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Original Article

Bacterial Diversity of Ny-Ålesund, Arctic Archipelago Svalbard

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Abstract

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The bacterial diversity of the water sample, collected from Ny-Ålesund, Arctic Archipelago Svalbard was analyzed by a phenotypic as well as a genotypic approach. Pure colonies of the culturable bacteria were established and grown at a range of temperatures: 4ºC, 15ºC, 22ºC and 37ºC. Optimum growth was found at 15ºC, and around 28 colonies were obtained. The library was dominated by 16S rDNAs of Gram-negative bacteria (γ-Proteobacteria). Twenty two isolates exhibited sequences were similar to that of known bacterial isolates (>97% sequence similarity), represented by the species of the genera *Psychrobacter*, *Pseudomonas*, and *Acinetobacter*. Six isolates exhibited sequences showed less affiliation with known taxa (<97% sequence similarity), and may represent novel taxa.

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Introduction

An enormous amount of effort is being made worldwide by microbial ecologists to identify microorganisms from environmental samples. In recent years, growing attention in research has been devoted to cold-adapted microorganisms. They successfully colonize cold habitats, which compose more than 80% of the earth's biosphere, and play a major role in the processes of nutrient turnover at low temperatures (Kottmeier & Sullivan, 1990; Rivkin *et al*., 1989). Polar regions are of interest since they provide diverse terrestrial and marine habitats for psychrophilic microorganisms. Several authors have isolated a number of psychrophiles from Arctic sea that showed considerable phylogenetic diversity. Physiological types include proteolytic, cellulolytic, amylotic, lipolytic, acetogenic and sulfate-reducing bacteria (Tatiana *et al*., 2004)

Among sea-ice prokaryotes, members of eight phylogenetic groups, subclasses α, β and γ of Proteobacteria, the Cytophaga–Flavibacterium–Bacterioides (CFB) phylum group, the high- and low-G+C Gram positives, and the orders Verrucomicrobiales and Chlamydiales have been detected by using the 16S rDNA approach (Brown & Bowman, 2001; Petri & Imhoff, 2001).

Plankton communities of polar oceans appeared to be more diverse than sea-ice bacterial communities. Archaea, δ and ε Proteobacteria, and green non-sulfur bacteria were detected in seawater in addition to the phylogenetic groups known from sea ice (DeLong *et al*., 1994; Massana *et al*., 1998).

In the present study, diversity of culturable bacteria associated with Arctic seawater was investigated. Isolation and molecular phylogenetic analysis of Arctic strains were performed in order to expand our knowledge on culturable fraction of seawater microbial communities.

Materials and Methods

Samples. For the analysis of the bacteria from Ny-Ålesund, Arctic sea, samples were filtered and stored at -70°C at the Center for Cellular and Molecular Biology, Hyderabad, India. The deep sea sediment at the depth of 83 m was collected by the multi-corer from site Ny-Ålesund (78°53.539'N, 12°28.253'E) locating at Svalbard Archipelago, Arctic between 9 and 18 August 2007. The mean air temperature at the time of collection was 4.5°C.

Isolation and characterization of bacterial strains. Filters were placed on Zobell marine agar plates and incubated at 15°C for 10 days. Single colonies, which appeared after 10 days were picked up and restreaked 3 to 4 times, so as to obtain pure colonies. Morphological and growth characteristics were determined as described by Reddy *et al*. (2000). The colony diameter, color, form, elevation and the nature of the margin were noted. Further, the opacity of the colony, i.e. whether it is opaque, transparent or translucent was also noted. Psychrophily test was conducted to classify the low temperature-adapted bacteria based on the ability to grow at different temperatures (Radjasa *et al.*, 2001). Biochemical characteristics were checked with a Hi25 Enterobacteriaceae identification kit (KB003; HiMedia) and HiCarbohydrate kit parts A, B and C (KB009; HiMedia).

16S rDNA amplification. The small subunit rDNA gene was amplified using two primers namely PA (5'-AGA GTT TGA TCC TGG CTC AG-3') and PH (5'-TAA CAC ATG CAA GTC GAA CG-3'), complementary to the conserved regions at the 5' and 3' ends of the 16S rDNA of *Escherichia coli* corresponding to positions 9 to 27 and 1498 to 1477, respectively (Lane, 1991). The PCR amplification reaction mix of 50 ml, contained bacterial DNA (≈200 ng), 25 ml of 2x Red dye master mix and the 16SrDNA primers. Amplification was carried out in a Peltier thermocycler (Model No. PTC-200, MJ research, USA) programmed for 30 cycles. In each cycle denaturation was done at 94°C for 30 s, annealing was done at 48°C for 1 minute, and extension was done at 72°C for 2 minutes. A final extension of 5 min was carried out at 72°C at the end of 30 cycles. The amplified DNA fragment of approximately 1.5 kb was separated on a 1% agarose gel and purified by using Qiagen spin columns. The purified fragment was used directly for DNA sequencing.

The desired DNA band from agarose gel was excised, weighed and transferred to a sterile microfuge tube containing thrice the volume of Buffer QE (300 ml / 100 mg), and transferred to a water bath maintained at 55°C for 10 min. The contents were then transferred to a Qiagen column and spun at 10000 rpm for 2 min (Catalogue No. 28704, Qiagen Inc. USA). Subsequently the column was washed twice with 750 ml of Buffer PE and eluted with 30 ml of sterile water.

Sequencing of 16S rDNA. Sequencing of the purified PCR product (~200 ng/reaction) was carried out using sequencing primer, and 3 µl of ready reaction mix from the Big Dye Terminator sequencing kit (Perkin Elmer) in a total volume of 5 µl. Cycle sequencing was carried out in a Gene Amp PCR machine (Perkin Elmer, 9600). Sequencing reactions were analyzed on the Applied Biosystems 3700 DNA sequencer.

Results and Discussion

Properties of the isolated strains. The representative strains of the phylotypes showed colony morphology similar to that reported for the nearest phylogenetic neighbor (Table 1). Based on colony morphology, all isolates could be categorized into several groups. Colonies of T1, T11, T12, T13, T61, T82, T84, T101 and T102 were circular, smooth, convex and white in color with a diameter of 2–3 mm, whereas colonies of T14, T2, T62, T64, T103 and T104 were irregular, dry, and had a characteristic wrinkled appearance and yellow in color. The colony of almost all bacterial isolates were about 1-3 mm in size.

Low temperature adapted bacteria have been classified based on the occurring of growth at 4°C and 20°C, i.e. psychrophiles were those able to grow at 4°C, but unable to grow at 20°C, whereas psychrotrophs were those able to grow

Table 1. Colony morphology of isolates

Strain number	Colony morphology
T1, T11, T12, T13, T61, T82, T84, T101, T102	2-3 mm in diameter, white color, smooth and circular
T14, T2, T62, T64, T103, T104	1-2 mm in diameter, yellow in color, wrinkled appearance, irregular and dry
T3	3-4 mm in diameter, white, smooth and circular
T41, T43, T44, T45	4-5 mm in diameter, white, smooth and circular
T5, T63, T7, T91, T92	2-3 mm in diameter, brown, wrinkled appearance, irregular and dry
T85	1-3 mm in diameter, white, smooth and circular
T105	1-2 mm in diameter, pale-white, smooth and circular
T106	1-3 mm in diameter, pale-white, smooth and circular

both at 4°C and 20°C incubations (Urakawa *et al.*, 1999). Psychrophily test indicated that all isolates were able to grow at 4°C to 22°C, therefore they were regarded as psychrotrophic bacteria (Table 2).

Sequence analysis of the isolated strains. The 28 strains were selected for their 16S rRNA gene sequence analysis based on their colony morphology. The resultant sequence data were compared to nucleotide databases using basic local alignment search tools (BLAST). Sequence similarity of representative isolates compared to the nearest phylogenetic neighbor ranged from 96 to 99%. The primary structure of the 16S rRNA is highly conserved, and species having DNA similarity of more than 97% sequence identity. It is proposed that organisms that have less than 97% sequence homology at 16S rRNA level will not reassociate to more than 60% (Stackerbrandt & Goebel, 1994), and they are represented as new species.

In the present study, about 80% of the isolates shared 98–99% sequence similarity with the closest valid published species. The nine isolates (T1, T11, T12, T13, T61, T82, T84, T101 and T102) exhibited the greatest similarity to the species *Psychrobacter nivimaris* (99% sequence similarity). Six isolates (T2, T14, T62, T64, T103 and T104) are belong to the similar taxa to *Pseudomonas stutzeri* (99% sequence similarity). Furthermore, isolates T41, T43, T44 and T45 showed 99% identity to isolate called *Psychrobacter vallis partial*. The isolate T3 was affiliated with *Psychrobacter fozii*, and the other two strains (T85 and T105) belonged to *Psychrobacter aquimaris* and *Acinetobacter sp*., respectively.

Based on phylogenetic comparisons, 20% of sequenced isolates potentially represent novel species or genera, sharing less than 97% sequence similarity to the closest validly described species. Five strains (T5, T63, T7, T91 and T92) are likely to be novel species of *Halomonas*, and T106 likely to be novel species of *Sporosarcina* as they differ from the nearest phylogenetic neighbour at the 16S rRNA gene sequence (Table 3).

As indicated in Table 4, the members of

Table 2. Growth of the bacterial isolates at different temperatures

Strain number	4°C	15°C	22°C	37°C
T1, T11, T12, T13, T61, T82, T84, T101, T102	+	+	+	-
T14, T2, T62, T64, T103, T104, T105	+	+	+	+
T3	+	+	+	-
T41, T43, T44, T45	+	+	+	-
T5, T63, T7, T91, T92	+	+	+	+
T85	+	+	+	-
T106	+	+	+	+

+ growth observed; – no growth observed

genus *Psychrobacter* are the most dominant group followed by *Pseudomonas* and *Halomonas* groups. Out of the 28 strains, 15 were affiliated to the genus *Psychrobacter* representing as *P. nivimaris, P. fozii, P. vallis* and *P. aquimaris*.

Biochemical characteristics were determined for novel species, T7 and T106. These two isolates have been studied with respect to their abilities to utilize different carbon compounds, to ferment sugars, to reduce nitrate, production of H_2S, indol, and activities of the following enzymes, viz., urease, b-galactosidase, lysine decarboxylase, ornithine decarboxylase (Table 5).

The two strains were positive for citrate utilization, lysine decarboxylase and ornithine decarboxylase. But, tests for phenylalanine deaminase, b-galactosidase, methyl red and Voges–Proskauer reactions were negative. Indole and H_2S were not produced. Nitrate was not

Table 4. Generic composition of psychrotrophic isolates

Genera	Number of isolates
Psychrobacter	15
Pseudomonas	6
Halomonas	5
Acinetobacter	1
Sporosarcina	1

Table 3. Phylogenetic relationships of Arctic seawater isolates

Strain number	Sequence length	Nearest phylogenetic relative	16S rRNA gene sequences similarity (%)
T1	1500	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T11	1505	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T12	1500	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T13	1533	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T14	1475	*Pseudomonas stutzeri* 13635O (EU741087.1)	99
T2	1379	*Pseudomonas stutzeri* 13635O (EU741087.1)	99
T3	1362	*Psychrobacter fozii* NF23 (NR_025531.1)	99
T41	1539	*Psychrobacter vallis partial* CMS 39 (AJ584832.1)	99
T43	1463	*Psychrobacter vallis partial* CMS 39 (AJ584832.1)	99
T44	1381	*Psychrobacter vallis partial* CMS 39 (AJ584832.1)	99
T45	1478	*Psychrobacter vallis partial* CMS 39 (AJ584832.1)	99
T5	1533	*Halomonas alkantarctica* CRSS (AJ564880.1)	97
T61	1520	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T62	1500	*Pseudomonas stutzeri* 13635O (EU741087.1)	99
T63	1532	*Halomonas alkantarctica* CRSS (AJ564880.1)	97
T64	1500	*Pseudomonas stutzeri* 13635O (EU741087.1)	99
T7	1500	*Halomonas alkantarctica* CRSS (AJ564880.1)	97
T82	1500	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T84	1505	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T85	1290	*Psychrobacter aquimaris* KOPRI24929 (EF101547.1)	98
T91	1491	*Halomonas alkantarctica* CRSS (AJ564880.1)	97
T92	1531	*Halomonas alkantarctica* CRSS (AJ564880.1)	97
T101	1500	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T102	1540	*Psychrobacter nivimaris* 88/2-7 (NR_028948.1)	99
T103	1502	*Pseudomonas stutzeri* 13635O (EU741087.1)	99
T104	1500	*Pseudomonas stutzeri* 13635O (EU741087.1)	99
T105	1556	*Acinetobacter* sp. DSM590 (X81659.1)	99
T106	1525	*Sporosarcina soli* I80 DQ073394.1	95

reduced to nitrite. Malonate was not utilized. Esculin was not hydrolysed. Urease was negative for the T7, but positive for T106.

The strain T7 produced acid from D-arabinose, glucose, lactose, D-ribose, cellobiose, xylose, D-galactose, L-arabinose, D-mannose and D-melibiose. The strain T106 produced acid only from D-ribose. Both strains did not produce acid from remaining carbon sources, including D-trehalose, D-fructose, glycerol, L-sorbose, adonitol, rhamnose, sorbitol, α-methyl-D-glucoside, α-methyl-D-mannoside, melezitose, maltose, D-raffinose, salicin, dulcitol, inulin, mannitol, sucrose and inositol.

Both T7 and T106 utilized glucosamine, but not dextrose, saccharose, sodium gluconate and xylitol, and only T7 utilized D-lactose.

Our attention was focused on the occurrence of low temperature-adapted bacteria followed by PCR-based approach for estimating the richness of psychrotrophic bacteria. Understanding the indigenous low temperature-adapted bacteria has important implications for analyses of microbial function and biogeochemical processes in the extreme cold environments as well as their biotechnological potentials.

The overall phylogenetic distribution of the strains isolated in this study shows similarity to the results obtained by analysis of the Arctic sea-ice/seawater clone libraries (Bano & Hollibaugh, 2002). The diversity of Arctic strains is not evenly distributed among the major groups. Over 90% of the isolates are affiliated with the γ subclass of Proteobacteria. Within the γ

Table 5. Characteristics of differentiation between T7 and T106

Characteristics	T7	T106
Growth temperature range (°C)	4 - 22	4 - 37
Optimum growth temperature (°C)	15	15
Biochemical characteristics		
Urease	-	+
Phenylalanine deamination	-	-
H2S production	-	-
b-galactosidase	-	-
Voges Proskauer's	-	-
Methyl red	-	-
Citrate utilization	+	+
Indole	-	-
Nitrate reduction	-	-
Malonate utilization	-	-
Esculin hydrolysis	-	-
Ornithine decarboxylase	+	+
L-Lysine decarboxylase	+	+
Production of acid		
D-arabinose	+	-
L-arabinose	+	-
Glucose	+	-
Lactose	+	-
D-mannose	+	-
D-Ribose	+	+
D-Trehalose	-	-
D-fructose	-	-
D-galactose	+	-

Characteristics	T7	T106
glycerol	-	-
Xylose	+	-
L-sorbose	-	-
Adonitol	-	-
Rhamnose	-	-
Sorbitol	-	-
α-methyl-D-glucoside	-	-
α-methyl-D-Mannoside	-	-
Melezitose	-	-
Maltose	-	-
D-melibiose	+	-
D- raffinose	-	-
Cellobiose	+	-
Salicin	-	-
Dulcitol	-	-
Inulin	-	-
Mannitol	-	-
Sucrose	-	-
Inositol	-	-
Carbon source utilization		
Dextrose	-	-
D-lactose	+	-
Saccharose	-	-
Glucosamine	+	+
Sodium gluconate	-	-
Xylitol	-	-

+ positive test; – negative test

subclass of Proteobacteria, Arctic isolates fell into the genera *Psychrobacter*, *Pseudomonas*, *Halomonas* and *Acinetobacter*. Over 10% of seawater isolates were found to belong to the Gram-positive branch. A direct comparison of Gram positives from Arctic sea-ice/seawater libraries, and this study was not possible due to the lack of sequence information. However, Gram positives were successfully isolated previously from Antarctic sea-ice environments.

All strains demonstrated good growth in a wide temperature range of 4–20^{0}C. These results suggest that not only psychrophilic microorganisms, but also psychrotrophilic bacteria may play an important role in the matter cycles in Arctic.

The data obtained in this study on the biodiversity of culturable bacteria from Arctic seawater expand our knowledge on the extent of bacterial diversity in low-temperature environments.

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