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COMPARATIVE ANALYSIS OF BACTERIAL COMMUNITY COMPOSITION IN SIBERIAN PERMAFROST AND ANTARCTIC POND SEDIMENTS

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COMPARATIVE ANALYSIS OF BACTERIAL COMMUNITY COMPOSITION IN SIBERIAN PERMAFROST AND ANTARCTIC POND SEDIMENTS

By

Brad Chia-Kai Chang

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ABSTRACT

COMPARATIVE ANALYSIS OF BACTERIAL COMMUNITY COMPOSITION IN SIBERIAN PERMAFROST AND ANTARCTIC POND SEDIMENTS

By

Brad Chia-Kai Chang

Understanding the microbial diversity under very cold and usually frozen environments such as Siberia and Antarctica may lead to the discovery of traits important for life in extreme environments. We compared the bacterial community structure of Siberian permafrost and Arctic pond sediment by building 16S rRNA gene clone libraries, a culture-independent approach. Our goal was to explore the composition and to compare the diversity of these communities by utilizing an RDP high throughput pipeline tool and the following programs: DOTUR, EstimateS, Libshuff, MEGA and Classifier.

Four Siberia samples were selected according to the age which ranged from twelve thousand to three million years old, and from freshwater alluvial versus marine horizon. There were two samples from the Antarctic McMurdo Ice Shelf. They are pond surface sediments collected from Fresh pond and Brack pond on Bratina Island. These two ponds are next to each other but represent very different salinities in pond water. From the alpha diversity analysis, the Siberia samples showed lower diversities than Antarctica samples. Both Arctic and Antarctica sediments with the higher salinities showed lower diversity in bacterial community. The results from RDP Classifier showed that the *Proteobacteria* were the most dominant phylum in both regions.

This work is dedicated to my parents,

who have continuously

loved and supported me.

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Most of all, I wish to express my deepest gratitude to my parents and both my sisters' families for giving me so much support, financially and spiritually. I could not make it without their love.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

APPENDIX A

LIST OF FIGURES

CHAPTER I

APPENDIX A

CHAPTER I

COMPARATIVE ANALYSIS OF BACTERIAL COMMUNITY COMPOSITION IN SIBERIAN PERMAFROST AND ANTARCTIC POND SEDIMENTS BY THE CULTURE-INDEPENDENT METHOD

INTRODUCTION

The permafrost environment is composed of soil, bedrock, sands, and sediments that remain frozen for a period of two years or more (Mueller, 1973). Over 20% of the earth's land surface is subjected to these permanently cold conditions including, 85% of Alaska, 55% of Russia and Canada, 20% of China, and the majority of Antarctica (Pewe, 1995). Members of the genera *Psychrobacter* are commonly isolated from environments that are low in temperature or high in salinity (Juni, 2002), such as permafrost. *Psychrobacter arcticus 273-4* was isolated (Vishnivetskaya *et al.*, 2000) from polar region permafrost soil of the Kolyma-Indigirka lowland (152-162°E, 68-72°N), adjacent to the Siberian Sea. The sediment from the sampled permafrost layer has been frozen for at least 20-30,000 years.

Siberian permafrost soil represents an extreme environment that is characterized by low temperatures (-10 to -12°C), low water activity (a_w = 0.9), and low nutrient availability (Gilichinsky, 2001). The low water activity is due to the increased concentration of solutes in the remaining unfrozen water. The water activity of 0.9 corresponds to a salt concentration of 2.79 m NaCl (5 osm). The permafrost environment in contrast to many surface environments is very stable, with a constant set of stresses

that may have acted as selective factors on the survivors, including adaptation to a long-term frozen environment and the associated desiccation (Gilichinsky, 2001; Willerslev, *et al.*, 2004). The Kolyma permafrost temperatures of -10 to -12°C would not be expected to freeze the cytosol of bacterial cells (McGrath, 2004) and hence continued biochemical catalysis could be expected, albeit the fluid would be viscous and reaction rates very slow (Ponder, *et al.*, 2004).

The Antarctic ecosystem is also considered an extreme environment. The McMurdo Ice Shelf comprises an area of about 1500 km^2 with a thickness from 10 to 50 m (Howard-Williams, *et al.*, 1990). During the summer, the area is covered with a network of meltwater ponds that vary in size, shape and physicochemical conditions, even though some of them are only several meters apart (De Mora, *et al.*, 1991)

The goal was to analyze the bacterial community diversity from these two geologically separated but low temperature and usually frozen environments. Our approach was to use a culture-independent, 16S rRNA gene clone library comparison method. The 16S rRNA genes in bacteria are highly conserved. This character makes them a good target for establishing phylogenetic relationships of microbes in ecological studies (Lane, *et al.*, 1985). The analysis of the total-community genomic DNA via 16S rRNA gene sequence has been used to characterize Archaea within the permafrost sediments (Tiedje, *et al.*, 1998). A recent study of Vishnivetskaya et al. (Vishnivetskaya, *et al.*, 2006) used both culture-dependent and culture-independent methods of characterizing a Siberian permafrost bacterial community. The results of two methods from above study yield very different perspective about the bacterial community structure. Thus, additional community comparison of Antarctic and Siberian communities

is important for a greater understanding of cold adapted communities and to see if the composition of bacterial communities is similar for both habitats.

MATERIALS AND METHODS

Characterization of the Antarctica and Siberian sediments

The samples from Antarctic were collected by Peter Bergholz during January 2006. The sampling sites were on the south portion of Bratina Island (165°35′E, 78°00′S) on the McMurdo Ice Shelf. The samples are sediments from two ponds lying within 50 meters of each other. These two ponds are discrete and were designated with the unofficial names, Brack Pond and Fresh Pond. They have electrical conductivities of about 20,000 and 1,200 micro-Siemens per centimeter respectively (20,000 microS/cm = ~219 mmol/L of NaCl). The average temperature of Brack pond was 5.9°C, and ranged from 3°C to 9.5°C for January 2006. The Fresh pond had a lower average temperature, 3.7°C, and ranged from 1.5°C to 6.8°C (Table 1).

The samples from the Siberian permafrost were collected by David Gilichinsky and his team from the Cryobiology Laboratory, Russian Academy of Sciences, Pushchino. Four permafrost samples were collected from borehole 17/99, 04/01, 01/01 and 02/92. The second pair of digits identifies the year the core was collected. Borehole 17/99 is located in Lake Yakutskoe by East Siberian Sea coast (159°29′E, 69°51′N). This is a tundra zone in the Kolyma lowland. The texture of this sediment sample is described as sandy and sandy-loams. The core was from a depth of 16.8-16.9 meter and is a middle Pleistocene marine horizon that contains cryopegs, and dated as 100 – 120 thousand years old. Borehole 04/01 is located in a tundra zone in the Cape Svyatoi Nos by Laptev Sea coast (140°10′E, 72°55′N). This alluvial sample is sandy-loam with icy complex. The depth where the sample was collected is 9.0 meter and dated as 300 – 400 thousand years

old from middle Pleistocene epoch. Borehole 01/01 is located in a tundra zone on Cape Bykovskii by the Laptev Sea coast (129°30′E, 71°40′N). This sample was collected from 13.0 meter deep and is late Pleistocene alluvial sandy-loam with icy complex. It is dated as 12-40 thousand years old. Borehole 02/92 is also located in a tundra zone. It is from the right bank of the mouth of the Malaya Kon′kovaya River, Kolyma lowland (158°28′E, 69°23′N). This sediment sample was collected from 18.7 meter deep. It is lake-swamp loam from the late Pliocene epoch and dated as 2-3 million years old. All of these sediment samples were stored in a -20°C freezer and were not exposed to room temperature for more than 5 min while weighing the soil needed for DNA extraction.

Table 1. Characteristics of the sites sampled for DNA extraction.

Sample	Sampling Site	Depth (m.)	Temp. (°C)	Age (year old)	Other
AB	Antarctic Brack Pond	Surface	3~9.5*	< 100	High salt
AF	Antarctic Fresh Pond	Surface	1.5~6.8*	< 100	
S17M	Siberian borehole 17/99	16.85	-10~-12	100~120 thousand	Marine horizon
S04F	Siberian borehole 04/01	9	-10~-12	300~400 thousand	
S01Y	Siberian borehole 01/01	13	-10~-12	12~40 thousand	Young
S02O	Siberian borehole 02/92	18.7	-10~-12	2~3 million	Old

* Average temperature during austral summer 2006

Total DNA extraction from sediment

The sediment samples (0.8 g) were placed in sterile ceramic mortars. After several repeated rounds of freezing and grinding steps, they were ground to powder condition. The total DNA was extracted using the FastDNA SPIN KIT for Soil (BIO 101 system from Q-BIO Gene Company) according to the manufacturer's protocol. The concentration of DNA was estimated at 260 nm by NanoDrop spectrophotometer. In case of extraction failure, two replicates were performed for each sediment sample and the replicate with better DNA quality was used for 16S rRNA gene amplification.

16S rRNA gene amplification from genomic DNA

The 16S rRNA genes were amplified by the using universal primer set 27F and 1392R in six replicates. Amplifications were performed in a reaction mixture containing 5 µl of 5X GoTaq Flexi Buffer (Promega), 1.0 mM $MgCl_2$, 0.4 mM each deoxynucleotide triphosphate (Invitrogen), 5 µg of bovine serum albumin (BSA) (New England Biolab), 0.4 µM each of forward and reverse primers, 25 ng of total community genomic DNA, 1.25 U of GoTaq DNA polymerase (Promega), and molecular level DNase-free H_2O (Sigma) to a total volume of 25 µl. The temperature profile for the polymerase chain reaction (PCR) was set to an initial denaturation at 95°C for 9 min followed by 25 cycles of 95°C for 1 min, 59°C (optimum temperature of the primer set) for 1 min, and 72°C for 1 min 40 s, plus a final extension at 72°C for 10 min.

Cloning by using TOPO TA cloning kit

The amplified PCR products were verified in a 1% agarose gel electrophoresis and the three reactions with better quality were cut and purified by a Qiaquick Gel Purification kit (Qiagen). The concentration of purified PCR product was determined by a NanoDrop spectrophotometer at 260 nm. The purification steps resulted in loss of deoxyadenosine (A) at the 3' end of the PCR product that is needed for successful ligation; therefore, a reaction for adding the 3' end A was preformed. This reaction mixture contained 0.1 µl of Taq DNA polymerase (Invitrogen), 2.5 µl of 10X PCR Buffer, 1.5 µl of 50 mM $MgCl_2$, 1 mM of deoxyadenosine triphosphate (dATP), and molecular level DNase-free H_2O (Sigma) to a total volume of 25 µl. The reactions were incubated in a water bath at 72°C for 10 min and the products were used directly for cloning. The PCR products were ligated into pCR™ 4.0 vector using TOPO TA cloning kit (Invitrogen) and transformed into chemically competent *E. coli* according to the manufacturer's instructions. Luria-Bertani (LB) agar containing 100 µg/ml of kanamycin was used for clone selection. After overnight incubation at 37°C, selected colonies were picked with toothpicks and placed in a 96-well growth block which contained 1.2 ml LB selective (100 µg/ml of kanamycin) freezing buffer (4.4% glycerol). The growth block was covered by airpore tape and the cells allowed to grow overnight on a shaker (200 rpm) at 37°C. Transfer of 150 µl of the grown cell culture was made into a Grainer 96-well plate. An ethanol-flamed colony copier was used to stamp colonies from the Grainer plate onto an OmniTray LB agar plate containing 100 µg/ml of kanamycin. The OmniTray plates were incubated at 37°C overnight before sending to Macrogen Inc.

(Seoul, Korea; www.macrogen.com) for sequencing. The Grainer plates were sealed by aluminum tape and stored in -20°C freezer to retain stock cultures.

Clone Library Analyses

Raw sequence data were processed by using pipeline quality filter tools on the Ribosomal Database Project II (RDP-II) website (Cole et al., 2005; http://rdp.cme.msu.edu). The RDP pipeline tool automatically processes entire libraries of single sequences from raw sequencer output to analysis. The base-calling work is done by PHRED. The quality trimming and vector removal are done by LUCY program. The Q20 cutoff was set to 0.8 and the minimum sequence length of 400 was used. Sequence alignment, library distance matrix file and input file for EstimateS (Colwell, 2005; http://viceroy.eeb.uconn.edu/EstimateS) software were downloaded via RDP pipeline tool. The distance matrix file was used with Distance-Based Operational Taxonomic Unit and Richness (DOTUR) determination program (Schloss and Handelsman, 2005; http://www.plantpath.wisc.edu/fac/joh/dotur.html) for obtaining operational taxonomic units (OTUs) and alpha diversities, the biodiversity within a particular community. The EstimateS software was used for obtaining beta diversity, which is a measure of biodiversity between ecosystems. The MEGA (Kumar, Tamura and Nei, 2004) program was used for clustering libraries following the EstimateS analysis. The distance matrix was used for comparing similarity between clone libraries via a statistical test provided by the LIBSHUFF program (Singleton et al., 2001).

The Classifier tool provided by RDP II is used to assign 16S rRNA sequences to the taxonomical hierarchy. The default rank confidence threshold of 80% was used.

RESULTS

Total community genomic DNA yield

The total community genomic DNA extractions for Siberia permafrost samples had yields of 1.95, 1.89, 3.23 and 3.26 μg DNA per gram of sediment for borehole 17/99, 04/01, 01/01 and 02/92, respectively. The genomic DNA yields from Antarctic Brack pond and Fresh pond samples were 9.84 and 1.69 μg/g of sediment (Table 2).

16S rRNA gene sequences analyses for positive sequences and OTUs

Six plates, one plate (96 clones) for each sample, were sent to Macrogen Inc. for sequencing. The raw sequencer output is processed by RDP pipeline tools. The numbers of clones that passed the quality control of RDP pipeline ranged from 92 to 96 clones per plate. This provided a total of 561 16S rRNA gene sequences cloned from total-community genomic DNA extracted from the six sediment samples. DOTUR program generated the OTU numbers and calculated alpha diversity indices. The OTU difference threshold for all DOTUR calculations was set at 97% similarity. The OTU numbers were 6, 13, 43 and 6 OTUs for Siberia 17/99, 04/01, 01/01 and 02/92 samples, respectively. There are 41 and 58 OTUs for sediment samples from Antarctica Brack pond and Fresh pond (Table 2). Higher OTU numbers appeared in Siberia 01/01 (young sample) and both Antarctica samples.

Table 2. DNA concentration from studied sites and clone library information.

*Sample**	*DNA concentration (μg/g-soil)*	*Sequences*	*OTUs*
S17M	1.95	93	6
S04F	1.89	92	13
S01Y	3.23	92	44
S02O	3.26	95	6
AB	9.84	93	41
AF	1.69	96	58
Total		561	168

* See Table 1 for identification of sample code

The rarefaction curves showed that samples S17M, S04F and S02O were close to full coverage of the community (Fig. 1). On the other hand, samples AB, AF and S01Y need more clones for adequate coverage. Images in this thesis are presented in color.

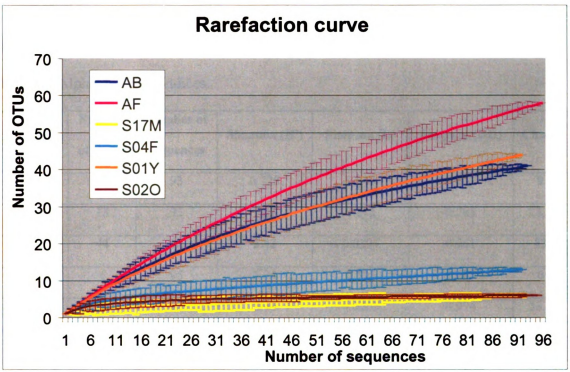

Figure 1. Rarefaction curve for Antarctic ponds and Siberian permafrost communities.

Alpha diversity indices and rank-abundance curves

Alpha-diversity indices include Shannon index, Simpson index and Chao 1 index. Alpha diversity indices are calculated for comparing diversity within a community and the values for indices were shown in Table 3.

Table 3. Alpha diversity indices.

SAMPLE	Number of OTUs	Number of sequences	Shannon (H')	Simpson (D)	Simpson (1-D)	Chao1
S17M	6	93	0.728	0.648	0.352	9
S04F	13	92	1.81	0.212	0.788	23.5
S01Y	44	92	3.42	0.0394	0.961	83
S02O	6	95	1.34	0.315	0.685	6
AB	41	93	3.49	0.0262	0.974	58.1
AF	58	96	3.9	0.0136	0.986	103

The Shannon index is most commonly used for presenting biodiversity. This index takes into account the number of species and the evenness of the species. The index (diversity) is increased either by having more unique species, or by having greater species evenness.

The results for Antarctica samples were 3.49 for Brack pond and 3.9 for Fresh pond. They were significantly higher than the value for three of the Siberia samples, 0.7 for sample S17M, 1.8 for S04F and 1.3 for S02O (Figure 2). However, the result for sample S01Y was 3.4, which was higher than other Siberian samples and was comparable to Antarctica samples.

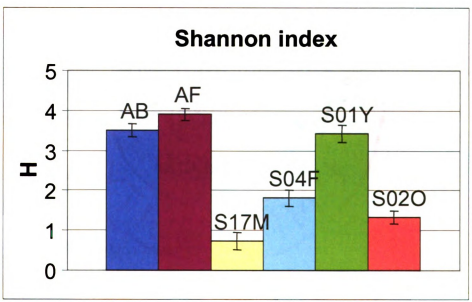

Figure 2. Shannon index result.

For the Simpson index, value (D) represents the probability that two randomly selected individuals in the community belong to the same species. Therefore, (1-D) represents the probability of two different species being picked. The higher the (1-D) value, the higher the diversity of the community. For example, sample S17M was low in biodiversity (1-D), the chance for picking up two individuals as same species (D) is as high as 64.8% (Figure 3).

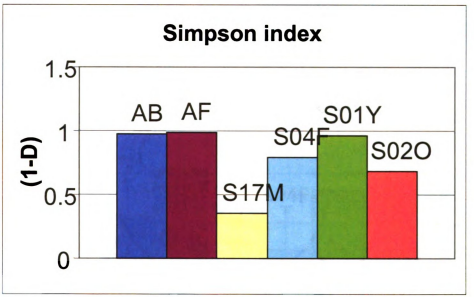

Figure 3. Simpson index result.

The Chao1 index is a nonparametric estimator suitable for microbial diversity analysis. It estimates the richness of a community.

Sample S17M and S02O had lower OTU numbers than others and thus had lower values for richness (Figure 4). Within the Antarctica communities, sample AB showed lower richness than sample AF.

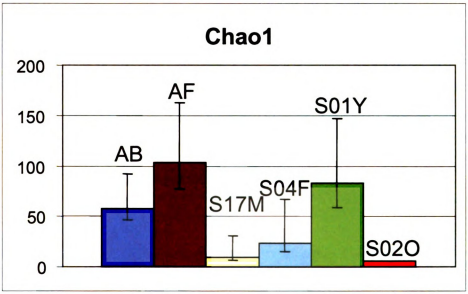

Figure 4. Chao1 index result.

Rank-abundance curves were generated for easier visualization of species abundance in a community (Figure 5). The figures clearly show that Siberian samples had higher abundance of species than Antarctica samples. There were 74 clone sequences from only 1 OTU in the Siberia marine sample; 31 clone sequences from only 1 OTU in the Siberia fresh sample; and 43 clone sequences from only 1 OTU in the Siberia 02 (old) sample.

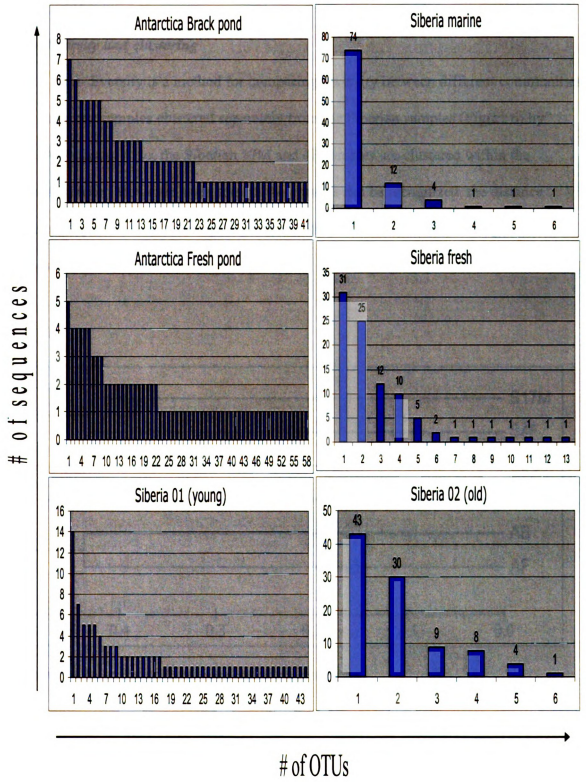

Figure 5. Rank-abundance curve for Antarctica and Siberia communities.

Beta-diversity and clustering

Beta-diversity is a method for comparing diversity between different communities. The Antarctic samples clustered separately from the Siberian samples (Figure 6) by MEGA program. Also, the Siberian 17M and 4F samples are clustered within the Siberian cluster. However, even though the clusters can be recognized, the distance between any two communities is larger than 0.86 (S17M and S4F), meaning that the highest similarity is about 14%.

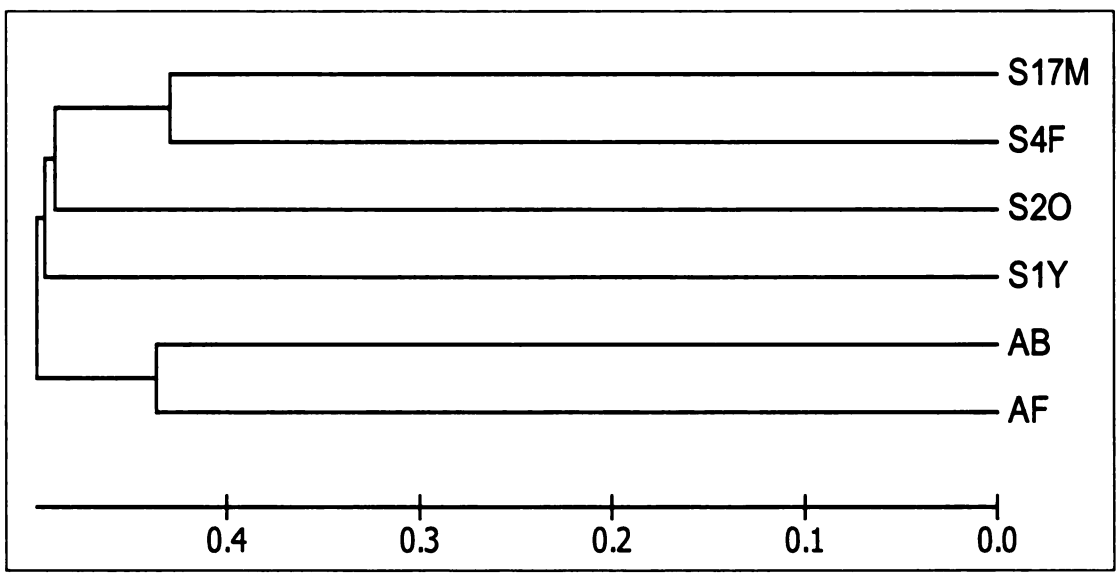

Figure 6. Clustering of Antarctica and Siberia samples.

LIBSHUFF

LIBSHUFF is a computer program designed to compare two libraries of 16S rRNA gene sequences and determine if they are significantly different. ∫-LIBSHUFF is another version of LIBSHUFF that can compare several libraries at once. The P value represents the rank of two communities being the same. When the P value is smaller than 0.05, samples are considered significantly different. The results from ∫-LIBSHUFF analysis showed that all six libraries from Siberian and Antarctic sites are significantly different from each other (Table 4).

Table 4. The result (P-value) for ∫-LIBSHUFF.

P - value	AB	AF	S17M	S04F	S01Y	S02O
AB	-	0.000	0.000	0.000	0.000	0.000
AF	0.000	-	0.000	0.000	0.000	0.000
S17M	0.000	0.000	-	0.000	0.000	0.000
S04F	0.000	0.000	0.000	-	0.000	0.000
S01Y	0.000	0.000	0.000	0.000	-	0.000
S02O	0.000	0.000	0.000	0.000	0.000	-

RDP classifier

From the total of 561 sequences cloned from the Siberian and Antarctic sediment samples, there were total of 167 OTUs differentiated by the 97% sequence identity cut-off. These OTUs were distributed among 10 phyla and at least 17 classes (Table 5). There were 7.3% of unclassified bacteria sequences and they only appeared in the Siberian-04 (young) sediment and in both Antarctic libraries.

Table 5. Overview of the clone result for Antarctica and Siberia bacteria diversity from sediments at class level.

Phylum	Class	AB	AF	S17M	S04F	S01Y	S02O
Proteobacteria	Alpha	16	2		1	2	8
	Beta	7	11				
	Gamma	1	5	90	61	1	86
	Delta	4	5				
Bacteroidetes	Sphingobacteria	3	1		1	1	
	Flavobacteria	3	16				
	Unclas. Bacteroidetes	15	6				
Firmicutes	Clostridia	5	14				1
	Mollicutes	1					
	Bacilli	5	1	1	1	3	
Actinobacteria	Actinobacteria	7	22	2	28	54	
Gemmatimonadetes	Gemmatimonadetes		1			2	
Cyanobacteria	Cyanobacteria		7				
Chloroflexi	Anaerolineae	12					
Deinococcus-Thermus	Deinococci	4					
Genera_incertae_TM7	TM7	1					
Spirocheta	Spirochaetes	1					
Thermomicrobia	Thermomicrobia					1	
Unclas. Bacteria		8	5			28	
Total		93	96	93	92	92	95

For Siberia samples, there were 69 OTUs in 372 cloned sequences. These OTUs were distributed among six known phyla and at least eight classes (Figure 7). The Proteobacteria and Actinobacteria were most dominant and contributed 66.9% and 22.6% of clone sequences, respectively. The Antarctica samples were more evenly distributed. They had 99 OTUs in 189 clone sequences among ten known phyla and at least sixteen classes.

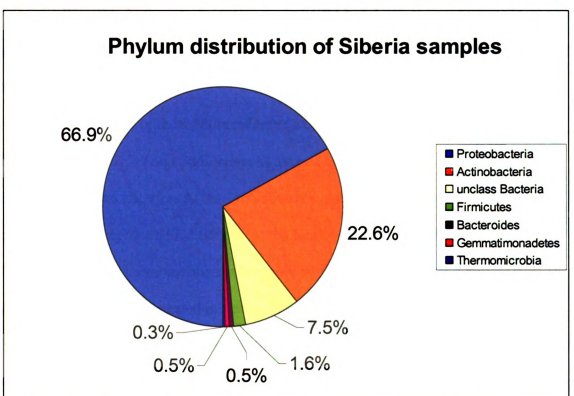

Figure 7. The phylum distribution of Siberia samples by clone sequence number. The Proteobacteria and Actinobacteria are significantly highly dominant.

Proteobacteria

The Proteobacteria were the most dominant phylum in both Siberian and Antarctic libraries, representing 67% and 27%, respectively. Gamma-Proteobacteria were the only class that was found in every library. A total of 96.8% of the clones for sample S17M, 66.3% of the clones for S04F and 90.5% of the clones for S02O were Gamma Proteobacteria. For communities S17M and S04F, all the Gamma-Proteobacteria were Pseudomonadales. For community S02O, 31.6% were Xanthomonadales, 45.3% were Pseudomonadales and 13.7% were Enterobacteriales. Pseudomonadales was not found in Antarctic libraries. At the family level, sample S17M had 4.3% of Pseudomonadaceae and 92.5% of Moraxellaceae, sample S04F had 12% of Pseudomonadaceae and 53.3% of Moraxellaceae, sample S02O had 45.3% of Pseudomonadaceae. At the genus level, the result showed that all of these Moraxellaceae were *Psychrobacter*. The *Psychrobacter* was dominant in S17M and S04F clone libraries. However, *Psychrobacter* was only found in these two libraries. Alpha-Proteobacteria was commonly found in these communities except the S17M library. At the family level, Hyphomicrobiaceae and Rhizobiaceae were found in Siberian libraries, while Beijerinckiaceae, Rhodobacteraceae and Caulobacteraceae appeared in Antarctic libraries. While Alpha- and Gamma Proteobacteria were common in both habitats, Beta- and Delta-Proteobacteria appeared in Antarctic libraries only. For Beta Proteobacteria, only Burkholderiales were present. For Deltaproteobacteria, Desulfobulbaceae was the only one represented in library AB. In sample AF, Geobacteraceae, Desulfuromonaceae, one unclassified Myxococcales and one unclassified Deltaproteobacteria were present.

Bacteroidetes

Clones of the Bacteroidetes phylum were common in Antarctic communities. They were among class Sphingobacteria, Flavobacteria and some unclassified Bacteroidetes. Total of 23.3% of Antarctic clones were Bacteroidetes. Only two clones from Siberian libraries were Bacteroidetes; they were Sphingobacteriales and appeared in S04F and S01Y libraries.

Firmicutes

Antarctic libraries contained 13.8% clones from the phylum of Firmicutes while the other four Siberian libraries only had 1.6% of clones in this phylum. Class of Bacillii was detected in all libraries, except library S02O. The other classes were Clostridia found in both Antarctic libraries and Siberian S02O, and class of Molicutes was found only in Antarctic Brack pond (AB) library.

Actinobacteria

Actinobacteria was another phylum widely distributed. This phylum was seen in five of the six libraries of this study. The Antarctic and Siberian libraries contain 15.3% and 22.6% of Actinobacteria. All of these clones fall into the class of Actinobacteria as well. At the order level, Actinomycetales was found in all five libraries. Acidimicrobiales was found in library AF and S01Y, Rubrobacterales was found in S01Y only. Two and five unclassified Actinobacteria clones were found in library AF and S01Y, respectively.

Overall, *Arthrobacter* was the most dominant genus within Actinobacteria by clone sequence number but was only found in sample S17M and S04F.

Other phyla

Clones from phylum Gemmatimonadetes appeared in both libraries AF and S01Y with one and two clones, respectively. There were seven clones from library AF that appeared in phylum Cyanobacteria. There were twelve clones in library AB appearing in phylum Chloroflexi and they were all genus *Anaerolinea*.

Phylogenetic tree for Psychrobacter

A bootstrap consensus phylogenetic tree was constructed that combines all published *Psychrobacter* type strains plus the *Psychrobacter* clones from this study by MEGA program (Fig. 8). The *Psychrobacter* type strains 16S rRNA gene sequences were collected and edited by Dr. Hector Ayala-del-Rio. The construction of a tree is for better understanding on the relationship of the clones to other Psychrobacter. Sib17-A11-12 represents one OTU from library S17M that contains 12 clones. This OTU is closely related to another OTU from library S04F (Sib4-A1-42). A nucleotide BLAST (Basic Local Alignment Search Tool) search indicated that these two OTUs were closely related to *Psychrobacter maritimus* and *P. aquaticus*. There were 74 clone sequences represented by Sib17-A1-74. The BLAST result on nucleotides showed that this OTU was closely related to *Psychrobacter psychrophilus* and the Sib4-C10-5 had very high possibility to be *P. glacincola. Psychrobacter arcticus 273-4,* which was isolated from another borehole in the Kolyma region, is also present in this phylogenetic tree. There was

another OTU that is less related to the majority and was more closely related to Pseudomonas.

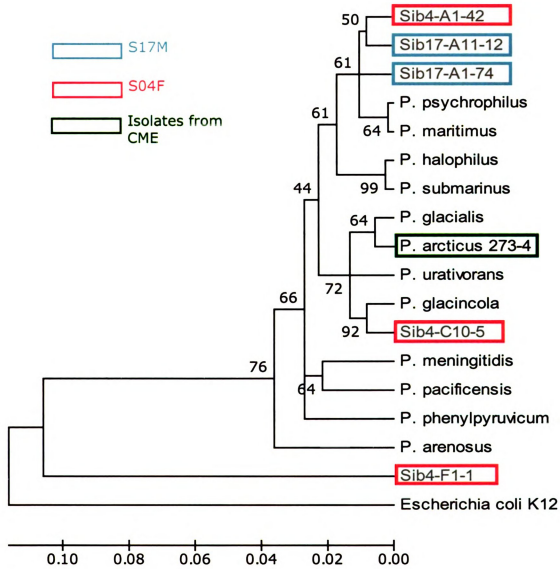

Figure 8. Phylogenetic tree shows the relationship of the *Psychrobacter* type strains and *Psychrobacter* clones from this study.

CONCLUSION AND DISCUSSION

When the age of sediment was plotted versus the number of OTUs, a trend of OTU number to the age of sediment sample is seen (Figure 9). The younger sediment samples had a higher yield of OTUs. Another noticeable trend from this figure and Shannon index (Fig. 2) or Chao1 index (Fig. 4) is the salinity effect. While comparing Antarctic communities, the AB community showed lower number in OTUs, lower diversity by Shannon index and lower community richness from Chao1 index. Same trend was also apparent in Siberian communities; the S17M sample presents the lowest OTUs, diversity and richness among other three Siberia samples.

However, the total community DNA yield does not show the same trend when compared to the data in Table 2. This could be due to the DNA degradation over time. After the 16S rRNA gene is degraded into fragments, the pieces can still be extracted from soil and contribute to the DNA yield. However, these fragments would not be amplified during PCR or would be cleaned out during the gel purification step I used.

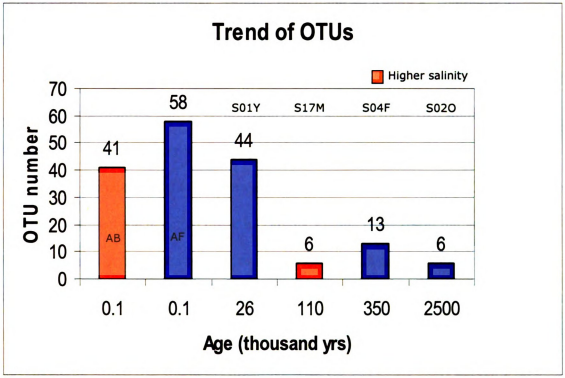

Figure 9. The relationship of OTU numbers to the age and salinity of samples.

One recently published paper by Tatiana A. Vishnivetskaya et al. (2006) used both culture-dependent and culture-independent methods for characterizing Siberian permafrost bacterial communities. Another study done by Aviaja A. Hansen et al. (Hansen, Ph.D. dissertation, University of Aarhus, 2006) used media enrichment to characterize the composition and diversity of the bacterial community in Spitsbergen soil, a high Arctic permafrost soil. A comparison of my study to these two studies shows a similar trend in composition of Siberia permafrost samples (Table 6).

The (+) marks indicate the presence of microbes at the taxonomic Class level. The (!) marks represent the negative result in Tatiana's clone study result but are positive in the isolate result and can be assumed as false negative. Alpha-, Gamma-Proteobacteria, Sphingobacteria, Bacilli and Actinobacteria are present in all studies. The Siberian clones from this study covered Tatiana's clone and isolate result at the class level, except Flavobacteria, which was found in Antarctica samples. My clone study agreed with Tatiana's study and added Thermomicrobia into the list, which was also found in Aviaja's media-enriched clone study.

Delta-Proteobacteria and Cyanobacteria were only seen in the Antarctic Fresh pond sediment sample. The reason for Cyanobacteria was seen in the Antarctica Fresh pond sample could be because the Antarctica ponds have algal growth each austral summer, which is not the case for the Siberian samples.

Over one-third of all sequences of permafrost strains from Tatiana's study belong to a single genus, Arthrobacter. This genus was dominant in phylum Actinobacteria from my study (Fig. 7).

Overall, Antarctica samples showed higher diversity. Despite that these samples are younger; this Antarctica habitat is a more dynamic environment due to the above zero temperatures each austral summer.

Table 6. Comparison of microbial community composition on three similar studies of Arctic-area permafrost plus the Antarctica pond sediments.

Phylum	Class	Arctic-area permafrost				Antarctica
		Tatiana Isolate	Tatiana Clone	Aviaja Clone	Brad Clone	Brad Clone
Proteobacteria	Alpha	+	!	+	+	+
	Beta			+		+
	Gamma	+	+	+	+	+
	Delta					+
Bacteroidetes	Sphingobacteria	+	!	+	+	+
	Flavobacteria	+	!	+		+
	Unclas. Bacteroidetes			+		+
Firmicutes	Clostridia		+	+	+	+
	Mollicutes			+		+
	Bacilli	+	+	+	+	+
Actinobacteria	Actinobacteria	+	+	+	+	+
Gemmatimonadetes	Gemmatimonadetes				+	+
Cyanobacteria	Cyanobacteria					+
Planctomycetes	Planctomycetacia			+		
Chloroflexi	Anaerolineae					+
Verrucomicrobia	Verrucomicrobiae			+		
Deinococcus-Thermus	Deinococci					+
Genera_incertae_TM7	TM7			+		+
Acidobacteria	Acidobacteria			+		
Spirocheta	Spirochaetes			+		+
Thermomicrobia	Thermomicrobia			+	+	
Unclass. Bacteria				+	+	+

Label of (!) assumes existence of microbe in column: Tatiana Clone according to the Tatiana Isolation evidence.

Appendix A

Appendix A

The Effects of Sodium Ion Concentration on the Growth of *Psychrobacter arcticus 273-4*

INTRODUCTION

Members of the genus *Psychrobacter* are commonly isolated from environments that are low in temperature or high in salinity (Juni, 2002), such as permafrost. *Psychrobacter arcticus* 273-4 was isolated (Vishnivetskaya, *et al.*, 2000) from polar region permafrost soil of the Kolyma-Indigirka lowland (152-162°E, 68-72°N), adjacent to the Siberian Sea collected by David Gilichinsky and team (Cryobiology Laboratory, Russian Academy of Sciences, Pushchino). The sediment from the sampled permafrost layer has been frozen for at least 20-30,000 years. This environment has very low water activity due to the concentration of solutes in water films as a result of freezing.

Psychrobacter arcticus 273-4 is a Gram negative, non-motile, Gamma-Proteobacteria (Bakermans *et al.*, 2006) which grows at low temperature; 22°C ~ -10°C, and is tolerant to long-term freeze. In order to grow, *P. arcticus* also requires a minimum of 5 mM NaCl in the culture media. Understanding how *P. arcticus* has adapted to life in the Siberian permafrost may lead to the discovery of traits important for life in extreme environments.

Monica Ponder compared the expression of a series of functional genes from *P. arcticus* under different conditions by microarray technology (Ponder, 2005). These conditions include two factors: temperatures (22 °C and 4 °C) and NaCl added to half

strength tryptic soy broth (TSB) (Difco, Detroit, MI). The microarray analysis showed an increased expression of the Na+ dependent symporter (ORF1600) and Na^+ pumps NADH dehydrogenases (ORF1687-ORF1691) under low temperature and high salinity condition (Fig. 1 and Fig. 2). Images in this thesis are presented in color.

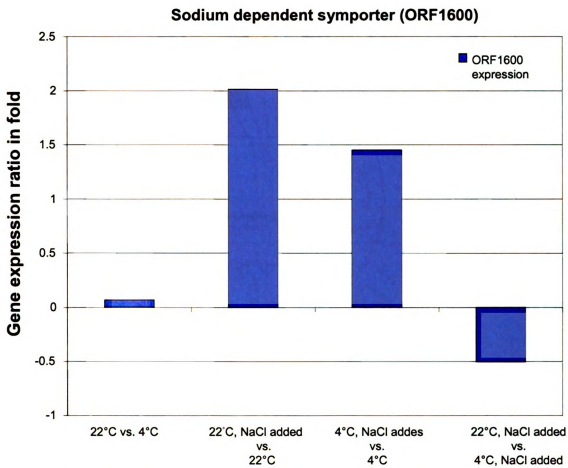

Figure 1. The gene expression ratio for sodium dependent symporter (ORF1600). The culture medium base is ½ TSB (from Ponder, 2005).

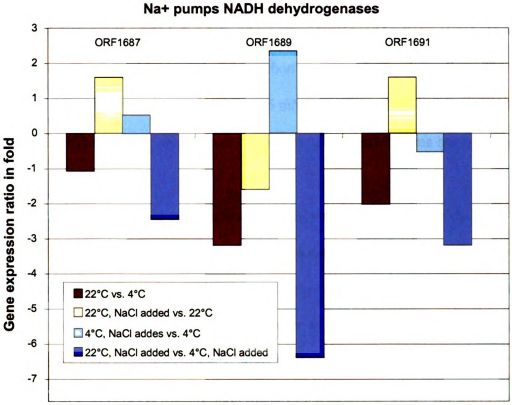

Figure 2. The gene expression ratio for Na^+ pumps NADH dehydrogenases (ORF1687-ORF1691). The culture medium base is ½ TSB (from Ponder, 2005).

These data suggest that *P. arcticus* may utilize the sodium motive force for energy production. Hence I propose that *P. arcticus* will show enhanced growth from the sodium motive force when grown under high salinity. This study tests the hypothesis from Dr. Monica Ponder's work that Na^+ provides an energy benefit to *Psychrobacter arcticus 273-4*.

MATERIALS AND METHODS

Growth and growth rate

In order to test the effects of sodium ion concentration on the growth of *Psychrobacter arcticus 273-4*, a defined medium developed by Peter Bergholz was chosen to maintain stable control of the carbon source and other ingredient. The medium contains 20 mM acetic acid (CH_3COOH), 5 mM NH_4Cl, 1 mM KH_2PO_4, 1:1000 volumes of Wolfe's Vitamins, 1:1000 volumes of trace metals, 25 mM trizma base ($C_4H_{11}NO_3$) in Milli-Q water.

NaCl was added to achieve four different concentrations: 10 mM, 30 mM, 100 mM and 300 mM. Under low concentration of sodium chloride, polystyrene vacuum filter systems were used for sterilization and polystyrene tissue culture flasks with vented cap were used for incubation in order to prevent addition of sodium ion from glassware. The cultures were incubated in a shaker at 22 °C. The optical density was recorded at 600 nm by a Cary 50 UV-Vis Spectrophotometer (VARIAN, CA, USA) for triplicate cultures.

Total protein yield

Another experiment was conducted to determine total protein yield of *Psychrobacter arcticus 273-4* at higher sodium concentrations. The same acetate medium with the different sodium concentrations stated above plus 1 M was used. These concentrations did not show growth inhibition by salt, which was shown to occur at or above 1.3 M Na^+ in preliminary experiments. Triplicate bacterial cultures were harvested for each Na^+ concentration when the maximum optical density for each sodium

concentration was achieved. Cells were centrifuged at 6,500x g for 10 min. The supernatant was decanted and cell pellets were washed by re-suspended in sterilized Milli-Q water and centrifuged again. The wash step was repeated to ensure removal of residual medium. The cell pellets were stored at -80 °C until all samples were collected. Aliquots of the frozen cells were mixed with an equal volume of solution containing 9 M urea, 2% 2-mercaptoethanol, 2% ampholytes (pH 8-10, BioRad), and 4% Nonidet P40 (a nonionic detergent). Soluble, denatured proteins were recovered in the supernatants after centrifugation of the samples at 435,000x g for 10 min using a Beckman TL100 tabletop ultracentrifuge. A modified Bradford protein assay was used to determine the concentration of total protein (Ramagli and Rodriguez, 1985).

RESULTS AND DISCUSSION

Growth and growth rate

The growth of *Psychrobacter arcticus* str. 273-4 under aforementioned four different salinities was measured by optical density (Fig. 3). Detectable growth under higher salinity occurs slightly later than for the lower salinity cultures. However, no significant difference in growth yield was shown in the higher sodium media.

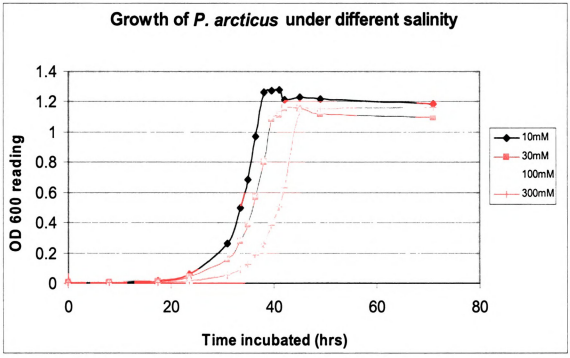

Figure 3. OD_{600} for *Psychrobacter arcticus* 273-4 grown in 20 mM acetate media with different sodium concentration

The growth rate for each different sodium concentration was calculated from the exponential phase of growth (Fig. 4). The results showed no consistent trend although the growth rate increased at the sodium concentration. However, the differences among these growth rates were not significant. Therefore, this experiment did not show a significant evidence to support that sodium can benefit the growth of *Psychrobacter arcticus* 273-4.

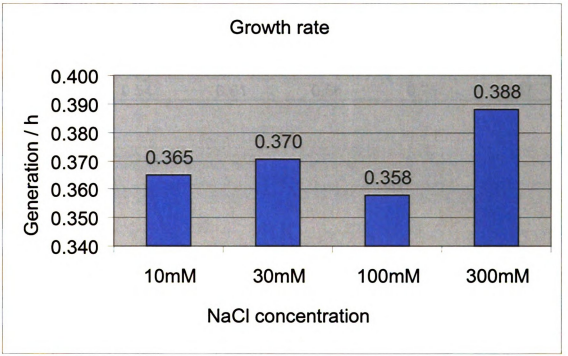

Figure 4. The growth rates (generation/h) for four different sodium concentrations of 20 mM acetate media.

Total protein yield

Preliminary experiments were undertaken to determine the acetate concentration that would provide a maximum OD_{600} of 0.6 to 0.8, to more sensitively measure the effect of sodium to the total protein yield (Table 1).

Table 1. The maximum OD_{600} reading for various concentrations acetate in the media with 30 mM NaCl.

	Acetate media concentration (with 30 mM NaCl)				
	8mM	10mM	11mM	12mM	14mM
OD_{600}	0.54	0.61	0.66	0.75	0.87

The 11 mM concentration of acetate was selected on the best fit for the range of cell yields expected for the total protein experiment. The results for protein yield are presented together with final bacterial culture maximum densities (Fig. 5).

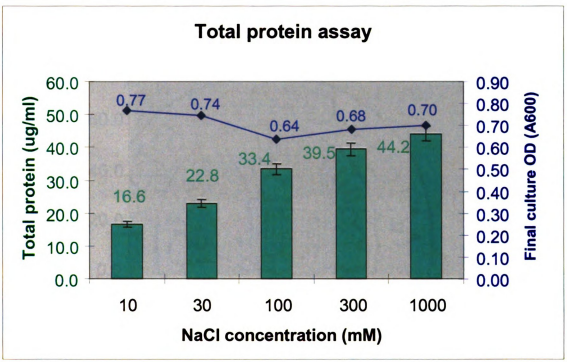

Figure 5. The total protein yields for different sodium concentrations in 11 mM acetate medium. The bars represent total protein concentration (μg/ml) and the blue dots represent the final (optimal) optical density for each different sample.

The protein yield can be present as protein concentration per cell optical density. The values show a clear trend in that higher sodium concentration yields higher total protein (Figure 6).

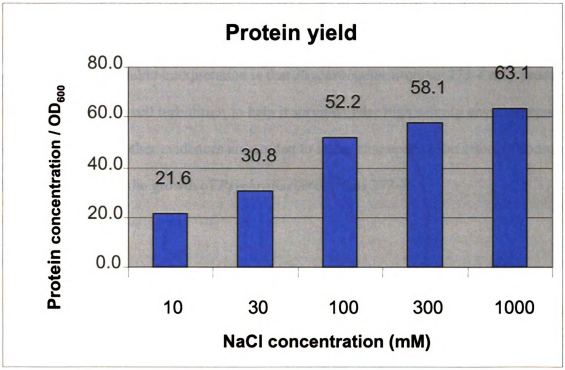

Figure 6. The protein yield presented by relative turbidity (OD_{600}). The values are shown on the top of the bars. The media used are 11 mM acetate media with the indicated NaCl concentrations.

In conclusion, the growth rate or the final optical density results were not consistent enough to answer the question whether sodium directly benefits *Psychrobacter's* energy for growth. This could be due to the fact that *Psychrobacter arcticus 273-4* has been selected in high salinity environment and thus sodium ion concentration does not show a strong effect on *P. arcticus*' growth. The total-protein yield result is significant. However, the final optical density did not support this conclusion. A reasonable interpretation is that *Psychrobacter arcticus 273-4* may produce more protein but not cell turbidities, to help it survive under high salinity environments. Nevertheless, more other evidences are needed to better characterize the effect of sodium ion concentration to the growth of *Psychrobacter arcticus* 273-4.

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