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E. M. D. Porporato, A. Lo Giudice, L. Michaud, E. De Domenico & N. Spanò

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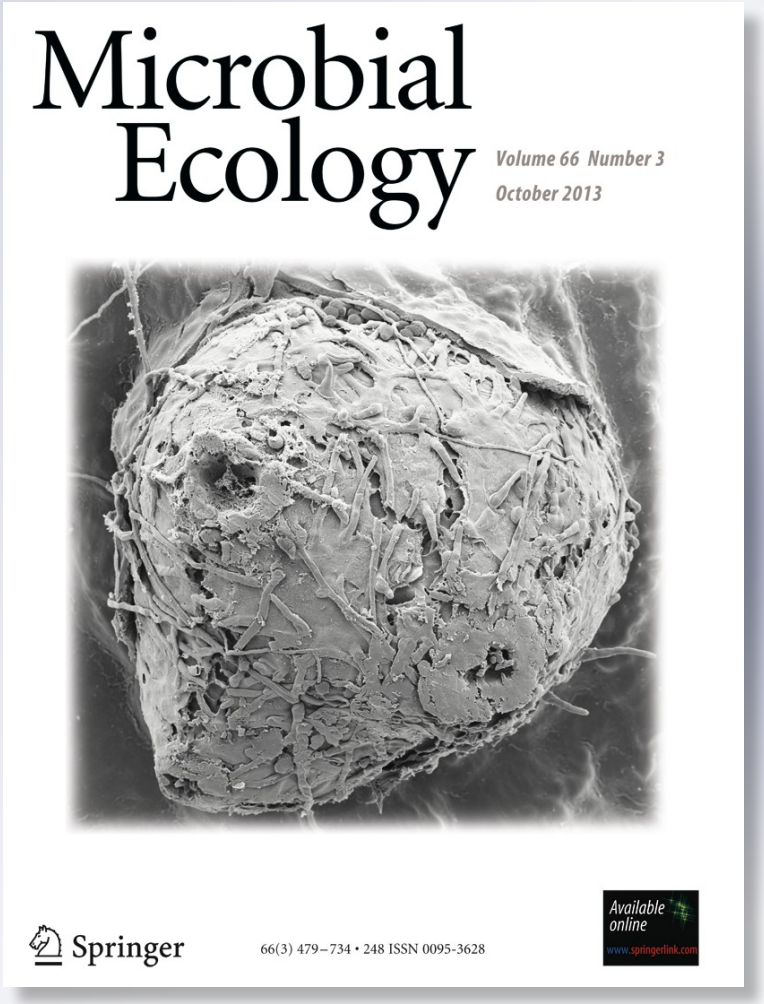

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INVERTEBRATE MICROBIOLOGY

Diversity and Antibacterial Activity of the Bacterial Communities Associated with Two Mediterranean Sea Pens, *Pennatula phosphorea* and *Pteroeides spinosum* (Anthozoa: Octocorallia)

E. M. D. Porporato · A. Lo Giudice · L. Michaud · E. De Domenico · N. Spanò

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Abstract A description of the bacterial communities associated with the Mediterranean pennatulids (sea pens) *Pennatula phosphorea* and *Pteroeides spinosum* from the Straits of Messina (Italy) is reported. The automated ribosomal intergenic spacer analysis showed a marked difference between coral (tissues and mucus) and non-coral (underlying sediment and surrounding water) habitats. The diversity of the coral-associated communities was more deeply analysed by sequencing the 16S rRNA genes of bacterial clones. *P. phosphorea* and *P. spinosum* harbour distinct bacterial communities, indicating the occurrence of species-specific coral-associated bacteria. In addition, only few phylotypes were shared between mucus and tissues of the same pennatulid species, suggesting that there might be a sort of microhabitat partitioning between the associated microbial communities. The predominance of *Alphaproteobacteria* was observed for the communities associated with both tissues and mucus of *P. phosphorea* (84 and 58.2 % of total sequences, respectively). Conversely, the bacterial community in the mucus layer of *P. spinosum* was dominated by *Alphaproteobacteria* (74.2 %) as opposed to the tissue library that was dominated by the *Gammaproteobacteria* and *Mollicutes* (40.6 and 35.4 %, respectively). The antibacterial activity of 78 bacterial isolates against indicator organisms was assayed. Active isolates (15.4 %), which predominantly affiliated to *Vibrio* spp., were mainly obtained from coral mucus. Results from the present study enlarge our knowledge on the composition and antibacterial activity of coral-associated bacterial communities.

E. M. D. Porporato and A. Lo Giudice equally contributed to the work.

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E. M. D. Porporato · A. Lo Giudice (✉) · L. Michaud · E. De Domenico · N. Spanò
Department of Biological and Environmental Sciences (DISBA), University of Messina, Viale F. Stagno d'Alcontrès 31, 98166 Messina, Italy
e-mail: alogiudice@unime.it

Introduction

Corals provide a multifaceted habitat with abundant and active microbial communities that reside in the host tissues and surface mucus layer [5]. There is a growing body of evidence that some of these microbial associates play important roles in coral physiology and health [4, 51, 56]. Microorganisms could be involved in different kinds of processes such as nutrient acquisition [63], processing of metabolic waste [46] and production of secondary metabolites, such as protective antibiotics and other biologically active compounds, which are able to select the holobiont communities [36, 53]. It has been demonstrated that the alterations of the environmental conditions could lead to a rapid change in the bacterial populations, allowing corals to overcome disease and perhaps develop resistance to certain microbial-driven diseases [44, 50, 56]. Rohwer et al. [49] suggested that microorganisms inhabiting the coral tissues and mucus may protect the holobiont from pathogens, and several coral-associated bacteria have been shown to possess antibacterial activity [39, 55, 56], antibiofilm [1, 41] and anti-pathogenic properties [2, 3].

Coral-associated bacterial communities are different from those that inhabit the surrounding environment (e.g. water column and sediment) and they seem to be largely coral species-specific. Similar bacterial populations were found on the same coral species that were geographically separated, whereas different populations were found on different coral species [15, 44, 47]. Further, the niches that are offered by corals (e.g. the coral tissue that represents a physiologically favourable niche for bacterial growth and the trapping of bacteria by mucus at the coral–seawater interface) harbour distinct and diverse bacterial species [5, 23, 26].

The study of the association between microorganisms and corals has been mainly conducted on specimens of hard corals, mostly collected from coral reefs, applying both culture-dependent and culture-independent methods (e.g. [5, 14, 48, 49]). In contrast, existing knowledge on the microbiology of soft corals is limited to a small number of reports that are mainly based on the application of culture-dependent techniques, therefore resulting in poor and fragmentary information [6, 17, 19, 37, 43, 61]. Among the soft corals, pennatulids (commonly known as sea pens) are the most complex and polymorphic members of the cnidarian class Anthozoa. They are approximately represented by 200 species in 34 genera [63] and display a wide distribution throughout all the oceans, largely restricted to soft bottoms, constituting a significant component of the sessile megafauna from intertidal to abyssal depths [12, 62–64, 65]. Nevertheless, the ecology and biology of sea pens remain poorly investigated, and a unique paper dealing with the associated microbial community exists [43].

The present study was aimed at describing the bacterial communities associated with the Mediterranean pennatulids *Pteroeides spinosum* Linnaeus, 1758 and *Pennatula phosphorea* Ellis, 1764. The bacterial communities on the coral body (tissues and mucus) and in the surrounding environments (underlying sediment and surrounding water) were firstly compared by a molecular fingerprinting technique, and the diversity of the coral-associated communities was more deeply analysed by sequencing the 16S rRNA genes of bacterial clones. Moreover, bacterial isolates from coral mucus and tissues were screened for antibacterial activity against indicator organisms.

Materials and Methods

Collection and Preliminary Treatment of Samples

Two replicates of each of the sea pen species *P. phosphorea* Ellis, 1764 (sample ID nos. A2 and A4) and *P. spinosum* Linnaeus, 1758 (sample ID nos. B1 and B6) were collected within a 10-m radius by scuba divers in the Straits of Messina, Italy (coordinates of 37°50′41.34″ N–15°17′31.417″ E; 35 m depth). The study was carried out on two specimens of each pennatulid species as the population density was very low (0.4 pennatulids m^{-2}) at sampling time.

Specimens were transferred directly to sterile plastic bags while underwater. Non-coral samples, i.e. seawater (W1 and W2) and sediment (ID nos. for sediment samples next to *P. phosphorea*: SA1 and SA2; ID nos. for sediment samples next to *P. spinosum*: SB1 and SB2), were also collected next to the pennatulid specimens. All samples were transported directly to the laboratory at 4 °C for microbiological processing (within 2 h after sampling). In particular, in the laboratory, specimens were immediately washed with filter-sterilised natural seawater to remove transient and loosely attached bacteria and/or debris and aseptically dissected using a sterile scalpel [35]. The mucus (ID nos. for mucus samples from *P. phosphorea*: A2M and A4M; ID nos. for mucus samples from *P. spinosum*: B1M and B6M) that was secreted by organisms during the dissection procedure was collected in sterile containers and treated, if possible, as described in the next paragraph for the homogenates.

The mucus was removed by centrifugation from the organism tissues which were subsequently aseptically weighted and homogenised in 0.22 μm filtered seawater using Ultraturrax. To avoid excessive heat production, such operation was carried out in ice.

Microbial Abundances

Total Counts

Samples for the estimation of total bacterial number were fixed with formaldehyde (final concentration, 2 %) and kept at 4 °C until processing. DAPI (4′,6-diamidino-2-phenilindole)-stained cells were counted by a Zeiss AXIOPLAN 2 imaging epifluorescence microscope as previously described [34]. More than 300 cells per sample were counted in randomly selected fields. Results were expressed in cells per gramme of organism tissue and sediment and in cells per millilitre of seawater and mucus.

Viable Counts

For viable counts, samples (tissue homogenates, mucus, sediment and seawater) were serially diluted using filter-sterilised seawater. Aliquots (100 μl) of each dilution were plated in triplicate on Marine Agar 2216 (MA; Difco). Such medium was chosen as it has been frequently used for the isolation of coral-associated bacteria (e.g. [22, 26, 30, 39]). Plates were incubated in the dark at 25 °C for 1 week. Data obtained are expressed as colony-forming units (CFU) per gramme of organism tissue and sediment and as CFU per millilitre of seawater and mucus.

Community Composition Analysis

DNA Extraction

Genomic DNA for automated ribosomal intergenic spacer analysis (ARISA) and clone library construction (see the next section) was extracted using the Ultra Clean Soil DNA Extraction Kit (MoBio) as recommended by Stepanauskas et al. [57] and Luna et al. [34]. In particular, seawater samples (5 L) were filtered on sterile 47-mm diameter, 0.22-μm pore size membranes (Millipore) and stored at −20 °C until processing. Extracted DNA was spectrophotometrically analysed for quantity and quality by Nanodrop device and amplified using an ABI 9600 thermocycler (PE; Applied Biosystems). Amplicons were purified from the polymerase chain reaction (PCR) mixture using the QIAquick PCR Purification Kit (Qiagen) according to the supplier's instructions.

Automated Ribosomal Intergenic Spacer Analysis

In order to study the bacterial community structure, we used the ARISA fingerprinting technique which amplifies the internal transcribed spacer (ITS) region in the rRNA operon plus approximately 282 bases of the 16S rRNA and 23S rRNA genes [7, 13]. Extracted DNA was amplified using universal bacterial primers 16S-1392F (5′-GYACACACCGCCCGT-3′) and 23S-125R (5′-GGGTTBCCCCATTCRG-3′). Primer 23S-125R was fluorescently labelled with the fluorochrome HEX (MWG; Biotech). PCRs were performed in triplicate in 50 μl volumes using an ABI 9600 thermocycler (PE; Applied Biosystems) with an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 90 s, with a final extension at 72 °C for 5 min.

A standardised amount of amplified DNA (100 ng) was purified with the QIAquik PCR Purification Kit (Qiagen, Germany) following the manufacturer's instructions and sent to an external sequencing service (BMR Genomics, Italy) in order to be resolved by capillary electrophoresis on an ABI PRISM 3130 Analyser (Applied Biosystems). The internal size standard was the ROX 2500 (Applied Biosystems), and the local southern size-calling method of the software GeneScan 3.7 (Applied Biosystems) was used.

To align the ARISA profiles of different runs, we binned the peaks in different fixed windows [18] depending on fragment length. All the peaks smaller than 350 bp and those longer than 1,300 bp size were not considered because the amplified fragments are composed by the ITS region plus 300–350 bp belonging to the next genes (about 200 bp from the 16S rRNA gene and 125 bp from the 23S rRNA gene) [8]. All peaks within 1.9 bp from a higher peak (commonly called "shoulder peaks") were eliminated. The detection threshold applied to the ARISA profiles was calculated according to the approach suggested by Luna et al. [34], and in our case, it was 0.2 % of the total fluorescence. The peak heights of all the ARISA profiles collected were standardised following the procedures described by Dunbar et al. [11].

Clone Libraries

Extracted DNA was also used to construct clone libraries. The PCR amplification of 16S rRNA genes was performed in triplicate using the forward primer 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer 907r (5′-CCGTCAATTCCTTTRAGTTT-3′). The reaction mixtures were assembled at 0 °C and contained 1–10 ng DNA, 10X buffer, 1.5 mmol L^{-1} $MgCl_2$, 150 ng of each forward and reverse primers, 250 μmol L^{-1} dNTP, 0.5 U of PolyTaq polymerase (Polymed) and sterile distilled water to a final volume of 20 μl. The PCR program was as follows: 3 min at 95 °C, followed by 30 cycles for 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C, with a final extension step of 10 min at 72 °C.

PCR products that derived from the same matrix (i.e. *P. spinosum* tissue, *P. phosphorea* tissue, *P. spinosum* mucus and *P. phosphorea* mucus) were pooled together prior to ligation, as follows. The 16S rDNA fragments were cloned into the pGEM Easy Vector System (Promega) according to the manufacturer's instructions. The resulting ligation products were used to transform *Escherichia coli* ElectroMAX DH10B cells (Invitrogen). Between 100 and 200 inserts were subsequently PCR-amplified from lysed white colonies using vector-specific primers, M13f (5′-GTAAAACGACGGCCAGT-3′ and M13r (5′-CAGGAAACAGCTATGACC-3′), under the same previously described PCR conditions. The results of all the amplification reactions were checked by agarose gel electrophoresis (1 %, *w/v*) in TAE buffer (0.04 M Tris–acetate, 0.02 M acetic acid, 0.001 M EDTA), containing 1 μg ml^{-1} of ethidium bromide.

Cultivable Bacteria Associated with Pennatulids

Bacterial Isolation

Bacteria were isolated from tissues and mucus of both pennatulid species. Colonies were randomly selected from MA plates used for viable counts, picked and subcultured almost three times under the same conditions (incubation at 25 °C for 1 week).

Screening for Inhibitory Activity Against Indicator Organisms

Indicator microorganisms used throughout this study were as follow: *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* ATTC 4698, *Bacillus subtilis* ATCC 6633, *Proteus mirabilis* ATCC 12453 and *Salmonella enterica* ATCC 14028, all maintained at +37 °C on nutrient agar (NA; Oxoid).

Three different screening methods were used, i.e. the cross-streaking method, overlay with soft agar and drop test. For the cross-streaking method, isolates were streaked across one third of an MA plate and incubated at 25 °C. After good growth was obtained (generally in 5–7 days, depending on the growth of the isolates), indicator organisms were streaked perpendicular to the initial streak. Plates were further incubated at 37 °C overnight and checked afterwards for inhibition zones. Each indicator organism was preliminarily grown individually on MA to ensure that any lack of growth depended on the medium used for screening. The antagonistic effect was indicated by the failure of the target strain to grow in the confluence area [32, 33].

For the overlay and drop tests, marine isolates were pre-cultured in Marine Broth 2216 (MB; Difco) with constant shaking at 120 rpm and allowed to reach an $OD_{600}=1.2$ prior to processing (about 48–72 h). For the overlay test, aliquots (100 µl) of the pre-culture were spread on MA plates and incubated at 25 °C for 5–7 days. Then, 8 ml of soft NA (0.7 % agar) mixed with 400 µl of an overnight culture at 37 °C of individual indicator bacteria were poured over MA plates. Inhibitory activity was defined by the absence of the growth of indicator organisms around the colonies of marine isolates after 18–24 h incubation at +37 °C. For the drop test, 10 µl of each marine isolate pre-culture were placed on NA plates containing 100 µl of an overnight culture at 37 °C of indicator bacteria spread as a lawn, followed by a further overnight incubation at 37 °C. Cell-free supernatant, produced by filtering marine isolate cultures through sterile 0.2-mm filters, was also tested for inhibitory activity using this technique. Activity was detected by the formation of an inhibition zone where the drop was placed [55]. Uninoculated MB was used as a negative control.

Each experiment was performed in duplicate. Inhibition had to be observed at least twice to be considered positive. If the first two assays showed ambiguous results, an additional assay was performed to re-assess inhibitory activity.

Amplification of 16S rRNA Gene from Isolates with Inhibitory Activity

A single colony of each isolate with inhibitory activity was lysed by heating at 95 °C for 10 min. PCR amplification of 16S rDNA was carried out under the conditions described previously for clones, with the exception of the two primers. In this case, the forward primer 27f (5′-AGAGTTTGATC CTGGCTCAG-3′) was used in addition to the reverse primer 1492r (5′-CTACGGCTACCTTGTTACGA-3′).

Sequencing of 16S rRNA Gene from Clones and Isolates

Automated sequencing of 16S rRNA gene from clone/isolates was carried out by cycle sequencing using the dye terminator method. Sequencing was carried out at the Sequencing Service of the Macrogen Laboratory (Korea). Closest relatives of isolates/clones were determined by comparison to 16S rRNA gene sequences in the NCBI GenBank and the EMBL databases using BLAST and the "SeqMatch" and "Classifier" programs of the Ribosomal Database Project II (http://rdp.cme.msu.edu/). Sequences were further aligned using the program CLUSTAL W [59] to the most similar orthologous sequences retrieved from the database. Each alignment was checked manually, corrected and then analysed using the neighbour-joining method [52] according to the model of Jukes–Cantor distances. The phylogenetic tree was constructed using the MEGA 5 (Molecular Evolutionary Genetics Analysis) software [28]. The robustness of the inferred trees was evaluated by 500 bootstrap re-samplings.

Nucleotide Sequence Accession Numbers

Nucleotide sequences have been deposited in the GenBank database under the accession nos. JX966192–JX966230.

Data Analyses and Diversity Indices

Data from ARISA were semi-automatically processed with the GeneScan software (Applied Biosystems). To obtain a significant quality resolution of the signal of the artificial community peaks with a distance of <1.9 bp (shoulder peaks) were excluded from major peaks.

The semi-quantitative analysis of the ARISA peaks was carried out, as suggested by Dunbar et al. [11]. A cluster analysis was carried out on ARISA peak profiles (based on a pre-computed Bray–Curtis similarity matrix) and results were super-imposed to a non-metric multidimensional scaling (nMDS) plot using the Primer 6 software, version 6β R6 (Copyright 2004, PRIMER-E Ltd.). To test differences in community composition, analysis of similarities (ANOSIM, based on Bray–Curtis similarity) was carried out. ANOSIM is a permutation-based statistical test, an analogue of the univariate ANOVA, which tests for differences between groups of (multivariate) samples. Results produce a sample statistic, *R*, which represents the degree of separation between test groups. A value close to 1 indicates that the community composition is totally different, whereas a value of 0 indicates no difference. For these calculations, as well as for the computation of diversity indices (see the next paragraph), it was assumed that the number of ARISA peaks represented the species number (genotype richness) and that the peak height represented the relative abundance of each bacterial species [10].

For clone libraries, the relative distribution of phylotypes in each sample was used to calculate coverage values [46] and the non-parametric Chao1 estimator [8], using the freely downloadable software SPADE [9]. The non-parametric Chao1 estimator

estimates the probable total number of phylotypes present in the sample [31].

Finally, the Shannon–Wiener index (H') and the Simpson reciprocal index ($1/D$) were computed on both ARISA and clone library results. The Shannon index (H') is a general diversity index that is positively correlated with species richness and evenness and is more sensitive to change in abundance of rare species. Simpson's index (D) is a dominance measure and shows the probability that two individuals chosen at random will be from the same species. The index is weighted towards the abundance of the commonest species. The use of $1/D$ instead of the original formulation of Simpson's index ensures that the value of the index ($1/D$) increases with increasing diversity. Calculations were performed using the Primer 6 software, version 6β R6 (Copyright 2004, PRIMER-E Ltd.).

Results

Microbial Abundances

Results from microbial abundance determinations are reported in Table 1. Total counts were generally two orders of magnitude higher than viable counts. Viable and total counts that were determined in association with pennatulids were in the ranges 0.6–19.6×10^3 CFU g^{-1} and 11.3–113.4×10^5 cells g^{-1}, respectively. Due to difficulty during the microscopical observation, microbial numbers in mucus samples were determined only by viable counts which were very low, being comprised between 0.2 and 1.3×10^3 CFU ml^{-1}. Abundances in seawater were in the ranges 10.1–18.9×10^3 CFU ml^{-1} and 5.4–8.6×10^5 cells-ml^{-1} for viable and total counts, respectively. Higher values were determined in sediment samples which were characterised by total and viable counts that ranged from 2,164.7 to 3,843.9×10^5 cells g^{-1} and from 168.3 to 202.5×10^3 CFU g^{-1}, respectively.

Table 1 Bacterial abundances (mean±standard deviation) that were determined in pennatulid specimens and environmental matrices

Matrix	Sample ID	Viable counts (CFU g^{-1}×10^3)	Total counts (cells g^{-1}×10^5)
P. phosphorea tissues	A2	19.6±8.4	11.3±4.9
	A4	7.4±3.6	38.0±17.6
P. spinosum tissues	B1	0.6±0.1	83.5±44.0
	B6	3.4±2.8	113.4±3.3
Sediment	SA1	202.5±70.0	3,843.9±2,669.1
	SA2	168.3±15.9	2,912.1±1,235.4
	SB1	175.9±18.5	2,164.7±1,045.9
	SB2	185.6±22.4	3,122.8±963.5
Matrix	Sample ID	Viable counts (CFU ml^{-1}×10^3)	Total counts (cells ml^{-1}×10^5)
P. phosphorea mucus	A2M	0.3±0.2	nd
	A4M	1.3±0.3	nd
P. spinosum mucus	B1M	1.2±0.2	nd
	B6M	0.2±0.1	nd
Water	W1	10.1±1.6	5.4±1.2
	W2	18.9±2.2	8.6±0.3

nd not determined

Table 2 Number of ARISA ribotypes, Shannon index and Simpson reciprocal index per sample

Matrix	Sample ID	Ribotypes (no.)	H'	$1/D$
P. phosphorea tissues	A2	60	3.13	11.46
	A4	52	3.11	10.37
P. spinosum tissues	B1	71	3.41	17.60
	B6	54	3.47	22.02
P. phosphorea mucus	A2M	92	3.87	33.01
	A4M	70	3.40	15.63
P. spinosum mucus	B1M	60	3.55	20.23
	B6M	66	3.55	20.76
Sediment	SA1	74	3.57	21.78
	SA2	67	3.60	22.30
	SB1	70	3.39	16.25
	SB2	65	3.33	17.36
Water	W1	61	3.24	12.94
	W2	44	3.04	14.76

Community Composition Analysis

Automated Ribosomal Intergenic Spacer Analysis

Overall, a total of 184 ribotypes emerged from ARISA. In detail, the number of ribotypes ranged from 52 to 92 in pennatulid samples (coral habitats) and from 44 to 74 in abiotic samples (non-coral habitats) (Table 2).

The combined representation of nMDS plots and cluster analysis of ARISA profiles from coral and non-coral habitats indicated a pronounced separation between three larger clusters (30 % Bray–Curtis similarity): (1) a cluster that was made of *P. phosphorea* and *P. spinosum* tissues, (2) a cluster that grouped sediments and animal mucus and (3) a cluster that was composed only by water samples (Fig. 1). Moreover, pennatulid tissues were grouped separately at a similarity of 50 %, as well as mucus and the four sediment samples. ANOSIM test computed with matrix/sample as a factor indicated a global R of 0.968 (P=0.001), thus confirming the separation of communities.

Clone Libraries

The composition of the microbial communities was investigated by the analysis of 16S rRNA gene sequences. Overall, a total of

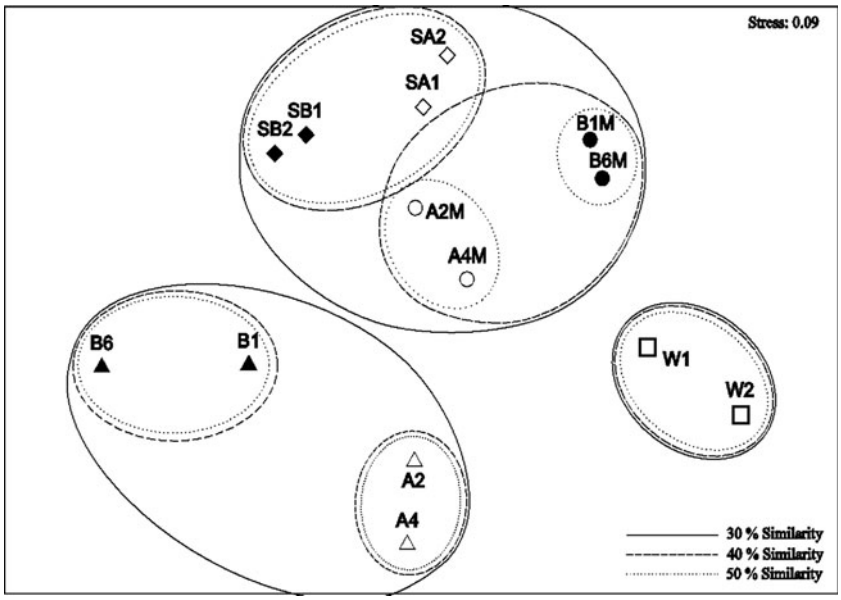


Fig. 1 nMDS ordination plot (Bray–Curtis distance matrix) of ARISA profiles for coral (mucus and tissues) and non-coral (sediment and water) samples. *Open triangles P. phosphorea* tissue samples, *filled triangles P. spinosum* tissue samples, *open circles P. phosphorea* mucus samples, *filled circles P. spinosum* mucus samples, *open squares* water samples, *open diamonds P. phosphorea* sediment samples, *filled diamonds P. spinosum* sediment samples)

374 clones (96 and 93 from *P. spinosum* tissues and mucus, respectively; 91 and 94 from *P. phosphorea* tissues and mucus, respectively) were screened. Clones were named with the suffix PP (*P. phosphorea*), PS (*P. spinosum*) and M (mucus), followed by a number. All sequences with similarity ≥97 % were considered to represent one phylotype.

Results in Table 1S and Fig. 2 are based on the BLAST and SeqMatch/Classifier analyses of the sequences and also on the phylogenetic affiliation of clones as depicted in Fig. 3. Overall, the phylogenetic analysis of sequences from clones revealed the distribution of pennatulid-associated bacteria within 29 separated phylotypes (listed in Table 1S) that predominantly fell into the *Proteobacteria* (including *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria*; 86.4 %), followed by the *Mollicutes* (9.6 %), *Spirochaetes* (2.9 %), *Actinobacteria* (0.8 %) and the *Cytophaga–Flavobacteria–Bacteroides* (CFB) group of *Bacteroidetes* (0.3 %). In particular, the phylotypes within the *Alphaproteobacteria* formed two distinct clusters: the first one included unidentified organisms, whereas the second cluster branched into two sub-clusters that were represented by bacteria closely related to *Rhodobium* spp., *Sphingomonas* spp. and *Paracoccus* spp. or, again, by unidentified bacteria (Fig. 3a). The *Betaproteobacteria* also clustered into two main groups

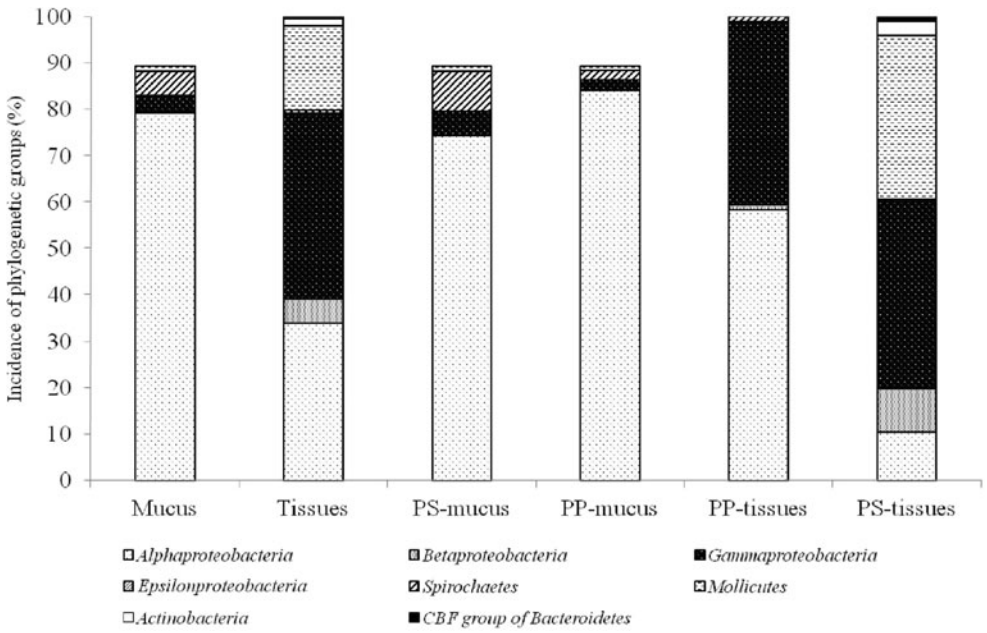


Fig. 2 Percentage of sequences from clones determined per matrix and pennatulid species. *Mucus* mucus from both pennatulid species, *Tissues* tissues from both pennatulid species, *PS-mucus* mucus from *P. spinosum*, *PP-mucus* mucus from *P. phosphorea*, *PS-tissues* tissues of *P. spinosum*, *PP-tissues* tissues of *P. phosphorea*

(Fig. 3a). The first one was composed by a single phylotype (i.e. PP6) which was strongly related to an unidentified bacterium. The larger group branched into two sub-clusters represented by *Ralstonia* spp. and *Janthinobacterium* spp., respectively (Fig. 3a). The *Gammaproteobacteria* contained nine phylotypes distributed among several genera of the phylum, e.g. *Pseudomonas*, *Thalassolituus*, *Pseudoalteromonas*, *Halomonas*, *Alcanivorax* and *Psychrobacter*, with latter forming a separate branch. The remaining phylogenetic groups (i.e. *Spirochaetes*, *Actinobacteria*, *Mollicutes*, *Epsilonproteobacteria* and CFB group of *Bacteroidetes*) were represented by few clone sequences, which were generally related to unidentified organisms (Fig. 3a, b).

Phylotype sharing between mucus and tissues of the same pennatulid species was rarely observed (Table 1S). In addition, ten bacterial phylotypes were shared between the two pennatulids with some sequences that appeared to be exclusively associated to their mucus or tissues. As it is shown in Fig. 2, sequences affiliated to the *Alphaproteobacteria* and *Gammaproteobacteria* were detected in all samples (both tissues and mucus), although to different extents (range of 10.4–84.0 and 2.1–40.6 %, respectively). In particular, the *Alphaproteobacteria* generally predominated in all the analysed samples. An exception was the *P. spinosum* mucus that hosted a higher percentage of *Gammaproteobacteria* and *Mollicutes* (40.6 and 35.4 %, respectively). The *Betaproteobacteria* and *Epsilonproteobacteria* occurred in both host species only in tissues or mucus, respectively. Finally, the *Actinobacteria* and CFB group of *Bacteroidetes* were exclusively detected in *P. spinosum* tissue samples.

Table 3 shows the diversity indices and coverage values calculated for each library. The high diversity coverage (90.1 to 94.6 %) confirmed the sufficient number of clones that were analysed (*P. phosphorea* tissues and *P. spinosum* mucus, respectively). The Shannon diversity (H') index ranged from 0.78 to 1.93 (*P. phosphorea* mucus and tissues, respectively), while the reciprocal Simpson dominance ($1/D$) index ranged from 1.5 to 4.77 (*P. phosphorea* mucus and *P. spinosum* tissues, respectively).

Cultivable Bacteria Associated with Pennatulids

Bacterial Isolation, Inhibitory Activity and Phylogenetic Identification

A total of 78 strains were isolated from pennatulids and their mucus. Among them, 44 isolates were obtained from *P. phosphorea* (i.e. 26 from tissues and 18 from mucus) and 34 from *P. spinosum* (i.e. 20 from tissues and 14 from mucus).

All isolates were screened for antibacterial activity against seven indicator organisms using three different methods, i.e. the cross-streaking, overlay and drop tests. No activity was put on evidence neither by the drop test when using cell-free supernatant or the cross-streaking method. Conversely, the overlay and drop tests that were both carried out using culture broths allowed the identification of 12 isolates (detection rate of 15.4 %) that expressed inhibitory activity against at least 1 indicator organism (Table 4). Among them, eight isolates showed activity by the overlay technique, two by the drop test and two by both methods. Active strains were mostly isolated from tissues (six isolates) and mucus (two isolates) of *P. spinosum*. Conversely, active isolates associated with *P. phosphorea* derived exclusively from mucus (four isolates).

Selected isolates were mainly placed within the *Gammaproteobacteria* and they predominantly belonged to the genus *Vibrio*. A unique isolate (strain PS-62) was strongly related to the species *Bacillus pumilus* among the *Firmicutes*. Each of them inhibited the growth of a single indicator organism. An exception was the isolate *Vibrio* sp. PP-106 that showed inhibitory activity against all the targets used in this study.

Overall, *M. luteus*, *S. aureus* and *P. mirabilis* appeared to be the most sensitive targets. In particular, inhibition of *M. luteus* and *P. mirabilis* was mainly observed using the overlay technique, whereas *S. aureus* appeared to be sensitive to marine bacteria mainly when it was assayed by the drop test.

Discussion

The present study characterises the bacterial communities associated with the Mediterranean pennatulids *P. phosphorea* and *P. spinosum* (Anthozoa: Octocorallia) which are commonly known as sea pens. To the best of our knowledge, information on the microbial community associated with soft corals is restricted to a few reports [6, 17, 19, 37, 61], with only one that reports on the isolation of a luminous *Vibrio* from *Pteroeides* sp. [43].

Two culture-independent molecular techniques (ARISA and clone libraries) were applied with the aim to investigate the structure and composition of the bacterial communities associated with the two coral species. The statistical nMDS performed on ARISA data showed that ribotypes mainly clustered according to the sample origin (coral and non-coral habitats). Coral-associated ribotypes and ribotypes recovered from the underlying sediments and the surrounding seawater (non-coral habitats) showed a no evident overlap, even if all the analysed matrices were assumed to be exposed to the same pool of bacterial colonisers. Additionally, different bacterial communities were harboured by the two analysed species of pennatulids. Despite the limitation of ARISA (including PCR bias), this method provided, with statistically relevant data, sufficient experimental evidence to demonstrate that a minimal or null influence of transient microbes that derive from the surrounding environment

probably occurs, thus supporting the hypotheses of the existence of symbiotic relationships between corals and the associated bacterial communities [5, 14, 16, 17, 27, 29, 42, 48, 54, 61].

Mucus and tissue samples were deeply analysed by clone library construction. Overall, the predominance of *Alphaproteobacteria* and *Gammaproteobacteria* was observed, supporting the observation by Bourne and Munn [5] on their possible symbiotic function related to nutrient uptake. The *Alphaproteobacteria* and *Gammaproteobacteria* have been previously reported as the most common group in other corals [5, 6, 14, 17, 24, 25, 29, 48, 49, 61]. Despite the predominance of the *Alphaproteobacteria* observed for both the bacterial communities associated with mucus and tissues of *P. phosphorea* (84.0 and 58.2 %, respectively), the *Gammaproteobacteria* also constituted a large portion (39.6 %) of sequences within the *P. phosphorea* tissue clone library. Conversely, the bacterial community in the mucus layer of *P. spinosum* was dominated by the *Alphaproteobacteria* (74.2 % of total sequences) as opposed to the tissue library that was dominated by the *Gammaproteobacteria* and *Mollicutes* (40.6 and 35.4 %, respectively). Interestingly, this latter group was particularly abundant in tissue samples of *P. spinosum*. The specific association between tissues of the scleractinian coral *Lophelia pertusa* and the "*Candidatus* Mycoplasma corallicola" was recently observed by Kellogg et al. [20] and Neulinger et al. [38]. These authors suggest that these *L. pertusa*-associated mycoplasmas live as commensals, in contrast to many of their parasitic relatives, by profiting from the prey capture activity of the host.

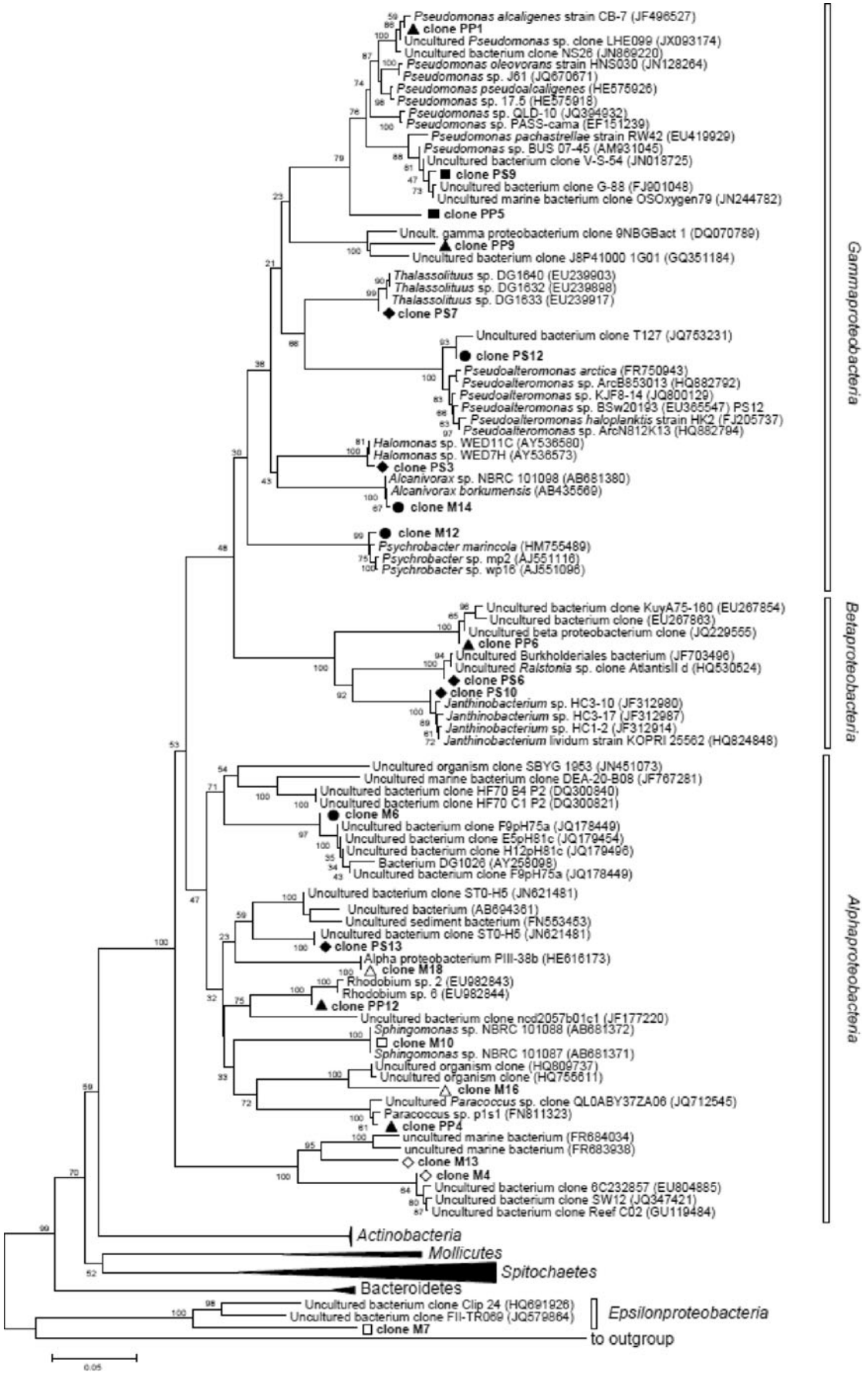
The remaining bacterial groups in *P. phosphorea* and *P. spinosum* were *Betaproteobacteria* and *Epsilonproteobacteria*, *Spirochaetes*, *Actinobacteria* and the CFB group of *Bacteroidetes*, which have been all reported also as a minor fraction of the bacterial community of other corals [6, 14, 20, 25, 26, 49]. In particular, *Spirochaetes* are generally free-living bacteria. However, their association with corals was also reported by Kellogg et al. [20] in the scleractinian coral *L. pertusa*. Interestingly, all the *Betaproteobacteria* (three phylotypes) and *Epsilonproteobacteria* (a single phylotype) occurred in both host species, but they were restricted to tissues or mucus. Similar results were also obtained for few additional phylotypes within the *Alphaproteobacteria* (clone *Sphingomonas* sp. M10 which was restricted to mucus samples), *Gammaproteobacteria* (clones *Pseudomonas* spp. PP5 and PS9 which were restricted to tissue samples) and *Spirochaetes* (unidentified clone M3, restricted to mucus samples). Such phylotypes may represent bacteria specifically adapted to these particular microbial niches [47, 58].

At the phylotype level, consistently with the nMDS elaboration of the data from ARISA, the composition of the associated bacterial community of *P. phosphorea* and *P. spinosum* by 16S rRNA gene sequencing appeared to be very different, even if the specimens were physically adjacent to one another during sampling, with only ten phylotypes that were shown to be common to both coral species. As it was stated by Neulinger et al. [38], a truly "specific" coral–microbial association has to be constant over time and space. Nevertheless, the results of the present study indicate the occurrence of species-specific coral-associated bacteria, and they are in agreement with the findings about other coral species [30, 48, 49].

The phylogenetic analysis of the clone libraries displayed a marked difference between the bacterial communities of mucus and tissues of each individual coral species, with only five phylotypes that were shown to be common to both coral compartments (i.e. *Alcanivorax* sp. M14, *Pseudoalteromonas* sp. PS12, *Psychrobacter* sp. M12, the unidentified alphaproteobacterial phylotype M6 and the unidentified *Mollicutes* phylotype M15). Such results suggest that there might be a sort of microhabitat partitioning between the associated microbial communities and strengthen previous results on other coral species [5, 54, 58]. The mucus layer on the surface of coral constitutes a special microenvironment that is rich in nutrients supporting bacterial growth. It is continuously produced and removed to facilitate the elimination of detrital particles from the body surface, thus creating a highly unstable environment for bacteria associated with it. Nevertheless, a distinct and specialised resident bacterial community, rather than a passive settlement and entrapment of water-borne bacteria, is supported by the mucus layer [5, 14, 30, 58].

Experimental data suggest that soft corals, rather than hard corals, can produce antibiotic substances as prevention against fouling organisms or defence against microbes which are often the causes of coral diseases [21, 22, 55, 58]. However, the resistance to disease is among the hypothesised roles that are played by coral-associated bacteria [44, 47, 50], rather than by the coral itself, via competition for nutrients and/or space and/or production of antibiotics [36]. Moreover, bacteria–bacteria interactions could drive the composition of

Fig. 3 Rooted phylogenetic tree calculated by Jukes–Cantor distance estimation algorithm showing the affiliation of clones to the closest-related sequences from either cultivated or cloned bacteria. Percentages of 500 bootstrap re-sampling that supported the branching orders in each analysis are shown above or near the relevant nodes. The tree was out-grouped with 16S rRNA gene sequence of *Methanocaldococcus jannaschii* DSM 2661. *Filled triangles* sequences only from *P. phosphorea* tissue samples, *open triangles* sequences only from *P. phosphorea* mucus samples, *filled diamonds* sequences only from *P. spinosum* tissue samples, *open diamonds* sequences only from *P. spinosum* mucus samples, *filled squares* sequences common to mucus from *P. spinosum* and *P. phosphorea*, *open squares* sequences common to tissues of *P. spinosum* and *P. phosphorea*, *filled circles* sequences common to almost all samples. **a** Phylogenetic tree for *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria*; **b** phylogenetic tree for other groups



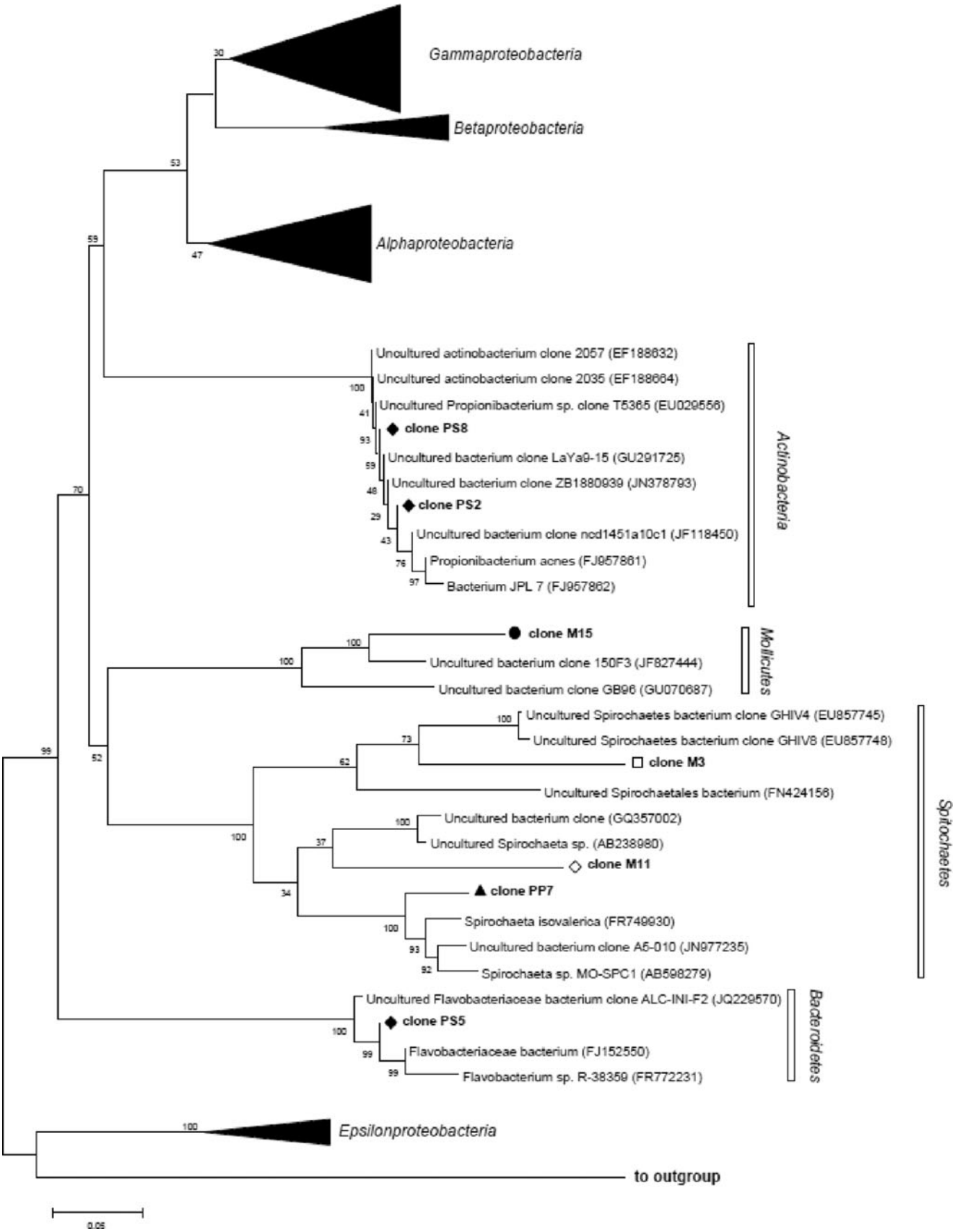


Fig. 3 (continued)

Table 3 Diversity indices, coverage values and estimators calculated for each library

Index	Mucus		Tissues	
	P. spinosum	*P. phosphorea*	*P. spinosum*	*P. phosphorea*
Taxa (*S*)	10	9	15	12
Individuals	93	94	96	91
Shannon (*H'*)	1.11	0.78	1.36	1.93
Simpson (1/*D*)	1.91	1.50	4.77	2.65
Coverage (%)	94.6	93.6	92.7	90.1
Chao	22.5	27.0	23.2	48.0

coral-associated microbial communities. Several studies have demonstrated the antibacterial activity of coral-associated bacteria against indicator microorganisms and putative pathogens of corals, such as *Vibrio shiloi*, *Vibrio coralliilyticus* and *Serratia marcescens* [39, 45, 55]. In the present study, the culturable members on MA of the microbial community associated with *P. phosphorea* and *P. spinosum* were screened in order to determine their activity against indicator bacteria. Only two strains (i.e. *Bacillus* sp. PS-62 and *Vibrio* sp. PP-93) gave positive responses by both the overlay and the drop tests, suggesting different mechanisms of antibiotic activity. The lack of antibiotic activity of the *cell-free* supernatants from the active strains suggests that the observed antibiotic activity could be due to the production of cell-linked substances rather than to their release into the external medium or the alteration of the culture medium features (in terms of nutrient availability or pH) [55].

The incidence of activity that was obtained using three different screening methods was higher among strains that were isolated from mucus (6 isolates out of 32; 18.8 %) rather than among those obtained from tissues (6 isolates out of 46; 13.0 %). A variety of percentages of active coral-associated bacteria are reported in the literature. For example, in a study carried out on bacteria associated with the mucus of *Acropora palmata*, Ritchie [45] reported a detection rate of 20 %. Higher percentages of bacteria displaying antibiotic activity were found by Nithyanand and Pandian [40] for bacteria associated with *Acropora digitifera* (25 and 48 % in mucus and tissues, respectively). Conversely, bacteria showing inhibitory activity accounted only for 5.8 % of the community associated with *Oculina patagonica* against *V. shiloi* [39]. However, it could be noted that the differences among the detection rates reported in the literature strongly depend on both the isolation and assay procedures (including the organisms used as a target and the media employed), as well as on the sources (e.g. mucus or tissues) of bacterial isolates.

Strains with inhibitory activity mostly belonged the genus *Vibrio* (10 out of 12), with only 2 strains that clustered within

Table 4 Inhibitory activity of pennatulid-associated isolates against indicator organisms

Test	Matrix	Strain	AN	Next relative by GenBank alignment (AN, organism, similarity)	Inhibition of						
					Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Micrococcus luteus*	*Bacillus subtilis*	*Proteus mirabilis*	*Salmonella enterica*
Overlay	*P. spinosum*	PS-59	JX966202	EU660364, *Vibrio parahaemolyticus* strain CT12, 99 %	−	−	−	+	−	−	−
		PS-60	JX966203	EU660364, *Vibrio parahaemolyticus* strain CT12, 99 %	−	−	−	+	−	−	−
		PS-62	JX966213	EF638794, *Bacillus pumilus* strain A55, 99 %	−	−	+	−	−	−	−
		PS-87	JX966204	EU660364, *Vibrio parahaemolyticus* strain CT12, 99 %	−	−	−	+	−	−	−
		PS-88	JX966205	EU660364, *Vibrio parahaemolyticus* strain CT12, 99 %	−	−	−	+	−	−	−
		PS-89	JX966206	EU660364, *Vibrio parahaemolyticus* strain CT12, 99 %	−	−	−	+	−	−	−
	P. phosphorea mucus	*PP-93*	JX966207	EF587978, *Vibrio* sp. UST061013-037, 99 %	−	−	−	−	−	+	−
		PP-96	JX966208	EU660364, *Vibrio parahaemolyticus* strain CT12, 99 %	−	−	−	−	−	+	−
	P. spinosum mucus	PS-150	JX966211	HM771345, *Vibrio chagasii*, 100 %	−	−	−	−	−	+	−
		PS-152	JX966212	EU277001, *Enterovibrio* sp. 4.5.0412DS11, 98 %	−	−	−	−	−	+	−
Drop	*P. spinosum*	*PS-62*	JX966213	EF638794, *Bacillus pumilus* strain A55, 99 %	−	−	+	−	−	−	−
	P. phosphorea mucus	*PP-93*	JX966207	EF587978, *Vibrio* sp. UST061013-037, 99 %	−	−	+	−	−	−	−
		PP-106	JX966209	AJ421444, *Vibrio penaeicida* strain DSM 14398T, 99 %	+	+	+	+	+	+	+
		PP-124	JX966210	EU660364, *Vibrio parahaemolyticus* strain CT12, 99 %	−	−	+	−	−	−	−

Strains that gave positive responses by both the overlay and drop tests are in italics

AN accession number

the genera *Bacillus* and *Enterovibrio* (PS-62 and PS-152, respectively). This is in accordance with previous reports that showed several members within such genera from corals exhibiting antibacterial activity [10, 40, 55]. *Bacillus* spp., which are well-known producers of antimicrobial compounds, seem to play a protective role in the coral host [55]. *Enterovibrio* spp. have been reported in association with corals [60], and their antibacterial activity has been recently reported by Chen et al. [10]. However, Ritchie [45] indicated *Enterovibrio* spp. as transient and/or potentially invasive microbes in *A. palmata*. This is not to be excluded for *Enterovibrio* sp. PS-52 that was isolated from *P. spinosum* mucus (in direct contact with the bulk water). Interestingly, among vibrios isolated in the present study, seven strains (five from *P. spinosum* and two from *P. phosphorea*) were closely related to *Vibrio parahaemolyticus* strain CT12 (AN EU660364), which was previously retrieved from tissues of *A. digitifera* [40]. This finding could confirm that the same bacterial species may be present in association with different species of coral, although geographically distant [55], indicating also that a beneficial association between such corals and *V. parahaemolyticus* could be established. In particular, the *V. parahaemolyticus* strains from tissue samples were able to inhibit the growth of *M. luteus* rather than *P. mirabilis* and *S. aureus*, as it was observed for all *V. parahaemolyticus* isolated from mucus. This result suggests that closely related strains that derive from different matrices can acquire different skills on the basis of the interactions developing within the associated bacterial community. It should be noted that vibrios are often the cause of disease in corals [39, 51]. However, according to Ritchie [45], the presence of vibrios in healthy corals is probably due to the ability of these bacteria to establish mutualistic relationships with their hosts by providing nutrients and secondary metabolites. The same author also argues that coral-associated vibrios are able to produce antibacterial compounds against several pathogens, thereby protecting the host.

This study provides a description of the bacterial communities associated with the Mediterranean pennatulids *P. phosphorea* and *P. spinosum*. The results show a non-evident overlap between coral (mucus and tissues) and non-coral habitats (underlying sediments and surrounding seawater), even if they were exposed to the same pool of bacterial colonisers. The analysed pennatulids not only harbour distinct bacterial communities but also in each only few phylotypes were shared between mucus and tissues, suggesting that there might be a sort of microhabitat partitioning between the associated microbial communities.

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