The Journal of Microbiology, June 2002, p.161-165 Vol. 40, No. 2

NOTE

Morphological Diversity of Marine Microorganisms on Different Isolation Media

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(Received April 1, 2002 / Accepted May 28, 2002)

Isolation frequency of microorganisms from marine sources was examined with different media and samples collected from the coastal area of Cheju Island. From sea water samples, about 1% of microorganisms from the total number of bacteria were recovered. Microorganisms were cultured at the much lower frequency of 10^{-4}-10^{-6} from other marine sources, such as sediment, sponges and corals. The frequency of duplicated isolation was examined with 140 morphologically different colonies isolated on different media. Fourteen percent of them exhibited the same morphology on two different media. The duplication frequency of the isolates among three different media was 33%.

Key words: diversity, marine microorganisms, isolation

The oceans represent a resource for discovery of novel pharmaceuticals, nutritional material, cosmetics and enzymes, as well as a rich source of biological diversity. Many natural products have been isolated from marine environments. However, only a small fraction of them was derived from marine microorganisms (Munro *et al.*, 1999; Pomponi, 1999). A great percentage of marine microorganisms have not been described (Pomponi, 1999), although marine microorganisms have been increasingly of interest as a source of new bioactive molecules (Fenical and Jensen, 1994; Bernan *et al.*, 1997). Some marine bacteria secret exopolysaccharides (Ikeda *et al.*, 1982; Worawattanmateekul and Okutani, 1992; Raguenes *et al.*, 1995), and many marine free-living and sediment-inhabiting bacteria produce secondary metabolites possessing antibacterial properties (Burgess *et al.*, 1991; Burgess *et al.*, 1999; Sponga *et al.*, 1999). Furthermore, a bacterium isolated from a seaweed produces anticancer molecules (Imamura *et al.*, 1997).

To discover novel bioproducts from marine environments, 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 (Kogure *et al.*, 1980; Staley and Konopka, 1985; Eguich and Ishida, 1990; Naoni *et al.*, 1996).

In this study, we report distribution and morphological diversity of microorganisms in seawater, sediments and marine organisms collected in coastal Cheju Island during 1998. We have also examined duplication of the isolates among multiple isolation media. 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 supplements, such as algal powder were examined. As for solidifying agents, agar and gellan gum were tested.

The media used for isolation of microorganisms were ZoBell (ZB; peptone 5 g, yeast extract 1 g, $FePO_4 4H_2O$ 0.01 g, agar 15 g, aged seawater 750 ml, distilled water 250 ml, pH 7.2), diluted ZB (DZ; 10% of ZB containing 15 g/l agar), super ZB (SZ; ZB containing 20 g of glucose) and ADF (algal powder 20 g, diatomaceous earth 20 g, fish meal 20 g, agar 15 g, and aged sea water 250 ml, distilled water 750 ml, pH 7.2). DZG, SZG, and ADFG media contained 15 g/l of gellan gum instead of agar, with compositions the same as those of DZ, SZ and ADF, respectively.

Collection of the samples was performed in January and June 1998 in coastal Cheju Island, Korea. In January, divers collected seawater and marine organisms at a depth of 10 m. Of the samples collected in June, seawater and sponges were obtained at 16 m of depth, while corals were obtained at 22 m. Marine sediments were collected

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at both 16 and 22 m. The samples were stored on dry ice until use.

One mililiter of sea water or sediments was mixed with 9 ml of sterile seawater, followed by serial dilution with sterilized seawater in a range of 10^{-1}-10^{-4}. The samples of sponges and corals were torn to small pieces and ground with sterilized seawater to 0.1 g (wet weight)/ml. One mililiter of ground samples was suspended in 9 ml of sterile sea water and subsequently diluted (10^{-2} through 10^{-6}). The diluted samples (0.1 ml) were seeded onto isolation agar media.

Colonies formed on the isolation media (colony forming units, CFU) were counted after incubation for 3 weeks at room temperature. Total number of microorganisms in the samples was determined by staining with 4',6'-diamidino-2-phenylindole (DAPI). The microorganisms in seawater were fixed with 1% formalin, and fixing of the microorganisms in sediments and the marine organisms prepared as above was carried out with Lugol's iodine filtered with a 0.2 μm-pore-size filter at the final concentration of 1%. Then cells in 1 to 10 ml of the sample solutions were collected on 0.2 μm-pore-size black polycarbonate filters (Nuclepore). Cells were destained with 1% of $Na_2S_2O_3$, followed by staining with 5 μg/ml of DAPI solution (Bianchi and Giuliano, 1996). The bacteria on the filters were observed under a fluorescence microscopy (Axioplan, Carl Zeiss, Germany) and counted from at least 12 fields.

As shown in Table 1 and Fig. 1, the ratio of bacteria cultured on the isolation media from seawater was higher than that of other samples. 1-2% and less than 1% of the total bacteria in seawater collected in January and June, respectively, were grown on various ZoBell media. 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. This suggests that the higher ratio of CFU of seawater was based on the composition of ZoBell medium, which has been devised by C. E. ZoBell and most popularly used for microbial evaluation of marine watercolumns. Bacteria from sediments at 22 m depth were less abundant but more culturable on the isolation media tested than bacteria from sediments from 16 m. The reason for the difference could

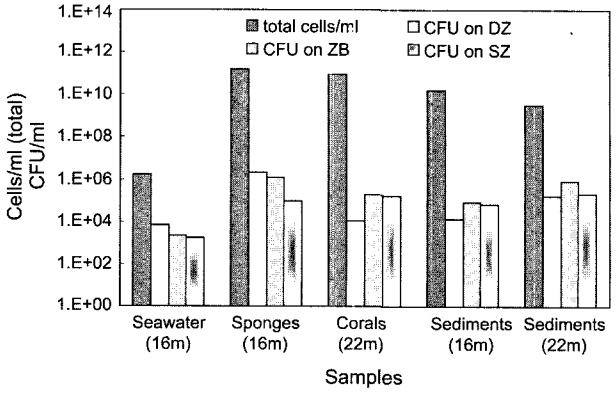

Fig. 1. Distribution of bacteria in marine sources collected in June 1998 calculated as total bacterial cells and colonies on the isolation media, DZ, ZB and SZ.

be either the sort (character) of sediments or seasonal difference (Fig. 1). The numbers of total bacterial cells per biomass of corals 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 media have to be developed to obtain microorganisms efficiently from marine sources.

There was no distinguishable difference between CFU on DZ and SZ media, when CFUs from seawater and corals collected in January were observed. Furthermore, a distinction between agar and gellan gum was not observed, when they were used as the solidifying agents of media tested. However, fewer bacteria were grown on ADF media than on DZ and SZ (Table 1).

Colonies obtained after 3 weeks incubation at room temperature on each isolation medium with morphology different from each other, were transferred to medium SZ. Morphologically different colonies on the SZ medium were isolated. The diversity of colonies was examined based on morphological characteristics such as size, color, opacity, texture, form, elevation, margin and surface (Table 2). Of samples collected in January, 74 and 61 isolates were obtained from two samples of sediments and a coral, *Plexauridae*, respectively. Among samples collected in

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	2.0×10^{6}	3.2×10^{4}	3.3×10^{4}	1.5×10^{4}	4.2×10^{4}	3.0×10^{3}	3.0×10^{3}
Antipathes	2.4×10^{8}	3.5×10^{3}	5.5×10^{3}	3.0×10^{3}	7.0×10^{3}	4.0×10^{3}	1.0×10^{2}
Alcyonium	2.4×10^{8}	4.5×10^{3}	3.0×10^{3}	1.0×10^{3}	2.5×10^{3}	1.0×10^{3}	3.0×10^{3}
Plexauridae	4.7×10^{8}	2.5×10^{4}	1.9×10^{4}	6.0×10^{3}	1.1×10^{4}	8.0×10^{3}	7.0×10^{3}
Dendronephtya	5.0×10^{8}	5.0×10^{3}	2.0×10^{3}	8.0×10^{3}	5.0×10^{3}	1.0×10^{2}	1.0×10^{2}
Dendrophylla	N.D.[b]	5.5×10^{4}	4.3×10^{4}	4.7×10^{4}	4.0×10^{4}	9.0×10^{3}	1.0×10^{3}

[a]media containing gellan gum instead of agar
[b]not detected

Table 2. Diversity of isolates based on colony morphology

Samples (depth)	Isolation media	Isolates on isolation medium	Colonies grown after transfer to SZ	Morphologically different isolates after transfer to SZ
Seawater (10 m)[a]	DZ	7	7	5
	DZG[c]	13	13	9
	SZ	12	12	10
	SZG[c]	12	9	7
	ADF	6	5	4
	ADFG[c]	4	4	3
Seawater (10 m)[a]	DZ	9	9	6
	DZG[c]	8	7	6
	SZ	5	5	4
	SZG[c]	13	13	11
	ADF	8	6	6
	ADFG[c]	4	3	3
Seawater (16 m)[b]	DZ	20	19	10
	ZB	20	17	9
	SZ	16	16	4
Coral (10 m)[a]	DZ	39	33	28
	DZG[c]	20	12	9
	SZ	15	14	12
	SZG[c]	13	5	4
	ADF	10	6	4
	ADFG[c]	8	6	4
Coral (22 m)[b]	DZ	12	8	7
	ZB	15	10	9
	SZ	13	11	9
Sponge (16 m)[b]	DZ	11	7	6
	ZB	4	8	3
	SZ	4	3	3
Sediments (16 m)[b]	DZ	14	12	9
	ZB	26	18	15
	SZ	12	10	10
Sediments (22 m)[b]	DZ	24	22	15
	ZB	31	25	18
	SZ	36	31	18

[a]Samples were collected in January 1998.
[b]Samples were collected in June 1998.
[c]Media containing gellan gum instead of agar.

June, 23 strains were isolated from seawater, and 85, 15 and 25 isolates were obtained from sediments, sponges, and corals, respectively.

A large number of morphologically different colonies were observed on ZB solid medium, where the sample of sediments was seeded. From seawater, more isolates as well as higher CFU were obtained on the diluted medium, DZ agar, than SZ mdium, generally. This suggests that more bacteria in seawater could be cultured on DZ medium because of low nutrient status of seawater. However, there was no exact correlation between organic content of sample sources and the nutrient level of the isolation media. Of the samples from marine organisms, that from corals collected in June provided a large number of isolates on the nutrient-rich media, ZB and SZ, while fewer isolates were obtained from corals collected in January and sponges on SZ medium than on DZ. It can be concluded that suitable medium for isolating abundant microorganisms from marine environment depends on the source of microorganisms.

The multiplicity of the isolates from each sample, based on colony morphology, was examined with three different isolation media (Table 3). A total of 47 isolates were observed on DZ medium from the 5 samples collected in June. From these, 6 and 9 isolates were morphologically undistinguished from isolates on ZB and SZ agar plates, respectively. Of 49 isolates on ZB agar plates, 6 and 5 were morphologically overlapped with the isolates on DZ

Table 3. Duplication of isolates among the isolation media

Samples	Isolates on DZ				Isolates on ZB				Isolates on SZ			
	Total	ZB[a]	SZ[a]	ZB/SZ[b]	Total	DZ[c]	SZ[c]	DZ/SZ[d]	Total	DZ[e]	ZB[e]	DZ/ZB[f]
Seawater	10	2	3	1	9	2	3	1	4	3	3	1
Sediments (16 m)	9	3	0	0	15	3	0	0	10	0	0	0
Sediments (22 m)	15	0	5	0	18	0	2	0	18	5	2	1
Sponge	6	1	0	0	6	1	0	0	3	0	0	0
Coral	7	0	1	0	9	0	0	0	9	1	0	0
Total	47	6	9	1	49	6	5	1	44	9	5	1

Samples collected in June 1998 were examined.
[a]number of isolates which exhibit colonies on ZB and SZ
[b]number of colonies with morphology the same as colonies on both ZB and SZ
[c]number of morphologically identical colonies with colonies on DZ and SZ
[d]number of colonies with morphology the same as colonies on both DZ and SZ
[e]number of isolates which show the same colony morphology on DZ and ZB, respectively
[f]number of isolates which exhibit identical colony morphology both on DZ and on ZB

and SZ media. From 44 isolated on SZ medium, 20.4% (9 isolates) were obtained repeated on DZ medium compared with 11.3% (Isolates) on ZB media. Fourteen percent of isolates exhibited the same colony morphology from two different media, and 33% of isolates were duplicated among three different media. Duplicated isolation was also observed in the case of isolation of actinomycetes from soil using multiple isolation media (Kim *et al.*, 1994).

By the isolation method using various media, more isolates could be obtained, while some of them were not morphologically distinguishable. Therefore, it can be concluded that isolation of microorganisms with a multiplicity of media can offer a number of microbial strains for screening of new biomaterials from marine sources. Epoch-making media have to be developed to obtain diverse microorganisms efficiently from marine sources, especially from marine organisms and sediment.

This study was supported by the National Research Laboratory Program and by the Life Phenomena & Function Research Project of the Ministry of Science and Technology, Korea.

References

Bernan, V.S., M. Greenstein, and W.M. Maiese. 1997. Marine microorganisms as a source of new natural products. *Adv. Appl. Microbiol.* 43, 57-90.

Bianchi, A. and L. Giuliano. 1996. Enumeration of viable bacteria in the marine pelagic environment. *Appl. Environ. Microbiol.* 62, 174-177.

Burgess, J.G., E.M. Jordan, M. Bregu, A. Mearns-Spragg, and K. G. Boyd. 1999. Microbial antagonism: a neglected avenue of natural products research. *J. Biotech.* 70, 27-32.

Burgess, J.G., H. Miyashita, H. Sudo, and T. Matsunaga. 1991. Antibiotic production by the marine photosynthetic bacterium Chromatium purpuratum NKPB 031704: localization of activity to the chromatophores. *FEMS Microbiol. Lett.* 84, 301-304.

David, M.W., J.F. Michael, C.N. Stephen, and M.B. Mary. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol. Mol. Biol. Rev.* 62, 1353-1370.

Eguich, M. and Y. Ishida. 1990. Oligotrophic heterotrophic bacteria and in situ heteroptrphic activity in pelagic seawater. *FEMS Microbiol. Ecol.* 73, 23-30.

Fenical, W. and P.R. Jensen. 1994. Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. *Ann. Rev. Microbiol.* 48, 559-584.

Fry, J.C. 1988. Determination of biomass. pp. 27-72. *In* B. Austin (ed.). Methods in aquatic bacteriology. John Willey Sons, Chichester

Ikeda, F., H. Shuto, T. Fukui, and K. Tomita. 1982. An extracellular polysaccharide produced by *Zoogloea ramigera* 115. *Eur. J. Biochem.* 123, 437-445.

Imamura, N., M. Nishijima., T. Takadera, and K. Adach. 1997. New anticancer antibiotics pelagiomicins, produced by a new marine bacterium *Pelagiobacter variablis*. *J. Antibiot.* 50, 8-12.

Kim, C.-J., K.-H. Lee, A. Shimazu, and I.-D. Yoo. 1994. Reisolation Frequency of soil Actinomycetes on multiple isolation media (Korean). *Kor. J. Appl. Microbiol. Biotechnol.* 22, 329-331.

Kogure, K, U. Simida, and N. Taga. 1980. Distribution of viable marine bacteria in neritic seawater around Japan. *Can. J. Microbiol.* 36, 318-323.

Munro, M.H.G., J.W. Blunt, E.J. Dumdei, S.J.H. Hickford, R.E. Lill, S. Li, C.N. Battershill, and A.R. Duckworth. 1999. The discovery and development of marine compounds with pharmaceutical potential. *J. Biotech.* 70, 15-25.

Naoni, W.R., A.R. Fred, and S. Erko. 1996. A study of the bacterial flora associated with *Holothuria arta*. *J. Exp. Mar. Biol. Ecol.* 203, 11-26.

Pomponi, S.A. 1999. The bioprocess-technological potential of the sea. *J. Biotech.* 70, 5-13.

Raguenes, G., P. Pignet, G., A. Peres, R. Christen, H. Rougeaux, G. Barbier, and J. Guezennec. 1996. Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, *Alteromonas macleodii* subsp. *fijiensis*, and preliminary characterization of the polymer. *Appl. Environ. Microbiol.* 62, 67-73.

Sponga, F., L. Cavaletti, A. Lazzarini, A. Borghi, I. Ciciliato, D. Losi, and F. Marinelli. 1999. Biodiversity and potentials of marine-derived microorganisms. *J. Biotech.* 70, 65-69.

Staley, J.T. and A. Konopka. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Ann. Rev. Microbiol.* 39, 321-346.

Worawattananmateekul, W. and K. Okutani. 1992. Isolation and characterization of a sulfated polysaccharide produced by a marine bacterium. *Nippon Suisan Gakkaishi* 58, 1729-1933.