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【Original Article】

Isolation and characterization of oligotrophic marine bacteria

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Although there are many bacteria surviving in seawater, the current consensus is that many bacteria have not been isolated and cultured yet. In addition, it is considered that seawater has a few nutrient source and unknown oligotrophic bacteria still exist. Analyzing oligotrophic bacteria is useful from the academic and industrial viewpoint.

In this study, marine oligotrophic bacteria were isolated and their properties were investigated. Thirty oligotrophic bacteria were isolated, and three of them were examined. These bacteria were identified as *Neptunomonas* sp., *Serratia* sp., *Pseudoalteromonas* sp. by 16s rRNA gene sequence, and growth was observed with 0.0001% succinic acid which could not be confirmed in isolated bacteria of the same genus. The shape of the three bacteria was bacilli, and almost all carbon source used in this study were available. Detailed characteristics of these bacteria are still unknown, and future studies are necessary.

1. Introduction

There are a wide variety of microorganisms in the environment. It plays a very important role in material circulation and environmental conservation such as decomposition of organic matter, nitrogen fixation, carbon fixation and so on.

Among the microorganisms in the environment, the metabolic mechanism of bacteria has many parts in common. However, bacteria with unique metabolic mechanisms exist due to mutation and environmental adaptation.

In normal seawater, nutrients exist only in extremely low concentrations. Therefore, it is thought that bacteria growing in such a low nutrient environment survive by very efficiently metabolism and uptake of nutrient source. Fluorescence microscopy[1)] and direct viable counting methods[2)] have shown that only 0.01 to 0.1% of all the microbial cells from marine environments form colonies on standard agar plates[3)]. Much of the discrepancy between direct counts and plate counts has been explained by measurements of microbial diversity that employed 16S rRNA gene sequencing without Cultivation[4, 5, 6)]. The present consensus is that many of the most abundant marine microbial groups are not yet cultivated.

An oligotrophic bacterium can grow at very low concentrations of carbon and has been defined as one that develops at the first cultivation from nature on a medium with minimal organic matter content of about 1-15mg of carbon per liter[7)]. Many researchers have focused on their growth at extremely low carbon concentrations and have actually isolated many oligotrophic bacteria from various natural environments, although biochemical and genetic studies on these organisms have been limited.

By marine bacteria isolation, identifying species and investigating what kind of nutrient sources these bacteria can utilize, it is thought that it is possible to obtain findings of new metabolic pathways, knowledge of bacteria positioning in substance circulation and biodiversity.

In addition, oligotrophic bacteria are useful in industry as well. Its can be used as a low cost catalyst in substance production and can minimize the nutrient necessary for growing in environmental purification using microorganisms.

2. Materials and methods

2.1 Seawater samples and culture condition.

Two different seawater samples were collected from surface waters at the Choshi marina (35°42'11.6"N,

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140°50'25.7"E) and Choshi marina beach (35°42'29.6"N, 140°50'14.9"E). Seawater samples were immediately used for isolation of bacteria.

Artificial seawater (ASW) used in this study was marine art SF-1 (Tomita Pharmaceutical Co., Ltd.). ASW basal medium (ASWB medium) was prepared by adding 0.01% of K_2HPO_4, 0.01% of KH_2PO_4 and 0.1% (vol/vol) mineral solution to ASW or two-fold diluted ASW (ASWB2 medium). The composition of the mineral solution was as follows: 119 mg $CoCl_2$, 9.7 g FeCl3 $6H_2O$, 118 mg $NiCl_2$ $6H_2O$, 62 mg $CrCl_2$ $6H_2O$ and 156 mg $CuSO_4$ $5H_2O$ in 1,000 ml of 0.1 M HCl. Purified agar (Nacalai Tesque) was used to prepare agar plates.

For bacteria isolation, the following two composition of medium were used: 1. ASW and 1.5% agar, 2. ASW, 1 mM NH_4Cl and 1.5% agar. Seawater samples were applied to agar plates and then incubated at 20-30˚C. A single colony formed on the plate was suspended with ASW and cultivated again, and finally samples that formed colonies having the same characteristics were regarded as isolated bacteria.

The size and morphology of the bacteria were examined with a JEOL JSM-6060LV scanning electron microscope (SEM) after culturing in ASW-dLB medium.

For measurement of colony forming units (cfu) and the examination of optimum growth temperature, ASW-dLB medium was used, which consisted of 2-fold diluted ASW, 100-fold diluted LB medium and 1.5% agar. In order to investigate the influence of the concentration of ASW on growth, 1 mM NH_4Cl and 0.1% sodium succinate were added to ASWB medium prepared using 10-fold and 100-fold diluted ASW. For the examination of carbon sources, 1 mM NH_4Cl and 0.1% carbohydrate (glucose, fructose) or organic acid (sodium succinate, marte, sodium acetate, sodium butyrate) were added to ASWB2 medium. For the examination of nitrogen sources, 0.1% sodium succinate and 1 mM NH_4Cl, $NaNO_2$ or $NaNO_3$ were added to ASWB2 medium. All cultures were done in the dark, usually incubated at 25˚C. To confirm that it is an oligotrophic bacterium, 1 mM NH_4Cl and 0.0001% sodium succinate were added to ASWB2 medium.

2.2 16S rRNA gene sequencing and phylogenetic analyses.

To identify the isolated bacteria, 16S rRNA gene fragments were amplified by colony PCR with the following set of primers: 27F (5'-AGAGTTTGATCMTGGCTCA-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR conditions were as follows: 2 min of denaturing at 94˚C followed by 35 cycles consisting of 10 sec at 98˚C, 1 min at 55˚C, and 1 min at 68˚C and finally by 10 min of extension at 68˚C. A partial sequence of the 16S rRNA gene was obtained by Sanger sequencing of a purified PCR product with the 337F (5'-GACTCCTACGGGAGGCWGCAG-3') primer.

The 16S rRNA sequences analysis was conducted using National Center for Biotechnology Information (NCBI) BLAST. Phylogenetic analysis was performed by a Clustal W algorithm using sequence analysis software, Genetyx Ver. 10 (Hitachi). A phylogenetic tree was constructed using the neighbor-joining (NJ) method. NJ analysis, a distance matrix was calculated according to Kimura' s two-parameter correction and bootstraps were done using 1,000 replications.

3. Results and Discussion

3.1 Isolation of oligotrophic marine bacteria

To isolate oligotrophic bacteria, more than 100 single colonies were initially cultured, but only 30 strains could eventually be isolated. This decrease is due to prolonged or stopped colony formation during repeated culture, it is considered that the reason is due to a decrease in trace components contained in seawater samples or components accumulated in bacteria. Thirty isolated strains may utilize a trace amount of carbon source contained in agar but at least it is thought to belong to oligotrophic bacteria.

Among 30 bacteria, 8 bacteria forming colonies within one week were identified by analysis of 16s rRNA gene sequence (Table 1). For the purpose of species differentiation, partial length 16S rRNA gene sequences (865-974 nt) of new isolates were determined. The phylogenetic tree was constructed based on 16S rRNA gene sequences of isolates and deposited in GenBank (Fig. 1). Analysis

Table 1. Closely related species based on 16S rRNA gene sequence

Strain	Species	Identities
MW2	*Pseudoalteromonas* sp.	99.79
MW3	*Neptunomonas* sp.	99.43
MW4	*Pseudoalteromonas carrageenovora*	100
MW5	*Pseudoalteromonas marina*	99.68
MW8	*Serratia* sp.	99.65
MW11	*Pseudoalteromonas* sp.	100
MW17	*Vibrio* sp.	99.69
MW19	Vibrio gigantis	99.69

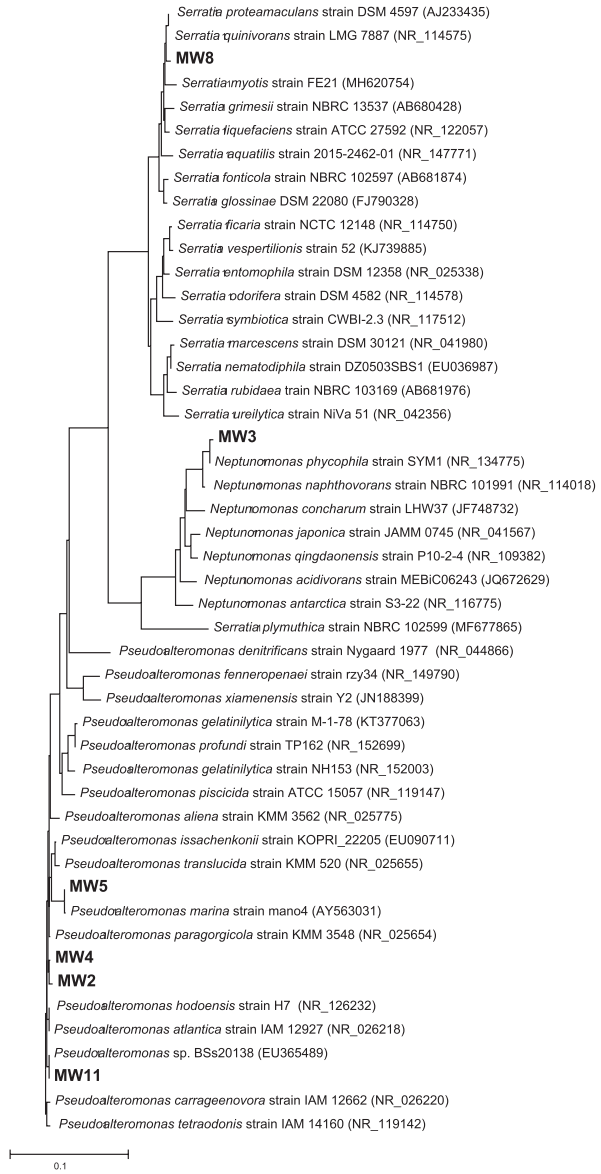

Fig. 1 Phylogenetic position of isolates of 8 strains. The unrooted tree was obtained using a NJ algorithm, Kimura's two-parameter correction for distance calculations and 1,000 replications in a bootstrap analysis. Scale bar indicates 10% nucleotide sequence divergence.

of the 16S rRNA gene sequence revealed that the novel 8 bacteria are members of the γ-subclass of the proteobacteria. BLAST and phylogenetic analyses of the 16S rRNA of strains that can be grown using low concentrations of succinic acid were employed to determine the closest related species. The phylogenetic tree based on partial 16S rRNA sequences showing the phylogenetic affiliations of the 3 isolates into 3 different bacterial genera. MW3, MW8 and MW11 were organized in the clade of the genus *Neptunomonas*, *Serratia* and *Pseudoalteromonas* respectively. However, since it was not possible to identify species any of the 3 isolates, MW3, MW8 and MW11 suggest the possibility of novel species also from their biochemical characteristics. For that reason, in order to identify species, more detailed physiological and biochemical characterization is required. In addition, it was considered necessary to classify the strain by using multigene or multilocus sequence analysis of the housekeeping genes. Housekeeping genes exhibit high sequence variation and are feasible alternatives to the 16S rRNA gene in accurately classifying and identifying bacteria[8]. It could accurately locate taxonomic positions for closely related species and strains.

3.2 Characterization of bacteria

In 8 bacteria (Table 1), an increase of growth was observed when 0.1% sodium succinate was added to the ASWB2 medium, but 3 bacteria (MW3, MW8, MW11) that gave similar results even at 0.0001% were examined in detail (Fig. 2). Compared with MW2 and MW4, these 3 bacteria showed clear growth, and from the definition it is considered to be a bacterium belonging to oligotrophic bacteria.

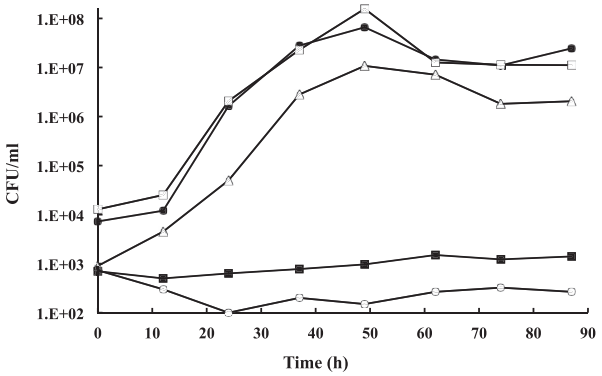

Fig. 2 Bacterial growth upon addition of 0.0001% succinic acid. All bacteria were cultured in ASWB2 medium containing 1 mM NH_4Cl and 0.0001% sodium succinate at 25°C. ■: MW2, △: MW3, ○: MW4, ●: MW8, □: MW11

As a result of electron microscopic (SEM) observation, the shape of the three strains was bacilli, the majority of the lengths were 0.5 to 1.5 μm for MW3, 1 to 2 μm for MW8, and 2 to 3 μm for MW11 (Fig. 3). These shapes are consistent with the characteristics of closely related species shown in the 16s rRNA gene sequence.

The effect of seawater concentration on bacterial

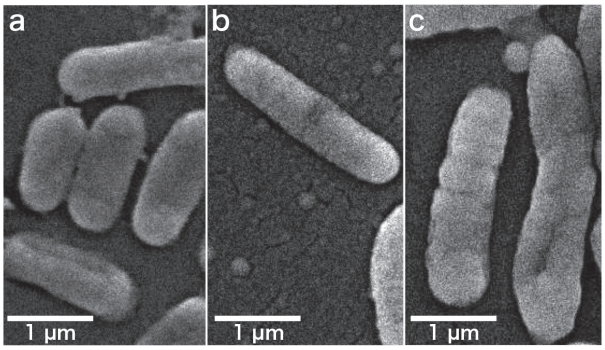

Fig. 3 SEM analysis of bacteria.
a: MW3, b: MW8, c: MW11

Table 2.
The effect of seawater concentration on growth

	Strain		
ASW con. (%)	MW3	MW8	MW11
100	+	+	+
50	+	+	+
10	+	++	±
1	-	++	-

++: increased growth, +: growth, ±: decreased growth, -: not growth

growth was as follows (Table 2). MW 3 grew at 10-fold dilution seawater but did not grow at 100-fold dilution. MW8 showed an increase in growth by 10-fold and 100-fold dilution, respectively. MW11 showed a declining growth at 10-fold dilution and did not grow at 100-fold dilution. These results indicate that bacterial growth is not affected by osmotic pressure due to salt concentration of seawater. However, low osmotic pressure in MW 8 is considered to be advantageous for growth. It is highly probable that any component contained in seawater is not essential in the growth of MW 8, but it is considered important for the growth of MW 3 and MW 11.

The growth temperature that can grow is 4 to 30˚C for MW 3, 4 to 40˚C for MW 8, and 4 to 30˚C for MW 11. All bacteria had the highest growth rate at 20 to 30˚C and formed colonies on the agar plate in about 24 hours. MW 3 and MW 8 required colony formation for less than 15˚C for about one week, but MW 11 grew in 2 days even at 4˚C (Table 3). Considering the industrial usefulness, MW 11 having a high growth rate even at low temperature is a very interesting bacterium in a different aspect from an oligotrophic bacterium.

As a result of examining whether various carbohydrate and organic acids can be used as the sole carbon source, some compounds that could not be used existed, but almost

Table 3. Growth temperature

	Culture time	Strain		
		MW3	MW8	MW11
Growth (˚C)	2 days	20～30	20～35	4～30
	1 week	4～30	4～40	4～30
Optimum (˚C)		25	25	20

Bacterial growth was confirmed by formation of colonies on agar plate.

Table 4. Bacterial growth was confirmed by formation of colonies on agar plate.

Nutrition		Strain		
		MW3	MW8	MW11
Carbon source	Glucose	+	+	+
	Fructose	+	-	+
	Succinate	+	+	+
	Malate	+	+	+
	Acetate	+	+	+
	Butyrate	+	+	-
Nitrogen source	Ammonium	+	+	+
	Nitrite	+	+	+
	Nitrate	+	+	+

+: growth, -: not growth

all compound used in this study were available.

Moreover, it was found that all nitrogen sources could be used. When glucose was used as the carbon source, MW 3 and MW 11 grew once and then immediately died (Table 4). Since such phenomena cannot be seen with other carbon sources, it is considered that the metabolic products of glucose are affecting in closed culture system like flasks. From these results, it is seems that 3 bacteria are not specific nutritional sources, but use various substances to increase the survival rate in the environment.

In this study, three marine oligotrophic bacteria were isolated and analyzed. Although it is unlikely that these bacteria are novel species, there is a possibility that the bacteria may have a unique metabolic mechanism due to their properties as oligotrophic bacteria, possibly leading to industrial applications. In the future, we will clarify what kind of metabolic mechanism exists on the gene level after examining further detailed characteristics.

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