S2-5

Phylogenetic Analysis of Culturable Arctic Bacteria

Yoo Kyung Lee[1]*, Hyo Won Kim[1], Sung-Ho Kang[2], Hong Kum Lee[1]
[1] NRL for Marine Microbial Diversity, Korea Ocean Research & Development Institute (KORDI)
[2] Polar Sciences Laboratory, Korea Ocean Research & Development Institute (KORDI)
Ansan PO Box 29, Seoul 425-600, Korea

Abstract

We isolated and identified culturable Arctic bacteria that have inhabited around Korean Arctic Research Station Dasan located at Ny-Alsund, Svalbard, Norway (79°N, 12°E). The pure colonies were inoculated into nutrient liquid media, genomic DNA was extracted, and phylogenetic analysis was performed on the basis of 16S rDNA sequences. Out of total 227 strains, 198 strains were overlapped or unidentified, and 43 bacteria were finally identified: 31 strains belonged to *Pseudomonas*, 7 strains *Arthrobacter*, two *Flavobacterium* sp., an *Achromobacter* sp., a *Pedobacter* sp., and a *Psychrobacter* sp. For isolation of diverse bacteria, we need more effective transport method than 3M petri-films, which were used for convenience of transportation that was restricted by volume. We also need to use other culture media than nutrient media. We expect these Arctic bacteria can be used for screening to develop new antibiotics or industrial enzymes that are active at low temperature.

Introduction

The range of temperatures in which growth of organisms has been detected reaches from −12°C to approximately 112°C. Within this frame, the velocity of chemical reactions and the physical properties of biomolecules change dramatically, such that microorganisms are only able to grow within a limited thermal range. At low temperature, the rate of enzymatic reactions, the fluidity of cellular membrane, and the affinity of uptake and transport systems decrease (Phadtare et al. 2000). Therefore, biomolecules of microorganisms living in cold habitat may show distinctive physical properties.

The Arctic is a representative cold habitat, which remains one of the least explored, studied and understood places on earth. The potential benefits from the exploration of the microbial diversity of the Arctic derive from the future biotechnological exploitation of the Arctic gene pool and from new insights into the biological mechanisms of adaptation to and tolerance of extreme environments by microorganisms.

In this study, we isolated and identified culturable Arctic bacteria that have inhabited around Korean Arctic Research Station Dasan located at Ny-Alsund, Svalbard, Norway.

Materials and Methods

Sample collection

The sampling site is in the near-by area of Korean Arctic Research Station Dasan located at Norwegian Polar Institute's Research Station in Ny-Alesund (78°55′N, 11°56′E), Svalbard, Norway. Soil samples were collected from the upper melted layer of soil with 0.1 m depth using sterile 50 ml conical tubes on 5 ~ 15 August 2002. The samples collected from 6 different sites were sealed and transferred to Dasan station. Aliquote of 0.2 g of the collected soils were diluted in distilled water, the diluted water was spread on the 3M petri-films *E. coli* Count Plate, which were kept at 4°C for 1 – 7 days until transportation and transferred to the laboratory at KORDI under the cold condition. The remained soil samples were frozen at -20°C in conical tubes, transferred to laboratory at KORDI in package with dry ice and icepack, and stored at -20°C.

Culture conditions

For isolation in the laboratory, the petri-films were cultured at 4°C for 1 month; colonies formed on petri-films were succeedingly cultured on nutrient agar plate (Difco 72063JD) at 4°C for every 7 days. Distinct colony types on the plates were purified by streaking and restreaking on fresh nutrient agar plates. The purified isolates were cultured in nutrient broth media at 20°C for 1 day frozen, and stored at -80°C in fresh medium that contained 15% (v/v) sterile glycerol.

DNA extraction and PCR amplification

Total genomic DNA for 16S rDNA amplification was extracted using AccuPrep genomic DNA Extraction kit (Bioneer, Korea) from 1 mL of isolates cultured in nutrient broth. From the genomic DNA nearly full-length 16S rDNA sequences were amplified by PCR using primers 27F(5'-AGA GTT TGA TCM TGG CTC AG-3') and 1522R(5'-AAG GAG GTT ATC CAN CCR CA-3'). The PCR mixture consisted of 5 μl of 10× PCR mix (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl of each primer, 1 μl of template DNA, and 2.5 units of *Taq* polymerase (TaKaRa, Japan) in a final volume of 50 μl. The PCR was performed in a thermal cycler (Biometra, Germany) using cycling conditions that consisted of an initial denaturation at 95°C for 5 min and then 30 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. A final extension was performed at 72°C for 7 minutes. The PCR products were analyzed by agarose gel electrophoresis, purified with Highpure PCR product Purification Kit (Roche, Germany), and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The nucleotide sequence data were deposited in GenBank of the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov).

Sequence Analysis

Sequences of the 16S rDNA were submitted to Advanced BLAST search program of the NCBI to identify sequences of closely related organisms. The related sequences were preliminarily aligned with the default settings of CLUSTAL W (Thompson et al. 1994), and complete sequence

alignments were performed using PHYDIT (Chun 1995) and manual comparison to secondary structures. The phylogenetic analysis was performed with PHYLIP (Felsenstein, 1993), and phylogenetic trees were inferred using the neighbor-joining method (Saitou & Nei 1987).

Results & Discussion

We isolated total 227 strains, 198 strains were overlapped or unidentified, and 43 bacteria were finally identified: 31 strains belonged to genus *Pseudomonas*, 7 strains genus *Arthrobacter*, two *Flavobacterium* sp., an *Achromobacter* sp., a *Pedobacter* sp., and a *Psychrobacter* sp (Table 1). *Pseudomonas* and *Arthrobacter* were the dominant bacterial groups isolated in most of the tundra soil (Zhou et al. 1997).

1. *Pseudomonas*

The majority of clones clustered with *Pseudomonas*. Most of clones showed the highest similarity with *Pseudomonas,* but aligned outside of the *Pseudomonas* branch, and could not be identified at the level of species. The genus *Pseudomonas* belongs to γ-Proteobacteria is ubiquitous and diverse bacteria in nature (Spiers et al. 2000). They possess variable metabolic abilities that utilize a wide range of organic compounds with significant ecological position in the carbon cycle, and they are also important as pathogens of animals and plants (Yamamoto et al 2000). Even though full genome have been revealed from several *Pseudomonas* (Stover 2000; Nelson 2002), the classification of *Pseudomonas* strains is not fully established due to the lack of an accurate taxonomic system. Sequence analysis of 16S rDNA is frequently used (Moore *et al.*, 1996). However, the degree of resolution obtained with 16S rRNA sequence analysis is not sufficiently discriminatory to permit resolution of intrageneric relationships because of the extremely slow rate of evolution of 16S rRNA. Due to the gap between the valid genetic ranges of the two methods, a detailed intrageneric structure of the genus *Pseudomonas* remains to be resolved.

2. *Arthrobacter*

Seven clones clustered with *Arthrobacter* that belongs to high G+C Gram-positive bacteria. Three clones were closely aligned with *A. polychromogenes* , a clone with *A. psychrolactophilus,* and three clones with *A. sulfureus.* Despite the high 16S rDNA sequence similarity (> 97%), new species had been assigned on the basis of DNA-DNA relatedness or phenotypic difference (Wauters et al. 2000; Reddy et al. 2002). Three species were reported from polar habitat such as Antarctica and Greenland (Osorio et al. 1999; Reddy et al. 2000, 2002). Therefore, the Artic clones isolated in this study need further study to clarify their taxonomic status.

Table 1. Culturable Artic bacteria identified from 16S rDNA sequences.

Clone No.	Site	The closest species	Similarity
5-4	Moss of puddle	*Achromobacter ruhlandii*	98
32-3	Verticar sediment of red river	*Arthrobacter polychromogenes*	99
41-1	North coastal sediment	*Arthrobacter polychromogenes*	99
7-1	Soil of coast	*Arthrobacter polychromogenes*	99
7-10	Soil of coast	*Arthrobacter psychrolactophilus*	99
19-1	Soil of tundra	*Arthrobacter sulfureus*	98
23-5	Soil of mountain	*Arthrobacter sulfureus*	98
7-7	Soil of coast	*Arthrobacter sulfureus*	98
4-4	Moss of puddle	*Flavobacterium hydatis*	99
4-6	Moss of puddle	*Flavobacterium hydatis*	99
7-6	Soil of coast	*Pedobacter cryoconitis*	99
26-8	Soil under polar icecaps	*Pseudomonas anderso*	95
21-9	Moss of tundra	*Pseudomonas borealis*	99
23-14	Soil of mountain	*Pseudomonas borealis*	99
4-5	Moss of puddle	*Pseudomonas borealis*	99
5-5	Moss of puddle	*Pseudomonas borealis*	99
15-5	Moss of puddle	*Pseudomonas chloror*	95
23-7	Soil of mountain	*Pseudomonas chloror*	97
16-4	Soil of coast	*Pseudomonas corrugata*	95
19-5	Soil of tundra	*Pseudomonas corrugata*	95
4-2	Moss of puddle	*Pseudomonas corrugata*	96
7-13	Soil of coast	*Pseudomonas corrugata*	99
4-13	Moss of puddle	*Pseudomonas frederiksbergensis*	100
25-19	Sediment of puddle	*Pseudomonas lini*	99
17-5	Soil of coast	*Pseudomonas mandeli*	98
19-2	Soil of tundra	*Pseudomonas mandeli*	97
21-7	Moss of tundra	*Pseudomonas mandeli*	98
25-11	Sediment of puddle	*Pseudomonas mandeli*	99
26-9	Soil under polar icecaps	*Pseudomonas mandeli*	99
4-1	Moss of puddle	*Pseudomonas mandeli*	99
6-20	Soil of coast	*Pseudomonas mandeli*	99
7-5	Soil of coast	*Pseudomonas mandeli*	99
6-21	Soil of coast	*Pseudomonas marginalis*	99
23-9	Soil of mountain	*Pseudomonas meridiana*	98
16-2	Soil of coast	*Pseudomonas migulae*	99
21-19	Moss of tundra	*Pseudomonas migulae*	99
23-2	Soil of mountain	*Pseudomonas migulae*	99
25-2	Sediment of puddle	*Pseudomonas migulae*	98
6-11	Soil of coast	*Pseudomonas migulae*	99
6-17	Soil of coast	*Pseudomonas syringae*	99
15-3	Moss of puddle	*Pseudomonas taetrol*	97
19-12	Soil of tundra	*Pseudomonas tolaasi*	96
12-5	Attached on a marine alga	*Psychrobacter glacincola*	98

3. Other strains

Two clones were closely related and they were aligned with *Flavobacterium hydatis* that belongs to Bacteroidetes (Cytophaga-Flexibacter-Bacteroides group). Several *Flavobacterium* species were isolated from Antarctica (McCammon et al. 1998; McCammon and Bowman 2000; Humphry et al.

2001). A clone was closely aligned with *Achromobacter ruhlandii* that belongs to β-Proteobacteria. A clone was closely aligned with *Pedobacter cryoconitis* that belongs to Bacteroidetes (Cytophaga-Flexibacter-Bacteroides group). Genus *Pedobacter* was recently isolated from *Sphingobacterium* (Steyn et al. 1998). A clone was closely aligned with *Psychrobacter glacincola* that belongs to γ-Proteobacteria. It was collected from a marine alga. Several species originated from marine habitats (Maruyama et al. 2000; Denner et al. 2001; Romanenko et al. 2002).

Polar habitat is a good source of new bacterial species and genera (Irgens et. al. 1996; Bowman et al. 1997a, 1997b, 1997c, 1997d, 1998a, 1998b, 1998c; Gosink et al. 1998; Junge et al. 2002). In polar habitat, cold-adapted bacteria should be the dominant organisms. For isolation of more diverse bacteria, we need more effective transport method than 3M petri-films, which were used for convenience of transportation that was restricted by volume. We also need to use other culture media than nutrient media. Polar bacteria can be good source for carotenioids that may provide protection against UV radiation (Jagannadham 2000), and cold-active protease that have biotechnological potential for novel applications, including food processing, additives in detergents, or pharmacy (Zeng 2003). We expect these Arctic bacteria can be used for screening to develop new antibiotics or industrial enzymes that are active at low temperature.

References

1. Bowman JP, Gosink JJ, McCammon SA, Lewis TE, Nichols DS, Nichols PD, Skerrat JH, Staley JT, McMeekin TA (1998a) *Colwellia demingae* sp. nov., *Colwellia hornerae* sp. nov., *Colwellia rossensis* sp. nov. and *Colwellia psychrotropica* sp. nov.: psychrophilic Antarctic species with the ability to synthesize docohexaenoic acid (22:6 n-3). Int J Syst Bacteriol 48:1171-1180
2. Bowman JP, McCammon SA, Brown JL, McMeekin TA (1998b) *Glaciecola punicea* gen. nov., sp. nov., and *Glaciecola pallidula* gen. nov., sp. nov.: psychrophilic bacteria from Antarctic sea-ice habitats. Int J Syst Bacteriol 48:1205-1212
3. Bowman JP, McCammon SA, Brown JL, Nichols PD, McMeekin TA (1997a) *Psychroserpens burtonensis* gen. nov., sp. nov., and *Gelidibacter algens* gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea-ice habitats. Int J Syst Bacteriol 47:670-677
4. Bowman JP, McCammon SA, Brown MV, Nichols DS, McMeekin TA (1997b) Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl Environ Microbiol 63:3068-3078
5. Bowman JP, McCammon SA, Lewis TE, Brown JL, Nichols PD, McMeekin TA (1998c) *Psychroflexus torquis* gen. nov., sp. nov., a psychrophilic bacterium from Antarctic Sea ice with the ability to form polyunsaturated fatty acids and the reclassification of *Flavobacterium gondwanense* Dobson, Franzmann 1993 as *Psychroflexus gondwanense* gen. nov., comb. nov. Microbiology 144:1601-1609
6. Bowman JP, McCammon SA, Nichols DS, Skerrat JH, Rea SM, Nichols PD, McMeekin TA (1997c) *Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov.—novel species with the ability to produce eicosapentaenoic acid (20:5w3) and grow anaerobically by dissimilatory Fe(III) reduction. Int J Syst Bacteriol 47:1040-1047

7. Bowman JP, Nichols DS, McMeekin TA (1997d) *Psychrobacter glacincola* sp. nov., a halotolerant, psychrophilic bacterium isolated from Antarctic sea ice. Syst Appl Microbiol 20:209-215
8. Chun J (1995) Computer-assisted classification and identification of actinomycetes. Ph.D. Thesis, University of Newcastle, Newcastle upon Tyne, UK
9. Denner EB, Mark B, Busse HJ, Turkiewicz M, Lubitz W (2001) *Psychrobacter proteolyticus* sp. nov., a psychrotrophic, halotolerant bacterium isolated from the Antarctic krill Euphausia superba Dana, excreting a cold-adapted metalloprotease. Syst Appl Microbiol 24:44-53
10. Felsenstein, J (1993). PHYLIP (Phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, WA, USA
11. Gosink JJ, Woese CR, Staley JT (1998) *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov., and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the Cytophaga–Flavobacterium–Bacteroides group and reclassification of *Flectobacillus glomeratus* as *Polaribacter glomeratus* comb. nov. Int J Syst Bacteriol 48:223-235
12. Humphry DR, George A, Black GW, Cummings SP (2001) *Flavobacterium frigidarium* sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. Int J Syst Evol Microbiol 51:1235-1243
13. Irgens RL, Gosink JJ, Staley JT (1996) *Polaromonas vacuolata* gen. nov., sp. nov., a psychrophilic, marine, gas vacuolate bacterium from Antarctica. Int J Syst Bacteriol 46:822-826
14. Jagannadham MV, Chattopadhyay MK, Subbalakshmi C, Vairamani M, Narayanan K, Rao CM, Shivaji S (2000) Carotenoids of an Antarctic psychrotolerant bacterium, *Sphingobacterium antarcticus*, and a mesophilic bacterium, *Sphingobacterium multivorum*. Arch Microbiol 173:418-424
15. Junge K, Imhoff F, Staley T, Deming JW (2002) Phylogenetic diversity of numerically important Arctic sea-ice bacteria cultured at subzero temperature. Microb Ecol 43:315-328
16. Maruyama A, Honda D, Yamamoto H, Kitamura K, Higashihara T (2000) Phylogenetic analysis of psychrophilic bacteria isolated from the Japan Trench, including a description of the deep-sea species *Psychrobacter pacificensis* sp. nov. Int J Syst Evol Microbiol. 50:835-846
17. McCammon SA, Bowman JP (2000) Taxonomy of Antarctic *Flavobacterium* species: description of *Flavobacterium gillisiae* sp. nov., *Flavobacterium tegetincola* sp. nov., and *Flavobacterium xanthum* sp. nov., nom. rev. and reclassification of *Flavobacterium salegens* as *Salegentibacter salegens* gen. nov., comb. nov. Int J Syst Evol Microbiol 50:1055-1063
18. McCammon SA, Innes BH, Bowman JP, Franzmann PD, Dobson SJ, Holloway PE, Skerratt JH, Nichols PD, Rankin LM (1998) *Flavobacterium hibernum* sp. nov., a lactose-utilizing bacterium from a freshwater Antarctic lake. Int J Syst Bacteriol. 48:1405-1412
19. Moore ERB, Mau M, Arnscheidt A, Bottger EC, Hutson RA, Collins MD, Van de Peer Y, De Wachter R, Timmis KN (1996) The determination and comparison of the 16S rRNA gene sequence of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intrageneric relationships. Syst Appl Microbiol 19:478-492
20. Nelson K, Paulsen I, Weinel C, Dodson R, Hilbert H, Fouts D, Gill S, Pop M, Martins Dos

Santos V, Holmes M, Brinkac L, Beanan M, DeBoy R, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen J, Timmis K, Duesterhoft A, Tummler B. Fraser C. (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4:799-808

21. Osorio CR, Barja JL, Hutson RA, Collins MD (1999) *Arthrobacter rhombi* sp. nov., isolated from Greenland halibut (Reinhardtius hippoglossoides). Int J Syst Bacteriol 49:1217-1220
22. Phadtare S,Yamanaka K, Inouye M (2000) The cold shock response. pp. 33-45. *In* Storz G, Hengge-Aronis R (eds.), *Bacterial stress responses*. American Society for Microbiology, Washington, D.C.
23. Reddy GS, Aggarwal RK, Matsumoto GI, Shivaji S (2000) *Arthrobacter flavus* sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antarctica. Int J Syst Evol Microbiol 50:1553-1561
24. Reddy GS, Prakash JS, Matsumoto GI, Stackebrandt E, Shivaji S (2002) *Arthrobacter roseus* sp. nov., a psychrophilic bacterium isolated from an antarctic cyanobacterial mat sample. Int J Syst Evol Microbiol 52:1017-1021
25. Romanenko LA, Schumann P, Rohde M, Lysenko AM, Mikhailov VV, Stackebrandt E. (2002) *Psychrobacter submarinus* sp. nov. and *Psychrobacter marincola* sp. nov., psychrophilic halophiles from marine environments. Int J Syst Evol Microbiol 52:1291-1297
26. Spiers AJ, Buckling A, Rainey PB (2000) The causes of *Pseudomonas* diversity. Microbiology 146:2345-2350
27. Steyn PL, Segers P, Vancanneyt M, Sandra P, Kersters K, Joubert J.J. (1998) Classification of heparinolytic bacteria into a new genus, *Pedobacter*, comprising four species: *Pedobacter heparinus* comb. nov., *Pedobacter piscium* comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. Proposal of the family *Sphingobacteriaceae*. Int. J. Syst. Bacteriol 48:165-177
28. Stover CK, Pham X-QT, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim RM, Smith KA, Spencer DH, Wong GK-S, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406(6799):959-964
29. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680
30. Wauters G, Charlier J, Janssens M, Delmee M (2000) Identification of *Arthrobacter oxydans*, *Arthrobacter luteolus* sp. nov., and *Arthrobacter albus* sp. nov., isolated from human clinical specimens. J Clin Microbiol 38:2412-2415
31. Yamamoto S, Kasai H, Arnold DL, Jackson RW, Vivian A, Harayama S (2000) Phylogeny of

the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology 146:2385-2394

32. Zhou J, Davey ME, Figueras JB, Rivkina E, Gilichinsky D, Tiedje JM. (1997) Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. Microbiology 143 :3913-3919