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Bacterial Diversity of Culturable Isolates from Seawater and a Marine Coral, *Plexauridae* sp., near Mun-Sum, Cheju-Island

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Fifty-eight strains showing different colony morphological characteristics on various media were isolated from marine coral (*Plexauridae* sp.) and ambient seawater near Mun-Sum, Cheju Island in 1998. Bacterial diversity was studied by phylogenetic analysis of the partial 16S rRNA gene sequences. All isolates representing the bacterial domain included affiliates of the high G+C (59%) and low G+C (3%) subdivision of Gram positive bacteria, and the alpha (33%) and gamma (5%) subdivision of the *Proteobacteria*. The 16S rDNA sequence similarity of the isolates was in the 88.3 to 100% range (average, 95.6%) to reported sequence data. In the comparison of the isolates from marine coral and ambient seawater, more diverse groups belonging to α-*Proteobacteria* were preferentially obtained from seawater.

Key words: Bacterial diversity, seawater, marine coral, 16S rDNA

Microbial communities are the most complex, diverse, and important assemblage of organisms in the natural environment, but unfortunately, more than 99% of microorganisms are not culturable by conventional culture techniques (2). Thus, molecular tools such as PCR and phylogenetic analysis of 16S rRNA gene (rDNA) sequences provide useful means for characterizing the types of organisms that occur in microbial communities without the need for cultivation. The molecular approaches exploring microbial diversity in many different environments have identified new microorganisms that may be abundant or physiologically significant (3, 8, 10, 13, 14, 15).

Studies on comparison of bacterial diversity between culturable isolates and 16S rDNA clones from the same seawater and soil sample have shown that the sequences of genes cloned directly from environmental DNA do not correspond to the genes of cultured bacteria (6, 21). On the contrary, in the study of biodiversity of human colonic biota, there was strong correlation between culturing bacteria and sampling rDNA directly (22). Also, a majority of the cultivated aerobic heterotrophic bacteria in a subsurface sediment could be described by 16S rDNA clones obtained directly from extracted DNA, though PCR-based methods could not account for all organisms from a given sample (5). These contradictory results appear to depend on the complexity of the environment being examined, the discrepancy between plate counts and direct counts, and/or the sample size of 16S rDNA clones (6). Suzuki *et al.* (21) showed that heterotrophic bacteria culturable on media with high organic contents include many strains for which 16S rDNA sequences are not available in sequence databases. This means that there are many unknown culturable microorganisms. Therefore, although direct rDNA sequencing clearly indicates undiscovered microbial diversity, it is an ongoing task to identify, isolate, and characterize new microorganisms, with respect to phylogeny and physiology (2).

The composition of bacterial communities of estuarine and coastal regions is largely unknown, despite substantial roles many coastal bacteria play in critical biogeochemical cycles and the potential utility of such bacteria for bioremediation and other biotechnological applications (11, 12). It was shown that phylogenetic analysis of 16S rDNA sequences was more efficient than classical phenotypic methods for the identification of bacterial strains freshly isolated from natural habitats (4). In this study, we have made an attempt to isolate diverse bacterial strains using different culture media and compared the bacterial diversity of

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culturable isolates between marine coral, *Plexauridae* sp., and ambient seawater surrounding Mun-Sum, Cheju Island based on molecular identification using 16S rDNA sequences. We have isolated 58 bacterial strains which were successfully classified into known groups by 16S rDNA sequence analysis.

Materials and Methods

Sampling site

Marine coral, *Plexaurida* sp., and seawater sample near the coral habitat 10 m deep were collected by direct SCUBA at a station, Mun-Sum (33°12'N, 126° 37'E), located 1 km off the coast of Seoguipo-Si, Cheju-Do on 21 Jan. 1998. This coastal area is rather free from pollution. The samples were stored on ice, and bacterial isolation procedures were carried out immediately after transportation at the day of sampling.

Isolation of bacterial strains

For the isolation of epibiotic bacteria from the *Plexauridae* sp., a portion of the tissue sample (about 0.1 g in weight) was aseptically placed into 1 ml of sterilized seawater and ground using mortar and pestle. Subsamples (each 100 μl) of seawater and ground live specimen of *Plexauridae* sp. were spread onto plates of 3 different media and incubated 20°C in the dark. The media for bacterial isolation were 10-fold diluted ZoBell 2216 marine agar (23), ZoBell 2216 marine agar supplemented with 2% glucose, and an agar medium consisting of 0.2% algal powder, 0.2% fish meal, 0.2% diatomaceous earth in 75% aged seawater. Colonies showing different morphologies (size, pigmentation, opacity, texture, form, elevation, margin, surface) were isolated and their Gram staining characteristics were determined by the procedure of the KOH test (20). The culturable isolates from seawater and marine coral, *Plexauridae* sp. were assigned the prefix SW and Plex, respectively. One ml of actively grown aliquots in ZoBell 2216 broth were mixed with the same volume of sterile 40% glycerol and were stored at -70°C.

Cell counts

Direct counts were carried out by epifluorescence microscopy with DAPI (4',6-diamidino-2-phenylindole) to stain cells according to the protocol (19) except for fixation with 1% Rugol's iodine. The heterotrophic cell counts were estimated from the number of CFU appearing in each media after three weeks.

Determination of 16S rDNA sequences

Based on their colony morphology, 58 isolates were selected for determination of 16S rDNA sequences. The isolates were grown in ZoBell broth and harvested by centrifugation (10,000× *g*, 5 min). The genomic DNAs were prepared by using a Wizard genomic DNA kit (Promega, Madison, WI) according to the manufacturer's instructions. The 16S rDNA was amplified from genomic DNA by PCR using two general bacterial 16S rDNA primers 27F (5'-AGAGTTTGATCM TGGCTCAG-3'; *Escherichia coli* nucleotides 8 to 27) and 1522R (5'-AAGGAGGTGATCCANCCRCA-3'; *E. coli* nucleotides 1541 to 1522) (9). The reaction mixtures for PCR contained 1 × PCR buffer, each deoxynucleotide triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.2 μM, 50~100 ng DNA template, and 2.5 U of *Taq* DNA polymerase (Promega) in a final volume of 100 μl. DNA amplification was performed on a model 2400 thermal cycler (PE, Applied Biosystems) with 5 min initial denaturation at 94°C, and 35 cycles consisting of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (2 min at 72°C), and final extension at 72°C for 7 min. Amplified DNA was visualized by electrophoresis of PCR products (5 μl) in 0.8% agarose in 1 × TAE buffer. The PCR products were purified with a Wizard PCR Preps kit (Promega) and sequencing was performed directly from the PCR products. The partial 16S rDNA sequence was determined using sequencing primers (27F and 525R; 5'-TTACCGCGGCNGCTGGCAC-3'; *E. coli* nucleotides 533 to 515) and a BigDye terminator cycle sequencing kit (PE, Applied Biosystems) by an Applied Biosystems model 377 automatic DNA sequencer.

Analysis of sequences

Analyses of the 16S rDNA sequences were performed using SIMILARITY_RANK from RDP (16) and Basic Local Alignment Search Tool (BLAST) (1). These analyses were used to determine whether the sequences were 16S rDNA sequences and to estimate the degree of similarity to other 16S rDNA sequences. The partial 16S rDNA sequences were manually aligned to pre-aligned 16S rDNA data from the RDP. The Phylogenetic Inference Package (PHYLIP), version 3.57c (7), was used to further analyze the sequence data, DNADIST, performed with the Jukes-Cantor option, and employed to determine sequence similarities. FITCH was used to create a phylogenetic tree.

Results and Discussion

The number of total bacteria in seawater and *Plexauridae* sp. by DAPI direct counting method were 2.0 $\times 10^6$ ml^{-1} and 4.7 $\times 10^9$ g (dry wt.)$^{-1}$, respectively. The aerobic heterotrophic bacteria in seawater and marine

Table 1. Nearest neighbors and inferred phylogenetic affiliations of isolated bacterial strains from the coastal area of Munsum, Cheju-Island

Strain No.	Neareast RDP-determined phylogenetic neighbor (S_{ab})[a]	Affiliation	% similarity
SW10567	*Micrococcus luteus* (0.852)	Gram positive-high G+C	98.7
SW10569	*Sphingomonas subarctica* (0.843)	*α-Proteobacteria*	96.1
SW10574	*Erythrobacter longus* (0.785)	*α-Proteobacteria*	94.4
SW10576	*Erythrobacter longus* (0.888)	*α-Proteobacteria*	99.6
SW10577	*Sinorhizobium meliloti* (0.753)	*α-Proteobacteria*	90.6
SW10579	*Rhizobium loti* (0.651)	*α-Proteobacteria*	89.3
SW10582	*Streptomyces tendae* (0.897)	Gram positive-high G+C	98.3
SW10583	*Erythrobacter longus* (0.760)	*α-Proteobacteria*	96.5
SW10584	*Sphingomonas paucimobilis* (0.805)	*α-Proteobacteria*	88.3
SW10585	*Aureobacterium testaceum* (0.826)	Gram positive-high G+C	96.9
SW10587	*Microbacterium imperiale* (0.851)	Gram positive-high G+C	97.1
SW10589	*Erythrobacter longus* (0.839)	*α-Proteobacteria*	96.5
SW10590	*Kytococcus sedentarius* (0.657)	Gram positive-high G+C	93.5
SW10592	*Paracoccus aminophilus* (0.823)	*α-Proteobacteria*	97.3
SW10593	*Kocuria rosea* (0.786)	Gram positive-high G+C	95.0
SW10594	*Nocardioides simplex* (0.774)	Gram positive-high G+C	94.1
SW10596	*Sphingomonas* sp. str. DhA-33 (0.882)	*α-Proteobacteria*	90.7
SW10597	*Cellulomonas cellullans* (0.863)	Gram positive-high G+C	99.1
SW10602	*Erythrobacter litoralis* (0.850)	*α-Proteobacteria*	94.4
SW10603	*Mycobacterium chlorophenolicum* (0.804)	Gram positive-high G+C	96.3
SW10604	*Erythrobacter longus* (0.822)	*α-Proteobacteria*	95.7
SW10605	*Erythrobacter longus* (0.858)	*α-Proteobacteria*	96.5
SW10606	*Dietzia maris* (0.883)	Gram positive-high G+C	99.6
SW10609A	*Erythrobacter longus* (0.765)	*α-Proteobacteria*	94.8
SW10610	*Dietzia maris* (0.891)	Gram positive-high G+C	99.7
SW10618	*Psychrobacter immobilis* (0.724)	*α-Proteobacteria*	95.0
SW10619	*Kytococcus sedenterius* (0.711)	Gram positive-high G+C	93.7
SW10621	*Arthrobacter globiformis* (0.849)	Gram positive-high G+C	96.4
SW10624	*Aurebacterium testaceum* (0.837)	Gram positive-high G+C	96.1
SW10625	*Micrococcus luteus* (0.872)	Gram positive-high G+C	94.7
SW10626	*Erythrobacter longus* (0.825)	*α-Proteobacteria*	94.4
SW10627	*Aureobacterium testaceum* (0.867)	Gram positive-high G+C	95.9
SW10629	*Brevundimonas diminuta* (0.885)	*α-Proteobacteria*	95.7
SW10630	*Micrococcus luteus* (0.834)	Gram positive-high G+C	99.5
Plex10635	*Psychrobacter immobilis* (0.762)	*α-Proteobacteria*	96.0
Plex10636	*Paracoccus aminovorans* (0.857)	*α-Proteobacteria*	95.3
Plex10639	*Kytococus sedentarius* (0.747)	Gram positive-high G+C	92.4
Plex10640	*Aurebacterium testaceum* (0.852)	Gram positive-high G+C	95.6
Plex10641	*Kytococcus sedenterius* (0.674)	Gram positive-high G+C	92.6
Plex10650	*Kytococcus sedenterius* (0.664)	Gram positive-high G+C	92.1
Plex10651	*Kytococcus sedenterius* (0.711)	Gram positive-high G+C	92.6
Plex10653	*Caulobacter* sp. str. FWC14 (0.825)	*α-Proteobacteria*	97.2
Plex10657	*Dermacoccus nishinomiyaensis* (0.843)	Gram positive-high G+C	97.7
Plex10659	*Gordona terrae* (0.904)	Gram positive-high G+C	100
Plex10660	*Aureobacterium testaceum* (0.719)	Gram positive-high G+C	92.9
Plex10665	*Brevibacterium linens* (0.828)	Gram positive-high G+C	98.1
Plex10666	*Staphylococcus warneri* (0.910)	Gram positive-low G+C	99.5
Plex10672	*Arthrobacter globiformis* (0.826)	Gram positive-high G+C	94.3
Plex10673	*Kytococcus sedentarius* (0.697)	Gram positive-high G+C	92.4
Plex10674	*Xanthomonas campestris* (0.702)	*α-Proteobacteria*	93.3
Plex10677	*Micrococcus luteus* (0.872)	Gram positive-high G+C	99.7
Plex10678	*Micrococcus luteus* (0.879)	Gram positive-high G+C	100
Plex10679	*Micrococcus luteus* (0.866)	Gram positive-high G+C	100
Plex10680	*Kytococcus sedentarius* (0.729)	Gram positive-high G+C	92.4
Plex10681	*Bacillus* sp. IF O12605 (0.872)	Gram positive-low G+C	98.0
Plex10683	*Terrabacter tumescens* (0.729)	Gram positive-high G+C	94.6
Plex10690	*Paracoccus aminovorans* (0.849)	*α-Proteobacteria*	97.7
Plex10691	*Terrabacter tumescens* (0.751)	Gram positive-high G+C	92.2

[a]The closest matching sequence from a cultivated strain was identified using SIMILARITY_RANK option of RDP. S_{ab}; similarity coefficient used by RDP.

coral (*Plexauridae* sp.) as the number of CFU on 3 different media were in the range of 3.0×10^3 to 4.2×10^4 ml^{-1} and $7.0 \times 10^3 \sim 2.5 \times 10^4$ g (dry wt.)$^{-1}$, respectively. The ratio of aerobic heterotrophic culturable bacteria to total bacteria from seawater and marine coral ranged from 1 to 0.001%, respectively.

Fifty-eight culturable isolates (34 from seawater and 24 from marine coral) showing different colony characteristics (size, pigmentation, opacity, texture, form, elevation, margin, and surface) were selected for molecular identification. The 1.5 kb DNA fragments corresponding to nearly full length 16S rDNAs were amplified by PCR from all strains using the primer set (27F and 1522R). Partial sequences (approximately 600~700 bp from 5' position) of these fragments were determined for approximate phylogenetic affiliation and the nearest neighbor of individual isolates using the SIMILA- RITY_RANK option of the RDP service. Phylogenetic analyses of 58 partial 16S rDNA sequences of culturable isolates revealed that Gram positive bacteria with high G+C (n=34) was the predominant group, followed by the *α-Proteobacteria* (n=19), *γ-Proteobacteria* (n=3) and Gram positive bacteria with low G+C (n=2) (Table 1). All isolates could be assigned to known cultured bacteria in RDP database except only one isolate, SW10594 of which the nearest neighbor was the FJ21A clone. However, it could be classified into a member of the *Nocardioides* group (94.1% in sequence similarity with *Nocardioides simplex*). Interestingly in this study, there were no isolates belonging to the *Flexibacter-Cytophaga-Bacteriodes* group that were found in a higher proportion in seawater (8, 18, 21).

The range of S_{ab} value of the partial sequences obtained from culturable isolates was 0.651 to 0.910 and mean S_{ab} value was 0.804. This is comparable with the results (median S_{ab} value, 0.84; S_{ab} value range, 0.32 to 0.98) where 35 partial sequences of cultivated isolates were obtained from arid soil (6). Sequence similarity of 100% between two isolates (Plex10678, Plex10679) and *Micrococcus luteus*, and between Plex 10659 and *Gordona terrae* (Table 1) were shown. Also, 8 culturable isolates corresponded well with the *Kytococcus* group (92.1~93.7% in sequence similarity), but 7 of them showed >99% in sequence similarity among them. This means that in spite of selection based on different colony morphology, there were some redundancy among the cultured isolates.

The 16S rDNA sequence similarity ranged from 88.3 to 100% with known cultivated organisms and average level was 95.6% based on analysis of partial sequences. Nineteen (33%) bacterial isolates showed >97% in sequence similarity and 27 (46%) isolates ranged from 93 to 97% in sequence similarity. It has been assumed that 0.97 and 0.93 represent levels of identity at the species and genus levels, respectively (17, 18). By using these criteria, 46 strains (79%) among 58 isolated strains could be classified into the previously described strains in the genus level. The remaining 12 isolates (21%) have shown sequence similarity values below 0.93. Due to high sequence similarity (>97%) among them, only 5 isolates (SW10579, SW10596, Plex10650, Plex10660, and Plex10691) were representatives showing sequence similarity below 0.93. Although further studies on biochemical and genetic characterization are needed, these isolates may represent a new species or genus. Consequently, phylogenetic analysis of partial 16S rDNA sequences have shown that although most 16S rDNA sequences from culturable isolates were phylogenetically related to groups of described species, a significant fraction of the isolates may be previously unidentified species. This result implies that microbial cultivation has not been employed extensively for determining the taxonomic identification and distribution of marine bacteria or that many of the systematically described marine bacterial strains have not yet been represented in the rDNA sequence database.

Fig. 1A shows phylogenetic analysis of representative cultivated isolates within the α-subdivision of the *Proteobacteria*. The most abundant *α-Proteobacteria* group were members of the *Sphingomonas* group (12 of the 19 isolates) containing *Erythrobacter* and *Sphingomonas* in the genus level (Table 2; Fig. 1A). The remaining cultivated isolates belonging to the *α-Proteobacteria* were members of the *Rhodobacter* group (3 isolates), *Caulobacter* group (2 isolates) and *Rhizobium-Agrobacterium* group (2 isolates). This result is consistent with the conclusion that the most abundant *α-Proteobacteria* cellular isolates from seawater were members of the *Sphingomonas* and *Rhodobacter* groups (21). Phylogenetic analysis of 3 isolates within the γ subdivision of the *Proteobacteria* was shown in Fig. 1B. Two isolates were members of the *Pseudo- monas* group and one isolate was classified into the *Xanthomonas* group. The sequence similarity were in the 93.3 to 96.0% range. Phylogenetic analysis of the representative isolates included in Gram positive, high G+C and low G+C division are shown in Fig. 1C. The isolates were classified into 17 diverse groups in the genus level. Most belonging to the Gram positive phylum were members of *Arthrobacter* relatives containing the *Dermatophilus*, *Arthrobacter*, *Microbacterium*, and *Cellulomonas* group.

In comparison of culturable isolates between marine coral and ambient seawater, most isolates (16 of the 19 isolates) belonging to the *α-Proteobacteria* were obtained from seawater (Table 2). In the case of Gram positive phylum, culturable isolates included in the *Dermatophilus* group containing two genera (*Ky- tococcus, and Dermatococcus*) were obtained from marine coral pref-

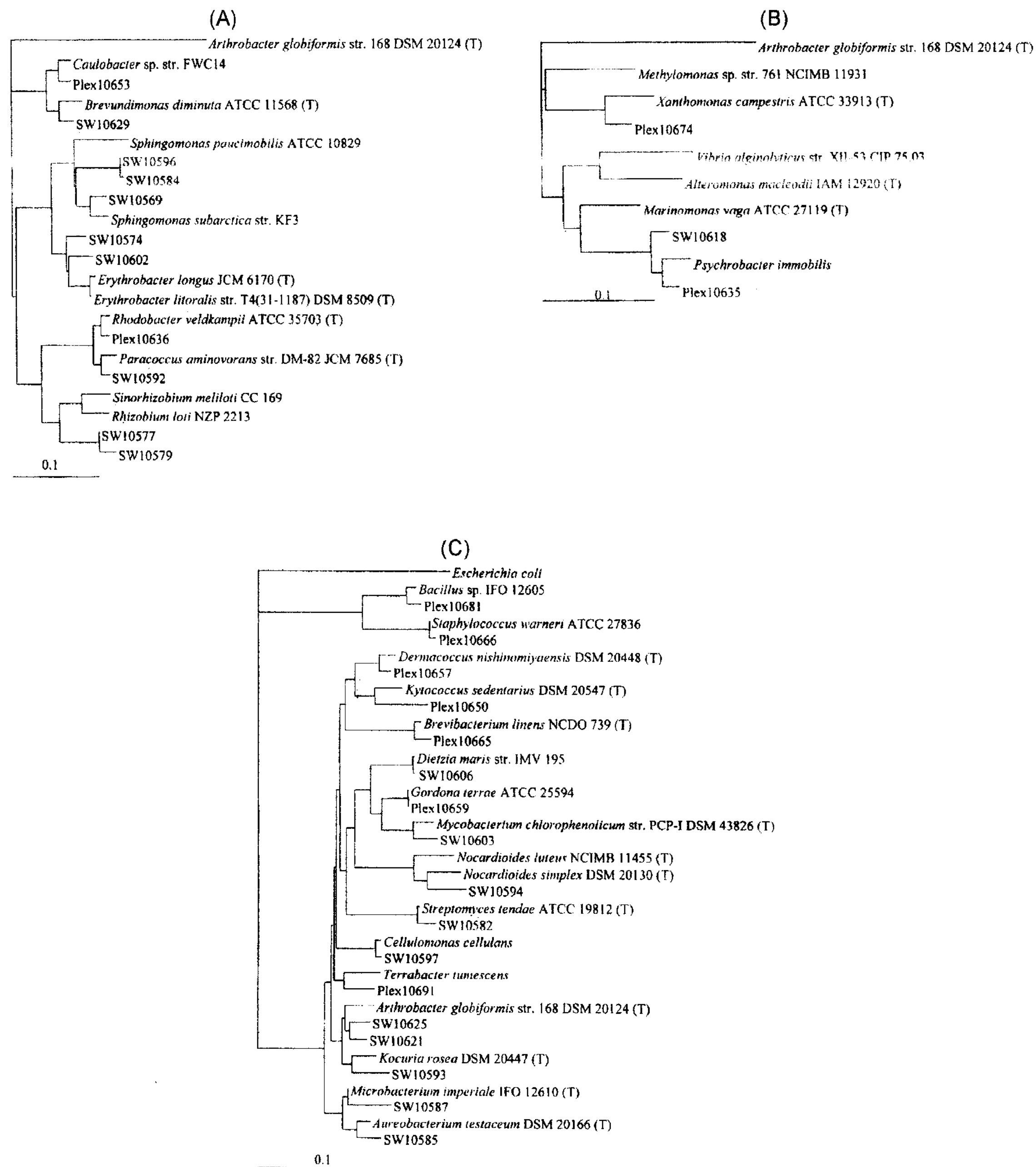

Fig. 1. (A) Phylogenetic tree of representative isolates within the subdivision of *Proteobacteria* based on positions 44 to 337 (*E. coli* numbering system) of the 16S rDNA gene. (B) Phylogenetic tree of isolates within the subdivision of *Proteobacteria* based on positions 52 to 611 (*E. coli* numbering system) of 16S rDNA. (C) Phylogenetic tree of isolates within the Gram positive phylum based on positions 41 to 598 (*E. coli* numbering system) of 16S rDNA. The scale bar indicates the number of substitutions per sequence position.

erentially (Table 2). This result implied that the bacterial community between seawater and marine coral was different based on comparisons of culturable isolates that were a minor fraction of total bacterial counts. A recent comparison of cultivation and 16S rDNA cloning for analysis of bacterial community diversity in soils has shown that 16S rDNA cloning and cultivation method generally describe similar relationships between soil microbial communities, while significant discrepancies could occur (6).

In conclusion, the molecular method based on comparisons of 16S rDNA sequences has been applied to identify bacterial strains isolated from natural environments at the genus level successfully. Also, the recovered isolates were closely related to several microbial species that had been described previously.

Table 2. Comparison of culturable isolates between seawater and marine coral (*Plexuaridae* sp.)

Affiliation	Group[a]	Genus	No. of isolates	
			Seawater	Marine coral
α-Proteobacteria	*Sphingomonas*	*Erythrobacter*	9	
		Sphingomonas	3	
	Rhodobacter	*Paracoccus*	1	2
	Rhzobium-Agrobacterium	*Rhizobium*	1	
		Sinorhizobium	1	
	Caulobacter	*Caulobacter*		1
		Brevundimonas	1	
α-Proteobacteria	*Pseudomonas*	*Psychrobacter*	1	1
	Xanthomonas	*Xanthomonas*		1
Gram positive, high G+C	*Dermatophilus*	*Kytococcus*	2	6
		Dermacoccus		1
	Arthrobacter	*Micrococcus*	3	3
		Arthrobacter	1	1
		Kocuria	1	
		Brevibacterium		1
	Microbacteium	*Aureobacterium*	3	2
		Microbacterium	1	
	Cellulomonas	*Cellulomonas*	1	
		Terrabacter		2
	Corynebacterium	*Dietzia*	2	
		Gordona		1
	Mycobacterium	*Mycobacterium*	1	
	Streptomyces	*Streptomyces*	1	
	Propionibacterium	*Norcardioides*	1	
Gram positive, low G+C	*Bacillus*	*Bacillus*		1
	Staphylococcus	*Staphylococcus*		1

[a]Based on RDP classfication.

It is evident that there are some differences in culturable isolates between seawater and marine coral at the same site in spite of biases against culturing techniques for recovering representative bacteria from most natural bacterial communities.

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