Microb Ecol (2013) 66:972–985
DOI 10.1007/s00248-013-0267-3

INVERTEBRATE MICROBIOLOGY

Bacterial Communities of the Gorgonian Octocoral *Pseudopterogorgia elisabethae*

Hebelin Correa · Brad Haltli · Carmenza Duque · Russell Kerr

Received: 17 July 2012 / Accepted: 9 July 2013 / Published online: 4 August 2013
© Springer Science+Business Media New York 2013

Abstract *Pseudopterogorgia elisabethae* is a common inhabitant of Caribbean reefs and is a well-known source of diterpenes with diverse biological activities. Notably, this octocoral is the sole source of the pseudopterosin family of anti-inflammatory diterpenes and is harvested to supply commercial demand for these metabolites. We have characterized the composition of the bacterial community associated with *P. elisabethae* collected from Providencia Island, Colombia, using both culture-dependent and culture-independent approaches. Culture-independent analysis revealed that the bacterial communities were composed of eight phyla, of which Proteobacteria was the most abundant. At the class level, bacterial communities were dominated by Gammaproteobacteria (82–87 %). Additionally, operational taxonomic units related to *Pseudomonas* and *Endozoicomonas* species were the most abundant phylotypes consistently associated with *P. elisabethae* colonies. Culture-dependent analysis resulted in the identification of 40 distinct bacteria classified as Bacilli (15), Actinobacteria (12), Gammaproteobacteria (9), Alphaproteobacteria (3), and Betaproteobacteria (1). Only one of the 40 cultured bacteria was closely related to a dominant phylotype detected in the culture-independent study, suggesting that conventional culturing techniques failed to culture the majority of octocoral-associated bacterial diversity. To the best of our knowledge, this is the first characterization of the bacterial diversity associated with *P. elisabethae*.

H. Correa · C. Duque
Departamento de Química, Universidad Nacional de Colombia, Bogotá, District of Colombia, Colombia

H. Correa · B. Haltli · R. Kerr
Department of Chemistry, University of Prince Edward Island, Charlottetown, Canada

R. Kerr (✉)
Department of Biomedical Sciences, University of Prince Edward Island, Charlottetown, Canada
e-mail: rkerr@upei.ca

Introduction

Studies examining bacterial diversity associated with marine invertebrates have demonstrated that these bacterial communities are highly diverse and distinct from the surrounding environment. The composition of invertebrate-associated bacterial communities has been observed to vary according to both host species and collection location, suggesting that the host invertebrate and environmental factors influence the establishment and maintenance of invertebrate–microbe associations [1–3]. The biological role of invertebrate-associated bacteria is largely unknown; however, they may play important roles in nutrient cycling, response and adaptation to environmental changes and defense against predators, pathogenic microbes, and fouling agents [4–8]. While the majority of studies regarding invertebrate microbial ecology have been conducted on scleractinian corals and sponges, octocorals have also been shown to host diverse bacterial communities [9–12]. Among octocorals, *Pseudopterogorgia elisabethae* is unique due to its importance as the sole commercial source of the pseudopterosins, a family of diterpene natural products (NPs) used in a variety of topical cosmetic products due to their potent anti-inflammatory properties [13]. Despite the economic importance of *P. elisabethae*, the microbial community associated with this invertebrate has yet to be studied. Exploring the microbiome of *P. elisabethae* will provide important information regarding the microbial community of healthy specimens, which may be invaluable for the future population management of this commercially harvested octocoral species.

Marine invertebrates are a rich source of bioactive metabolites, with nearly 10,000 NPs identified between 1990 and 2009 [14, 15]. Sponges (Porifera) and corals (Cnidaria) are the primary sources of invertebrate-derived marine NPs, accounting for 49 and 29 % of discovered metabolites, respectively. Within Cnidaria, approximately 94 % of metabolites have been isolated from organisms belonging to the subclass Octocorallia [15]. Despite the reported diversity of octocoral bacterial communities and the wealth of NPs isolated from octocorals [9–12, 14, 15], few studies have investigated octocoral-associated bacteria for the ability to produce bioactive NPs [16–19]. In contrast, bioprospecting from other marine ecotopes, such as sponges and sediments, has shown marine bacteria to be a rich source of bioactive metabolites [20–24]. Consequently, octocoral-associated microorganisms may represent an untapped resource for the discovery of novel marine NPs.

To gain insight into the bacterial community associated with Colombian *P. elisabethae* specimens collected at Providencia Island, we have characterized the associated bacterial community using both culture-dependent and culture-independent approaches. Culture-independent bacterial community analysis was accomplished by bacterial tag-encoded FLX amplicon pyrosequencing of small subunit ribosomal gene (16S) amplicons generated from genomic DNA purified from *P. elisabethae*. Culture-dependent microbial diversity was investigated by dilution plating of octocoral samples on agar media. Taxonomic affiliations of purified isolates were determined by partial small subunit ribosomal (16S rRNA) gene sequencing.

Materials and Methods

Sample Collection

Samples from three *P. elisabethae* colonies (*Pe*A, *Pe*B, and *Pe*C) were collected within a 20 m radius by SCUBA (ca. 20–30 m depth) at El Planchon (13°25′0″N and 81°23′0″W), Providencia Island, Colombia. Previous research determined that NP content is greatest in the tips of *P. elisabethae* branches [25]; thus, samples were collected from this region. Six samples, each possessing 8–12 branchlets, were obtained from each colony. Voucher specimens (INV CNI 1612–1614) were deposited at the invertebrate collection of Museo de Historia Natural Marina Colombiana at Instituto de Investigaciones Marinas de Punta Betín. Specimens were transported to the laboratory within 2 h of collection and either immediately processed aseptically for culture-based analysis or preserved for DNA extraction. To preserve tissue for DNA extraction ca. 2 g of tissue from each sample was rinsed briefly with sterile Instant Ocean® (IO) to remove loosely associated bacteria, aseptically transferred to sterile tubes containing biological grade phenol (pH 8.0), and stored at −80 °C. A seawater sample (100 ml) adjacent to *Pe*B (depth, 26 m) was collected in a sterile plastic bag to serve as a seawater control for culture-dependent analyses.

Culture-Independent Characterization of *P. elisabethae*-Associated Bacterial Diversity

DNA Extraction and Purification

DNA was extracted from preserved octocoral tissue using a modified phenol–chloroform procedure [26]. Thawed tissue samples (0.5 g) were removed from phenol, pulverized in liquid nitrogen, and suspended in 5.0 ml of lysis buffer. Large tissue fragments were removed by centrifugation (100×*g*, 2 min), and the supernatant was amended with 1 μl RNase One (Promega, Madison, WI), 6 mg lysozyme (Thermo Scientific, Hampton, NH), 1 ml polyvinylpyrrolidone (9.0 mg ml^{-1}), and 0.3 ml sodium dodecyl sulfate (10 %*w*/*v*) and incubated for 20 min at 37 °C. Proteinase K (6 mg; Promega, Madison, WI) was added, and the solution was incubated at 55 °C for 2.5 h. Insoluble material was removed by centrifugation at 4,500×*g* for 15 min. Sodium acetate was added to the supernatant (0.3 M), and the supernatant was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase by the addition of 0.7 vol of isopropanol followed by centrifugation (10,000×*g*, 20 min). To remove co-extracted PCR inhibitors, DNA samples were further purified using the PowerClean® DNA Clean-Up Kit (MO BIO Laboratories, Inc., Carlsbad, CA). DNA was eluted with 50 μl of 10 mM Tris–HCl, and samples were diluted to 200 ng μl^{-1} prior to pyrosequencing.

Pyrosequencing of 16S rDNA Amplicons and Data Processing

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed by Research and Testing Laboratories (Lubbock, TX) (www.researchandtesting.com) as described previously [27]. To reduce the effect of sequencing artifacts on downstream data analysis, sequence data were processed using mothur version 1.28.0 [28] according to published recommendations [29, 30]. Briefly, sequence data were denoised using the mothur implementation of PyroNoise (shhh.flows), and sequences were removed if they contained homopolymers greater than 8 bp in length, ambiguous bases, more than one mismatch to barcode sequences and more than two mismatches to the forward primer sequence, or were shorter than 250 bp. Sequences passing these quality control criteria were aligned in mothur using the Silva reference alignment obtained from the mothur

website (http://www.mothur.org/wiki/Silva_reference_alignment). Alignments were screened to ensure the sequences overlapped in the same alignment space. Alignments were filtered to remove gaps, and then sequences were pre-clustered using a "diffs" setting of 2. Chimeras were identified using UCHIME and removed from the analysis. Sequences were classified using the mothur Bayesian classifier (80 % confidence) utilizing the mothur-formatted version of the Ribosomal Database Project (RDP) training set (v. 9) [31]. Sequences classified as chloroplasts, mitochondria, or "unknown" (sequences not classified at the kingdom level) were removed from the analysis. Analysis of the taxonomic affiliations of the remaining sequences by nucleotide basic local alignment search tool (BLASTN) [32] searching of GenBank revealed the presence of several chloroplast sequences which were identified as "unclassified bacteria" in mothur. These sequences were manually identified as chloroplast sequences and removed from the analysis. Sequence data have been archived in the NCBI Short Read Archive under accession number SRA043548.

Bacterial Diversity Analysis

Operational taxonomic units (OTUs) were identified using mothur as described previously [30]. OTUs were classified as described above, but using the quality filtered dataset. Graphs of order level taxonomic composition of *P. elisabethae* microbial communities were prepared using Microsoft Excel (2007). Observed richness (S_{obs}), estimated richness (Chao1), and the Shannon diversity (H') and equitability (E) indices were calculated using mothur. To enable more accurate comparisons between coral colonies, calculations were performed on datasets normalized to 1,947 sequences (equivalent to the smallest sample size). To assess richness using another commonly used analysis program, OTUs were also identified using the RDP Pyrosequencing Pipeline (http://pyro.cme.msu.edu/index.jsp) [33]. Processed sequences generated via mothur were aligned using the INFERNAL Aligner program in the RDP Pyrosequencing Pipeline and then clustered using the RDP Complete Linkage Clustering application.

Identification of Ten Most Abundant OTUs

OTUs identified by mothur (without normalization of the number of sequences in each sample) at the species level (distance of ≤0.03) were sorted based on OTU abundance. Representative sequences of each cluster were obtained using mothur, and BLASTN searching of the GenBank database was used to infer the taxonomic affiliations of representative sequences [32].

Characterization of the Culturable Bacterial Community Associated with *P. elisabethae*

Isolation

A 10 cm length of tissue from each specimen was rinsed with sterile IO to remove loosely associated bacteria. Tissue was cut into thin sections using a sterile scalpel and was homogenized under sterile conditions for 3 min (20 °C) in 100 ml of sterile phosphate-buffered saline (pH 7.2) using a commercial Universal blender. To isolate potential epiphytes, the surface of each colony (~10 cm^2) was swabbed with a sterile cotton bud, and the swabbed material was dispersed in 1 ml of sterile IO. Serial dilutions of homogenized octocoral tissue samples, surface swab suspensions, and the seawater sample were prepared using sterile IO, and 100 μl of the 10^{-4} and 10^{-6} dilutions were spread on triplicate plates of Difco Marine Agar 2216 (MA) and Difco Sabouraud Dextrose Agar (SD) prepared with deionized water according to the manufacturer's recommendations and amended with cycloheximide (250 μg ml^{-1}). The Parafilm®-sealed plates were incubated at room temperature and monitored for the appearance of colonies for 3 months. Representatives of unique colony morphotypes were serially subcultured until axenic on MA. Purified bacteria were examined for gross cell morphology and motility via light microscopy in combination with standard Gram-staining procedures. For long-term storage, purified bacterial isolates were grown in Difco Marine Broth 2216 and preserved in 50 % (*v*/*v*) glycerol at −80 °C.

Identification of Bacteria

Small subunit rRNA genes were amplified using a EconoTaq® Plus Green 2X Master Mix (Lucigen, Middleton, WI), the primers pA (5′-agagtttgatcctggctcag; 0.5 μM) and pH (5′-aaggaggtgatccagccgca; 0.5 μM), and template DNA (5 %*v*/*v*) [34]. Template DNA was prepared by dispersing pure colonies in 50 μl of PCR-grade DMSO (Sigma-Aldrich, Canada). PCR cycling conditions consisted of 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 54 °C for 1.5 min, 72 °C for 2 min, and a final cycle at 72 °C for 7 min. Duplicate bacterial isolates were identified by restriction fragment length polymorphism analysis of 16S rDNA amplicons. Amplicons were digested with *Hha*I and *Hae*III individually, and duplicate cultures were identified as those cultures having identical restriction patterns with both enzymes. Partial 16S rDNA sequences were obtained using the 16SR530 primer (5′-ccgcggctgctggcacgta), while nearly full-length sequences were obtained for selected isolates by sequencing with the primers pA, pH, 16SR530, and 16SF514 (5′-gtgccagcagccgcggtaa) [35]. All sequencing was performed using an ABI 3730xl DNA Analyzer (Applied

Biosystems) at the McGill University and Genome Quebec Innovation Centre DNA sequencing facility. Sequences were analyzed using Vector NTI Advance 10 (Invitrogen, Carlsbad, CA) and aligned using ClustalX v.2.0.10 [36]. To determine taxonomic affiliation of cultured isolates, 16S rDNA sequences were compared to those of related strains represented in the list of prokaryotic names with standing in nomenclature [37]. Estimates of divergence between sequences were determined by calculating the number of base differences per site (*p* distance). Ambiguous bases were excluded from these calculations.

Phylogenetic Analysis

Phylogenetic analysis was conducted using the neighbor-joining algorithm [38] based on distances estimated by Kimura's two-parameter model [39] using MEGA 4.0 [40]. All gaps and missing data were eliminated from the dataset, and the robustness of the inferred tree topology was evaluated with 1,000 bootstrap replicates [41].

Nucleotide Accession Numbers

DNA sequences were deposited in GenBank under accession numbers JQ282802 to JQ282841 (cultured isolates) and KC561023 to KC561051 (ten most abundant OTUs from each sequence library).

Results

Culture-Independent Bacterial Diversity

After denoising, quality filtering, and removal of nonbacterial 16S rDNA sequences, sequence libraries from three *P. elisabethae* samples (*Pe*A, *Pe*B, and *Pe*C) contained between 1,947 and 5,730 sequences with an average length of 253–260 bp. The diversity of *P. elisabethae* bacterial communities (*S*, Chao1, *H*′, and *E* indices) at the species and class level distances (D=0.03 and 0.15, respectively) is summarized in Table 1. Non-normalized S_{obs} values varied from 87 to 327 OTUs at the species level and from 30 to 134 at the class level. Richness (D=0.03) calculated using the RDP and mothur differed by 9–19 %, indicating the range of richness obtained using different computational methods. As diversity measures can be strongly influenced by sampling depth [29], we calculated the observed and predicted richness as well *H*′ and *E* from normalized datasets. Comparison of these values revealed that *Pe*A and *Pe*B possessed similar richness, while *Pe*C exhibited more than double the observed (2.2–2.7-fold more) and estimated (Chao1, 2.4-fold more) richness than the other coral colonies. The *H*′ and *E* index values were similar across all three coral samples, indicating similar levels of overall diversity.

Eight phyla (Lentisphaerae, Planctomycetes, Verrucomicrobia, Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Fusobacteria) were identified in *P. elisabethae* sequence libraries. Five of these (Verrucomicrobia, Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria) were found in all three sequence libraries. Proteobacteria were the dominant phylum, accounting for 87.1–94.8 % of sequences, followed by unclassified bacteria (sequences which could not be assigned to a phylum; 4.1–10.0 %), Actinobacteria (0.7–1.2 %), Bacteroidetes (0.2–0.9 %), and Firmicutes (0.1–0.2 %). At the class level, *P. elisabethae* bacterial communities were dominated by Gammaproteobacteria (82.2–87.1 %), unclassified bacteria (4.1–10.0 %), and Alphaproteobacteria (4.0–7.45 %). To more precisely represent similarities and differences between *P. elisabethae* bacterial communities, community composition was further examined at the order level and is summarized in Fig. 1. The *P. elisabethae* bacterial communities were dominated by Pseudomonadales (37.0–51.1 %), Oceanospirillales (18.3–29.02.3 %), unclassified bacteria (4.1–10.0 %), unclassified Gammaproteobacteria (5.4–13.7 %), Xanthomonadales (3.1–6.6 %), and Caulobacterales (1.6–6.7 %). Collectively, these six groups accounted for 90.7–91.8 % of sequences in *P. elisabethae* sequence libraries. All other individual orders represented less than 2.4 % of each community.

To examine the contribution of species level phylotypes (D=0.03) to the bacterial communities of *P. elisabethae*, the ten most abundant OTUs from each individual bTEFAP sequence library were identified (Table 2). These OTUs accounted for the majority of sequences in each library (85.0–93.1 %). Conversely, rare phylotypes (<1 % abundance) accounted for the majority of bacterial diversity present in *P. elisabethae* sequence libraries as the majority of OTUs were present at abundances below 1 % (*Pe*A, 92.0 %; *Pe*B, 89.7 %; *Pe*C, 97.6 %). Interestingly, *Pseudomonas*-related OTUs were the most abundant phylotypes in each library (36.9–51.0 %). Phylotypes related to *Endozoicomonas* spp. sequences originating from octocorals, scleractinian corals, and sponges were also a major component (24.3–31.8 %) of each sequence library (Table 2; *Pe*A-OTUs 2, 4, 8, and 9; *Pe*B-OTUs 2, 5, and 8; *Pe*C-OTUs 2, 3, 6, and 10). *Brevundimonas*- and *Stenotrophomonas*-related phylotypes were found in all sequence libraries and were the major alphaproteobacterial component of the *P. elisabethae* microbiome, accounting for 3.1–6.5 and 1.0–6.6 % of sequences, respectively. Four of the abundant phylotypes listed in Table 2 exhibited limited sequence identity to characterized bacteria. Two of these OTUs (*Pe*A-OTU6 and *Pe*B-OTU3) were identical to each other and closely related to an uncultured gammaproteobacterium identified from the octocoral *Muricea elongata* (DQ917904.1;

Table 1 Composition, richness and diversity of bacterial communities associated with three colonies (A, B and C) of *P. elisabethae* determined via analysis of 16S rDNA sequence libraries

Sample	Total sequences[a]	Average length (bp)	Classified sequences (OTUs[b])	Unclassified sequences (OTUs[b])	Distance (D)	RDP richness[c]	Mothur richness[d]	Richness[e] (normalized)	Chao1[f]	H'[g]	E[h]
A	5,730	253	5,256 (97)	474 (41)	0.03	112	138	71	201	1.93	0.45
					0.15	55	–	–	–	–	–
B	1,947	260	1,868 (54)	79 (33)	0.03	95	87	87	197	2.17	0.49
					0.15	30	–	–	–	–	–
C	4,560	256	4,102 (202)	458 (125)	0.03	298	327	190	475	2.3	0.44
					0.15	134	–		–	–	–

Chao1, H', and E were calculated from data normalized to 1,947 sequences

[a] Total number of sequences after denoising, removal of chimeras and removal of sequences not related to bacterial 16S rRNA genes

[b] Species level OTUs (D=0.03)

[c] Observed richness (S_{obs}) at the indicated distance determined using the RDP

[d] S_{obs} at the indicated distance determined using mothur

[e] S_{obs} normalized to 1,947 sequences

[f] Nonparametric statistical estimate of total richness based on distribution of singletons and doubletons

[g] Shannon diversity index, a higher number represents greater diversity (sensitive to richness)

[h] Shannon equitability index, a higher number represents greater diversity (sensitive to evenness)

Ranzer et al., direct submission); a related OTU was also present in *Pe*C and accounted for 0.4 % of sequences in this sample (data not shown). *Pe*B-OTU3 and *Pe*C-OTU4 were only observed in a single sequence library. *Pe*A-OTU3 was closely related to an unclassified bacterium identified from the octocoral *Gorgonia ventalina* [12] but exhibited limited sequence identity (<85 %) to cultured *Spiroplasma* and *Mycoplasma* strains. *Pe*C-OTU4 exhibited the greatest sequence identity (97.3 %) to an uncultured cyanobacterium reported from *Montipora hispida* (JX022138.1; Sato et al., direct submission). The mothur classifier did not classify *Pe*C-OTU4 as Cyanobacteria, but rather as an unclassified bacterium; thus, this OTU is represented in the unclassified bacteria category in Fig. 1. A high degree of congruence was observed between the ten most abundant species level OTUs in each *P. elisabethae* library. Six of the ten most abundant OTUs present in each library shared >99.2 % identity with an OTU from each of the other libraries (e.g., OTU1 from all three libraries was identical). Collectively, these six groups accounted for 77–81.2 % of each sequence library, indicating an overall high degree of similarity between the microbial communities of the three *P. elisabethae* colonies. The phylogenetic relationship of OTUs reported in Table 2 and observed in two or more sequence libraries is shown in Fig. 2.

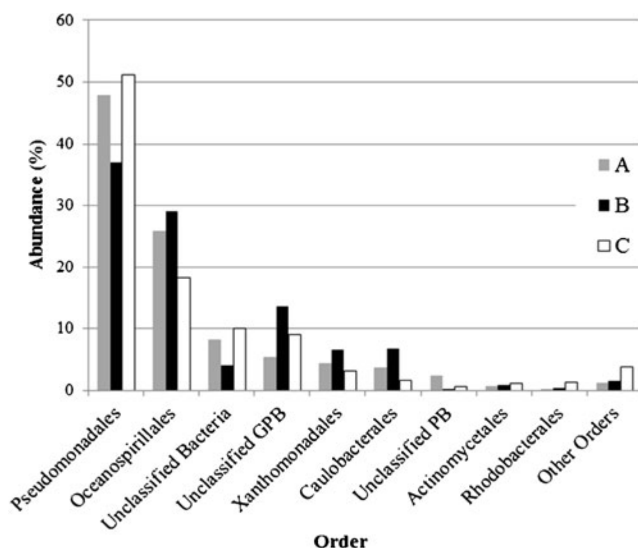

Fig. 1 Order level bacterial community composition determined from 16S rRNA gene sequence libraries of three *P. elisabethae* colonies (*A*, *B*, and *C*). Abundance (in percent) of each order is indicated on the *y*-axis for orders representing ≥1 % of the total community. Orders comprising less than 1 % of the total community were grouped into an artificial "other orders" category. *GPB* Gammaproteobacteria, *PB* Proteobacteria

Culturable Bacterial Diversity

A total of 214 bacteria were isolated from *P. elisabethae*: 150 from surface swabs and 64 from homogenates. Only four isolates were obtained from the seawater sample. The low number of isolates obtained from the seawater is likely due to the low starting dilution (10^{-4}) used for dilution plating. These isolates formed 40 sequence groups sharing <99 % 16S rDNA sequence identity. The isolation source and

Table 2 Top ten most numerous OTUs (D=0.03) in three *P. elisabethae* 16S rDNA sequence libraries (*Pe*A, *Pe*B, and *Pe*C).

Sample[a]	OTU	No. of seq.	Abundance (%)	Accession no.	Length (bp)	OTU classification			
						Accession no.	Phylogenic association	% identity	Closest related GenBank sequence (source)
*Pe*A (93.1 %)	1	2,739	47.8	KC561023	249	HE586387.1	γ-Proteobacteria	100	*Pseudomonas asplenii* LMG5147 (milk)
	2	1,189	20.8	KC561024	269	JF925006.1	γ-Proteobacteria	95.2	Uncultured *Endozoicomonas* sp. (octocoral)
	3	356	6.2	KC561025	248	GU118339.1	Unclassified	99.2	Uncultured bacterium *Gven*_K23 (octocoral)
	4	295	5.1	KC561026	253	AY897105.1	γ-Proteobacteria	98.4	Uncultured *Endozoicomonas* sp. (sponge)
	5	175	3.1	KC561027	257	JQ918382.1	γ-Proteobacteria	100	Uncultured *Stenotrophomonas* sp. (fish)
	6	136	2.4	KC561028	255	DQ917904.1	γ-Proteobacteria	100	Uncultured Gammaproteobacterium ME42 (octocoral)
	7	129	2.3	KC561029	223	JX680801.1	α-Proteobacteria	100	Uncultured *Brevundimonas* sp. (fish)
	8	125	2.2	KC561030	254	AB611839.1	γ-Proteobacteria	99.2	Uncultured *Endozoicomonas* sp. (coral)
	9	111	1.9	KC561031	266	AF365796.1	γ-Proteobacteria	98.9	Uncultured *Endozoicomonas* sp. (coral)
	10	79	1.3	KC561032	221	JX992642.1	α-Proteobacteria	99.0	Uncultured *Brevundimonas* sp. (soil)
*Pe*B (90.7 %)	1	719	36.9	KC561032	256	HE586387.1	γ-Proteobacteria	100	*Pseudomonas asplenii* LMG5147 (milk)
	2	496	25.5	KC561033	277	JF925006.1	γ-Proteobacteria	95.7	Uncultured *Endozoicomonas* sp. (octocoral)
	3	173	8.9	KC561034	262	DQ917904.1	γ-Proteobacteria	95.8	Uncultured Gammaproteobacterium ME42 (octocoral)
	4	106	5.4	KC561035	263	JN909545.1	γ-Proteobacteria	100	Uncultured *Stenotrophomonas* sp. (hydrothermal vent)
	5	84	4.3	KC561036	260	AY897105.1	γ-Proteobacteria	99.2	Uncultured *Endozoicomonas* sp. (sponge)
	6	59	3.0	KC561037	231	JX680801.1	α-Proteobacteria	99.6	Uncultured *Brevundimonas* sp. (fish)
	7	59	3.0	KC561038	231	JN067021.1	α-Proteobacteria	99.1	Uncultured *Brevundimonas* sp. (human bowel)
	8	39	2.0	KC561039	262	AB611839.1	γ-Proteobacteria	99.6	Uncultured *Endozoicomonas* sp. (coral)
	9	22	1.1	KC561040	262	HQ120389.1	γ-Proteobacteria	100.0	Uncultured *Stenotrophomonas* sp. (soil)
	10	11	0.6	KC561041	230	JN907774.1	α-Proteobacteria	99.1	Uncultured *Brevundimonas* sp. (hydrothermal vent)
*Pe*C (85.0 %)	1	2,324	51.0	KC561042	255	HE586387.1	γ-Proteobacteria	100	*Pseudomonas asplenii* LMG5147 (milk)
	2	686	15.0	KC561043	275	JF925006.1	γ-Proteobacteria	95.3	Uncultured *Endozoicomonas* sp. (octocoral)
	3	317	7.0	KC561044	259	AY897105.1	γ-Proteobacteria	98.5	Uncultured *Endozoicomonas* sp. (sponge)
	4	221	4.8	KC561045	226	JX022138.1	Cyanobacteria[b]	97.3	Uncultured Cyanobacterium (coral)
	5	83	1.8	KC561046	261	HQ120389.1	γ-Proteobacteria	100.0	Uncultured *Stenotrophomonas* sp. (soil)
	6	79	1.7	KC561047	260	AB611839.1	γ-Proteobacteria	98.9	Uncultured *Endozoicomonas* sp. (coral)
	7	60	1.3	KC561048	262	GQ468194.1	γ-Proteobacteria	100.0	Uncultured *Stenotrophomonas* sp. (milk)
	8	45	1.0	KC561049	229	JX680801.1	α-Proteobacteria	100.0	Uncultured *Brevundimonas* sp. (fish)
	9	36	0.8	KC561050	252	JN695033.1	Actinobacteria	100.0	*Rhodococcus* sp. (soil)
	10	27	0.6	KC561051	274	JF925006.1	γ-Proteobacteria	96.75	Uncultured *Endozoicomonas* sp. (octocoral)

[a] Percentage of bacterial communities represented by the top ten most abundant OTUs indicated in brackets

[b] Classified by the mothur classifier as unclassified bacteria (Fig. 2)

taxonomic affiliations of the 40 sequence groups are summarized in Table 3. Comparison of isolation media revealed that MA was the most effective isolation medium, with 82.5 % of the sequence groups obtained exclusively on MA. In contrast, only 7.5 % of the sequence groups were cultured only on SD (Table 3). SD was used to mimic

conditions potentially encountered in *P. elisabethae* tissues, such as reduced salinity, slight acidity, and increased nutrient availability. However, the low salinity, high nutrient concentration, and acidity (pH 5.6) of SD clearly did not promote the growth of the majority of bacteria associated with *P. elisabethae*. Examination of the distribution of sequence groups between coral and water samples identified only two sequence groups consisting of coral- and water-derived isolates (groups 6 and 18). These data suggest that the majority (92.5 %) of sequence groups identified in this study have a specific association with *P. elisabethae* colonies *Pe*A, *Pe*B, and *Pe*C; however, this comparison should be viewed with caution given the low number of isolates obtained from the seawater sample. The 39 distinct bacterial groups isolated from *P. elisabethae* were evenly distributed among the phyla Actinobacteria, Firmicutes, and Proteobacteria (Table 3). At the class level, Bacilli, Actinobacteria, and Gammaproteobacteria were most common. The greatest genus level diversity was observed among the Actinobacteria, which was comprised of nine genera, followed by Gammaproteobacteria and Bacilli with

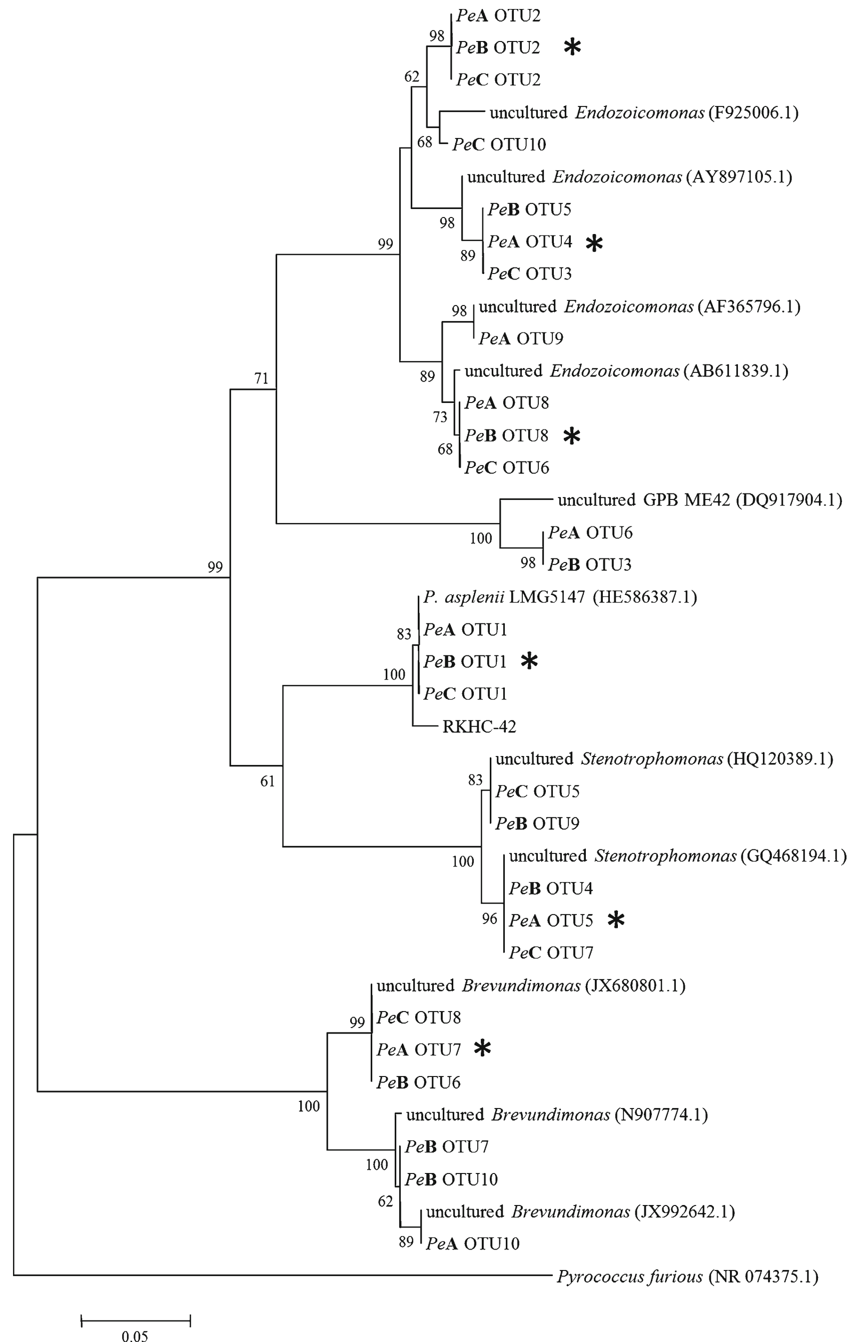

Fig. 2 Phylogenetic relationships of dominant OTUs (*Pe*-prefix) present in two or more *P. elisabethae* sequence libraries. The cultured isolate, RKHC-42, is included in the tree to illustrates its relationship to dominant OTUs. Clades composed of OTUs present in all three sequence libraries are indicated with an *asterisk*. *GPB* Gammaproteobacteria. Bootstrap values greater than 50 % are shown at the *nodes*. The *scale bar* represents the number of base substitutions per site. A total of 212 positions were used in the final dataset. *Pyrococcus furious* was used to root the tree. GenBank accession numbers are in *brackets*

Table 3 Bacteria isolated from *P. elisabethae* and the seawater control

	Sequence group	Representative isolate	Source[a]			Isolation media[b]	Accession no.	Length (bp)	Phylogenetic association[c]	Closest related type strain (GenBank accession number)	Similarity %
			S	H	W						
Actinobacteria	1	RKHC-35	1	0	0	SD	JQ282807	1,453	Corynebacterinea	*Corynebacterium afermentas* ATCC 51403 (X82054.1)	100
	2	RKHC-59B	1	1	0	MA	JQ282810	439	Corynebacterinea	*Dietza schimae* DSM 45139 (EU375845.1)	100
	3	RKHC-26	1	0	0	MA	JQ282804	438	Corynebacterinea	*Rhodococcus baikonurensis* DSM 44587 (AB071951.1)	100
	4	RKHC-66	6	1	0	MA	JQ282811	455	Micrococcineae	*Brevibacterium casei* ATCC 35513 (AJ251418.1)	100
	5	RKHC-70A	1	0	0	MA	JQ282812	451	Micrococcineae	*Brevibacterium casei* ATCC 35513 (AJ251418.1)	99.5
	6	RKHC-33	2	0	1	MA	JQ282806	444	Micrococcineae	*Brachybacterium conglomeratum* DSM 10241 (AB537169.1)	100
	7	RKHC-11	1	0	0	MA	JQ282803	443	Micrococcineae	*Microbacterium phyllosphaerae* DSM 13468 (AJ277840.1)	98.8
	8	RKHC-45	3	0	0	MA	JQ282808	445	Micrococcineae	*Microbacterium luteolum* ATCC 51474 (AB004718.1)	99.5
	9	RKHC-27	0	1	0	MA	JQ282805	459	Micrococcineae	*Arthrobacter koreensis* NBRC 16787 (AY116496.1)	100
	10	RKHC-10	0	1	0	MA	JQ282802	435	Micrococcineae	*Kocuria palustris* DSM 11925 (Y16263.1)	100
	11	RKHC-49	0	1	0	MA	JQ282809	464	Micrococcineae	*Kocuria kristinae* ATCC 27570 (X80749.1)	99.8
	12	RKHC-71A	1	1	0	MA	JQ282813	450	Micrococcineae	*Micrococcus luteus* ATCC 4698 (AJ536198.1)	99.7
Firmicutes	13	RKHC-68A	7	13	0	MA/SD	JQ282823	1,501	Bacillaceae	*Bacillus horneckiae* MTCC 9535 (EU861362.1)	100
	14	RKHC-13	2	0	0	MA	JQ282815	1,514	Bacillaceae	*Bacillus horti* ATCC 7007780 (D870351.1)	98.7
	15	RKHC-21	1	0	0	MA	JQ282816	1,485	Bacillaceae	*Bacillus licheniformis* ATCC 14580 (CP000002.3)	99.7
	16	RKHC-37	8	6	0	MA	JQ282818	498	Bacillaceae	*Bacillus mojavensis* ATCC 51516 (AB021191.1)	99.2
	17	RKHC-57A	1	0	0	MA	JQ282821	498	Bacillaceae	*Bacillus pumilus* ATCC 7061 (AY876289.1)	100
	18	RKHC-69A	69	5	2	MA/SD	JQ282824	462	Bacillaceae	*Bacillus anthracis* ATCC 14578 (AB190217.1)	99.7

Table 3 (continued)

	Sequence group	Representative isolate	Source[a]			Isolation media[b]	Accession no.	Length (bp)	Phylogenetic association[c]	Closest related type strain (GenBank accession number)	Similarity %
			S	H	W						
	19	RKHC-9	5	3	0	MA	JQ282814	489	Bacillaceae	*Bacillus cereus* ATCC 14579 (AE016877.1)	100
	20	RKHC-62B	9	8	0	MA/SD	JQ282822	1,516	Bacillaceae	*Oceanobacillus profundus* DSM 18246 (DQ386635.1)	99.9
	21	RKHC-82A	0	1	0	SD	JQ282828	367	Bacillaceae	*Oceanobacillus iheyensis* DSM 14371 (AB010863.2)	99.5
	22	RKHC-8	3	1	0	MA	JQ282827	433	Paenibacillaceae	*Paenibacillus glucanolyticus* ATCC 49278 (AB073189.1)	100.0
	23	RKHC-78A	1	0	0	MA	JQ282825	491	Staphylococcaceae	*Staphylococcus epidermidis* ATCC 14990 (D83363.1)	98.9
	24	RKHC-78B	1	0	0	MA	JQ282826	499	Staphylococcaceae	*Staphylococcus warneri* ATCC 27836 (L37603.1)	99.4
	25	RKHC-54	0	1	0	MA	JQ282820	500	Staphylococcaceae	*Staphylococcus equorum* ATCC 43958 (AB009939.1)	99.2
	26	RKHC-48	1	0	0	SD	JQ282819	1,511	Staphylococcaceae	*Staphylococcus auricularis* ATCC 33753 (D83358.1)	99.4
	27	RKHC-28	0	1	0	MA	JQ282817	1,272	Staphylococcaceae	*Jeotgalicoccus halophilus* NBRC 105788 (FJ386517.1)	99.9
Proteobacteria	28	RKHC-77B	0	1	0	MA	JQ282841	1,270	Alphaproteobacteria	*Paracoccus chinensis* NBRC 104937 (EU660389.1)	100
	29	RKHC-14	1	7	0	MA	JQ282831	1,368	Alphaproteobacteria	*Ruegeria atlantica* ATCC 700000 (D88526.1)	97.9
	30	RKHC-1	0	0	1	MA	JQ282830	1,443	Alphaproteobacteria	*Erythrobacter flavus* JCM 11808 (AF500004.1)	99.3
	31	RKHC-60A	2	0	0	MA/SD	JQ282835	491	Betaproteobacteria	*Tetrathiobacter kashimirensis* LMG 22695 (AJ864470.1)	98.9
	32	RKHC-12	0	4	0	MA	JQ282829	1,351	Gammaproteobacteria	*Acinetobacter lwoffii* ATCC 15309 (X81665.1)	100
	33	RKHC-61A	5	0	0	MA	JQ282836	1,382	Gammaproteobacteria	*Pseudomonas libanensis* CCUG 43190 (AF0576451.1)	99.7
	34	RKHC-25	1	0	0	MA	JQ282833	476	Gammaproteobacteria	*Pseudomonas oleovorans* ATCC 8062 (D84018.1)	98.8
	35	RKHC-42	0	5	0	MA	JQ282834	495	Gammaproteobacteria	*Pseudomonas asplenii* ATCC 238358 (AB021397.1)	99.4

Table 3 (continued)

Sequence group	Representative isolate	Source[a] S	H	W	Isolation media[b]	Accession no.	Length (bp)	Phylogenetic association[c]	Closest related type strain (GenBank accession number)	Similarity %
36	RKHC-23	2	0	0	MA	JQ282832	1,497	Gammaproteobacteria	*Photobacterium rosenbergii* LMG 22223 (AJ842344.1)	99.8
37	RKHC-63B	0	1	0	MA	JQ282837	497	Gammaproteobacteria	*Vibrio hollisae* ATCC 33564 (AJ514909.1)	95.6
38	RKHC-67B	1	0	0	MA	JQ282838	446	Gammaproteobacteria	*Vibrio sinaloensis* CAIM 797 (DQ451211.1)	100
39	RKKC-76A	1	0	0	MA	JQ282839	1,396	Gammaproteobacteria	*Vibrio maritimus* ATCC 15381 (AB038033.1)	99.6
40	RKHC-76B	12	1	0	MA	JQ282840	1,399	Gammaproteobacteria	*Vibrio communis* LMG 25430 (GU078672.1)	100

[a] Numbers indicate the number of isolates obtained from each source: S, octocoral surface swab; H, octocoral homogenate; W, sea water

[b] Isolation media: MA, marine agar; SD, Sabouraud dextrose agar

[c] Phylogenetic associations: Actinobacteria, suborder; Firmicutes, family; Proteobacteria, class

five and four genera, respectively. The majority of bacteria isolated from *P. elisabethae* were closely related to previously cultured bacteria as indicated by high 16S rRNA gene similarities to those of type strains (Table 3). Two isolates, RKHC-14 and RKHC-63B, exhibited the least amount of similarity to previously described bacterial species (97.9 and 95.6 %, respectively) and may represent new species of *Ruegeria* and *Vibrio*, respectively. Further polyphasic taxonomic characterization would be required to conclusively determine the taxonomic novelty of the bacteria isolated from *P. elisabethae*.

Analysis of the origins of bacteria isolated from *P. elisabethae* revealed that the majority of isolates were associated with the octocoral surface (Table 3). Of the 214 isolates, 70 % were obtained from surface swabs, suggesting an epiphytic association with *P. elisabethae*. Surface-associated strains were also dominant (70 %) among the 39 *P. elisabethae*-associated sequence groups: 44 % were only isolated from surface swabs and 26 % were isolated from surface swabs and homogenates. Twenty-five percent of the sequence groups were composed of isolates exclusively obtained from octocoral homogenates, suggesting that these isolates may have an endophytic association with *P. elisabethae*.

To determine if the bacteria cultured from *P. elisabethae* were represented in bTEFAP sequence libraries, we compared representative 16S rDNA sequences of the cultured bacteria sequence groups to the bTEFAP sequence libraries. The majority of the cultured bacterial sequence groups were either not found (70 %) in bTEFAP sequence libraries or found at very low frequencies (<0.26 %) in one or two sequence libraries (based on a >97 % sequence identity cutoff). Only sequence group 35, represented by RKHC-42, shared significant sequence identity (97.2–97.4 %) with abundant OTUs present in all sequence libraries (*Pe*A, *Pe*B, *Pe*C-OTU1). The phylogenetic relationship between RKHC-42 and abundant OTUs is shown in Fig. 2.

Discussion

Culture-Independent Bacterial Diversity

Symbioses between microorganisms and marine invertebrates are essential to the functioning of reef ecosystems, as they facilitate photosynthetic productivity, nutrient cycling, and NP production [4]. Elucidation of the nature of bacterial associations with scleractinian corals and sponges are relatively well studied [1, 2, 8, 12, 20]; however, few investigations have explored the bacterial diversity associated with octocorals using modern high-throughput sequencing approaches [12, 42]. This study elucidates, for the first time, the structure and diversity of bacterial communities associated with healthy specimens of *P. elisabethae* using a 16S rDNA amplicon pyrosequencing approach [32].

The results obtained in this study revealed that *P. elisabethae* colonies from Providencia Island (Colombia) host moderately diverse bacterial assemblages as indicated by Shannon diversity index values ranging between 1.93 and 2.3 and observed (S_{obs}) and predicted (S_{Chao1}) richness levels ranging between 87–327 and 197–475 species level OTUs

(D=0.03), respectively. The structure of *P. elisabethae* bacterial communities is similar to that of other marine invertebrates in that the communities are dominated by a few abundant OTUs, while the majority of the diversity is comprised of numerous rare OTUs [12, 43, 44]. These rare phylotypes may serve as important reservoirs of metabolic diversity, which enable the coral to more rapidly adapt to abiotic and biotic changes in their environment [12]. According to the "probiotic hypothesis," rare microbes increase in abundance when conditions favor their growth (e.g., increased temperature), thereby replacing the metabolic functions provided to the invertebrate by previously abundant microbes less adapted to the new environmental conditions [6]. In terms of composition, *P. elisabethae* bacterial communities were dominated by Gammaproteobacteria, a result which has been observed in several studies of octocorals and other marine invertebrates [1, 9, 44, 45]. Interestingly, the bacterial communities of *P. elisabethae* were dominated by two groups of gammaproteobacterial OTUs belonging to the genera *Pseudomonas* and *Endozoicomonas*. Collectively, OTUs belonging to these genera accounted for 68.7–75.3 % of each sequence library. *Pseudomonas*- and *Endozoicomonas*-related sequences are frequently detected in culture-independent studies of coral microbial diversity [46–48] and have been found to constitute a major proportion of the bacterial communities of other octocorals [12, 42]. Recent research has determined that an abundance of Gammaproteobacteria-related sequences (including *Pseudomonas*- and *Endozoicomonas*-related sequences) in invertebrate microbial communities correlates with the presence of photosymbionts in the invertebrate holobiont (invertebrate host and all associated microbes) [42]. This association is believed to be driven by the large quantities of dimethylsulfoniopropionate produced by invertebrate photosymbionts, which can serve as carbon and sulfur sources for marine bacteria [48]. *P. elisabethae* hosts photosymbiotic dinoflagellates (*Symbiodinium*) which it relies on for much of its nutritional requirements [49]. Consequently, it is likely that interactions between photosymbiotic *Symbiodinium* and heterotrophic bacteria may play an integral role in shaping the microbial communities of *P. elisabethae*. In addition to nutrient cycling, both *Endozoicomonas*- and *Pseudomonas*-related members of the *P. elisabethae* bacterial community may also play a role in regulating bacterial colonization of *P. elisabethae* via the production of bioactive secondary metabolites or probiotic mechanisms such as competitive exclusion of pathogenic bacteria [3, 46, 50, 51].

Numerous studies have demonstrated that invertebrates maintain specific microbial communities, which drastically differ from the surrounding ocean environment and are species specific and geographically stable [1, 2]. While we did not analyze the bacterial diversity present in seawater surrounding Providencia Island, the composition of *P. elisabethae* bacterial communities differs significantly from those reported for seawater collected from the South West Caribbean Sea (Panama) and Eastern Atlantic Ocean, which is typically dominated by phototropic Cyanobacteria (e.g., *Synechococcus* sp., and *Prochlorococcus*) and Alphaproteobacteria (e.g., *Pelagibacter*) [12, 52]. Additionally, a high degree of similarity was observed between bacterial communities of the three *P. elisabethae* colonies, with 77–81 % of each sequence library attributed to six OTUs common to all samples. These data suggest that *P. elisabethae* maintains a microbial consortium distinct from its environment. However, further studies are needed to elucidate how environmental factors influence the composition of *P. elisabethae* bacterial communities and to determine if they are species specific and geographically stable.

Culturable Bacterial Diversity

Forty distinct bacterial groups sharing <99 % 16S rDNA sequence identity were obtained from octocoral and seawater samples (Table 1). While the broadly accepted definition of a bacterial species on the basis of 16S rDNA sequence identity is >98.5 % [53], 99 % was chosen as the cutoff for this study, as many bacteria sharing this degree of sequence identity can possess significant genomic variation, which can manifest itself in the form of species- and strain-specific production of bioactive secondary metabolites [54]. A majority of the bacterial groups obtained from *P. elisabethae* samples were associated with the octocoral surface, while only 25 % appeared to be associated with octocoral tissue. Nutrient-rich coral mucous has previously been shown to host a diverse array of bacteria; thus, it is not surprising that the majority of isolates were surface associated [7, 55]. Additionally, some bacteria may be excluded from *P. elisabethae* tissues due to the presence of endogenous antimicrobial diterpenes [56]. For putative endosymbiotic isolates, further experimentation using in situ hybridization approaches would be required to prove their localization within octocoral tissues. The octocoral-associated bacteria isolated in this study were closely related to widely distributed, metabolically diverse genera commonly encountered in the marine environment. Several of the bacteria isolated from *P. elisabethae* are also commonly encountered in terrestrial habitats (e.g., *Bacillus* species), raising the possibility that these bacteria may merely be dormant terrestrial visitors to the marine environment. However, bacteria traditionally considered terrestrial in origin are frequently isolated from marine habitats, indicating that these bacteria are metabolically flexible and may be adapted to both marine and nonmarine habitats [17, 35, 57]. The diversity of bacterial groups cultured from *P. elisabethae* was similar to that reported in other studies of octocorals; however, the *P. elisabethae* culture library was less dominated by Proteobacteria and more evenly distributed between

phyla [9, 10, 45]. Interestingly, comparison of the 16S rDNA sequences of cultured isolates to culture-independent 16S rDNA sequence libraries revealed a nearly total lack of overlap between cultured bacteria and bacteria detected in DNA isolated from the octocoral holobiont. Only one group of isolates (represented by RKHC-42) exhibited >97 % sequence identity to the dominant, *Pseudomonas*-related OTUs identified in 16S rDNA sequence libraries. Notably, isolates belonging to this group were only isolated from coral homogenates, suggesting that these bacteria may have an endophytic association with *P. elisabethae*. The phylogenetic relationship of this bacterium to the OTUs present in the *P. elisabethae* culture-independent sequence library is illustrated in Fig. 2. Although closely related to OTU1 from each sequence library, RKHC-42 forms a well-supported sister clade to these sequences, suggesting that RKHC-42 may be a different strain or species of *Pseudomonas*. However, given the limited nature of the dataset (212 positions), further research is needed to fully elucidate the relationship between RKHC-42 and the dominant members of the *P. elisabethae* bacterial community. Our inability to culture the vast majority of the bacterial diversity observed in *P. elisabethae* culture-independent analyses is consistent with the "great plate count anomaly" observed in many studies of culturable bacterial diversity, in which rare microbes capable of growing on complex media are preferentially cultured over abundant and potentially symbiotic bacteria [58–60]. The narrow range of isolation media and dilutions used in this study likely contributed, at least in part, to our inability to culture a more diverse and representative selection of the *P. elisabethae* microbiome. A greater diversity of marine bacteria may also have been cultured had marine salts been added to SD medium and the pH adjusted to that of the local seawater. The use of isolation media low in nutrients and/or containing specific host factors derived from *P. elisabethae* is a promising approach that could aid in the recovery of potential bacterial symbionts of *P. elisabethae* [61]. Additionally, the use of isolation techniques aimed at sequestering slow-growing microbes, to prevent their overgrowth by fast-growing bacteria, is another strategy that could be used to increase the diversity of bacteria isolated from *P. elisabethae* [61, 62].

Conclusions

This is the first report of the bacterial diversity associated with the octocoral *P. elisabethae*. The data presented here demonstrate that *P. elisabethae* hosts a diverse assemblage of bacteria comparable to that observed in other coral species. This study also represents a valuable starting point for further characterization of the microbiome of *P. elisabethae* to answer fundamental questions regarding the specificity and stability of *P. elisabethae* bacterial communities over larger geographic scales. Finally, we have established a diverse library of 39 distinct bacteria associated with *P. elisabethae,* which can serve as a future resource for the discovery of novel secondary metabolites.

Acknowledgments This work was partially financed by grants from the Colciencias and Universidad Nacional de Colombia. The Ministerio de Ambiente, Vivienda y Desarrollo Territorial granted collection and research permits (permission no. 4 of 10/02/2010). HC, BH, and RK gratefully acknowledge the financial support from the Natural Sciences and Engineering Council of Canada, the Canada Research Chair Program, the University of Prince Edward Island, the Atlantic Innovation Fund, and the Jeanne and Jean-Louis Lévesque Foundation.

References

1. Bourne DG, Munn CB (2005) Diversity of bacteria associated with the coral *Pocillopora damicornis* from the Great Barrier Reef. Environ Microbiol 7:1162–1174
2. Kvennefors EC, Sampayo E, Ridgway T, Barnes AC, Hoegh-Guldberg O (2010) Bacterial communities of two ubiquitous Great Barrier Reef corals reveals both site- and species-specificity of common bacterial associates. PLoS One 5:e10401
3. Littman RA, Willis BL, Pfeffer C, Bourne DG (2009) Diversities of coral-associated bacteria differ with location, but not species, for three acroporid corals on the Great Barrier Reef. FEMS Microbiol Ecol 68:152–163
4. Mouchka ME, Hewson I, Harvell CD (2010) Coral-associated bacterial assemblages: current knowledge and the potential for climate-driven impacts. Integr Comp Biol 50:662–674
5. Neulinger SC, Järnegren J, Ludvigsen M, Lochte K, Dull WC (2007) Phenotype-specific bacterial communities in the cold-water coral *Lophelia pertusa* (Scleractinia) and their implications for the coral's nutrition, health, and distribution. Appl Environ Microbiol 74:7272–7285
6. Reshef L, Koren O, Loya Y, Zilber-Rosenberg I, Rosenberg E (2006) The coral probiotic hypothesis. Environ Microbiol 8:2068–2073
7. Ritchie KB (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Mar Ecol Prog Ser 322:1–14
8. Sacristán-Soriano O, Banaigs B, Casamayor EO, Becerro MA (2011) Exploring the links between natural products and bacterial assemblages in the sponge *Aplysina aerophoba*. Appl Environ Microbiol 77:862–870
9. Brück TB, Brück WM, Santiago-Vazquez LZ, McCarthy PJ, Kerr RG (2007) Diversity of the bacterial communities associated with the azooxanthellate deep water octocorals *Leptogorgia minimata, Iciligorgia schrammi,* and *Swiftia exertia*. Mar Biotechnol 9:561–576
10. Gray MA, Stone RP, McLaughlin MR, Kellogg CA (2011) Microbial consortia of gorgonian corals from the Aleutian Islands. FEMS Microbiol Ecol 76:109–120
11. Santiago-Vazquez LZ, Brück TB, Brück WM, Duque-Alarcon AP, McCarthy PJ, Kerr RG (2007) The Diversity of the bacterial communities associated with the azooxanthellate hexacoral *Cirrhipathes lutkeni*. ISME J 1:654–659
12. Sunagawa S, Woodley CM, Medina M (2010) Threatened corals provide underexplored microbial habitats. PLoS One 5:e9554
13. Mayer AMS, Glaser KB, Cuevas C, Jacobs RS, Kem W, Little RD, McIntosh JM, Newman DJ, Potts BC, Shuster DE (2010) The

odyssey of marine pharmaceuticals: a current pipeline perspective. Trends Pharmacol Sci 31:255–265
14. Berrue F, Kerr RG (2009) Diterpenes from gorgonian corals. Nat Prod Rep 26:681–710
15. Leal MC, Puga J, Serôdio J, Gomes NCM, Calado R (2012) Trends in the discovery of new marine natural products from invertebrates over the last two decades—where and what are we bioprospecting? PLoS One 7:e30580
16. Chen YH, Kuo J, Su JH, Hwang TL, Chen YH, Lee CH, Weng CF, Sung PJ (2012) Pseudoalteromone B: a novel 15C compound from a marine bacterium *Pseudoalteromonas* sp. CGH2XX. Mar Drugs 10:1566–1571
17. Boya CA, Herrera L, Guzman HM, Gutierrez M (2012) Antiplasmodial activity of bacilosarcin A isolated from the octocoral-associated bacterium *Bacillus* sp. collected in Panama. J Pharm Bioallied Sci 4:66–69
18. Zhang XY, He F, Wang GH, Bao J, Xu XY, Qi SH (2013) Diversity and antibacterial activity of culturable actinobacteria isolated from five species of the South China Sea gorgonian corals. World J Microbiol Biotechnol 29:1107–1116
19. Gao CH, Tian XP, Qi SH, Luo XM, Wang P, Zhang S (2010) Antibacterial and antilarval compounds from marine gorgonian-associated bacterium *Bacillus amyloliquefaciens* SCSIO 00856. J Antibiot 63:191–193
20. Thomas TR, Kavlekar DP, LokaBharathi PA (2010) Marine drugs from sponge-microbe association—a review. Mar Drugs 8:1417–1468
21. Roué M, Quévrain E, Domart-Coulon I, Bourguet-Kondracki ML (2012) Assessing calcareous sponges and their associated bacteria for the discovery of new bioactive natural products. Nat Prod Rep 29:739–751
22. Blunt JW, Copp BR, Hu WP, Munro MHG, Northcote PT, Prinsep MR (2008) Marine natural products. Nat Prod Rep 25:35–94
23. Debnath M, Paul AK, Bisen PS (2007) Natural bioactive compounds and biotechnological potential of marine bacteria. Curr Pharm Biotechnol 8:253–260
24. Fenical W, Jensen PR (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. Nat Chem Biol 2:666–673
25. Mydlarz LD, Jacobs RS, Boehnlein JM, Kerr RG (2003) Pseudopterosin biosynthesis in *Symbiodinium* sp, the dinoflagellate symbiont of *Pseudopterogorgia elisabethae*. Chem Biol 10:1051–1056
26. Rowan R, Powers DA (1992) Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae). Proc Natl Acad Sci U S A 89:3639–3643
27. Callaway TR, Dowd SE, Wolcott RD, SunY MRJL, Edrington TS, Byrd JA, Anderson RC, Krueger N, Nisbet DJ (2009) Evaluation of the bacterial diversity in fecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing. Poult Sci 88:298–302
28. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541
29. Schloss PD, Gevers D, Westcott SL (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS One 6:e2731
30. Mothur wiki (2013) Schloss SOP. http://www.mothur.org/wiki/Schloss_SOP. Accessed January 2013
31. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267
32. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
33. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:D141–D145
34. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17:7843–7853
35. Gontang EA, Fenical W, Jensen PR (2007) Phylogenetic diversity of gram-positive bacteria cultured from marine sediments. Appl Environ Microbiol 73:3272–3282
36. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948
37. Euzéby JP (1997) List of bacterial names with standing in nomenclature: a folder available on the Internet. Int J Syst Bacteriol 47:590–592
38. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
39. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
40. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
41. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
42. Bourne DG, Dennis PG, Uthicke S, Soo RM, Tyson GW, Webster N (2013) Coral reef invertebrate microbiomes correlate with the presence of photosymbionts. ISME J 7:1459
43. Barott KL, Rodriguez-Brito B, Janouškovec J, Marhaver KL, Smith JE, Keeling P, Rohwer FL (2011) Microbial diversity associated with four functional groups of benthic reef algae and the reef-building coral *Montastraea annularis*. Environ Microbiol 13:1–13
44. Jackson SA, Kennedy J, Morrissey JP, O'Gara F, Dobson AD (2012) Pyrosequencing reveals diverse and distinct sponge-specific microbial communities in sponges from a single geographical location in Irish waters. Microb Ecol 64:105–116
45. Webster N, Bourne D (2007) Bacterial community structure associated with the Antarctic soft coral, *Alcyonium antarcticum*. FEMS Microbiol Ecol 59:81–94
46. Bourne DG, Iida Y, Uthicke S, Smith-Keune C (2008) Changes in coral-associated microbial communities during a bleaching event. ISME J 2:350–363
47. Penn K, Wu D, Eisen JA, Ward N (2006) Characterization of bacterial communities associated with deep-sea coral on Gulf of Alaska seamounts. Appl Environ Microbiol 72:1680–1683
48. Raina JB, Tapiolas D, Willis BL, Bourne DG (2009) Coral-associated bacteria and their role in the biogeochemical cycling of sulfur. Appl Environ Microbiol 75:3492–3501
49. Santos SR, Gutiérrez-Rodriguez C, Lasker HR, Coffroth MA (2003) *Symbiodinium* sp. association in the gorgonian *Pseudopterogorgia elisabethae* in the Bahamas: high levels of genetic variability and population structure in symbiotic dinoflagellates. Mar Biol 143:111–120
50. Berdy J (2005) Bioactive microbial metabolites. J Antibiot 58:1–26
51. Kvennefors EC, Sampayo E, Kerr C, Vieira G, Roff G, Barnes AC (2011) Regulation of bacterial communities through antimicrobial activity by the coral holobiont. Microb Ecol 63:605–618
52. Friedline CJ, Franklin RB, McCallister SL, Rivera MC (2012) Microbial community diversity of the eastern Atlantic Ocean reveals geographic differences. Biogeosci Discuss 9:109–150
53. Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 4:152–155

54. Jensen PR (2010) Linking species concepts to natural product discovery in the post-genomic era. J Ind Microbiol Biotechnol 37:219–224
55. Klaus JS, Janse I, Heikoop JM, Sanford RA, Fouke BW (2007) Coral microbial communities, zooxanthellae and mucus along gradients of seawater depth and coastal pollution. Environ Microbiol 9:1291–1305
56. Correa H, Aristizabal F, Duque C, Kerr R (2011) Cytotoxic and antimicrobial activity of pseudopterosins and seco-pseudopterosins isolated from the octocoral *Pseudopterogorgia elisabethae* of San Andrés and Providencia Islands (SW Caribbean). Mar Drugs 9:334–344
57. Ivanova EP, Vysotskii MV, Svetashev VI, Nedashkovskaya OI, Gorshkova NM, Mikhailov VV, Yumoto N, Shigeri Y, Taguchi T, Yoshikawa S (1999) Characterization of *Bacillus* strains of marine origin. Int Microbiol 2:267–271
58. Rypien KL, Ward JR, Azam F (2010) Antagonistic interactions among coral-associated bacteria. Environ Microbiol 12:28–39
59. Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science 276:734–740
60. Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345:63–65
61. Vartoukian SR, Palmer RM, Wade WG (2010) Strategies for culture of 'unculturable' bacteria. FEMS Microbiol Lett 309:1–7
62. Connon SA, Giovannonis SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl Environ Microbiol 68:3878–3885