Original Article

MARINE BIOTECHNOLOGY

Isolation of Marine Bacteria by In Situ Culture on Media-Supplemented Polyurethane Foam

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Received 31 January 2005 / Accepted 18 September 2005 / Published online: 26 April 2006

Abstract

Polyurethane foam (PUF) supplemented with various agar media was used *in situ* to trap marine bacteria and it consequently provided a substrate on which they could be cultivated while exposed to natural seawater in the coral reef area. The bacterial population on the PUF blocks was analyzed by denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 16S rDNA fragments. Changing the composition of the cultivation medium in the PUF blocks and selecting different sampling sites resulted in different bacteria being detected on the PUF blocks. For example, iron-utilizing (IU) bacteria, siderophore-producing (SP) bacteria, and petroleum-degrading (PD) bacteria were isolated from PUF blocks and it was discovered that IU and SP contained iron and PD contained hydrocarbon. This method opens up the possibility for isolating novel and useful marine bacteria.

Keywords: Coral reef — denaturing gradient gel electrophoresis — marine bacteria polyurethane foam (PUF) — siderophore

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Introduction

More than 99% of naturally occurring microorganisms remain uncultured (Hugenholtz et al., 1998), because methods for isolation of microorganisms from natural environments still remain to be established. Previous work reported that carriers such as glass submerged in the sea become populated by periphytic bacteria (Corpe, 1973). However, there are few reports that the carrier soaked with agar media can be used as a tool for the isolation of marine bacteria.

This article describes a potentially useful enrichment culture method using polyurethane foam soaked with various culture media (PUF-media). This method may provide a three-dimensional culture environment that is composed of various different subenvironments for microorganisms. The subenvironments have different conditions for growth of microorganisms, such as pH, oxygen concentration, and so forth. PUF has been used as an effective microbe carrier in a down-flow microfilm bioreactor (Araki et al., 1999).

Enrichment culture is a method for isolating bacteria from the environment. We suggest that it may be possible to stimulate the growth of microorganisms possessing such expected functions as hydrocarbon degradation or metal utilization by supplementing the cultivation medium with hydrocarbons or some metals. This method is good for isolating novel and useful marine bacteria.

Materials and Methods

On-Site PUF Culture. PUF blocks (5 × 5 × 7 cm) were autoclaved in a glass beaker, soaked in 100 ml of a hot agar medium, pressed several times with a sterile spoon so that the medium could penetrate the center of each block, and then they were cooled

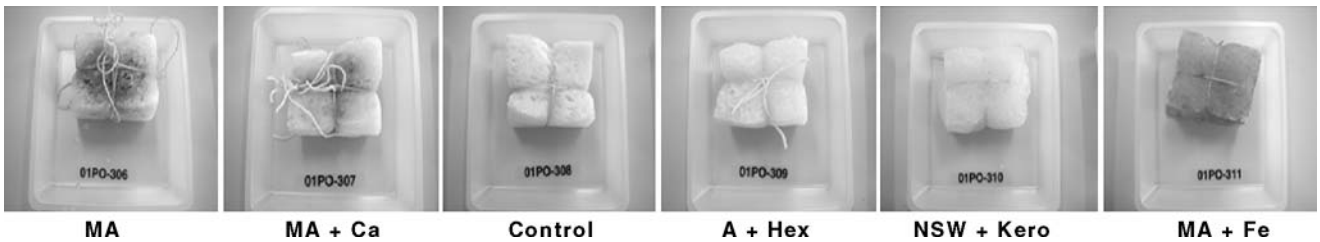

Fig. 1. PUF samples loaded with the different media displayed distinct differences in color and smell when compared with the control samples (sampling point 2).

and stored in a sterile package. The composition of each agar medium used was as follows: (1) MA, marine broth (Difco) containing 1.5% agar; (2) MA + Ca, marine broth (Difco) with 1% $CaCO_3$ containing 1.5% agar; (3) A + Hex (*n*-hexadecane), 30.0 g of NaCl, 0.5 g of KH_2PO_4, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.3 g of KCl, 0.01 g of $FeCl_2 \cdot nH_2O$, 1000 ml of distilled water, 1.0 g of L-alanine, 15.0 g of agar, and 1 ml of *n*-hexadecane at pH 7.5; (4) NSW + Kero (kerosene), 1.0 g of NH_4NO_3, 0.02 g of ferric citrate, 0.02 g of K_2HPO_4, 0.5 g of yeast extract, 800 ml of filtered seawater, 200 ml of distilled water, 15.0 g of agar, and 5 ml of kerosene at pH 7.8; and (5) MA + Fe, marine broth (Difco) with 0.3% iron (III) citrate hydrate containing 1.5% agar. Enrichment cultures using A + Hex (*n*-hexadecane) and NSW + Kero (kerosene) liquid media have been reported as a method for isolating hydrocarbon-degrading bacteria (Fujisawa et al., 1977; Higashihara et al., 1978).

PUF blocks loaded with agar media were tied to a rope at intervals of 80 cm with one PUF block unsupplemented with agar medium as a blank control. This rope with the series of PUF blocks was positioned about 5 to 6 m below the surface of the sea at Pohnpei in Micronesia for 3 days from December 4, 2001. The sampling points over the coral reefs were as follows: point 1, the channel near the reef edge (6°58′75″ N, 158°07′60″ E); point 2, near the mangroves (6°59′30″ N, 158°11′ E); and point 3, around the patch reef between 1 and 2 (6°58′85″ N, 158°10′ 02″ E). Three days after the deployment, PUF blocks were recovered, immersed in TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8.0) and stored at ambient temperature for 4 days until their return to the laboratory with subsequent storage at –20°C.

Scanning Electron Microscopy (SEM). After *in situ* incubation, PUF cubes were fixed in 2.5% glutaraldehyde solution (in seawater) for 24 h. The samples were dehydrated using ethanol series after post-fixation using osmium tetroxide. Dehydrated samples were critical-point dried (CO2), mounted, and sputter-coated with platinum-palladium. SEM observation was performed on a Hitachi S-2500 electron microscope at 15 kV.

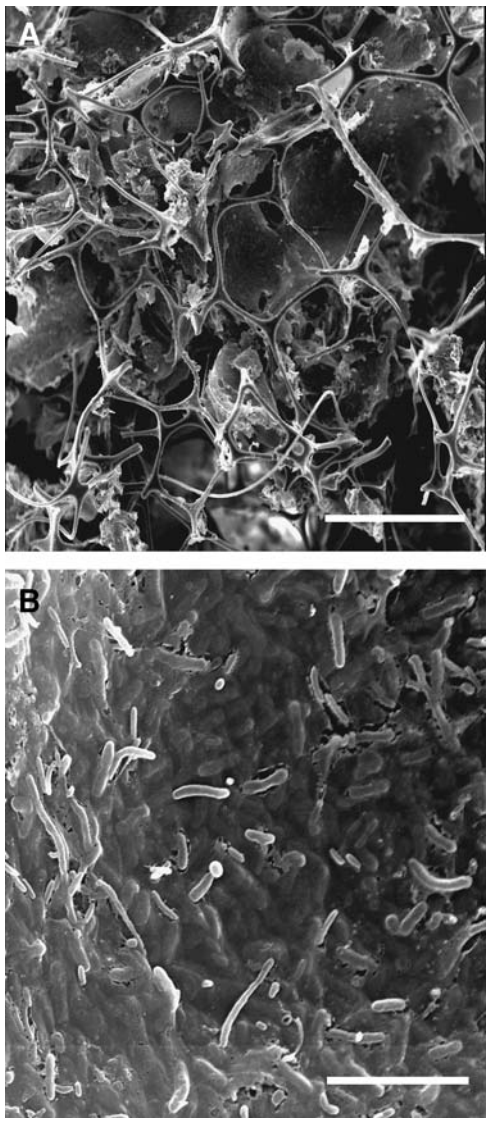

Fig. 2. SEM image of PUF-MA + Ca. The PUF cube was obtained in Okinawa, 2002. (**A**) Scale bar = 150 μm. (**B**) Scale bar = 7.5 μm. This is the surface of the cross section.

DNA Extraction. Each PUF block (1 cm^3) was ground with a mortar and pestle in the TE buffer (1 ml) and transferred to a 2-ml sample tube. DNA extraction was carried out by using TE-equilibrated phenol (pH 7.5 to 8.0), phenol–chloroform–isoamyl alcohol (25:24:1, by vol), and chloroform–isoamyl alcohol (24:1, vol/vol). The DNA was precipitated with 2-propanol, before the extracted DNA was purified using a GFX genomic blood purification kit (Amersham Pharmacia Biotech).

Polymerase Chain Reaction (PCR) for Denaturing Gradient Gel Electrophoresis (DGGE) Analysis. The PCR-DGGE method was used to analyze the bacterial population of each PUF block, with a PCR primer set and touchdown PCR protocol similar to those used by Muyzer et al. (1993, 1996). Variable region 3 of 16S rDNA from the bacterial community in each PUF block was amplified by touchdown PCR with two primers. These primers for PCR were 341F, (5′-CCTACGGGAGGCAG CAG-3′), of which the 5′ end was attached to a GC-rich sequence (5′-CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCACGGGGGG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′). They respectively corresponded to 341–357 and 517–534 of *Escherichia coli* 16S rDNA sequences. The reaction mixtures used for PCR contained (per 50 μl) 5 μl of a 10× PCR buffer containing MgCl2, 4 μl of a dNTP solution, the primers (341F-GC and 534R) at a concentration of 25 μM, 0.25 U of Ampli Taq Gold (Applied Biosystems), and 10 ng of DNA. The touchdown PCR protocol was as follows: The initial denaturation stage was started at 94°C for 9 min, followed by 18 cycles at 94°C for 1 min, 64°C for 1 min (this annealing temperature was decreased by 1°C every two cycles), and then at 72°C for 2 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, before the final extension was carried out at 72°C for 10 min. After the amplified PCR products were checked using 3% agarose gel electrophoresis, DGGE was carried out with the D-code system (Bio-Rad Laboratories), using a TAE buffer and 10% acrylamide gel [a 40% acrylamide stock solution (acrylamide–bis-acrylamide of 37.5:1)] with a 30% to 55% denaturing gradient of urea and formamide at 60°C for 3.5 h at a constant voltage of 200 V. After electrophoresis, the gel was soaked in SYBR™ Green I nucleic acid gel stain (1:10,000 dilution;

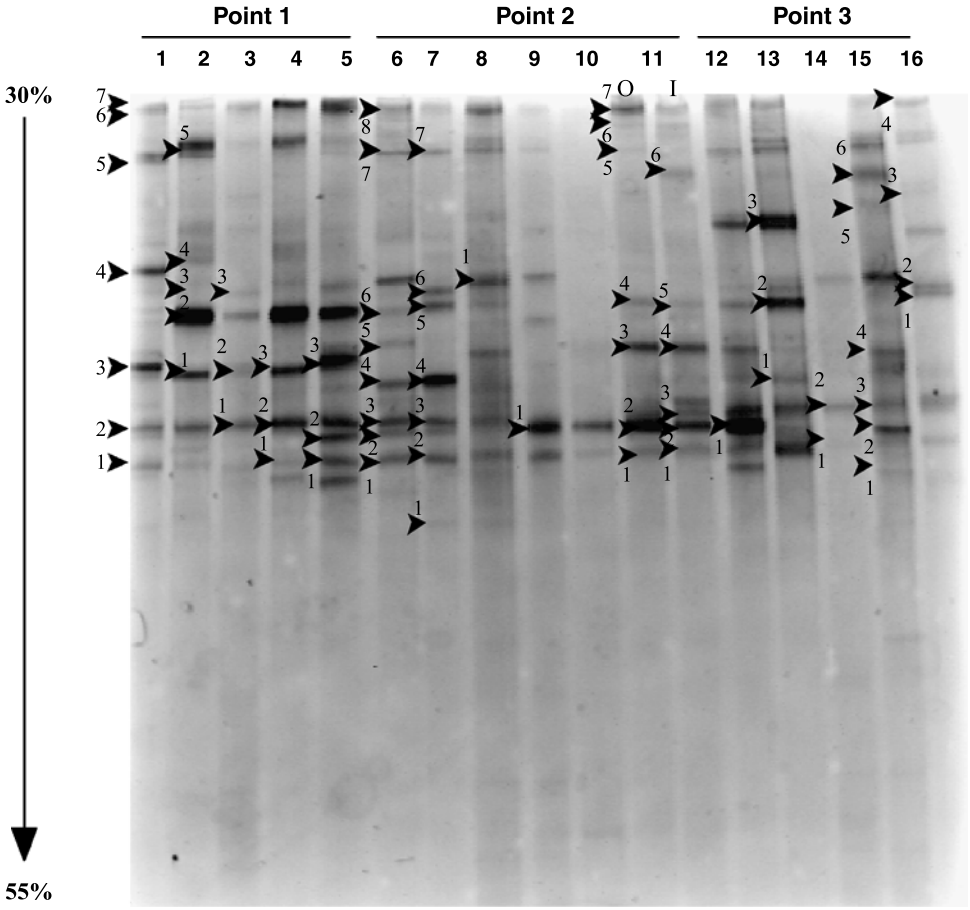

Fig. 3. DGGE analysis of bacterial 16S rDNA fragments from the PUF samples at Pohnpei. *Lanes: 1–5*, sampling point 1; *6–11*, sampling point 2; *12–16*, sampling point 3; **1**, **6**, and **12**, MA; **2**, **7**, and **13**, MA + Ca; **3**, **8**, and **14**, control; **4**, **9**, and **15**, A + Hex; **5**, **10**, and **16**, NSW + Kero; **11o**, MA + Fe (outside of PUF); **11i**, MA + Fe (inside of PUF).

Table 1. 16S rDNA Sequences Most Closely Related to the Major Bacterial Populations Detected on the PUF Samples by DGGE and Shown in Figure 2

	Band	*Media*	*Closest relative*	*% Identity*	*Accession no.*	*Taxonomic group*
	PO-1-1	MA	*Shewanella algae*	100	X81621	*gamma*
	PO-1-2	MA	Alpha proteobacterium MBIC3368	97	AF218241	*alpha*
	PO-1-3	MA	*Persicobacter diffluens*	100	M58765	*Bacteroidetes*
	PO-1-4	MA	*Persicobacter diffluens*	100	M58765	*Bacteroidetes*
	PO-1-5	MA	*Arcobacter* sp. D1a1	96	AJ271654	*epsilon*
	PO-1-6	MA	uncultured epsilon proteobacterium clone CD5B11	95	AY038410	*epsilon*
	PO-1-7	MA	uncultured epsilon proteobacterium clone CD5B11	98	AY038410	*epsilon*
	PO-2-1	MA+Ca	*Vibrio* sp. VI737/19	96	X97987	*gamma*
	PO-2-2	MA+Ca	*Vibrio* sp. VI737/19	100	X97987	*gamma*
	PO-2-3	MA+Ca	*Vibrio* sp. VI737/19	96	X97987	*gamma*
Point 1	PO-2-4	MA+Ca	*Vibrio* sp. VI737/19	97	X97987	*gamma*
	PO-2-5	MA+Ca	uncultured epsilon proteobacterium 34-49	98	AF473974	*epsilon*
	PO-3-1	Control	*Vibrio furnissii* (ATCC 35016 T)	93	X76336	*gamma*
	PO-3-2	Control	*Vibrio* sp. VI737/19	94	X97987	*gamma*
	PO-3-3	Control	*Vibrio* sp. VI737/19	95	X97987	*gamma*
	PO-4-1	A+Hex	*Vibrio* sp. VI737/19	97	X97987	*gamma*
	PO-4-2	A+Hex	*Vibrio* sp. VI737/19	100	X97987	*gamma*
	PO-4-3	A+Hex	*Vibrio* sp. VI737/19	100	X97987	*gamma*
	PO-5-1	NSW+Kero	*Sulfurospirillum* sp. 18.1	97	AF357199	*epsilon*
	PO-5-2	NSW+Kero	Marine bacterium ATAM173a_36	91	AF359543	*gamma*
	PO-5-3	NSW+Kero	*Vibrio* sp. (A075)	92	Z22978	*gamma*
	PO-6-1	MA	*Fusibacter paucivorans*	92	AF050099	*Firmicutes*
	PO-6-2	MA	*Fusibacter paucivorans*	98	AF050099	*Firmicutes*
	PO-6-3	MA	*Fusibacter paucivorans*	96	AF050099	*Firmicutes*
	PO-6-4	MA	*Vibrio vulnificus* (ATCC 27562 T)	97	X76333	*gamma*
	PO-6-5	MA	*Vibrio* sp. (A065)	98	Z22999	*gamma*
	PO-6-6	MA	*Desulfovibrio acrylicus*	96	U32578	*delta*
	PO-6-7	MA	uncultured epsilon proteobacterium 34–49	98	AF473974	*epsilon*
	PO-6-8	MA	uncultured epsilon proteobacterium 34–49	95	AF473974	*epsilon*
	PO-7-1	MA+Ca	*Vibrio* sp. (A081)	94	Z22980	*gamma*
	PO-7-2	MA+Ca	*Fusibacter paucivorans*	96	AF050099	*Firmicutes*
	PO-7-3	MA+Ca	delta proteobacterium RS64	90	AJ289756	*delta*
	PO-7-4	MA+Ca	Marine bacterium PWF3	98	AY082667	*gamma*
	PO-7-5	MA+Ca	uncultured Ruegeria CtaxAus-6	98	AF259591	*alpha*
	PO-7-6	MA+Ca	*Persicobacter diffluens*	98	M58765	*Bacteroidetes*
Point 2	PO-7-7	MA+Ca	uncultured bacterium clone CD4D1	95	AY038542	unclassified
	PO-8-1	Control	Marine bacterium SCRIPPS_413	99	AF359548	*Bacteroidetes*
	PO-9-1	A+Hex	*Fusibacter paucivorans*	97	AF050099	*Firmicutes*
	PO-11 (o)-1	MA+Fe	*Fusibacter paucivorans*	97	AF050099	*Firmicutes*
	PO-11 (o)-2	MA+Fe	*Fusibacter paucivorans*	98	AF050099	*Firmicutes*
	PO-11 (o)-3	MA+Fe	*Vibrio diazotrophicus* (ATCC 33466T)	90	X74701	*gamma*
	PO-11 (o)-4	MA+Fe	*Fusibacter paucivorans*	98	AF050099	*Firmicutes*
	PO-11 (o)-5	MA+Fe	*Shewanella algae*	98	X81621	*gamma*
	PO-11 (o)-6	MA+Fe	*Vibrio* sp. (A065)	98	Z22999	*gamma*
	PO-11 (o)-7	MA+Fe	uncultured bacterium clone CD4D1	87	AY038542	unclassified

Continues

Table 1. Continued

	Band	Media	Closest relative	% Identity	Accession no.	Taxonomic group
	PO-11 (i)-1	MA+Fe	*Fusibacter paucivorans*	95	AF050099	*Firmicutes*
	PO-11 (i)-2	MA+Fe	*Vibrio diazotrophicus* (ATCC 33466T)	99	X74701	*gamma*
	PO-11 (i)-3	MA+Fe	*Shewanella algae*	98	X81621	*gamma*
	PO-11 (i)-4	MA+Fe	*Vibrio* sp. UST991130-011	99	AF465368	*gamma*
	PO-11 (i)-5	MA+Fe	uncultured bacterium clone BM89MF5BA11	98	AF365487	*epsilon*
	PO-11 (i)-6	MA+Fe	*Vibrio vulnificus* (ATCC 33147)	99	Z22992	*gamma*
	PO-12-1	MA	*Pseudomonas putida* 5IIANH	90	AF307869	*gamma*
	PO-13-1	MA+Ca	uncultured bacterium NoosaAW70	87	AF269024	TM7
	PO-13-2	MA+Ca	*Thalassospira lucentensis*	94	AF358664	*alpha*
	PO-13-3	MA+Ca	uncultured CFB group bacterium clone CD13H9	93	AF441869	*Bacteroidetes*
	PO-14-1	Control	*Fusibacter paucivorans*	97	AF050099	*Firmicutes*
	PO-14-2	Control	*Fusibacter paucivorans*	96	AF050099	*Firmicutes*
	PO-15-1	A+Hex	uncultured gamma proteobacterium TIHP302-28	98	AB031614	*gamma*
Point 3	PO-15-2	A+Hex	*Fusibacter paucivorans*	98	AF050099	*Firmicutes*
	PO-15-3	A+Hex	*Shewanella algae*	96	X81621	*gamma*
	PO-15-4	A+Hex	*Desulfovibrio acrylicus*	96	U32578	*delta*
	PO-15-5	A+Hex	uncultured epsilon proteobacterium clone CD5B11	99	AY038410	*epsilon*
	PO-15-6	A+Hex	uncultured epsilon proteobacterium clone CD5B11	98	AY038410	*epsilon*
	PO-16-1	NSW+Kero	unidentified eubacterium	95	AJ011042	*Bacteroidetes*
	PO-16-2	NSW+Kero	unidentified eubacterium	95	AJ011042	*Bacteroidetes*
	PO-16-3	NSW+Kero	Propionigenium maris strain ML-1	97	Y16800	*Fusobacteria*
	PO-16-4	NSW+Kero	*Fusibacter paucivorans*	97	AF050099	*Firmicutes*

Molecular Probes, Inc.) for 30 min and then photographed on a UV transilluminator with a CCD camera. Selected DGGE bands were excised from the gel with a surgical blade and transferred into fresh sterile microtubes. DNA was extracted from the excised gel with TE buffer. The extracted DNA was purified, and DGGE was repeated until there was only a single band without any trace from other bands. The partial sequence of the DNA from this band was analyzed by an ABI 3700 automated sequencer (Applied Biosystems), using the ABI Prism(R) BigDye™ primer cycle sequencing kit (Applied Biosystems). The closest relative to each sequence was obtained by a BLAST (Altschul et al., 1997) in DNA database (DDBJ/EMBL/Genbank). Multiple alignment and construction of phylogenetic trees by the neighbor-joining method (Saitou and Nei, 1987) were performed with the CLUSTAL W computer program (Thompson et al., 1994).

Isolation of Microorganisms. Each PUF was cut into small pieces (1 cm^3) and homogenized with 5 ml of sterile seawater using a glass rod. The homogenate was diluted 1:100 with sterile seawater. Fifty microliters of the diluted homogenate was spread onto four types of agar medium: MA + CA; A + Hex; NSW + Kero; and 1/10MA: 3.74 g of marine broth, 750 ml of filtered seawater, 250 ml of distilled water, and 15.0 g of agar.

Identification of Isolated Bacteria. Genomic DNA of the isolated strains was extracted by using a Puregene DNA extraction kit (Gentra Systems). PCR amplification of the 16S rDNA was performed by using forward primer 341F and reverse primer 907R (5′-CCGTCAATTCATTTGAGTTT-3′) under the same conditions as those used for touchdown PCR. After purifying the PCR product with a QIAquick PCR purification kit (QIAGEN), the purified PCR products were sequenced and identified via the same procedure as that described for DGGE.

Detection of Siderophores. The chrome azurol S (CAS) assay was used to detect siderophores (Schwyn and Neilands, 1987). On CAS agar plates,

siderophore-producing bacteria formed colonies with an orange halo.

Competitive PCR. The competitor DNA that has a sequence of two bacterial primers (9F and 534R) each attaching to one end was constructed by using a competitive DNA construction kit (Takara Bio) according to the manufacturer's manual. Bacteria specific primer, 9F and universal primer, 534R were used to amplify bacterial 16S rDNA. One nanogram of extracted DNA from each sample and known copies of the competitor DNA were added into the same PCR tube and amplified with AmpliTaq Gold (Applied Biosystems) under the following conditions: denaturation at 94°C for 9 min, and then 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, and a final extension step of 72°C for 10 min. The amplified PCR products were stained with ethidium bromide and checked using 3% agarose gel electrophoresis. The gel image was taken with an FMBIO II Multi-View fluorescent image analyzer (Hitachi Software Engineering Co.), and the fluorescence intensity of the amplified DNA band was analyzed by FMBIO Analysis Version 6.0 software (Hitachi Software Engineering Co.). Based on the obtained fluorescence intensity, the copy number of 16S rDNA in each sample was calculated via the following equation:

$$\log C = -a \log (T/C) + \log C_0$$

where C is the copy number of the DNA competitor, T is the copy number of the objective (template) DNA, a is the gradient of the plotted line, and $\log C_0$ is the y-intercept.

Results and Discussion

On-Site PUF Block Culture. A preliminary examination of on-site PUF block culture was carried out in Okinawa. The agar that had been loaded into the PUF blocks was almost completely retained after 3 days of the on-site culture, apparently protected from environmental bacterial degradation. The PUF blocks cultured on site with the agar medium displayed distinct colors (Figure 1) and distinct smells when compared to the PUF blocks without the medium. It is assumed that agar filled the internal pores of the PUF of the PUF blocks, enabling environmental microbes to attach to the agar surface and to grow on the components in the medium. The center of each PUF block seemed to be anoxic, indicated by black coloration and the presence of anaerobic bacteria, which was suggested by 16S rDNA gene sequencing that showed strains related to known anaerobes. Growth of the bacteria on the PUF was examined by SEM (Figure 2).

Population Analysis by the PCR-DGGE Method. Partial 16S rDNA fragments were separated by DGGE, and 67 bands were sequenced (Figure 3). Table 1 lists the bacterial species that exhibited the highest nucleotide identity to each band from DGGE. Two sequences, *PO-11(o)-7* (sampling point 2, MA + Fe medium) and *PO-7-7* (sampling point 2, MA + Ca medium), were identified as close relatives (although only 87% and 95% similarity, respectively) of the uncultured bacterium [AY038542] which had previously been identified as a likely candidate division. The DGGE band, *PO-13-1* (sampling point 3, MA + Ca medium) was closely related to the TM7 candidate division [AF269024] (Hugenholtz et al., 2001).

We selected $CaCO_3$ as substance for PUF media because Ca is required for the bones of various marine organisms in different habitants in the coral reef area. When MA + Ca was applied as media, the microbial diversity increased. *Alphaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes*, TM7, and unclassified bacteria were detected. We plan to investigate the relationships between the habitants and the bacteria that concentrate in the habitats in the near future.

Vibrio spp. were predominant at both sampling points 1 and 2, but could not be detected at sampling point 3. *Alphaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* were each detected as DGGE bands at all three sampling points.

The difference in division level of the bacterial community associated with the PUF samples by sampling point is shown in Figure 4. *Firmicutes* were predominant at sampling points 2 and 3, but could not be detected at sampling point 1 by DGGE. The *Firmicutes* detected as DGGE bands were almost all (13 out of 14) related to *Fusibacter paucivorans* [AF0 50099] which had been reported to be an anaerobic thiosulfate-reducing bacterium (Ravot et al., 1999).

Isolated Bacteria. One hundred and twenty-two isolates were obtained, and were identified via 16S rDNA gene sequence analysis.

All the bacteria (19 strains, 14 of which sequenced) isolated from the PUF samples soaked with the MA + Fe medium were found to produce siderophores determined by CAS assay (Schwyn and Neilands, 1987), whereas 6 out of the 14 strains (12 of which were sequenced) of bacteria isolated from the PUF soaked in the MA medium were found to be

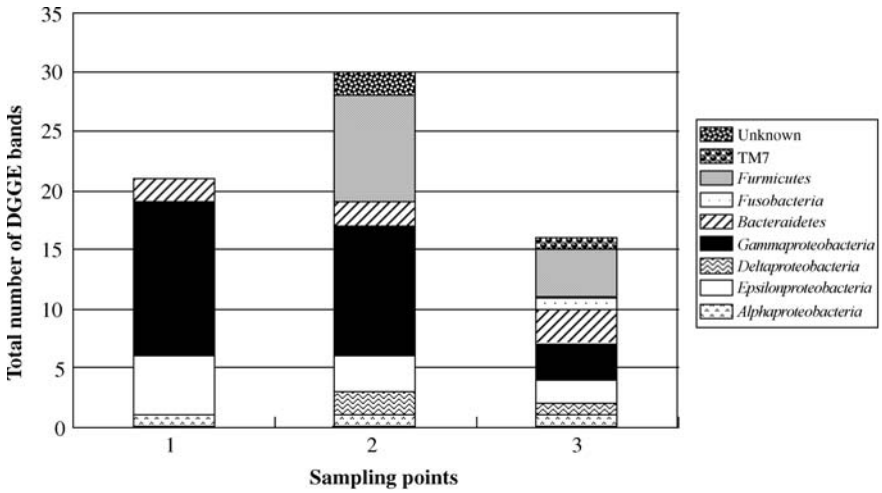

Fig. 4. Histogram illustrating the division-level diversity of the partial 16S rDNA bacterial sequence of DGGE bands associated with the PUF samples from the three sampling points. Sampling point 1, the channel near the reef edge; sampling point 2, near the mangroves; sampling point 3, around the patch reef between 1 and 2.

CAS-positive. Siderophores are low-molecular-mass, iron-chelating compounds that have medical and agricultural applications. Guan et al. (2001) have also reported on the isolation of siderophore-producing bacteria from the marine environment. Bacterial strain PO-47 [AB235413] was isolated from PUF MA + Fe block. The PO-47 was identified as being closely related (99% similarity) to *Shewanella alga* [AF006669], which has been reported to grow anaerobically, by dissimilatory Fe(III) reduction (Bowman et al., 1997). The PO-47 strain produced the cyclic dihydroxamate siderophore (about 1 g of bisucaberin from 4 liters of culture broth) (Yasumoto-Hirose et al., unpublished data). Bisucaberin was previously isolated from a marine bacterium, *Alteromonas haloplanktis* (Kameyama et al., 1987; Takahashi et al., 1987).

n-Alkane-degrading bacteria belonging to the *Alcanivorax* group were isolated from the PUF soaked in A + Hex (at sampling points 2 and 3) and in NSW + Kero (sampling point 3). The *Alcanivorax* genus has been reported to play an important role in the first step of crude oil biodegradation in a marine environment (Harayama et al., 1999). The *Alcanivorax* sp. was isolated from PUF containing hydrocarbon. These were isolated from various plates: the A + Hex, the NSW + Kero, and the Ca plates. These results prompt speculation that bacteria grew on the PUF blocks by hydrocarbon degradation. It is possible that the bacteria were concentrated in the natural marine environment by the PUF. However, the bacteria on the PUF did not result simply from such concentration, but from enrichment by the media employed.

Ten sequences detected as DGGE bands were related to *Epsilonproteobacteria*. Bacterial strains PO-40 [AB235414] was identified as closely related (96% similarity) to an uncultured epsilon bacterium

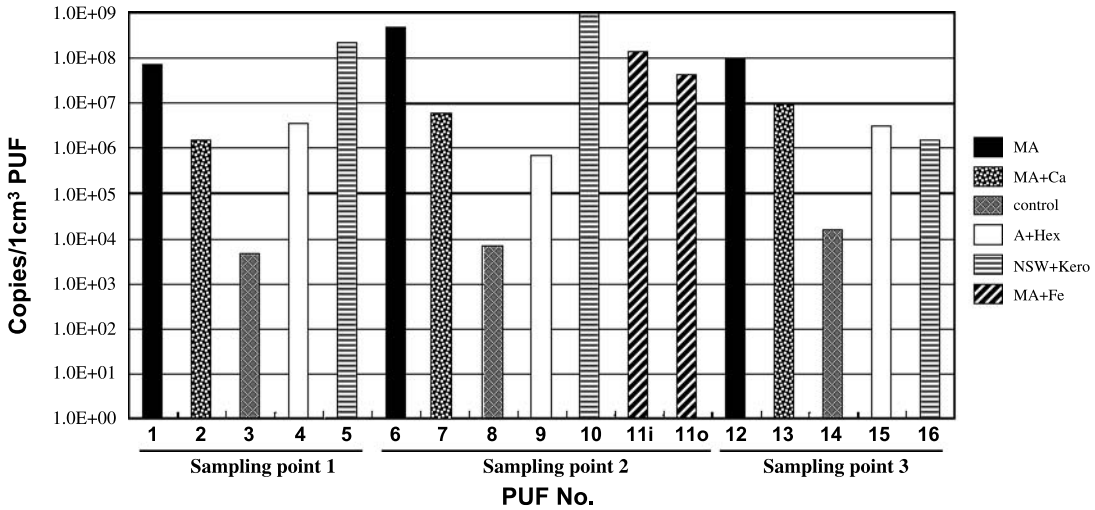

Fig. 5. The copy number of 16S rDNA of the microbial consortia attached to each medium-soaked PUF (1–16) was determined by competitive PCR. The control PUF samples without any medium (3, 8, and 14).

Table 2. 16S rDNA Sequences Most Closely Related to the Major Bacterial Populations Isolated on the PUF Samples

	Strain no.	*Sponge no. media*	*Isolate plate*	*Closest relative*	*% Identity*	*Accession no.*	*Taxonomic group*
	PO-3	1. MA	Kero.	*Vibrio shiloi*	98	AF007115	*gamma*
	PO-4	1. MA	Ca	*Bacillus firmus*	94	D16268	*Firmicutes*
	PO-9	2. MA+Ca	Hex.	*Pseudoalteromonas* sp.	94	U80834	*gamma*
	PO-11	2. MA+Ca	Hex.	*Vibrio* sp.	98	AF319769	*gamma*
	PO-13	4. A+Hex.	Ca	*Vibrio gazogenes* (ATCC 29988T)	98	X74705	*gamma*
	PO-18	4. A+Hex.	Hex.	*Vibrio* sp.	98	AF319769	*gamma*
	PO-23	4. A+Hex.	1/10MA	MBIC3368	99	AB012864	*alpha*
Point 1	PO-24	4. A+Hex.	1/10MA	MBIC3368	97	AB012864	*alpha*
	PO-25	4. A+Hex.	1/10MA	*Roseobacter* sp.	95	AF398495	*alpha*
	PO-27	4. A+Hex.	Kero.	*Pseudoalteromonas flavipulchra*	98	AF297958	*gamma*
	PO-29	4. A+Hex.	Ca	*Vibrio* sp.	99	AF319769	*gamma*
	PO-28	5.NSW+Kero.	Kero.	*Bacillus circulans*	99	AY043084	*Firmicutes*
	PO-73	5.NSW+Kero.	1/10MA	*Pseudoalteromonas flavipulchra*	99	AF297958	*gamma*
	PO-74	5.NSW+Kero.	1/10MA	*Photobacterium* sp.	96	AB038032	*gamma*
	PO-100	5.NSW+Kero.	1/10MA	*Vibrio nigripulchritudo*	98	X74717	*gamma*
	PO-32	6. MA	1/10MA	*V.campbellii*	99	X56575	*gamma*
	PO-33	6. MA	1/10MA	*V.alginolyticus*	99	X56576	*gamma*
	PO-34	6. MA	Ca	*Vibrio* sp.	99	AF319769	*gamma*
	PO-35	6. MA	Kero.	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-37	6. MA	Kero.	*Vibrio* sp.	99	AF319769	*gamma*
	PO-38	6. MA	Kero.	*Bacillus firmus*	98	D16268	*Firmicutes*
	PO-39	6. MA	Hex.	marine bacterium PP-145.98 partial	98	AJ296157.1	*gamma*
	PO-40	6. MA	Hex.	Uncultured epsilon proteobacterium	96	AF235116	*epsilon*
	PO-41	6. MA	Hex.	*Vibrio* sp.	99	AF319769	*gamma*
	PO-42	6. MA	Ca	*V.orientalis*	95	X74719	*gamma*
	PO-43	6. MA	Ca	*Vibrio* sp.	99	AF319769	*gamma*
	PO-44	6. MA	Ca	Alpha proteobacterium MBIC1887	99	AB026492	*alpha*
	PO-76	7.MA+Ca	Ca	*V.natriegens* (ATCC 14048T)	98	X74714	*gamma*
	PO-78	7.MA+Ca	Ca	*V.fluvialis*	98	X76335	*gamma*
	PO-79	7.MA+Ca	Kero.	*Ruegeria* sp.	98	AJ391197	*alpha*
	PO-80	7.MA+Ca	Kero.	*Vibrio* sp.	99	AF319769	*gamma*
	PO-110	7.MA+Ca	Hex.	*B.licheniformis*	99	X68416	*Firmicutes*
	PO-111	7.MA+Ca	Kero.	MBIC1887	99	AB026492	*alpha*
	PO-112	7.MA+Ca	Kero.	*Bacillus firmus*	99	D16268	*Firmicutes*
	PO-133	7.MA+Ca	Kero.	*B.licheniformis*	100	X68416	*Firmicutes*
	PO-140	7.MA+Ca	Kero.	*Bacillus pumilus*	99	AY030327	*Firmicutes*
Point 2	PO-66	9. A+Hex.	Hex.	*Vibrio campbellii*	99	AY035896	*gamma*
	PO-67	9. A+Hex.	1/10MA	*Alteromonas macleodii*	99	Y18228	*gamma*
	PO-68	9. A+Hex.	1/10MA	*Vibrio* sp.	98	AF319769	*gamma*
	PO-69	9. A+Hex.	1/10MA	*Curacaobacter baltica*	96	AJ002006	*gamma*
	PO-70	9. A+Hex.	1/10MA	*Bacillus firmus*	98	D16268	*Firmicutes*
	PO-71	9. A+Hex.	Ca	*V.tubiashi* (ATCC 19109T)	98	X74725	*gamma*
	PO-72	9. A+Hex.	Ca	*Alteromonas alvinellae*	98	AF288360	*gamma*
	PO-102	9. A+Hex.	Hex.	*Alteromonas alvinellae*	99	AF288360	*gamma*
	PO-104	9. A+Hex.	Kero.	*Pseudoalteromonas* sp.	99	AF227238	*gamma*
	PO-105	9. A+Hex.	Ca	*Pseudoalteromonas* sp.	99	AF227238	*gamma*
	PO-106	9. A+Hex.	Ca	*Vibrio* sp.	99	AF319769	*gamma*
	PO-108	9. A+Hex.	Hex.	*Vibrio* sp.	99	AF319769	*gamma*
	PO-109	9. A+Hex.	Kero.	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-153	9. A+Hex.	Kero.	*Alcanivorax* sp. Mho1	99	AB053124	*gamma*

Continues

Table 2. Continued

	Strain no.	Sponge no. media	Isolate plate	Closest relative	% Identity	Accession no.	Taxonomic group
	PO-154	9. A+Hex.	Ca	*Alcanivorax* sp. Mho1	99	AB053124	*gamma*
	PO-155	9. A+Hex.	Kero.	*Alcanivorax* sp. Mho1	99	AB053124	*gamma*
	PO-45	11. MA+Fe	Kero.	*Vibrio* sp.	98	AF319769	*gamma*
	PO-46	11. MA+Fe	Kero.	*Vibrio* sp.	99	AF319769	*gamma*
	PO-47	11. MA+Fe	Kero.	*Shewanella alga*	99	AF006669	*gamma*
	PO-49	11. MA+Fe	1/10MA	*Vibrio* sp.	99	AF319769	*gamma*
	PO-50	11. MA+Fe	1/10MA	*Pseudoalteromonas* sp.	99	AJ391204	*gamma*
	PO-51	11. MA+Fe	1/10MA	*V.campbelli*	99	X74692	*gamma*
	PO-52	11. MA+Fe	1/10MA	*Pseudoalteromonas flavipulchra*	99	AF297958	*gamma*
	PO-55	11. MA+Fe	Hex.	*Vibrio* sp.	99	AF319769	*gamma*
	PO-56	11. MA+Fe	Ca	*V.tubiashi* (ATCC 19109T)	99	X74725	*gamma*
	PO-57	11. MA+Fe	Ca	*Pseudoalteromonas flavipulchra*	99	AF297958	*gamma*
	PO-58	11. MA+Fe	Ca	*Vibrio* sp.	99	AF319769	*gamma*
	PO-59	11. MA+Fe	1/10MA	*Vibrio* sp.	99	AF319769	*gamma*
	PO-60	11. MA+Fe	Ca	*Vibrio campbellii*	98	AY035896	*gamma*
	PO-64	11. MA+Fe	Hex.	*V.campbelli* (ATCC 25920T)	98	X74692	*gamma*
	PO-81	12. MA	Ca	*Vibrio* sp.	98	AF319769	*gamma*
	PO-82	12. MA	Ca	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-83	12. MA	1/10MA	*Vibrio* sp.	99	AF319769	*gamma*
	PO-84	12. MA	Kero.	*Pseudoalteromonas porphyrae*	98	AF475096	*gamma*
	PO-85	12. MA	Hex.	*Vibrio campbellii*	97	AY035896	*gamma*
	PO-86	12. MA	Hex.	*Vibrio* sp.	99	AF319769	*gamma*
	PO-87	12. MA	Kero.	MBIC1876	97	AB026194	*alpha*
	PO-88	12. MA	Ca	*Ruegeria algicola (ATCC 51440 T-FF3)*	95	X78315	*alpha*
	PO-89	12. MA	1/10MA	*Vibrio* sp.X76335.1 V.fluvialis*97	98	AF410778	*gamma*
	PO-91	12. MA	Ca	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-92	12. MA	Ca	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-93	12. MA	Ca	*Pseudoalteromonas* sp.	98	AJ391204	*gamma*
	PO-103	12. MA	Ca	*Bacillus pumilus*	98	AY030327	*Firmicutes*
	PO-115	12. MA	Ca	MBIC3368	98	AB012864	*alpha*
	PO-116	12. MA	Ca	MBIC3368	99	AB012864	*alpha*
	PO-117	12. MA	Ca	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-123	12. MA	Ca	*Bacillus circulans*	99	AY043084	*Firmicutes*
	PO-124	12. MA	Ca	*Pseudoalteromonas* sp.	99	AF227238	*gamma*
	PO-141	12. MA	1/10MA	*Bacillus subtilis*	99	Z99108	*Firmicutes*
	PO-142	12. MA	Kero.	*Bacillus subtilis*	99	Z99108	*Firmicutes*
	PO-143	12. MA	1/10MA	*Bacillus subtilis*	99	Z99108	*Firmicutes*
	PO-145	12. MA	Ca	*Bacillus subtilis* N5	99	AF270793	*Firmicutes*
	PO-146	12. MA	Ca	*Vibrio* sp.	98	AF410778	*gamma*
	PO-147	12. MA	Hex.	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-101	13. MA+Ca	1/10MA	*Pseudoalteromonas flavipulchra*	100	AF297958	*gamma*
	PO-107	13. MA+Ca	Hex.	*Pseudoalteromonas* sp.	94		*gamma*
	PO-119	13. MA+Ca	Ca	*Bacillus Licheniformis*	99	X68416	*Firmicutes*
	PO-120	13. MA+Ca	Ca	*Bacillus pumilus*	99	AY030327	*Firmicutes*
	PO-121	13. MA+Ca	Kero.	*Bacillus firmus*	99	D16268	*Firmicutes*
	PO-122-1	13. MA+Ca	Hex.	*Bacillus pumilus*	99	AY030327	*Firmicutes*
Point 3	PO-148	13. MA+Ca	Ca	*Bacillus thuringiensis*	98	Z84594	*Firmicutes*
	PO-149	13. MA+Ca	1/10MA	*Bacillus pumilus*	99	D55731	*Firmicutes*
	PO-150	13. MA+Ca	1/10MA	*Bacillus licheniformis*	100	X68416	*Firmicutes*
	PO-151	13. MA+Ca	Kero.	*Bacillus pumilus*	99	AY030327	*Firmicutes*

Continues

Table 2. 16S rDNA Sequences Most Closely Related to the Major Bacterial Populations Isolated on the PUF Samples (Continued)

Strain no.	*Sponge no. media*	*Isolate plate*	*Closest relative*	*% Identity*	*Accession no.*	*Taxonomic group*
PO-97	15. A+Hex.	Kero.	Crassostrea virginica symbiont	97	F246615	*alpha*
PO-98	15. A+Hex.	Kero.	*Tenacibaculum mesophilum*	98	AB032502	*Bacteroidetes*
PO-99	15. A+Hex.	Ca	*Vibrio* sp.	99	AF319769	*gamma*
PO-125	15. A+Hex.	Kero.	*Alteromonas* sp.	97	AJ391191	*gamma*
PO-131	15. A+Hex.	Kero.	*Alteromonas* sp.	98	AJ391191	*gamma*
PO-132	15. A+Hex.	Hex.	MBIC3368	99	AB012864	*alpha*
PO-134	15. A+Hex.	Hex.	*Alteromonas alvinellae*	98	AF288360	*gamma*
PO-135	15. A+Hex.	Ca	*Alteromonas alvinellae*	98	AF288360	*gamma*
PO-136	15. A+Hex.	Ca	MBIC3368	99	AB012864	*alpha*
PO-137	15. A+Hex.	1/10MA	MBIC3368	98	AB012864	*alpha*
PO-138	15. A+Hex.	Kero.	MBIC3368	98	AB012864	*alpha*
PO-139	15. A+Hex.	Kero.	*Alteromonas* sp.	98	AJ391192	*gamma*
PO-156	15. A+Hex.	Ca	*Pseudomonas* sp.	98	AJ007005	*gamma*
PO-157	15. A+Hex.	Ca	*Alcanivorax* sp. Mho1	99	AB053124	*gamma*
PO-158	15. A+Hex.	Kero.	*Alcanivorax* sp. Mho1	99	AB053124	*gamma*
PO-159	15. A+Hex.	Kero.	*Alcanivorax* sp. Mho1	99	AB053124	*gamma*
PO-161	15. A+Hex.	Hex.	*Alcanivorax* sp. Mho1	98	AB053124	*gamma*
PO-113	16. NSW+Kero.	Kero.	*Roseobacter* sp.	95	AF107210	*alpha*
PO-114	16. NSW+Kero.	1/10MA	*Erythrobacter* sp. MBIC4118	99	AB035545	*alpha*
PO-162	16. NSW+Kero.	Ca	*Alcanivorax* sp. Mho1	98	AB053124	*gamma*
PO-163	16. NSW+Kero.	Ca	*Alcanivorax* sp. Mho1	98	AB053124	*gamma*

[AF235116], and also to DGGE bands PO-1-6 [AB235415].

One liter of seawater at each PUF sampling area was collected, filtered through a sterile 0.22-μm filter, DNA extracted, and DGGE analyzed, and the sample was compared with the PUF samples in the marine environment, showing different band patterns (Yasumoto-Hirose et al., unpublished data). This media-supplemented PUF method likely provides a three-dimensional culture environment that is composed of various different subenvironments for microorganisms. The microenvironment in the media-supplemented PUF has different conditions for growth of microorganisms, such as pH, oxygen concentration, and so forth. Many strains detected as DGGE bands were related to anaerobic bacteria, but isolation of the anaerobic bacteria remains to be done.

Competitive PCR. Competitive PCR was carried out for quantifying 16S rDNA to estimate the bacterial population on each PUF block. The DNA extracted from each PUF was used. The number of copies of 16S rDNA per 1 cm^3 of PUF was calculated (Figure 5). Although the number of copies did not equal the number of bacteria, the difference in the numbers of copies on PUF blocks with or without a medium was observed. Although the same amount (1 ng) of extracted DNA was used as template DNA for the competitive PCR, the PUF blocks without a medium (control PUF blocks) showed fewer than 10 copies per 1 ng DNA by competitive PCR; that is, the number of copies of bacteria 16S rDNA in 1 cm^3 PUF was less than 10^3 to 10^4. This low number of copies/cm^3 might be a result of attachment of eukaryotes on the control PUF. On the other hand, the PUF blocks soaked with media contained approximately 10^5 to 10^9 copies per 1 cm^3 of PUF. Among medium-soaked PUFs, a larger number of copies 16S rDNA (10^8 order) was detected in PUF-MA (Figure 5). PUF-A + Hex showed a smaller number among tested media. PUF-NSW + Kero showed a large number at points 1 and 2, but a small number at point 3. It was interesting that PUF-MA + Fe showed a larger number of copies (PUF-MA + Fe(i), 1.3×10^8 copies/1 cm^3 of PUF; PUF-MA + Fe(o), 4.2×10^7 copies/1iter cm^3 of PUF) than PUF-MA + Ca (5.8×10^6 copies/1 cm^3 of PUF) at sampling point 2. The two kinds of PUFs used MA for basal medium. In addition, both the bacterial diversity detected on DGGE bands and the diversity of the isolated strains on PUF-MA + Ca were higher than the diversity of bacteria on PUF-MA + Fe (Tables 1 and 2). Different media containing various compounds have different selective pressures on bacterial growth on the PUF. The growth of hydrocarbon-degrading bacteria was likely enhanced on the PUF

with hydrocarbons (hexadecane and kerosene) more than the growth of other heterotrophic bacteria.

The methods and procedures reported here make it possible to collect naturally occurring marine bacteria relatively simply and economically. Even if the population of the bacteria in seawater is quite low, the PUF block is a powerful tool to enrich various types of bacteria with interesting properties in natural environments. After enrichment culture using PUF, the target bacteria can be isolated by conventional agar plate media containing certain selective substrate. In our study, we isolated hydrocarbon-degrading bacteria from PUF blocks containing hydrocarbon and siderophore-producing bacteria from those containing iron. We intend to study further whether the method can be applied to other varieties of bacteria in different natural environments.

Acknowledgments

We thank Izumi Yamashima for technical assistance. We also thank Minoru Yasumoto for sampling. This work was part of The Industrial Science and Technology Project for Technology Development of Biological Resources in Bioconsortia, which is supported by the New Energy and Industrial Technology Development Organization of Japan, as part of the project on Constructing the Genetic Resource Library of Unidentified Microbes Based on Genome Information which is supported by the Ministry of Economy, Trade and Industry of Japan.

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