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Study on the Useful Marine Bacteria from the Coastal Waters of Iriomote Island, Japan

(Kajian Mengenai Bakteria Marin yang Berguna dari Pesisir Pantai Pulau Iriomote, Japan)

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ABSTRACT

The screening of 86 marine bacterial strains associated with marine invertebrates, algae, sediment, artificial sponges and sea water from the coastal waters of the Iriomote Islands (Japan) has been carried out. The purpose of this study was to get various strains of marine bacteria that are beneficial to the production of bioactive compounds. The screening of antibacterial activity produced by strains of marine bacteria was carried out using the paper disk method. Antibacterial activity test results showed that one strain of bacteria showed some activity against Bacillus subtilis and Cautobacter halobacteroides, two strains of bacteria showed some activity against Staphylococcus aureus, four strains of bacteria showed some activity against Cytophaga marinoflava, 63 strains of bacteria showed some activity against Vibrio harveyi and 14 strains of bacteria showed some activity against Pseudovibrio sp., while Salinivibrio costicola showed no antibacterial activity. This indicates that about 78% of all the bacterial strains isolated have the potential to produce bioactive compounds.

Keywords: Antibacterial, marine bacteria, Iriomote Island, Japan

ABSTRAK

Saringan pada 86 strain bakteria marin yang berkaitan dengan invertebrata marin, alga, sedimen, span tiruan dan air laut dari pesisir pantai Iriomote Islands (Jepun) telah dijalankan. Tujuan kajian ini adalah untuk mendapatkan pelbagai strain bakteria marin yang bermanfaat untuk menghasilkan sebatian bioaktif. Saringan aktiviti antibakteria yang dihasilkan oleh strain bakteria laut dibawa dengan menggunakan kaedah cakera kertas. Keputusan ujian aktiviti antibakteria menunjukkan bahawa salah satu strain bakteria mempunyai aktiviti terhadap Bacillus subtilis dan halobacteroides Cautobacter, dua jenis bakteria mempunyai aktiviti terhadap Staphylococcus aureus, empat jenis bakteria mempunyai aktiviti terhadap Cytophagamarinoflava, 63 jenis bakteria mempunyai aktiviti terhadap Vibrio harveyi dan 14 jenis bakteria mempunyai aktiviti terhadap Pseudovibrio sp., manakala pada Salinivibriocosticola tidak menunjukkan aktiviti antibakteria. Ia menunjukkan bahawa kira-kira 78% daripada semua strain bakteria yang terpencil mempunyai potensi untuk menghasilkan sebatian bioaktif.

Katakunci: Antibakteria, bakteria marin, Pulau Iriomote, Japan

INTRODUCTION

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Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from microorganisms, many based on traditional medicines. In the past century, however, an increasing role has been played by microorganisms in the production of antibiotics and other drugs for the treatment of a number of serious diseases (Jha & Zi-rong, 2005).

The marine surface environment is the site of competition for marine living space by a wide variety of organisms. Bacteria are generally recognized as primary colonizers of this habitat and are able to rapidly form biofilms over freshly exposed surfaces (James et al. 1999). The marine environment is a rich source of both biological and chemical diversity. This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agrochemicals. In recent years, a significant number of novel compounds with potent pharmacological properties have been discovered from marine organisms (Kijjoa & Sawangwong 2004). Over the past 20 years, a core group of marine natural product chemists from several countries, in collaboration with both academic pharmacologists and the pharmaceutical industry, reported a very large number of novel metabolites with useful and sometimes sensational pharmacological properties (Faulkner 2000).

This paper will describe the isolation and screening of a crude antibacterial compound from marine bacteria that inhibits the growth of both gram-positive and gram-negative bacteria. The objective of this study is to collect the many kinds of useful marine bacteria for the production of biologically active compounds.

MATERIALS AND METHODS

Samples used in this research were collected by Marine Biotechnology Institute (MBI) researchers at the Iriomote islands in Japan. Samples consist of invertebrates, seaweeds, sediments, artificial sponges (Yasumoto-Hirose et al. 2006) and sea water. All chemicals containing Nutrient Broth and Marine Broth were obtained commercially. Test strains shown in Table 1 were purchased from each microbial collection such as ATCC.

Isolation of Useful Marine Bacteria by Special Media

A piece of each organism was dipped into sterilized seawater (5 ml) contained in 15 ml of a centrifuge tube. After homogenizing, the suspended solution was diluted 10-fold and 100-fold with sterilized seawater. Similary, the sand and mud were collected into 15 ml of centrifuge. After standing for two hours, the supernatant was diluted 10-fold and 100-fold with sterilized seawater. These stock solutions were used for the isolation of the microorganisms.

Two kinds of special media are used for bacterial screening in research, namely 1/10NA (Nutrient Agar) and lignan enrichment liquid medium. The 1/10 NA consists of a nutrient broth (0.8g), Agar (15g) and distilled water (1L). Lignan enrichment liquid medium consists of a mineral stock solution (5.5mL), a vitamin stock solution (0.05mL), NaCl (2.2g), lignan (0.05g) and distilled water (100mL).

The 50 µL aliquots of the sample solutions were spread on isolation plates on the day the samples were collected. After several days, each distinct strain of bacteria forms colonies on the isolation plates, and these are then each further isolated to new plates by a

single-use needle. For enrichment culture, 100 µL aliquots of the sample solutions were added into 900 µL of the liquid medium. After 2 weeks, 50 µL aliquots of the enrichment solution were spread on the MA or 1/3MA plates for isolation. Each bacterial strain was harvested with a sterilized loop and suspended in seawater containing 10% glycerol. These bacterial solutions were stored at -80°C

Sample Preparation for Screening

The isolated strains were cultured in 4 ml of a liquid medium containing distilled water, 37.4g of Marine Broth (Difco) and 3.3g tryptophan per liter of solution. The culture tube was incubated for 4 - 6 days at 30 °C on a shaker. After harvesting, each culture was freeze-dried and extracted with 2 mL of 60 % ethanol.

Antibacterial Test

The antibacterial test against terrestrial and marine bacteria on a Nutrient Agar plate or Marine Agar (Difco) plate was tested using the paper disk method. The samples were dissolved in methanol or 60 % ethanol and 15 µL of the solution was applied to a disk 6 mm in diameter. All bacteria were incubated at 30 ºC. In this study, useful marine bacteria was screened by antibacterial assay using 7 bacterial test strains as shown in Table 1.

Table 1. Test strains in the antibacterial test

No.	Test Strains	Gram	Origin	Taxon
1	*Bacillus subtilis* IFO 3134	+	Terrestrial	Firmicutes
2	*Staphylococcus aureus* IFO 12732	+	Terrestrial	Firmicutes
3	*Cytophaga marinoflava* IFO 1417	-	Marine	Bacteriodetes
4	*Salinivibrio costicola* ATCC 33508	-	Marine	Gammaproteobacteria
5	*Vibrio harveyi* IFO 15631	-	Marine	Gammaproteobacteria
6	*Pseudovibrio* sp. MBIC 3368	-	Marine	Alphaproteobacteria
7	*Cautobacter halobacterioides* NCIMB 2022	-	Marine	Alphaproteobacteria

RESULTS AND DISCUSSION

Halophilicity Test

Bacteria that require salt for growth are referred to as halophilic. Certain bacteria do not require salt for growth but can tolerate the presence of salt are referred to as halotolerant. In order to avoid the use of terrestrial bacteria, the growth of the 86 bacterial strains selected was tested in the absence of sodium chloride. The results of the analysis showed that the lignan enrichment liquid medium (64 strains) consisted of halophiliic bacteria (31 strains) and halotolerant bacteria (33 strains). On the other hand, in the case of the 1/10 nutrient agar medium, all the strains were halotolerant. The 1/10 nutrient agar medium did not contain sodium chloride, so the results were acceptable.

Tabel 2. Results of the halophilicity test

No. of strains	Medium	Halophilic	Halotolerant
22	1/10NA	-	22

64	Lignan enrichment	31	33

Screening of useful marine bacteria by antibacterial assay

1/10NA was used for the selection of halotolerant bacteria such as Firmicutes and Actinobacteriodes. The lignan enrichment liquid medium was originally designed for the collection of bacteria converting sesame lignan to useful sesamin. In this study, this medium was used for the collection of new bacteria utilizing lignan as a carbon source.

For the easy selection of the active strains, samples were dispensed on an agar plate inoculated with an indicator microorganism, and after incubation a clear circular zone of growth inhibition surrounding the sample was visible against the partially opaque background of the growth. The approach employed for most indicator organisms is very similar although the details of inoculums, medium petri dish size, sample application, and zone of inhibition measurement can vary widely. In recent years, many different pieces of equipment have become commercially available to mechanize and automate the agar diffusion assay, thereby greatly increasing the number of samples that can be processed. However, these methods sometimes resulted in erroneous data because the clear circular zone of growth inhibition appeared as a result of a lack of theneutients (Hunter-Cevera et al. 1986). Thus in this study, the agar diffusion assay used paper disks, on which the extract of the bacterial culture broth was applied.

The results of the antibacterial activity test of 86 strains are shown in the Table 3.The results of the antibacterial activity test for 86 strains consist of 1 strain against *B. subtilis,* 2 strains against *S. aureus,* no strains against *S. costicola,* 4 strains against *C. marinoflava,* 63 strains against *V. harveyi,* 14 strains against *Pseudovibrio* sp. and 1 strain against *C. halobacteroides.* Many strains have antibacterial activities but pattern were different. For the comparison, authentic antibiotic were tested in the same manner. The strains which have highest activity among the tested strains were selected for the future investigation.

Table 3: Results of the antibacterial activity test on 86 strains

		Diameter clear of zone (mm)						
No.	**Strain No.**	**1**	**2**	**3**	**4**	**5**	**6**	7
1	A5S-5-1	-	-	-	-	10	-	-
2	A5S-10-1	-	-	-	-	-	-	-
3	A5S-12-1	-	-	-	-	12	-	-
4	A5S-15-1	-	-	-	-	12	-	-
5	A5S-22-1	-	-	-	-	13	-	-
6	A5S-27-1	-	-	-	-	13	-	-
7	A5S-32-1	-	-	-	-	15	-	-
8	A5S-33-1	-	-	-	-	-	-	-
9	A5S-35-1	-	-	-	-	12	-	-
10	A5S-36-1	-	-	-	-	11	-	-
11	A5S-39-1	-	-	-	-	-	-	-
12	A5S-40-1	-	-	-	-	-	-	-
13	A5S-42-1	10	30	40	-	14	15	16
14	A5S-44-1	-	-	-	-	-	-	-
15	A5S-45-1	-	-	-	-	13	-	-

16	A5S-47-1	-	-	-	-	-	-	-
17	A5S-51-1	-	-	-	-	-	-	-
18	A5S-5	-	-	-	-	11	-	-
19	A5S-58	-	-	-	-	10	-	-
20	A5S-59	-	-	-	-	12	-	-
21	A5S-65	-	-	-	-	11	-	-
22	A5S-67	-	-	-	-	12	-	-
23	A5T-1	-	-	-	-	13	-	-
24	A5T-2	-	-	-	-	12	-	-
25	A5T-3	-	-	-	-	11	-	-
26	A5T-4-1	-	-	-	-	16	-	-
27	A5T-5	-	-	-	-	16	-	-
28	A5T-6	-	-	-	-	13	-	-
29	A5T-7	-	-	-	-	14	-	-
30	A5T-8	-	-	-	-	-	23	-
31	A5T-9-1	-	-	-	-	13	-	-
32	A5T-10	-	-	-	-	-	18	-
33	A5T-11-1	-	-	-	-	12	-	-
34	A5T-11-2	-	-	-	-	14	-	-
35	A5T-12	-	-	-	-	-	-	-
36	A5T-13	-	-	-	-	16	-	-
37	A5T-14	-	-	-	-	14	-	-
38	A5T-15	-	-	-	-	-	-	-
39	A5T-16	-	-	-	-	15	-	-
40	A5T-17	-	-	-	-	12	-	-
41	A5T-18	-	-	-	-	16	-	-
42	A5T-19	-	-	-	-	13	-	-
43	A5T-20	-	-	-	-	12	-	-
4	A5T-21	-	-	-	-	12	-	-
45	A5T-22	-	-	-	-	11	-	-
46	A5T-23	-	-	-	-	12	-	-
47	A5T-24	-	-	-	-	12	-	-
48	A5T-25	-	-	-	-	13	-	-
49	A5T-26	-	-	-	-	12	-	-
50	A5T-27	-	-	-	-	12	-	-
51	A5T-28	-	-	-	-	12	-	-
52	A5T-29	-	-	-	-	12	-	-
53	A5T-30	-	-	-	-	11	-	-
54	A5T-31	-	-	-	-	-	-	-
55	A5T-32	-	-	-	-	14	-	-
56	A5T-33	-	-	-	-	11	-	-
57	A5T-34	-	-	-	-	13	-	-
58	A5T-35	-	-	-	-	13	-	-
59	A5T-36	-	-	-	-	14	-	-
60	A5T-37	-	-	-	-	13	-	-
61	A5T-38	-	-	-	-	12	-	-
62	A5T-39-1	-	-	-	-	12	17	-
63	A5T-39-2	-	-	-	-	14	-	-
64	A5T-40	-	-	-	-	11	-	-
65	A5T-41	-	-	-	-	14	-	-
66	A5T-42	-	-	10	-	12	13	-
67	A5T-43	-	-	-	-	12	14	-
68	A5T-44	-	-	-	-	11	15	-
69	A5T-45	-	-	-	-	-	-	-
70	A5T-46	-	-	13	-	13	13	-
71	A5T-47	-	-	-	-	14	12	-

72	A5T-48		-	10	-	14	14	-
73	A5T-49	-	-	-	-	-	-	-
74	A5T-50	-	-	-	-	-	-	-
75	A5T-51	-	-	-	-	-	24	-
76	A5T-52	-	-	-	-	-	-	-
77	A5T-53	-	-	-	-	-	13	-
78	A5T-54	-	-	-	-	-	-	-
79	A5T-55	-	-	-	-	15	-	-
80	A5T-56	-	-	-	-	13	-	-
81	A5T-57	-	-	-	-	-	20	-
82	A5T-58-1	-	-	-	-	14	-	-
83	A5T-58-2	-	-	-	-	11	-	-
84	A5T-59	-	-	-	-	-	-	-
85	A5T-60	-	-	-	-	-	-	-
86	A5T-62	-	-	-	-	-	-	-
87	Polymyxin B (200 ppm)	24	24	-	10	-	-	-
88	Penicillin G (200 ppm)	8	13	-	-	-	26	-
89	Chloramphenicol (200 ppm)	19	14	-	17	13	-	-
90	Kanamycin (200 ppm)	-	-	-	-	-	-	-
91	Kanamycin (2000 ppm)	25	31	-	-	12	-	-
92	CCCP* (200 ppm)	-	-	-	-	-	-	-
93	CCCP* (2000 ppm)	34	30	32	36	14	33	38

*CCCP (carbonyl cyanide m-chlorophenylhydrazone)

The most bacterial activity emerged with the use of *V.harveyi*. Another experiment on the dependence of the medium showed that the strains grown in the marine broth showed no activity, on the other hand the strains grown on the medium containing sucrose showed some activity. Therefore, it is thought that the organic acids metabolized from sucrose may show some activity against *V. harveyi.* It was found that the growth of *Vibrio harveyi* was suppressed by weak acids produced by the bacteria.

Based on the Table 3, many strains have antibacterial activities but pattern were different. For the comparison, authentic antibiotic were tested in the same manner. The strains which have highest activity among the tested strains were selected for the future investigation. In previous studies has been isolated Actinomycin D from marine derived *Streptomyces.* Except *S. costicola,* this compound showed antibacterial activity against six antibacterial test strains (Rasyid & Adachi 2007). Two new antibiotic depsipeptides unnarmicins A and C were isolated from the fermentation broth of a marine bacterium, *Photobacterium* sp. strain MBIC06485. Both compounds selectively inhibited the growth of two strains belonging to the genus Pseudovibrio, one of the most prevalent genera in the marine environments within the class Alphaproteobacteria (Oku et al. *2008*). The result of this study is expected to produce new antibiotics that can provide benefits to address diseases that are horrible and even deadly.

CONCLUSION

Screening for 86 bacterial strains collected from the Iriomote Islands, Japan showed that 78% of the bacterial strains were found to be biologically active compound producers. No bacterial strains were against *Salinivibrio costicola.*

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