[SIV-4]

Sustainable Use of Marine Microorganisms

Yoo Kyung Lee, Jung Hyun Lee, Kae Kyoung Kwon, Hong Kum Lee*
Microbiology Laboratory, Korea Ocean Research & Development Institute,
Ansan P. O. Box 29, Seoul 425-600, Korea

Abstract

The oceans cover about 71% of the Earth's crust and contain nearly 300,000 described species. Free-living bacteria in the sea and symbiotic bacteria of marine invertebrates are proving to be valuable sources of useful bioactive compounds. Marine sponges, in particular, which contain diverse communities of bacteria, produce many classes of compounds that are unique to the marine environment. Uncultured microorganisms are commonly believed to represent 99.9% of the whole microbial community. They have been investigated for the possiblity of isolating and over-expressing genes in viable microorganisms. Strict symbiotic species that have beeen adapted to the host are candidate unculturable species. With the enormous potential for discovery, development, and market value of marine derived compounds, supply of the products is a major limiting factor for further development.

Isolation of Microorganisms

For the approach to biotechnological application of microbial diversity, maintenance of not simply abundant but diverse microorganisms is necessary. Efficient methods for isolation of microorganisms from the oceans are required, since only a small percentage (<1%) of the bacteria in seawater can be cultured. Frequency of microorganisms from marine sources was examined with different media and samples collected from the coastal area of Cheju Island. We tested several media, among which ZoBell medium is very popular for the isolation and cultivation of marine microorganisms. Diluted ZoBell medium, enriched ZoBell medium, and enriched medium with other organic supplement, such as algal powder were examined. As for solidifying agent agar and gellan gum were tested. About 1% of microorganisms from the total number of bacteria were recovered from sea water samples (Fig. 1). In comparison to this, 10^{-5}~10^{-4} of the total bacteria in corals and sponges were recovered, and 10^{-6}~10^{-4} of the total bacteria was cultured from sediments collected. The numbers of total bacterial cells per corals biomass were not very different. However, the ratios of culturable bacteria to total cells observed on the isolation media were different depending on the kinds of corals (Table 1). These results indicate that epoch-making media have to be developed to obtain diverse microorganisms efficiently from marine sources, especially from marine organisms and sediment.

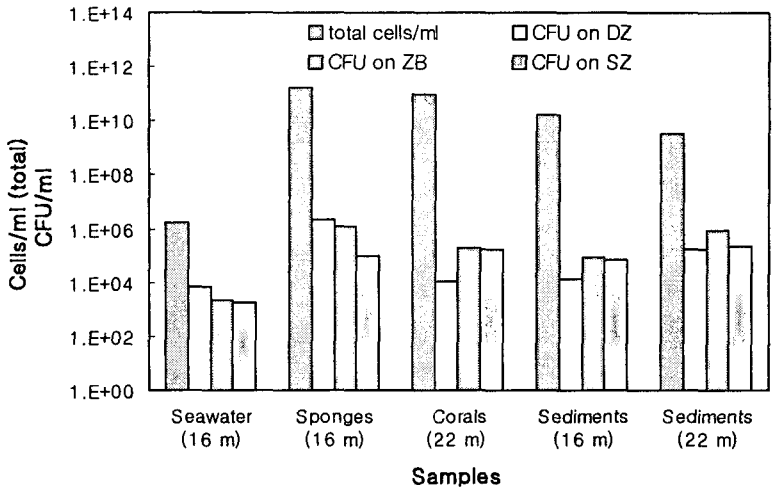

Fig. 1. Distribution of bacteria in marine sources collected June 1998, calculated as total bacterial cells and colonies on the isolation media, DZ, ZB and SZ.

Table 1. Distribution of bacteria in seawater and corals in January 1998 calculated as total cells and colonies on the isolation media

Samples	Cells/ml (total)	CFU/ml					
		DZ	DZG[a]	SZ	SZG[a]	ADF	ADFG[a]
Seawater	$2.0x10^6$	$3.2x10^4$	$3.3x10^4$	$1.5x10^4$	$4.2x10^4$	$3.0x10^3$	$3.0x10^3$
Antipathes	$2.4x10^8$	$3.5x10^3$	$5.5x10^3$	$3.0x10^3$	$7.0x10^3$	$4.0x10^3$	$1.0x10^2$
Alcyonium	$2.4x10^8$	$4.5x10^3$	$3.0x10^3$	$1.0x10^3$	$2.5x10^3$	$1.0x10^3$	$3.0x10^3$
Plexauridae	$4.7x10^8$	$2.5x10^4$	$1.9x10^4$	$6.0x10^3$	$1.1x10^4$	$8.0x10^3$	$7.0x10^3$
Dendronephtya	$5.0x10^8$	$5.0x10^3$	$2.0x10^3$	$8.0x10^3$	$5.0x10^3$	$1.0x10^2$	$1.0x10^2$
Dendrophylla	N.D.[b]	$5.5x10^4$	$4.3x10^4$	$4.7x10^4$	$4.0x10^4$	$9.0x10^3$	$1.0x10^3$

[a] media containing gellan gum instead of agar

[b] not detected

Preservation of Microorganisms

Because microbial cultures are extremely vulnerable, minimizing the loss of viability during the processing and storage of a library of strains is as important as collecting the microorganisms. Microorganisms can be successfully maintained by freeze-drying through the removal of water under reduced pressure and storage at -70℃ or at the temperature of liquid nitrogen in the presence of cryoprotectants. Freezing is usually the method of choice for both short- and long-term storage of microorganisms based on user requirements, such as the expense, number of cultures, frequency of use of cultures, etc. This method is very quick and easy, and requires no subsequent manipulation during storage. It is ideally suited to the storage of large collections of isolates in screening programs. However, few papers on methods for maintaining marine bacteria have been.

We evaluated a freezing method for maintaining marine bacteria at -70□ using halophiles and halotolerants isolated from seawater, sediment and corals. Distilled water and artificial seawater were tested and compared as the cell suspension solutions, while glycerol and DMSO were examined and compared as the cryoprotective agents. From the preservation test using specific marine isolates, no significant distinction was found between the procaryoprotectants used on halophiles and halotolerants. It was also confirmed that the tested marine bacteria needed to stored using a more efficient deep-freezing method because only around one percent of the preserved marine bacterial cells were recovered after one year of storage at -70□.

Laboratory culture of symbiotic microorganisms

The culture of symbiotic microorganisms has important implications for the screening and production of symbiont-derived bioactive natural products (Table 2). Sponges are host organisms for various symbiotic microorganisms such as archaea, bacteria, cyanobacteria and microalgae. Sponges are also sources of a wide variety of useful natural products like cytotoxins, antifouling agents, antibiotics, and anti-inflammatory and antiviral compounds. Symbiotic microorganisms in sponges can be sources of various natural products, because metabolites previously ascribed to sponges have recently been demonstrated to be biosynthesized by symbionts. If a symbiotic microorganism from which some natural products are derived can be cultured, the microorganism could be used in a mass production of the bioactive compounds.

Table 2. Bioactive compounds isolated from marine symbiotic microorganisms.

Microorganisms	Macroorganisms	Compounds
Ateromonas sp.	*Halichondria okadai*	Alteramide A
Alteromonas sp.	*Palaemon macrodactylus*	Isatin
Coryneform bacteria	*Babylonia japonica*	Surugatoxin
Cyanobacterium	*Dysidea herbacea*	Chlorinated metabolires
Filamentous bacterium	*Aciculites orientalis*	Theonegramide
Filamentous bacterium	*Theonella swinhoei*	Theopalauamide
Flavobacterium ulginosum	Algae	Marinatan
Micrococcus luteus	*Xenospongia* sp.	Antimicrobial compounds
Micrococcus sp.	*Tedania ignis*	Diketopiperazine
Micrococcus sp.	*Tedania ignis*	Benzpthiazole
Oscillatoria spongeliae	*Dysidea herbacea*	Polybrominated biphenyl ethers
Procentrum lima	*Halichondria okadai*	Okadaic acid
Pseudomonas sp.	*Homophymia* sp.	Antimicrobial compounds
Pseudomonas sp.	*Suberea creba*	Quinolones
Pseudomonas sp.	*Fugu poecilonotus*	Tetrotoxin
Psychroserpens burtonensis	*Halichondria panicea*	Neuroactive compounds
Streptomyces sp.	Tropic coral	Octalactin
Vibrio sp.	*Dysidea* sp.	Bisbromophenol

Isolation of interesting symbiotic microorganisms is the first step of a laboratory culture. Symbiotic microorganisms can be separated from sponge cells by differential centrifugation, by density gradient centrifugation, by Ficoll/Percoll density gradient centrifugation, or by Ficoll gradient centrifugation. For more effective cultivation, several culture media have been developed. After over 100 attempts to culture the symbionts, Schmidt *et al.* could cultivate some filamentous bacteria using J agar with sodium thiosulfate, aqueous sponge extract, and sodium silicate. To improve recoverability of microorganisms from natural marine sponges, Olson added catalase or sodium pyruvate to solid growth and isolation media. By adding sponge extracts to marine agar 2216, Webster *et al.* isolated bacterial isolates not previously cultured. Innovative culture techniques should be explored to obtain additional sponge-symbiotic microorganisms in cultures.

Diversity of symbiotic microorganisms

Only very little information exists about the taxonomic affiliation of sponge-symbiotic microorganisms. In spite of these successful cultures, most symbiotic microorganisms are difficult to isolate and cultivate. Because most symbiotic microorganisms cannot be cultured using current and traditional techniques, culture-based techniques are inadequate for studying microbial diversity and identifying unculturable symbiotic microorganisms. To overcome this problem, symbiotic microorganisms are identified directly using culture-independent methods such as molecular taxonomy. Phylogenetic analysis of 16S rRNA genes gives data on microbial diversity and the phylogenetic position of each symbiotic microorganism. These potentially novel and diverse isolates would be a useful resource for screening for bioactive natural products.

Archaea is a predominant microorganism, inhabiting in various environments, moderate to extreme and free-living to symbiotic. We surveyed archaeal communities closely associated with sponges based on molecular analysis of 16S rDNA sequences. Archaeal 16S rDNAs were partially amplified from total DNAs of the sponges collected at the coast of Cheju Island. Terminal restriction fragment length polymorphism (T-RFLP) analysis showed that some sponge had its specific archaeal communities. To confirm the community components, 170 archaeal 16S rDNA clones were obtained and characterized by restriction fragment length polymorphism (RFLP) typing. Representative 19 clones were sequenced and phylogenetic analysis has been performed (Fig. 2). As a result, archaeal sequences showed the highest similarity to 16S rDNAs of the Crenarchaeotes. In the generated tree, the clade of clones was distinguished from the previously known orders within Crenarchaeota.

Future studies on symbiotic microroganisms

There are several potential drawbacks for future studies on sponge-symbiotic microorganisms and related natural products. First, identification and isolation of symbiotic microorganisms producing bioactive natural products are a crucial step for the future culture and production of these metabolites. Second, the origin of natural products can be assigned to symbiotic microorganisms only when synthesis has been demonstrated in cultures isolated from the host species. Third, the cultures for metabolites production are uncertain about whether or not the symbiotic microorganism will continue to produce the target compound in the absence of the sponge host. These drawbacks can be overcome by the molecular approach. Molecular cloning and analysis of

the genes responsible for synthesizing the target compound also offer useful information for genetic engineering of the biosynthesis. *In situ* hybridization with specific probes allows the direct, cultivation-independent analysis of most natural symbiotic microbial communities. FISH (fluorescence *in situ* hybridization) based on the 16S rRNA can visualize the localization and abundance of a specific symbiont and the composition of the symbiotic microorganisms. These studies could yield new insights in the true abundance of well-known or new symbiotic microorganisms and increase our knowledge on the species diversity and specificity of symbiotic microorganisms. But the sequence variation among 16S rRNA genes is so small that it is inadequate to distinguish exact species at the level of species or strain. To resolve this low level of sequence variation, more variable sequences such as ITS (internal transcribed spacer) region can be used, because it contains genetic variation sufficient for differentiating species of microorganisms.

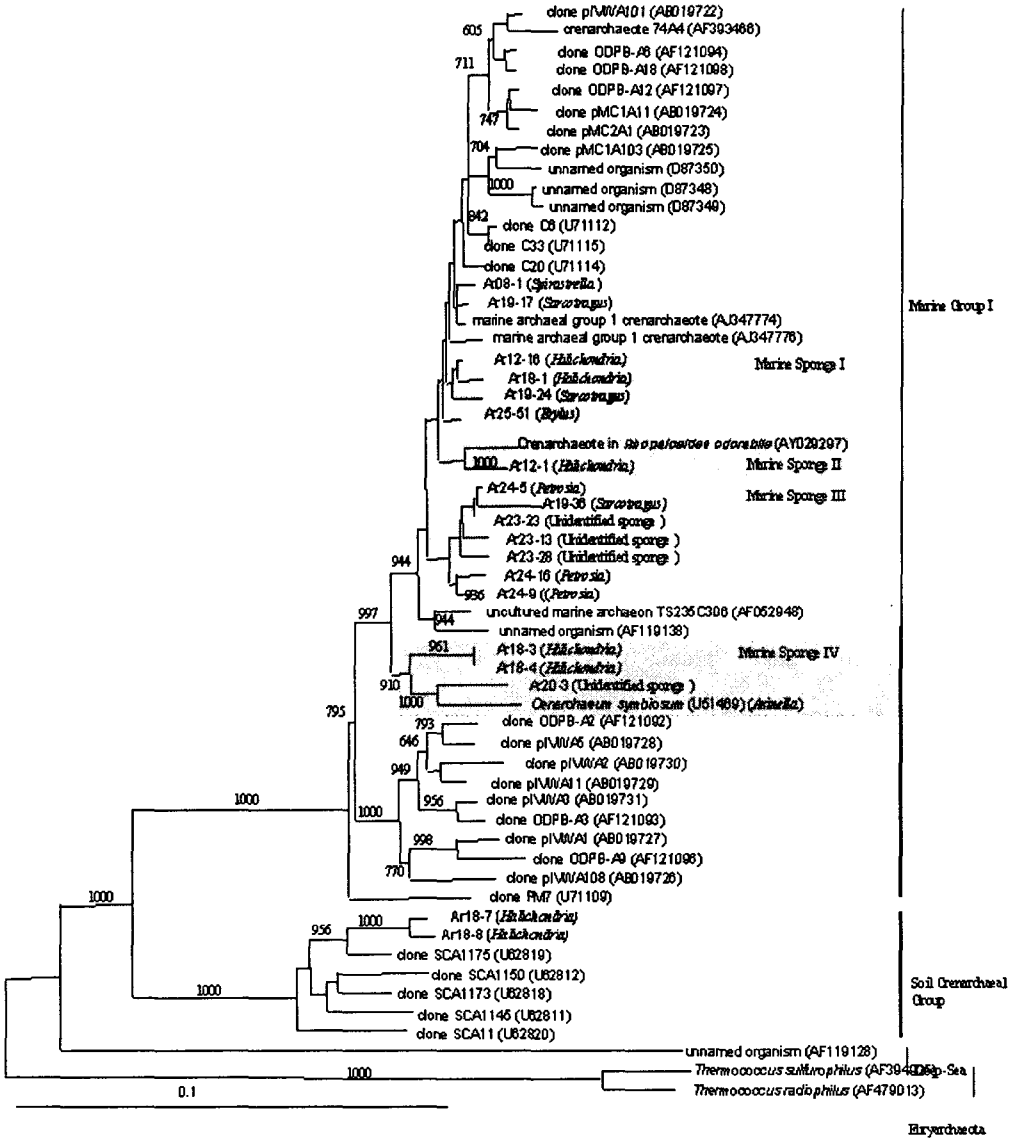

Fig. 2. Neighbor-joining tree based on partial 16S rDNA sequence of archaeal bacteria associated with the sponges. Color box indicates each clade including archaeal clones. Bootstrap values above 500 are presented near the branches.

Detection of the origin of natural product synthesizing organisms is also an important step. Some specific natural products can be detected directly using fluorescence, therefore the localization of the metabolites can be observed *in situ*. Salomon et al. detect dercitamide in cells using a combination of visualization methods, including laser-scanning confocal, epifluorescence, and transmission electron microscopy, as well as cell-separation techniques, and chemical analysis. Development of *in situ* detection methods can help identification of the cell type producing bioactive natural products.

References

1. Beran, V.S., M. Greenstein, and W.M. Maise. 1997. Marine microorganisms as a source of new natural products. *Adv. Appl. Microbiol.* 43, 57-90.
2. Haygood, M.G., E.W. Schmidt, S.K. Davidson, and D.J. Faulkner. 1999. Symbiosis of marine invertebrate and microorganism. *J. Mol. Microbiol. Biotechnol.* 1, 33-43.
3. Lee, J.-H., H.-H. Shin, D.-S. Lee, K.K. Kwon, S.-J. Kim, and H.K. Lee. 1999. Bacterial diversity of culturable isolates from seawater and a marine coral, *Plexauridae* sp., near Mun-Sum, Cheju-Island. *J. Microbiol.* 37, 193-199.
4. Lee, Y.K, J.-H. Lee, and H.K. Lee. 2001 Microbial symbiosis in marine sponges. *J. Microbiol.* 39, 254-264.
5. Pietra, F. 2002. Biodiversity and Natural Product Diversity. Pergamon, Amsterdam.
6. Pomponi, S.A. 1999. The bioprocess-technological potential of the sea. *J. Biotech.* 70, 5-13.
7. Webster, N.S., K.J. Wilson, L.L. Blackall, and R.T. Hill. 2001. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile. Appl. Environ. Microbiol.* 67, 434-444.