some clones from the adult and embryo libraries shared highly similar or even identical sequences (clones A59 versus E60, A81 versus E15, A16 versus E25, A95 versus E96, and A60 versus E163), indicating substantial overlap of the bacterial communities associated with *S. zeai* adults and embryos. Cross-contamination of the adult bacterial community with that in embryos during sample collection was ruled out because of extremely careful washing procedures. Vertical transfer of microbial symbionts has also been implicated for another Caribbean sponge, *Ircinia felix* (48). Interestingly, bacterial communities associated with adult *I. felix* sponges more closely resembled those of their own larvae than those of other adult *I. felix* sponges (48). This was not found to be true in the present study, as the symbiont communities found in *Svenzea zeai* were highly similar among adults and larvae.

Sequencing analysis was performed with common bands obtained in DGGE gels and for all clones from the clone libraries to identify bacterial associates that might have been passed vertically from adult sponge to embryo. Our results indicated that adults and embryos of *S. zeai* harbored highly similar yet diverse bacterial communities covering eight different phyla, which were also encountered in gene libraries of other sponges (reviewed in references 32 and 58). The most dominant groups of sponge-derived microbial sequences were closely related to sequences from uncultured clones belonging to the phyla *Acidobacteria* and *Chloroflexi*. This is in good agreement with other studies showing abundant uncultured *Acidobacteria* and *Chloroflexi* bacteria in different sponge species (20, 58, 63). In addition, about 77% of our sponge-derived sequences were closely clustered with sequences from other sponge species, for instance, *Corticium* sp. (52), *Theonella swinhoei*, *Aplysina aerophoba* (20), *Plakortis* sp., and *Agelas dilatata* (58), collected from different locations around the world. Of the 77% of sponge-related sequences, nearly 70% fell into the sponge-specific clusters suggested by Hentschel et al. (20) and Taylor et al. (58). These results further strengthen the argument that there are sponge-specific clusters of bacteria present in geographically and phylogenetically diverse sponge species. An-

other discrepancy of the results obtained in the present study compared with previous research was that the largest and best-known sponge-specific microbial clusters, "*Candidatus* Synechococcus spongiarum" (60) and "*Poribacteria*" (11), were absent from our sponge libraries. One possible reason may be due to mismatches in the target region of the "*Poribacteria*" 16S rRNA genes which make our universal primers not able to amplify them (11). Another possibility is that the abundance of these microbial groups may be too low to be recovered by clone library analysis. Sequencing of all minor bands in the DGGE gels was experimentally not feasible. In the future, a metagenomic approach may be a better way to reveal the most complete picture of the sponge-associated microbial community. It would also be interesting to correlate the metabolically active bacteria in the sponge and embryo tissues by using FISH or quantitative PCR so as to understand how they are transferred between generations and their possible roles in host sponges.

An important difference between this study and others in which the vertical transfer of microbial symbionts between sponge adults and embryos has been implicated is the inclusion of a comparison with the indigenous bacterioplankton community. Analysis of the microbes in the water column adjacent to adult sponges allowed us to assess the possibility that horizontal transfer of symbionts was occurring. As indicated by the DGGE and clone library analyses, the bacterial communities in the surrounding seawater were less diverse than and completely different from the bacterial communities associated with *S. zeai* adults and embryos. Sequences belonging to the phyla *Alpha-* and *Gammaproteobacteria* dominated the bacterioplankton community, and most were closely related to sequences recovered from the water column in geographically distinct locations, such as the Red Sea (14), the Mediterranean Sea (1), the east coast of South Korea (5), Monterey Bay, and Cocos Island. Distinct planktonic bacterial communities were also recorded in many other studies (16, 17, 42). Hill (23) argued that considering the powerful filtration capacity of sponges, with up to $2.4 \times 10^{13}$ bacterial cells filtered per day, one might expect that even an extremely rare bacterium in the water column could possibly be concentrated in a sponge to levels detectable by PCR-based methods. As such, it would be risky to completely rule out the possibility that the bacterial symbionts found in sponge samples can be present in seawater samples, even though we did not detect them in the seawater in this study. However, our observation that none of the bacteria detected in the seawater samples resembled any of the bacteria recovered from *S. zeai* adults or embryos strongly argues against the horizontal transfer hypothesis. Therefore, environmental acquisition of bacterial associates is probably not an important factor in maintaining the sponge-microbe association of *S. zeai*.

If vertical transmission of bacterial associates in sponges is the rule, then the implication for the coevolution of sponges and sponge-associated microbes is considerable (21). However, the lack of a clearly resolved phylogeny of sponges (6) still hampers our understanding of symbiont evolution in sponges. Although vertical transmission of *Chloroflexi* and *Acidobacteria* from adult to embryo was possible for *S. zeai*, the cellular mechanisms for the transfer remain to be explored. One technique may be to design specific probes for in situ qualitative and quantitative tracking of the presence of specific bacteria during different developmental stages of the sponge.

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