# STUDIES ON MARINE MICROORGANISMS. II

## ACTINOMYCETES IN SAGAMI BAY AND THEIR ANTIBIOTIC SUBSTANCES

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In all, 136 strains of actinomycetes were isolated from 37 samples collected at three inlets in Sagami Bay. The salt tolerance of terrestrial actinomycetes was examined. Most of actinomycetes tested were able to grow in media containing 3.0~3.5 % NaCl eqivalent to the average salt concentration of sea water, with the exception of two strains of *Microbispora*, which failed to grow in media containing less than 2 % NaCl. A strain of *Actinomyces aureoverticillus*, of *Chainia violens* and of *Nocardia asteroides* were able to grow in media containing more than 10 % NaCl. The production of antibiotic substances by four strains of these isolated actinomycetes was studied in various media with varied temperatures. It was remarkable that the antibacterial spectra changed with varied cultural conditions. In all, 27 % of the isolates had antimicrobial activity and 17 % showed inhibition against YOSHIDA sarcoma cell.

As reported in the previous paper[1] marine microorganisms seem to be one of the important sources for the screening of biologically-active substances, although actinomycetes were not isolated. In this report the authors isolated 136 strains of actinomycetes from the mud of Sagami Bay and examined their properties with reference to antibiotic production.

### Materials and Methods

Samples were collected at 3 inlets in Sagami Bay (7 samples at Kawana Inlet and 20 at Koajiro Inlet in August 1970 and 10 at Odawa Inlet in October 1971) (Fig. 1).

Sampling Methods: Two types of samplers were used, including the device conical head sampler (Fig. 2) and mini sediment sampler (Fig. 3). Collected samples were brought back to the laboratory soon after their collection within a half day.

Isolation Methods and Media: Collected samples were directly spread over the following five agar media and kept at 20°C for 2~6 weeks.

| Medium | Components (%) | | Medium | Components (%) | |
|---|---|---|---|---|---|
| K | Glucose | 1.0 | GG | Glycerol | 2.0 |
| | Asparagine | 0.05 | | Glycine | 0.25 |
| | $K_2HPO_4$ | 0.05 | | NaCl | 0.1 |
| | Agar | 1.7 | | $K_2HPO_4$ | 0.1 |
| | pH 7.4 | | | $FeSO_4$ | 0.01 |
| MYS | Maltose | 1.0 | | $MgSO_4$ | 0.01 |
| | Yeast extract | 0.4 | | $CaCO_3$ | 0.01 |
| | Agar | 1.7 | | Agar | 1.7 |
| | pH 7.2 | | | pH 7.4 | |

Fig. 1. Locations in Sagami Bay for the collection of sea mud.

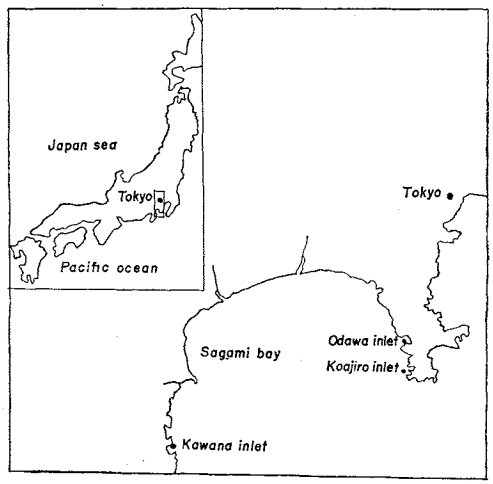



Fig. 2. Conical head sampler.[4]
Length : 350 mm
Weight : 2.5 kg

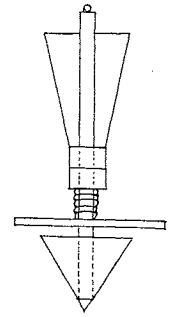
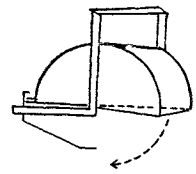


Fig. 3. Mini sediment sampler.[4]
Length : 80 mm
Weight : 0.2 kg



| Medium | Components (%) | |
|---|---|---|
| SC | Soluble starch | 1.0 |
| | Casein | 0.1 |
| | Agar | 1.7 |
| | Artificial mixed sea water* 500 ml+dist. water 500 ml pH 7.4 | |

| Medium | Components (%) | |
|---|---|---|
| Z | Bacto-pepton | 0.5 |
| | Bacto-yeast extract | 0.1 |
| | Ferric phosphate | 0.01 |
| | Agar | 1.7 |
| | Artificially mixed sea water* 750 ml and dist. water 250 ml pH 7.8 | |

* Jamarine Co., Osaka.

Cultural Conditions: At the first, 4 strains of isolated actinomycetes were arbitrary selected and shaked reciprocally with 130 rpm strokes at 15°C for 10 days, 21°C for 7 days and 27°C for 5 days in the following 12 media, and examined their antimicrobial activity by cylinder plate method.

| Medium | Components (%) | |
|---|---|---|
| I | Starch | 1.0 |
| | Glucose | 1.0 |
| | Malt-extract | 0.75 |
| | Polypeptone | 0.75 |
| | NaCl | 0.3 |
| | $MgSO_4$ | 0.1 |
| | $CuSO_4 \cdot 5H_2O$ | 0.007 g/liter |
| | $FeSO_4 \cdot 7H_2O$ | 0.001 g/liter |
| | $MnCl_2 \cdot 4H_2O$ | 0.008 g/liter |
| | $ZnSO_4 \cdot 7H_2O$ | 0.002 g/liter |
| II | Starch | 1.0 |
| | Glucose | 1.0 |
| | Soybean-meal | 1.5 |
| | $K_2HPO_4$ | 0.1 |
| | $MgSO_4$ | 0.1 |

| Medium | Components (%) | |
|---|---|---|
| II | NaCl | 0.3 |
| | $CuSO_4 \cdot 5H_2O$ | 0.007 g/liter |
| | $FeSO_4 \cdot 7H_2O$ | 0.001 g/liter |
| | $MnCl_2 \cdot 4H_2O$ | 0.008 g/liter |
| | $ZnSO_4 \cdot 7H_2O$ | 0.002 g/liter |
| III | Starch | 2.0 |
| | Glucose | 2.0 |
| | Soybean-meal | 2.0 |
| | Yeast-extract | 0.5 |
| | NaCl | 0.25 |
| | $CaCO_3$ | 0.32 |
| | $CuSO_4 \cdot 5H_2O$ | 0.005 g/liter |
| | $MnCl_2 \cdot 4H_2O$ | 0.005 g/liter |
| | $ZnSO_4 \cdot 7H_2O$ | 0.005 g/liter |
| | pH 7.4 | |

| Medium | Components (%) | |
|---|---|---|
| SIII | Same as III medium cxcept NaCl concentration | |
| | NaCl | 1.5 |
| PS | Soluble starch | 3.0 |
| | Pharmamedia* | 1.5 |
| | Cornsteep-liquor | 2.0 |
| | Beef-extract | 1.0 |
| | pH 7.4 | |
| V | Glucose | 0.5 |
| | Peptone | 1.0 |
| | Yeast extract | 0.5 |
| | $K_2HPO_4$ | 0.005 |
| | $MgSO_4$ | 0.005 |
| | NaCl | 2.0 |
| | pH 7.8 | |
| SV | Same as V except NaCl | |
| | NaCl | 1.5 |
| X | Glucose | 0.5 |
| | Soybean-meal | 1.5 |
| | $K_2HPO_4$ | O0.05 |

* Traders Oil Mill Co., U.S.A.

| Medium | Components (%) | |
|---|---|---|
| X | $MgSO_4$ | 0.05 |
| | NaCl | 2.0 |
| | pH 7.8 | |
| SX | Same as X except NaCl | |
| | NaCl | 1.5 |
| Y | Glucose | 0.5 |
| | Asparagine | 0.5 |
| | Glutamate-Na | 0.5 |
| | $K_2HPO_4$ | 0.05 |
| | $MgSO_4$ | 0.05 |
| | NaCl | 2.0 |
| | pH 7.8 | |
| SY | Same as Y except NaCl | |
| | NaCl | 1.5 |
| SZ | Glucose | 1.0 |
| | Bacto-peptone | 0.5 |
| | Bacto-yeast extract* | 0.1 |
| | Ferric-phosphate | 0.01 |
| | Artificial mixed sea water 750 ml and dist. water 250 ml | |
| | pH 7.4 | |

* Difco Co., Baltimore

Antimicrobial and Antimalignant Cell Activity of Marine Actinomycetes: Marine actinomyces (136 strains) were inoculated and shake-cultured in three media, SIII, PS and SZ of above media at 27°C for 5 days and at 15°C for 10 days. The antimicrobial activity as examined by the cylinder plate method. The antimalignant activity was examined by its inhibition of YOSHIDA sarcoma cell in respect to the UV absorption as described previously by HORI *et al.*[2]

## Results and Discussion

### Salt Tolerance of Terrestrial Actinomycetes

Various kinds of actinomycetes were isolated at each inlet in Sagami Bay. Since these samples were collected at the sea coast, and the average depth was only about 10 meters, some of those marine microorganisms are thought to be derived from terrestrial origin carried into the sea. When terrestrial actinomycetes reach the sea environment, their tolerance to salt concentration would be one of the factors for their survival. Therefore, 20 strains of ter-

Fig. 4. Salt tolerance of actinomycetes. (Basal medium. MYS, 11 days)

Solid line shows good growth. Dotted line shows fair growth.

Four lines for each organisms designate incubation at 20°, 27°, 30° and 37°C from upper to lower respectively.

S; *Streptomyces* A; *Actinomyces* Sv; *Streptoverticillium* Ch; *Chainia* Ap; *Actinoplanes* Mb; *Microbispora* N; *Nocardia*

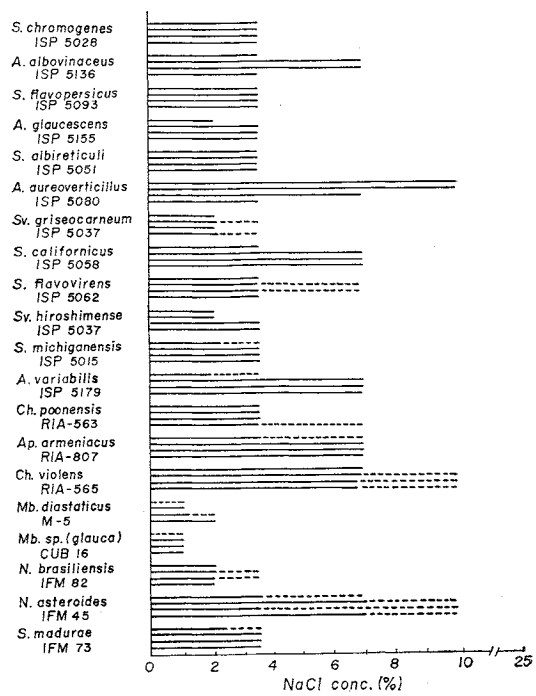


Table 1. Antimicrobial activity of marine actinomycetes under various conditions

| Strain No. | | SS-5 | SS-7 | SS-11 | SS-12 |
|---|---|---|---|---|---|
| Med. | Temp. | Active to* | Active to | Active to | Active to |
| I | 15 | | | | Sa |
| | 21 | | | Sa | |
| | 27 | Ec Kp Mf | Sa | Ec | |
| II | 15 | | | | |
| | 21 | | | Sa | |
| | 27 | Kp Mf | | Sa | Mf |
| III | 15 | Kp | Ec | | Xo |
| | 21 | | | Sa | |
| | 27 | Ec Kp Va | | | |
| SIII | 15 | | | | |
| | 21 | | Sa | Sa | |
| | 27 | | Sa | | Ps |
| PS | 15 | | | | Xo |
| | 21 | | | | |
| | 27 | Mf | | | Mf |
| V | 15 | | | Xo | Xo |
| | 21 | | | | Pc |
| | 27 | Ps Mf | | | Mf |
| SV | 15 | | | | Ps |
| | 21 | | Sa | | |
| | 27 | Ps | Sa Ps | Sa | |
| X | 15 | | | | |
| | 21 | | | | |
| | 27 | Ps Mf | Mf | | Mf |
| SX | 15 | Mf | | | |
| | 21 | | Sa | Sa | |
| | 27 | Mf | Mf | Mf | Sa Kp |
| Y | 15 | | | | |
| | 21 | | | | |
| | 27 | Mf | | | Ps Mf |
| SY | 15 | Mf Xo | | Ec Ps Xo | Mf Xo |
| | 21 | | | | |
| | 27 | Mf | | | Mf |
| SZ | 15 | Xo | | Ec Ps Xo | Bs Sa Xo Xc |
| | 21 | | Sa | | Mf |
| | 27 | | | Mf | Mf |

* Inhibition diameter more than 15 mm by cylinder plate method.

Ec : *Escherichia coli* Xo : *Xanthomonas oryzae*
Kp : *Klebsiella pneumoniae* Sa : *Staphylococcus aureus*
Mf : *Micrococcus flavus* Xc : *Xanthomonas citri*
Va : *Vibrio anguillarum* Bs : *Bacillus subtilis*
Ps : *Pseudomonas fluorescens*

Table 2. Antimicrobial activity of marine actinomycetes

| Strain No. | Active against* | | | | Production (Identified) |
|---|---|---|---|---|---|
| | Gram − | Gram + | Candida | Fungi | |
| SS-2 | | | + | + | |
| 3 | | | | + | |
| 4 | | | | + | |
| 12 | | | | + | |
| 14 | + | | | + | |
| 17 | | + | | + | pluramycin like |
| 18 | + | | | + | |
| 21 | + | + | | + | xanthomycin |
| 22 | | + | | + | |
| 28 | + | + | | + | xanthomycin like |
| 30 | | | | + | actinoleukin like |
| 31 | + | + | | | xanthomycin |
| 35 | | | | + | |
| 37 | | | | + | |
| 38 | | + | | | xanthomycin |
| 41 | + | | | | |
| 45 | | | | + | |
| 48 | | | | + | |
| 50 | | | | + | |
| 52 | | + | | | luteomycin |
| 54 | | | | + | |
| 55 | | | | + | |
| 58 | + | + | | + | |
| 61 | | | | + | |
| 66 | | | | + | |
| 91 | + | + | + | + | |
| 94 | + | + | | | |
| 99 | | + | | + | |
| 104 | | | | + | |
| 105 | + | + | | | |
| 107 | | | | + | |
| 109 | | | | + | |
| 112 | + | + | | + | |
| 118 | + | | | + | |
| 124 | | | | + | viomycin |
| 129 | | + | | + | |
| 133 | | | | + | |
| 37/136 | 12 | 14 | 2 | 31 | |

* Inhibition diameter more than 20 mm by cylinder plate method.

restrial actinomycetes belonging to various genera were examined and their growth under various salt concentrations (0.5, 1.0, 2.0, 3.5, 7.0, 10.0 and 25.0 %) and temperatures (20, 27, 30 and 37°C) determined. As shown in Fig. 4, most of them were able to grow in media containing 3.0~3.5 % NaCl, equivalent to the average salt concentration of sea water. On the other hand, there were actinomycetes such as *Actinomyces aureoverticil* which were not affected by more than 10 % NaCl concentration. Some

Table 3. Anti-malignant cell activity of marine actinomycetes

| Strain No. | YOSHIDA Inhib*. (UV) % | Strain No. | YOSHIDA Inhib*. (UV) % | Strain No. | YOSHIDA Inhib*. (UV) % |
|---|---|---|---|---|---|
| SS-10 | 58.0 | 36 | 41.0 | 94 | 47.5 |
| 16 | 67.8 | 37 | 43.0 | 99 | 49.0 |
| 17 | 89.8 | 38 | 72.8 | 105 | 50.2 |
| 18 | 70.2 | 42 | 85.3 | 109 | 50.2 |
| 21 | 88.5 | 52 | 40.0 | 112 | 32.2 |
| 27 | 73.1 | 56 | 31.5 | 124 | 25.9 |
| 31 | 94.9 | 58 | 92.9 | 129 | 90.8 |
| 34 | 79.0 | 76 | 35.5 | | |
| 35 | 61.4 | 91 | 67.3 | | |

$$\frac{\text{Active } 25}{\text{Tested } 136} = 17.0\ \%$$

* Inhibition of YOSHIDA sarcoma cell measured by UV adsorption after HORI's method[2].

actinomycetes such as *Microbispora* sp. were sensitive to less than two percent concentration of salt. TRESNER *et al.*[3] have proposed a salt tolerance as a criterion in the classification of streptomyces. The data obtained suggested agreement with the above, and it can be extended to actinomycetes other than streptomycetes. All of isolates from Sagami Bay in this experiment were able to grow in media containing 3.0% NaCl.

## Cultural Conditions and the Antimicrobial Activity of Marine Actinomycetes

Strains designated as Nos. 5, 7, 11 and 12 were selected for these studies. These strains were shake-cultured under various conditions, and the antimicrobial activity examined by the cylinder plate method. The activities shown by an inhibition zone of more than 15 mm diameter are listed in Table 1. The production of antimicrobial substance varied with cultural conditions as would be expected. For example, strain No. 12 in medium V was active to Xo (*Xanthomonas oryzae*) at 15°C, but active only to Pc (*Phytophthora capsici*) at 21°C and moreover active of Mf (*Micrococcus flavus*) at 27°C. It was also noted that these four strains grew well in SIII and SZ medium containing NaCl and artificially mixed sea water better than in other media.

Among 136 strains tested, 37 strains (27%) showed very high activity to test microorganisms (more than 20 mm diameter of inhibitory zone in the cylinder plate method), and they were more frequently active against fungi than bacteria, as well as bacteria isolated from Japan Sea (reported in the previous paper) (Table 2). Known antibiotics such as xanthomycin and pluramycin were found as products of marine actinomycetes. Also active substances, supposedly new substances, having a unique spectrum of antibacterial activity, have been noted. Moreover, the varieties of microorgrnisms found in a marine sample are more varied, comparing with that found in one soil sample. These facts suggest that marine microorganisms particularly actinomycetes would be important sources for the discovery of new active principles as well as terristrial actinomycetes. As shown in Table 3, the fact that 25 strains of actinomycetes isolated inhibited YOSHIDA sarcoma cell, suggests marine microorganisms are very useful screening sources of anti-cancer substances as well as of antimicrobial substances.

Acknowledgement

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## References

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