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Exploring the Links between Natural Products and Bacterial Assemblages in the Sponge *Aplysina aerophoba*▽†

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The sponge *Aplysina aerophoba* produces a large diversity of brominated alkaloids (BAs) and hosts a complex microbial assemblage. Although BAs are located within sponge cells, the enzymes that bind halogen elements to organic compounds have been exclusively described in algae, fungi, and bacteria. Bacterial communities within *A. aerophoba* could therefore be involved in the biosynthesis of these compounds. This study investigates whether changes in both the concentration of BAs and the bacterial assemblages are correlated in *A. aerophoba*. To do so, we quantified major natural products using high-performance liquid chromatography and analyzed bacterial assemblages using denaturing gradient gel electrophoresis on the 16S rRNA gene. We identified multiple associations between bacteria and natural products, including a strong relationship between a *Chloroflexi* phylotype and aplysinamisin-1 and between an unidentified bacterium and aerophobin-2 and isofistularin-3. Our results suggest that these bacteria could either be involved in the production of BAs or be directly affected by them. To our knowledge, this is one of the first reports that find a significant correlation between natural products and bacterial populations in any benthic organism. Further investigating these associations will shed light on the organization and functioning of host-endobiont systems such as *Aplysina aerophoba*.

Many sponges are known to be associated with large amounts of bacteria (46) that can amount up to 40% of the sponge biomass (35, 76). These types of sponges have been referred to as high-microbial-abundance sponges (36, 38, 40, 87, 88). While a fraction of the bacterial assemblage is used for sponge nutrition (56), another fraction can be permanently associated with sponges (35). This fraction is highly diverse and phylogenetically complex, with representatives from several phyla (e.g., *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Actinobacteria*, *Acidobacteria*, and *Bacteroidetes* [36, 38, 40, 65, 87, 88]). Variability in sponge-associated microbial communities has been assessed at several levels, including within and between specimens of the same host species (2, 38, 66, 67, 69, 87), between sponge species (38, 66), and temporal or spatial distribution within sponges (31, 35, 41, 66, 78, 86, 87).

Sponges are also known as a rich source of natural products or secondary metabolites (29, 30, 46, 89), many of which have the potential to be used in pharmaceutical and biotechnological applications (51, 61, 62, 65). Understanding the true origin of sponge-derived compounds may help obtain the required amount of specific potential drugs to set up clinical trials. However, the actual biosynthetic pathways and true producers of most sponge natural products are uncertain and remain to be investigated. Some natural products seem to have a sponge origin since they are located within sponge cells (74, 77). Other compounds are associated with microbial symbionts, suggesting that microbes are the true producers (2, 29, 30, 32, 76). However, location does not necessarily imply production. Microbes are known to actively excrete their natural products into the surrounding medium (46), and the synthesis of natural products could be accomplished in several of the multiple cell components present in sponges (45).

Sponges of the *Aplysinidae* family are a rich source of microorganisms (35, 72, 80) and also contain high concentrations of brominated alkaloids (BAs) (35, 36, 39). These BAs seem to be located within sponge cells, suggesting a biosynthesis by the sponge (74). However, bromoperoxidase enzymes (responsible for the halogenation reaction that incorporates the bromine into the compound) have only been reported for bacteria, algae, fungi, and plants (3, 81–83, 85). Ebel et al. (27) suggest that bacteria may produce the enzymes necessary to transform some of the secondary metabolites in *Aplysina aerophoba*. The possibility that sponge-associated bacteria, algae, or fungi might be taking part in the biosynthesis of these compounds cannot be ruled out, making it possible that the production of these BAs is a joint effort of multiple cell components in the sponge (45). To date, the majority of studies report the presence of compounds in specific cell compartments or show qualitative associations between secondary metabolites and bacteria (33, 74–76). Quantitative data supporting a relationship between bacterial abundance and concentration of natural products is lacking, let alone experimental evidence for this hypothesis, which would benefit from a better understanding of the links between bacterial strains and concentration of natural products.

In the present study, we assess the relationship between microbial community structure and natural products in the

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sponge *Aplysina aerophoba* (Nardo, 1843). BAs are known to vary within this sponge (45, 74), and we sought to detect whether the bacterial community structure covaried with the concentration of natural products at an intraspecimen scale. To achieve our goal, we used high-performance liquid chromatography (HPLC) and denaturing gradient gel electrophoresis (DGGE) to investigate the relative abundance of major secondary metabolites and the complex bacterial assemblages found in multiple tissues of *Aplysina aerophoba*. The resulting chemical and bacterial matrices were analyzed with a variety of uni- and multivariate statistical methods that can prove useful in the field of microbial ecology.

MATERIALS AND METHODS

Sampling. In spring 2006, the sponge *Aplysina aerophoba* (formerly known as *Verongia aerophoba* [Nardo, 1843]) was collected by scuba diving at four locations along the Costa Brava, northwestern Mediterranean, between 3 and 14 m in depth. We collected several specimens from each location to obtain enough material for the bulk chemical extraction necessary to set up the chemical methods. We also collected one specimen from each location to assess intraspecimen variation in both natural products and the bacterial community.

To investigate intraspecimen variability, we randomly chose two chimney-like structures for each sponge. We cut the chimneys underwater and placed them in plastic bags with seawater. Then, we placed the sealed plastic bags in a cooler with ice to transfer them to the laboratory (2 to 3 h). Each chimney was divided into apical and basal zones, and we took samples from the ectosome and choanosome of the sponge from both zones with a sterilized scalpel under seawater (see Fig. S1 in the supplemental material). Apical and basal zones refer to the first top and bottom centimeter of a chimney-like structure. Typically, a distance of 2 to 3 cm separates the apical and basal zones. Ectosome refers to the 2 to 3 mm outer layer of sponge, with a greener or more purple color due to the presence of cyanobacteria (10, 74), while choanosome refers to the cyanobacterium-free inner layer of the sponge. Sponge samples were always under water to prevent the compound degradation that this sponge experiences in contact with air (as the species name "*aerophoba*" indicates). For each individual sponge, we had a total of eight samples (2 chimneys × 2 zones × 2 tissues) for natural product quantification and bacterial analyses.

Secondary metabolite isolation and identification. Sponge tissues were frozen at −20°C, freeze-dried, and extracted three times (1 h, 1 h, and overnight) with methanol (MeOH; 20 ml of MeOH per 1 g of sponge). The combined extracts were concentrated by vacuum rotary evaporation, leaving a powdery organic residue. To isolate the major compounds observed in preliminary HPLC analyses, the organic extract was first fractionated by flash chromatography using VWR LaFlash equipment on an Analogix Sepra C18 (SF25-55g) cartridge eluted with increasing amount of methanol in water, resulting in four fractions.

Fraction 1 (eluted with 30% MeOH) and fraction 3 (eluted with 100% MeOH), contained the major compounds and were further purified by semipreparative reversed-phase HPLC (Waters 1525 binary HPLC pump and Waters 2487 dual λ absorbance detector) on a Phenomenex Gemini RP-18 (250 by 10 mm, 5 μm) column. The elution conditions consisted of 30% MeOH in water (peaks 1 to 5) and 75% MeOH in water (peak 6) and a flow rate of 2.5 ml min^{-1} with UV detection at 245 nm. These conditions led to purification of the four known compounds aerophobin-1 (5 mg), aerophobin-2 (5 mg), aplysinamisin-1 (7 mg), and isofistularin-3 (12 mg). These four compounds were characterized by proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR; JEOL EX 400 spectrometer), liquid chromatography-mass spectrometry (LC-MS; Thermo Scientific LCQ Fleet), UV spectrometry (Hewlett-Packard diode array spectrophotometer), and comparison of spectroscopy data with published values from the literature.

HPLC analysis and compound quantification. HPLC analyses were performed with a system from Waters, including the Alliance separations module 2695, the column heater, and the 2998 photodiode array detector. The equipment was controlled and the data were handled by the Empower Chromatography Data software (Waters). The HPLC conditions consisted of two eluents (eluent A [0.1% aqueous trifluoroacetic acid] and eluent B [acetonitrile]) and an elution profile based on a linear gradient from 30% eluent B to 80% eluent B within 18 min and then to 100% eluent B for an additional 10 min. Flow rate was kept constant at 0.4 ml min^{-1}. We used a Phenomenex Synergi Max-RP (80 Å, 250 by 3.0 mm, 4 μm) analytical column with a fixed temperature of 30°C.

For the quantification of the natural products, 30 mg of freeze-dried sponge tissue from each ectosomal and choanosomal samples were extracted three times with 1.5 ml of MeOH in an ultrasonic tank for 15 min each time. The crude extract was filtered through a 20-μm-pore-size polytetrafluoriethylene filter (PTFE) and added in a 5-ml beaker. The final volume was adjusted to 5 ml of crude extract solution, and an aliquot of 1.5 ml was passed through a 13-mm, 0.2-μm-pore-size PTFE syringe-filter before HPLC injection. Then, 10 μl of this filtered solution was injected into the HPLC system described above. The brominated compounds were detected at 245 nm from the data collected across the 210- to 800-nm wavelength range. Peaks were integrated by applying the detector response based on peak areas to calibration curves obtained using the previously purified and characterized compounds as external standards. The final amount of natural compounds was calculated by averaging three replicate injections. Concentrations of brominated compounds were expressed as mg per g (dry mass) of sponge tissue.

DNA extraction and PCR amplification. DNA was extracted from ~1 mm^3 of ethanol-preserved (100% final concentration) sponge tissue (2 to 3 mg [wet mass]) using a DNeasy tissue kit (Qiagen), the effectiveness of which has been tested for environmental samples (60), according to the manufacturer's instructions with the following modifications recommended in the troubleshooting guide of the Qiagen kit: (i) lower amount of sample processed (2 to 5 mg), (ii) higher proteinase K digestion time (3 to 5 h, until the tissue looked well digested), and (iii) a final elution step with 40 μl of elution buffer (10 mM Tris-Cl, 0.5 mM EDTA [pH 9.0]) and incubation at room temperature for 10 min. DNA extracts were run in an agarose gel to check integrity and concentration using a standard mass ladder (DNA Smart Ladder; Eurogentec, Belgium). Small differences in yield extraction were visualized among samples. However, such differences were not expected to produce qualitative changes in the DNA mixtures, and the results were normalized using relative abundances in the fingerprinting analysis for an accurate intersample comparison.

PCR amplification of bacterial 16S rRNA gene suitable for subsequent genetic fingerprinting analysis was carried out using the universal bacterial primer combination BAC358F (5′-CCT ACG GGA GGC AGC AG-3′) with a 40-nucleotide GC-rich sequence attached to the 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) and BAC907RM (5′-CCG TCA ATT CMT TTG AGT TT-3′), which amplify a fragment approximately 568 bp long as described elsewhere (20). The cycling conditions were as follows: one initial denaturing step for 5 min at 94°C; 10 touchdown cycles of 1 min at 94°C, 1 min at 70°C (with a 1°C decrease every cycle), and 3 min at 72°C; 20 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C; and one final elongation step for 5 min at 72°C. The PCR mix consisted of 34 μl of sterilized MilliQ water, 5 μl of 10× reaction buffer, 1.5 μl of $MgCl_2$ (50 mM), 1 μl of deoxyribonucleoside triphosphates (10 mM each), 2.5 μl of each primer (10 μM), 2.25 μl of bovine serum albumin (6 mg/ml), 0.25 μl of EcoTaq polymerase (Ecogen; 5 U/μl), and 1 μl of DNA template. The amounts of DNA template ranged between 10 and 100 ng of DNA for the different samples. Within such range we did not observe any remarkably loss/gain of DGGE bands in the fingerprinting analysis. PCR products were run in an agarose gel using a standard mass ladder (DNA Smart Ladder; Eurogentec, Belgium) to quantify the PCR product obtained in each case (20, 25).

DGGE analysis of PCR products and sequencing. DGGE was performed by using a Bio-Rad DCode universal mutation detection system (Bio-Rad) on a 6% polyacrylamide gel in 1× TAE (40 mM Tris base, 20 mM sodium acetate trihydrate, 1 mM EDTA). A comparable amplicon mass for each sample (c. 600 ng of PCR product) was added on the DGGE, and the gels were run for 4 h at a constant voltage of 200 V and 60°C in a 45 to 70% vertical denaturant gradient (100% denaturant agent is 7 M urea and 40% deionized formamide) (20). After electrophoresis, gels were stained for 45 min with SYBR Gold nucleic acid stain (Molecular Probes) and photographed with the UV GelDoc system (Uvitec). Image files were processed with the NIH Image software (National Institutes of Health, Bethesda, MD), and the relative band intensities were measured (25). Although not free of limitations, the signal intensity of the DGGE bands has been shown to be a useful tool for calculating the relative percentages of the different groups (20), and analysis of absence/presence data offered the same conclusions here (see Results). Obviously, absolute quantitative data on the abundance of specific bacteria requires the use of other techniques (see reference 20 and references therein).

Prominent bands were excised from the gel, resuspended in 25 μl of MilliQ water, and stored at 4°C overnight. An aliquot (2 to 5 μl) of the supernatant was used for PCR reamplification with the original primer set, and the PCR product was sequenced by using external sequencing facilities (Macrogen). Sequences were sent to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) to get a first indication of what sequences were retrieved. Sequences with >97% sequence

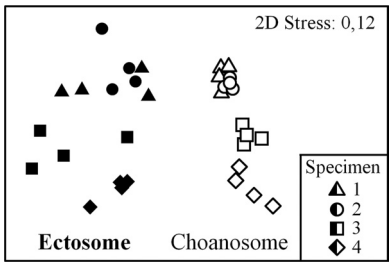

FIG. 1. Nonmetric multidimensional scaling based on Bray-Curtis similarity matrices from standardized and square root-transformed abundances of chemical and bacterial data. Significant differences between tissues and specimens (obtained by PERMANOVA) are shown. See the text for details on PERMANOVA.

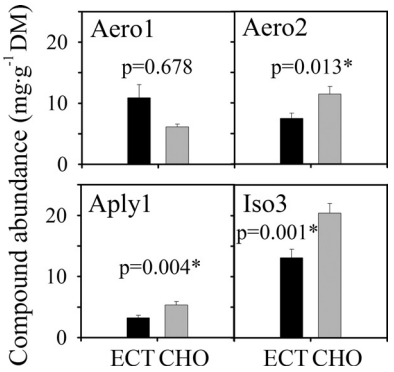

FIG. 2. Secondary metabolite concentrations (mg g [dry mass] of sponge tissue^{-1} ± 1 standard error) of the sponge *A. aerophoba* between ectosome (ECT) and choanosome (CHO) layers. Asterisks (*) indicate significant differences between tissues ($P \leq 0.05$). Aero1, aerophobin-1; Aero2, aerophobin-2; Aply1, aplysinamisin-1; Iso3, isofistularin-3.

identity to a cultured nearest phylogenetic neighbor in GenBank database were named at the species level. A band (operational taxonomic unit [OTU]) was defined as a stained signal whose intensity was >0.2% of the total intensity for each lane.

Data analysis. We used several statistical methods available in PRIMER 6 software (22) to analyze data on secondary metabolites and bacteria of *Aplysina aerophoba* as a function of specimen (four specimens), chimney (two chimneys for each specimen), chimney zone (top and bottom), and tissue type (ectosome and choanosome). Standardized and square root-transformed data were used to calculate Bray-Curtis similarity, and permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in secondary metabolite and bacterial data together across the four factors. We also performed two independent PERMANOVAs on chemical and bacterial data using exclusively factors that proved significant in the previous analysis. In addition, we tested the effect that the DGGE gel might impose in the analysis of the bacterial community with a PERMANOVA with DGGE gel as a factor. Taking into account the PCR bias and the limitations of the DGGE as a quantitative technique (49), we also used presence/absence data to validate the method.

We used ANOVA and the nonparametric Mann-Whitney U test from Systat 12 software (63, 64) to analyze separately each secondary metabolite and each OTU across significant factors. We also performed a Mantel test (based on similarity matrices) to investigate whether there was a relationship between secondary metabolites and bacteria. We constructed two independent matrices from secondary metabolites and bacterial data and calculated a measure of similarity between each point and all of the others. The Mantel test correlates the two $n \times n$ similarity matrices (34) and detects any association between the components of both matrices (secondary metabolites and bacteria in our study). Mantel tests cannot point to any specific subset of data (i.e., any specific secondary metabolites and bacteria) responsible for an overall association, so further analyses are required to identify specific relationships.

Because of the large number of secondary metabolites and bacteria analyzed in the present study, we used factor analysis to look for coherent groups of chemical and bacterial variables that were correlated with one another within groups but largely independent between groups (64). These groups of correlated variables or factors help interpret the underlying mechanisms that have created the relationship between variables. Specifically, we used a principal component analysis extraction with a minimum eigenvalue of 1 to estimate the number of factors. To facilitate interpretation, we used varimax rotation since it minimizes the number of variables that load highly on a factor and maximizes the loading variance across factors. The resulting independent factors were used as variables in a canonical correlation analysis to test whether any chemical and bacterial factors were correlated. We then used simple correlation analysis from Systat 12 software (63, 64) to establish the quantitative relationship behind the actual chemical and bacterial variables in the correlated factors.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences were deposited in GenBank under accession numbers AM905024 to AM905030.

RESULTS

Natural product and bacterial profiles of *A. aerophoba*. We quantified a total of 32 samples (4 individual sponges × 2 chimneys for each sponge × 2 zones for each chimney × 2 tissues for each zone) to characterize the chemical profile. We found six major peaks in the crude extract of *A. aerophoba*. We labeled the peaks according to their retention times: peak 1, peak 2, peak 3, peak 4, peak 5, and peak 6 (see Fig. S2 in the supplemental material). We identified four of the six major peaks: aerophobin-1 (Aero1; peak 3), aerophobin-2 (Aero2; peak 4), aplysinamisin-1 (Aply1; peak 5), and isofistularin-3 (Iso3; peak 6) according to their retention times and their UV profiles in comparison with purified and characterized compounds. We used two DGGE gels to characterize the bacterial profiles of the same 32 samples previously analyzed for the chemical profile. We identified a total of 24 different band positions, and we assigned each position to an OTU (see Fig. S3 in the supplemental material). Of these 24 OTUs, 15 were shared by all specimens except for OTUs 4, 11, and 19, which were restricted to three specimens. The remaining 9 OTUs were uncommon and were restricted to a particular sample or gel.

Using both chemical and bacterial data, we found significant differences between specimens (PERMANOVA, $P = 0.001$) and between ectosome and choanosome tissues (PERMANOVA, $P = 0.018$) (Fig. 1). We found no differences between chimneys or between the chimney top and bottom zones (PERMANOVA, $P = 0.118$ and $P = 0.155$, respectively). We therefore ran subsequent independent PERMANOVAs on chemical and bacterial data using the significant factors "specimens" and "tissue type."

Natural product variation in *A. aerophoba*. Ectosome and choanosome tissues significantly differed in their secondary chemistry (PERMANOVA, $P = 0.002$). Aero2, Aply1, and Iso3 were more abundant in choanosome tissues than in ectosome tissues (ANOVA, $P = 0.013$, $P = 0.004$, and $P = 0.001$, respectively), while Aero1 showed no differences between tissues (Mann-Whitney U test, $P = 0.678$) (Fig. 2). We found nonsignificant differences in the secondary chemistry between specimens (PERMANOVA, $P = 0.371$).

Bacterial community variation in *A. aerophoba*. We found highly significant differences in the bacterial assemblage between ectosome and choanosome tissues (PERMANOVA, $P = 0.001$) but differences between specimens were not significant (PERMANOVA, $P = 0.542$).

We found a gel effect when we compared the bacterial com-

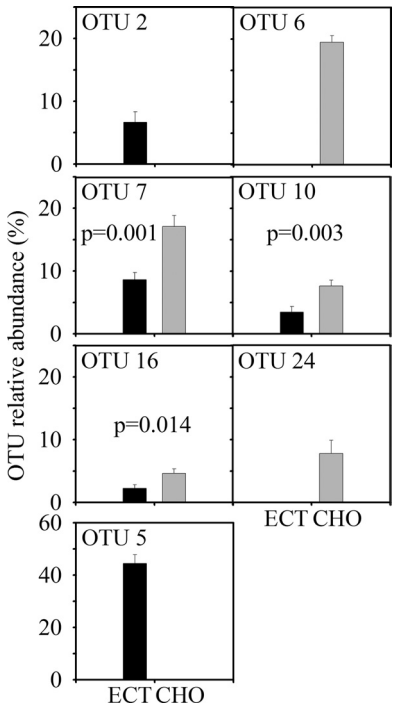

FIG. 3. OTU relative abundances of the sponge *A. aerophoba* between ectosome (ECT) and choanosome (CHO) tissue layers. Only OTUs with significant differences between tissues ($P \leq 0.05$) are shown.

munity between DGGE gels (PERMANOVA, $P = 0.004$), so we analyzed both gels separately. In both gels, we found significant differences between ectosome and choanosome tissues (PERMANOVA, $P = 0.001$ for both gels) and nonsignificant differences between specimens (PERMANOVA; gel 1, $P = 0.695$; gel 2, $P = 0.638$). Analysis of presence/absence data showed the same results, i.e., significant differences between ectosome and choanosome tissues (PERMANOVA; gel 1, $P = 0.018$; gel 2, $P = 0.008$) and no differences between specimens (PERMANOVA; gel 1, $P = 0.437$; gel 2, $P = 0.470$), so the bacterial differences between tissues and the homogeneity between specimens were quantitatively well supported despite the gel effect. Of 24 bacterial types, 7 were responsible for the differences found between the two sponge tissues (Fig. 3). OTUs 2 and 5 were exclusively found in the ectosome, while OTUs 6 and 24 were restricted to the choanosome (Fig. 3). OTUs 7, 10, and 16 were distributed over both tissues but were more abundant in the choanosome of the sponge (Mann-Whitney U tests; $P = 0.001$, $P = 0.003$, and $P = 0.014$, respectively) (Fig. 3).

Relationship between natural products and bacterial community. We detected a significant correlation between the chemical and bacterial profiles regardless of the gel analyzed or their combination (Mantel test; bacterial data from both gels, $R = 0.209$, $P = 0.001$; gel 1, $R = 0.249$, $P = 0.025$; and gel 2, $R = 0.315$, $P = 0.005$).

We used factor analysis to reduce the number of individual compounds and bacterial strains to a few consistent groups of compounds and OTUs that were highly correlated with one another within groups and independent between groups. Factor analysis resulted in two chemical and eight bacterial factors (Tables 1 and 2) that explained 85.31 and 79.99% of the total variance, respectively. Canonical correlation analysis on the factor analysis scores resulted in four significant correlations that included three bacterial factors (BF1, BF2, and BF7) and the two chemical factors (Table 3). Individual correlation analysis of the secondary metabolites and bacterial types included in the correlated factors resulted in 15 uncorrected significant correlations (of 37 possible correlations [Table 4]), which is an extremely unlikely event to be explained by chance (Bernoulli equation, $P = 9.25E^{-11}$). Significant correlations after Bonferroni corrections were drastically reduced and restricted to the positive relationships between OTU19 and the compounds Aero2 and Iso3 ($R = 0.606$, $P = 0.007$, and $R = 0.569$, $P = 0.020$, respectively) and between OTU 7 (*Chloroflexi*; see below) and Aply1 ($R = 0.593$, $P = 0.010$) (Fig. 4; Table 4).

Phylogenetic analysis of excised 16S rDNA-DGGE bands. To identify the potential bacterial populations that were related to secondary metabolites of *Aplysina aerophoba*, we excised from the gels and successfully sequenced 24 16S ribo-

TABLE 1. Bacterial factors obtained from the factor analysis of the bacterial data[a]

OTU	Loading value obtained with bacterial factor:							
	BF1	BF2	BF3	BF4	BF5	BF6	BF7	BF8
OTU5	–0.86							
OTU6	0.84							
OTU7	0.81							
OTU2	–0.74							
OTU18		–0.86						
OTU19		–0.84						
OTU24		–0.83						
OTU12		–0.69						
OTU9		0.64						
OTU20		–0.62						
OTU22			–0.86					
OTU11				0.82				
OTU4				0.75				
OTU16				0.67				
OTU13					0.82			
OTU23						–0.81		
OTU15						–0.79		
OTU8							–0.93	
OTU3								–0.80

[a] To facilitate interpretation, we exclusively show OTU loadings with absolute values greater than 0.60. The closer the absolute loading values to 1.0, the stronger the association between the variables (OTUs or compounds) and the factors. The sign of the values represents the positive or negative nature of their association. BF, bacterial factor.

TABLE 2. Chemical factors obtained from the factor analysis of the chemical data[a]

Compound	Loading value obtained with chemical factor:	
	CF1	CF2
Iso3	0.97	
Aero2	0.94	
Aply1	0.77	
Aero1		0.99

[a] The compounds aerophobin-1 (Aero1), aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), and isofistularin-3 (Iso3) were evaluated. CF, chemical factor.

TABLE 3. Canonical correlation analysis between chemical and bacterial factors

Bacterial factor	*R* (*P*)[a]	
	CF1	CF2
BF1	0.516 (0.002)	NS
BF2	–0.332 (0.032)	0.359 (0.036)
BF3	NS	NS
BF4	NS	NS
BF5	NS	NS
BF6	NS	NS
BF7	NS	–0.440 (0.012)
BF8	NS	NS

[a] The correlation coefficient (*R*) and *P* value of significant correlations ($P \leq 0.05$) are given. NS, nonsignificant correlations. BF, bacterial factor; CF, chemical factor.

somal DNA (rDNA)-DGGE bands. These bands belonged to seven different OTUs (see Fig. S3 in the supplemental material), five of which were very abundant (>44% of the total bacterial abundance) and were shared by both gels. Comparison of the 16S rRNA gene sequences with GenBank database showed a large range of bacterial taxa present in *Aplysina*. The bacterial community included representatives of the *Chloroflexi* (OTU6, accession no. AM905025; OTU7, accession no. AM905026; and OTU9, accession no. AM905027), *Cyanobacteria* (OTU5, accession no. AM905024), and *Bacteroidetes* (OTU2, accession no. AM905030), as well as members of the *Gammaproteobacteria* (OTU24, accession no. AM905029) and *Actinobacteria* (OTU8, accession no. AM905028) (Table 5). Six sequences were previously reported from marine sponges (OTU5 from *Aplysina aerophoba*, OTU6 from *Spongia agaricina*, OTU7 from *Xestospongia testudinaria*, OTU8 and OTU9 from *Ancorina alata*, and OTU 24 from *Ircinia felix*) and closely related to those reported in another *Aplysina* species (Table 5). The remaining sequence was closer to sequences from plankton (OTU2 from seawater).

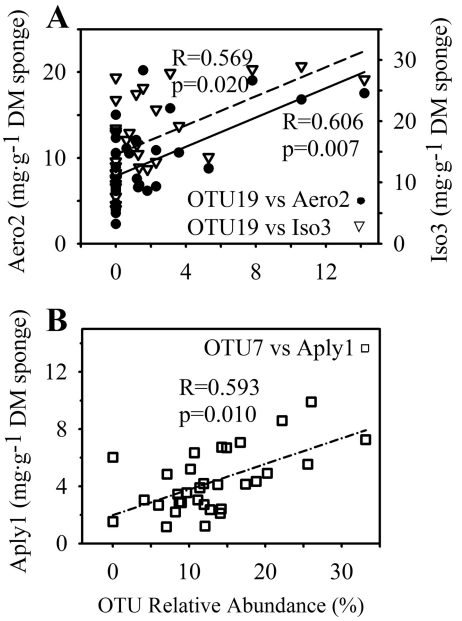

FIG. 4. Relationship between (A) the concentrations of aerophobin-2 (Aero2) and isofistularin-3 (Iso3) and the relative abundance of the unidentified OTU19 and (B) the concentration of aplysinamisin-1 (Aply1) and the relative abundance of a *Chloroflexi* clade (OTU7). Concentrations of compounds in mg g (dry mass) of sponge tissue^{-1} ± 1 standard error. *P* values are significant after Bonferroni corrections (see the text for details).

DISCUSSION

Over the last decades, many sponges have been investigated in an attempt to independently describe both the large diversity of natural products (14–17, 46) and potential symbiotic microorganisms (37, 40, 65). Sponge microbial ecology is receiving intense and increasing attention (4), and one area of particular interest is to assign the true origin of these natural products, whether of sponge or microbial origin (33, 46–48,

TABLE 4. Correlation analyses of the specific brominated alkaloids and bacterial types (OTU) included in the correlated chemical and bacterial factors

Bacterial factor	OTU	Bacterial group	*R* (*P*)[a]			
			CF1			CF2 (Aero1)
			Aero2	Iso3	Aply1	
BF1	2	*Bacteroidetes*	NS	NS	NS	–
	5	*Cyanobacteria*	–0.432 (0.013)	–0.519 (0.002)	–0.507 (0.003)	–
	6	*Chloroflexi*	0.386 (0.029)	0.499 (0.004)	0.511 (0.003)	–
	7	*Chloroflexi*	NS	NS	0.593 (<0.001)*	–
BF2	9	*Chloroflexi*	NS	NS	NS	NS
	12	UI[b]	NS	NS	NS	NS
	18	UI	NS	0.378 (0.033)	NS	–0.365 (0.040)
	19	UI	0.606 (<0.001)*	0.569 (0.001)*	NS	NS
	20	UI	0.374 (0.035)	NS	NS	NS
	24	*Gammaproteobacteria*	0.363 (0.041)	0.386 (0.029)	NS	NS
BF7	8	*Actinobacteria*	–	–	–	0.442 (0.011)

[a] The correlation coefficients (*R*) and uncorrected *P* values of all significant correlations are shown. Asterisks indicate significant correlations after Bonferroni correction. NS, nonsignificant correlations; –, correlations not tested; BF, bacterial factor; CF, chemical factor.
[b] UI, unidentified OTU.

TABLE 5. 16S rDNA sequence identities of representative bands excised from DGGE gels[a]

DGGE band	OTU	Division	Closest relative in database (isolation source; AN)	% Identity	Closest *Aplysina* host species (% identity; AN)
19	2	*Bacteroidetes*	Uncultured *Flavobacterium* (seawater; FN433428)	92.9	Not found
1	5	*Cyanobacteria*	*Synechococcus spongiarum* (*Aplysina aerophoba*; EF656449)	99.6	*Aplysina aerophoba* (99.6; EF656449)
6	6	*Chloroflexi*	Uncultured clone DGGE band bd29 (*Spongia agaricina*; AM849595)	100	*Aplysina fulva* (98.7; GU982064)
7	7	*Chloroflexi*	*Chloroflexus* sp. (*Xestospongia testudinaria*; FJ481354)	99.8	*Aplysina fulva* (99.6; GU982078)
13	8	*Actinobacteria*	Uncultured clone AncD18 (*Ancorina alata*; FJ900572)	98.1	*Aplysina fulva* (97.3; FM160879)
12	9	*Chloroflexi*	Uncultured clone AncB18 (*Ancorina alata*; FJ900579)	97.2	*Aplysina fulva* (96.8; GU982098)
18	24	*Gammaproteobacteria*	Uncultured clone DGGE band IF6-3 (*Ircinia felix*; DQ661847)	97.4	*Aplysina fulva* (96.4; FM160910)

[a] AN, accession number.

74–77). The question is not trivial because it has potential applications in biotechnology and pharmacognosy, but it remains to be rigorously addressed. The traditional approach consists in locating secondary metabolites in cell components using a variety of methods including X-ray microanalysis, density gradients of dissociated cells, and others (48, 74, 77). Although these methods can locate secondary metabolites (or a characteristic element of the metabolite, e.g., a bromine atom), they reveal only limited information on the true production of these compounds. This is particularly true if several parts of the compound are assembled in multiple cell compartments. This possibility cannot be ruled out and would provide additional support for the true collaboration between the sponge and its bacterial assemblage. In the present study we explored the association between bacteria and secondary chemistry by investigating relationships between the concentration of secondary metabolites and significant changes in the relative abundance of bacterial populations. Our study provides one of the first reports of a significant correlation between specific secondary metabolites and bacterial types (32). Although we cannot infer the true functional nature of these associations, they provide more explicit and simplified paths to explore the links between secondary metabolites and sponge-associated bacteria in *A. aerophoba*.

The characterization of chemical and bacterial profiles in *A. aerophoba* has already been reported by numerous authors (21, 28, 38, 39, 71, 89). The relative composition of BAs present in *A. aerophoba* seems to be highly conserved in both spatial and temporal scales. *Aplysina* individuals sampled thousands of kilometers apart showed indistinguishable secondary metabolite profiles (28). At a small spatial scale, bromoisoxazoline patterns of two *Aplysina* species proved to be remarkably stable and unaffected either by changing the light conditions or depth (54). Similarly, the alkaloid content remained unchanged after cultivation of *Aplysina* specimens in starvation conditions and antibiotics exposure in aquaria (35). Nevertheless, other studies with emphasis on natural products quantification show considerable variability in the concentration of secondary metabolites within *Aplysina* species (55) and between specimens of the same *Aplysina* species (62). The relative composition of the main brominated compounds under *ex situ* and *in situ* cultivation of *A. aerophoba* remained stable, although the total alkaloid content increased twice as much *ex situ* than *in situ* (43). There is a similar concern about the variation of bacterial assemblages in sponges. The microbial community seems to be uniform over time in *A. aerophoba* (35) and in other sponge species (41, 66).

Our results show evidence for a significant variation in the concentration of secondary metabolites in *A. aerophoba* both within and between specimens, which is consistent with the pronounced variability of secondary metabolite concentrations found not only in *Aplysina* spp. but also in other sponge species (9, 13, 52, 58) and other sessile organisms such as bryozoans (53). Our results also show evidence for changes in the relative abundances of bacterial populations in *A. aerophoba*. Signal intensity of DGGE bands is a useful tool to estimate the relative abundance of predominant bacterial groups (i.e., those >0.1 to 1% of total abundance) and to have a rough picture of microbial changes but does not provide detailed quantitative estimations (20). We were cautious in the number of PCR cycles run to avoid the "plateau" phase and in using the same amount of template in each reaction. The samples that we compared were run under the same PCR and DGGE conditions, and we carefully estimate the error among replicates. If there was any PCR bias it should be the same in all lanes, and therefore comparison among samples is still valid. The use of normalized relative abundances in the analyses also allowed for accurate intersample comparison.

Except for aerophobin-1, we found higher concentrations of every compound in the choanosome of the sponge, making a picture of a chemically rich sponge nucleus surrounded by a not-so-rich tissue layer. Traditionally, significant differences in secondary metabolites between sponge tissues are usually interpreted as evidence of their roles against predation, competition, or both (8, 9, 11, 58). The ectosome of *A. aerophoba* is packed with cyanobacteria (10, 79, 80; the present study) and is the preferred tissue preyed upon by the opisthobranch nudibranch *Tylodina perversa* (10). Thoms et al. (73) failed to detect aerophobin-1 in *A. aerophoba* but reported aerophobin-2 as one of the major constituents and the major deterrent compound against generalist predators together with isofistularin-3. Although the deterrence of aerophobin-1 is not known, chemical differences from aerophobin-2 are so minor that aerophobin-1 would be expected to be similarly deterrent against generalist predators too. If so, the high concentration of aerophobin-1 in the outer tissues of the sponge in the present study could serve as a defensive barrier against generalist predators, which have never been observed feeding on *A.*

aerophoba (unpublished data). High chemical concentrations in the outer tissues could also be a defense against foulers (6, 11). The higher concentration of isofistularin-3 (and other compounds) in the choanosome could be acting as a final deterrent against the specialist predator *T. perversa*, which is similar to what has been described for other opisthobranch-sponge feeding interactions (5, 7). Other roles, such as antibacterial activities, are far less investigated but could also explain the observed variation in secondary chemistry (6). The natural products found in *A. aerophoba* can be rapidly converted into aeroplysinin-1 and dienone, which show stronger antibiotic activity than their precursors and may protect this sponge from invasion by bacterial pathogens (27, 68, 73).

The predominant bacterial community in *A. aerophoba* seemed to be fairly constant between and within individuals, although some bacteria significantly varied their relative abundance between the ectosome and choanosome tissues of the sponge. These observations are in agreement with those previously reported by Thiel et al. (69), who found a distinct bacterial community between the outer (ectosome) and the inner (choanosome) tissues of the sponge *Tethya aurantium*. To date, the most noticeable microbial difference between ectosome and choanosome tissues of *A. aerophoba* is the presence of photosynthetic cyanobacteria in the outer tissues (10). Our study provides evidence for a specific distribution of *Cyanobacteria* and *Bacteroidetes* in the ectosome of the sponge. Some *Chloroflexi* and *Gammaproteobacteria* were restricted to the choanosome, while other bacterial bands had dissimilar distributions between tissues. The differences between the ectosome and the choanosome seem to be greater than previously reported. Apart from these bacterial groups, the microbial assemblage included representatives of other *Chloroflexi* and *Actinobacteria*. Overall, the sponge-associated bacterial community investigated in our study is as highly diverse and phylogenetically complex as described by other authors (36, 38, 40, 87, 88).

Our study also found significant differences in secondary chemistry between specimens of *Aplysina aerophoba* that were collected at a regional scale (<100 km). Thoms et al. (71) also found significant differences in the concentrations of secondary metabolites between specimens of *A. aerophoba* collected in Croatia and France, supporting the idea that chemical variation seems to be common within species of the genus *Aplysina* (55). Similarly, several bacteria differed between *A. aerophoba*, *Geodia barretti*, and *Cymbastela concentrica* (35, 41, 66). However, bacterial communities can be fairly constant between sponge species both within and between geographic regions (38, 87). Further research is necessary to understand these contrasting differences in bacterial variability within and between sponges.

Beyond the actual chemical or bacterial variation reported in this or previous studies, our study is the first attempt to correlate changes in both secondary chemistry and bacterial assemblage. A total of 15 of the 37 correlations evaluated resulted in significant positive and negative correlations between bacterial populations and natural products. This high number of significant correlations is extremely unlikely to occur by chance alone and suggests strong interactions between bacteria and between bacteria and natural products. Some *Chloroflexi* strains were positively related to the concentration of aplysinamisin-1, while a cyanobacterial strain was negatively related to the same compound. These *Chloroflexi* and *Cyanobacteria* strains are found in the choanosome and ectosome of the sponge, respectively, and were clearly negatively associated with each other. Other bacterial types, such as the *Gammaproteobacteria* strain and the unidentified OTU19, seemed to be positively related to the abundance of aerophobin-2 and isofistularin-3 but not to aplysinamisin-1. An *Actinobacteria* strain was associated with the concentration of aerophobin-1. Our results show how phylogenetically distinct bacterial groups are related to multiple natural products, which is also supported by a genomic study in *A. aerophoba* that assigned secondary metabolite gene clusters to a *Chloroflexi* clade and to the candidate phylum *Poribacteria* (59).

These correlations could be driven by multiple factors. Larger compound concentrations could lead to larger abundance of particular bacteria because of specific habitat conditions or preferences. For example, the abundance of brominated compounds in the sponge tissue would facilitate bacteria with the ability to metabolize these compounds (84). Ahn et al. (1) demonstrated that anaerobic bacteria harbored within the *A. aerophoba* carried out an anaerobic reductive dehalogenation of brominated aromatic compounds, suggesting that natural products would determine the presence or absence of these bacteria. Other hypotheses that could produce the same type of correlations between secondary chemistry and bacteria could include reduced competitive interactions with other bacterial species or preferential abiotic conditions. Under these hypotheses, the increasing abundance of bacteria could be either a spurious correlation or a consequence of the higher concentration of the compound. We cannot rule out that low abundant bacteria can produce metabolites that accumulate over time, although this would imply a low turnover of these compounds and the spherulous cells where they concentrate (74). However, it is well known that spherulous cells degenerate and are frequently released into the environment (11, 12, 70, 74), suggesting rapid cell turnover (24).

Alternatively, bacteria might be involved in the production of the natural product leading to higher concentration of compounds with increasing bacterial abundance. Various secondary metabolites of *A. aerophoba* are found in multiple sponge compartments, raising the possibility that distinct sponge compartments play a role at specific steps of the biosynthetic pathway (45). Interestingly, the haloperoxidase or halogenase enzymes responsible for halogenation processes have only been reported for bacteria, algae, and fungi (3, 81–84), which suggests that the bacterial endobionts of *A. aerophoba* could be producing these enzymes (24). Thus, *Aplysina* sponge cells may produce the inactive precursors of secondary metabolites, while bacteria may provide the enzymes necessary to activate them. Alternatively, the halogenation reaction could be attributed to either fungi or microalgae, which can also be associated with sponges.

Our phylogenetic analysis reveals that multiple bacterial strains were involved in these relationships. Populations of *Chloroflexi*, *Actinobacteria*, and *Cyanobacteria* are associated with the production of bioactive compounds (42, 50). The phylum *Chloroflexi* is an early branching lineage of bacteria about which little is known, especially about non-free-living microbes (18, 50). We now know that prominent enzymes

involved in the biosynthesis of bioactive secondary metabolites are assigned to *Chloroflexi* (nonribosomal peptide synthetase) and to *Poribacteria* clades (polyketide synthases) in *A. aerophoba* (59). Mat-forming *Cyanobacteria* are a rich source of natural products (19, 44, 47), and symbiotic cyanobacteria seem to be involved in the production of halogenated compounds in the sponge *Dysidea herbacea* (33, 75, 76). *Gammaproteobacteria*, *Deltaproteobacteria*, and *Actinobacteria* carry out halogenation or dehalogenation reactions (81, 82, 84), and the former phylum is the most likely producer of bryostatins in the bryozoan *Bugula neritina* (23). In our specimens, we found many of these bacterial types and they were correlated with some of the brominated compounds investigated in our study. Taken together, our data and those from the literature seem to support that sponge-associated bacteria are related to natural product synthesis.

Unraveling the true origin of natural products is challenging. The current body of evidence is too limited to make broad generalizations, but it suggests complex chemical and biological interactions that are far from being resolved. The diversity of secondary metabolites in any particular sponge species is usually large and so is the diversity of their associated bacterial communities. These multiple levels of complexity result in a demanding field, where solutions must include tools from multiple areas and disciplines. Further experimentation and new molecular techniques are needed to advance in this research area (57, 59). Our study presented evidence for a positive correlation between natural products and certain bacterial strains, which provides a simplified version of the sponge-microbe interactions and could be used as a starting point for hypothesis testing. Experimentally modifying the concentrations of secondary metabolites or the abundance of endobiotic bacteria would be critical to elucide their true association and to infer functional relationships, and such research is under way in our laboratory. A further step would be to use quantitative reverse transcriptase PCR (26) to study the expression of genes involved in the production of secondary metabolites and the enzymes responsible for the biotransformation of natural products. Applying modern chemical, molecular, and ecological techniques will substantially improve our understanding of the organization and functioning of these truly complex host-endobiont ecosystems.

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