Short Communication

Isolation of Halotolerant Microorganisms from Seawater around the Inland Sea in Western Japan

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Twenty-three microorganisms taken from the Inland Sea in Western Japan formed colonies on plates containing 2 M NaCl, of which 15 isolates showed growth rates of more than 0.5 (doublings/h) at 30°C in the presence of 2 M NaCl. The isolates thus selected were classified into two groups by their growth patterns in response to NaCl concentrations added externally. Four isolates (group I) did not grow in the presence of 20 mM and 3 M NaCl, but they showed a peak growth of 1.67–2.14 doublings/h at 0.5 M. Three isolates (group IIA) grew in the presence of 20 mM (0.92–0.95 doublings/h) and 3 M (0.29–0.33 doublings/h) NaCl, indicating that they are halotolerant microorganisms. Another 8 microorganisms (group IIB) also showed the halotolerant growth pattern. These findings indicate that halotolerant microorganisms are widely distributed in seawater.

Key words: halotolerant microorganisms, halophiles, seawater, growth rate

Microorganisms, especially those living in marine environments, can be divided into two categories, i.e., halophilic and halotolerant, on the basis of their ability to grow in the presence of NaCl¹⁾. The halophilic microorganisms require a certain concentration of NaCl for their growth, while the halotolerant ones have the ability to grow in the absence or presence of higher concentrations of NaCl. In a solar salt field and its periphery, Takashina *et al.*¹⁰⁾ isolated two extremely halotolerant strains, which grew in the absence and presence of NaCl up to about 5 M at pH 7.0. *Brevibacterium* sp. JCM 6894, isolated from seawater in Aburatsubo, Kanagawa, Japan, is another marine halotolerant microorganism that is able to grow in the presence of up to 2 M NaCl⁶⁾.

Since seawater contains about 0.5 M NaCl, slightly

halophilic microorganisms are mainly found there, although they cannot be defined as real marine microorganisms²⁾. Extremely halophilic microorganisms, however, can not survive in seawater because cell lysis occurs due to osmotic shock.

We are interested in the physiology of the halotolerant *Brevibacterium* sp. JCM 6894^{3–9)}. Ecological study also seems to be necessary to expand our knowledge of the nature of halotolerant marine microorganisms. Therefore, in this study, we tried to isolate other halotolerant microorganisms from seawater, and examine their growth in response to the external NaCl concentrations.

The sampling positions are shown in Fig. 1. Seawater was collected from the deck at the stern of the University-Ship “Fukae-maru” by autoclaved test tube (10.9 × 1.7 cm ϕ) attached with a rope. The collected seawater of 23–28°C seemed to be a mixture of surface and an about 3-m depth, because the sampling was

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Fig. 1. Sampling positions of seawater around the Inland Sea located in Western Japan, and viable cell numbers in the sample seawater grown on plates containing 2 M NaCl.

The letters from A to S indicate the different sampling positions. Closed bars show the sampling positions where one kind of halophile alone was isolated.

carried out when the ship was moving by propeller. Fifty mM potassium-phosphate buffer, pH 7.5, containing 0.5 M NaCl was used for the serial dilution of seawater samples. One ml was spread onto an agar plate containing 10 g Tryptone (Difco, Michigan, USA), 5 g Yeast extract (Difco), 117 g NaCl, and 15 g agar per liter. Medium pH was adjusted to 7.5 by tetramethylammonium hydroxide. These plates were

stored in a cool and dark space in the cabin at 20–25°C. After total colony numbers on the plates were counted for each sampling position (Fig. 1), comparison of the color, shape, and size of the individual colonies was performed. We did not compare colonies obtained from different sampling sites. A total of 23 isolates thus obtained were kept at 4°C on the agar medium containing 0.5 M NaCl.

From the sampling positions at Tomogashima channel (position C), Hyuga-nada (position F) and Bingo-nada (position N), we did not isolate any microorganisms that grew in the presence of 2 M NaCl (Fig. 1). Except for these positions, the total num-

ber of colonies varied from 10 cfu/ml at Osumi channel (position I), Harima-nada (position P) and West of Akashi-Kaikyo straits (position Q) to 110 cfu/ml South of the Bungo channel (position E).

To test halotolerance, we measured the growth rate

Fig. 2. Growth rate for each isolate as a function of NaCl added externally.

The isolates of M-2 (○), P-1 (△), Q-1 (□), and S-1 (◇) were in group I (I). The isolates of L-1 (○), P-2 (△), and R-1 (□) were in group IIA (IIA). *Brevibacterium* sp. JCM 6894 (◇) was also classified in this group. The isolates of A-1 (○), A-2 (△), B-1 (□), and D-1 (◇) (left side) were in group IIB (IIB). The isolates of E-1 (○), G-1 (△), H-2 (□), and I-1 (◇) (right side) were in group IIB.

Each value shown is an average of two independent experiments. The maximum difference between the two values obtained was 0.04 (doublings/h).

Table 1. Some taxonomic characteristics of microorganisms isolated from seawater.

Group	Gram stain	Glucose utilization	Cytochrome oxidase	Catalase	Motility	Color of colony
I (all isolates)	—	F	+	+	+	ivory
IIA (all isolates)	+	—	—	+	W	light-orange
IIB (others)	+	—	—	+	W	milky-white
(A-1)	+	—	—	+	W	light orange

+: Positive; —: Negative; F: Fermentation of glucose; W: Weak.

in the presence of various concentrations of NaCl. The composition of liquid medium used for the cell growth was the same as that for the isolation of microorganisms, except for the NaCl concentrations added externally. Pre-incubation was carried out for 2 days at 30°C in the presence of 2 M NaCl. Then a 0.5 ml cell suspension was added to 50 ml fresh medium containing various concentrations of NaCl. Even when the NaCl was not added externally, the growth medium contained 27 mM total NaCl, because the medium itself contained 7 mM NaCl, and 20 mM NaCl was supplemented with the addition of cell suspension to start the growth.

The isolated microorganisms were incubated aerobically in an Erlenmeyer flask, and the growth rate (doublings/h) was calculated from the turbidity change that was monitored by spectrophotometry (DU 640, Beckman, California, USA). The optical densities between 0.05 and 0.5 at 650 nm were chosen for linearity on a logarithmic scale.

A primary screening was carried out to detect isolates that showed high growth rates of more than 0.5 (doublings/h) in the presence of 2 M NaCl at 30°C, since a higher growth rate at 2 M NaCl indicates the possibility that an isolate possesses a higher salt tolerance. We selected a total of 15 isolates from 23 samples.

By the growth rate pattern for each isolate drawn as a function of the externally added NaCl from 20 mM to 3 M, 15 isolates were classified into two groups of I and II (Fig. 2). The isolates of M-2, P-1, Q-1, and S-1 (group I) did not grow in the presence of NaCl at lower than 20 mM or at higher than 3 M. A peak was observed at 0.5 M, where the growth rates of P-1, Q-1, and S-1 were more than 2 (doublings/h) and M-2

showed 1.67 (doublings/h). The growth rates for the isolates of L-1, P-2, and R-1 (group IIA) were reduced gradually with an increase in NaCl concentration. The values at 20 mM and 3 M NaCl were 0.92–0.95 and 0.29–0.33 (doublings/h), respectively. The growth pattern of *Brevibacterium* sp. JCM 6894 was the same as those in group IIA. Plateaus were observed at 20 mM and 0.5 M NaCl for eight isolates in group IIB, A-1, A-2, B-1, D-1, E-1, G-1, H-2, and I-1, where the growth rates were within 1.11–1.34 (doublings/h). These values were reduced by less than one-tenth in the presence of 3 M NaCl.

Some of the taxonomic characteristics for each microorganism are shown in Table 1. Gram staining and the glucose oxidation-fermentation tests were carried out following standard procedures. We added 29 g NaCl to the growth medium. To create fermentation conditions, sterile liquid paraffin was loaded on the surface of the soft-agar medium in the test tube (12.0 × 1.3 cm ϕ) after inoculation. When an isolate metabolizes glucose, the medium pH is shifted to the acidic region, which is indicated by the changing color of Bromothymol blue from blue to yellow. After 2 days of incubation at 30°C, we judged the medium color. By spreading the intact cells on the test chip (Eiken kagaku Co., Ltd) we tested cytochrome oxidase activity. We also examined the catalase activity by adding cells to a 3% H₂O₂ solution. Motility was judged from the level of cell growth in the soft-agar medium, where 3.0 (g/liter) agar was added.

Gram staining for the isolates in group I showed negative, whereas it was positive for all isolates in group II. The isolates in group I metabolized glucose both in aerobic and anaerobic conditions. None of the isolates in group II metabolized it

regardless of the oxygen concentrations. Cytochrome oxidase activity was observed for the isolates in group I, but not in group II. Groups I and II both showed catalase activity. The isolates in group I showed high motility.

According to the grouping of microorganisms by Larsen¹⁾ on the basis of NaCl requirements for growth, marine microorganisms have been considered slight halophiles that show a sharp growth pattern at concentrations of NaCl between 0.35 and 0.51 M. The isolates in group I grew rapidly in the presence of 0.5 M NaCl. Even in the presence of 2 M NaCl, they still grew. These findings indicate that the isolates of M-2, P-1, Q-1, and S-1 are halophiles and have the ability to grow in a relatively high range of NaCl. *Brevibacterium* sp. JCM 6894⁶⁾ was used for comparing the salt tolerance growth patterns with the isolates. By comparison to *Brevibacterium* sp. JCM 6894, the isolates of R-1, P-2, and L-1 in group IIA showed similar growth patterns, and their growth rates were higher in all NaCl concentrations examined. A moderately halotolerant microorganism was defined as one that demonstrates the ability to grow in the presence of NaCl up to 3.4 M¹⁾, indicating that the isolates in group IIA as well as *Brevibacterium* sp. JCM 6894 belong to this group. The growth rates of the isolates in group IIB were very low in the presence of 3 M NaCl, but they could still grow. In the presence of 20 mM and 0.5 M NaCl, they showed growth rates of over one doubling/h. These findings indicate that the isolates are also moderately halotolerant microorganisms.

The halotolerant microorganisms isolated in this study seem to have little opportunity of being exposed to higher salinity environments than 0.5 M NaCl, because near the coast, the concentration of NaCl is diluted by the influx of river water. Therefore, having ability to grow over a wide range of NaCl concentrations might allow them to survive when they are ex-

posed to concentrations of NaCl lower than that of natural seawater.

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