Cultivation and Characterization of Microorganisms in Antarctic Lakes

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Abstract

Microorganisms were isolated from Antarctica lakes and identified based on 16S rDNA sequence analysis. The lake samples were collected from Antarctic lakes in Skavrvsnes near Syowa Station area. When cultivation of lake water was performed at 4 ºC and 20 ºC in several selection media, most of microorganisms could not grow at 20 ºC, but they could efficiently grow at 4 ºC, indicating that low incubation temperature was more suitable for microorganisms in Antarctica lake. 16S rDNA of each isolate was sequenced. Homology search on GenBank showed that some clones were closely related to class of *Flavobacteria*, *Bacilli*, *Actinobacteria*, *Alpha-proteobacteria*, and *Gamma-proteobacteria*. A strain which grew at 20 ºC was identical to previously characterized bacterium (*Gillisia limnaea*) with 93% of 16S rDNA similarity, which suggested that this strain might be representative of novel genera.

I. INTRODUCTION

Antarctic zone pristine biotopes are subject to long periods of ice and snow-cover, low temperatures, and low levels of photosynthetically active radiation. As several research techniques including the skill of crossing an Antarctic sea and land, sampling system, and analysis method are advancing in recent year, psychrotrophs/psychrophiles have been understood in these permanent cold environments. Especially, the development of molecular biological technique have contributed to the understanding a certain knowledge of these organisms' characteristics, and a lot of studies about biological diversity using this technique without cultivation have been accumulated in last decades, overcoming the inability to cultivate the majority of microorganisms from environmental samples [1,2]. However, this approach also has limitation because only sequence data is clarified.

Therefore, cultivating and characterizing isolates are also thought to be particularly important for providing a more comprehensive view of diversity because these analyses make the characteristics of psychrotrophs/ psychrophiles microorganisms clear, and we might get several interesting information such as the low temperature adaptability of organisms. Moreover, some psychrotrophs/ psychrophiles microorganisms' application to industry might develop commercial product working at low temperature. Unfortunately, the recovery of culturable organisms from Antarctica is very difficult, and the development of new methods to resuscitation and culturability of Antarctic microorganisms is very important. In order to understand the diversity, survival, and activity of microorganisms in Antarctic zone, we cultivated and characterized bacterial isolates from Antarctic lakes.

II. MATERIALS AND METHODS

Sampling site

Water samples were collected from Antarctic lakes in Skavrvsnes near Syowa Station area (A-6 Ike, depth 0 m, sample date 19th January 2005; A-7 Ike, depth 0 m, sample date 29th January 2005; B-1 Ike, depth 0 m, sample date 21th January 2005; B-3 Ike, depth 0 m, sample date 21th January 2005; Hunazoko Ike, depth 4 m, 22th January 2005; Tokkuri Ike, depth 4 m, sample date 22th January 2005; Suribati Ike, depth 10 m, sample date 24th January 2005 in Fig.1).

Media

LB Agar medium (g/liter): Bacto tryptone 10; Bacto-yeast extract 5; Agar 15 with different NaCl concentration (0 M, 1 M, 2 M, 3 M, 4 M) [3]. Beef extract Agar medium (g/liter): Beef extract 10; Peptone 10; Agar 15 with different NaCl concentration (0 M, 1 M, 2 M, 3 M, 4 M) [3]. R2A Agar medium (g/liter): Yeast extract 0.5; Protease Peptone no. 3 0.5; Casamino Acids 0.5; Glucose 0.5; Soluble starch 0.5; Sodium pyruvate 0.3; K_2HPO_4 0.3; $MgSO_4 \cdot 7H_2O$ 0.05; Agar 15.0 [3]. YM Agar medium (g/liter): Yeast extract 3; Malt extract 3; Peptone 5; Glucose 10; Agar 15 with different NaCl concentration (0 M, 1 M, 2 M, 3 M, 4 M) [3]. PYMV Agar medium (g/liter): Mineral salt sol. ("Hutner / Cohen-Bazire") 20 ml; Peptone 0.25; Yeast extract 0.25; Agar 15; Glucose sol.

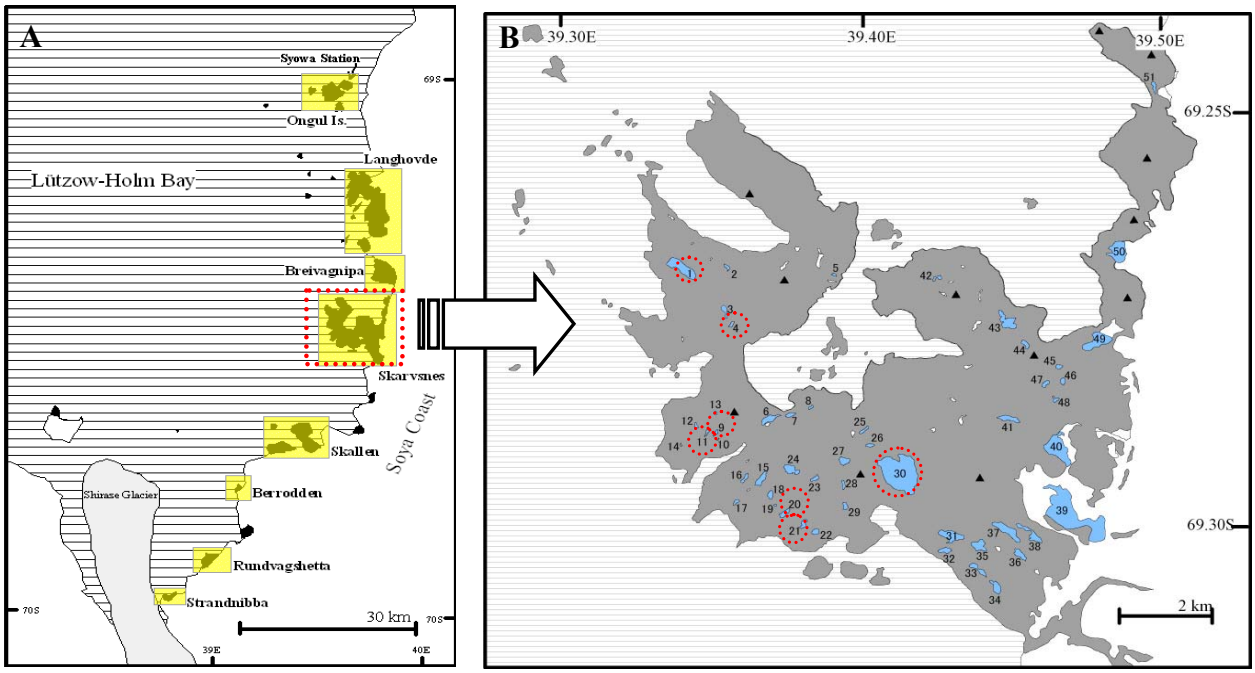

Fig.1 A. The location of Skavrvsnes; B. Lakes on Skavrvsnes. 1. Hunazoko Ike, 4. Tokkuri Ike, 9.B1 Ike, 11. B3 Ike, 20. A6 Ike, 21. A7 Ike, 30. Suribati Ike.

(2.5%, sterile-filtered) 10 ml; Vitamin sol. (double conc.) 5 ml; Adjust pH to 7.5 (the medium is only weakly buffered; one needs approx. 10 drops/liter medium of 6 N KOH) [3].

Culture condition

Antarctic samples collected in 2005 from 7 lakes were directly spread on several selection agar-media (LB Agar medium, Beef extract Agar medium, and YM Agar medium), developed for bacterial isolation, and incubated at 20 ºC for 15 days. Some samples were concentrated with 0.2 µm Nuclepore Track-Etch Membrane filtration products (Whatman Inc., Kent, UK), washed with PBS Buffer (pH 7.4), spread on several selection agar-media (R2A Agar medium, PYMV Agar medium) and incubated at 4 ºC for 2 months. The grown bacterial colonies on each media were re-streaked several times, incubated by two selection media, R2A and PYMV specific for eutrophic bacterial isolation, at 20 °C for up to 20 days, and developed as isolates [3-5].

Preparation of DNA and PCR

Colonies of each isolate were suspended in 20 µl of TE buffer (pH 8.0), boiled for 10 minutes and placed on ice for 5 minutes. The solutions were used as DNA template solutions for amplification of 16S rDNA.

Partial 16S rDNA sequences (about 500 bp) were amplified by PCR with the common primers EUB 27F 5'-AGAGT TTGATCMTGGCTCAG-3' [6] and EUB 533R: 5'-TTACC GCGGCKGCTGRCAC-3' [6]. PCR was performed in 20 µl volumes containing 1.25 u of Go Taq polymerase (Promega, Madison, USA), 1×Green Go Taq Reaction Buffer, 0.2 mM dNTPs, 0.2 µM of each primers set, and 1 µl of template DNA under the conditions: heating at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, and elongation at 72 °C for 1 minute. After cycling, extension was carried out at 72 ºC for 10 minutes [4].

Cloning

The PCR products were purified with the Wizad SV Gel and PCR Clean-up System Kit (Promega, Madison, USA) for use as sequencing templates. The 16S rDNA amplicons were sequenced, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The 16S rDNA sequences were examined for similarity using FASTA [7] and BLAST [8, 9] at the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp) and National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/bl2seq).

III. RESULT AND DISCUSSION

Cultivation of Antarctic lake samples and isolation of microorganisms

After first incubation, we could recover 22 bacterial strains (strain No. 15, 20, 22-33, 35-37, and 41-45) on the basis of the difference of colony size, shine and color. While twenty bacteria strains (strain No. 22-33, 35-37, and 41-45) were isolated from incubation at 4 °C only two bacteria strains (strain No. 15, 20) were isolated from incubation at 20 °C. Different colorful colonies (orange, white, yellow, buff, and sandy beige) were appeared on incubation agar-media, and some strains (strain No. 15, 41) had a slimy and gummy appearance. As one of these reasons that there were only two strains isolated from incubation at 20 °C

TABLE I

HOMOLOGY ANALYSIS OF ISOLATES FROM ANTARCTICA A-6 IKE, A-7 IKE, B-1 IKE, B-3 IKE ,SURIBAT I IKE, TOKKURI IKE, AND HUNAZOKO IKE

Strain No.	Lake	16S rDNA				Incubation temperature (°C)	Colony description
		Close representative Organisms/ group	Sequence accession number	Similarity (%)	E-Value		
15	Hunazoko Ike	*Gillisia limnaea* (R-8282)	AJ440991	93	0.0	20	Orange, slimy
20	B-1 Ike	*Gracilibacillus halotolerans* (NN)	AF036922	99	0.0	20	White
22	A-6 Ike	*Psychrobacter fozii* (S2-83)	AY771717	99	0.0	4	White
23	Tokkuri Ike	*Psychrobacter maritimus* (Pi2-20T)	AJ609272	99	0.0	4	White
24	B-3 Ike	*Psychrobacter alimentarius* (S3-15)	AY771725	99	0.0	4	White
25	Tokkuri Ike	*Flavobacterium frigidarium* (S3-9)	AY771722	99	0.0	4	Yellow
26	Tokkuri Ike	*Flavobacterium frigidarium* (S3-9)	AY771722	99	0.0	4	Yellow
27	A-7 Ike	*Flavobacterium aquatile* (DSM1132)	AM230485	97	0.0	4	Orange
28	A-7 Ike	*Flavobacterium aquatile* (DSM1132)	AM230485	97	0.0	4	Orange
29	B-3 Ike	*Psychrobacter cryohalolentis* (K5)	CP000323	99	0.0	4	White
30	B-3 Ike	*Psychrobacter alimentarius* (S3-15)	AY771725	99	0.0	4	White
31	B-1 Ike	*Methylobacterium adhaesivum* (AR27)	AM040156	99	0.0	4	Dark red
32	A-7 Ike	*Flavobacterium aquatile* (DSM1132)	AM230485	97	0.0	4	Orange
33	A-6 Ike	*Cryobacteriu psychrophilum* (DSM4854)	AJ544063	99	0.0	4	Orange
35	Tokkuri Ike	*Psychrobacter maritimus* (Pi2-20)	AJ609272	99	0.0	4	White
36	Tokkuri Ike	*Flavobacterium frigidarium* (S3-9)	AY771722	99	0.0	4	Buff
37	Tokkuri Ike	*Psychrobacter maritimus* (Pi2-20)	AJ609272	99	0.0	4	White
41	Suribati Ike	*Psychroflexus torques* (BSi20642)	DQ007442	97	0.0	4	Orange, slimy
42	A-7 Ike	*Brevundimonas variabilis* (ATCC15255)	AJ227783	99	0.0	4	Nigger-brown
43	A-7 Ike	*Brevundimonas variabilis* (ATCC15255)	AJ227783	99	0.0	4	Sandy beige
44	A-7 Ike	*Flavobacterium aquatile* (DSM1132)	AM230485	97	0.0	4	Brown
45	A-7 Ike	*Blastomonas natatoria* (2.3.)	AJ250435	98	0.0	4	Puce

by spreading the Antarctica lake samples directly onto agar-media plates but twenty strains were isolated at 4 °C by filtration Antarctica lake samples, it was thought that microorganisms in Antarctica lakes had been subjected to low temperature and limiting nutrient for long time, high incubation temperature and rich nutrient medium might be stressor for Antarctic microorganisms. Therefore, incubation at low temperature with media not containing rich nutrition were more suitable for Antarctica lake microorganisms because the isolated microorganisms were most likely to be adapted to the often oligotrophic conditions of many cold habitats. Although the developing method of dealing with Antarctica lake sample was difficult, the procedure refracting the Antarctic environment led to the good result. The concentration of bacterial density with filtration increased the number of recovered isolates, which indicated that it was also important factor of concentration of cell number because the bacterial density in Antarctica lakes might be very low. In order to isolate microorganisms strictly and know its temperature adaptability of bacteria incubated at 4 °C, strains were re-streaked. As a result, strain No. 23, 25, and 26 formed each colony on PYMV agar plates after only 2 days incubation at 20 °C; however, most of other strains formed each colony on PYMV or R2A agar plates with slower growth requiring incubation for 1-3 weeks at 20 °C. According to the definition and category of psychrophiles by Morita [10], psychrophiles has been further subdivided into psychrophiles *sensu stricto*, which have optimal growth temperature below 15 °C and an upper limit of 20 °C, and psychorotrphs (psychrotolerants) which are able to divide at 0 °C or below and grow optimally at temperatures around 20-25 °C. It has the possibility that strain No. 23, 25, and 26 belonged to psychorotrphs (psychrotolerants), whereas most of other strains belonged to psychrophiles *sensu stricto*.

Characterization based on 16S rDNA sequence

As a result (see Table I) of sequencing and homology analysis, four clones (strain No. 31, 42, 43, and 45) showed the high similarity to *Alpha-proteobacteria*, seven clones (strain No. 22, 23, 24, 29, 30, 35, and 37) were similar to *Gamma-proteobacteria*, one clone (strain No. 33) was similar to *Actinobacteria* and one clone (strain No. 20) was similar to *Bacilli*. Nine clones (strain No. 15, 25, 26, 27, 28, 32, and 41) were similar to *Fibrobacter*. Strain No. 25 had 99%, 100% of 16S rDNA sequence similarity with strain No. 26 and strain No. 36, separately. Also, strain No. 27 had 100% of 16S rDNA sequence similarity with strain No. 28 and strain No. 44. Strain No. 42 had

100% of 16S rDNA sequence similarity with strain No. 43. These results showed that some strains might be similar or same species. The analysis of the community by obtaining and comparing isolates not only contributes to the general knowledge of Antarctic microorganisms but also a large collection of organisms that can be further characterized and useful to several fields. Naganuma *et al*. have isolated some euryhaline halophilic strains from Suribati Ike [3]. Some strains have 100% 16S rDNA (about 1465 bp) sequence similarity with *Marinobacter sp.* in GenBank Database; however the physiological characteristics were different such as growth in different temperature, anaerobic or aerobic, and survival in different NaCl concentration. Comparison of the genomic and physiological characteristics among our isolates also might increase much understanding of the microbial diversity of this environment.

16S rDNA sequencing results suggested that three of the isolates may represent new species. Strain No. 15, 41, and 27 (28, 32, and 44) has the highest 93%, 97%, and 97% 16S rDNA sequence similarity with the GenBank database respectively. Strain No. 15, 41, and 27 (28, 32, and 44) are likely novel species in the genus *Gillisia*, *Psychroflexus*, and *Flavobacterium* which belong to the *Flavobacteria* group. The potential novel strains isolated constitute a unique collection for further taxonomic analysis, physiological characterization and screening. More studies of these microorganisms in Antarctic lakes may increase the molecular and biochemical several advantages such as antifreezing proteins.

From this survey, we overcame the difficulty of recovery culturable organisms from Antarctica with comparison several incubation conditions of the microorganism such as temperature, nutrient concentration, and the natural growth habitat, and could get the representative microbial microorganisms from low temperature environments, proposing the possibility that several useful microorganisms might be isolated from low temperature zone in the future using techniques like this study.

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