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ORIGINAL ARTICLE

Phylogenetic Diversity and Spatial Distribution of the Microbial Community Associated with the Caribbean Deep-water Sponge *Polymastia* cf. *corticata* by 16S rRNA, *aprA*, and *amoA* Gene Analysis

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Abstract Denaturing gradient gel electrophoresis (DGGE)-based analyses of 16S rRNA, *aprA*, and *amoA* genes demonstrated that a phylogenetically diverse and complex microbial community was associated with the Caribbean deep-water sponge *Polymastia* cf. *corticata* Ridley and Dendy, 1887. From the 38 archaeal and bacterial 16S rRNA phylotypes identified, 53% branched into the sponge-specific, monophyletic sequence clusters determined by previous studies (considering predominantly shallow-water sponge species), whereas 26% appeared to be *P.* cf. *corticata* specifically associated microorganisms ("specialists"); 21% of the phylotypes were confirmed to represent seawater- and sediment-derived proteobacterial species ("contaminants") acquired by filtration processes from the host environment. Consistently, the *aprA* and *amoA* gene-based analyses indicated the presence of environmentally derived sulfur- and ammonia-oxidizers besides putative sponge-specific sulfur-oxidizing *Gammaproteobacteria* and *Alphaproteobacteria* and a sulfate-reducing archaeon. A sponge-specific, endosymbiotic sulfur cycle as described for marine oligochaetes is proposed to be also present in *P.* cf. *corticata*. Overall, the results of this work support the recent studies that demonstrated the sponge species specificity of the associated microbial community while the biogeography of the host collection site has only a minor influence on the composition. In *P.* cf. *corticata*, the specificity of the sponge–microbe associations is even extended to the spatial distribution of the microorganisms within the sponge body; distinct bacterial populations were associated with the different tissue sections, papillae, outer and inner cortex, and choanosome. The local distribution of a phylotype within *P.* cf. *corticata* correlated with its (1) phylogenetic affiliation, (2) classification as sponge-specific or nonspecifically associated microorganism, and (3) potential ecological role in the host sponge.

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Introduction

Sponges constitute the phylum *Porifera*, which is taxonomically divided into the three classes: *Calcarea*, *Hexactinellida*, and *Demospongiae* with the latter encompassing approximately 85% of the recognized species [23]. They are benthic, sessile filter feeders that depend on (dissolved) organic particle uptake from a continuous stream of water passing through their aquiferous canal system of channels and chambers. Particles including bacteria and single-celled eukaryotes can be captured by epithelial pinacocytes (forming the ectosome) at the sponge surface but are predominantly trapped from the filtered water within the choanocyte chambers-containing region of the sponge, the choanosome (or endosome). The food particles are transferred via transcytosis to the sponge cells of the interior mesohyl matrix. In some sponge species, a specialized area of the mesohyl that lacks choanocyte chambers but contains mineral deposits or collagen fibrils is located immediately below the exopinacoderm, the cortex. As part of the ectosome, the cortex has

been presumed to act as a special supportive device for the openings of the canal system and as a protective layer of the endosome against superficial injury or strong currents [1, 56]. The genus *Polymastia* Bowerbank, 1864 (*Demospongiae*, *Tetractinomorpha*, *Hadromerida*, *Polymastiidae*) with approximately 50 described species is characterized by their encrusting, spherical- or cushion-shaped sponges always with pore-bearing, inhalant and exhalant papillae and a cortex of the ectosomal skeleton that is composed of at least two layers [4, 43]. The species *Polymastia* cf. *corticata* Ridley and Dendy, 1887, is distinctive by its dense and leathery cortex (2 mm thickness; two distinct layers; intermediary layer collagenous), 100 inhalant and 4 to 5 exhalant papillae of approximately 8 mm height, and choanosomal bundles of spicules that stop below the cortex (the latter can be easily detached from the endosome). The species has been collected off the coast of Brazil and east of the Azores in a bathymetric range from 200 to 1,385 m [4, 43].

Early microscopic and culture-based examinations demonstrated that large numbers of microbes populate the mesohyl matrix of many demosponges (termed "bacteriosponges") forming up to 40% of the sponge volume with densities of 10^8 to 10^{10} bacteria per gram of sponge wet weight [64, 72]. The hypothesis of a widespread, sponge-specific microbial community that is distinctly different from the marine bacterioplankton that was postulated by Hentschel *et al.* [15, 16] and has been supported by others (for recent reviews, see [17, 19, 59, 67]). In contrast, the results of several recent diversity surveys contradict the existence of a general uniform sponge-associated microbial community regardless of sponge species and location. Instead, they provided evidence that the composition of the sponge-inhabiting microbial consortium depends on the host species [18, 22, 33, 60–63, 68]. A correlation between the presence of certain major bacterial taxonomic groups in the sponge microbiota and the geographical location of the host sponge was proposed by Hill *et al.* [18]. However, the presence of distinct microbial consortia in different sponge species sharing the same habitat indicated a low impact of the surrounding marine plankton for the sponge–bacteria associations [60, 62, 63, 68]. As recently demonstrated by Thiel *et al.* [63], the specificity of sponge–microbe associations extends to the spatial distribution of microbial populations within the sponge body: Distinct bacterial communities were found to inhabit the endosome and cortex of marine sponge *Tethya aurantium*; specifically associated phylotypes (e.g., cortex-associated members of the *Bacteroidetes* and *Alphaproteobacteria*) were identified for both regions.

The current knowledge about sponge–microbe associations is restricted to investigations of shallow-water species with the only exception of the bacterial 16S rRNA gene-based analysis published by Olson and McCarthy [48]. The aim of the present study was to examine the phylogenetic composition and spatial distribution of the archaeal and bacterial community associated with a deep-water sponge *P.* cf. *corticata* by DGGE-based 16S rRNA and functional gene analyses. The analyses of functional genes that encode key enzymes of the dissimilatory sulfate reduction, sulfur oxidation, and ammonia oxidation pathway, e.g., *aprA* and *amoA* (coding for the alpha subunits of the dissimilatory APS reductase, AprA, and ammonia monooxygenase, AmoA), allow diversity surveys of certain physiological groups, e.g., sulfate-reducing, sulfur-oxidizing, and ammonia-oxidizing prokaryotes. The polyphyly of these physiological groups (see [5, 11, 13, 24, 28, 29, 50, 73] and references therein) restricts the concomitant detection of all recognized members by the use of single 16S rRNA gene-targeting probes or primer pairs and limits the identification of novel lineages in environmental analyses. In addition, the analysis of 16S rRNA genes cannot provide an unambiguous link between the genetic identity of an uncultured microorganism and its physiological or metabolic capacity. Analyses of functional genes like *aprA* and *amoA* circumvent these limitations and (although complicated by lateral gene transfer events of *aprA* [39, 40]) have been successfully applied for biodiversity studies [2, 14, 31, 41, 46, 47]. They were used to determine the phylogenetic complexity of the *P.* cf. *corticata*-associated microbial communities putatively involved in sulfur and nitrogen cycling within the sponge.

Materials and Methods

Sampling

The deep-water sponge was collected by a chain bag dredge from a depth of 1,127 m at the Kahouanne Basin (Lesser Antilles, Caribbean Sea) (16°28.80′ N, 61°58.66′ W) in January 2001 (RV Sonne cruise SO-154). Ambient seawater (temperature 5°C) was sampled from the surface of undisturbed sediment cores with a sterile syringe; all samples were immediately frozen and stored at −20°C until further molecular investigation. In the laboratory, the sponge was washed carefully three times in autoclaved artificial seawater before cutting. Small subsamples (2–3 cm^3 tissue cubes) were fixed in 4% formaldehyde, placed in 70% ethanol, and sent to the Senckenberg Museum, Frankfurt am Main, Germany, for sponge identification. Examination of its general morphological features including spicules geometry of the tissue sample (deposited under the registration number SMF 9633) classified this deep-water specimen as *P.* cf. *corticata* Ridley and Dendy, 1887, a member of the *Polymastiidae* (*Demospongiae*, *Hadromerida*) (D. Janussen, pers. comm.). For molecular analy-

sis, two sponge tissue subsamples were separated into papillae, outer cortex (including ectopinacoderm), inner cortex, and endosome (choanosome) sections (Fig. 1) with a sterile scalpel. The tissue sections were gently rinsed in autoclaved artificial seawater to remove loosely attached bacteria from the surfaces and cut into small pieces before DNA extraction. Two seawater samples (each 200 ml) were filtered through a Sterivex filter (0.2 μM pore size, Millipore).

Genomic DNA Extraction

Total genomic DNA were extracted from the four different sponge tissue sections in duplicate and purified using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for Gram-positive bacteria and animal tissue. Genomic DNA from both filtered seawater samples was extracted after five cycles of thawing at 30°C and freezing in liquid nitrogen using the protocol of Zhou *et al.* [74]. The DNA concentrations were estimated spectrophotometrically, whereas its integrity was examined visually by gel electrophoresis on 0.8% (*w*/*v*) agarose gels run in 1x Tris–borate–EDTA (TBE) buffer followed by ethidium bromide staining (0.5 mg l^{-1}). The extracted DNA (dissolved in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5) was stored at −20°C until further analysis.

PCR Amplification of Partial 28S rRNA Gene

Besides its morphological identification, 28S rRNA gene fragments (C1, D1, C2, and D2 domains at the 5′-end) were amplified from the sponge-derived genomic DNA using the primers and polymerase chain reaction (PCR) protocol of Chombard *et al.* [6] to confirm the former morphological classification by molecular systematics (for PCR primer sequences and detailed thermocycling conditions see Table S1 of the Electronic Supplementary Material).

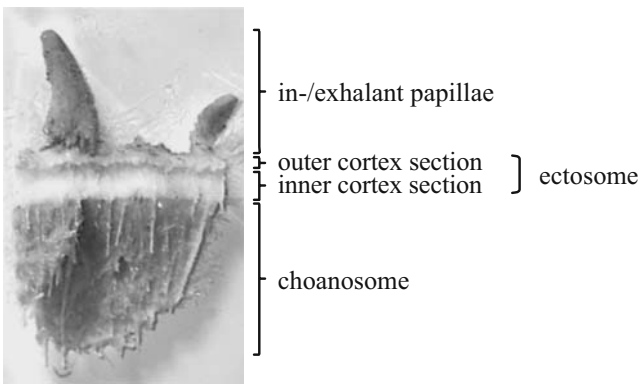

Figure 1 Photograph of a cross-section of *Polymastia* cf. *corticata* showing the examined morphologically different tissue regions, (1) papillae, (2) outer section of the cortex including the exopinacoderm and (3) inner (collagenous) section of the cortex (comprising the ectosome), and (4) the choanosome (endosome)

PCR Amplification of Partial 16S rRNA, *aprA*, and *amoA* genes for Subsequent Double Gradient Denaturing Gradient Gel Electrophoresis Analysis

In general, PCR mixtures (50 μl total volume) contained 1x RED*Taq* PCR reaction buffer (Sigma-Aldrich, St. Louis, Missouri, USA), 0.3 mg ml^{-1} bovine serum albumin (BSA), 200 μM deoxynucleoside triphosphates (dNTPs) mixture, 1 μM of each primer, 0.05 U μl^{-1} RED*Taq* DNA Polymerase, and 100 ng genomic DNA as template (negative controls with water). Partial 16S rRNA gene amplification for subsequent double gradient denaturing gradient gel electrophoresis (DG-DGGE) analysis was performed using the primer sets (1) GM5F-GC clamp (341F) and 907R for *Bacteria* [45], (2) Arch516F-GC clamp (K. Knittel, unpublished) and Arch958R [7] for *Archaea*, and (3) CTO189f-GC clamp and CTO654r specific for betaproteobacterial ammonia-oxidizers [30]. An approximately 0.4-kb *aprA* gene fragment was amplified using the primer set AprA-1-FW and AprA-5-RV with GC clamp [41]. An approximately 0.5-kb *amoA* gene fragment was yielded applying the primer pair AmoA-1F and AmoA-2R-TC [46] (for details see Table S1 of the Electronic Supplementary Material). Duplicate amplifications were performed from each sponge tissue section and seawater DNA sample. The PCR products were visually analyzed by electrophoresis of aliquots (10% of the reaction volume) on 2% agarose gels (*w*/*v*) run in 1x TBE buffer stained with ethidium bromide (0.5 mg l^{-1}) to verify correct amplicon size. If necessary, amplicons of the expected gene fragment size were purified before further analysis using either the QIAquick gel extraction kit (Qiagen, Hilden, Germany) or the Perfectprep gel cleanup sample kit (Eppendorf, Hamburg, Germany) following the supplier's recommendations.

DG-DGGE Analysis

The DG-DGGE analyses of the aforementioned duplicate 16S rRNA, *aprA*, and *amoA* amplicons from each sponge tissue type and seawater DNA sample were performed using the D-GENE™ and D-CODE™ system (Bio-Rad, Munich, Germany). DG-DGGE gels (1.0 mm thick) were poured with an polyacrylamide gradient from 6% to 8% of acrylamide/bis-acrylamide stock solution, 37.5:1 (*v*/*v*) (Bio-Rad) superimposed over a colinear denaturant gradient [100% denaturant corresponds to 7 M urea and 40% (*v*/*v*) formamide, deionized with AG501-X8 mixed bed resin (Bio-Rad)], which varied for the different genes analyzed in this study (see Table S2 of the Electronic Supplementary Material). Gradients were formed using a Bio-Rad Gradient

Former Model 385. Twenty microliters of the PCR samples were mixed with 6 µl of dye solution [0.1% bromphenol blue (*w*/*v*), 70% glycerol (*v*/*v*)] and applied to the gels. Triplicate gel runs were carried out as reported elsewhere (for references see Table S2 of the Electronic Supplementary Material) followed by ethidium bromide staining (0.5 mg l^{-1}) for 15 min and subsequent destaining in double-distilled water for 10 min. The DNA bands were visualized on a UV transillumination table (Biometra, Göttingen, Germany); persisting and dominant bands were excised from multiple lanes of the polyacrylamide gels with ethyl alcohol-sterilized scalpel, incubated in 50 µl Tris–HCl, pH 8.0, overnight at 4°C, and reamplified using 1 µl of the eluate as template and PCR conditions as described above. The purity and migration behavior of the reamplification products of the bands were checked by DG-DGGE. The reamplification products were purified from free PCR primers using either the QIAquick gel extraction kit (Qiagen) or the Perfectprep gel cleanup sample kit (Eppendorf) following the supplier's recommendations.

Nucleotide Sequencing

All reamplification products of the DG-DGGE bands were sequenced directly in both directions using the respective amplification primers and the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. Sequencing reactions were run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis

The partial 16S rRNA sequences obtained from DGGE analysis were checked for chimeras with the program CHECK_CHIMERA of the Ribosomal Database Project, added to the 16S rRNA sequence database of the Technical University Munich (Germany) using the automatic alignment function ARB_Align implemented in the ARB software program package (http://www.arb-home.de), and manually corrected. The sequences were maintained separately according to their source (sponge tissue type, seawater); each set of sequences was grouped into phylotypes, i.e., operational taxonomic units (OTUs), based on a >99% identity cutoff. Only one sequence per OTU and tissue type/seawater was used for further analysis. The closest phylogenetic relatives of each phylotype were identified by comparison to the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) analysis tools (www.ncbi.nlm.nih.gov/BLAST/). For phylogenetic analysis, the online version of PhyML (http://atgc.lirmm.fr/phyml) was used. Maximum-likelihood trees were calculated based on the near full-length 16S rRNA reference sequences received from the database; their robustness was tested by bootstrap analysis with 100 resamplings. Subsequently, these trees were imported into ARB and the DGGE analysis-derived short sequences were individually added by using the QUICK_ADD parsimony tool of ARB without allowing changes in the overall tree topology. The phylogenetic positions of the short sequences were additionally verified by bootstrap analysis with 100 resamplings (maximum-likelihood method). To determine the specificity of the microbial associations, the detected phylotypes from *P.* cf. *corticata* were classified as (1) "specialists", (2) "sponge associates", or (3) "generalists" depending on their presence in (1) this host species only, (2) several sponge species but absent from seawater, or (3) several sponge species and seawater in accordance to Taylor *et al.* [60]; the enumeration of the sponge-associated bacterial (SAB) and archaeal (SAA) phylogenetic clusters is based on the previous studies of Hentschel *et al.* [16], Thiel *et al.* [63], Holmes and Blanch [22], and Lee *et al.* [32].

The *aprA* and *amoA* nucleotide sequence data obtained from DGGE analysis were assembled and manually corrected using the Bioedit (version 7.0.5) sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were maintained separately according to their source (sponge tissue type, seawater); each set of sequences was grouped into phylotypes based on a >99% identity cutoff. Only one sequence per OTU and tissue type/seawater was used for further analysis. BLAST searches in the public databases for homologous sequences of the partial AmoA sequences were performed. The partial and complete AmoA sequences were automatically aligned using the Web server Tcoffee@igs (http://igs-server.cnrs-mrs.fr/Tcoffee/); the initial alignment was refined manually. The partial AprA sequences were integrated into the persisting Apr alignment of sulfate-reducing and sulfur-oxidizing reference strains [39, 40] including all full-length Apr sequences available from the public databases. The AmoA and AprA data sets were phylogenetically analyzed using PhyML. Regions of insertions and deletions (indels) were omitted. The maximum-likelihood method-based phylogenetic trees were constructed using the global rearrangement and randomized species input order options and the JTT matrix as amino acid replacement model. Statistical support is given by bootstrap analysis with 100 resamplings.

GenBank Accession Numbers

The nucleotide sequence data reported in this article are available under the GenBank accession numbers EU005552 (28S rRNA gene), EU005553-EU005595 and EU005641-EU005648 (16S rRNA gene), EU005596-EU005620 (*aprA* gene), and EU005621-EU005640 (*amoA* gene).

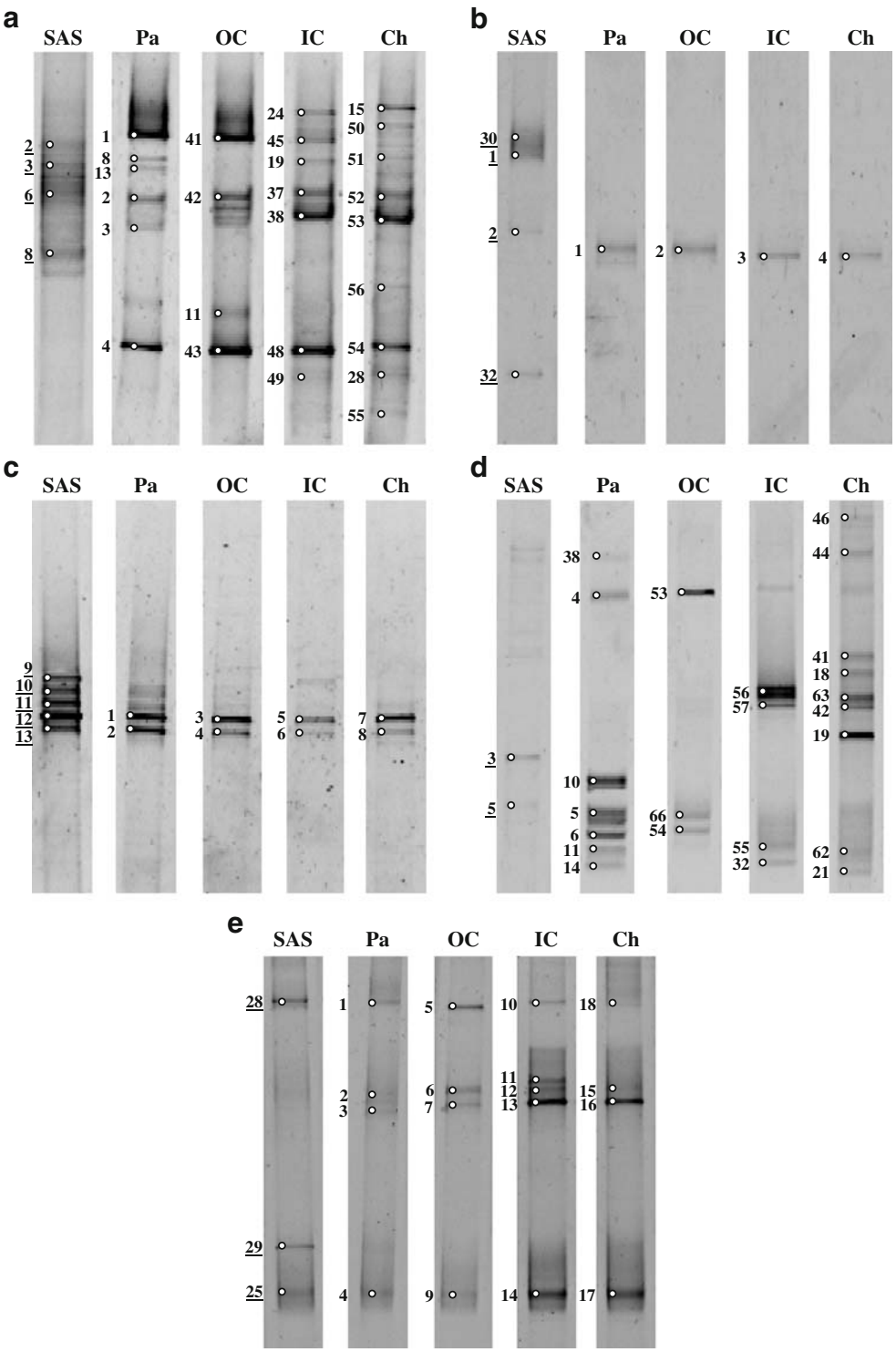

Figure 2 DGGE banding patterns of amplified 16S rRNA, *aprA*, and *amoA* gene fragments using DNA samples from sponge tissue sections, papillae (*Pa*), outer cortex (*OC*), inner cortex (*IC*), and choanosome (*Ch*) of Caribbean *P.* cf. *corticata* and sponge ambient seawater (SAS) collected at the Kahouanne Basin (see Fig. 1). 16S rRNA gene-specific diversity analysis was performed applying universal bacterial (**a**), universal archaeal (**b**), and betaproteobacterial ammonia-oxidizer-specific (**b**) gene-targeting primer pairs in PCR. Diversity analysis of functional genes was performed using SRP- and SOB-specific *aprA* gene-targeting primers (**d**) and betaproteobacterial ammonia-oxidizer-specific *amoA* gene-targeting primers (**e**). *Numbered* DGGE bands were excised from replicate gels and successfully sequenced (phylogenetic analyses of retrieved sequences, see Figs. 3, 4, and 5)

Results

DGGE

DGGE was used to fingerprint the microbial community in *P.* cf. *corticata* (Fig. 1). The banding patterns of the same tissue type were identical, indicating no genomic DNA extraction and PCR amplification bias. The banding patterns of DGGE analyses using universal archaeal, bacterial, and *aprA* gene-targeting primers showed the presence of different microbial communities in the inner and outer regions of *P.* cf. *corticata* (Fig. 2a, b, d). In addition, all tissue

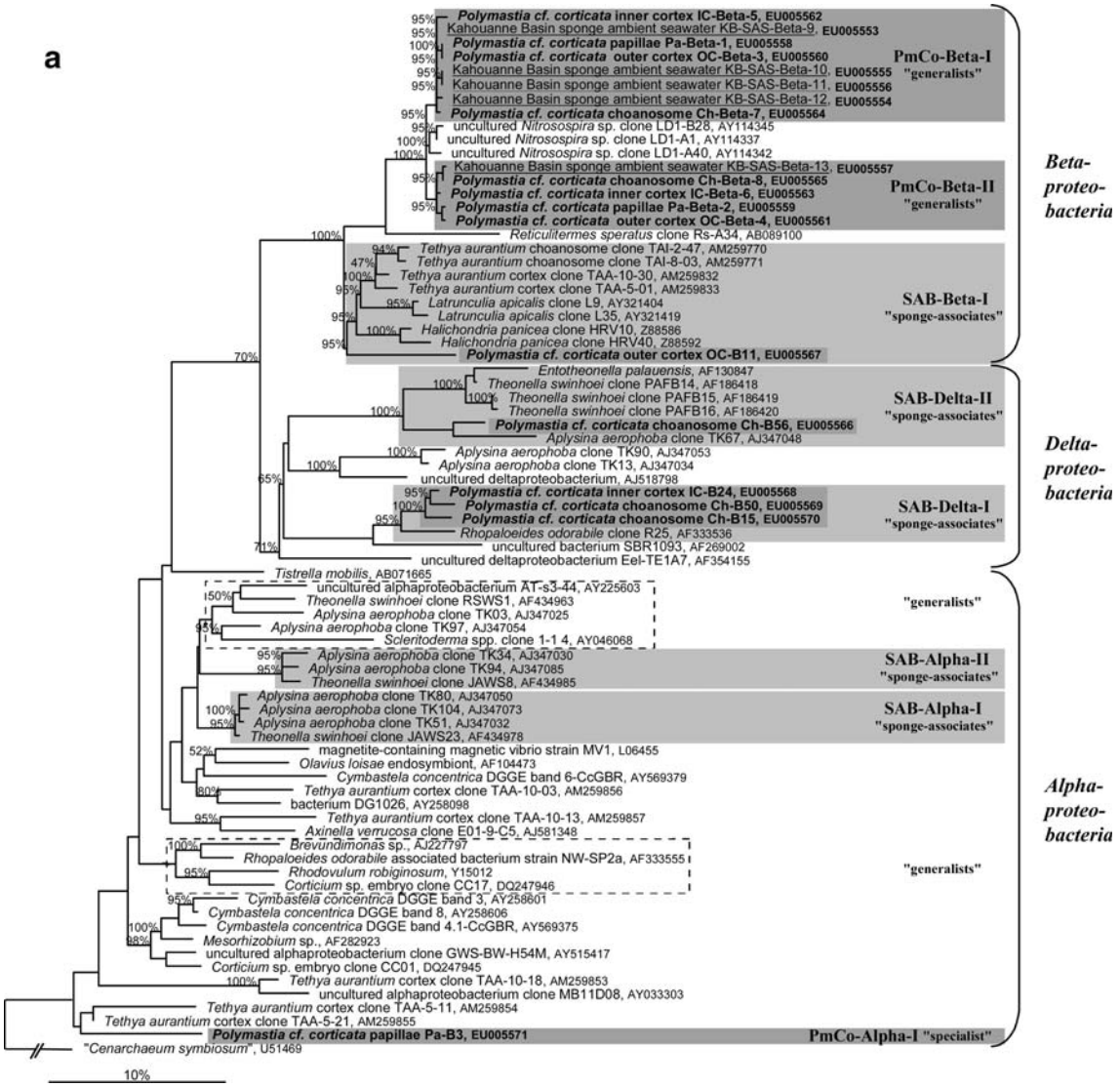

Figure 3 Phylogenetic trees based on the 16S rRNA sequences obtained from the *P.* cf. *corticata*-associated microbial community and host sponge surrounding seawater with the three 16S rRNA gene-targeting primer sets used in this study and related sequences belonging to the *Alphaproteobacteria*, *Betaproteobacteria*, and *Deltaproteobacteria* (**a**); *Gammaproteobacteria* (**b**); *Acidobacteria* and *Nitrospira* (**c**); and *Actinobacteria* and *Archaea* (**d**). Sequences obtained from *P.* cf. *corticata* are shown in *bold type* and highlighted by *dark-gray boxes*, whereas those obtained from the host sponge ambient seawater are *underlined*. The monophyletic, sponge-associated bacterial and archaeal 16S rRNA clusters are highlighted by *light-gray boxes*. The *P.* cf. *corticata*-derived sequences are classified into clusters of *P.* cf. *corticata* specialists ("PmCo specialist"), general "sponge-associated" *Bacteria* and *Archaea* (SAB, SAA) and "generalists" ("generalist" groups formed by other sponge species-derived sequences are framed by *dashed lines*) according to Taylor *et al.* [60]. Maximum-likelihood bootstrap resampling values greater than 50% (100 resamplings) are indicated near the nodes. The 16S rRNA gene sequence of *Cenarchaeum symbiosum* was used as outgroup reference. The scale bar corresponds to 10% estimated sequence divergence

sections of the sponge differed in their DGGE banding pattern from the sponge ambient seawater (SAS) samples. Archaeal and bacterial phylotypes specifically associated with the distinct sponge regions were represented by DGGE bands that were exclusively present in all papillae, outer cortex, inner cortex, or choanosome samples, respectively, but absent in seawater (Fig. 2a, b, d). Other bands were found in all sponge tissue samples. Contrarily, the DGGE banding patterns of the betaproteobacterial ammonia-oxidizer-specific 16S rRNA and *amoA* gene-based analyses were highly similar (1) between the distinct sponge tissue types and (2) between sponge and ambient seawater (Fig. 2c and e). Phylotypes specifically associated with *P.* cf. *corticata* that were represented by DGGE bands exclusively found in the sponge samples (but not in seawater) were only detected by the *amoA* gene-based analysis (Fig. 2e).

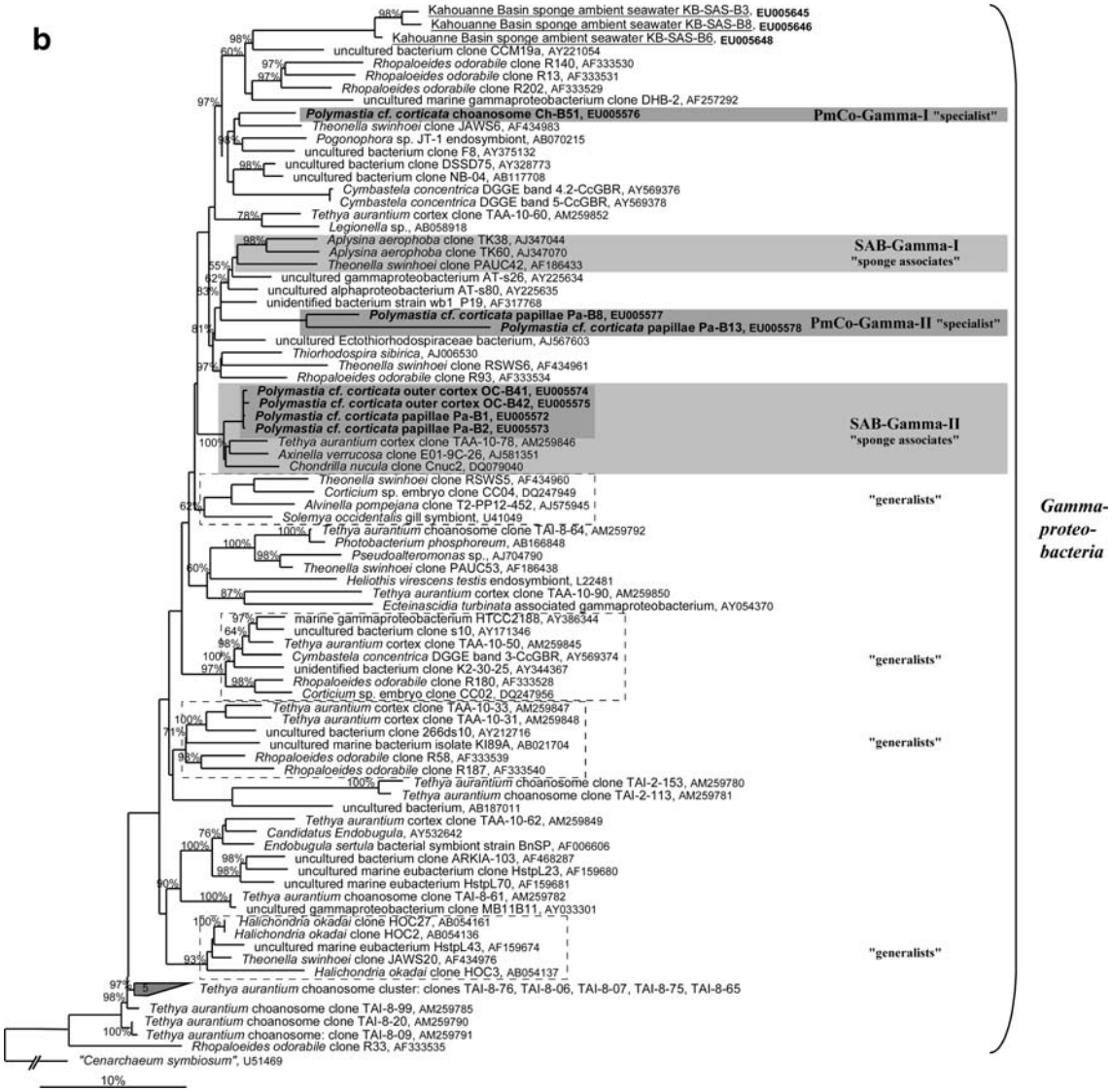

Figure 3 (continued)

Phylogenetic Analysis of Partial 16S rRNA Gene Sequences

A total of 38 OTUs was identified in *P.* cf. *corticata* forming 18 distinct sequence clusters. The sequences fell into eight different archaeal and bacterial divisions, the *Crenarchaeota*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Nitrospira* (Fig. 3). In agreement with microbial communities associated with other examined deep-water sponges [48], no phylotype related to the cyanobacterial lineage was identified in the deep-water specimen of *P.* cf. *corticata*. Members of the latter phototrophic group are abundant in shallow-water sponges [16, 18, 51, 60, 61, 63]. Twenty of the aforementioned 38 phylotypes (53%) were most closely affiliated with other sponge-derived sequences previously found in *Tethya aurantium*, *Rhopaloides odorabile*, *Theonella swinhoei*, *Latrunculia apicalis*, *Halichondria panicea*, *Aplysina aerophoba*, *Sclerotiderma* sp., *Chondrilla nucula*, *Petrosia* sp., *Suberites* sp., and *Axinella verucosa* from different geographical locations (predominantly shallow-water habitats). These phylotypes comprised the eight sponge-specific, monophyletic clusters SAB-Beta-I [63], SAB-Gamma-II, SAB-Delta-I and SAB-Delta-II, SAB-Acido-III, SAB-Actino-III, SAB-Nitrospira-I [16], and SAA-Cren-I (sponge group C) [22, 32] (classified as "sponge associates"). It is interesting to note that the detected alphaproteobacterial, most gammaproteobacterial, actinobacterial, and crenarchaeal phylotypes appeared to represent *P.* cf. *corticata* host-

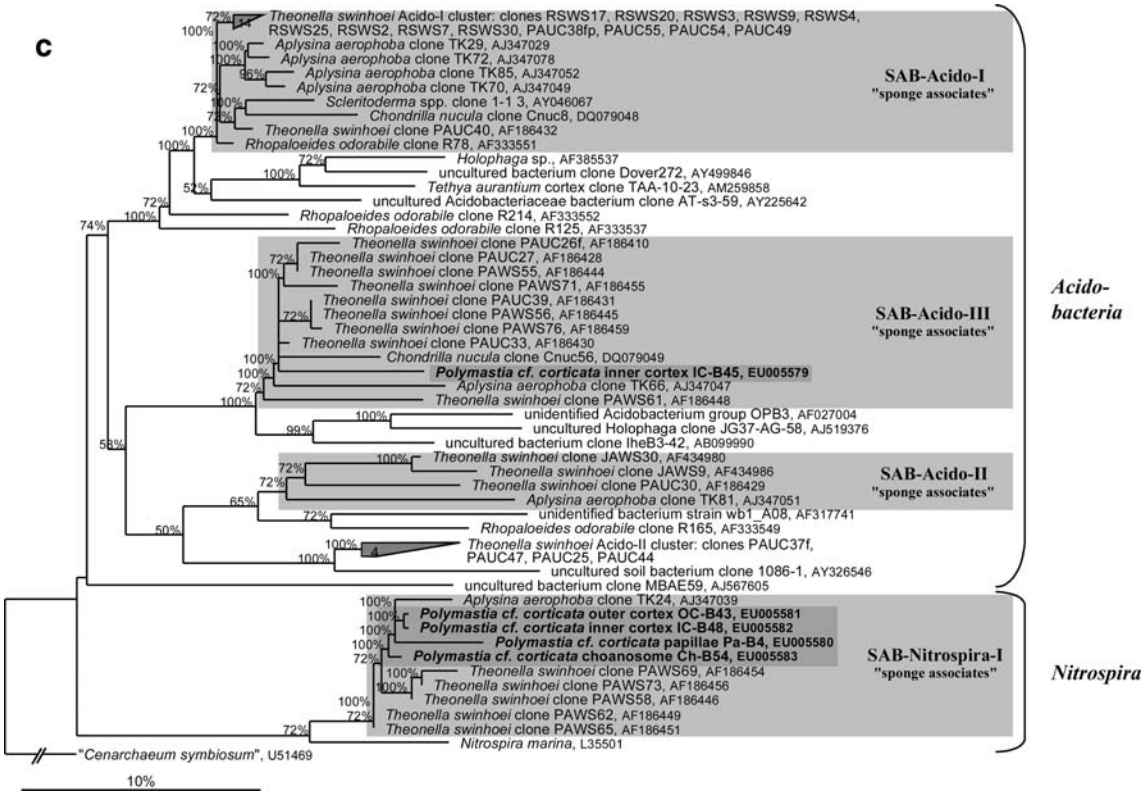

Figure 3 (continued)

specific microorganisms ("specialists" PmCo-Alpha-I, PmCo-Gamma-I/-II, PmCo-Actino-I/-II/-III, and PmCo-Cren-I) not present in the ambient seawater and other sponge species. Based on the current database, these 10 phylotypes (26%) were only moderately related to other sponge-derived sequences but affiliated to environmental sequences received from other marine macroorganisms (*Pogonophora* endosymbionts; PmCo-Gamma-II), aquatic microbial formations in Nullarbor caves [21] (PmCo-Gamma-I, PmCo-Actino-I), inactive deep-sea hydrothermal vent chimneys [57] (PmCo-Actino-II), and deep-sea sediments [65] (PmCo-Cren-I). Indeed, by the use of betaproteobacterial ammonia-oxidizer-specific primers [30], close relatives of uncultured marine *Nitrosospira* [10] were detected in the sponge tissue and in the host surrounding seawater (classified as "generalists" PmCo-Beta-I and PmCo-Beta-II).

The bacterioplankton assemblage of the sponge ambient seawater at the Kahouanne Basin (1,127 m depth) was phylogenetically less diverse with respect to the complex microbial community associated with *P.* cf. *corticata* (Fig. 3). The detected phylotypes were most closely affiliated with seawater- and deep-sea sediment-derived sequences of uncultured members of the marine group-I *Crenarchaeota* (MG-I, group C1a-α) [65], *Euryarchaeota* [38], *Nitrosospira* [10], uncultured *Gammaproteobacteria* and *Bacteroidetes* (respective SAS-B2 16S rRNA sequence is not shown in Fig. 3; closest relative is AJ567605; M.X. Xu, P. Wang, F.P. Wang, and X. Xiao, unpublished results).

Phylogenetic Analysis of Partial AprA and AmoA Sequences

The *aprA* gene-based analysis allowed the detection of six phylotypes that are indicative for the presence of five sulfur-oxidizing alphaproteobacterial and gammaproteobacterial species and one sulfate-reducing archaeon in the tissue of *P.* cf. *corticata* (Fig. 4). The alphaproteobacterial SOB-phylotypes were most closely related to AprA sequences of cultured members of the SAR11 clade [12] (PmCo-sulfur-oxidizer-I to PmCo-sulfur-oxidizer-III) and the LGT-affected *Thiobacillus plumbophilus* (PmCo-sulfur-oxidizer-IV). Because representatives of the alphaproteobacterial clusters I to III have also been found to be abundant in deep-sea sediments of the Kahouanne Basin and the sponge ambient seawater (Fig. 4), the corresponding SOB species were classified as "generalists". The gammaproteobacterial SOB-phylotype (PmCo-sulfur-oxidizer-V) was moderately affiliated to AprA sequences of uncultured *Thiothrix* species, which have been found in cold seep *Beggiatoa*-mats at the Hydrate Ridge, Oregon [41]. All identified SOB-phylotypes could not be assigned to any detected 16S rRNA gene-

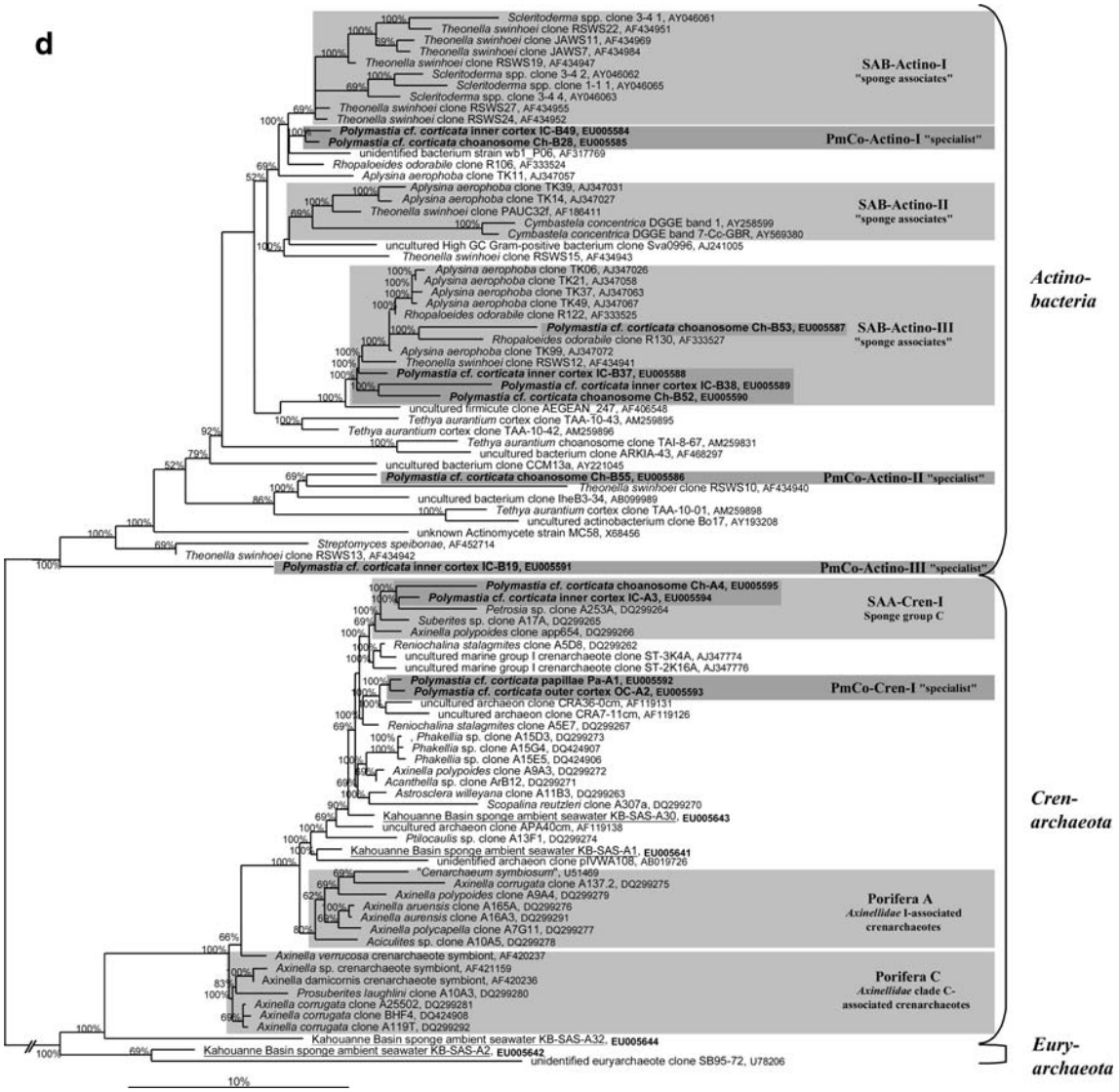

Figure 3 (continued)

based OTU. Besides, a SRP-phylotype was detected that is most closely related to the AprA sequences of *Archaeoglobus* members; a corresponding euryarchaeal species was not identified by the 16S rRNA gene-based analysis.

The *amoA* gene-based analysis (*Betaproteobacteria*-specific primers) allowed the detection of nearly identical phylotypes (PmCo-ammonia-oxidizer-I and PmCo-ammonia-oxidizer-II) in the sponge tissue and the host surrounding seawater (Fig. 5); closely related environmental sequences have also been reported from diverse sediment or seawater–sediment interface samples of other habitats [2, 14, 31]. The AmoA sequence clusters corresponded to the uncultured *Nitrosospira* sequence clusters that have been identified by the group-specific 16S rRNA gene analysis (Fig. 3).

Spatial Distribution of the Sponge-Associated Microbial Community

The phylogenetic investigations of different tissue regions from *P.* cf. *corticata* (Fig. 1) demonstrated high variability in the microbial communities associated with the sponge papillae/outer cortex and the inner cortex/choanosome (Figs. 3, 4, and 5, summarized in Table 1). The 16S rRNA gene-based analysis indicated that the highest phylogenetic diversity is present in the inner region of the sponge, the choanosome. From the total of 38 different 16S rRNA gene-based phylotypes, 12 and 10 OTUs were detected in the choanosome and the inner cortex, respectively, whereas only 7 and 9 OTUs were found in the outer cortex and in the papillae tissue sections, respectively. The members of

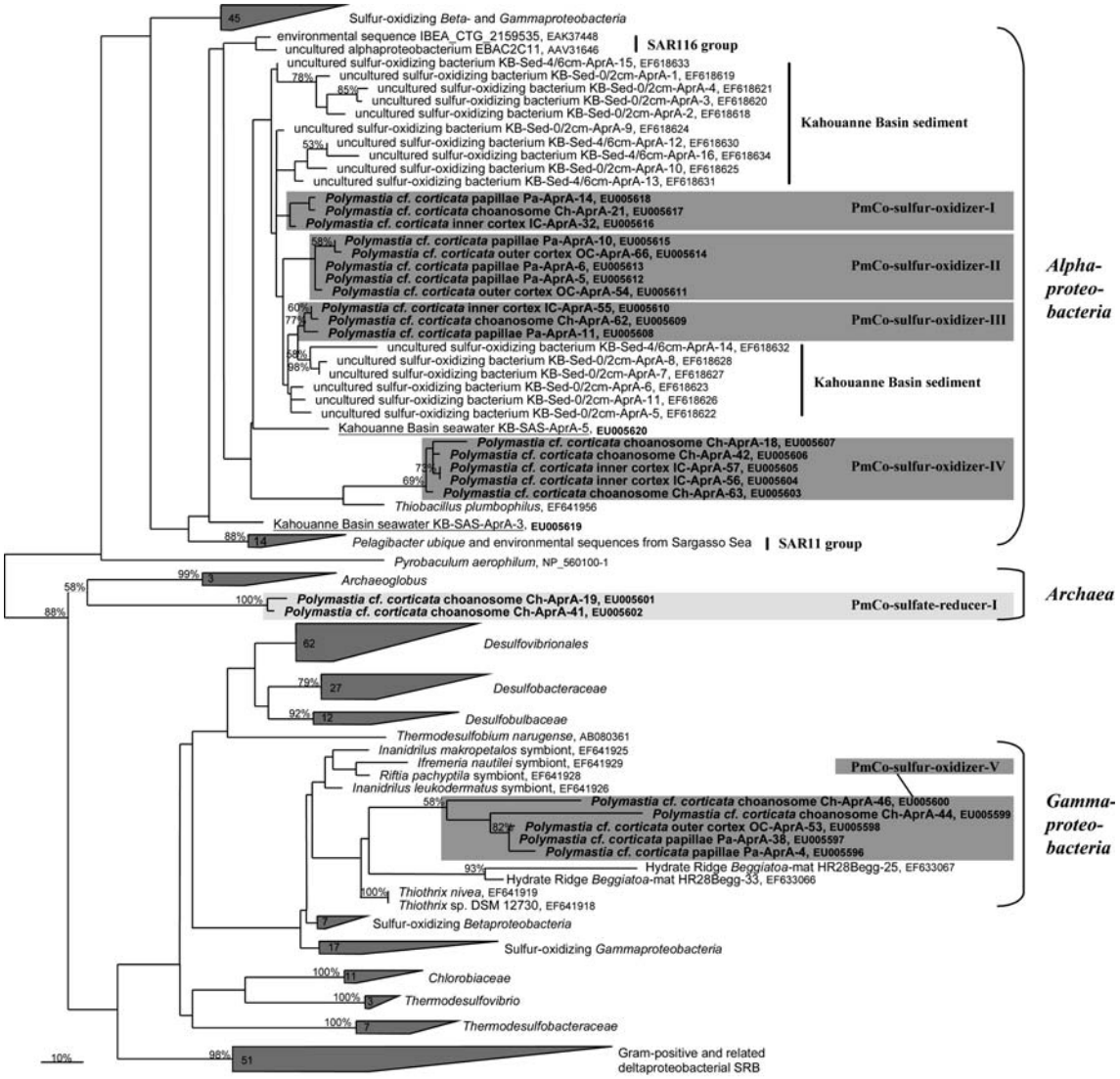

Figure 4 Phylogenetic tree based on the AprA sequences obtained from the *P.* cf. *corticata*-associated microbial community, the host sponge surrounding seawater, and adjacent sediment samples from the Kahouanne Basin. SRP- and SOB-type sequences received from *P.* cf. *corticata* are shown in *bold type* and highlighted by *light-gray* and *dark-gray boxes*, respectively; those obtained from the host sponge ambient seawater are *underlined*. The taxonomic classification of the SRP and SOB reference strains and affiliated sponge-derived sequences is indicated. Maximum-likelihood bootstrap resampling values greater than 50% (100 resamplings) are indicated near the nodes. The scale bar corresponds to 10% estimated sequence divergence

the different archaeal and bacterial divisions were not evenly distributed within the sponge body but appeared to be associated with distinct parts of the host tissue. Five of the eight general sponge-specific clusters (SAB-Delta-I and SAB-Delta-II, SAB-Acido-III, SAB-Actino-III, SAA-Cren-I, 11 phylotypes) and all *P.* cf. *corticata*-specifically associated actinobacterial phylotypes (PmCo-Actino-I, PmCo-Actino-II, and PmCo-Actino-III, 4 phylotypes) were exclusively present in the inner cortex and the choanosome. Thus, the members of the *Deltaproteobacteria*, *Acidobacteria*, and *Actinobacteria* were restricted to the inner parts of this sponge. In contrast, four of the five *P.* cf. *corticata*-derived sequence clusters that were exclusively identified in the sponge surface regions belonged to the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (8 phylotypes). The OTUs comprised only two general sponge-specific clusters (SAB-Beta-I, SAB-Gamma-II) besides four putative "specialists" clusters (PmCo-Alpha-I, PmCo-Gamma-II, PmCo-Cren-I) whose phylotypes were affiliated with nonsponge-derived sequences received from diverse habitats. The *Crenarchaeota* associated with the papillae and outer cortex sections were distinct to those found in the inner cortex and choanosome. Notably, with the exception of PmCo-Gamma-I, no phylotype obtained from the choanosome or the inner cortex section was closely related to environmental sequences found in seawater and sediment samples.

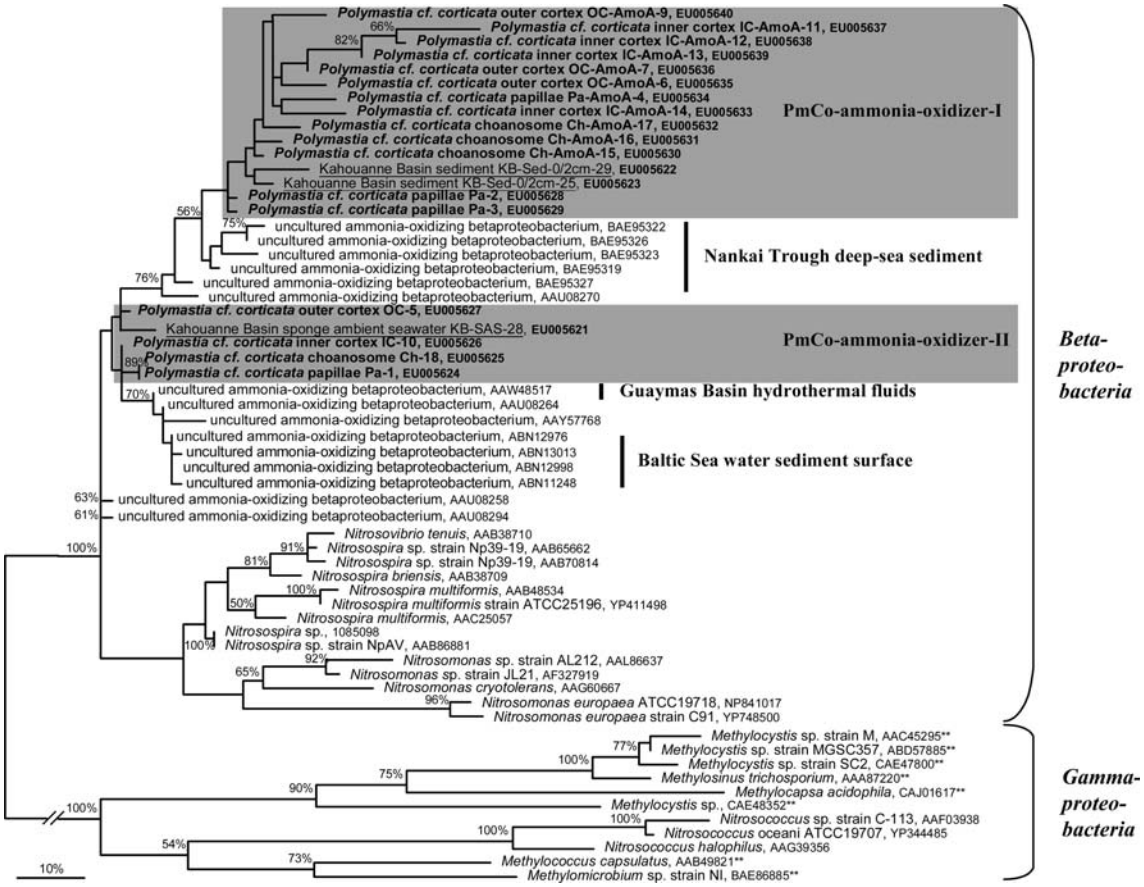

Figure 5 Phylogenetic tree based on the AmoA sequences obtained from the *P.* cf. *corticata*-associated microbial community and host sponge surrounding seawater including related (reference strain and environmental) sequences belonging to the *Betaproteobacteria* and *Gammaproteobacteria*. Sequences received from *P.* cf. *corticata* are shown in *bold type* and highlighted by *dark-gray boxes*, whereas those obtained from the host sponge ambient seawater are *underlined*. The taxonomic classification of the ammonia-oxidizing proteobacterial reference strains and affiliated sponge-derived sequences is indicated. Maximum-likelihood bootstrap resampling values greater than 50% (100 resamplings) are indicated near the nodes. The AmoA sequences of the *Gammaproteobacteria* were used as outgroup reference. The scale bar corresponds to 10% estimated sequence divergence

Table 1 Spatial distribution of phylotypes from the major microbial taxonomic/physiological groups identified in different tissue sections of *P.* cf. *corticata* by DGGE analysis of PCR-amplified 16S rRNA, *aprA*, and *amoA* gene fragments

Taxonomic/physiological group	Papillae	Outer cortex	Inner cortex	Choanosome
16S rRNA gene-based analysis				
Crenarchaeota	+	+	+	+
Alphaproteobacteria	+	+	−	−
Betaproteobacteria[a]	+	++	++	++
Gammaproteobacteria	++	++	−	+
Deltaproteobacteria	−	−	++	++
Actinobacteria	−	−	++	++
Acidobacteria	−	−	+	+
Nitrospira	+	+	+	+
aprA gene-based analysis				
Sulfate-reducing prokaryotes	−	−	−	+
Sulfur-oxidizing bacteria	++	++	++	++
amoA gene-based analysis				
Ammonia-oxidizing *Betaproteobacteria*	+	+	+	+

−: no phylotype affiliated with this major taxonomic/physiological group identified, +: phylotype affiliated with this major taxonomic/physiological group identified, ++: two or more distinct phylotypes affiliated with this major taxonomic/physiological group identified

[a] Phylotypes retrieved from universal bacterial and betaproteobacterial-specific 16S rRNA gene analysis

Consistent with the 16S rRNA analysis, the *aprA* gene-based analysis revealed that the highest phylogenetic complexity was present in the choanosome of *P.* cf. *corticata*. In accordance to the 16S rRNA gene-observed local variability of the microbial community structure, the members of the six identified sulfur-oxidizing and sulfate-reducing lineages differed significantly in their distribution within the host sponge. Whereas the gammaproteobacterial SOB (*Thiothrix* spp.-affiliated cluster V) and most alphaproteobacterial SOB species (potential "generalist" clusters PmCo-sulfur-oxidizer-I to PmCo-sulfur-oxidizer-III) were present in the entire sponge body, the putative sponge-specific alphaproteobacterial sulfur-oxidizers (*Thiobacillus plumbophilus*-affiliated cluster IV) and the archaeal sulfate-reducing strains (PmCo-sulfate-reducer-I) were restricted to the inner tissue sections. In support of the 16S rRNA analysis results obtained with the group-specific primers, the *amoA* gene-based analysis showed the ubiquitous presence of two potential ammonia-oxidizing betaproteobacterial species in the sponge body.

Discussion

Microbial Diversity in the Caribbean Deep-water Sponge *Polymastia* cf. *corticata* in Comparison to Other Sponge Species

The 16S rRNA gene-based diversity analysis of the deep-water sponge *P.* cf. *corticata* collected at the Kahouanne Basin in the Caribbean Sea revealed its associated microbial community to be as phylogenetically complex and diverse as reported for the shallow-water sponge species [16, 18, 33, 60–63, 68]. Of the *P.* cf. *corticata*-associated phylotypes, 53% belonged to 16S rRNA sequence clusters that were strictly confined to sponges ("sponge associates" groups SAB-Beta-I, SAB-Gamma-II, SAB-Delta-I and SAB-Delta-II, SAB-Acido-III, SAB-Actino-III, SAB-Nitrospira-I, and SAA-Cren-I; see Fig. 3). The residual 47% of the detected OTUs, however, did not branch into any of the recognized sponge-specific, monophyletic clusters [15, 16, 19, 22, 59, 67]. Whereas the PmCo-Beta-I and PmCo-Beta-II members have been confirmed to be sponge-unspecific, the PmCo-Gamma-I, PmCo-Gamma-II, PmCo-Alpha-I, PmCo-Actino-I, PmCo-Actino-II, PmCo-Actino-III, and PmCo-Cren-I phylotypes might represent *P.* cf. *corticata*-specifically associated microorganisms ("specialists"). By the increasing number of environmental sequences available in the databases, identical free-living relatives of these currently designated "specialists" might be identified in the future and change their classification status into "generalists." Indeed, most sponge-derived alphaproteobacterial and gammaproteobacterial OTUs share low sequence similarity but are more closely affiliated with 16S rRNA sequences received from other marine organisms, sediment, or the water column [16, 18, 33, 60–63, 68, 71]. In addition, the *aprA* gene-based diversity analysis indicated the presence of one sulfate-reducing archaeal species and two *Thiothrix* spp.- and *Thiobacillus plumbophilus*-related potential SOB strains that might be general sponge-specific microorganisms; however, with respect to the limited sequence information available concerning sponge–SRP/SOB associations, their specificity might also be restricted to a certain group of sponges (e.g., genus or family) or possibly only to the species *P.* cf. *corticata* itself.

Overall, the bacterial community resident in *P.* cf. *corticata* was significantly distinct to those of the examined deep-water specimens of *Sclerotiderma* spp. [48], which were also collected from the Lesser Antilles area of the Caribbean Sea (coast of Curaçao and Bonnaire; 242–255 m depth). The phylotypes of *Sclerotiderma* spp. comprised representatives of the *Alphaproteobacteria*, *Actinobacteria*, *Acidobacteria*, *Spirochaeta*, and *Chloroflexaceae*, while members of the latter two groups were not present in *P.* cf. *corticata*. The identified OTUs of the three deep-water sponge species were only distantly related: The acidobacterial and actinobacterial phylotypes belonged to different sponge-specific clusters (SAB-Acido-I and SAB-Actino-I in *Sclerotiderma*, SAB-Acido-III and SAB-Actino-III in *P.* cf. *corticata*, see Fig. 3c and d), whereas the alphaproteobacterial OTUs represented unrelated bacteria classified as "generalist" and *P.* cf. *corticata*-"specialist" (PmCo-Alpha-I, see Fig. 3a). Nevertheless, as both studies were based on DGGE analysis of PCR products obtained with different primers, the nonoverlapping in their associated bacterial diversity might also be caused by primer-introduced different amplification bias [58, 66] and the sensitivity limitation of the DGGE method detecting only populations that comprise 1% or more of a sampled community [44]. The archaeal community of the *Sclerotiderma* spp. has not been assessed by Olson and McCarthy [48]. In *P.* cf. *corticata*, four crenarchaeotic phylotypes (SAA-Cren-I, PmCo-Cren-I) were detected that belonged to the cosmopolitan group C1a-α of MG-I [27, 38]; only two of them branched into the sponge-specific, monophyletic "sponge C group" [22, 32] (SAA-Cren-I, Fig. 3d). It is interesting to note that all sponge-derived C1a-α sequences were recently disputed to represent sponge-specific phylotypes because of the absence of significant host-clade specificity in clones of this group. Truly specific (potential mutualistic) associations between sponges and *Archaea* were postulated to exist only for *Cenarchaeum symbiosum* relatives (group C1a, *Porifera* A and C cluster) and axinellid sponges based on their parallel evolution (cospeciation) [22, 35]. Besides the aforementioned *P.* cf. *corticata*-associated *Crenarchaeota*, a putative sponge-

specific sulfate-reducing euryarchaeon was identified by the *aprA* gene analysis (Fig. 4). Euryarchaeal 16S rRNA clones have so far only been reported from *Axechina raspailoides*, *Reniochalina stalagmites*, *Ptilocaulis* sp. [22], and *Rhopaloides odorabile* (*Methanomicrobiales* relative) [69]. In contrast to our results, Holmes and Blanch reported the general absence of *Archaea* in *Polymastia invaginata* [22], which is a close relative of *P.* cf. *corticata*.

The 16S rRNA gene analysis with the universal primer sets demonstrated that the microbial consortium of *P.* cf. *corticata* and the ambient bacterioplankton were distinctly different, indicative for the low impact of the surrounding seawater on the sponge–microbe associations. In contrast, the usage of group-specific 16S rRNA, *aprA*, and *amoA* gene-targeting primers confirmed that seawater- and sediment-derived "generalist" phylotypes were present in *P.* cf. *corticata* (PmCo-Beta-I and PmCo-Beta-II; PmCo-ammonia-oxidizer-I and PmCo-ammonia-oxidizer-II; PmCo-sulfur-oxidizer-I, PmCo-sulfur-oxidizer-II, and PmCo-sulfur-oxidizer-III). These phylotypes most likely corresponded to ammonia-oxidizing *Nitrosospira* relatives and putative sulfur (or sulfite)-oxidizing, yet uncultivated *Alphaproteobacteria*. The nondetection of the *Nitrosospira* relatives with the universal 16S rRNA gene-targeting primer pair might indicate that the abundance of the respective populations was below the detection limit of approximately 1% to 5% [44]. Vice versa, the failure to amplify the 16S rRNA genes of the sponge-specific SAB-Beta-I members with the group-specific primer pair (CTO) [46] might indicate that these *Betaproteobacteria* were no chemolithoautrophic ammonia-oxidizers. Nevertheless, the CTO primer set [30] has been documented to bias the PCR-based analysis toward *Nitrosospira* relatives [36]. Because sponges are powerful filter-feeders (see [17, 19, 59, 67] and references therein) and are not able to discriminate between different types of food bacteria [70], even less abundant seawater- or sediment-derived microorganisms might be enriched by the stable and nutritionally rich microhabitat "sponge" ("microbial fermenter" [17]) and become part of the sponge-associated microbiota. As close relatives of the *P.* cf. *corticata*-associated *Nitrosospira* and *Alphaproteobacteria* were demonstrated to be abundant in the seawater–sediment interface [2, 10, 14, 41, 47], they might have represented transient bacterial populations that either served as "food bacteria" or resisted the phagocytosis process. In consequence, although a subset of the *P.* cf. *corticata*-associated microbial community (comprising especially the "sponge associates") will be transferred vertically as documented for several other sponges [9, 54, 55], the ambient seawater had an influence on the composition of its associated microbial community (see presence of proven "generalists").

A correlation between the geographical location of sponges and the presence of major bacterial taxonomic groups in their associated microbiota was proposed by Hill *et al.* [18]. However, this postulated geographical impact appears to be of minor importance with respect to the results obtained from the Caribbean sponges ([18, 48] and this study). *Acidobacteria*, *Deltaproteobacteria*, and *Nitrospira* relatives that were proposed to be characteristic for warm-water sponges were also present in the *P.* cf. *corticata* specimen collected from 5°C cold deep-sea habitat. However, representatives of *Bacteroidetes* and *Planctomycetes*, which were suggested to be typical for microbial consortia of cold/temperate sponge, were absent. Indeed, several phylogenetic surveys demonstrated that the microbial consortia associated with different sponge species are highly diverse even if the hosts share one habitat [33, 51, 60, 62, 63, 68]. In contradiction to the hypothesis of a uniform microbial signature of sponges across spatial and temporal scales [15, 16], the abundance, phylogenetic composition, and diversity of the host-associated microbial community mainly depends on the sponge-species and host state-depending interior factors as demonstrated by results of previous studies [18, 22, 33, 60–63, 68] and confirmed by this work. The proportion of the "sponge associates," "specialists," and "generalists" populations appears to be a sponge-specific feature.

Spatial Differences in the *Polymastia* cf. *corticata*-associated Microbial Community

The 16S rRNA, *aprA*, and *amoA* gene-based phylogenetic investigation of the tissue sections, papillae, inner and outer cortex, and choanosome revealed that distinct bacterial and archaeal populations are associated with the different tissue regions of the deep-water specimen of *P.* cf. *corticata*. In accordance, spatial differences in the microbial community have recently been documented for the shallow-water sponge *Tethya aurantium* [63]. However, in contrast to the latter work, our study indicated that the local distribution of a phylotype in the host tissue depended on its phylogenetic affiliation (bacterial/archaeal division) and correlated with its classification as "sponge associate," "specialist," and "generalist," and its potential ecological role in the sponge. Indeed, compartmented sponges might provide distinct microenvironments as ecological niches for the different bacterial and archaeal populations. Nutrient-rich conditions within the mesohyl of *P.* cf. *corticata* (extensive phagocytosis by archaeocytes) might explain that the choanosome- and inner cortex-associated microbiota was the most complex. Notably, all identified *Acidobacteria*, *Actinobacteria*, and *Deltaproteobacteria* were (1) general sponge-specific members or at least specifically associated with *P.* cf. *corticata* and (2) restricted in their distribution to the choanosome. Sponge-associated *Actinobacteria* and *Deltaproteobacteria* (e.g., *Entotheonella paulensis*) have been reported to be prolific secondary metabolite producers

[34, 42, 52, 53] and suggested to attribute largely to the chemical defense mechanisms of their host sponges against predators with biologically active compounds (repellents) and biofouling (see [15, 25] and references therein). Thus, their association with *P.* cf. *corticata* could have represented true mutualistic sponge–symbiont interactions. The functional role of the sponge-specific *Acidobacteria* is still unresolved because information concerning the physiology and metabolism of the marine acidobacterial members are not yet available and even limited for the abundant terrestrial species [26, 49]. In further support of a correlation between spatial distribution, sponge specificity, and the ecological role of the associated microorganisms, the *aprA* gene analysis demonstrated that the presumed general sponge-specific archaeal SRP and SOB strains (PmCo-sulfate-reducer-I, PmCo-sulfur-oxidizer-IV; Fig. 4) were also restricted to the host inner tissue regions. Consistently, potential sulfate-reducing strains (relatives of *Desulfovibrionaceae* and the *Desulfarculus*/*Desulfomonile*/*Syntrophus* cluster) were only present in the choanosome sections of two shallow-water sponge species [20, 37]. As postulated for *Geodia barretti* [20], the associations of sponges with sulfate-reducing microorganisms might generally be synergistic. Most sponges alternate between periods of high water-pumping velocity and periods of low water circulation [1, 56]; during the latter periods, oxygen becomes limited by the ongoing active respiration of sponge cells and aerobic microorganisms with the consequence of intermittent tissue anoxia. It was postulated that the anaerobic SRPs might benefit from the metabolic end products of sponge cells that switch to fermentation in these anoxic zones of the mesohyl; in turn, the microbial biomass might be consumed by the sponge cells ("bacterial farming") [20]. If oxygen is present again, the SRP-derived reduced sulfur compounds could be reoxidized by the activity of sulfur-oxidizing chemolithoautotrophs. Because potential members of both physiological groups were identified in the choanosome of *P.* cf. *corticata* by functional gene analysis, a sponge-specific, endosymbiotic sulfur cycle as described for marine oligochaetes [3, 8] may also be present in *P.* cf. *corticata*. As their closest related and cultivated species, *Pelagibacter ubique* (SAR11 clade) [12], the ubiquitous *Alphaproteobacteria* (cluster I to III; Fig. 4) might not rely on a sulfur-based chemolithoautotrophic lifestyle. As "generalists," they most likely represented opportunistic, transient bacterial populations obtained from the seawater and sediment.

In contrast to the aforementioned choanosome/inner cortex-associated bacteria, most microbes that were exclusively present in the outer tissue regions of *P.* cf. *corticata* were not general sponge-specific (exception SAB-Gamma-II representatives) but presented putative host-specific members of the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Crenarchaeota*. These "specialists" might have been acquired by the settlement of free-living, opportunistic populations at the sponge surface with no benefit for the host. Indeed, the papillae- and outer cortex-associated microbial communities reflected the phylum level structure of the planktonic and surface sediment-associated microbiota at the Kahouanne Basin (Fig. 3).

The ubiquitous presence of potential ammonia- and nitrite-oxidizing bacteria and archaea in the entire sponge tissue probably reflected the overall availability of nitrogenous host waste products (e.g., ammonia, urea) in the sponge body. The coordinated metabolism of *Nitrosospira* and *Cenarchaeum symbiosum*/*Nitrosopumilus maritimus* relatives as ammonia-oxidizing bacteria/archaea [13, 28, 73] together with members of the genus *Nitrospira* as nitrite-oxidizing bacteria [29] (PmCo-Beta-I and PmCo-Beta-II and corresponding PmCo-ammonia-oxidizer-I and PmCo-ammonia-oxidizer-II; SAA-Cren-I and PmCo-Cren-I; SAB-Nitrospira-I) might be responsible for the process of nitrification in *P.* cf. *corticata* as it has been proposed for other sponge species (see [17, 59] and references therein). In analogy to the mutualistic sponge–SRP/SOB interactions ("bacterial farming"), the chemolithoautotrophic CO_2 fixation by the nitrifying microbial community could provide new carbohydrates for the host cells via microbe–sponge exchange. The members of the *Nitrospira* and inner cortex/choanosome-associated SAA-Cren-I-*Crenarchaeota* belong to sponge-specific clusters, which might be indicative for long-term mutualistic interactions, whereas the *P.* cf. *corticata*-associated *Nitrosospira* relatives were most likely colonizers acquired by filtration processes from free-living populations that are generally abundant in marine habitats [10, 47].

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