Chemistry & Biology, Vol. 12, 397–406, March, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.02.003

Speciation and Biosynthetic Variation in Four Dictyoceratid Sponges and Their Cyanobacterial Symbiont, *Oscillatoria spongeliae*

Christian P. Ridley,[1] Patricia R. Bergquist,[2] Mary Kay Harper,[3] D. John Faulkner,[1,5] John N.A. Hooper,[4] and Margo G. Haygood[1,*]
[1]Center for Marine Biotechnology and Biomedicine
Scripps Institution of Oceanography
University of California at San Diego
La Jolla, California 92093
[2]Department of Anatomy
University of Auckland
Private Bag 92019
Auckland
New Zealand
[3]Department of Medicinal Chemistry
University of Utah
Salt Lake City, Utah 84112
[4]Queensland Centre for Biodiversity
Queensland Museum
South Brisbane, QLD 4101
Australia

Summary

Four species of marine sponges (Phylum Porifera, Order Dictyoceratida), which contain the filamentous cyanobacterial symbiont *Oscillatoria spongeliae*, were collected from four locations in Palau. The halogenated natural products associated with the symbiont were characterized from each sample, revealing that each species contained either chlorinated peptides, brominated diphenyl ethers, or no halogenated compounds. Analysis of the host sponges and the symbionts indicated that each species of sponge contained a distinct strain of morphologically similar cyanobacteria. Although cospeciation may be present in this group, we have identified that at least one host switching event has occurred in this symbiosis. Only the strain of *O. spongeliae* in the sponge containing the chlorinated compounds possessed genes involved in the biosynthesis of chlorinated leucine precursors, indicating that the chemical variation observed in these animals has a genetic foundation.

Introduction

The marine sponge *Lamellodysidea herbacea* (= *Dysidea herbacea*, Order Dictyoceratida) is often found in shallow waters of the Indo-Pacific. The consistent association of this sponge and other related sponges with the filamentous cyanobacterium *Oscillatoria spongeliae* has been frequently observed [1–4]. *O. spongeliae* is an intercellular symbiont that is found in the sponge mesohyl (connective tissue between epithelial tissues), and accounts for 30%–50% of the tissue volume in these sponges. Research has shown that the cyanobacterium fixes carbon through photosynthesis [5], and probably transfers organic carbon to the host. It has been proposed by natural product chemists that *O. spongeliae* might play another role in the symbiosis by producing halogenated natural products that have been isolated from the sponge. These compounds include chlorinated amino derivatives such as dysidenin (**1**) and dysideathiazole (**2**) [6] that contain a trichloromethyl functionality on leucine-derived precursors and are very similar to pseudodysidenin (**3**) and barbamide (**4**), which have been isolated from the marine cyanobacterium *Lyngbya majuscula* (Figure 1) [7, 8]. Dysideathiazole analogs are fish-feeding deterrents to the lunar wrasse, *Thalassoma lunare*, and therefore these chlorinated metabolites may support the symbiosis in that manner [9]. Other specimens of *L. herbacea* along with *Lamellodysidea chlorea* (= *Dysidea chlorea*) and *Phyllospongia* spp. did not have the chlorinated peptides but contained antibacterial brominated diphenyl ethers such as **5**–**7** (Figure 1) [10–12]. This class of compounds had been isolated from unrelated organisms such as the green alga *Cladophora fascicularis* (**8**, Figure 1) [13] and an unidentified sponge in the family Callyspongiidae (Order Haplosclerida) [14], and so were also thought more likely to be produced by a symbiont than the marine invertebrates themselves.

Experimental support for these hypotheses was gained when Unson and Faulkner investigated a specimen of *L. herbacea* which contained the chlorinated metabolite 13-demethylisodysidenin (**9**, Figure 1) and were able to separate the autofluorescent cyanobacterium from the sponge cells using a fluorescence activated cell sorter (FACS). Chemical examination revealed that only *O. spongeliae* contained the chlorinated metabolite, and therefore was likely the source of the compound [15]. Using a Percoll density gradient to separate dissociated sponge and cyanobacterial cells from *L. herbacea*, others have shown that the chlorinated diketopiperazines **10** and **11** (Figure 1) were also only found in the cyanobacterium [16]. A further cell separation study conducted on a specimen of *L. herbacea* containing the brominated diphenyl ether (**12**, Figure 1) demonstrated that only *O. spongeliae* contained the metabolite, and therefore the filamentous cyanobacterium is likely the biosynthetic source of the brominated compounds as well as the chlorinated metabolites [17]. In our experience, the two classes of metabolites have never been found together in the same sponge. The results of a large-scale collection of 43 samples of five *Dysidea* morphotypes containing *O. spongeliae* from Indonesia and Papua New Guinea followed by characterization of the halogenated metabolites found in each specimen were in agreement with this observation [18].

The putative gene cluster for barbamide (**4**, Figure 1) has been reported by Chang et al. [8]. Similar to the chlorinated peptides found from *L. herbacea*, it contains a trichloromethyl functionality on an isovaleric acid unit derived from leucine. Two hypothetical/putative halogenase genes (*barB1, barB2*) in this cluster were identified whose products were similar to the syr-

*Correspondence: mhaygood@ucsd.edu
[5]Deceased.

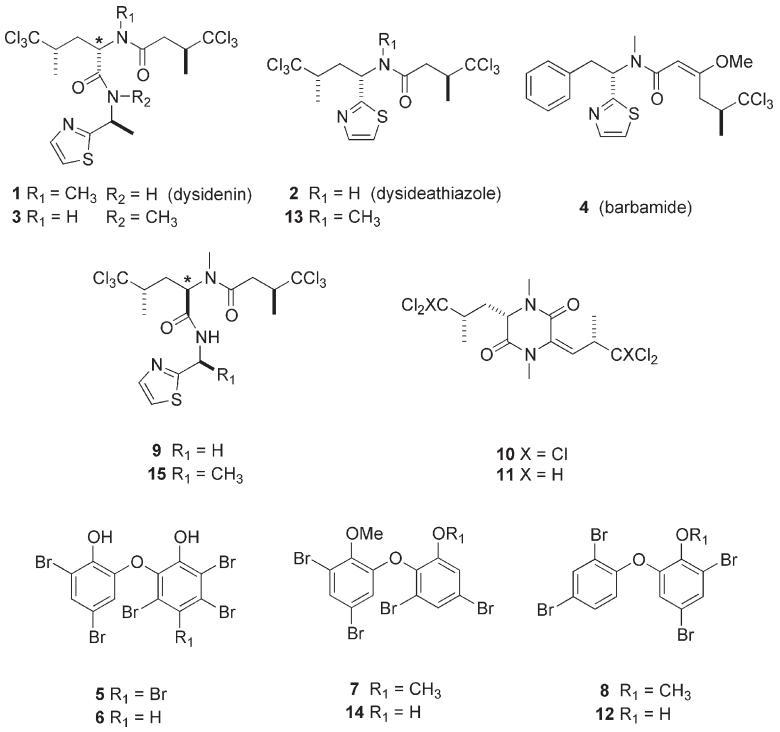

Figure 1. Structures of Halogenated Secondary Metabolites Isolated from Dictyoceratid Sponges (**1–2**, **5–7**, **9–15**), the Cyanobacterium *Lyngbya majuscula* (**3–4**), or the Green Alga *Cladophora fascicularis* (**8**)

The asterisk indicates the epimeric stereocenter of dysidenin (**1**) and isodysidenin (**15**).

ingomycin protein SyrB2 [19], an enzyme possibly involved in the chlorination of a threonine methyl group. The BarB1/BarB2 and SyrB2 enzymes are not similar to other known halogenating enzymes, and it is proposed that they form a unique group in this class [8]. Although function has not yet been demonstrated for these enzymes, their distant relationship to other known enzymes has enabled the design of PCR primers to probe other organisms for the presence of homologous genes found in gene clusters producing chlorinated leucine derivatives. Gerwick and coworkers have been able to obtain *barB* homologs from genomic DNA isolated from a sample of *L. herbacea* which contained the chlorinated peptides, and a fluorescent in situ hybridization experiment targeting mRNA encoding for the *barB1* homolog has also identified *O. spongeliae* as the source of the chlorinated amino acid derivatives (P. Flatt and W. Gerwick, personal communication).

A molecular phylogeny has been constructed for *O. spongeliae* and three species of host sponges in Guam, although the natural products contained in the invertebrates were not reported [20]. The sponges, identified as *Dysidea* n. sp. aff. *herbacea* (ridged form), *D.* n. sp. aff. *herbacea* (smooth form), and *Dysidea* n. sp. aff. *granulosa*, were collected from various sites around the island and phylogenetic analysis was done using the small subunit 16S rRNA gene of the cyanobacteria and the second internally transcribed spacer region (ITS-2) with some surrounding sequence from the sponge. Thacker and Starnes reported that the three sponge species host distinct clades of cyanobacteria, and that cospeciation may be occurring between the sponges and the cyanobacteria [20].

It was not clear from the previous studies whether or not there is a correlation between strains of *O. spongeliae* and halogenated natural products or alternatively whether one strain is capable of selective expression of the halogenated metabolites. Additionally, the proposal of cospeciation between the cyanobacterium and the host sponges deserved further investigation. Therefore, we collected four species of dictyoceratid sponges (*L. herbacea*, *L. chlorea*, *Lendenfeldia chondrodes*, and *Phyllospongia papyracea*) that contained *O. spongeliae* (Figures 2A–2F) from four distant collection sites in Palau. Where possible, samples were collected both from different locations and on the same reef to determine whether there is geographic diversity and whether two adjacent species would have the same symbionts (Figure 3). We characterized the halogenated natural products and investigated the evolutionary relationships of hosts and symbionts as well as the biosynthetic potential of the *O. spongeliae* symbionts. To determine a valid outgroup in the phylogenetic analysis of the host sponges, three additional sponges (*Chelonaplysilla delicata*, *Hyrtios erectus*, and *Dysidea* sp.) shown in Figure 2 were collected in Palau and included in the analysis.

Results and Discussion

Examination of the 5.8S, ITS-2, and 28S gene sequences obtained from the sponges indicated that there was no significant variation among specimens of the same species. Within *L. herbacea* or *L. chondrodes*, the intraspecific gene sequences were identical, and the only divergence in *P. papyracea* was detected in the highly variable ITS-2 region (3/200 bp). A phylogram of these four species using the dendroceratid sponge *Chelonaplysilla delicata* as the outgroup and incorpo-

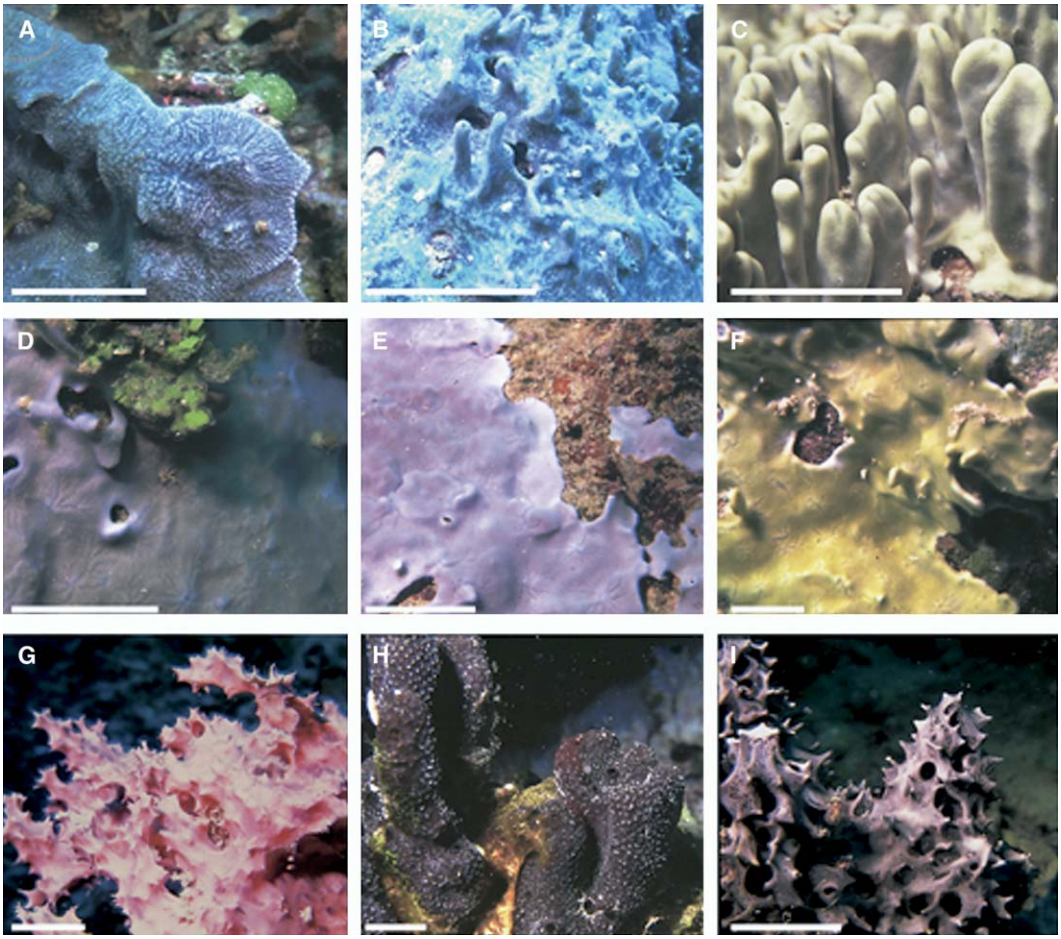

Figure 2. Underwater Photographs of the Marine Sponges in This Study

(A) *Lamellodysidea herbacea*; (B) *Lamellodysidea chlorea*; (C) *Lendenfeldia chondrodes*; (D) *Phyllospongia papyracea* site 3 (1); (E) *P. papyracea* site 3 (2); (F) *P. papyracea* site 1; (G) *Dysidea sp.*; (H) *Hyrtios erectus*; (I) *Chelonaplysilla delicata*. Note that *L. herbacea* and *P. papyracea* can form fingery projections as shown for *L. chlorea*, which were usually present, and underwater photographs of *L. herbacea* and *L. chlorea* alone are not sufficient to distinguish the two. A picture is provided for all three samples of *P. papyracea* as it was the only species where the surface coloration varied. All scale bars, approximately 2 cm.

rating sequence obtained from *Hyrtios erectus* (Thorectidae) and a *Dysidea* sp. (Dysideidae) is shown in Figure 4. It appears that genetically, the four species of sponge containing the *Oscillatoria* symbionts in this study may be more closely related than present classification indicates since they formed a well-supported clade that excludes *H. erectus* and the *Dysidea* sp. However, *Phyllospongia* and *Lendenfeldia*, when compared to all species currently grouped in Dysideidae (i.e., *Lamellodysidea* and *Dysidea*), have fundamental differences in morphology such as choanocyte chamber structure and fiber structure. Therefore, *P. papyracea* and *L. chondrodes* remain as currently classified in the family Thorectidae. Separation at the family level of the two *Lamellodysidea* spp. and those that have the morphology of the type seen in *Dysidea* sp. could be justified based on the molecular results but would require study of a greater range of species. The taxonomy of this order of sponges is recognized to be extremely challenging based on morphology alone [21], but our work indicates that analysis of the genetic and morphological characteristics in conjunction may help resolve these difficulties. Further refinement of the classification of the Dysideidae will be introduced by P.R.B. and Cook when describing the New Zealand Dysideidae (in preparation). Until that work is completed, the taxonomic arrangement of the family remains as currently defined [21]. As a result of this analysis, we were able to designate the *Dysidea* sp. as the outgroup for these *O. spongeliae*-containing sponges when investigating hosts and symbionts for the presence of cospeciation.

The ultrastructure of the *Oscillatoria* filaments from one specimen of each sponge species matched previous descriptions [2, 3], and appeared similar when the filaments were compared to each other. A superficial difference existed between those samples collected at greater depth than the shallow water samples, as the cyanobacteria have a greater number of thylakoids and are more electron dense than the shallow water *Oscillatoria*. The filaments in the *L. herbacea* sample were the only ones observed to contain "stellate bodies," which were first reported by Berthold et al. [2]. The identity of

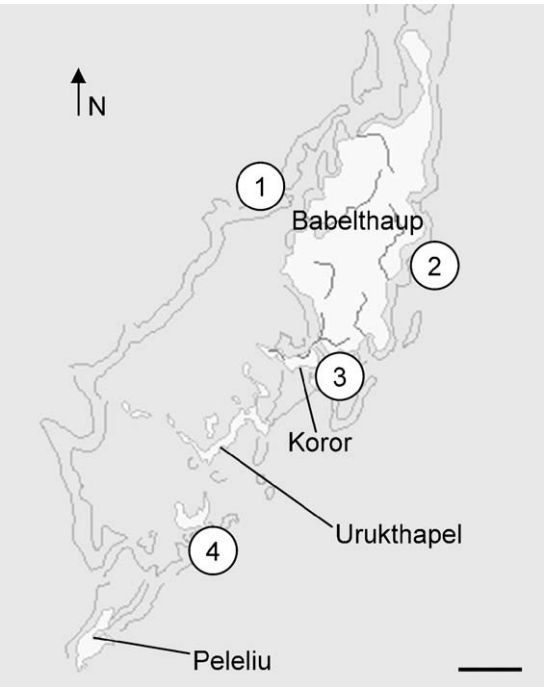

Figure 3. Map of the Republic of Palau (Excluding Anguar and the Southwest Islands) with the Sponge Collection Sites Shown

From site 1, four sponges were collected: *L. herbacea* (n = 1), *L. chondrodes* (n = 2), and *P. papyracea* (n = 1). From site 2, one sample of *L. herbacea* was collected, and two samples of *P. papyracea* were obtained from site 3. One sample each of *L. chlorea* and *L. herbacea* was collected from site 4. Scale bar is approximately 7.5 km. Map was provided by ReefBase (http://www.reefbase.org).

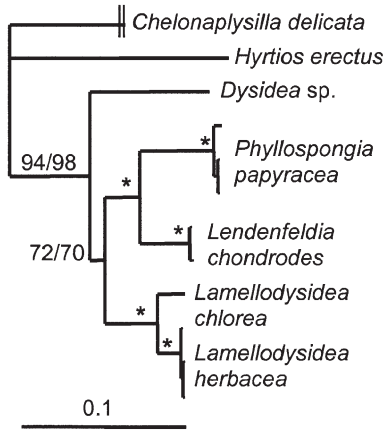

Figure 4. Bayesian Phylogram Derived from Sponge rRNA and ITS-2 Gene Sequences

Replicate samples of individual species had very similar or identical gene sequences irrespective of collection site, so for clarity, the clades are only labeled with species names. A maximum parsimony (MP) tree had identical topology. Support for the clades is indicated by posterior probability (Bayesian)/bootstrap value (MP), and the asterisk indicates 100% support for both methods. The scale bar indicates substitutions per nucleotide position.

the stellate bodies is not known, but it has been proposed that they may be viral in nature [3]. Despite the morphological similarity, examination of the 16S rRNA gene sequence revealed that each species of sponge had its own strain of *O. spongeliae*, as was seen in related sponges in Guam [20]. This host specificity was not affected by proximity to other sponges containing different strains of the symbiont or by geographic separation in Palau. Since cyanobacteria can contain multiple rRNA operons with 16S rRNA gene heterogeneity in their genomes [22], we obtained DNA-dependent RNA polymerase gene (*rpoC1*) sequence as well, which exists in single copy in prokaryote genomes [23]. No divergence within strains of *O. spongeliae* was detected with either gene. Comparison of the 16S rRNA gene sequence showed only about 1%–2.5% divergence between the *O. spongeliae* strains, and 1.5%–4% divergence in *rpoC1*. Apparently *rpoC1* is relatively conserved in *O. spongeliae* strains compared to *Synechococcus* spp., as *Synechococcus* isolates WH7805 and WH8103 that are 1.4% divergent by 16S sequence are 17% divergent by *rpoC1* sequence [23].

To determine whether cospeciation between the cyanobacteria and the Palauan sponges was occurring, composite phylogenetic trees were compared (Figure 5A). Interestingly, strict cospeciation is not supported by the molecular phylogeny. In this analysis based on 16S rRNA gene and *rpoC1* sequence, the *O. spongeliae* strains in *L. herbacea* and *L. chondrodes* appear to share a common ancestor with strong clade support, as did the strains in *L. chlorea* and *P. papyracea*. In contrast, the hosts have the opposite affiliations. Further phylogenetic analysis was completed incorporating sponges and symbionts from the Guam study without *rpoC1* sequence (Figure 5B) [20]. In this dataset, only the *L. chondrodes* strain of *O. spongeliae* was not in agreement with the proposal of cospeciation. To determine if one of these models is more probable then the other, we estimated the likelihoods using the maximum likelihood estimation function of PAUP [24] of the parsimony trees that were obtained for Figures 5A and 5B and constrained trees which were forced to match the outcome predicted by the other data set. For the 16S rRNA gene and *rpoC1* data set, the –ln L = 8724.67 for the symbiont tree in Figure 5A and 8726.03 for a tree constrained to have the symbionts from *L. herbacea*, *L. chlorea*, and *L. chondrodes* in the same clade. For the larger data set which only contained 16S rRNA gene sequences, the –ln L = 2521.78 for the symbiont tree in Figure 5B and 2523.28 for a tree where the *O. spongeliae* sequences from *L. herbacea* and *L. chondrodes* were forced in one clade and the sequences from *L. chlorea* and *P. papyracea* were restricted to another clade. Since these differences are not significant, it is not clear to which clade the *L. chlorea* strain belongs based on these data. Regardless, the position of the *L. chondrodes* symbiont in the trees from both data sets indicates that the speciation of these cyanobacteria and their hosts is more complex than currently thought. The most parsimonious scenario is that at least one host switching event has occurred in this sponge/cyanobacterium symbiosis. These species of sponges are occasionally found in contact with each other on reefs, so presumably there is opportunity for

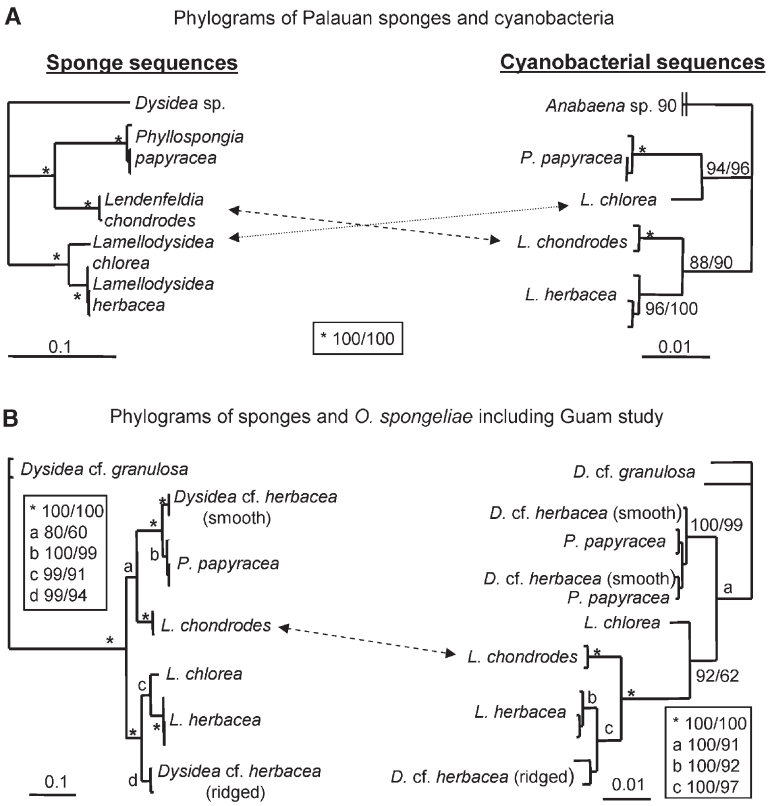

Figure 5. Bayesian Phylogenetic Trees of Marine Sponges and Cyanobacteria

Replicate samples of individual sponge species had very similar or identical gene sequences irrespective of collection site, so for clarity the clades are only labeled with species names. Maximum parsimony trees had identical topologies. Support for the clades is indicated by posterior probability (Bayesian)/bootstrap value (MP). The scale bars indicate the substitutions per nucleotide position.

(A) Trees derived from Palauan sponge rRNA and ITS-2 gene sequences and *Oscillatoria spongeliae* 16S rRNA gene and *rpoC1* sequences. The GenBank accession numbers for *Anabaena* sp. 90 used as the root for the *O. spongeliae* phylogram are AY424996 for *rpoC1* and AJ133156 for 16S rRNA gene.

(B) Trees from Palauan sponge rRNA and ITS-2 gene sequences and *O. spongeliae* 16S rRNA gene sequences including two representative samples of the three sponge species in the Guam study and their symbionts (GenBank accession #s AF420446, AF534687, AF420441, AF420443, AF420444, AF534689, AF534691, AF534692, AF534694, AF534695, AF534699, and AF534702). The trees were rooted with *Dysidea* n. sp. aff. *granulosa* or their symbiont without implying polarity.

the symbionts to migrate between species of sponge. Further analysis of a larger data set of sponges containing *O. spongeliae* would be required to determine whether *L. chondrodes* and its symbiont is just an unusual exception, or if these potential host switching events are common in the evolution of this symbiosis.

Comparative phylogenetic analysis of the sponge sequences for our specimens from Palau and those from Guam [20] indicated that the Guam sponge previously identified as *D.* n. sp. aff. *herbacea* (smooth form) appears to be the same species as *P. papyracea* in this study, consistent with comparison of the underwater photographs. The divergence between the gene sequences of the Palauan and Guam sponges is significant at 2%, but all the genetic variability except for 1 bp was located in the ITS-2 region. Since the ITS-2 has a fast molecular clock, it is likely reflecting the geographic separation of the sponges. Interestingly, the sponges contain the same strain of *O. spongeliae* with 16S rRNA gene sequence identity of 99.9%–100%, despite the geographic separation. The Guam sponge identified as *L.* n. sp. aff. *herbacea* (ridged form) is closely related to the Palauan *L. herbacea* and *L. chlorea* (Figure 5B), with genetic divergence of 4.5% and 4.3%, respectively. These three sponges are quite similar in appearance and gross morphology, yet they appear to be separate species that contain genetically distinct strains of *O. spongeliae*.

The chemical investigations revealed that all four species of sponges had a different secondary metabolite composition. Through comparison of spectral data with previously reported compounds, the dominant halogenated secondary metabolites contained in *L. herbacea*, *L. chondrodes*, and *P. papyracea* were identified. *L. herbacea* from site 1 contained N-methyldysideathiazole (**13**) [15], from site 2 contained dysideathiazole (**2**) [6] and **13**, and from site 4 contained dysidenin (**1**) [25] (Figure 1). All three samples of *P. papyracea* contained the brominated diphenyl ethers **5–7** [11], and both specimens of *L. chondrodes* had **14** [10] as the only brominated compound (Figure 1). Chemical analysis of 22 other specimens of these sponges collected from various sites in Palau over a span of three years revealed that *L. herbacea* contained chlorinated amino acid derivatives, and *P. papyracea* and *L. chondrodes* contained brominated diphenyl ethers. No examples were found in these species where the reverse was true, and the halogenated compounds were always present. Neither class of metabolite was detected in the one sample of *L. chlorea* investigated in this study. Instead, the organic extract was dominated by sterols.

Using primers designed from *barB1* and *barB2* sequence, homologs designated *dysB1* and *dysB2* were successfully amplified using PCR on genomic DNA isolated from the three samples of *L. herbacea* collected at site 1, site 2, and site 4 (Figure 3). The *dysB* sequences obtained from the three samples of *L. herbacea* and an additional sample of *L. herbacea* containing dysidenin had 93% sequence identity or higher to the *L. majuscula* genes. Interestingly, the gene homologs that were amplified from *L. herbacea* samples which contain dysidenin were not identical to those from the dysideathiazole-containing sponges, suggesting that the biosynthesis of the two metabolites is encoded by

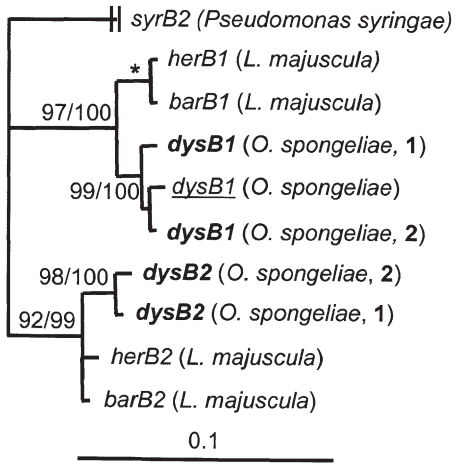

Figure 6. Phylogenetic Tree of Available Hypothetical/Putative Halogenase DNA Sequences

Sequences from *Lyngbya majuscula* (GenBank Accession nos. AF516145, AY648944, and AY648945) and *O. spongeliae* (AY648943) are included with those obtained in this study in bold. The corresponding chlorinated peptide is indicated: **1** = dysidenin, **2** = dysideathiazole).

The underlined sequence has been localized to *O. spongeliae* using FISH. The *syr*B2 gene (AAD50521) from *Pseudomonas syringae* was used as an outgroup. A maximum parsimony (MP) tree had identical topology. Support for the clades is indicated by posterior probability (Bayesian)/bootstrap value (MP), and the asterisk indicates 100% support for both methods. The scale bar indicates substitutions per nucleotide position.

different gene clusters. The nucleotide divergence in these genes isolated from dysidenin- and dysideathiazole-containing sponges was 1% out of 855 bp for *dysB1*, and for *dysB2* was 1.5% out of 846 bp. This divergence results in translation differences of three amino acids for DysB1 and two for DysB2.

The *dysB1* and *dysB2* sequences were obtained from total sponge/cyanobacterial genomic DNA, so further analysis was completed to identify the source of the sequence. A phylogenetic tree with all available *barB1/barB2* homologs was constructed (Figure 6). The *dysB1* and *dysB2* genes obtained in this study formed distinct clades which did not include their counterparts from *L. majuscula*. The *dysB1* clade included the sequence obtained by Gerwick and coworkers which was localized to *O. spongeliae*, indicating the sequences we obtained were from the cyanobacterium. We were able to fractionate host and symbiont DNA from genomic DNA using a cesium chloride/hoechst dye gradient, and from the lower band of the *L. herbacea* samples, we amplified the following: clean *O. spongeliae* 16S rRNA gene sequence using general eubacterial primers, clean *rpoC1* sequence, and *dysB* homologs. Therefore, the *O. spongeliae* in the Palauan sponges is the source of the *dysB* sequence and the chlorinated peptides as found in the previous studies. For the other three species of sponges, no *barB1/barB2* homologs were amplified. Based on these PCR screen results, we propose that the existence of chlorinated peptides in some *Oscillatoria*-containing sponges is not a case of selective expression of the metabolites, but rather that the respective gene pathway is not present in the *Oscillatoria* symbionts of other sponges which do not contain the compounds.

The identification of different gene clusters for dysideathiazole or dysidenin is intriguing, as the *O. spongeliae* in these sponges had identical 16S rRNA gene and *rpoC1* sequence. If both gene clusters were present in the genome, the *dysB* PCR products would be expected to be

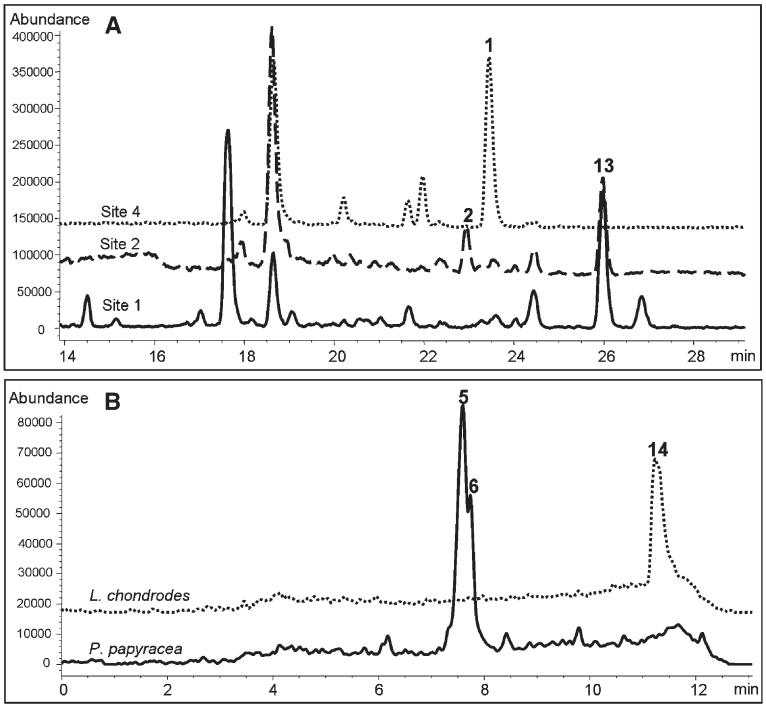

Figure 7. Ethyl Acetate Extract LC/MS Traces from the Palauan Sponges Containing Chlorinated Peptides or Brominated Diphenyl Ethers

Peaks representing halogenated compounds are labeled with a number corresponding to the respective compound given in Figure 1.

(A) Traces from the three *L. herbacea* samples with their collection site indicated in positive detection mode with a gradient of 10% ACN/H_2O to 100% ACN over 30 min.

(B) Representative traces from *Lendenfeldia chondrodes* and *Phyllospongia papyracea* in negative detection mode with a gradient of 10% ACN/H_2O to 100% ACN over 15 min.

the same regardless of which compound was produced, with the areas of divergence in the sequence present as ambiguous sequence in the sequence chromatograms. Since this is not the case, it appears that only one or the other gene cluster is present. As these biosynthetic pathways yield such similar natural products, it is possible that one gene cluster was derived from the other through the gain or loss of an alanine NRPS module. This event could have occurred in *O. spongeliae* in the recent past and would result in the chemical diversity observed in these cyanobacteria. Another possibility is that the ancestral strain had both gene clusters and evolutionary pressure only required the production of one of the compounds, and the ability to produce both natural products was recently lost. No sample from Palau was ever found that contained both dysideathiazole and dysidenin analogs when nine total specimens of *L. herbacea* collected over a period of three years were analyzed, providing further support that only one or the other compound is produced.

Significance

The chemical and molecular investigations of four dictyoceratid sponges from Palau provide insight into the symbiosis that these marine invertebrates maintain with a filamentous cyanobacterium, *Oscillatoria spongeliae*. Instead of one ubiquitous cyanobacterium selectively producing either chlorinated peptides or brominated diphenyl ethers, it appears that the four sponge species contain their own genetically distinct strains of which only one strain can produce the chlorinated secondary metabolites. Within this strain, there is biosynthetic variability indicated by the presence of one of two sets of biosynthetic genes and either the chlorinated compound dysideathiazole and its analogs or the chlorinated metabolite dysidenin and its derivatives. This indicates that the strain contains just one chlorinated peptide encoding gene cluster despite having identical small subunit 16S rRNA gene and DNA-dependent RNA polymerase *rpoC1* sequence, and cannot produce both compounds. This result raises the question of whether gain or loss of an alanine NRPS module resulted in formation of the two gene clusters, or whether the ancestral strain lost the ability to produce both chlorinated peptides. The phylogeny of the *O. spongeliae* strains when compared to the host sponges suggests that host switching can occur, providing further information about the complex nature of this symbiosis.

This study has demonstrated the utility of analyzing the ultrastructure of the symbionts, characterizing the natural products that are present, and probing biosynthetic capabilities when investigating the evolutionary relationships of microbial symbiosis with marine invertebrates. As found here, morphologically similar symbionts can be identified that vary in their ability to produce secondary metabolites and therefore fulfill different roles in their environments.

Experimental Procedures

Sponge Collection and Description

Specimens of the dictyoceratid marine sponges *Lamellodysidea herbacea* (Keller, 1889; family Dysideidae), *Lamellodysidea chlorea* (de Laubenfels, 1954), *Lendenfeldia chondrodes* (de Laubenfels, 1954; family Thorectidae), and *Phyllospongia papyracea* (Esper, 1806; family Thorectidae) were collected from four reef sites using SCUBA in the Republic of Palau. Site 1 was at West Channel Buoy 5 (07°32.33′N, 134°28.30′E) at a depth of 15–20 ft, site 2 was an inner reef entrance to Ngatpaet (07°27.94′N, 134°37.65′E) at a depth 45 ft, site 3 was at seamount 2 in the KB channel (07°20.30′N, 134°31.07′E) at a depth of 45 ft, and site 4 was in Ngerechong channel (07°06.90′N, 134°22.78′E) at a depth of 20 ft. While collecting care was taken to ensure that the entire sample was one piece so that no sample contained more than one species.

Full taxonomic descriptions of *Lamellodysidea herbacea*, *Lamellodysidea chlorea*, and *Lendenfeldia chondrodes* have been previously reported [26]. The species *Phyllospongia papyracea* was fully redescribed by Bergquist et al. [27]. *L. herbacea* and *L. chlorea* are gray to green-gray in coloration, grow in sheets on reefs or rocks, and have a ridged surface structure (Figures 2A–2B). *L. chondrodes* is yellow to yellow-green in coloration, grows in a spreading sheet on the reef with numerous vertical fingers, and has a smooth surface (Figure 2C). *P. papyracea* varies in coloration from blue-green to golden-yellow, grows in sheets on reefs or rock, and has a smooth surface (Figures 2D–2F). Identification of these four species requires analysis of fiber structure and organization, choanocyte chamber structure, mesohyl texture and histology, and morphology of the sponge surface.

The *Dysidea* sp. (*Dysidea* cf. *pallascens*, Schmidt, 1862; Order Dictyoceratida, family Dysideidae) was found at a site locally known as Wonder Channel (7°10.83′N, 134°21.67E) at a depth of 13 min, *Hyrtios erectus* (Keller, 1889; Order Dictyoceratida, family Thorectidae) was collected at a depth of 3 min from a small lagoon at Ngermeuangel Island (07°20.27′N, 134°27.43E), and *Chelonaplysilla delicata* (Pulitzer-Finali and Pronzato, 1999; Order Dendroceratida, family Darwinellidae) was collected from site 3. The *Dysidea* sp. (Figure 2G) is probably new to science, and unlikely to be conspecific with the Mediterranean population of *D. pallascens*. Nevertheless, it resembles *D. pallascens* closely in external morphology, growth form, and surface characteristics, but a comprehensive revision of the massive, erect, and ramose species of *Dysidea* is required to place this species accurately. *Hyrtios erectus* (Figure 2H) is a black upright conulose sponge found growing on rock or coral [26], and *Chelonaplysilla delicata* (Figure 2I) is a gray frilly sponge with a smooth surface found growing on reefs or rock [28]. Voucher specimens have been deposited in the SIO Benthic Invertebrate Collection for all the sponges in this study (P1192–P1203), and vouchers of the *Dysidea* sp. (QM G316990) and *Chelonaplysilla delicata* (QM G316991) were deposited at the Queensland Museum repository as well.

Chemical Analysis

For each specimen of *L. herbacea*, *L. chlorea*, *P. papyracea*, and *L. chondrodes* considered in this study, 14–40 g of sponge was frozen immediately after collection and stored at −20°C until extraction. The sponges were individually lyophilized, extracted with methanol (3 × 100 ml), dried in vacuo, and partitioned between ethyl acetate and water to obtain the organic extracts of each sample (80 mg to 1 g). ^{1}H NMR spectra on the extracts were recorded in $CDCl_3$ on a Varian Gemini 400 MHz spectrometer, and gradient COSY experiments were performed on a Varian Inova 300 MHz NMR spectrometer. Liquid chromatography-mass spectrometry (LC/MS) analysis was performed on each extract using a Hewlet Packard series 1100 mass spectrometer and an Agilent C_{18} analytical HPLC column (Figure 7). Molecular ions of the chlorinated compounds were obtained in the positive detection mode, and were found for the brominated metabolites in the negative mode. Only two brominated diphenyl ethers were present in the LC/MS traces of *P. papyracea*, but analysis of the ^{1}H NMR spectra revealed the presence of a third which was not detected. This compound was isolated by chromatography of the organic extract on silica gel (200–430 mesh) using 10% ethyl acetate/hexane as the eluent, and was identified as **7** (Figure 1) based on comparison of spectral data with the authentic compound [11]. Since dysidenin and isodysidenin are epimers (Figure 1) that have similar ^{1}H NMR spectra and identical MS data, the extract containing dysidenin was subjected

to HPLC using a Varian preparative C_{18} column with a gradient of 50% ACN/H_2O to 100% ACN to yield the pure compound. The optical rotation of the natural product was obtained using a Rudolph Research Autopol III polarimeter. The measured $[\alpha]_D$ –95.2° (*c* 0.66, $CHCl_3$) was consistent with the reported value for dysidenin, $[\alpha]_D$ –98°, and not isodysidenin, $[\alpha]_D$ +47° [7].

DNA Isolation and Sponge Gene PCR

DNA was isolated from sponge tissue from each sample stored in RNAlater (Ambion) at –20°C using the animal tissue protocol of a DNeasy kit (Qiagen). In all cases, the optional RNase treatment was carried out. Since PCR inhibitors were present, the isolated DNA was further purified using a Qiaquick PCR purification kit (Qiagen). The D3 variable loop of the 28S rRNA of the host species was amplified using D3A and D3B primers [29], and the ITS-2 was amplified with the primers SP58bF and SP28cR [20]. Twenty-five microliter PCR reactions were performed for both genes using Taq DNA polymerase (Promega) with a 1 min denaturing step at 95°C followed by 35 cycles of 95°C for 1 min, 50°C for 0.5 min, and 72°C for 1.5 min. For each sample, 4–6 PCR reactions were carried out to minimize PCR errors and later combined.

16S rRNA Gene Clone Library Analysis

Using the eubacterial primers 27F and the 1492R [30] and the high-fidelity DNA polymerase *pfu* turbo (Stratagene), 50 μl PCR reactions were carried out on genomic DNA from each specimen of *L. herbacea*, *L. chlorea*, *L. chondrodes*, and *P. papyracea*. The reaction conditions were a 1 min denaturing step at 95°C followed by 25 cycles of 95°C for 1 min, 60°C for 0.5 min, and 72°C for 2 min. In an attempt to minimize heteroduplex formation, reconditioning PCR [31] was then performed on each sample by running another 50 μl PCR reaction using *pfu* turbo and 5 μl of obtained PCR product as the DNA template. The PCR conditions were 3 cycles of 95°C for 1 min, 60°C for 0.5 min, and 72°C for 2 min. These "reconditioned" PCR products were then purified using a PCR cleanup kit (Qiagen), and adenylated through incubating of the PCR product at 72°C for 10 min with Taq DNA polymerase and dATP. This adenylated product was cloned directly into a pCR4-TOPO plasmid vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen) following Invitrogen's instruction manual. The 16S rRNA gene inserts in the resulting colonies were amplified directly from *E. coli* cells using the T3 and T7 primers provided in the TOPO TA Cloning kit (Invitrogen). The PCR conditions were as follows: 25 μl reactions using Taq polymerase, 1 min 95°C denaturing step followed by 35 cycles of 95°C for 1 min, 60°C for 0.5 min, and 72°C for 2 min. The clone libraries were screened by incubating the PCR products with RsaI (Invitrogen), MspI (NEB), and EcoRI (NEB) for a period of 3 hr. Seventeen to twenty-seven clones were screened from the three samples of *L. herbacea* for a total of 66, while 25 clones were screened from *L. chlorea*. From the two samples of *L. chondrodes*, 19 and 34 clones were screened. Thirty-one to forty clones were screened from the three samples of *P. papyracea* for a total of 107. One restriction fragment digestion pattern was recognized on a 1.2% agarose gel for each sample which accounted for 63%–97% of the clones recovered from the four species. Plasmids were isolated using a QIAprep Miniprep kit (Qiagen), and sequenced using the T3 and T7 plasmid primers as well as internal 338F [32] and 536R [33] 16S rRNA gene primers. At least two clones of the dominant pattern were sequenced from each sponge for a total of 9 from *L. herbacea*, 2 from *L. chlorea*, 4 from *L. chondrodes*, and 7 from *P. papyracea*. The sequences were identified as *O. spongeliae* based on their identity to posted *O. spongeliae* sequences when compared to other bacteria using the basic local alignment search tool (BLAST) [34]. Since no variation was detected within a sponge sample, one sequence from each species was used to represent the 16S rRNA gene of the symbiont. At least one clone of the minor restriction digest patterns were sequenced from each sample, which were from other bacteria not related to *O. spongeliae* based on BLAST and ribosomal database project (RDP-II) searches [35].

Biosynthetic Gene PCR

Using primers designed to amplify *barB1* and *barB2* homologs, all 9 sponge samples containing *O. spongeliae* were screened for the presence of those genes through attempted PCR amplification from genomic DNA. The *barB1* primers were BarB1-S-NdeI (5′-GGAATTCCATATGAAAACTAGGGCAAAAGCTG- 3′) and B-Hali-AS-1 (5′-CCGCTCGAGCTGTCCCAAAGGTCGTCTAAT-3′), and the *barB2* primers were B-Halii-S-1 (5′-GGAATTCCATATGAAAACTATCACTG CTGAAC-3′) and B-Halii-AS-1 (5′-CCCCTCGAGCAGAGGTTGTC TCACTTTG-3′). These primers have restriction digest sites which were not considered in the subsequent analysis. The PCR conditions for both sets of primers were a 1 min denaturing step at 95°C followed by 35 cycles of 95°C for 1 min, 45°C for 0.5 min, and 72°C for 1.5 min. For the *barB1* primers, PCR products of the appropriate size were obtained from genomic DNA from all the sponges. However, the sequence data obtained from the sponges that did not contain the chlorinated peptides were of poor quality and clearly not *barB1* homologs. Further PCR experiments conducted under the same PCR conditions using B-Hali-AS-1 and an internal primer DysB1-S-1 (5′-ATTGAGTTGTGGCAGGAAATTAG-3′) designed to bind to *dysB1* homologs resulted in the expected PCR product for sponges that contained the chlorinated compounds, and no product for those which did not. To rule out the presence of PCR inhibitors in those samples where *barB* homologs were not amplified, PCR reactions were carried out on the samples using 16S and 28S rRNA gene primers and the expected amplicons were obtained.

Separation of Host and Cyanobacterial DNA

High molecular weight (HMW) DNA, 30–50 kb in size, was isolated from tissue stored in RNAlater using a Blood and Cell Culture DNA kit Midi (Qiagen). Yields ranged from 30–75 μg per sample of sponge containing *O. spongeliae*, of which the entire amount of HMW DNA was added to a 53% CsCl solution with 13 μg/ml Hoechst 33258 dye (EMD Biosciences). Centrifugation was performed on a Beckman L8-M ultracentrifuge using a VTi65 rotor (Beckman) at 267,000 × g (55,000 rpm) at 20°C for 24 hr. Two distinct bands were achieved for each sample of sponge; these were individually extracted from the gradient with an 18 gauge needle and syringe. The Hoechst dye was removed through 2 extractions of water-saturated *n*-butanol, and the DNA was precipitated with *i*-propanol, washed with 70% EtOH, resuspended in 10 mM tris-HCl (pH 8.5), and vortexed at maximum speed for 0.5 min. From DNA isolated from the lower band, 25 μl PCR reactions using the published C-terminal and N-terminal (X = G) *rpoC1* primers [23] were carried out 4 to 6 times per sample to minimize PCR errors using Taq polymerase and combined afterwards. No product of the right size was obtained from PCR on DNA isolated from the upper band. The conditions were a 1 min 95°C step followed by 35 cycles of 95°C for 1 min, 50°C for 0.5 min, and 72°C for 1.5 min. It was necessary to excise the appropriate band from a 1.2% agarose gel, and purify the DNA from the agarose using a QIAquick gel extraction kit (Qiagen) prior to sequencing.

Sequencing

For sequencing, all PCR products were purified using a Qiaquick PCR purification kit (Qiagen) and sequenced using both forward and reverse primers. All sequencing was performed on an ABI 3100 Analyzer using the ABI Prism BigDye Terminator kit. All DNA sequences obtained were assembled and checked by eye using Sequencher 4.0.5 (Gene Codes Corp.). The GenBank accession numbers for the DNA sequences used in the phylogenetic analysis are AY613960–AY613983 and AY615501–AY615518, and for the *dysB* genes are AY628171–AY628174.

Phylogenetic Analysis of Sponge Sequence

For Figures 3 and 5A, the 5.8S, ITS-2, and 28S sequences obtained from the Palauan sponges were combined, aligned using Clustal X 1.83 [36], and checked by eye. Composite phylogenetic trees were constructed using Mr. Bayes 3.0 [37] and the maximum parsimony (MP) algorithm in PAUP 4.0b10 [24] with the sequence data divided into an rRNA (all 5.8S and 28S sequence) and ITS-2 partitions. Default priors were used for the Bayesian analysis, and the general time reversible (GTR) model was used with a γ distribution of rate variation across sites. The partitions were unlinked to allow for independent estimations of likelihood. Four markov chain monte carlo (MCMC) chains were run for three million generations sam-

pling every 100, and the first 2000 trees were discarded. For MP analysis, transversions were weighted 3 times transitions (maximum likelihood estimation of ratio), the partitions rRNA:ITS2 were weighted 3:1 in the analysis, and a 10 replicate heuristic search with random addition of sequences was completed. Bootstrap analysis was performed using 1000 replicates. For Figure 5B, the tree was constructed using sequence obtained using the SP58bF-SP28cR primer set (no D3 sequence available from Guam sponges). Otherwise, the analysis was the same as Figure 5A.

Phylogenetic Analysis of *O. spongeliae* Sequence

For Figure 5A, composite phylogenetic trees for the *Oscillatoria* strains were constructed based on the 16S rRNA gene and *rpoC1* sequence using Mr. Bayes and MP (PAUP) with the data partitioned into 4 blocks: 16S rRNA and *rpoC1* divided into 3 (first position, second position, and third position). The Bayesian analysis was carried out with unlinked partitions excluding the third position of the *rpoC1* gene using the GTR model and γ distribution across sites. Four MCMC chains were run for three million generations, sampling every 100 with the first 2000 trees rejected from the analysis. For the MP phylogeny, transversions were weighted 2 times transitions, the partitions were weighted 2:2:3:1 (16S, first position, second position, and third position), and a heuristic search with 10 replicates was performed with sequences added randomly. Bootstrap analysis was conducted using 1000 replicates. For Figure 5B, the tree was constructed with 16S rRNA gene sequence (no *rpoC1* sequence available from Guam symbionts), and no partitioning and weighting of the data was employed. Otherwise the same methodology was employed as in Figure 5A. The GTR model with γ distribution across sites was used (in agreement with the Bayesian analysis) for the maximum likelihood estimations of constrained and unconstrained trees.

Phylogenetic Analysis of Biosynthetic Gene Sequence

The gene sequences were aligned using Clustal X, and corrected based on comparison to the alignment of the protein sequences encoded by these genes. The phylogenetic trees for the hypothetical/putative halogenase sequences were constructed using Mr. Bayes and MP (PAUP) with the data partitioned into 3 blocks (first position, second position, and third position). The Bayesian analysis was carried out with unlinked partitions excluding the 3rd position of the codon using the GTR model and γ distribution across sites. Four MCMC chains were run for three million generations sampling every 100 with the first 2000 trees rejected from the analysis. For the MP phylogeny, transversions were not weighted to transitions based on the likelihood estimation. The codon positions were weighted 2:3:1 (first position, second position, and third position), and a heuristic search with 10 replicates was performed with sequences added randomly. Bootstrap analysis was conducted using 1000 replicates.

Supplemental Data

Supplemental Data consists of five elements and can be found online at http://www.chembiol.com/cgi/content/full/12/3/397/DC1/.

Acknowledgments

We thank the Coral Reef Research Foundation (CRRF) for their invaluable assistance in collecting these sponges, the Republic of Palau for the research permits, Catherine Sincich and Koty Sharp for taking the underwater photographs, and Christine Salomon for assistance with molecular tools and field work. Mark Hildebrand assisted with the cesium chloride/Hoechst dye gradient, and Grace Lim provided advice on experiments and phylogenetic analysis. We especially thank Bill Gerwick and Patty Flatt (Oregon State University) for generously providing the halogenase primers prior to publishing them and Bill Fenical for his support of this project. The transmission electron microscopy was completed with Pat Reid at the VA San Diego Hospital (La Jolla), and Matt Woolery assisted with the mass spectrometry. The DNA sequencing was performed by the DNA Sequencing Shared Resource, UCSD Cancer Center, which is funded in part by NCI Cancer Center Support Grant 2 P30 CA23100-18. This research was supported by a grant from the National Science Foundation (CHE 98-16169).

Received: November 30, 2004
Revised: January 21, 2005
Accepted: February 1, 2005
Published: March 25, 2005

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