Occurrence and Generic Composition of Protease-producing Moderately Halophilic Bacteria in Neritic Seawater around Japan

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A large proportion of moderately halophilic bacteria exist in considerable numbers in marine environments, just off the coast of Japan. Viable counts of the marine moderate halophiles and general heterotrophic marine bacteria varied from station to station. A large proportion of protease-producing bacteria was found among heterotrophic bacteria and a considerable proportion of protease-producers was also found among moderate halophiles in the various marine environments studied. Mean value of the occurrence of total protease producers among heterotrophic marine bacteria was generally higher than that of the moderately halophilic bacteria; that is, the proportions were 70% in Tokyo Bay, 80% in Sagami Bay, 60% in the Kuroshio region and 30% in the sediments of Sagami Bay in the case of the heterotrophic marine bacteria; 30% in Tokyo Bay, 45% in Sagami Bay, 20% in the Kuroshio region and 15% in the sediments of Sagami Bay in the case of the moderately halophilic bacteria.

Among the moderately halophilic isolates tested, *Pseudomonas* spp. were always found to be dominant in seawater samples. However, *Acinetobacter* spp. were dominant in the moderate halophiles in the sediments. Only a small percentage of *Flavobacterium* spp. and *Caulobacter* spp. were found in the microflora.

SHAH and DE SA²⁾ reported that a few marine bacteria could grow slowly in media containing 21% NaCl, other surveys, involving a total of 27 species of marine bacteria showed that 8-12% NaCl were the highest concentrations that permitted growth^{3,4)}. To distinguish marine bacteria from most non-marine species, the Na⁺ requirement for their growth was studied in detail, because characteristics of their Na⁺ requirement are of special interest⁵⁾. FORSYTH *et al.*⁶⁾ showed that most marine bacteria off the coast of New Brunswick isolated in nutrient agar containing 3% NaCl could grow in 30% NaCl solution. These results point out that some marine bacteria can grow in much higher salt concentrations than those found in seawater, and some of them are moderately halophilic or at least very salt tolerant microorganisms.

In the course of studies on heterotrophic activities of marine bacteria, we found that a large proportion of bacteria in the sea could grow in media containing 3-20% NaCl and produce extracellular proteases. In this paper, an assessment has been made of the occurrence and generic composition of moderately halophilic bacteria isolated from seawater and bottom sediments of

Sagami and Tokyo Bays and from the region of the Kuroshio close to Japan. Studies have also been made of their proteolytic properties.

Materials and Methods

Water and Sediment Samples

Seawater and sediment samples were collected at the locations shown in Fig. 1 during cruises of the Tansei Maru, Ocean Research Institute, University of Tokyo in 1977. Surface seawater samples were collected with sterile 500 ml glass bottles and deep water samples for microbiological analysis were taken using a sterilized ORIT sampler⁶⁾ attached to Nansen bottles. Sediment samples were obtained with a Phleger-type gravity corer.

Isolation and Enumeration of Bacteria

Modified medium 2216E⁷⁾ was used for enumeration and isolation of bacteria in samples. The composition of the medium was as follows: Bacto-peptone (Difco), 0.5 g; Bacto-yeast extract, 0.1 g; ferric citrate, 0.01 g and Bacto-agar, 1.5 g in 100 ml of artificial seawater containing MgSO₄ · 7H₂O, 0.2%; KCl, 0.3%; CaCl₂, 0.005%; MgCl₂ · 6H₂O, 0.2%; NaCl, 3.5% for the isolation of

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Fig. 1. Location of sampling stations.

general heterotrophic marine bacteria and 7, 14, 20 and 24.5% for that of halophilic bacteria.

A suitable volume of seawater sample was filtered through a sterilized membrane filter (Millipore HA-47 mm, 0.45 μ m pore diameter) as soon as possible after the samples were taken aboard ship. The filter was then incubated on plates of modified medium 2216E.

Sediment samples were stirred up with a suitable volume of sterilized seawater and the portions were spread onto agar plates of the above-mentioned medium using sterile spreading rods⁹⁾.

After the inoculated plates were incubated at 20°C for two weeks, the bacterial colonies on the plates were counted and the numbers were expressed as colony-forming units (c.f.u.). The colonies on the 3.5% NaCl, 7–14% NaCl and 24.5% NaCl agar plates were enumerated as the c.f.u. of general marine bacteria, moderately halophilic bacteria and that of extremely halophilic bacteria, respectively. After enumeration, bacteria were isolated and purified by picking all the colonies from appropriate plates.

Proteolytic Activity of Isolated Bacteria

The hydrolysis of casein by isolated bacteria was examined by inoculating them on the agar plate of a modified medium 2216E which contained 1% of casein. A preliminary estimate of the enzyme activity was based on the clear zone around the colony formed on the casein agar plates.

The proportions of isolated bacteria giving positive results in this test were used to calculate the numbers of protease-producing bacteria in each sample examined.

Identification of the Bacteria

The generic composition of the isolates was determined according to the schemes of SHEWAN *et al.*⁹⁾ and of SIMIDU¹⁰⁾. The *Vibriostatic* compound 0/129 (2, 4-diamino-6, 7-diisopropylpteridine) was not used as the means to distinguish *Vibrio* from *Aeromonas*. Their separation was made on the basis of gas production from glucose. A few Gram negative oxidase tests were assigned to *Vibrio* or *Aeromonas* but not to *Enterobacteriaceae*. The mode of glucose metabolism of the isolates was determined using the Hugh-Leifson's medium¹¹⁾ and artificial seawater.

Results

Viable counts (c.f.u.) of the general heterotrophic marine bacteria were generally found to be higher than those of moderately halophilic bacteria in the seawater of the Kuroshio region (St. K-1) and in Sagami Bay (St. A-1), and the viable count of moderately halophilic bacteria was vertically distributed in the range from 10^0 to 10^4 /100 ml in seawater of the Kuroshio region and from 10^4 to 10^5 /100 ml in that of Sagami Bay (Fig. 2).

A large proportion of the heterotrophic marine bacteria in seawater samples examined vertically in the present study had proteolytic activity. However, the proportions of proteolytic bacteria among moderately halophilic bacteria were generally smaller than those among heterotrophic marine bacteria in vertical profiles of seawater at station (K-1) and station (A-1) (Fig. 3).

Figure 4 shows the occurrence rates of proteolytic bacteria among the heterotrophic bacteria at different sampling stations. The average rates did not vary greatly with the sampling stations; i.e., 70% in Tokyo Bay, 80% in Sagami Bay, 60% at stations (K-1), (K-2) and (K-3) in the Kuroshio region and 30% in the bottom sediments of Sagami Bay (St. A-1).

Fig. 2. Vertical distribution of general heterotrophic marine bacteria (●) and moderately halophilic bacteria (○) in seawater of the Kuroshio region (A) and Sagami Bay (B).

Fig. 3. Occurrence of protease-producing bacteria among general heterotrophic marine bacteria and moderately halophilic bacteria, distributed vertically in seawater of the Kuroshio region (A) and of Sagami Bay (B).

Open column: Heterotrophic marine bacteria; Oblique-line column: moderately halophilic bacteria.

The occurrence rates of proteolytic bacteria among moderate halophiles isolated from different sampling stations are shown in Fig. 5. The average rates were obviously smaller than those among heterotrophic marine bacteria; i.e., 30% in Tokyo Bay, 45% in Sagami Bay, 20% in the Kuroshio region and 15% in the sediments of Sagami Bay (St. A-1).

As summarized in Table 1, 77% of the heterotrophic marine bacteria, 33% of the moderately halophilic bacteria and 27% of the extremely halophilic bacteria examined in the present study were found to have proteolytic activity.

The moderately halophilic bacterial flora occurring in seawater at various stations was examined taxonomically by making observations of

the isolates: 109 isolates in Tokyo Bay, 100 isolates in Sagami Bay, 48 isolates in the bottom sediments of Sagami Bay and 156 isolates from the Kuroshio region. As summarized in Fig. 6, taxonomical compositions of the halophilic bacterial flora in seawater samples were fairly similar, revealing the dominance of *Pseudomonas* spp. Thus, among the moderate halophiles in seawater of the Kuroshio region (St. K-1, 2 and 3), Tokyo Bay (St. T-3) and Sagami Bay (St. A-1), *Pseudomonas* spp. constituted 70%, 40% and 48%; and *Vibrio* spp. 25%, 24% and 20%, respectively.

In addition to *Pseudomonas* spp. being the dominant halophile in seawater, the proportions of protease producers in this genus were remarkably higher than in other genera of halophiles. The

Fig. 4. Occurrence of protease-producing bacteria among heterotrophic bacteria isolated from different seawater and sediment samples.

Open column: Heterotrophic bacteria; Oblique line column: protease-producing bacteria among heterotrophic bacteria.

Fig. 5. Average occurrence rate of protease-producing bacteria among moderately halophilic bacteria from different seawater and sediment samples.

Open column: moderately halophilic bacteria; Oblique line column: protease-producing bacteria among moderately halophilic bacteria.

Table 1. Percentages of protease-producing bacteria in the strains isolated in media with various concentrations of NaCl

	Concn. of NaCl in medium (%)		
	3.5	7-14	24.5
Protease positive (%)	77.0	33.0	27.4
No. of strains tested	212	413	18

Fig. 6. Taxonomic composition of halophilic bacteria and protease-producing bacteria in seawater and bottom sediments investigated. Open column: moderately halophilic bacteria; Oblique line column: protease-producing bacteria.

proportions of protease producers in *Pseudomonas* spp. were 30% in the Kuroshio region, 60% in Tokyo Bay, 63% in Sagami Bay and 43% in the sediments (St. A-1). At station (T-3), 13% of *Vibrio* spp. also produced proteases. The proportion of the protease producers among *Vibrio* spp. of the Kuroshio region, Sagami Bay and its sediments (St. A-1) were 20%, 36% and 33%, respectively.

Discussion

The existence of moderately halophilic bacteria, which can grow in medium containing 3-20% NaCl has been reported by LARSEN¹²⁾ and by ONISHI *et al.*¹³⁾. ZOBELL¹⁴⁾ reported the requirement of NaCl for growth of bacteria in seawater, sea bottom sediments, saltern, Great salt-lake and land soil. But the occurrence of moderately halophilic bacteria in natural environments has been

studied less, possibly because they appeared to be less unusual organisms. In marine environments, there have so far been few attempts to study moderately halophilic bacteria¹⁴⁾.

Results of the present study show that moderately halophilic bacteria exist in considerable numbers in marine environments. Viable counts of the marine moderate halophiles and general heterotrophic marine bacteria varied according to the sampling stations. A large proportion of protease-producing bacteria was found among moderately halophilic bacteria in the various marine environments studied here.

Mean value in the occurrence rates of total protease-producing bacteria among the heterotrophic marine bacteria in seawater samples was generally high; i.e., 70% in Tokyo Bay and 80% in Sagami Bay, 60% in the Kuroshio region and 30% in sediments of Sagami Bay. In addition, that among the moderately halophilic bacteria in seawater samples was usually lower than that among the former; i.e., 30% in Tokyo Bay, 45% in Sagami Bay, 20% in the Kuroshio region and 15% in the sediments of Sagami Bay.

The study of halophilic bacteria distributed in the sea has centered on the origin of extremely halophilic bacteria^{15,16)}. Many extremely halophilic bacteria live in the salterns, that arise from evaporation of natural seawater. On the other hand, it is obvious from the present study that in natural seawater there exists a considerable number of bacteria which are able to grow best in the range of 7–14% NaCl and sometimes to grow in saturated concentrations (Table 1). Therefore, these results support the assumption that the origin of extremely halophilic bacteria in salterns is the sea.

Among the moderately halophilic bacteria isolated from seawater, *Pseudomonas* spp. were always found to be dominant, while *Acinetobacter* spp. were rather slightly dominant than *Pseudomonas* spp. in the halophiles from sediments in Sagami Bay. Only a small percentage of *Flavobacterium* spp. and *Caulobacter* spp. were found in the microflora. It is interesting that Gram positives such as *Bacillus* spp. and *Micrococcus* spp. were present in the microflora of Tokyo Bay, *Micrococcus* spp. in Sagami Bay and *Bacillus* spp. in the bottom sediments.

Although *Vibrio* spp. are most frequently encountered among the heterotrophic bacterial population in the open sea, their percentage in the moderately halophilic population is far lower than that of *Pseudomonas* spp. The significance of the

Vibrio population in the marine ecosystem has been discussed by SIMIDU *et al.*¹⁷⁾ They suggested that *Vibrio* spp. play a role as the first decomposer of dead or dying plants and animals because of their wide distribution in marine environments, their rapid growth under favorable cultural conditions and their ability to degrade proteins, DNA¹⁸⁾, chitin, urea¹⁹⁾ and other organic compounds of high molecular weights. On the other hand, in the case of moderate halophiles isolated from seawater, *Pseudomonas* spp. were dominant and the main protease producers. The proportion of protease producers among isolated halophilic *Pseudomonas* spp. was 30% in the Kuroshio region, 60% in Tokyo Bay and 63% in Sagami Bay. This implies that the halophilic *Pseudomonas* group is physiologically better adapted for environments with high concentrations of nutrients.

MAEDA *et al.*¹⁸⁾ and KOBORI *et al.*²⁰⁾ reported that the ratios of phosphatase-producing bacteria as well as DNA-hydrolyzing bacteria in *Pseudomonas* groups increased with the transition from coastal to oceanic regions. AKAGI *et al.*²¹⁾ found that the ratio of *Pseudomonas* spp. was higher in oligotrophs than in ordinary heterotrophs at Nanseishoto area, Aburatsubo Inlet and Ozuchi Bay, while the proportion of *Vibrio* spp. was lower in the former than in the latter. These results imply that various types of *Pseudomonas* populations exist in different marine environments.

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