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ABSTRACT

ECOLOGY, PHYSIOLOGY AND METABOLISM OF COLD-ADAPTED MICROORGANISMS FROM THE SIBERIAN PERMAFROST

By

Debora Frigi Rodrigues

In our solar system, seven out of nine planets, including Earth, are considered to be cold. Even though cold temperatures are lethal to many microorganisms, some successfully colonize cold habitats. I studied the distribution, abundance and diversity of two cold-adapted genera, *Exiguobacterium* and *Psychrobacter*. Total microbial community DNA extracted from 54 sediment and soil samples from Siberia, Antarctica, Michigan, Iowa, Brazil, Puerto Rico and Hawaii was analyzed with specific primer sets for each genus by quantitative real-time PCR and by 16S rRNA gene clone libraries. Both genera were more commonly found and have higher densities in polar regions, but they were also detected in some temperate and tropical sites. The later was more likely the case when physicochemical conditions such as salinity, K^+ and pH were similar to their polar habitat conditions.

Exiguobacterium isolates obtained from the Siberian permafrost were analyzed using a polyphasic approach, and yielded a new species, *Exiguobacterium sibiricum*. The genome of the type strain of the new *E. sibiricum* species was sequenced by the DOE Joint Genome Institute. The genome is approximately 3 Mb in size, has a GC content of 47.7% and includes 2,978 putative protein-encoding genes (CDS). I used the genome and transcriptome analysis along with the organism's known physiology to better understand

its thermal adaptation. A total of about 27%, 3.2% and 5.2% of *E. sibiricum* strain 255-15 CDS spotted on the DNA microarray yielded differentially expressed genes in cells grown at -2.5°C, 10°C and 39°C, respectively, when compared to cells grown at 28°C. The hypothetical and unknown genes represented 10.6%, 0.89% and 2.3% of the CDS differentially expressed when grown at -2.5°C, 10°C and 39°C versus 28°C. The transcriptome analyses showed that *E. sibiricum* is constitutively adapted to cold temperatures since little differential gene expression was observed at growth temperatures of 10°C and 28°C, but at the extremities of its Arrhenius growth profile, namely -2.5°C and 39°C, much more differential gene expression occurred. The genes that responded were more typically associated with stress response.

I showed that *Exiguobacterium* and *Psychrobacter* are more prevalent in but not restricted to cold, polar environments, and that this ecology is consistent with the *Exiguobacterium*'s transcriptional response, i.e., little differential gene expression between 10°C and 28°C.

This work is dedicated to my husband
and my family who have continuously
loved and supported me.

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PREFACE

The life processes of all organisms are adapted for ecological success in their respective "normal" physiological environments. Any change in environmental conditions is experienced as a stress that threatens the normal metabolic balance and produces a response designed to counter the effects of the disturbing influence. Among all the natural stress conditions on our planet and solar system, cold is by far the most widespread from the perspective of Earth's most visable, albeit, mesophilic organisms. In our solar system, seven (including Earth) out of nine planets are cold. For instance, 90% of the Earth's oceans have a temperature of 5°C or less. When terrestrial habitats are included, over 80% of the Earth's biosphere is permanently cold. Since we inhabit a cold planet and numerous microorganisms found on Earth have the ability to cope with low temperatures, this makes them appear to be the Earth's most successful colonizers.

Among the cold habitats on our planet, the permafrost environments are strong candidates for astrobiological studies because they have characteristics similar to other cold planets in our solar system. The permafrost habitat underlies an estimated 20 to 25% of the Earth's terrestrial surface. The mean annual temperature is between -10 and -12°C in the Arctic and between -18 and -27°C in the Antarctic. Permafrost microorganisms have been living in the frozen sediments for as long as 2 to 3 million years in our primary study aerea, northeastern Siberia, without gene flow and microbial dispersal from any outside source.

In previous studies, four of the six major taxa isolated from the Siberian permafrost had strains from the Antarctic or other cold habitats as their closest known phylogenetic relatives. This pattern suggests the selection for more than a few traits for cold adaptation. One derivative of this hypothesis is that such strains may be restricted to cold environments. In this study, I studied two taxa, *Exiguobacterium* and *Psychrobacter,* to establish their distribution, abundance and biodiversity in polar, temperate and tropical locations on our planet. The results demonstrated that both genera had a patchy distribution in warmer environments, but were ubiquitous in polar regions.

Exiguobacterium isolates from the Siberian permafrost were further studied to better understand their physiology and the basis of their adaptation to permafrost. I found that permafrost harbors *Exiguobacterium* species with several metabolic and physiological features that seem to be necessary for survival and growth under subzero temperatures. Hence, the present study provides new data on the ecology, physiology and metabolism of psychrotroph microorganisms from permafrost.

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CHAPTER 1

COPING WITH OUR COLD PLANET

ABSTRACT

In our solar system, seven out of nine planets, including Earth, are considered to be cryogenic. Since water is the medium for all biochemical reactions in biological systems, changes in water properties due to cold temperatures directly affect biochemical reactions and hence, the physiology of microorganisms. Even though cold temperatures can affect survival of many microorganisms, this has not impeded cold-adapted microorganisms to successfully colonize and grow in polar, deep-sea waters and alpine regions that are at, or close to, sub-zero temperatures. In this brief review I will summarize how physical properties of water can affect the physiology of microorganisms at cold temperatures. I will also summarize the adaptations microorganisms use to live at low temperatures.

INTRODUCTION

Several factors can generate physiological stress conditions. Of all the natural stress conditions on this planet and in our solar system, cold is by far the most widespread, from the perspective of mesophilic and thermophilic organisms. In our solar system, seven out of nine planets, including Earth, are cold. For instance, 90% of the Earth's oceans have a temperature of 5°C or less. When terrestrial habitats are included, over 80% of the Earth's biosphere is permanently cold (78).

The fact that water is the major constituent of life, it is expected that the manner in which its physical properties depend on temperature must be understood to better understand the mechanisms that govern cold tolerance and acclimation. Since we inhabit a cold planet and several microorganisms found on Earth have the ability to cope with low temperatures, this makes them appear to be the Earth's most successful colonizers (28, 29). In this brief review I summarize how physical properties of water at cold

temperatures affects the physiology of microorganisms. I also summarize the mechanisms microorganisms have used to adapt to cold temperatures and hence successfully live in cold habitats.

Effects of cold on physical properties of water and on biochemical reactions. In a subzero environment, water can be supercooled or frozen (21). The first condition does not involve separation of water as a pure phase (ice). Thus supercooling conditions are not accompanied by changes in the concentrations of water-soluble components, except in rare cases where such substances might be near saturation at normal temperatures and precipitate under supercooling conditions. Supercooling conditions in aqueous systems can persist down to - 40°C and even below (21). Under cold and subzero temperatures the physical properties of liquid water exhibit substantial changes. These changes are not linear but become more pronounced at lower temperatures. Some of the changes at lower temperatures involve increase in viscosity, dielectric permittivity and pK_w; and reduction of diffusion (1). However, the most dramatic effect, at least from the biochemistry viewpoint, is the degree of ionization (35). As H^+ and OH^- ions are involved in most life reactions (condensation, hydrolysis, oxidation and reduction) it is likely that the decrease of K_w will affect equilibrium and kinetic process (21).

Besides the supercooled water condition, some cells can also endure freezing. The cooling velocity is one of the major factors that determines whether or not a cell will be viable after freezing. Cooling either too slowly or too rapidly can be damaging (59). If cooling is sufficiently slow and ice forms extracellularly, the cell is able to lose water rapidly enough by exosmosis resulting in increased concentration of intracellular solutes to maintain the chemical potential of intracellular water in equilibrium with that of

extracellular water. The result is that the cell dehydrates and does not freeze intracellulary. But if the cell is cooled too rapidly, it is not able to lose water fast enough to maintain equilibrium and can eventually freeze intracellulary. Ideally, for better cell survival at subzero temperatures, it is necessary to cool the cells slowly enough to avoid intracellular ice formation by increasing the solutes concentration intracellularly and to permit the cell to physiologically adapt to the new temperature condition (59).

Besides the fact that the change in water properties can affect cell integrity, water is also essential for most biochemical reactions. In virtually all natural environments, energy or sometimes other substrates necessary for growth (phosphates, sulfates, nitrogenous compounds, among others) is present in rate-limiting concentrations (65). Growth and survival depend, therefore, upon the ability of sequestering these sparse resources more successfully than the competing species. The reduction of the substrate diffusion rates and the increase in the viscosity of the water in the cells' environment at cold temperatures make it more difficult for the cell to acquire resources. The efficiency of active uptake at low concentration depends on substrate affinity of uptake mechanisms (65). Affinity for substrate uptake is frequently described by Monod type of saturation curves relating growth rate to the concentration of the rate-limiting substrate (32). The general paradigm is that when environmental temperatures decrease below the optimum for growth there is concomitant decreased affinity for substrates as measured by μ_{max}/K_s. The trend of decreased affinity with lowered temperatures applies to the uptake of both organic and inorganic substrates (36, 66, 70).

A common strategy used to sustain cell activity at a permanently low temperature is to produce cold-adapted enzymes with enhanced catalytic efficiency (14). In general,

extracellular enzymes for substrate uptake at low temperatures requires mainly increased specific activity (k_{cat}) (14, 20, 68). Alternatively, for secreted enzymes in marine environments or intracellular enzymes that could face low substrate concentrations, a decrease in K_m (Michaelis-Menten constant) provides a higher substrate affinity (25, 37). Enzymes are stabilized largely by noncovalent bonds, which have low energies of stabilization (80). Decreases in temperature will change intramolecular bonds, interactions with water and its associated ions, both leading to a possible compaction of the protein core and hence, a reduction of enzyme flexibility (80). Hydrogen bonds and electrostatic interactions are formed exothermically, so they are stronger at low temperatures. In contrast, hydrophobic bonds are formed endothermically and are weaker at low temperatures. These general considerations are reflected in more specific terms by changes in frequency of particular molecular bonds and hence, amino acids, in cold-active proteins, i.e., more polar and less hydrophobic residues, additional glycine residues, a lower arginine/lysine ratio, additional surface loops with increased polar residues and/or decreased proline content, lack of salt bridges, modified alpha-helix dipole interactions, reduced hydrophobic interactions between subunits, and fewer hydrogen bonds, aromatic interactions and ion pairs. All these features are not present in the same cold-active enzymes, but are some of the characteristic changes found in cold-active enzymes relative to their mesophilic/thermophilic counterparts (80). These cold-adaptive features are illustrated in several reviews comparing cold-adapted enzymes with mesophilic/thermophilic counterparts, such as lactate dehydrogenase, citrate synthase, alpha-amylase and malate dehydrogenase among others (13, 14, 37, 80).

Microbial responses to temperature stress. Cold is a physical stress that drastically modifies all physical-chemical parameters of a living cell, and thereby influences solute diffusion rates, enzyme kinetics, membrane fluidity and conformation, flexibility, topology and interactions of macromolecules such as DNA, RNA and proteins. In order to overcome these physical effects caused by cold stress, cellular modifications may occur (Table 1.1). The DNA becomes more negatively supercoiled and nucleoid - associated proteins such as, Gyrase A (gyrA) and DNA-binding proteins HU-β (*hup*B) and H-NS (*hns*) are suggested to be necessary for its relaxation (34, 81, 93). However, studies in *Escherichia coli*, demonstrated that *hns* is essential for extended growth in the cold, but not for cell survival immediately after cold shock. Additionally, null mutants for *hns* display severe morphological alterations and eventually lyse in the cold (18).

The transcription system is also affected by the cold, for instance, the transcription factor NusA is known to be involved in both termination and antitermination of transcripts in *E. coli* and it is highly expressed at lower temperatures (22, 73). Besides the transcription and translation of mRNA, the cells need to cope with all the transcripts produced. The system responsible for mRNA degradation during cold stress depends on the activity of 3'-5'Exonuclease PNPase (*pnp*) and possibly of the DeaD RNA helicase (*csd*A) (4, 96, 97). Defects in mRNA degradation produce a cold-lethal phenotype displayed by *pnp* mutants in *Yersinia enterocolica* (67) and *E. coli* (96). A DeaD RNA helicase deletion mutant of *E. coli* has reduced growth rate and filamentous growth at low temperature (34, 44).

Table 1.1. Summary of genes important for adapting to cold-shock stress

Protein	Functions	References
AceE	Decarboxylation of pyruvate (Pyruvate dehydrogenase)	(41, 45)
AceF	Dihydrolipoyltransacetylase (Pyruvate dehydrogenase)	(41)
CspA	RNA chaperone	(30, 69, 95)
CspB	RNA/DNA chaperone (?)	(54, 69)
CspE	Regulation of CspA	(3)
CspG	RNA/DNA chaperone (?)	(64, 69)
CspI	Unknown	(92)
CsdA	RNA unwinding activity	(44, 88)
DnaA	DNA binding and replication (initiation); transcriptional regulator	(2)
RbfA	30S ribosomal binding factor	(42)
InfA	Initiation factors: Binding of charged tRNA-fmet to the 30 S ribosomal subunit	(45)
InfB		(45)
PNP	Degradation of DNA	(4, 45, 57, 96)
Hsc66	Molecular chaperone	(55)
HscB	DnaJ homologue	(55)
HUβ	Nucleoid protein; DNA supercoiling	(26, 62)
Trigger Factor	Prolyl-isomerase activity and other functions	(50)
RecA	Recombination factor	(45)
GyrA	DNA topoisomerase	(33, 43, 56)
H-NS	Nucleoid-associated DNA binding protein	(18, 53, 69)
NusA	Involved in termination and antitermination	(45)
OtsA	Trehalose phosphate synthase	(49)
OtsB	Trehalose phosphatase	(49)
Desaturases	Unsaturation of membrane lipids	(33, 53, 63)
Dihydrolipoamide acetyltransferase	Decarboxylation of pyruvate	(45)
Alpha-glutamyltranspeptidase	Glutathione metabolism	(73)

For translation, ribosome assembly may be affected by cold stress, as suggested by the cold-sensitive phenotype displayed by many ribosome assembly mutants in *E. coli* and in yeast (16). In light of these considerations, it seems likely that the resumption of

this process, when interrupted by a sudden cold stress or when starting *de novo* ribosome assembly at a suboptimal temperature, requires the presence of new types and /or additional copies of proteins, RNA chaperones and helicases. Among the genes that encode ribosome-associated proteins translated during cold stress there are cold-shock protein A (*csp*A), DeaD-box RNA helicase (*csd*A), ribosome binding factor A (*rbf*A) and initiation factor 2 (IF-2). The *csp*A product was proposed to act as a chaperone to make translation more efficient during cold shock because it can bind to RNA and destabilize its secondary structure (40, 71). The gene product of *csd*A exhibits RNA helicase activity, which may help in unwinding RNA secondary structure for efficient translation at low temperature (19, 73). Initiation factor 2 (IF2) mediates the binding of charged $tRNA^{fmet}$ to the 30S subunit for initiation of translation (41). After translation of the proteins, correct protein folding in the cold could be problematic and require the presence of chaperones such as the DnaK homologue Hsc66 (55) and perhaps IF2 (10) while a direct or indirect participation of Trigger factor (TF) in this process has also been suggested (34).

Besides the production of cold induced proteins, several other known cold stress cellular responses occur to maintain cell viability, such as the cell membrane composition. To avoid membranes from becoming less fluid under cold temperatures (77, 79), cells synthesize unsaturated and branched fatty acids. This phenomenon has been studied in detail in two cyanobacteria, *Anabaena variabilis* and *Synechocystis* PCC 6803, in which mutants defective in the desaturation of fatty acids (*des*A) have a lower rate of growth at low temperature (91). Grau and collaborators (33) proposed that DNA supercoiling may regulate unsaturated fatty acid synthesis in *Bacillus subtilis* since the

desaturation of fatty acids induced after transfer to low temperature is inhibited by addition of novobiocin, an antibiotic that affects DNA gyrase activity.

Although increased unsaturation and decreased chain length of fatty acids constitute the major modifications of cell membrane, other membrane associated molecules may also play important roles in low temperature adaptation of Antarctic psychrotrophic bacteria (12, 38, 73). One such molecule is a carotenoid pigment, which remains associated with the cell membrane. Studies *in vitro* have indicated that the pigments interact with cell membrane and increase its rigidity (38). Since a large number of Antarctic bacteria have been found to contain carotenoid pigments in their membrane, it has been speculated that these pigments may have a role in buffering membrane fluidity when there is an up shift in environmental temperature (38). In such an up-shift, the caroteinoid pigments may maintain the homeoviscosity of membrane prior to the *de novo* synthesis of saturated fatty acids. However, experimental evidence is still lacking for this hypothesis.

Cells undergo many cellular modifications in order to survive and grow at extreme temperatures. A network of genes that are activated simultaneously or in cascade fashion generates these modifications. However, how the cascade of genes is activated and the effect of some of these genes in different microorganisms is unexplored, especially in psychrotrophs and psychrophilic microoganisms.

Biodiversity in cold habitats. The lowest temperature reported for microbial activity is -20°C in permafrost soil and sea ice (15). Cold-adapted microorganisms have been found in Antarctic sub-glacial soil and permanently ice-covered lakes, cloud droplets, ice cap cores from considerable depth, snow, glaciers and cold deep-sea environments (17, 85).

Both Bacteria and Archaea have been surveyed in polar and several other cold environments (9, 11, 24, 51, 90). The Bacteria are usually present in greater number and diversity than the Archaea. The exceptions are the cold interior and deep waters of the ocean, well below the upper mixed layer. There, the Archaea appear in numbers elevated above their counterparts in surface waters (17, 72) and above the Bacteria at the same depths (51). In the deep Pacific Ocean, the populations of Crenarchaeota largely account for the Archaeal with population densities matching or exceeding the Bacterial densities (51).

Even though most of the biodiversity studies in cold habitats focus on the domain Bacteria, this domain still holds surprises, since conventional cultivation produces isolates from cold environments that grow at -1°C, containing many novel 16S rRNA sequences (46, 52). Most isolates from Arctic and Antarctic permafrost were psychrotrophic rather than psychrophilic, although mesophiles and even a limited number of thermophiles were detected (85). A wide diversity of bacteria including aerobic and anaerobic heterotrophs, methanogens, iron reducers, sulfate reducers, nitrifying and nitrogen-fixing bacteria representing over 30 bacterial genera, including *Arthrobacter*, *Aeromonas*, *Bacillus*, *Cellulomonas*, *Exiguobacterium*, *Flavobacterium*, *Micrococcus*, *Myxococcus*, *Nitrobacter*, *Pseudomonas*, *Psychrobacter*, *Rhodococcus*, *Streptomyces*, and others have been isolated from Arctic permafrost (29, 70, 76, 82, 90). In Antarctic permafrost the genera *Arthrobacter*, *Bacillus*, *Streptomyces* were predominant (85), even though *Exiguobacterium* (23) and *Psychrobacter* (8, 83, 84), among others, were also found.

The interface between the solid and liquid phase has the coldest ice forms so far examined (sea ice at -20°C) and it is called eutectic interface. Microscopic studies *in situ* have shown that living bacteria can be found in this eutectic interface (47), these microorganisms are called eutectophiles (17). Kanavarioti and collaborators (48) by exploring ice eutectic phase, found that the ice matrix with its concentrated solutes in the spaces between ice crystals can propitiate oligonucleotide polymerization reactions. These findings suggest that microorganisms may be able to survive and have metabolic activity in these eutectic interfaces.

Besides, Archaea and Bacteria, viruses are also found in cold environments, however this domain is the least explored in cold habitats. A marine virus-host system, unique in its cold adaptation, was obtained from Arctic sea ice (5, 6, 89, 94). This knowledge gives us insight into the dynamics of polar microbial ecosystems and explores lateral gene transfer at cold temperatures.

Conclusions. Understanding how cold adapted microorganisms survive cold temperatures is of ecological importance, as well as biotechnological and evolutionary significance. Ecologically, these microorganisms are vital to nutrient recycling and for production and consumption of greenhouse gases (86). The fact that these organisms can survive in permanently frozen environments may indicate that they possess unique mechanisms that allow them to maintain viability for very long periods of time. Also, some enzymes may have value in biotechnology. Low temperature enzymes may be used in industry, agriculture, bioremediation, and in food microbiology for preservation or avoidance of food spoilage (31). Additionally, recent evidence of massive amounts of shallow ground ice near the surface of Mars (7, 85) and the realization that the majority

of cosmic bodies beyond Earth are cold have made cold terrestrial environments significant extraterrestrial analog sites (27, 39, 74, 75). Studies in frigid environments on Earth will aid in the testing and refinement of instruments and procedures for looking for life on other planets, in the development of overall concepts for cold-adapted biology, and will allow the collection of baseline data for comparison to results from Mars (60).

Microorganisms that are able to survive in frozen soil on our planet and on other planets may have ancient traits since they have very low metabolic activity at such temperatures and consequently have evolved more slowly than mesophilic counterparts. Therefore, analysis of their features may be a window into microbial life as it was several million years ago. Even though studies in cold environments are advancing rapidly, the complete understanding of the microbial community and its functions is still in its infancy.

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CHAPTER 2

BIOGEOGRAPHY, DIVERSITY AND ABUNDANCE OF *EXIGUOBACTERIUM* SPP. AND *PSYCHROBACTER* SPP.

ABSTRACT

The biogeography and phylogenetic diversity of *Psychrobacter* and *Exiguobacterium* was studied in 54 soil and sediment samples from polar, temperate and tropical environments to determine their habitat preferences. Quantitative PCR revealed that both genera were more prevalent in but not restricted to the polar environments. Three sites from each climatic region and with high abundance of both genera were selected to build 16S rRNA gene libraries. The clone libraries were analyzed using FastGroupII, DOTUR and *∫*-libshuff to determine their diversity, structure and similarity. *Exiguobacterium* spp. and *Psychrobacter* spp. both had patchy distributions, which showed correlation to salinity, pH, potassium and Cu.

INTRODUCTION

Ecologists describing microbial biogeography typically invoke Baas-Becking's statement: "Everything is everywhere, but, the environment selects" (7). Few studies have, however, attempted to verify this statement (14, 20, 35), as well as, to determine which environmental factors exert the strongest influences on a population's distribution in nature (12, 21). It recently has been argued that the ubiquitous distribution of some extremophile species is implausible due to barriers to their surviving dispersal (19, 35). Studies on biogeography of microbes from hot springs or geothermal sources have shown endemism (21, 35). Studies with sea ice bacteria indicate that members of some genera occur at both poles but the species found in the poles are not in warmer environments, hence at the species level they are not cosmopolitan (30).

Considering that 80% of Earth's biosphere is permanently cold, it is surprising that few biogeography studies have been done with microorganisms from cold

environments (30). Among the cold habitats of our planet, microorganisms inhabiting permafrost are strong candidates for biogeography and biodiversity studies. Permafrost microorganisms have been living in frozen Siberian sediments for as long as 2 to 3 million years, conditions that have prevented gene flow from any outside biota (34) and prevented dispersal to other environments.

Previous studies in Siberian permafrost soils yielded isolates of *Exiguobacterium* and *Psychrobacter* from geological strata frozen for 20 thousand to 3 million years (1, 23, 33). Another study demonstrated ubiquity of the *Exiguobacterium* genus in the Siberian permafrost (24). In addition to Siberian permafrost, species of *Exiguobacterium* and *Psychrobacter* have also been isolated from several other cold environments, such as Antarctica, sea ice and the Himalayan mountains (2, 3, 28, 29) . The fact that they seem to be successful in cold environments and that one is a Gram-positive (*Exiguobacterium*) and the other is a Gram-negative (*Psychrobacter*), make them good models to test Baas-Becking's statement. The results show that these genera are not resctricted to the poles, but are favoured in cold and polar environments.

MATERIAL AND METHODS

Sample collection. A total of six sediments and forty-eight soil samples were collected from a wide range of ecosystems with diverse climates and site characteristics (Table 2.1). All samples were stored frozen at -20^{o}C until processed.

Table 2.1. Location and information of sampling sites.

Sample	Description Sample Sites	Age (years)	Depth (m)	Geographic Coordinates
P1	Puerto Rico: Ballena beach	Recent	0 to 0.1m	17° 57.243'N, 66° 50.858'W
P2	Puerto Rico: Carmelitas underground cave system - Ortencia cave	Recent	0 to 0.1m	18° 26'30''N 66° 21'15''W
P3	Puerto Rico: Carmelitas underground cave system - Reina cave	Recent	0 to 0.1m	18° 26'30''N 66° 21'15''W
P4	Puerto Rico: Ceiba mangrove	Recent	0 to 0.1m	18°13'40.40"N 65°35'21.31"W
P5	Puerto Rico: San Juan mangrove	Recent	0 to 0.1m	18°21'06.85"N 65°36'53.22"W
P6	Puerto Rico: Carmelitas underground cave system - Murciélago cave	Recent	0 to 0.1m	18° 26'30''N, 66° 21'15''W
P7	Puerto Rico: Guanica dry forest	Recent	0 to 0.1m	17°58'N, 66°51'W
HW	Hawaii: Kohala forest	Recent	0 to 0.1m	20°03'N, 155°41'E
B1	Brazil: Forest, Guanabara II, 1-5	Recent	0 to 0.2 m	4° 24' 20.0'' S, 69° 54' 27.7''W
B2	Brazil: Forest, Guanabara II, 1-10	Recent	0 to 0.2 m	4° 24' 20.0''S, 69° 54' 27.7''W
B3	Brazil: Forest, Guanabara II, 1-16	Recent	0 to 0.2 m	4° 24' 20.0''S, 69° 54' 27.7''W
B4	Brazil: Pasture, Benjamin Constant, 6-84	Recent	0 to 0.2 m	4° 22' 24.2''S, 70° 01' 39.4''W
B5	Brazil: Pasture, Benjamin Constant, 6-91	Recent	0 to 0.2 m	4° 22' 24.2''S, 70° 01' 39.4''W
B6	Brazil: Pasture, Benjamin Constant, 6-92	Recent	0 to 0.2 m	4° 22' 24.2''S, 70° 01' 39.4''W
M1	Michigan: KBS deciduous Forest - DF-1	Recent	0 to 0.1m	42° 24' N, 85° 24' W
M2	Michigan: KBS deciduous Forest - DF-2	Recent	0 to 0.1m	42° 24' N, 85° 24' W
M3	Michigan: wheat plantation KBS -T1R1	Recent	0 to 0.1m	42° 24' N, 85° 24' W
M4	Michigan: wheat plantation KBS -T1R2	Recent	0 to 0.1m	42° 24' N, 85° 24' W
M5	Michigan: mid-successional community KBS -T8R1	Recent	0 to 0.1m	42° 24' N, 85° 24' W

Table 2.1 (cont'd).

M6	Michigan: mid-successional community KBS –T8R2	Recent	0 to 0.1m	42° 24' N, 85° 24' W
M7	Michigan: Baker Woodlot, Rachana Rajendra Bird Sanctuary – MSU	Recent	0.1m	42°44'N, 08°42'W
IA	Iowa: Prairie wetland, Jasper county	Recent	0 to 0.1m	41°48'N, 09°30W
A1	Antarctica: Bratina Island – fresh pond	Recent	0 to 0.1 m	78° 01'S, 165° 32'E
A2	Antarctica: Bratina Island – brack pond	Recent	0 to 0.1 m	78° 01'S, 165° 32'E
A3	Antarctica marine sediment: Commandante Ferraz station	Recent	20m	62° 05,1255'S, 58° 23,390'W
A4	Antarctica marine sediment: Punta Ulmann	Recent	20m	62° 04,91' S 58° 20,15' W
A5	Antarctica marine sediment: Botany Point	Recent	20m	62° 05,845'S, 58° 20,144'W
A6	Antarctica marine sediment: Macchu Picchu	Recent	30m	62° 05,4317' S 58° 28,5578' W
A7	Antarctica marine sediment: Arctowski	Recent	20m	62° 10,3337' S 58° 31,3476' W
A8	Antarctica marine sediment: Arctowski	Recent	30m	62° 10,3337' S 58° 31,3476' W
S1	Siberian Permafrost: Kolyma Lowland, Cape Chukochii, East Siberian Sea coast, tundra zone	5-10 tds	2.55m	70°05'N, 159°55'E
S2	Siberian Permafrost: Kolyma Lowland, Cape Chukochii, East Siberian Sea coast, tundra zone	5-10 td	8.55m	70°05'N, 159°55'E
S3	Siberian Permafrost: Kolyma Lowland, Cape Chukochii, East Siberian Sea coast, tundra zone	5-10 td	14.95m	70°05'N, 159°55'E
S4	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	5-10 td	6.3m	129° 30'E, 71° 40'N
S5	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	5-10 tds	6.8m	130° 00'E, 72° 00'N
S6	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	5-10 td	3m	130° 00'E, 72° 00'N

Table 2.1 (cont'd)

S7	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	5-10 td	7.5m	130° 00'E, 72° 00'N
S8	Siberian Permafrost: Kolyma Lowland, Cape Chukochii, East Siberian Sea coast, tundra zone	20-30 td	20m	70°05'N, 159°55'E
S9	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	12-40 td	2.8m	129° 30'E, 71° 40'N
S10	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	12-40 td	8m	129° 30'E, 71° 40'N
S11	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	12-40 td	13m	129° 30'E, 71° 40'N
S12	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	12-40 td	23.3m	129° 30'E, 71° 40'N
S13	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	12-40 td	33m	129° 30'E, 71° 40'N
S14	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	12-40 td	37.5m	129° 30'E, 71° 40'N
S15	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	15-20 td	4.8m	130° 00'E, 72° 00'N
S16	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	15-20 td	8.15m	130° 00'E, 72° 00'N
S17	Siberian Permafrost: Kolyma Lowland, forest-tundra zone, left bank of Kolyma river, Khalarchinskaya tundra sandy plain	20 td	11m	68°50'N, 161°02'E
S18	Siberian Permafrost: Cape Svyatoi Nos, Laptev Sea coast, tundra zone	300-400 td	22.5m	140°10'E, 72°55'N
S19	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	3 million	18.1m	130° 00'E, 72° 00'N
S20	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	3 million	24m	130° 00'E, 72° 00'N

Table 2.1 (cont'd)

S21	Siberian Permafrost: Cape Bykovskii Laptev Sea coast, tundra zone	3 million	24.4m	130° 00'E, 72° 00'N
S22	Siberian Permafrost: Kolyma Lowland, Krestovka River, right inflow of Kolyma River (north taiga)	2-3 million	16.5m	66°50'N, 62°30'E
S23	Siberian Permafrost: Kolyma Lowland, tundra zone, Mouth of Malaya Kon'kovaya River, right bank	2-3 million	18.7m	66°50'N, 62°30'E
S24	Siberian Permafrost: Kolyma Lowland, tundra zone, Mouth of Malaya Kon'kovaya River, right bank	2-3 million	19.9m	66°50'N, 62°30'E

KBS: Kellog biological station, MSU: Michigan State University

DNA extraction. Total community genomic DNA was extracted using the Fast DNA SPIN kit for soil (Bio 101, Q BIOgene) with some modifications, namely the first step of the extraction was a grinding step using liquid nitrogen followed by treatment with 100 µl of lysozyme (50 µg μl^{-1}) at room temperature before using the kit. The quality of the extracted DNA was analyzed by electrophoresis on a 1% agarose gel. DNA concentrations were measured by absorbance at 260 nm.

Quantitative real-time PCR assays (Q-PCR). The primer sets for *rpo*B, *gyr*B and hypothetical genes and conditions for *Exiguobacterium* spp. quantitative real-time PCR were described previously (24). Detection was only considered positive when at least two genes were amplified by these primer sets. Data are represented as copy number averages and standard deviations. The primers used for *Psychrobacter* spp. quantification were 432-F (5'-GCA CTT TAA GCA GTG AAG AAG A- 3') and 476-R (5'-TAT TCT GCA GCT AAT GTC ATC G- 3'). These primers were designed by Dr. Hector Ayala-del-Rio. The reaction mixture contained 1X SYBR green PCR buffer, 1.75 mM $MgCl_2$,

0.2 mM dNTPs, 0.05 U μl^{-1} Amplitaq Gold, 0.15 μM and 0.4 mg ml^{-1} of bovine serum albumine (BSA) and the amplification settings were the standard for real-time PCR with annealing and extension at 59°C for 1 min. The real-time PCR reactions and primer optimizations for both genera were done as described previously (24). The increase in fluorescence emission was monitored during PCR amplification using the 7700 Sequence Detector (PE Applied Biosystems). The C_T values obtained from each sample were compared with the standard curve to determine the initial copy number of the target gene.

Principal component analysis was performed with 22 samples described in Table 2.2. The variables for the principal component analysis consisted of Q-PCR results for each genus as well as nine environmental variables selected among 14 variables measured (phosphorus, potassium, magnesium, calcium, cation exchange capacity, copper, manganese, zinc, iron, pH, organic matter, moisture, soluble salts and average annual temperature) that had statistically significant Pearson correlation with at least another variable. The variables were transformed by Box-Cox transformation to improve normality of the sample, the principal component analysis was performed with a correlation matrix and the results were displayed by a correlation biplot. The program XLSTAT was used for data transformation and principal component analysis.

Genus specific clone library construction and sequencing. Primers for conserved regions of the 16S rRNA genes for the genera *Exiguobacterium* spp. and *Psychrobacter* spp. were designed using ARB based on alignments of reference strains for each genus and 50 other Gram-positive and Gram-negative microorganisms (16).

Table 2.2. Physicochemical results from the sites used for the principal component analysis

Samples[a]	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	CEC (meq/ 100g)	Cu (ppm)	Mn (ppm)	Zn (ppm)	Fe (ppm)	pH	O.M.[b] %	Moisture %	Salinity (mmhos)	Mean Annual T°C
A3	19	726	1017	6185	41.3	28.1	46.7	6.2	1230.6	7.5	0.70	37.10	11.75	-2
A5	400.7	549	6.3	3.3	11	3.5	127.5	1.3	960.6	8.4	2	31.16	6.51	-2
S1	34	358	582	3685	24.2	7.7	159.8	7.8	3515.4	7.5	1.80	16.60	8.23	-10
S23	8	89	600	1770	14.1	5.1	154.8	6.2	479.7	7.7	3.30	39.90	1.53	-10
S4	34	86	351	543	5.9	7.1	103.3	8.9	2538	7.8	2.50	19.00	0.26	-10
M1	70	44	88	427	11.4	1.6	95.3	5	38.7	5.1	6.10	21.30	0.11	9.7
M2	22	94	61	307	10.7	1.3	55.8	5	52.2	4.6	5.80	20.40	0.23	9.7
M3	29	88	140	553	5.4	2	37.3	2.5	35	5.3	2.50	13.10	0.07	9.7
M4	34	97	187	611	6.1	1.4	42.1	2.7	43.2	5.4	2.70	12.70	0.08	9.7
M5	37	163	119	635	10.6	2.3	104.4	6.2	41.7	5.6	8.25	27.40	0.16	9.7
M6	46	157	124	691	8.5	1.9	90	5.1	49.1	5.7	7.60	25.80	0.14	9.7
M6	82	56	18	169	17.9	3.5	33.2	3.1	133.7	4.3	2	10.20	0.11	7.8
IA	12	192	444	2063	18.1	5.3	14.3	3	25.8	6.4	4.40	17.70	0.21	7.8
B1	4.9	74	2.7	4.9	12.2	1.2	57.7	5.4	158	4.7	1.50	37.69	0.08	26.1
B2	5.5	47	1	3	10.2	1.3	137.7	2	208.8	4.7	1	36.87	0.13	26.1
B3	4.6	53	1.6	4.3	10.2	1	72.1	17.9	191	4.5	2.20	44.71	0.1	26.1
B4	3.1	67	2.1	1.8	6.4	2.1	20.9	2.5	300.9	5.1	1.80	31.34	0.06	26.1
B5	2.5	30	0.6	1.5	4	1.4	59	3.1	798	5.3	1.30	39.44	0.06	26.1
B6	2.5	48	2.2	2.8	9	2.3	28.6	10.6	558.5	4.9	1.60	40.68	0.06	26.1
HW	7	118	68	143	8	2.9	11.6	3.3	366.2	5	52.10	62	0.21	23.9
P3	8	101	130	4654	24.6	58.2	1.3	1.6	2.3	7.3	3.90	11.50	2.94	26
P5	5	843	2511	6185	55.2	28.5	49.9	4.6	812.8	6	1.50	33.70	15.27	26

[a]See table 1 for sample code; [b] O.M.% = percentage of organic matter in the sample

The annealing temperatures for *Exiguobacterium* spp. and *Psychrobacter* spp. primer sets were 59°C and 57°C, respectively. Amplification, for all primers, was by standard procedures, except $MgCl_2$ concentrations that were optimized to 1.75 mM for *Psychrobacter* spp. and 1.5 mM for *Exiguobacterium* spp. (8). Primer sequences used for the 16S rRNA gene were 432-F and 823-R (5'-TCA AGG GAC CCA ACG ACT AGT A- 3') for *Psychrobacter* spp. and Exi16S-F (5'- GAT GAA AGG CGC TYC GGC G- 3') and Exi16S-R (5'- CGG TCA RGG GGA TGT CAA GAG TT- 3') for *Exiguobacterium* spp. Amplicon sizes were approximately 400 bp and 800 bp, respectively.

The specific 16S rRNA gene amplicons for each genus were cloned using the TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies) and sent to Macrogen Inc. (Seoul, Korea; www.Macrogen.com) for sequencing with primers for the cloning vector (M13F). Sequences generated in the present study were deposited in GenBank.

Clone library analysis. The 16S rRNA gene sequences from the clone libraries were processed by using the pipeline quality filter tools on the Ribosomal Database Project (RDP-II) website (5) (http://rdp.cme.msu.edu). The Classifier tool provided by RDP-II was used to assign the 16S rRNA gene sequences to the taxonomical hierarchy. Aligned sequences assigned to *Exiguobacterium* or *Psychrobacter* were downloaded from RDP-II for further analyses. The aligned sequences were uploaded to FastGroupII program (37) to cluster sequences with 99% similarity including gaps. One representative of each dominant cluster was used to construct the phylogenetic tree within MEGA 3.1

environment (15) by the neighbor-joining method. The robustness of the inferred tree was evaluated by applying 1000 bootstrap re-samplings.

Distance matrix files for each clone library were downloaded from the RDP-II pipeline to determine alpha diversity through Distance-Based Operational Taxonomic Unit and Richness (DOTUR) program (26), and to compare clone library similarity via statistical tests provided by ∫-libshuf program (27).

RESULTS AND DISCUSSION

Distribution and abundance data of *Exiguobacterium* spp. and *Psychrobacter* spp. show that both genera are more commonly found in both Antarctic and Arctic samples than in the non-polar samples (Figure 2.1). *Psychrobacter* spp., however, seems to be more successful in Antarctica than *Exiguobacterium* spp., as the quantity of *Psychrobacter* spp. is higher in Antarctica than in the Siberian permafrost. The Antarctic samples, however, are both from non-frozen sediments: ponds A1 and A2, and coastal marine sediments A3 to A8, which had the highest population densities. In temperate (Michigan and Iowa) and in tropical (Brazil, Puerto Rico and Hawaii) environments, the distribution and abundance of *Psychrobacter* spp. was patchy and when present, in very low density. For *Exiguobacterium* spp. the distribution was also patchy but when present its abundance was similar to that found in the colder habitats. The fact that both genera were not found in most of the non-polar soil samples suggests that they are not cosmopolitan, in the strict sense; however they are not restricted to the poles as previously suggested for some polar bacteria (30).

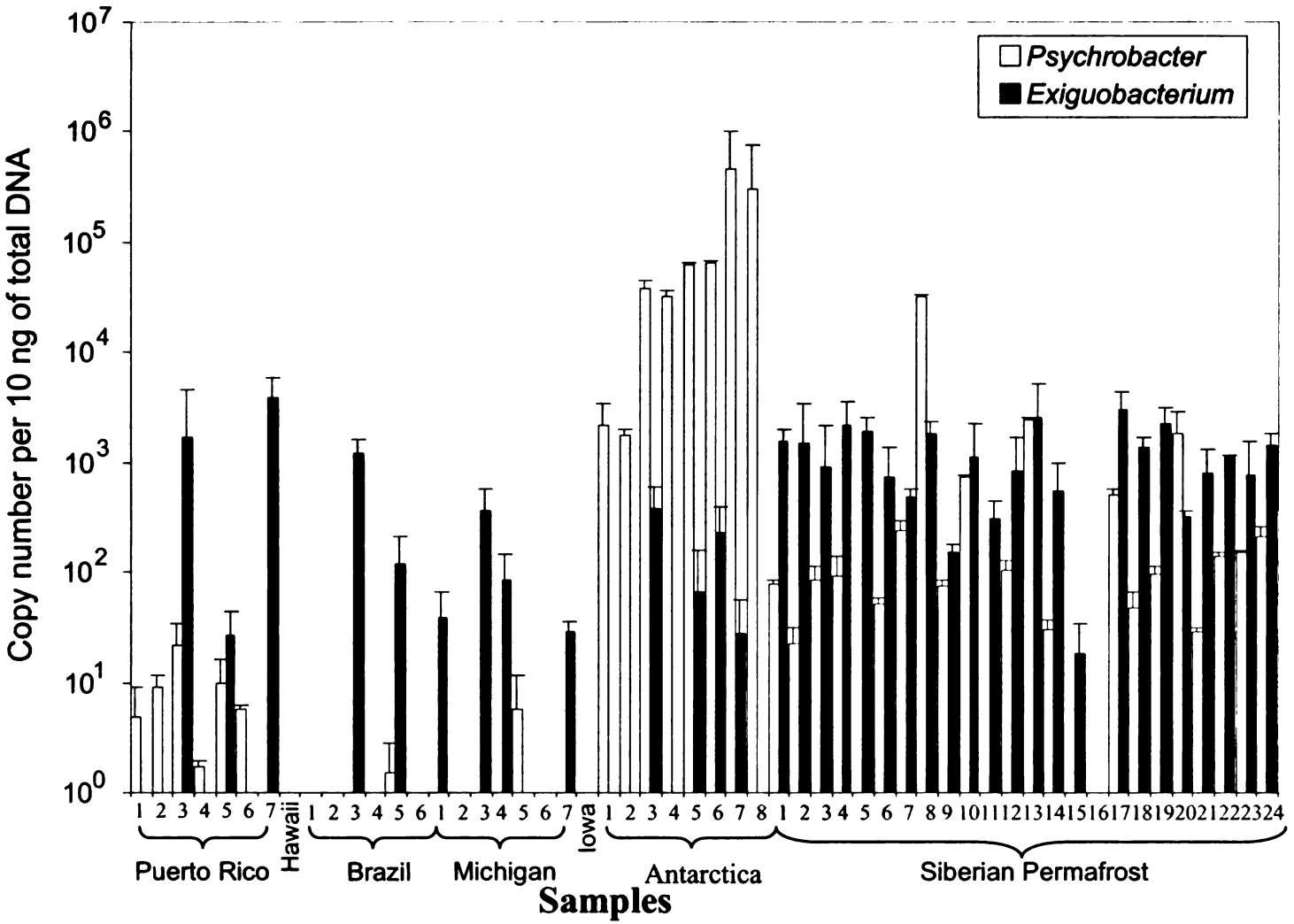

Figure 2.1. *Exiguobacterium* spp.and *Psychrobacter* spp. gene abundance in various habitats as measured by Q-PCR. The sample number corresponds to the sample numbers in Table 1.

The patchy distribution of these microorganisms correlates with some physico-chemical factors of their environment (Figure 2.2). *Exiguobacterium* spp. and *Psychrobacter* spp. populations have a high correlation with pH and salinity, as well as potassium (K^+), cation exchange capacity (CEC) and the micronutrient copper (Cu).

Copper is an essential micronutrient for respiring bacteria, e.g. cytochrome c oxidase and some peroxidase dismutases (17); both genera are aerobes. The fact that pH showed a strong correlation with the presence of these genera is not surprising, since this is a well known population determinant. Soils with near-neutral pH have from 26% to 60% higher richness than more acidic soils (9). *Exiguobacterium* spp. and *Psychrobacter* spp. abundance seem to follow the same pattern in regard to pH, preferring neutrality.

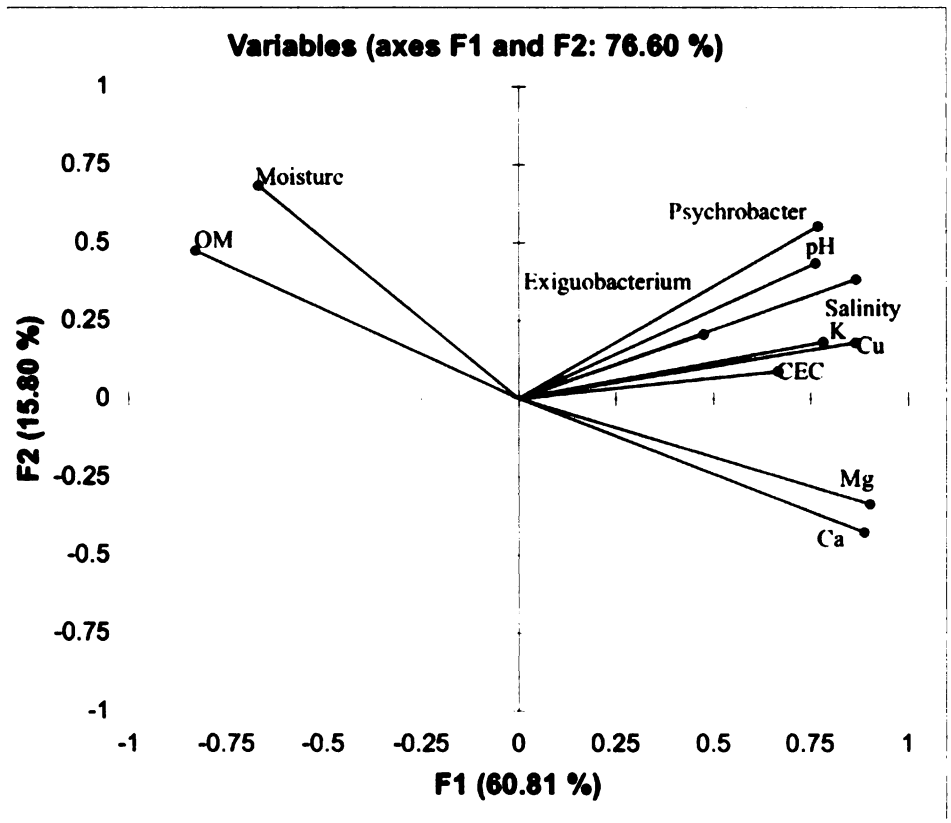

Figure 2.2. Correlation biplot from physicochemical characteristics of principal component analysis

Potassium is the principal cation in bacteria, and it not only plays a role as a cofactor for many enzymes and ribosomes, but is also responsible for pH and osmotic homeostasis (36). Environments with pH 6 to 8, which seems to be the optimum environmental pH range for these genera, triggers K^+ influx to raise the intracellular pH (36). This physiological need for K^+ to maintain the cell pH homeostasis, could explain their preference for higher environmental concentrations of K^+.

The effect of salinity on the abundance of these genera can be explained by their physiology. Studies with *Psychrobacter* spp. and *Exiguobacterium* spp. isolated from the Siberian permafrost have shown that these genera can tolerate increased osmolarity and showed associated changes in membrane composition, cell morphology and size (22). Besides, all isolates from *Exiguobacterium* spp. and *Psychrobacter* spp. can grow with the addition of NaCl at various concentrations in their medium (6, 23, 25, 28). This evidence suggests that these genera are adapted to salinity, which would explain the strong correlation of the presence and high abundance of these genera to moderate to high salinity habitats, i.e. Antarctic marine sediments, the Puerto Rican mangroves and the Siberian permafrost, which due to its frozen water status has low water activity (22).

While these genera are not restricted to the poles, it may be that species or subspecies may be specially adapted to particular temperature regions. We examined OTU (Operational Taxonomic Unit) diversity patterns from very different geographic locations for each genus in which they were abundant. The number of clones for each site was determined by rank abundance curves, which reached a plateau (data not shown) and ensured that most phylotypes were sampled (Table 2.3). To quantitatively measure diversity in the samples (Table 2.3), we used the Shannon and Simpson indices and

Chao1 estimator (11, 13, 18). The data from all the indices (Table 2.3), as well as the abundance of OTUs in each site (Figure 2.3) depicted similar results, showing that the diversity within each genus and sampling site was different. Even though in some sites the quantity of *Exiguobacterium* spp. was higher than of *Psychrobacter* spp. (Figure 2.1), the diversity within *Exiguobacterium* was never higher than for *Psychrobacter* (Figure 2.3). Furthermore, the results indicate an apparent latitudinal gradient in diversity at the species level for both genera: *Psychrobacter* spp. and *Exiguobacterium* spp. were more diverse in the Antarctica samples with less diversity in Michigan and even less diversity in Puerto Rican samples (Figure 2.3 and Table 2.3). Similar distance decay relationships are seen with macroorganisms (31) and with *Pseudomonas* genotype, in which genetic similarity of *Pseudomonas* isolates was negatively correlated with geographic distance at regional distances, but not at continental scales (4, 10). The higher abundance and diversity of these genera at the poles than in the other regions suggest that the preferred habitats for these genera are the cold, polar environments.

Table 2.3. Diversity comparisons of 16S rRNA environmental clone libraries

Samples[a]	Genus studied	Total No of sequences	No. of OTUs	Shannon Index	Simpson Index (D)	Chao 1 estimator
A3	*Psychrobacter*	129	21	2.60	0.09	21.43
M5	*Psychrobacter*	150	14	2.22	0.13	17.00
P5	*Psychrobacter*	137	11	1.84	0.21	11.50
A5	*Exiguobacterium*	149	7	1.53	0.26	7
M7	*Exiguobacterium*	109	4	0.34	0.84	5
P3	*Exiguobacterium*	135	1	0	1	1

[a] See table 2.1 for sample code

The result obtained by ∫-libshuff verifies whether two samples are drawn from the same populations or whether one is a subset of the other. The Puerto Rican and Michigan clone libraries for *Psychrobacter* displayed significantly different populations from these

from Antarctica (Figure 2.4). However, the *J*-libshuff analysis for Puerto Rico and Michigan clone libraries for *Psychrobacter* spp. revealed that the Puerto Rican population is a subset of the Michigan population, i.e., they have many OTUs in common; however, Michigan has more diverse OTUs. All the *Exiguobacterium* from the different sites had significantly different populations, i.e. all samples had different OTUs.

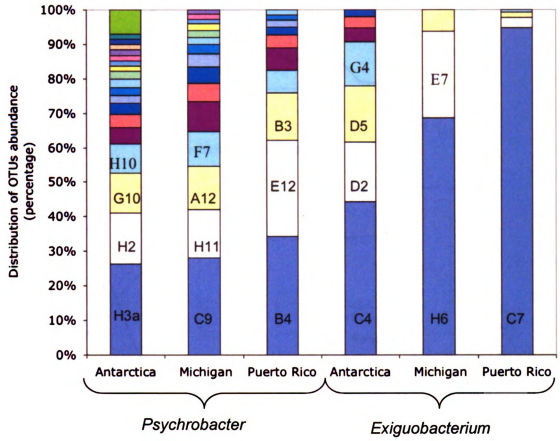

Figure 2.3. Distribution of OTUs abundance in different soils. The colors are ranking the most abundant OTU to the least abundant. The labels correspond to the clones represented in the phylogenetic trees for each genus.

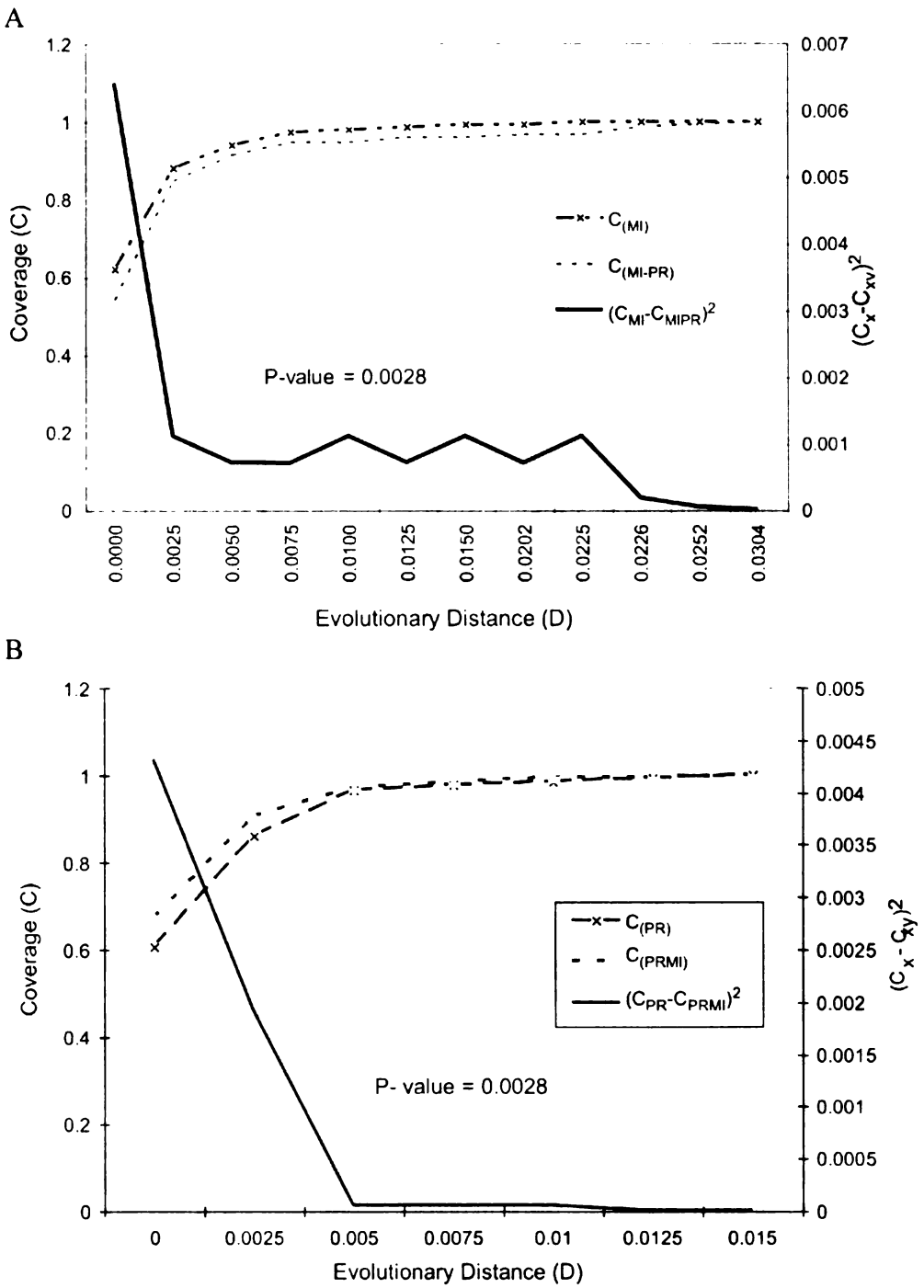

Figure 2.4. Results of selected ʃ-LIBSHUFF comparisons. Homologous (C_x) and heterologous (C_{xy}) coverage curves for 16S rRNA gene sequence libraries from *Psychrobacter* spp. in Michigan and Puerto Rico. Solid lines indicate the value of $(C_x-C_{xy})^2$ for the original samples at each value of Evolutionary Distance (D). D is equal the Jukes-Cantor evolutionary distance determined by RDP-II pipeline. Broken lines indicate

the 950th value (or P=0.05) of $(C_x-C_{xy})^2$ for the randomized samples. **(A)** Comparison of *Psychrobacter* clones from Michigan soil (x=MI) and from Puerto Rico soil (y=PR). **(B)** Comparison of *Psychrobacter* clones from Puerto Rico soil (x=PR) and from Michigan soil (y=PR).

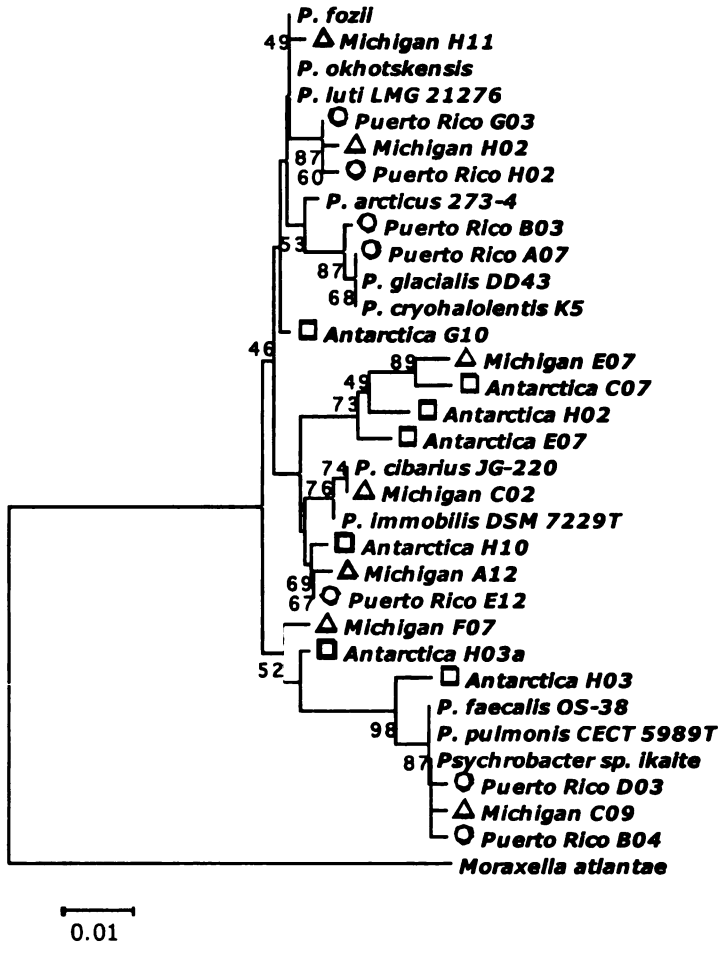

Figure 2.5. Phylogenetic tree based on 16S rRNA gene sequences of *Psychrobacter* strains and the top seven dominant OTUs in the clone libraries from Michigan (△), Puerto Rico (O) and Antarctica sediment (□). The trees were produced by the neighbor-joining method and rooted using the 16 S rRNA gene from *B. subtilis*. The nodes with less than 45% bootstrap values were not added to the tree. The scale bar represents changes per nucleotide.

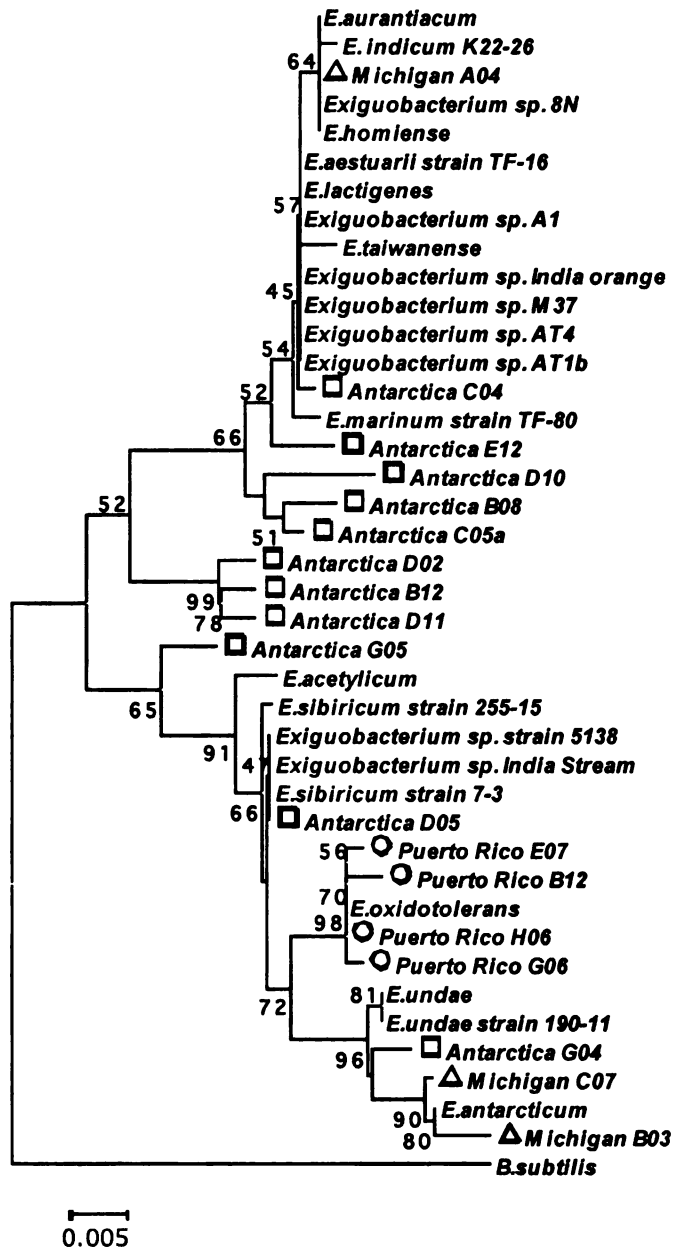

Figure 2.6. Phylogenetic tree based on 16S rRNA gene sequences of *Exiguobacterium* strains and clone libraries from Michigan (△), Puerto Rico (O) and Antarctica sediment (□). The trees were produced by the neighbor-joining method and rooted using the 16S rRNA gene from *B. subtilis*. The nodes with less than 45% bootstrap values were not added to the tree. The scale bar represents changes per nucleotide.

The phylogenetic trees from *Psychrobacter* spp. and *Exiguobacterium* spp. show that new species are yet to be identified, especially in Antarctica. There are many clusters

of clones that do not contain any known described species (Figure 2.5, 2.6). The *Psychrobacter* spp. tree, confirms the results from ∫-libshuff that Michigan and Puerto Rico share similar OTUs, since several clusters contain clones from both habitats. Additionally, some Puerto Rican clones also contained OTUs with high similarity to isolates from the Siberian permafrost, such as *P. cryohalolentis* K5 (1) but also to isolates found in warmer habitats, such as *P. pulmonis* (32) that was isolated from lamb lungs.

In the case of *Exiguobacterium* spp. all the Puerto Rican clones clustered with *E. oxidotolerans* isolated from a fish processing plant, a strain that tolerates 12% of NaCl, pH values from 7 to 10 and is a mesophilic (38). One group of clones from Antarctica had identical sequence to several isolates from the Siberian Permafrost, such as *E. sibiricum* strains 7-3 and 255-15, *Exiguobacterium* sp. strain 5138 (23), confirming that both poles share similar species. The *Exiguobacterium* phylogenetic tree also confirmed the results from ∫-libshuff, since the clones from the diverse geographic locations never clustered with one another.

This study shows that *Exiguobacterium* spp. and *Psychrobacter* spp. are more commonly found and have higher densities in polar regions, but they can be detected in temperate and tropical sites. The later is more likely the case when physicochemical conditions such as salinity, K^+ and pH are similar to these in their polar sites. In the context of the Baas-Becking statement, the data suggest that *Exiguobacterium* and *Psychrobacter* have been widely distributed in diverse sites but polar and other low water activity environments favor their growth.

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CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF *EXIGUOBACTERIUM* ISOLATES FROM THE PERMAFROST

Rodrigues, D. F., J. Goris, T. Vishnivetskaya, D. Gilichinsky, M. F. Thomashow, and J. M. Tiedje. 2006. Characterization of *Exiguobacterium* isolates from the Siberian permafrost. Description of *Exiguobacterium sibiricum* sp. nov. Extremophiles **10:**285-94.

ABSTRACT

Three Gram-positive bacterial strains, 7-3, 255-15 and 190-11, previously isolated from Siberian permafrost, were characterized and taxonomically classified. These microorganisms are rod-shaped, facultative aerobic, motile with peritrichous flagella and their growth ranges are from -2.5°C to 40°C. The chemotaxonomic markers indicated that the three strains belong to the genus *Exiguobacterium*. Their peptidoglycan type was A3α L-Lys-Gly. The predominant menaquinone detected in all three strains was MK-7. The polar lipids present were phosphatidyl-glycerol, diphosphatidyl-glycerol and phosphatidyl-ethanolamine. The major fatty acids were iso-C13:0, anteiso-C13:0, iso-C15:0, C16:0 and iso-C17:0. Phylogenetic analysis based on 16S rRNA and six diverse genes, *gyr*B (gyrase subunit B), *rpo*B (DNA-directed RNA polymerase beta subunit), *rec*A (homologous recombination), *csp* (cold shock protein), *hsp*70 (ClassI-heat shock protein – Chaperonin) and *cit*C (isocitrate dehydrogenase), indicated that the strains were closely related to *E. undae* (DSM 14481^T) and *E. antarcticum* (DSM 14480^T). On the basis of the phenotypic characteristics, phylogenetic data and DNA-DNA reassociation data, strain 190-11 was classified as *E. undae*, while the other two isolates, 7-3 and 255-15, comprise a novel species, for which the name *Exiguobacterium sibiricum* sp. nov. is proposed.

INTRODUCTION

Temperature is a major environmental condition that affects microbial physiology and growth. Viable microorganisms in permafrost survive and perhaps metabolize in their continuously frozen habitat. Earth's permafrost is characterized by low carbon

availability, low water availability and continuous exposure to gamma radiation (0.03 rad/year) originating from the soil minerals. Despite these challenging conditions, microorganisms in several microbial groups remain viable for 20,000 to 3 million years. Many of the studies demonstrating this point were performed by Russian scientists in the Kolyma Lowland, region of northeast Siberia. This area has tundra vegetation and an Arctic climate with a mean annual air temperature of -13.4°C and an annual precipitation of 229 mm (31). The ice content of the permafrost is 20-50 % with 2-5 % of liquid water in films adsorbed to soil particles (20, 39). The permafrost in this region is formed from deposits of sediment that came from shallow lake bottoms, alluvial deposits and marine sediments during the late Pliocene and Pleistocene periods (31), and can reach a thickness of 600 to 800 m. While the upper tundra layer, 0.5 to 1 m deep, freezes and thaws every year, the strata below have remained permanently frozen since they were buried by geological events. The temperature at a depth of 14 m below the surface is stable at -10 °C (11). This region is considered to be among the oldest continuously frozen localities on Earth (21) and make this environment ideal for studies of microorganisms that are adapted to survive at subzero temperatures.

Three *Exiguobacterium* strains were previously isolated from Kolyma Lowland permafrost cores of three different ages. *Exiguobacterium* strain 255-15 was isolated from a depth of 43.6 m from a geological layer estimated to be 2 to 3 million years old. Strains 7-3 and 190-11 were isolated from layers estimated to be 20 - 30 thousand years and 200 - 600 thousand years old, respectively. Because of their unique physiology and habitat, we characterized these strains and established their taxonomy. Two of the strains comprise a new species.

MATERIAL AND METHODS

Bacterial strains. *Exiguobacterium* strains 255-15 and 190-11 were isolated by Vishnivetskaya et al. (33) and strain 7-3 was isolated by Vera Soina (Moscow State University). They were compared to four reference strains obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), *E. acetylicum* (DSM 20416^T), *E. aurantiacum* (DSM 6208^T), *E. antarcticum* (DSM 14480^T) and *E. undae* (DSM 14481^T). The strains were grown and maintained on ½ Tryptic Soy Broth (TSB) and ½ Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, Michigan, USA) at 30 °C. The strains were stored at -80 °C in ½ TSB supplemented with 25% glycerol.

Genotypic characterization. Chromosomal DNA isolation and purification was performed as described by (19) from cells grown overnight at 30 °C in ½ TSB. DNA concentrations were quantified by UV spectrophotometry at 260 nm (Cary 50 Bio from Varian).

The genomic diversity of the strains was determined by repetitive element-PCR (14, 30) with BOXA1R primer as described previously (32). The patterns were analyzed using GelCompar II (Applied Maths Version 3.0) using the pairwise Pearson's product-moment correlation coefficient (25).

DNA-DNA reassociation was determined fluorometrically by the method of Ezaki et al. (7) using photobiotin-labelled DNA probes and black MaxiSorp microplates (Nunc). Hybridizations were performed at 37°C in eight replicates for each sample. The reported DNA-DNA reassociation values are the averages following exclusion of the outliers according to the criteria established for this method (12).

The phylogeny of the new isolates was determined from sequences of 16S rRNA gene and six other genes previously used for phylogenetic classification (1, 10, 15, 34, 35, 38): *gyr*B (gyrase subunit B), *rpo*B (DNA-directed RNA polymerase beta subunit), *rec*A (homologous recombination), *csp* (cold shock protein), *hsp*70 (ClassI-heat shock protein – Chaperonin) and *cit*C (isocitrate dehydrogenase). The primers (Table 3.1) were designed based on alignments of these genes found in *Exiguobacterium* 255-15 genome (http://genome.jgi-psf.org/draft_microbes/exigu/exigu.draft.html) with the same genes of other Gram-positive microorganisms using ARB (17), with the exception of the standard 16S rRNA (Lane 1991) and *csp* primers (8). Amplification, for all primers, was by standard procedures (6). The annealing temperatures for each primer sets were 56 °C for *csp,* 53 °C for *rpo*B, 49 °C for *gyr*B and *cit*C, 47 °C for *rec*A and *hsp*70. The 16S rRNA amplified product was purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced. For the other genes, the amplicons were cloned using the TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies) and the clones were extracted using the Qiagen Plasmid Mini kit (Qiagen, Valencia, CA, USA). Cycle sequencing was performed on a Perkin Elmer 9600 thermal cycler using ABI dye terminator chemistry (PE Applied Biosystems) and products were analyzed on an ABI 373a DNA sequencer. Five primers described in Table 3.1 were used for the 16S rRNA gene sequencing, and primers for the cloning vector (M13F and M13R) were used for sequencing the other genes. Assembled sequences were generated using the Sequencher program versions 3.1.1 (Gene Codes Corporation). Sequences were deposited in GenBank (accession numbers **DQ019127** to **DQ019169**). Sequences from strain 255-15 were obtained from the sequenced genome.

Table 3.1. Primers used for PCR amplification for MLST analysis. All the primers, except for the major cold-shock protein, where designed based on the *Exiguobacterium* sequences as well as other strains from the *Bacillus* genus.

Gene amplified	Sequence (5'- to -3')	Fragment size (bp)
DNA Gyrase – beta subunit		
*gyr*B –F	AAA CGT CCG GGT ATG TAT ATC GGA TCG AC	1539 bp
*gyr*B- R	CGG CGG CTG SGC AAT RTA SAC GTA	
Universal major cold-shock protein (Francis & Stewart 1997)		
CSPU5	CCC GAA TTC GGT AHA GTA AAA TGG TTY AAC KC	200 bp
CSPU3	CCC GGA TCC GGT TAC GTT ASC WGC TKS HGG DCC	
DNA-directed RNA polymerase beta subunit		
*rpo*B –F	CGA ACA TGC AAC GTC AGG C	1078 bp
*rpo*B-R	ACA TCY TCY TCA CGN GCA CC	
ClassI-heat shock protein – Chaperonin		
*hsp*70 –F	GGT ATT GAY TTA GGA ACA ACA AAC T	1455 bp
*hsp*70-R	CTT CTG CWT CTT TKA CCA T	
Homologous recombination protein		
*rec*A-F	GAR AAR CAA TTY GGB AAA GGT TC	869 bp
*rec*A-R	TGT TTY GMA TTT TCA CGK CCT TG	
Isocitrate dehydrogenase		
*cit*C-F	GGD GAY GGM ACW GGW CCW GAY ATT TGG	1165 bp
*cit*C-R	AAT TCW GAA CAT TTM ACT TCT GT	
16S rRNA gene universal primers (Lane 1991)		
8F	AGA GTT TGA TCC TGG CTC AG	
787R	TAC CAG GGT ATC TAA T	
802F	ATT AGA TAC CCT GGT A	
1100R	AGG GTT GCG CTC GTT G	
1525R	AAG GAG GTG WTC CAR CC	

N=A:T:C:G; H=A:T:C; D= T:G:A; K= T:G; Y= C:T; M=C:A; W=A:T; R= A:G; S=C:G ; all 1:1

The consensus sequences were aligned against the most similar sequences using ARB (17). Phylogenetic analyses were performed by the neighbor-joining (27) and distance (5) methods from within the ARB environment and by a maximum-likelihood method using fastDNAml (24). The robustness of the inferred tree was evaluated by applying 1000 bootstrap re-samplings.

Phenotypic characterization. Temperature requirements were determined by growing all the *Exiguobacterium* strains on ½ TSA. Inoculated agar plates were incubated for 24

h to 48 h at 30 °C, 4 to 6 days at 12 °C, 20 days at 0 °C, 32 days at -2.5 °C in order to reach a colony size of 1 to 2 mm of diameter. Cell morphology at different temperatures was examined by phase contrast microscopy and transmission electron microscopy (TEM) using colonies grown on the same plate. Flagellum type was observed by TEM at all temperatures. Cultural and physiological characteristics were determined by using API 50 CHE (bioMérieux) and Biolog GP2 microplate (26). Production of constitutive enzymes was assessed with API ZYM strips (bioMérieux) as recommended by the manufacturer. Cells for the fatty acid methyl ester analysis (FAME) (22) and chemotaxonomic markers were grown at 28°C on TSA and analyzed by DSMZ. The chemotaxonomic markers studied were cell wall amino acids using the methodology of Schleifer and Kandler (29), polar lipids by Minnikin et al. (23), peptidoglycan structure by (18), (28) and (18, 28, 29) and isoprenoid quinones by (3) and (13).

Cytochrome oxidase was tested using the Bactident Oxidase kit from Merck (1.13300) and catalase activity was tested using H_2O_2 as described by (16). Nitrate reduction was analyzed as described by Cowan and Steel (4). The ability of the new isolates and the four reference strains to grow in ½ TSB supplemented with 5%, 10 %, 12 %, 15 % and 17 % of NaCl was also determined. Results were only considered positive if an increased medium turbidity was observed for both duplicates.

RESULTS AND DISCUSSION

Genotypic analysis. Genotypic studies were performed on *Exiguobacterium* permafrost strains 7-3, 190-11 and 255-15 to determine their phylogenetic relationship with the most relevant set of reference strains. The rep-PCR profiles confirmed that the new isolates

and reference strains were all distinct genotypes (data not shown). Sequence analysis of the 16S rRNA genes of the strains placed them in the genus *Exiguobacterium* (Figure 3.1a). Strains 7-3, 190-11, 255-15, *E. undae*, *E. antarcticum*, *E. acetylicum* and *E. oxidotolerans* form a closely related group with a bootstrap value of 1000, while *E. aurantiacum*, *E. marinum*, and the two *E. aestuarii* strains form another closely related group. For a more robust analysis of the phylogenetic relationships of strains within *Exiguobacterium*, we analyzed the phylogeny of six protein-encoded genes (Figure 3.1b,c,d,e,f,g).

Since protein-encoding genes evolve much faster than ribosomal RNAs, they provide higher resolution than 16S rRNA gene sequences (37). Furthermore, such results were shown with *Acinetobacter* to fill the resolution gap between 16S rRNA gene sequence analysis and DNA-DNA reassociation values (36).

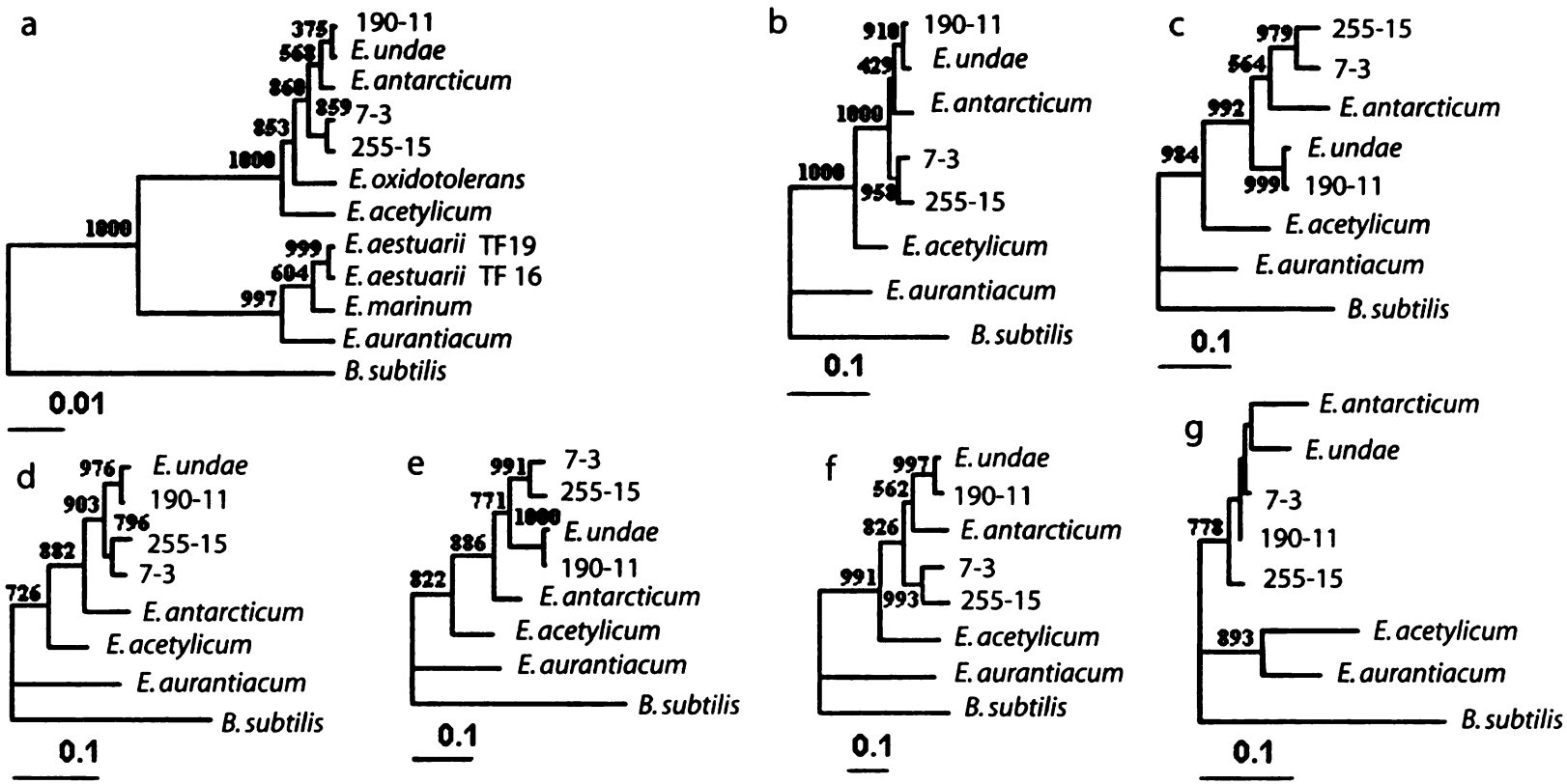

Figure 3.1. Phylogenetic trees based on 16S rRNA gene (a), *rpo*B (b), *rec*A (c), *hsp*70 (d), *gyr*B (e), *cit*C (f), major cold-shock proteins (g) sequences of the following genes of indicated *Exiguobacterium* strains. The trees were produced by a maximum-likelihood method using fastDNAml_loop and were rooted using those genes from *B. subtilis*. The nodes without bootstrap values represent either branching orders that were inconsistent between the phylogenetic tree generated by the maximum-likelihood method and the consensus tree generated from 1000 bootstraps or had bootstrap values of 40% or less. The scale bar represents 0.1 changes per nucleotide position.

The basic tree topologies of *rpo*B (Figure 3.1b), *rec*A (Figure 3.1c), *hsp* 70 (Figure 3.1d), *gyr*B (Figure 3.1e) and *cit*C (Figure 3.1f) were very similar. *E. undae* DSM 14481^T and strain 190-11 showed very similar sequences and always clustered together with very high bootstrap values. Strains 7-3 and 255-15, also clustered together, albeit at a slightly lower sequence similarity. As found for the 16S rRNA gene sequence analysis, *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T were always clearly separated from the other *Exiguobacterium* strains by long branches. *E. antarcticum* DSM 14480^T was the only strain for which the clustering was inconsistent over the different genes, either clustering with strain 190-11 and *E. undae* DSM 14481^T (*rpo*B, *cit*C) or with strain 7-3 and 255-15 (*rec*A). In other cases (*hsp*70, *gyr*B), *E. antarcticum* DSM 14480^T was separated from these four related *Exiguobacterium* strains. Notably, the branch nodes were often not well supported by the bootstrap values, which makes the affiliation of *E. antarcticum* DSM 14480^T uncertain.

In order to unequivocally determine the species status of the new isolates, DNA-DNA hybridization experiments were performed (Table 3.2). All reassociation values with strains *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T were well below the threshold for species identity (14 - 44 %). Strain 190-11 and *E. undae* DSM 14481^T showed a DNA relatedness of 91 %, which indicates that these two strains belong to the same species. This is in agreement with the multi-locus sequence typing analysis (MLST), and many of the phenotypic analyses (see below). Furthermore, strain 190-11 showed a relatively high reassociation (65 %) with *E. antarcticum* DSM 14480^T.

A DNA reassociation value of 71 % was found between the strains 7-3 and 255-15, suggesting that these strains maybe members of the same species. However, both 7-3 and 255-15 had a relatively high similarity (65-68 %) with the type strains *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T (Table 3.2). The DNA-DNA reassociation values are borderline for species delineation, making it difficult to classify the new strains as belonging to one or to the other species. Those two type strains are clearly distinct based on their phenotypic differences and DNA-DNA reassociation values of 51 % by (9), and confirmed by our results (59 %). Since the two isolates, 7-3 and 255-15, cannot be clearly classified as belong to either one of these type strains and have consistent MLST data supporting that they are related and distinct from the other species previously described, we suggest that they are a new species.

Phenotypic properties. Phenotypic studies were also performed to determine whether the new isolates are phenotypically distinguishable from each other and from the reference strains of *Exiguobacterium*. Allocation of the strains 7-3, 190-11 and 255-15 to the genus *Exiguobacterium* was confirmed by the presence of the peptidoglycan type A3α L-Lys-Gly, which is typically the genus (2).

The major isoprenoid quinone (Table 2.3) in all strains was an unsaturated menaquinone with seven isoprene units (abbreviated as MK7). Strains 7-3, 190-11, *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T contained minor but significant amounts of MK6 and MK8 quinones, while for 255-15 the major quinone was MK7 with trace amounts of MK6 and MK8, which provides a good distinction from the other closely related strains like *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T. In the analysis of polar lipids, none of the Siberian isolates (7-3, 190-11 and 255-15)

contained phosphatidyl-serine or phosphatidyl-inositol as was observed in *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T (Table 3.3). All strains were catalase positive. *E. antarcticum* DSM 14480^T and *E. aurantiacum* DSM 6208^T were the only oxidase negative strains (Table 3.3). *E. aurantiacum* DSM 6208^T was also the only strain positive for nitrate reduction. The major fatty acids in the new isolates (Table 3.4) were iso-C13:0, anteiso-C13:0, iso-C15:0, C16:0 and iso-C17:0, which were similar to these in *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T, further supporting the assignment of the new isolates as *Exiguobacterium*.

E. aurantiacum DSM 6208^T was the only strain that did not express any of the 19 enzymes tested (API ZYM), and 7-3 and 190-11 were the only strains that expressed esterase lipase (C8) and β-glucosidase respectively. All *Exiguobacterium* strains expressed α-glucosidase and β-galactosidase with the exception of *E. aurantiacum* DSM 6208^T, as mentioned above, and *E. acetylicum* DSM 20416^T, which only expressed α-glucosidase. Interestingly, even though 190-11 and *E. undae* DSM 14481^T were highly similar in all genotypic tests, only 190-11 and *E. antarcticum* DSM 14480^T did not express alkaline phosphatase, while 7-3, 255-15 and *E. undae* DSM 14481^T did express this enzyme.

Table 3.2. DNA-DNA similarity of *Exiguobacterium* strains using the microplate DNA-DNA hybridization technique. Data greater than 70% in bold.

Strains	190-11	*E. undae* DSM 14481^T	*E. antarcticum* DSM 14480^T	7-3	255-15	*E. acetylicum* DSM 20416^T
190-11	100					
***E. undae* DSM 14481^T**	**91.2 ± 7.2**	100				
***E. antarcticum* DSM 14480^T**	65.2 ± 13.4	59.9 ± 5.5	100			
7-3	62.0 ± 4.7	67.5 ± 3.6	65.3 ± 2.9	100		
255-15	56.4 ± 6.4	64.7 ± 5.8	65.3 ± 6.0	**71.2 ± 3.3**	100	
***E. acetylicum* DSM 20416^T**	41.7 ± 9.9	43.5 ± 7.2	38.8 ± 3.7	38.3 ± 6.9	37.7 ± 7.0	100
***E. aurantiacum* DSM 6208^T**	13.7 ± 2.6	16.2 ± 1.8	21.0 ± 3.0	15.3 ± 3.1	15.5 ± 2.4	18.0 ± 1.4

Table 3.3. Phenotypic characteristics of new isolates and type strains of *Exiguobacterium* strains: 1= 7-3; 2= 255-15; 3= 190-11; 4= *E. undae* DSM 14481^T (data obtained from this work and Frühling et al. 2002); 5= *E. antarcticum* DSM 14480^T (data obtained from this work and Frühling et al. 2002); 6= *E. aurantiacum* DSM 6208^T (data obtained from this work and Frühling et al. 2002); 7= *E. acetylicum* DSM 20416^T (data obtained from this work, Frühling et al. 2002, Collins et al., 1983); 8= *E. oxidotorerans* (Yumoto et al., 2004); 9= *E. aestuarii* (Kim et al, 2005); 10= *E. marinum* (Kim et al, 2005).

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Flagellum type	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	SP
Colour of colonies	BO	BO	O	O	BO	OY	OY	OY	PO	DO
Oxidase	+	+	+	+	-	-	+	+	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Growth in NaCl	10%	10%	12%	12%	5%	12%	10%	12%	>19%	>17%
Nitrate reduction	-	-	-	-	-	+	-	-	+	+
Growth range	-2.5-40°C	-2.5-40°C	-2.5-40°C	-2.5-40°C	-2.5-40°C	12-30°C	7-43°C	4-36°C	10-47°C	10-43°C
Enzymatic activity										
Alkaline phosphatase	+	+	-	+	-	-	+	-	nd	nd
Esterase Lipase (C8)	+	-	-	-	-	-	-	-	nd	Nd
β-galactosidase	+	+	+	+	+	-	-	+	Nd	Nd
α-glucosidase	+	+	+	+	+	-	+	-	Nd	Nd
β-glucosidase	-	-	+	-	-	-	-	-	Nd	nd
Acid phosphatase	-	-	-	-	-	-	-	+	Nd	nd

Table 3.3 (cont'd).

Isoprenoid quinines (ratio)										
MK8	8	Traces	12	15	20	-	nd	nd	nd	nd
MK7	85	100	83	75	71	Major	Nd	major	major	major
MK6	6	Traces	6	2	2	+	Nd	nd	Nd	Nd
Polar lipids										
Phosphatidyl glycerol	+	+	+	+	+	+	+	+	+	+
Diphosphatidyl glycerol	+	+	+	+	+	+	+	+	+	+
Phosphatidyl ethanolamine	+	+	+	+	+	+	+	+	+	+
Phosphatidyl serine	-	-	-	+	+	-	+/-	+	+	+
Phosphatidyl inositol	-	-	-	+	+	-	-	+	+	+

+, positive, -, negative, ND, not determined.

DP= Deep orange; BO= bright orange; O= orange; OY=orange-yellow, PO=Plale orange.

Pt=peritrichous; SP=single polar

Table 3.4. Fatty acid composition of the Siberian permafrost isolates and the type strains of *Exiguobacterium*. Values ≥ 5% are in bold.

	7-3	255-15	190-11	*E. undae* DSM 14481^T	*E. antarcticum* DSM 14480^T	*E. aurantiacum* DSM 6208^T	*E. acetylicum* DSM 20416^T
$iC_{11:0}$						2	
$iC_{12:0}$	2	2	2	2	3	3	
$C_{12:0}$	1		1		1	2	1
$iC_{13:0}$	**9**	**13**	**8**	**9**	**12**	**18**	**5**
$aiC_{13:0}$	**11**	**15**	**10**	**9**	**11**	**12**	**6**
$iC_{14:0}$	1	1	1	2	1		1
$C_{14:1\omega5c}$							2
$C_{14:0}$	3	1	2	2	2	3	**13**
$iC_{15:0}$	**13**	**12**	**13**	**10**	**11**	4	**8**
$aiC_{15:0}$	3	4	3	3	2		1
$C_{16:1\omega11c}$	**8**	3	**7**	**8**	**18**	**10**	**26**
$iC_{16:0}$	2	2	2	2			
$C_{16:1\omega5c}$	1		1				2
$iC_{16:1\ H}$			1				
$C_{16:1\omega7c}$	1		1	**7**	3		**13**
$C_{16:0}$	**17**	**20**	**12**	**17**	**13**	**27**	**10**
$C_{17:1\omega10c}$	2	2	3	2	3		1
$aiC_{17:1\omega9c}$			1				
$C_{17:0}$		1					
$iC_{17:0}$	**9**	**12**	**8**	**7**	**5**	**6**	1
$aiC_{17:0}$	3	3	3	2			
$C_{18:1\omega9c}$	2	1	2	3	**6**	2	**5**
$C_{18:1\omega7c}$	2	1	3	3			2
$C_{18:1\omega5c}$			1				
$C_{18:0}$	4	**5**	3	**6**	**5**	**5**	1

The BIOLOG and API 50 CH systems (Table 3.5) were used to assess the metabolic profile of *Exiguobacterium* strains. A dendrogram obtained with this data shows that 255-15 shares a slightly more similar carbon utilization pattern with *E. antarcticum* DSM 14480^T (76 % similarity) than with the other strains (Figure 3.2). In addition, 7-3, 190-11 and *E. undae* DSM 14481^T were clustered together and had more similar carbon utilization patterns, respectively with 73 % and 82 % similarity to *E. undae* DSM 14481^T.

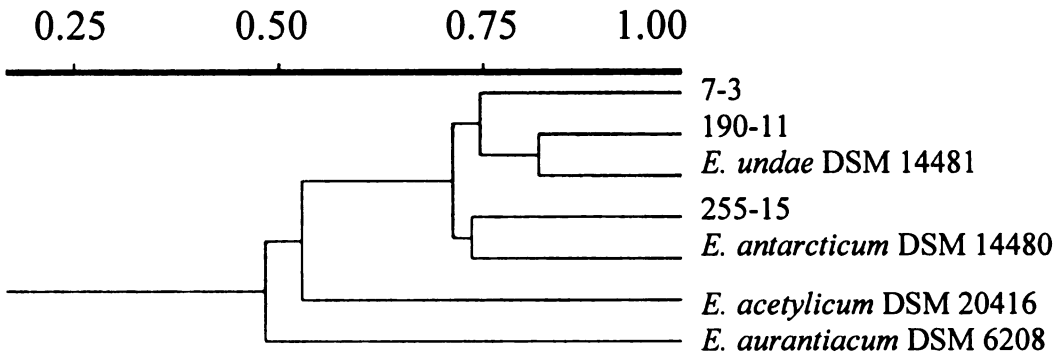

Figure 3.2. Phenogram obtained from the carbon source utilization of the seven *Exiguobacterium* strains based on the UPGMA method (unweighted pair-group method using arithmetic averages) after measuring similarity/dissimilarity among the strains using the coefficient of simple matching, which considers the same weight positive and negative similarities.

However, the higher similarity observed was between 190-11 and *E. undae* DSM 14481^T, which had 82 % similarity in the carbon source utilization, confirming the genotypic data that suggests that they are the same species. In the case of 7-3 and 255-15, even though they were clustered with two different reference strains in the phenogram, when we look at the matrix (data not shown) the pair-wise comparison of those two strains with the other reference strains (except for *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T) showed a similarity of 71 % and 73 % for 7-3 with *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T, respectively, and a similarity of 73

% and 76 % for 255-15 with *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T, respectively. Hence, this data is not conclusive with regard to a species assignment for these two new strains.

The strains were also tested for their ability to grow at different temperatures, from -2.5 to 30 °C. The reference strains *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T were unable to grow at 4 °C or below, but were able to grow at 30 °C and 12 °C. Strains 7-3, 190-11, *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T grew at all temperatures tested. The cell pigmentation remained unaltered at low growth temperatures, except for *E. acetylicum* DSM 20416^T, which at 12 °C, became much more orange and therefore more similar to the other *Exiguobacterium* strains. These data confirm results obtained with the *csp* gene (Figure 3.1g), i.e. that these two strains unable to grow below 12°C cluster together, while the others that are able to grow at lower temperatures formed a separate cluster. This observation suggests that CSP primers may be able to discriminate strains that grow at lower temperatures from the ones that cannot.

In regard to growth at different salt concentrations, 7-3 and 255-15 were able to grow in up to 10 % NaCl, as was the case for *E. acetylicum* DSM 20416^T. Strain 190-11 was able to grow at salinities of up to 12 % NaCl, like *E. undae* DSM 14481^T, *E. oxidotolerans* and *E. aurantiacum* DSM 6208^T (Table 3.3). These data show that *E. undae* DSM 14481^T and 190-11 have another phenotype in common. The same is observed for the strains 7-3 and 255-15 that also exhibit the same tolerance to salt.

Colonies of all the new isolates and the reference strains (except for DSM 20416^T, which was yellow colored) were orange-colored, smooth, circular, convex and shiny. The

orange pigment did not diffuse into the medium. The isolates also showed genus-specific characters such as rod-shape morphology, motility, peritrichous flagella and absence of spore formation at all temperatures tested (Table 3.3). Strains were inoculated on ½ TSA plates and cell morphology was observed in different temperatures with phase contrast microscopy and transmission electron microscope after negative staining. The cell shape and slime secretion of 190-11 and 255-15 strains did not change with temperature and were very similar to *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T. However, inclusions were observed inside the cells of strain 255-15 at 0 °C, which could be carbon storage polymer (Figure 3.4). Strain 7-3 was the only strain that showed temperature-dependent cell morphology. For temperatures from 0 to 12 °C, the cells became much more elongated in comparison to all the other strains and in comparison to its growth at 30 °C (Figure 3.3 and 3.4). This elongation state was not permanent, as cell size returned to normal when incubated at 30 °C. Interestingly, at -2.5 °C the cell size of 7-3 was similar to the other strains.

The phenotypic analyses confirmed that the new isolates are *Exiguobacterium*, that 190-11 and *E. undae* DSM 14481^T share similar properties and hence are the same species, consistent with the genotypic analyses; and that 255-15 and 7-3 have some phenotypic properties that are not consistent with any current species. The genotypic results were clearer cut for 7-3 and 255-15 classification, in that they had high DNA-DNA reassociation and were clustered together in all but one of the MLST analyses.

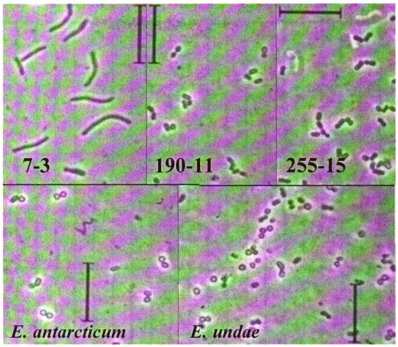

Figure 3.3. Cell morphology of new isolates and reference strains of *E. antarcticum* DSM14480 and *E. undae* DSM 14481 grown at 0°C by phase contrast microscopy. Bar scale corresponds to 10 µm.

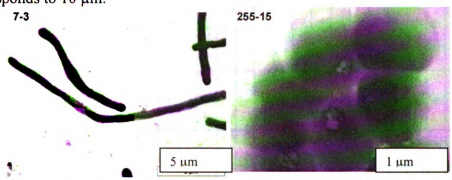

Figure 3.4. Negatively stained electron micrograph of 7-3 and 255-15 grown at 0°C. Strain 7-3 presents elongated cells and 255-15 presents intracellular granules.

Hence, we conclude that, 255-15 and 7-3 are members of the same species and that they belong to a different species from the reference strains described so far. The phenotypic results for all *Exiguobacterium* new and old members are not strikingly distinctive among the species, except for the most distantly related strains. Hence, the genotypic analyses are more conclusive for the species of this genus.

Table 3.5. Phenotype properties that differentiate the novel isolates from *Exiguobacterium* type strains using Biolog and API 50 CH

Substrates[a]	7-3	255-15	190-11	*E. undae* DSM 14481	*E. antarcticum* DSM 14480	*E. aurantiacum* DSM 6208	*E. acetylicum* DSM 20416
2,3-butanediol	-	+/-	-	-	-	-	-
3-Methyl-D-glucose	+/-	+	+	+	+	-	+
Acetic acid	+	+	+	+	-	-	+/-
a-cyclodextrin	+	-	-	+/-	-	+	-
Adenosine-5'-monophosphate	+	+	+/-	+	+	+	-
a-D-Glucose-1-phosphate	-	-	-	-	-	-	+
a-methyl-D-Glucoside	+	+	-	-	-	-	+
Amidon (starch)	+	+	+	+	+	+	-
b-cyclodextrin	+/-	-	-	-	-	+/-	-
b-hydroxybutyric acid	+	+/-	+	+/-	-	-	-
D- galactose	+	+	+	+	+	-	-
D- Melibiose	-	+/-	+	+	-	-	-
D-alanine	+/-	-	-	-	-	-	-
D-fructose-6-phosphate	-	-	-	-	-	-	+
D-glucose-6-phosphate	-	-	-	-	-	-	+
D-lactose (bovine origin)	+	-	-	-	-	-	-
D-L-a-glycerol phosphate	-	-	-	-	-	-	+
D-mannitol	+	+	+	+	+/-	-	+
D-mannose	+	-	+	+	-	-	-
D-melezitose	-	+/-	-	+	-	-	-
D-melibiose	-	-	+/-	+	-	-	-
D-raffinose	+	+	+	+	+	-	-
D-ribose	+	+	+	+	+	+	-
d-sorbitol	+	-	+	+	+	-	+

Table 3.5. (cont'd)

D-xylose	+	+/-	-	+/-	+	-	-
esculin Ferric citrate	+	-	+	+	+	+	+
Glycyl-L-Glutamic acid	+	+/-	+	+	+	-	+
L-alanine	+/-	-	-	-	-	-	-
L-alanyl-glycine	+	+	+	+	+	-	+/-
L-asparagine	+/-	+	-	-	+	-	+
L-glutamic acid	-	+/-	-	-	+	-	+
L-Lactic acid	-	-	-	-	-	+	-
L-Malic acid	-	+/-	-	+/-	-	-	-
L-Pyroglutamic acid	-	-	-	-	+	-	-
L-rhamnose	-	+/-	+/-	-	+	-	-
L-Serine	+/-	+/-	-	+/-	+	-	+/-
Methyl -alpha-D-glucopyranoside	±	-	-	-	-	+	-
N-acetyl-b-D-Mannosamine	-	+/-	-	+/-	-	-	-
N-acetyl-D- Glucosamine	+	+	+	+	+	+	+
N-acetyl-L-glutamic acid	-	-	-	+	-	-	-
Propionic acid	+	+	+/-	-	+	-	+
Pyruvic acid	+	+	+	+	+	-	+
Pyruvic acid methyl ester	+	+	+	+/-	+	+	-
salicin	+	+	+	+	+	+	-
Sedoheptulosan	-	+/-	-	-	+	-	-
stachyose	-	-	-	-	+/-	-	-
Succinic acid Mono-methyl ester	+	+	+	+	+	-	+
Thymidine-5'-Monophosphate	+	+	+	+	+	-	-
Turanose	+/-	+	+/-	+	+	-	+
Uridine-5'-monophosphate	+	+	+/-	+	+	+	-

[a]-Positive results for the following substrates: 2'-deoxy adenosine, Adenosine, a-D-Glucose, a-Ketovaleric acid, Amygdalin, arbutin, b-Methyl-D-glucoside, D- Mannose, D-cellobiose, Dextrin, D-Fructose, D-glucose, D-maltose, D-psicose, D-saccaharose, Gentiobiose, glycerol, Glycogen, Inosine, D-trehalose, Maltose, Maltotriose, Thymidine, uridine, Palatinose

Negative results for the following substrates: a-D-Lactose, a-hydroxybutyric acid, a-ketoglutaric acid, a-Methyl-D-Galactoside, a-methyl-D-mannoside, b-methyl-D-Galactoside, D-Arabitol, D-fucose, D-galacturonic acid, D-Gluconic acid, D-Lactic acid Methyl ester, D-lyxose, D-malic acid, D-sorbitol, D-tagatose, D-turanose, Dulcitol, g-hydroxybutyric acid, inositol, Inulin, Lactamide, Lactulose, L-alaninamide, L-arabinose, L-arabitol, L-Fucose, L-rhamnose, L-sorbose, Mannan, Methyl-alpha-D-mannopyranoside, p-Hydroxyphenylacetic acid, potassium 2-ketogluconate, potassium 5-ketogluconate, potassium gluconate, Putrescine, Succinamic Acid, Succinic acid, Tween 40, Tween 80, xylitol.

Description of Exiguobacterium *sibiricum* sp. nov.

Exiguobacterium sibiricum (si.bi.ri.cum M. L. – Sibir' from Siberia, a Russian region)

Cells are Gram-positive and facultatively anaerobic, non-spore-forming rods, motile with peritrichous flagella. The cells can vary in shape and size depending on growth temperature. At 30 °C, they can have 0.8 μm long and 0.6 μm in diameter, but can reach up to 15 μm long at temperatures between 12 °C and 0 °C. Surface colonies on ½ TSA are 3.5 to 4 mm in diameter after 50 h at 30 °C, bright orange, convex, entire and shiny. The orange pigment does not diffuse in the medium. Growth occurs at -2.5 °C to 40 °C, with optimum temperature growth rate at 36 °C. Growth does occur in ½ TSB with 10 % NaCl, but does not occur at 12 %. The peptidoglycan type is L-Lys-Gly and the predominant menaquinone is MK-7; MK-6 and MK-8 might or might not occur. The only polar lipids are phosphatidyl-glycerol, diphosphatidyl-glycerol and phosphatidyl-ethanolamine, and the major fatty acids are iso-C13:0, anteiso-C13:0, iso-C15:0, C16:0 and iso-C17:0. The G+C content of DNA of the type strain is 47.7 mol% and its genome size is 3 Mb. The type strain is strain 255-15 (= DSM 17290)

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CHAPTER 4

EXIGUOBACTERIUM GENUS: AN EXAMPLE OF PERMAFROST SURVIVOR

Rodrigues, D. F., and J. M. Tiedje. 2007. Multi-locus real-time PCR for quantitation of bacteria in the environment reveals *Exiguobacterium* to be prevalent in permafrost. FEMS Microbiol Ecol 59:489-99.

ABSTRACT

We developed a multi-locus quantitative PCR approach to minimize problems of precision, sensitivity and primer specificity for quantifying a targeted microbial group in nature. This approach also avoids a systematic error in population quantitation when 16S rRNA genes are used because of copy number heterogeneity. Specific primers were designed to assess the abundance of psychrotrophic and mesophilic *Exiguobacterium* spp. that excluded the thermophilic members of the genus. The chosen primers targeted genes for DNA gyrase B (*gyr*B), the beta subunit of the RNA polymerase gene (*rpo*B) and a hypothetical gene so far found only in this group. The results demonstrate that the multiple primer approach provides a more reliable estimate of population density; that the targeted *Exiguobacterium* group is found at a median density of 50,000 gene copies μg^{-1} of total community DNA in 27 of 29 permafrost soils but was found in only one of the four temperate and tropical soils tested.

INTRODUCTION

Quantification of microbial abundances is central to understand microbial community structure and population dynamics. Real-time PCR (qPCR) provides high sensitivity, is quantitative (29) and less laborious than whole-cell fluorescence in situ hybridization techniques (6, 16). Real-time PCR techniques have more commonly been developed for bacteria and viruses of clinical importance, c.f. for bacteria (11, 18, 21, 31) and viruses (9, 19). More recently this technique has been used for detection of microorganisms such as *Pseudomonas stutzeri* (10), *Rhodococcus* sp. (23) and *Geobacter* spp. (25) in terrestrial environments.

All studies using real-time PCR to quantify microorganisms in the environment use only one gene, typically the 16S rRNA gene or functional genes of interest, such as nitrate reductases (10), (bi) sulfite reductases (20) and aromatic dioxygenases (2). The conserved nature of the 16S rRNA gene can make it difficult if not impossible to design primers of sufficient specificity to track populations of interest, plus the variation in copy number of this gene leads to uncertainty in quantitation. If less conserved genes are used, the lack of sequence data, especially for the targeted species, makes specificity and the detection range of primers uncertain. Additionally, some primers yield less than desired sensitivity (10, 24). The above problems can lead to over or under estimation of the number of microorganisms in the environment, especially when dealing with complex communities.

Previous studies in Siberian permafrost soils yielded isolates of *Exiguobacterium* from four geological strata ranging from 20 thousand to 3 million years since they were frozen (22). This genus belongs to the family *Bacillaceae*. These microorganisms are Gram positive, rod-shaped, facultative aerobic, motile with peritrichous flagella. Colonies on ½ Tryptic soy agar medium are orange-yellow. The pigment does not diffuse in the medium. Since this genus was found in several layers of the Siberian permafrost, including the oldest, we sought to determine whether it is more prevalent in these soils than temperate or tropical soils. To do this we developed a multi-locus quantitative real-time PCR (ML-qPCR) method to achieve more specific and accurate quantification of psychrotrophic and mesophilic members of *Exiguobacterium*. The results demonstrate the importance of using multiple genes for more accurate quantification of the targeted group, and that *Exiguobacterium* preferentially resides in permafrost.

MATERIAL AND METHODS

Soils. Twenty-nine Siberian permafrost soil samples from sixteen sites were collected by David Gilichinsky's team, Russian Academy of Sciences, Pushchino, from depths varying from 2.55 m to 37.5 m. These soils had been frozen between 5 thousand to 3 million years depending on the location and depth (Table 4.1). Non-permafrost soils from Michigan, Iowa, Hawaii and Puerto Rico were sampled from 5 to 10 cm depth. All soils were stored frozen at -20°C.

Table 4.1. Location and additional information of sample sites [a].

Sample	Depth (m)	Collection Date (Year)	Age (years)	Location
1- Siberia	2.55	2000	5-10 thousand	Kolyma Lowland, Cape Chukochii, East Siberian Sea coast, tundra zone, 70°05'N, 159°55'E
2- Siberia	8.55			
3- Siberia	14.95			
4- Siberia	20.0		20-30 thousand	
5- Siberia	2.8	2001	12-40 thousand	Cape Bykovskii Laptev Sea coast, tundra zone 129° 30'E, 71° 40'N
6- Siberia	8.0			
7- Siberia	13.0			
8- Siberia	23.3-23.4			
9- Siberia	28.0			
10- Siberia	33.0			
11- Siberia	37.5			
12- Siberia	6.3	2001	5-10 thousand	
13- Siberia	6.8			
14- Siberia	18.1-18.2	2003	3 million	130° 00'E, 72° 00'N
15- Siberia	24.0			
16- Siberia	24.4			
17- Siberia	3.0	2003	5-10 thousand	
18- Siberia	7.5			
19- Siberia	4.8-4.9	2003	15-20 thousand	
20- Siberia	8.15-8.25			
21- Siberia	22.5-22.6	2001	300-400 thousand	Cape Svyatoi Nos, Laptev Sea coast, tundra zone,140°10'E, 72°55'N
22- Siberia	9.0			

Table 4.1 (cont'd)

23- Siberia	11.0	1990	20 thousand	Kolyma Lowland, forest-tundra zone, left bank of Kolyma river, Khalarchinskaya tundra sandy plain, 68°50'N, 161°02'E
24- Siberia	16.5	1990	2-3 million	Kolyma Lowland, Krestovka River, right inflow of Kolyma River (north taiga), 66°50'N, 62°30'E
25- Siberia	18.7	1992	2-3 million	Kolyma Lowland, tundra zone, Mouth of Malaya Kon'kovaya River, right bank, 69°23'N, 58°28'E
26- Siberia	19.9			
27- Siberia	20.1-20.2	1999	100-120 thousand	Kolyma Lowland, tundra zone, Lake Yakutskoe, East Siberian Sea coast, 69°51'N, 159°29'E
28- Siberia	23.3-23.45			
29- Siberia	16.8-16.9			
Puerto Rico	0.1	2002	Recent	Guanica dry forest, vereda cerca de casa guardia, 17°58'N, 66°51'W
Hawaii	0.1	1992	Recent	Kohala forest, 20°03'N, 155°41'E
Iowa	0.1	2005	Recent	Prairie wetland, Jasper county 41°48'N, 09°30W
Michigan	0.1	2005	Recent	Baker Woodlot, Rachana Rajendra Bird Sanctuary – Michigan State University, 42°44'N, 08°42W

[a] The permafrost sample information was provided by David Gilichinsky.

Bacterial strains and culture conditions. The following microorganisms were used as negative controls: *Achromobacter cyclocastes* ATCC 21321^T, *Streptococcus pyogenes* JCM 5674, *Rhodococcus opacus* ATCC 51881^T, *Arthrobacter* sp. 348-9 (Siberian permafrost isolate), *Pseudomonas fluorescens* ATCC 17575^T, *Bacillus azotoformans*

ATCC 29788^T, *Corynebacterium nephridii* ATCC 11425^T, *Psychrobacter arcticus* VKM B-2377 (Siberian permafrost isolate), *Escherichia coli* B and *Pseudomonas stutzeri* strain JM300 DSM 10701. Positive controls: *Exiguobacterium* spp. isolates (strains AT1b, M37, A1, A19, TC38-2b, 5138, India orange and India stream) *Exiguobacterium aurantiacum* DSM 6208^T, *Exiguobacterium oxidotolerans* JMC 12280^T, *Exiguobacterium acetylicum* DSM 20416^T, *Exiguobacterium antarcticum* DSM 14480^T, *Exiguobacterium undae* DSM14481^T, *Exiguobacterium undae* strain 190-11, *Exiguobacterium sibiricum* strain 255-15 DSM 17290 (Table 4.2). All culture collection strains and permafrost isolates used in this study were grown in ½ tryptic soy broth (TSB) at 28°C (Difco, Detroit, Mich.), except for *Psychrobacter arcticus* which was grown at 20°C. *E. coli* transformants carrying TOPO TA clones (Invitrogen, Life Technologies) were grown in LB amended with kanamycin (50 μg mL^{-1}) as recommended by the manufacturer.

DNA extraction and quantification. Genomic DNA from the isolates was extracted from late-exponential phase cultures using the QiAamp DNA mini kit (Qiagen Inc.). Plasmid DNA from *E.coli* transformants was extracted using the Qiagen Plasmid mini kit (Qiagen Inc.). Soil DNA was extracted using the Fast DNA SPIN kit for soil (Bio 101, Q BIOgene) with some modifications, namely the first step of the extraction was a grinding step using liquid nitrogen followed by treatment with 100 μl of lysozyme (50 μg μl^{-1}) before using the kit. The quality of the extracted DNA was analyzed by electrophoresis on a 0.8% agarose gel. DNA concentrations were measured by absorbance at 260 nm.

Table 4.2. Specificity of real-time PCR for *Exiguobacterium* spp.

Strains	Local of isolation	Temperature Ranges [a]	Real- time PCR		
			Hypothetical	RpoB	gyrB
E. sibiricum strain 255-15	Siberia permafrost	Psychrotroph	+	+	+
E. undae strain 190-11	Siberia permafrost	Psychrotroph	+	+	+
E. undae DSM 14481	Garden pond, Germany	Psychrotroph	+	+	+
E. antarcticum DSM 14480	Antarctica	Psychrotroph	+	+	+
Exiguobacterium sp. strain 5138	Siberia permafrost	Psychrotroph	+	+	+
Exiguobacterium sp. strain A19	Stem of potato plants infected with Erwinia carotova, Austria	Psychrotroph	-	-	+
Exiguobacterium sp. strain TC38-2b	Carpathian Mountains, Ukraine	Psychrotroph	-	+	+
Exiguobacterium aurantiacum DSM 6208	Alkaline effluent of potato wash, UK	Mesophilic	+	+	+
Exiguobacterium acetylicum DSM 20416	Creamery waste, UK	Mesophilic	+	+	+
E. oxidotolerans JMC 12280	Fish processing plant, Japan	Mesophilic	-	+	-
Exiguobacterium sp. strain India stream	Hot spring, India	Mesophilic	-	-	+
Exiguobacterium sp. strain A1	Yellowstone park, US	Moderate Thermophilic	-	-	-
Exiguobacterium sp. strain AT1b	Yellowstone park, US	Moderate Thermophilic	-	-	-
Exiguobacterium sp. strain M37	Yellowstone park, US	Moderate Thermophilic	-	-	-
Exiguobacterium sp. strain India orange	Kunds, India	Moderate Thermophilic	-	-	-

[a]- Information obtained from Vishnivetskaya and coworkers (28).
All strains used as negative controls did not amplify in the q-PCR with any of the primers

Primers. Specific primers were designed for gyrase subunit B (gyrB27-F 5'ACT GCA TCG AAG GGG CTT CA 3'; gyrB198-R 5'ACT GCC GGA CGT CCC ATC TT 3'), DNA-directed RNA polymerase beta subunit (rpoB2-F 5'TGG CAA CAT CGT TCA AGG TG; rpoB148-R 5' ATC GAT GGA CCA TCT GCA AGG 3') and a hypothetical gene from *Exiguobacterium sibiricum* strain 255-15 (hy926-F 5' TGA CCA TTA CCG ACG ACA TCG 3'; hy1070-R 5'CCG CGG ATC AGT AAA TTA CCG 3'). Alignments of gene sequences from *Exiguobacterium* spp. and unrelated strains were obtained from Rodrigues and coworkers (22) and the Joint Genome Institute's (JGI) website (http://genome.jgi-psf.org/draft_microbes/exigu/exigu.home.html) and compared using the ARB program (17) to select conserved regions that encompassed the cold adapted *Exiguobacterium* species. The primers were designed within the conserved regions using the program PrimerExpress (PE Applied Biosystems) and checked against GenBank for specificity. Integrated DNA Technologies (Coralville, Iowa) synthesized the primers.

Real-Time PCR assays. The Real-time PCR reactions and primer optimizations were done as described in Applied Biosystems protocol for SYBR Green PCR master mix and RT-PCR (3). The reagents used for the reaction were the 2X PCR master mix (Applied Biosystems) with addition of 0.4 mg ml^{-1} final concentration of Bovine Serum Albunine (Roche). The optimum primer concentrations for hypothetical and *gyr*B were 50 nM and 300 nM for *rpo*B. The annealing and extension temperatures for all primers were 60°C, as described by the Applied Biosystems protocol. The size of the amplicons for *gyr*B, *rpo*B and hypothetical were 141 bp, 148 bp, 147 bp, respectively.

The increase in fluorescence emission was monitored during PCR amplification using the 7700 Sequence Detector (PE Applied Biosystems). The C_T values obtained for each

sample were compared with the standard curve to determine the initial copy number of the target gene.

Primer specificity. The DNA extracted from the *Exiguobacterium* spp. and the other species described above were used as positive and negative controls to test the specificity of the primer sets. Template DNA (10 ng) was added to each reaction tube. Total soil DNA extracts from Puerto Rico, Michigan and three permafrost soils from different ages and locations were amplified with each primer set to construct clone libraries with the TOPO TA cloning kit (Invitrogen) to verify the specificity of the primers in detecting *Exiguobacterium* spp.

Primers for the cloning vector (M13F and M13R) were used for sequencing. Cycle sequencing was performed on a Perkin Elmer 9600 thermal cycler using ABI dye terminator chemistry (PE Applied Biosystems) and products were analyzed on an ABI 373a DNA sequencer. Sequences generated in the present study were deposited in GenBank (accession numbers are **DQ490772-DQ490823**, **DQ490824-DQ490868** and **DQ499826-DQ499885** for *rpo*B, *gyr*B and hypothetical, respectively) and sequences from strain 255-15 were obtained from the genome sequenced by JGI (http://genome.ornl.gov/microbial/exig/).

To construct the phylogenetic trees, the sequences were aligned in the ClustalW program (26). Analyses were performed by the neighbor-joining method from within the MEGA 3.1 environment (15).

Sensitivity and detection limit. *Exiguobacterium undae* strain 190-11, *Exiguobacterium sibiricum* strain 255-15 and *Exiguobacterium antarcticum* DSM 14480^T were chosen for determining the detection limit of the hypothetical, *gyr*B and *rpo*B primer sets. Clones of

each gene of *Exiguobacterium undae* strain 190-11 were used to measure sensitivity and to generate standard curves in subsequent determinations. Serial dilutions (10-fold) of each DNA were prepared with 1μg μl^{-1} *E. coli* B DNA as a carrier in water (Sigma – molecular biology reagent). Different volumes (2, 4, 10 and 20 μl) of template DNA plus *E. coli* B DNA were added to individual reaction tubes and the differences were made up with water. Calibration curves were constructed to determine the lower detection limit of each assay and our ability to discriminate two-fold difference in template concentration. All determinations were performed in triplicate and standard deviations were determined (shown as error bars). The maximum allowable error (MAE) in order to distinguish a twofold difference in copy number among C_T obtained from three different strains (*E. sibiricum* 255-15, *E. undae* 190-11 and *E. antarcticum* DSM 14480^T) was calculated as follow: $\triangle C_T = m \cdot \log(2)$, where $\triangle C_T$ is the difference between C_T obtained from samples with a twofold difference in target copy number, and "m" is the slope of the standard curve. The MAE, calculated as: MAE = $\triangle C_T$ /2, was computed for each standard curve, and the mean MAE and the 95% confidence interval were determined.

Quantification and accuracy of Exiguobacterium spp. in environmental samples. Reactions were performed using 10 ng of template DNA. The template copy number was determined from C_T values from triplicates by using standard curves.

In order to test for the presence of PCR inhibitors, 10^4 clone copies were added to 10 ng of DNA extracted from the environmental samples from each site. Samples, with and without the addition of positive control DNA, were compared and the percent recovery of the added DNA was calculated.

RESULTS

Primer selection and specificity. We previously used five housekeeping genes for phylogenetic studies (22) and from this set we could design specific primers for the genus *Exiguobacterium* from the *gyr*B and *rpo*B sequences. These genes are essential for bacterial growth and are present in low copy number. For the *rpo*B, we found that only 1.3% of 234 genomes on the JGI website have two copies in their genome, and for *gyr*B only 11.3% and 0.5% of 194 genomes have two and three copies, respectively (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). The hypothetical gene was selected since this category of gene is often specific for a genus or species. We tested several primers designed for five diverse hypothetical genes from the *Exiguobacterium sibiricum* 255-15 genome, and found one primer set that was able to amplify this gene from only the targeted group.

DNA extracted from a range of *Exiguobacterium* spp. isolated from different environments and with different growth temperature ranges were used to test the specificity of the three primer sets (Table 4.2). A logarithmic increase in fluorescence intensity was observed by qPCR for five psychrotroph isolates for all three primer sets tested. Even though two mesophilic isolates, *E. aurantiacum* DSM 6208^T and *E. acetylicum* DSM 20416^T, could also be detected with all primers, the logarithmic increase in fluorescence intensity was much lower when compared to the psychrotrophs (data not shown). Although the primers for the hypothetical gene (Table 4.2) did not detect two psychrotrophic strains from non-polar regions, it seems to be more specific for the psychrotrophs, and detected all the strains from the two Polar Regions. The primers for *gyr*B and *rpo*B detected additional mesophilic strains and a broader set of

Exiguobacterium overall. None of the primers could amplify the moderately thermophilic isolates.

The sequences of the cloned genes confirmed that only the targeted gene was detected. In the *rpo*B gene tree (Figure 4.1), the cluster with all the thermophilic strains, with 90% bootstrap value, did not contain any clone from the permafrost, temperate (Michigan and Iowa) or tropical environments (Hawaii and Puerto Rico). This tree also shows that mesophilic and psychrotrophic *Exiguobacterium* spp. cluster together with a 99% bootstrap value and separated from the thermophilic strains. Additionally, the majority of the clones obtained from the Siberian permafrost soils from geologic strata of 100-200 thousand years since being frozen were similar to *Exiguobacterium* sp. TC38-2b isolated from the Carpathian Mountain in Ukraine. Most of the other clones were similar to the strains found previously in the Siberia permafrost, such as *E. undae* strain 190-11 isolated in soils from geological strata of 200-600 thousand years and *E. sibiricum* strain 255-15 from soil dated 2 to 3 million years before present.

The phylogenetic tree for the hypothetical gene does not contain any clones related to mesophilic or thermophilic strains (Figure 4.1), consistent with its higher specificity for psychrotrophic strains. In addition, all clones obtained from temperate (Michigan) or tropical (Puerto Rico) soils clustered together, while the others obtained from the Siberian permafrost formed an independent cluster with 65% bootstrap. The phylogenetic tree for *gyr*B gene (Figure 4.2) had clones mostly similar to the strains from the species *Exiguobacterium undae* with a few clones similar to the other strains from cold environments (Siberia or Antarctica). No clones were similar to the mesophilic *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T.

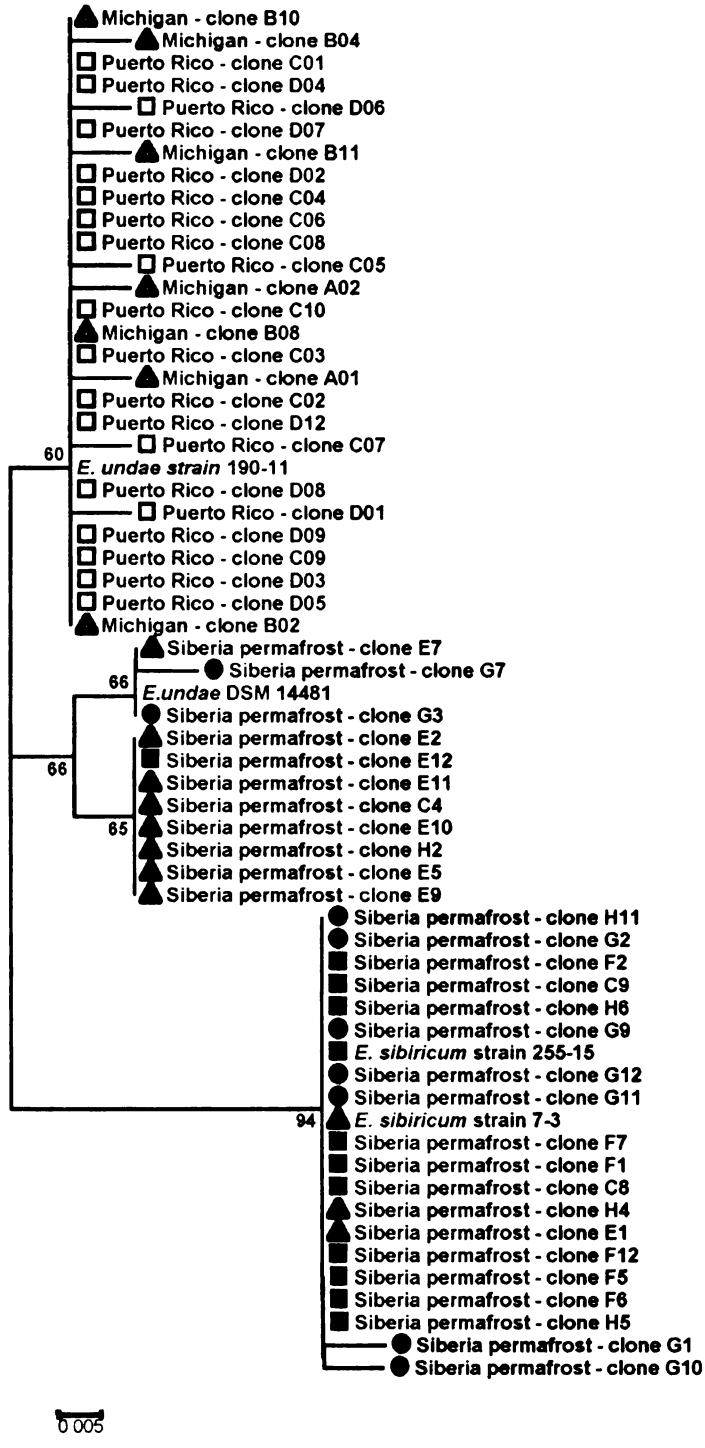

Figure 4.1 (A). Phylogenetic tree based on hypothetical (A) and *rpo*B (B) sequences of indicated *Exiguobacterium* strains and clone libraries from Michigan (△),Puerto Rico soil (□) and three Siberia permafrost soils dated of 2 to 3 million years (•), 100-200 thousand years (■) and 12 to 40 thousand years (▲). The trees were produced by the neighbor-joining method. The *rpo*B tree was rooted using the *rpo*B gene from *B. subtilis*. The nodes with less than 40% bootstrap values were not added to the tree. The scale bar represents changes per nucleotide.

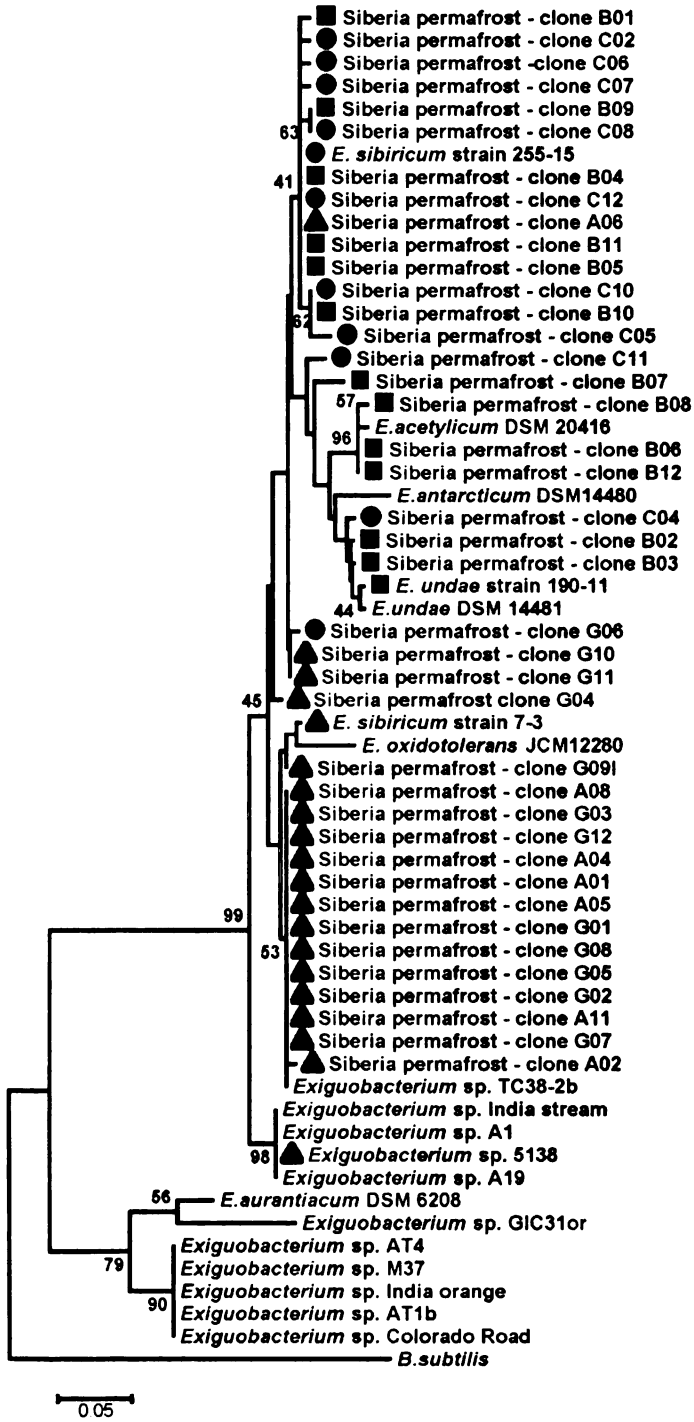

Figure 4.1 (B). Phylogenetic tree based on hypothetical (A) and *rpo*B (B) sequences of indicated *Exiguobacterium* strains and clone libraries from Michigan (△),Puerto Rico soil (□) and three Siberia permafrost soils dated of 2 to 3 million years (•), 100-200 thousand years (■) and 12 to 40 thousand years (▲). The trees were produced by the neighbor-joining method. The *rpo*B tree was rooted using the *rpo*B gene from *B. subtilis*. The nodes with less than 40% bootstrap values were not added to the tree. The scale bar represents changes per nucleotide.

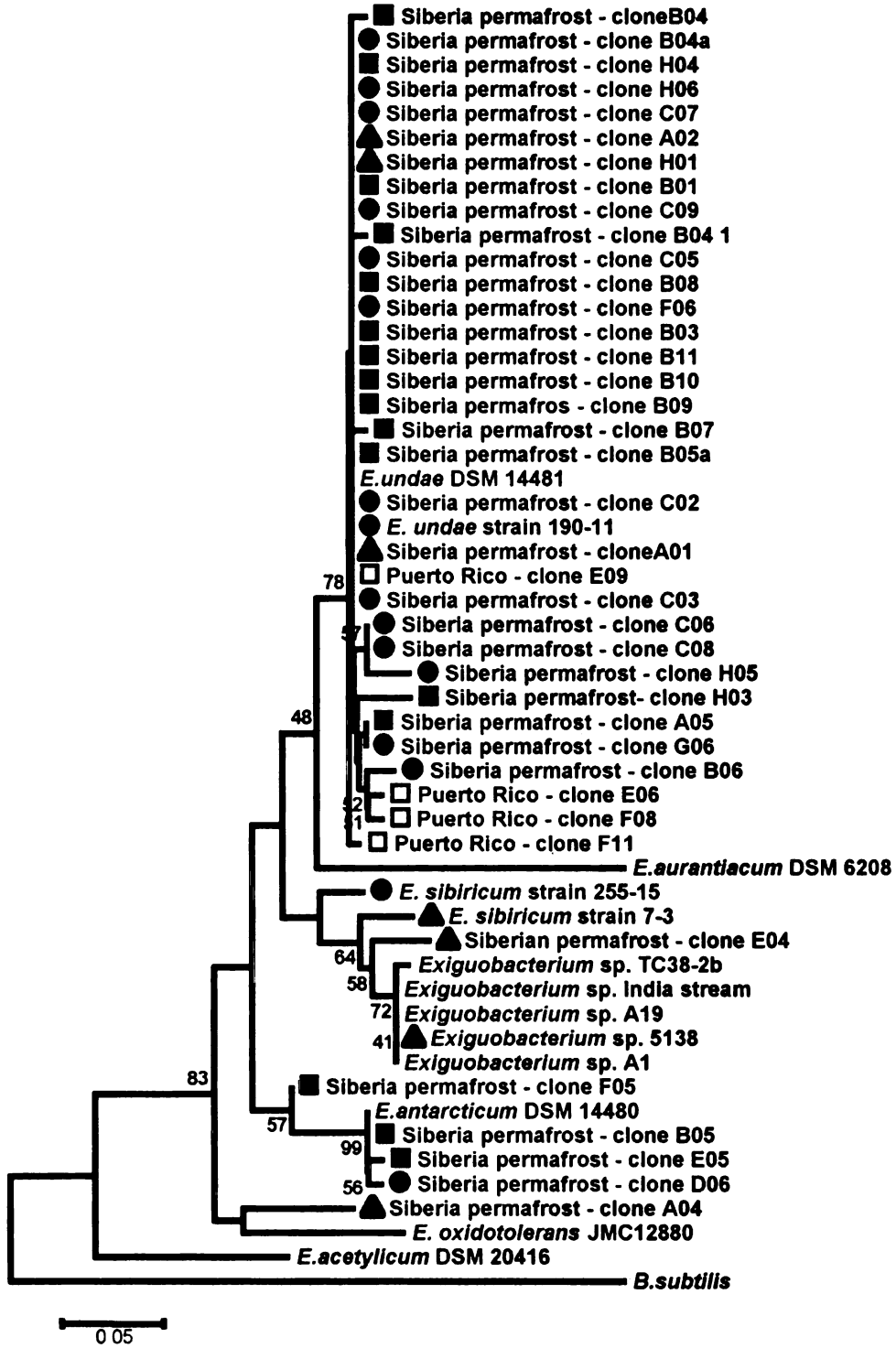

Figure 4.2. Phylogenetic tree based on *gyr*B sequences of indicated *Exiguobacterium* strains and clone libraries from Puerto Rico soil (□) and three Siberia permafrost soils dated of 2 to 3 million years (•), 100-200 thousand years (■) and 12 to 40 thousand years (▲). The tree was produced by neighbor-joining method and was rooted using the *gyr*B

gene from *B. subtilis*. The nodes with less than 40% bootstrap values were not added to the tree. The scale bar represents 0.05 changes per nucleotide.

Sensitivity, detection limit and accuracy. We tested the sensitivity of the real-time detection system using a dilution series of clone for each gene from *Exiguobacterium undae* strain 190-11 for each gene. To each dilution we added 1μg μl^{-1} of *E. coli* DNA to simulate the soil DNA in samples. The data obtained were used to construct a standard curve relating the C_T values to the added mass of *Exiguobacterium undae* gene clones. A regression line between the mean threshold cycle (C_T) values and the template DNAs from 1 to 10^6 dilution range gave an r^2 value superior to 0.98 for all genes tested (Figure 4.3). Detection of fluorescence could be observed in DNA concentrations as low as 10^0 gene copies per μl for the hypothetical gene and as low as 10^1 gene copies per μl for the *gyr*B and *rpo*B genes (Fig 4).

We also tested the detection limit and our ability to differentiate similar DNA concentrations of *E. undae* strain 190-11, *E. sibiricum* strain 255-15 and *E. antarcticum* DSM 14480^T for each gene. Different volumes (ranging from 2 to 20 μl) of a dilution series of template DNA (ranging from 1 to 10^6 gene copies μl^{-1}) were added to the PCR reactions. The variability associated with the C_T values was evaluated (Figure 4.5) for low and high copy numbers. Although the curves remained linear for all strains, the variability associated with C_T values at low copy numbers precluded our ability to distinguish concentrations for different strains in samples with similar amounts. Only when copy number was greater than 100 were we able to reliably differentiate a twofold difference in all the samples.

The increase in variability with cycle number is apparent when the upper 95% confidence interval for all determinations is plotted against the gene copy number. Based on the slope of each individual standard curve, we calculated the maximal error in the C_T value that would still allow detection of a twofold difference in gene copy number (MAE). This analysis was done for all three genes as illustrated in figure 4.5 for *gyr*B, since the other genes gave similar results. The average MAE value varied little for each gene tested, 0.51, 0.50 and 0.48 for hypothetical gene, *gyr*B (Figure 4.5) and *rpo*B, respectively. The corresponding 95% confidence interval was in all cases very small for all genes tested.

To test the accuracy of the system, 10^4 gene copies of *E. undae* strain 190-11 was added to 10 ng of different soil samples (Figure 4.6). Data for all genes showed that in most cases soil inhibitors for qPCR amplification did not inhibit the reaction by more than a factor of 10 (data not shown for *rpo*B and *gyr*B since results were similar to those for the hypothetical gene).

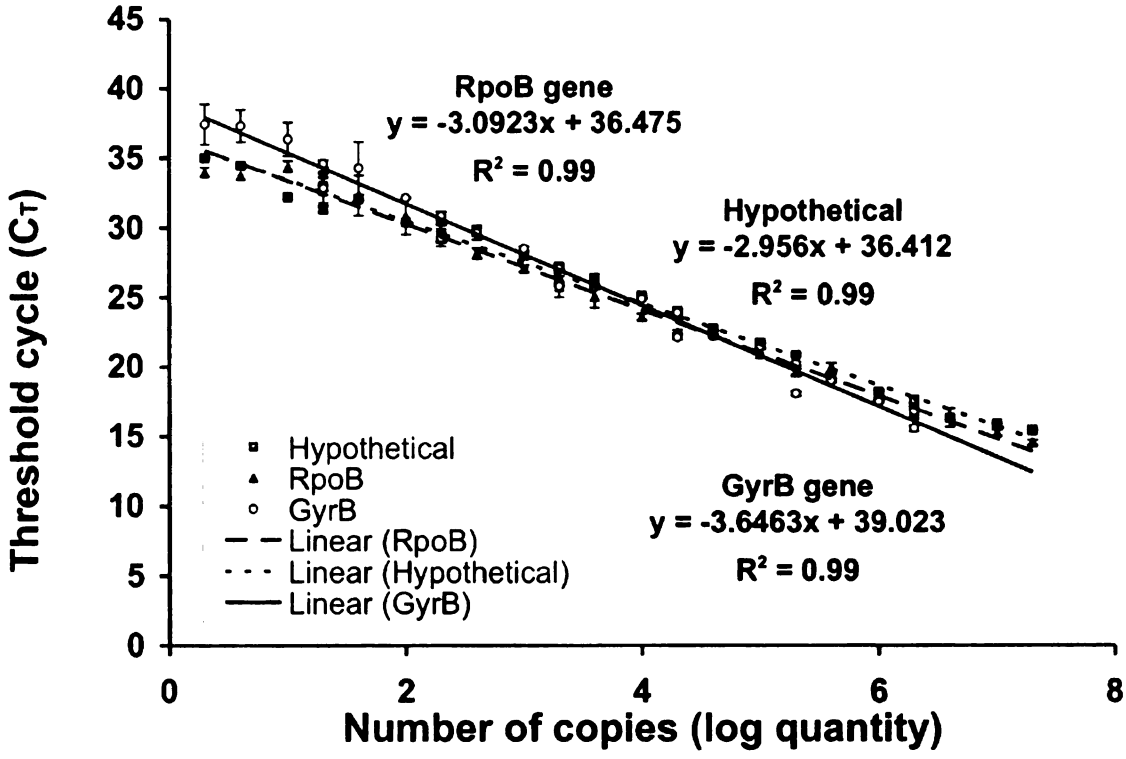

Figure 4.3. Calibration curves for each primer set.

Analyses of environmental samples. DNA extracted from the soil samples showed that *Exiguobacterium* spp. is widely distributed in the Siberian permafrost (Figure 4.7) and at a median density of 10^5 gene copies μg^{-1} of total community DNA. In none of the four temperate or tropical soils was *Exiguobacterium* detected by all three primers, which was the case in only two Siberian samples, numbers 19 and 20. Furthermore, *gyr*B had the lowest detection capability in the permafrost environment, but it gave the highest and only positive detection for these Exiguobacteria in the tropical environments. On the other hand, the *rpo*B primer was the only primer to detect *Exiguobacterium* spp. uniquely in the permafrost, even though it has the potential to detect both mesophilic and psychrotrophic ecotypes (Table 4.2).

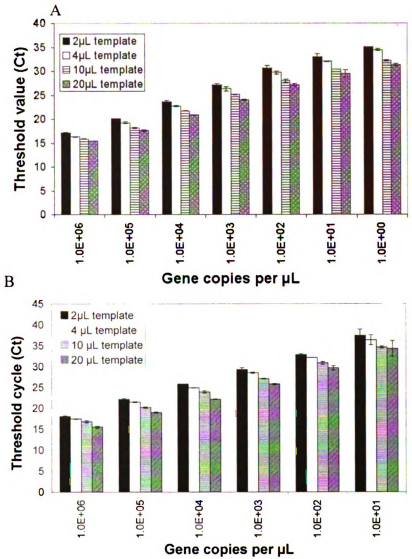

Figure 4.4. Detection limit of the *Exiguobacterium* spp. using real-time PCR. The denoted volumes of serially diluted gene clone were added to different reaction mixtures. (A) Detection limit of hypothetical gene. (B) Detection limit of *gyr*B gene.

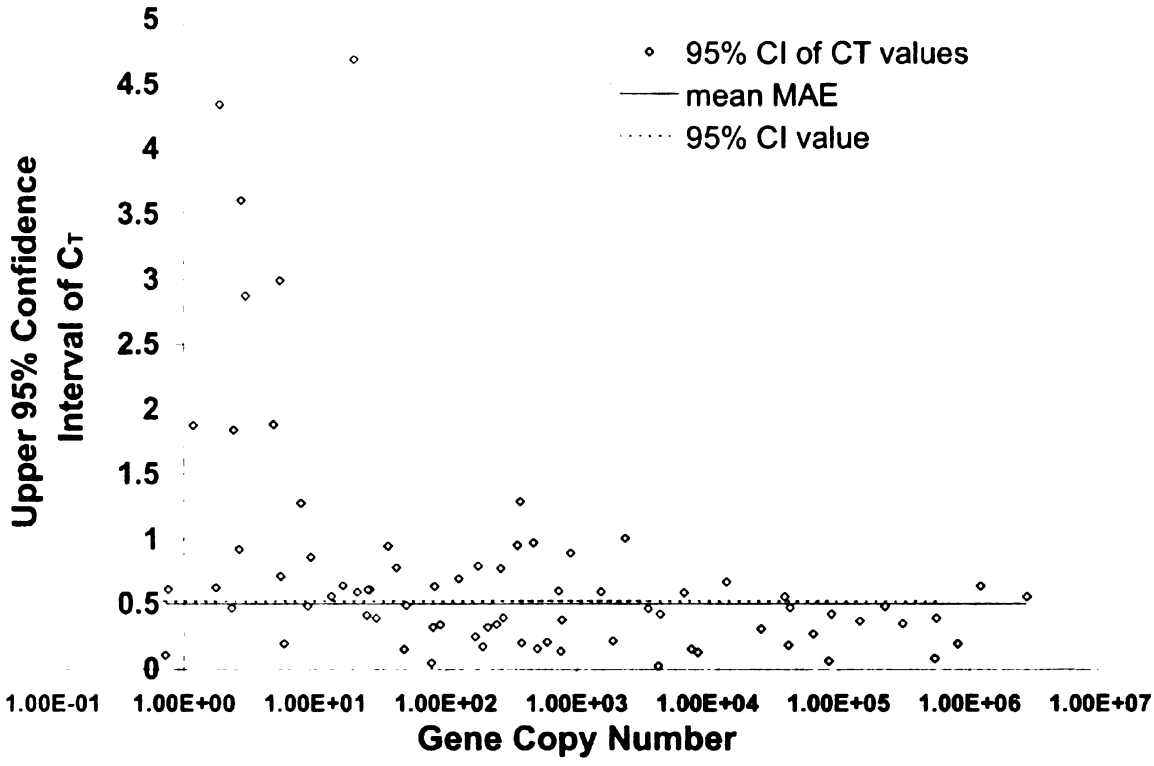

Figure 4.5. Relationship between gene copy number and error illustrated for *gyr*B. The horizontal line represents the MAE to discriminate between samples containing a twofold difference in *Exiguobacterium sibiricum* 255-15, *E. undae* 190-11 and *E. antarcticum* DSM 14480^T copy number. Values shown represent the mean from 16 independent standard curves. The 95% confidence intervals are shown as a dashed line.

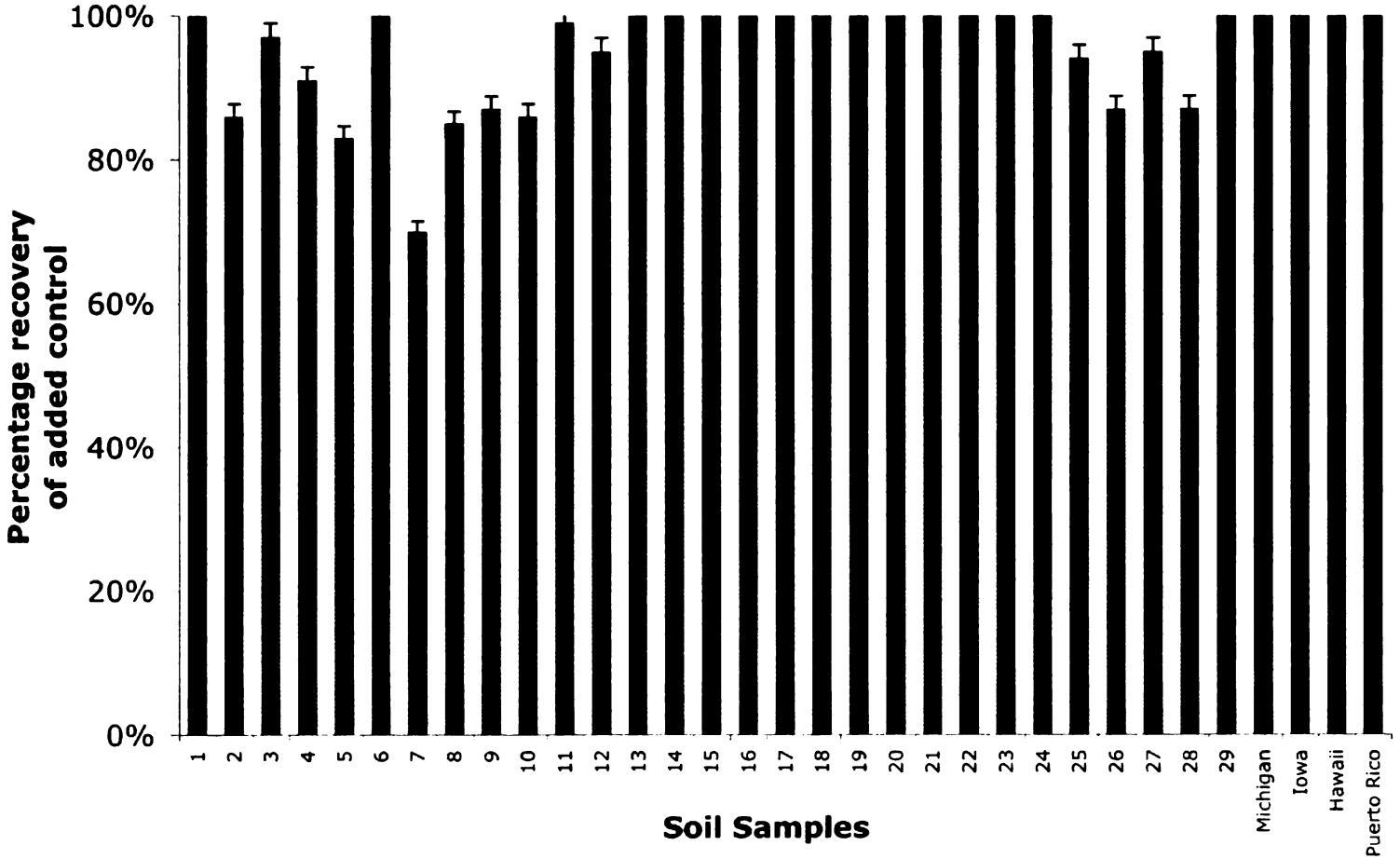

Figure 4.6. Percentage recovery of 10^4 hypothetical gene copies of *Exiguobacterium* spp. added to the soil DNA extracted from the different environments.

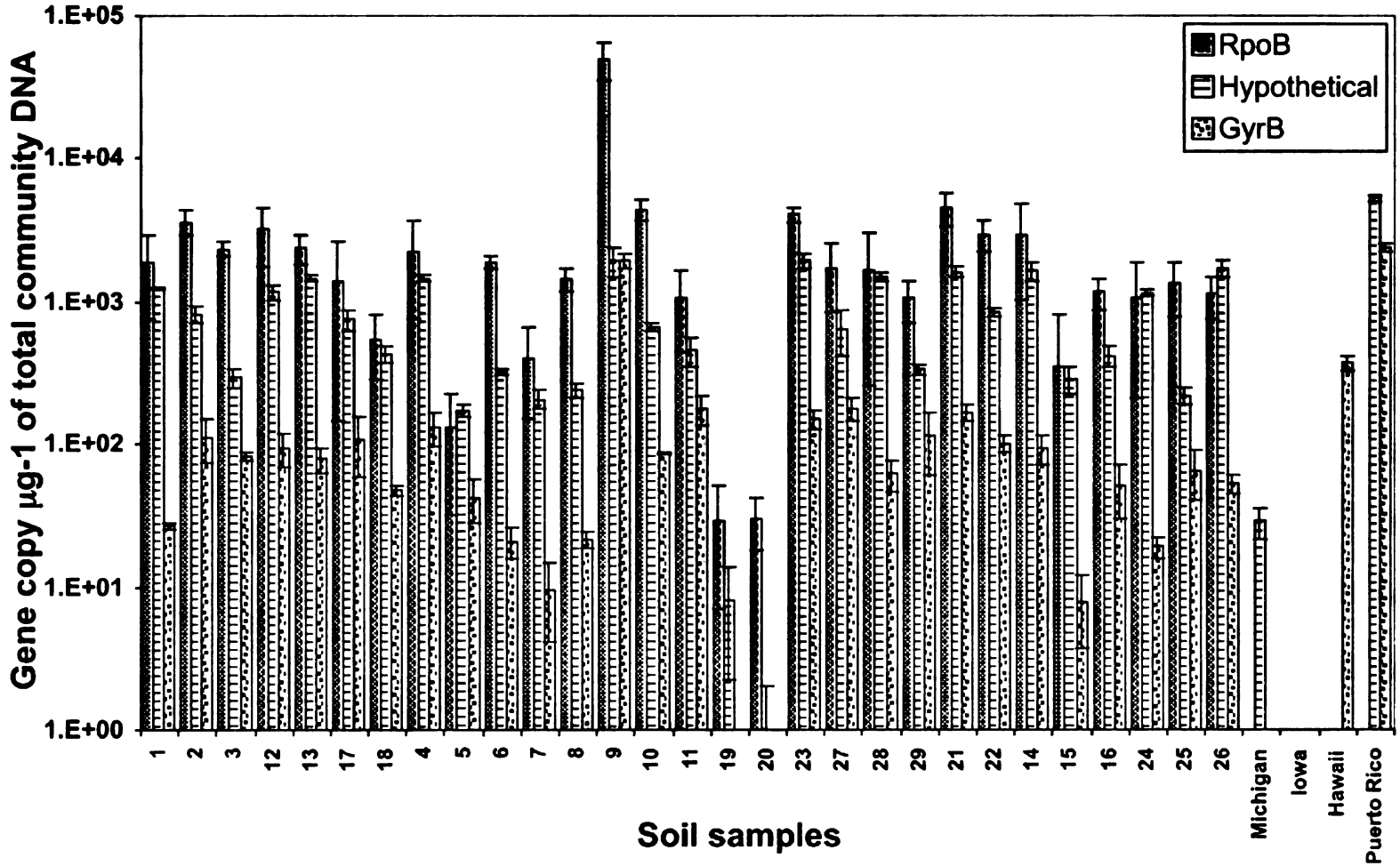

Figure 4.7. *Exiguobacterium* gene abundance in various habitats as measured by qPCR. The sample number corresponds to the permafrost sample number in Table 1.

DISCUSSION

Real-time PCR can provide specificity, sensitivity and precision for quantifying microbial populations (10, 19), provided that appropriate primers are identified. Using more than one gene to quantify a taxon will reduce the error in both the quantitation and specificity. The former is achieved if the genes are single locus, which is not the case for the 16S rRNA gene, and the later can be achieved by using multiple and less conserved genes as primer targets.

The copy number of rRNA operons per bacterial genome may vary from 1 to as many as 15 (1, 7, 13). Even within a genus the *rrn* copy number can vary, e.g. *Bifidobacterium* species have 1 to 5 copies of the *rrn* operon (5). Labrenz and coworkers (16) suggested using a standard DNA representing the bacteria most likely to predominate in a given habitat to reduce the copy number error, but this information is often unknown. The sequenced genome of *Exiguobacterium sibiricum* 255-15 has nine *rrn* copies, making the quantitation issue, if based on this gene, significant.

In the present study, we designed primers for three protein-coding genes (*gyr*B, *rpo*B and a hypothetical gene) to quantify the targeted *Exiguobacterium* group. The sequenced genome shows two copies of *gyr*B gene and one copy of *rpo*B and the hypothetical gene, which make them good candidates for study. The data show that the numbers of the hypothetical and *rpo*B genes are more consistent in the permafrost samples and in the Puerto Rican sample for the hypothetical and *gyr*B genes. This consistency, and similar and sensitive standard curves suggest that the use of both genes provides a more reliable quantitation of the targeted Exiguobacteria.

Certain protein-coding genes may have other advantages over use of rRNA-targeted primers. Usually, protein-coding genes have higher levels of sequence variation, which allow the differentiation of closely related strains (30), which is necessary for studies of different species from the same genus. Several publications have suggested that *gyr*B and *rpo*B are suitable genes for bacterial phylogeny, possessing essential attributes such as limited horizontal gene transfer and presence in all bacterial groups (12, 14, 30, 32). The hypothetical gene with unknown function was also used in this study because it was not found in any other genus when blasted against the NCBI database, which suggested that this gene is unique to our genus and not laterally transferred to other genera. The phylogenetic trees derived from the sequences of these three genes confirmed the ability of these primers to discriminate subgroups of the Exiguobacteria in clone libraries. The phylogenetic trees also show considerable diversity within the *Exiguobacterium* populations in different environments.

This study indicates that reliance on one primer set will not always give reliable quantitative results for the target group. Hence, multiple primers are advantageous. When multiple primers do not give the same results, criteria are needed to determine which primer sets best reflect the population density of the targeted group. Useful criteria are specificity with control strains, standard curve sensitivity, phylogeny of amplified clones and primer performance on a variety of habitat samples. While qPCR provides a fast and sensitive method for gene quantitation, the multi-locus addition provides greater specificity and in some cases sensitivity for more reliable quantitation of a targeted population. This approach is especially important where specific rRNA primers cannot be found, or where population resolution is important at the species or ecotype levels.

The fact that in some samples only one primer showed positive results suggests that if *Exiguobacterium* is present, that it is a substantially different genotype and probably species. The consistent and significant populations of *Exiguobacterium* detected in permafrost by the two primer pairs, and not generally in other soils, suggest that *Exiguobacterium* is selected in the Siberian permafrost. Furthermore, this genus may be ubiquitous in cold environments since a number of strains have been isolated from other cold environments such as Antarctica (8), Carpathian mountains (Ukraine) (4), Himalaya (India) (Mayilraj, S. personal communication and sequence **DQ450895**) and Siberian permafrost (27). *Exiguobacterium* ubiquity in cold versus other environments can now be tested with the qPCR method described herein.

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CHAPTER 5

ARCHITECTURE OF THERMAL ADAPTATION IN *EXIGUOBACTERIUM SIBIRICUM* STRAIN 255-15: A GENOME AND TRANSCRIPTOME APPROACH

ABSTRACT

Several microorganisms have wide temperature growth range and versatility to tolerate large thermal fluctuations in diverse environments. To better understand thermal adaptation of psychrotrophs, I used *Exiguobacterium sibiricum* strain 255-15, a psychrotrophic bacterium that grows from -5°C to 39°C. Its genome is approximately 3 Mb in size, has a GC content of 47.7% and includes 2,978 putative protein-encoding genes (CDS). I used the genome and transcriptome analysis along with the organism's known physiology to better understand its thermal adaptation. A total of about 27%, 3.2% and 5.2% of *E. sibiricum* strain 255-15 CDS spotted on the DNA microarray yielded differentially expressed genes in cells grown at -2.5°C, 10°C and 39°C, respectively, when compared to cells grown at 28°C. The hypothetical and unknown genes represented 10.6%, 0.89% and 2.3% of the CDS differentially expressed when grown at -2.5°C, 10°C and 39°C versus 28°C. The transcriptome analyses showed that *E. sibiricum* is constitutively adapted to cold temperatures since little differential gene expression was observed at growth temperatures of 10°C and 28°C, but at the extremities of its Arrhenius growth profile, namely -2.5°C and 39°C, much more differential gene expression occurred. The genes that responded were more typically associated with stress response.

INTRODUCTION

About 80% of Earth's surface is 15°C or colder (18). Psychrophilic together with psychrotolerant bacteria comprise the cold-adapted microorganisms. These microbes have been isolated and characterized from various environments such as polar sediments

and soils, as well as open oceans (37, 46). Psychrotolerant microorganisms are of special interest since they grow at a wide range of temperatures, between -5 and +40°C (26, 46), to tolerate large thermal fluctuations in diverse environments (12, 37).

A number of factors have been identified that provide physiological advantages for cold-adapted microorganisms (11, 37). A range of mechanisms that contribute to survival for mesophilic bacteria has been identified by examining the cold-shock response (28, 44). In contrast, to the best of my knowledge, no research has been devoted to exploring thermal adaptation of psychrotrophic bacteria over their entire growth temperature range.

The variation in specific growth rate as a function of temperature is commonly portrayed by Arrhenius equations (14). The Arrhenius profiles of most bacteria are characterized by a linear portion in a 20°C suboptimal growth range, which is called the normal or Arrhenius range (14). Below and above the normal range, deviations of the thermodependence of growth from the Arrhenius law reveals the inability of cells to maximize their growth rate (17), but little is known about the reasons why microbes are unable to maximize their growth rate at these extreme temperatures.

In the psychrotrophic bacterium *Exiguobacterium sibiricum* strain 255-15, growth occurs from -5°C to 40°C . This member of the *Bacillaceae* is Gram positive, rod-shaped, facultative aerobic, motile with peritrichous flagella (35). The relevance of studying this microorganism is that this genus has been shown to be prevalent in Siberian permafrost (36) and can be found in geological layers frozen for 20 thousands to up to 3 million years. Besides, this microorganism grows over an unusually broad temperature range and hence provides a good model for exploring molecular mechanisms of thermal adaptation.

The genome of *E. sibiricum* 255-15 has been sequenced to draft status by DOE's Joint Genome Institute. The genome has been assembled into 51 contigs of 20 reads or larger with an estimated genome size of 3 Mb. This study shows that few transcriptional changes were observed when the microbes were grown at 10°C and 28°C, but a number of changes were observed after growth at -2.5°C and 39°C, indicating that *E. sibiricum* strain 255-15 is constitutively adapted for growth at moderate range of temperatures.

MATERIAL AND METHODS

Bacterial strain and genome sequence. *E. sibiricum* 255-15 was isolated from a depth of 43.6 m in the permafrost sediment of the Kolyma Indigirka Lowland by Vishnivetskaya et al. [105]. At this depth, sediments are estimated to have been continuously frozen for 2 to 3 million years. A draft genome of 8X sequence coverage was produced by the Department of Energy's Joint Genome Institute and annotated by the automated pipeline operated by Oak Ridge National Laboratory's Computational Genomics Group. The annotation used in this work is available at JGI/ORNL dated April 1st, 2005 (http://genome.ornl.gov/microbial/exig/). The genome sequence is available on line at NCBI under the accession numbers: AADW02000001 to AADW02000051.

Arrhenius profile. *E. sibiricum* strain 255-15 was grown in flasks of ½ tryptic soy broth (TSB) (Difco, Detroit, MI) shaken at 200 rpm, at temperatures from 39°C to -2.5°C. Growth rates were calculated from the slope of optical densities for four or more time points for each temperature and summarized as an Arrhenius relationship.

Transmission electron microscopy. Inoculated agar plates were incubated for at 30°C, 12°C and -2.5°C, until they reached a colony size of 1 to 2 mm of diameter. Cell morphology and flagella were examined by transmission electron microscopy (TEM).

Probe design and array construction. The genome of *Exiguobacterium* strain 255-15 has a GC content of 47.7% and 2978 putative protein-encoding sequences (CDS). All 2978 CDS were used to select gene-specific or group-specific oligonucleotide (70 mer) probes using CommOligo (20) with group-specific probe design features. The design criteria were as follows: (i) 85% sequence similarity, 18-base stretch, and -35 kcal/mol free energy for gene-specific probes; and (ii) 96% sequence similarity, 55-base stretch, and -90 kcal/mol free energy for group-specific probes. Based on those criteria, 2931 CDS had gene-specific probes; 25 CDS were covered by six group-specific probes; no qualified probes were selected for 22 CDS. In addition, 10 human and 10 Arabidopsis probes were designed as controls. Those probes are expected to be very specific since the criteria used are even stricter than those previously suggested (15). All designed oligonucleotides were commercially synthesized without modification by MWG Biotech Inc. (High Point, NC). The concentration of oligonucleotides was adjusted to 100 pmol μl^{-1}. Oligonucleotide probes prepared in 50% DMSO (Sigma Chemical Co., MO) were spotted onto UltraGAPS glass slides (Corning Life Science, NY) using a Microgrid II robotic arrayer (Genomic Solutions Inc., MI). Each oligonucleotide probe had two spots on a single slide. Additionally, six different concentrations (5~300 ng μl^{-1}) of genomic DNA were also spotted (four duplicates on a single slide) as positive controls. After printing, the oligonucleotide probes were fixed onto the slide by UV cross-linking (600 mJ of energy) according to the protocol of the manufacturer (Corning Life Science, NY).

Growth conditions. All the cells for the DNA microarray experiments came from the same *E. sibiricum* 255-15 frozen stock that was used for the genome sequencing. All experiments were performed by first plating the cells in ½ Tryptic Soy Agar (TSA) and then transferred to ½ Tryptic Soy Broth (TSB) twice. In total, I had six samples grown independently at 39°C, 28°C, 10°C and -2.5°C. The cells were acclimated by growth at 39°C, 28°C, 10°C and -2.5°C. For the growth at 39°C and 28°C the plates were incubated overnight. At 10°C, the plates were incubated for 3 to 4 days. At -2.5°C the plates were transferred three times to new plates to acclimate the cells to lower temperature as follows: the first plates were incubated overnight at 22°C followed by incubation at 4°C for 3 days and then for 2-3 weeks at -2.5°C. After growing the cells in agar at the four temperatures, a loop from each plate was transferred to tubes containing 5 ml ½ TSB and grown in its respective temperatures until an optical density at 600 nm (OD_{600}) of 1.0 was attained. Then 1 ml of this culture was used to inoculate 100 ml of ½ TSB in a Nephlo Flask (Belco), except for the -2.5°C cultures that were inoculated in tissue flasks for better aeration. The samples were incubated until reaching mid-log growth ($0.1 < OD_{600} < 0.3$) when 100 ml of RNAlater (Ambion, Austin, Texas) was added at the same temperature as the grown cells. Cells were pelleted by centrifugation at 5,000 X *g* for 20 min at 4°C and resuspended in 1 ml of RNAlater, transferred to a 1.5 ml microcentrifuge tube, and re-pelleted at 5,000 X *g* at 4°C for 10 min.

RNA isolation. The cells were re-suspended in 100 µl of RNase-free 3 mg ml^{-1} Lysozyme in TE buffer pH 8 (50 mM Tris-Cl and 1 mM EDTA) by vortexing and then

incubated at room temperature for at least 20 min or until the pellet cleared. Lysozyme incubation at 37°C for 1 h was needed to lyze cells grown at -2.5°C. The RNA was then isolated using the RNeasy mini Prep kit (Qiagen) according to the manufacturer's instructions; the step of DNase digestion was included. The resulting RNA was checked by denaturing agarose gel electrophoresis for DNA contamination and for the presence and integrity of the rRNA bands. The amount of RNA was quantified using a UV-spectrophotometer at OD_{260}.

cDNA labeling and slide hybridization. Amino-allyl labeling was performed as adapted from a protocol of The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). Briefly, 10 μg of total RNA was used to synthesize cDNA overnight at 42°C using 0.5 mM of Random Hexamer Primers (Invitrogen, Carlsbad, CA), 3:2 ratio of 5-(3-amino-allyl)-dUTP and dTTP (Ambion), and Superscript II reverse transcriptase (Invitrogen), and subsequently labeled by coupling reactive Cy5 or Cy3 fluorophores (Amersham, Piscataway, NJ) to the amino-allyl groups. Purification after cDNA synthesis and chemical coupling were performed using QiaQuick PCR purification columns (Qiagen) as described in TIGR protocol. The quantity of labeled cDNA and the fluorophore incorporation efficiency were determined by using UV-visible spectrophotometry.

Microarray slides were incubated for 60 min at 46°C with prehybridization solution (50% Ultrapure formamide (Invitrogen), 5x SSC, 0.1% SDS and 0.1 mg ml^{-1}), washed three times in double-distilled water and one time in isopropanol, and dried by centrifugation at 50 X *g* for 3 min. Two cDNA's from different temperatures were mixed for direct comparisons for all temperature combinations. Each microarray received about

30 µl of hybridization solution (50% Ultrapure Formamide, 5x SSC, 0.1% SDS, 0.1 µg/µl Salmon sperm DNA) containing the two cDNAs. The solution was applied by capillary action under a coverslip (LifterSlip; Erie Scientific Company, Portsmouth, NH) placed over the microarray. The whole assembly was sealed in a hybridization chamber (CMT Hybridization Chamber; Corning Incorporated, Corning, NY) and submerged for 16 h in a 46°C water bath. Microarray slides were washed twice for 5 min at 46°C with 1X SSC-0.1%SDS; twice for 10 min at room temperature with 0.1X SSC-0.1%SDS and five times for 1 min at room temperature with 0.1 X SSC. Slides were dried by centrifugation at 50 X *g* for 3 min and were immediately scanned and analyzed.

Data analysis. Slides were scanned with an Axon 4000B scanner and GenePix 5.0 used for spot finding. Only spots with more than 80% of pixels greater than background plus 2 standard deviations in either Cy5 or Cy3 channel were used for analysis. Analysis was performed with *Limma* (Linear models for microarrays data) library in the CARMAweb environment (32). The background correction was done by background subtraction of the median value, followed by within and between arrays data normalization using the print tip Lowess method, and quantile method, respectively. A moderated t-test based on empirical Bayes approach (from the Bioconductors *Limma* package) with an adjustment of the calculated raw *P*-values was used with the following methods: Benjamini and Hochberg (1), Westfall and Young (49) as well as Bonferroni. Only *P*-values smaller that 0.01 for all these methods were considered statistically significant for further analysis.

RESULTS AND DISCUSSION

Genome analysis

E. sibiricum's genome sequence was compared by BLASTP with sequences from other microorganisms. The pathways and types of carbohydrate, amino acid, coenzyme, cofactor, nucleotide and energy metabolism of this microorganism were inferred from the genome to give a wholistic view of its metabolic potential (Figure 5.1). The genome showed that 27% of its CDS have top hits with *Bacillus halodurans*, while 25% and 24% have top hits with *B. anthracis* and *B. subtilis,* respectively (Figure 5.2). About 75% of the *Exiguobacterium* 255-15 CDS encode proteins that are putative homologs to *Psychrobacter arcticus* 273-4 proteins, which is a Gram negative microorganism also prevalent in Siberian permafrost.

The analysis of cluster of orthologous groups (COGs) distribution of *E. sibiricum* genome (Table 5.1) shows that it has a distribution similar to most microorganisms. Approximately 23% of the genes are poorly characterized. Since cold and heat stress related genes are not yet completely elucidated, it is possible that among these large numbers of poorly characterized genes there are those important for the cell acclimation to stress temperatures. The genome encodes apparent homologs of stress-related proteins as well as many novel proteins that may have unique roles in adaptation to the permafrost environment (Table 5.1).

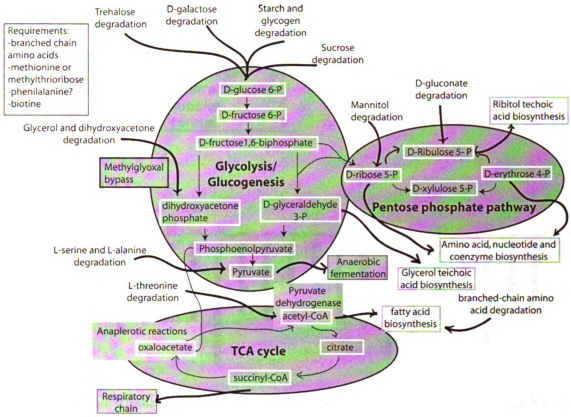

Figure 5.1. Metabolic pathway reconstruction of *Exiguobacterium sibiricum* 255-15 based on genome content (Figure provided by Natalia Ivannova).

I divided the genome analysis of *E. sibiricum* 255-15 into the following seven categories to more specifically analyze this psychroactive microorganism's metabolism (Figure 5.1): carbohydrate metabolism, amino acid biosynthesis, amino acid catabolism, coenzyme and cofactor biosynthesis, nucleotide biosynthesis, energy metabolism and miscellaneous observations, which includes the description of genes involved in temperature adaptation.

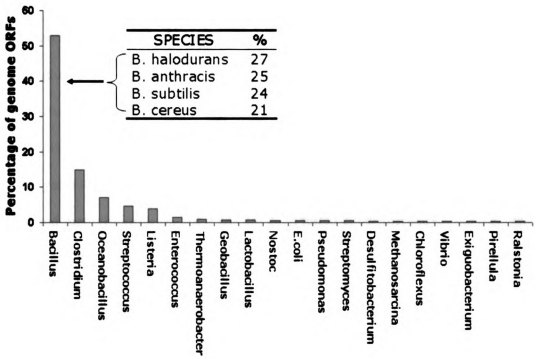

Figure 5.2. Distribution of top hits with other microorganisms of *Exiguobacterim sibiricum* 255-15 CDS by using BLASTP.

Carbohydrate metabolism. The genome analysis and physiological studies previously done showed that *E. sibiricum* 255-15 prefers sugar and carbohydrate polymers as carbon sources (29, 35). All the genes for Embden-Meyerhoff version of glycolysis such as glucose-specific PTS, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase, among others, are present. In addition to glycolysis, *E. sibiricum* should be capable of gluconeogenic growth on glycerol due to the presence of glycerol utilization operons and fructose-1,6-bisphosphatase and also due to its ability to grow on glycerol as sole carbon source (35). The presence of methylglyoxal synthase is another indicator that *E. sibiricum* prefers glycolytic substrates, since it allows bacteria to bypass the lower part of glycolysis in carbon-rich but phosphorus-limited conditions. *E. sibiricum* also has all enzymes from the non-oxidative pentose phosphate pathway, but

not from its oxidative branch (no glucose-6-phosphate dehydrogenase and phosphogluconolactonase). Among the sugars that *E. sibiricum* can utilize for growth are (35): D-glucose, D-gluconate, D-galactose (with two operons), maltose, D-fructose, sucrose, trehalose, beta-glucosides such as salicin and arbutin, mannitol (the last five sugars have the glucose-specific PTS), N-acetylglucosamine, D-ribose, glycerol and dihydroxyacetone. *E. sibiricum* might be able to utilize some other compounds, such as ethanol and methylthioribose, but it has not been tested experimentally.

Table 5.1. COGs (cluster of orthologous groups) classification of the genes in the genome of *Exiguobacterium sibiricum* strain 255-15.

CLUSTER OF ORTHOLOGOUS GROUPS CATEGORIES	**% in the total COGS**
Information storage and processing: 19%	
J Translation, ribosomal structure and biogenesis	6
K Transcription	7
L DNA replication, recombination and repair	6
Cellular processes: 21%	
D Cell division and chromosome partitioning	1
V Defense mechanisms	2
T Signal transduction mechanisms	6
M Cell envelope biogenesis, outer membrane	5
N Cell motility and secretion	3
U Intracellular trafficking, secretion, and vesicular transport	2
O Posttranslational modification, protein turnover, chaperones	3
Metabolism: 36%	
C Energy production and conversion	5
G Carbohydrate transport and metabolism	7
E Amino acid transport and metabolism	8
F Nucleotide transport and metabolism	3
H Coenzyme metabolism	4
I Lipid metabolism	2
P Inorganic ion transport and metabolism	5
Q Secondary metabolites biosynthesis, transport and catabolism	2
Poorly characterized: 23%	
R General function prediction only	13
S Function unknown	10

E. sibiricum 255-15 has a number of enzymes for degradation of carbohydrate polymers, mostly starch and starch-derived oligosaccharides, which were observed in the genome and experimentally (data not shown). These include several alpha-amylases, oligo-1,6-glucosidases, non-glucogenic alpha-amylase, exo-alpha-1,4-glucosidase, alpha-glucosidase, pullulanases, maltose phosphorylase. *E. sibiricum* may also store carbon as a non-branch glucose polymer, similar to glycogen, but without 1->6 branches, due to the absence of the branching enzyme. *E. sibiricum* might be capable of storing extracellular carbohydrate polymers such as glucans due to the presence of a putative glucansucrase, which catalyzes synthesis of insoluble alpha-D-glucans from sucrose. Most likely, the linkage pattern of the glucan product is 1->4/1->6 alpha-D-glucan based on the specificity of hydrolases found in the genome. Previous work has shown the presence of granules inside the cell (35), as well as the presence of exopolysaccarides (Figure 5.3), which could be result of these gene products.

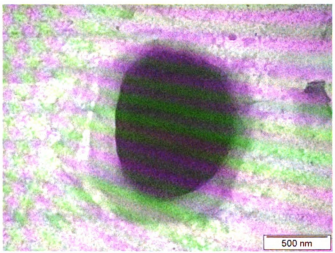

Figure 5.3. Negatively stained electron micrograph of *E. sibiricum* strain 255-15. Exopolysaccharide and no flagella are observed after growth at -2.5°C.

Amino acid biosynthesis. *E. sibiricum* should be auxotrophic for branched-chain amino acids (leucine, isoleucine and valine) and threonine due to the absence of any enzymes for their biosynthetic pathways. *E. sibiricum* might also be auxotrophic for phenylalanine, since there are no orthologs of the genes for phenylalanine and tyrosine biosynthesis. However, it is also possible that it might have an unusual pathway for phenylalanine biosynthesis, since it has the gene for the first enzyme (after chorismate) chorismate mutase, which is fused to 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase. In addition, there is a weak homolog of the periplasmic cyclohexadienyl dehydratase from *P. aeruginosa*. However, since Gram-positive bacteria do not have periplasmic space, it is unlikely that this protein is part of phenylalanine biosynthesis pathway. Besides, no ortholog of a periplasmic aromatic amino acid aminotransferase of *P. aeruginosa* is present. No genes from the sulfate activation pathway (ATP sulfurylase, APS reductase or APS kinase and PAPS reductase, any of sulfite reductases) were found, which indicates that *E. sibiricum* requires an organic sulfur source, such as methionine, cysteine or methylthioribose. Orthologs of a putative cysteine (cystine) ABC transporter and a methionine ABC transporter, such as in *Lactococcus lactis,* were found. *E. sibiricum* has a probable serine O-acetyltransferase and cysteine synthase for biosynthesis of cysteine from serine and sulfide. In addition, it seems to have a transsulfurylation pathway from methionine to cysteine represented by cystathionine beta-synthase and cystathionine gamma-lyase. No orthologs of homoserine O-succinyl(O-acetyl)transferase (metA) or O-acetylhomoserine sulfhydrylase (cysD) were found in the genome. *E. sibiricum* has cobalamin-independent homocysteine S-methyltransferase and a pathway for methylthioribose recycling to methionine. Thus, it appears that *E. sibiricum* requires

methionine or methylthioribose for growth and can produce cysteine by transsulfurylation from methionine. Tyrosine might be produced from phenylalanine by phenylalanine 4-hydroxylase and a complete pathway for tryptophan biosynthesis is present. Lysine biosynthesis appears to be proceeding via acetylated intermediates and employing a dapX-type diaminopimelate epimerase.

Conventional amino acid biosynthesis pathways are present for the remaining amino acids: arginine, histidine, glutamate, glutamine, aparagine, serine and even polyamines.

Amino acid catabolism. *E. sibiricum* has several amino acid degradation pathways and some of them can be used as energy sources. It has an operon, which includes NAD-dependent valine dehydrogenase, which catalyzes oxidative deamination of valine, isoleucine and leucine, branched-chain alpha-keto acid dehydrogenase, phosphotransbutyrylase and butyrate kinase. This pathway would allow use of branched-chain amino acids as nitrogen sources and convert branched-chain amino acids into corresponding free acids: isovalerate, isobutyrate, and methylbutyrate. The organism also has the genes to produce ATP through substrate-level phosphorylation under anaerobic conditions and provides branched-chain acyl-CoAs for fatty acid biosynthesis.

E. sibiricum also has pathways for degradation of the aromatic amino acids phenylalanine and tryptophan. Phenylalanine dehydrogenase is present, which catalyzes the oxidative deamination of phenylalanine to phenylpyruvate, a product which can be further converted to phenylacetate (e.g., by pyruvate dehydrogenase found next to phenylalanine dehydrogenase). Phenylacetate can be further degraded via a ring-hydroxylation/beta-oxidation pathway encoded by an operon next to phenylacetate-CoA

ligase. Tryptophan degradation genes encoding tryptophan dioxygenase, kynurenine formamidase and kynureninase are present and could produce enzymes to degrade L-tryptophan to anthranilate. There is no pathway for further degradation of anthranilate, so most likely tryptophan can be used as nitrogen, but not carbon source.

E. sibiricum has a pathway for anaerobic degradation of threonine, which includes threonine 3-dehydrogenase and 2-amino-3-ketobutyrate-CoA ligase, which converts threonine into glycine and acetyl-CoA and would allow using threonine as a nitrogen and probably carbon source. Glycine can be further degraded via a glycine cleavage system. L-serine dehydratase and alanine dehydrogenase are also present; they produce pyruvate out of L-serine and L-alanine, respectively.

Coenzyme and cofactor biosynthesis. *E. sibiricum* is auxotrophic for biotin (no KAPA synthase, KAPA aminotransferase, dethiobiotin synthase and biotin synthase genes). The rest of coenzyme biosynthesis pathways seem to be complete, with the exception of several genes missed either by annotation or sequencing. The missing genes include uroporphyrinogen-III synthase (the gene is present in the sequence, but it was missed by the gene prediction algorithm), riboflavin kinase/FAD synthase, GTP cyclohydrolase I, and pantothenate kinase.

Nucleotide biosynthesis. Both purine and pyrimidine biosynthesis pathways are complete. *E. sibiricum* has both aerobic and anaerobic ribonucleoside diphosphate/triphosphate reductases.

Energy metabolism. *E. sibiricum* has been shown to grow both aerobically and anaerobically (data not shown). For aerobic growth, one can observe in the genome a complete TCA cycle and an aerobic respiratory chain, which consists of monomeric

NADH-quinone oxidoreductase, and two quinol oxidases, one cytochrome caa3-dependent, another cytochrome ba3-dependent. Although there is no anaerobic respiratory chain observed in the genome, anaerobic growth has been shown to occur via fermentation of sugars (data not shown) and maybe via fermentation of branched-chain amino acids and threonine. Fermentation pathways found in the genome include pyruvate-formate lyase and acetoin (or butanediol).

Miscellaneous observations. *E. sibiricum* has genes that encode the production of both glycerol teichoic and ribitol teichoic acids. It has only one fatty acid desaturase, no fatty acid hydroxylases and no cyclopropane-fatty acid synthase, but it seems to produce mostly branched-chain fatty acids (29). It might be also capable of producing one or more different carotenoids (two squalene/phytoene synthases, one of them is clustered with likely diapophytoene desaturase and another phytoene dehydrogenase). The orange pigmentation observed in *Exiguobacterium* colonies suggests that it does caroteinoid (Monica Ponder personal communication).

This microorganism has several genes that may be involved in thermal adaptation, i.e. heat and cold-shock. The genes listed in Figure 5.4 are candidates that are present in *E. sibiricum* strain 255-15 genome and hence may be expressed under temperature stresses. These include several ribosomal binding proteins for the translational machinery, genes responsible to maintain the membrane homeoviscous adaptation, sigma factors, genes involved in DNA replication and metabolism genes that may have special temperature adaptations.

- **Ribosomal binding**
  - CspA,C,R,L
  - GroEL, GroES, GrpE
  - rpsF
  - rbfA
  - Ef-Tu
  - Trigger factor-chaperone
  - DnaK, DnaJ -chaperone
- **Membrane alteration**
  - Desaturase
  - β-ketoacyl carrier protein
- **Miscellaneous**
  - NusB
  - recA
  - IF2β Lon protease
  - General stress proteins
  - htrA
  - hrcA
- **Metabolism**
  - Isocitrate dehydrogenase
  - Cysteine synthase
  - Glyceraldehyde phosphate dehydrogenase
  - Triose phosphate isomerase
  - Pyruvate dehydrogenase
  - γ-glutamyltranspeptidase
  - Dihydrolipoamide acetyltransferase
- **Sigma factors**
  - rpoE
  - rpoH
  - rpoD
  - rseB (anti-sigma factor)
- **DNA replication**
  - GyrA

Figure 5.4. Known cold and heat stress response genes with homologs to *Exiguobacterium sibiricum* 255-15. The genes in red correspond to the heat expressed genes. The genes in blue are the cold expressed genes and the ones in pink are the ones that are expressed in both conditions. The Image in this dissertation is presented in color.

Transcriptome analysis

To gain further insight into which of the organism's genes may be involved in temperature adaptations, I performed a transcriptome analysis with cells at -2.5°C, 10°C, 28°C and 39°C (Figure 5.5). The 28°C and 10°C temperatures were chosen as the mean of the maximum and the minimum temperature where the biphasic shift in the growth rate occurs (Figure 5.6). The temperature of 39°C was selected because it is close to the upper limit of *E. sibiricum* 255-15 growth (40°) and -2.5°C was selected as a subzero temperature where the medium does not freeze.

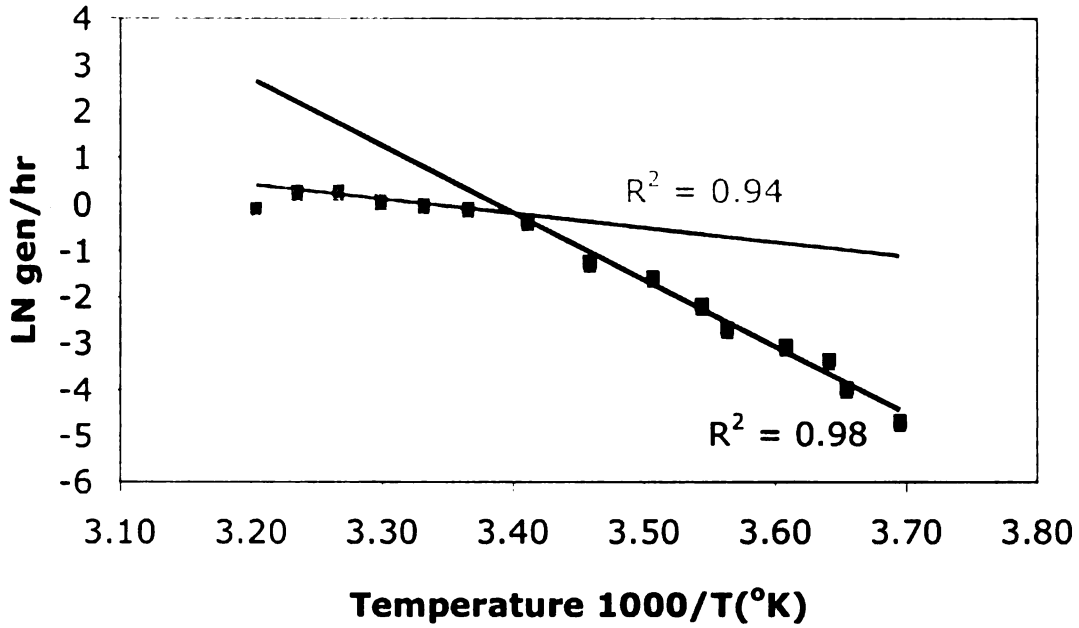

Figure 5.5. Arrhenius plot of *E. sibiricum* 255-15 growth rates in ½ TSB. The first phase of the biphasic response is in gray and the second phase is in black, each with its respective trend lines and R^2 values.

The transcriptome studies overall results are represented by Venn diagrams in Figures 5.6 and 5.7. A total of about 27%, 3.2% and 5.2% of *E. sibiricum* strain 255-15 CDS spotted on the DNA microarray yielded differentially expressed genes in cells grown at -2.5°C, 10°C and 39°C, respectively, when compared to cells grown at 28°C. The hypothetical and unknown genes represented 10.6%, 0.89% and 2.3% of the CDS differentially expressed when grown at -2.5°C, 10°C and 39°C versus 28°C. This data shows that many unknown genes are differentially expressed, especially at -2.5°C, suggesting that new genes important to thermal adaptation may be in this group. The results from the transcriptome comparisons are represented in Table 5.2.

Figure 5.6. Venn diagram of all differentially expressed genes at the indicated temperatures

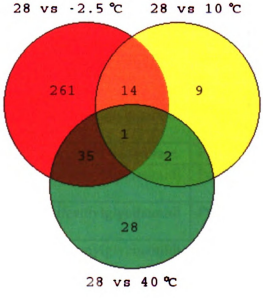

Figure 5.7. Venn diagram of unknown or hypothetical genes differentially expressed at the indicated temperatures

Table 5.2. Fold-change (log odds score) at different temperatures.

ID	Gene Product	Gene	28°C vs		
			10°C	-2.5°C	40°C
General metabolism					
278_1189	Orotate phosphoribosyl transferase	PyrE	-3.9	-5.6	-0.9
278_1190	Orotidine 5'-phosphate decarboxylase	PyrF	-3.8	-5.3	-0.8
278_1191	Dihydroorotate dehydrogenase 1	PyrD	-2.8	-4.1	-1.0
278_1192	dihydroorotate dehydrogenase electron transfer subunit	UbiB	-4.3	-6.0	-0.7
278_1193	Carbamoyl-phosphate synthase, large subunit, glutamine-dependent	CarB	-4.6	-5.8	-0.9
278_1194	Carbamoyl-phosphate synthase, small subunit	CarA	-4.1	-7.0	-1.2
278_1195	Dihydroorotase multifunctional complex type	PyrC	-3.8	-5.1	-1.5
278_1196	Aspartate carbamoyltransferase	PyrB	-3.9	-5.4	-1.3
278_1197	Xanthine/uracil permease	UraA	-2.6	-4.7	-0.5
278_1198	pyrimidine operon attenuation protein	PyrR	-2.2	-3.9	-0.3
283_2148	AICARFT/IMPCHase bienzyme	PurD	-1.1	-0.4	0.0
283_2149	Phosphoribosylglycinamide formyltransferase	PurH	-1.2	-1.6	0.1
283_2150	Phosphoribosylformylglycinamidine cyclo-ligase	PurN	-1.2	-2.5	0.4
283_2151	Amidophosphoribosyl transferase	PurM	-1.0	-2.8	0.1
283_2152	Phosphoribosylformylglycinamidine synthase II	PurF	-0.7	-2.8	-0.2
283_2153	Phosphoribosylformylglycinamidine synthase I	PurL	-1.3	-2.3	-0.5
283_2154	Phosphoribosylformylglycinamidine synthetase PurS	PurL	-0.8	-1.6	-0.3
283_2155	SAICAR synthetase	PurS	-0.8	-3.4	-0.4
283_2156	Adenylosuccinate lyase	PurC	-1.0	-2.5	-0.8
283_2157	Phosphoribosylaminoimidazole carboxylase, ATPase subunit	PurB	-0.6	-2.6	-0.4
283_2158	1-(5-Phosphoribosyl)-5-amino-4-imidazole-carboxylate (AIR) carboxylase	PurK	-1.0	-2.4	-1.1
277_1054	Adenylosuccinate synthetase	PurA	-0.9	-0.8	0.3

Table 5.2 (cont'd)

Energy metabolism					
245_40	NADH dehydrogenase	Ndh	-0.1	-2.0	-0.5
245_47	NADH dehydrogenase	Ndh	-0.6	-1.3	0.5
249_98	Ubiquinone/menaquinone biosynthesis methyltransferase	UbiE	-0.8	-2.2	-0.9
250_114	Nicotinate phosphoribosyltransferase related	PncB	0.2	1.0	0.4
272_751	oxidoreductase, putative		0.3	3.2	0.9
272_787	2-polyprenyl-3-methyl-5-hydroxy-6-metoxy-1 4-benzoquinol methylase	UbiQ	-0.1	-1.6	-0.1
279_1294	electron transfer flavoprotein beta-subunit	FixA	-0.2	1.8	-0.3
279_1295	electron transfer flavoprotein, alpha subunit	FixB	0.3	2.4	0.3
281_1741	NADH-dependent flavin oxidoreductase	NemA	0.2	1.2	-0.1
284_2268	Rieske 2Fe-2S iron-sulfur protein		-0.2	3.6	0.0
285_2440	Carbonic anhydrase	CynT	0.0	1.9	3.2
284_2338	H+-transporting two-sector ATPase, gamma subunit	AtpD	0.1	-0.9	-0.9
284_2339	ATP synthase F1, alpha subunit	AtpG	0.1	-1.6	-1.8
284_2340	H+-transporting two-sector ATPase, delta (OSCP) subunit	AtpA	-0.5	-1.2	-1.7
284_2341	ATP synthase F0, subunit B	AtpH	-0.2	-1.1	-1.0
284_2342	ATP synthase F0, C subunit	AtpF	0.2	-1.3	-1.2
284_2374	6,7-dimethyl-8-ribityllumazine synthase	RibH	-0.9	2.0	0.4
284_2375	3,4-Dihydroxy-2-butanone 4-phosphate synthase:GTP cyclohydrolase II	RibB	0.3	2.5	0.5
284_2376	Lumazine-binding protein	RibC	0.3	2.2	0.3
284_2377	Riboflavin biosynthesis protein RibD	RibD	-0.9	2.3	1.1
278_1235	cytochrome caa3 oxidase subunit III	cyoC	-1.7	-2.2	-1.2
278_1236	cytochrome c oxidase subunit I	cyoB	-1.2	-2.0	-1.2
278_1237	cytochrome c oxidase subunit II	cyoA	-0.5	-4.1	-1.7
278_1238	Protoheme IX farnesyltransferase	cyoE	-1.3	-2.0	-0.5
283_2015	Heme/copper-type cytochrome/quinol oxidases subunit 2	cyoA	-0.7	2.3	0.1

Table 5.2 (cont'd)

274_901	Dihydropteroate synthase	FolP	-0.3	1.1	-0.6
274_902	Dihydroneopterin aldolase family:Dihydroneopterin aldolase	FolB	0.5	2.2	0.5
257_279	Dihydrofolate reductase	FolA	0.2	3.0	-0.6
Carbohydrate metabolism					
260_327	glycerol-3-phosphate dehydrogenase	GlpA	0.5	2.6	1.1
247_71	Glycerol kinase	GlpK	0.6	2.4	-0.3
247_72	Aquaporin	GlpF	1.7	3.8	0.1
281_1712	glycerol-3-phosphate dehydrogenase, aerobic	GlpA	-0.2	-0.6	2.0
248_85	simple sugar transport system permease protein		-1.1	-2.3	-1.5
248_86	sugar ABC transporter (permease)		0.1	-1.6	-0.4
256_250	Phosphoenolpyruvate-protein phosphotransferase	PtsA	1.1	-0.1	-1.5
256_252	PTS system, glucose-specific IIBC component	PtsG	-0.4	-1.0	0.2
260_320	glucose uptake protein	GlcU	-0.7	1.3	1.9
260_321	glucose 1-dehydrogenase	gdh	0.0	2.5	2.3
262_369	fructokinase	RbsK	-0.3	-0.9	0.3
262_371	PTS system, sucrose-specific IIBC component	PtsG	-0.6	-2.6	-1.1
264_451	butyryl-CoA dehydrogenase	CaiA	0.4	2.7	-0.4
265_462	Phosphoenolpyruvate carboxykinase (ATP)	PckA	0.2	1.9	0.1
265_481	maltose permease		-1.1	-1.1	-1.4
281_1564	L-lactate dehydrogenase	Mdh	0.8	3.1	1.6
281_1578	Alpha amylase, catalytic subdomain	AmyA	1.6	3.9	1.4
281_1678	maltosaccharide ABC transporter, permease	MalG	0.0	2.9	1.2
281_1679	maltosaccharide ABC transporter, permease protein	UgpA	-0.3	1.6	1.5
281_1681	Alpha amylase, catalytic subdomain	AmyA	0.1	1.4	0.3
281_1713	Glycerol kinase	GlpK	-3.0	-0.4	-0.5
281_1715	Aquaporin	GlpF	-1.0	1.3	-0.9
282_1773	Alpha amylase, catalytic subdomain	AmyA	-0.1	0.3	1.7

Table 5.2 (cont'd)

282_1868	transcriptional repressor of the xylose operon		0.0	2.3	-0.3
282_1876	UDP-glucose 4-epimerase	GalK	-0.3	0.9	0.2
282_1878	aldose 1-epimerase	GalT	-0.6	1.1	0.2
282_1912	glycerol dehydrogenase	ARA1	-0.6	-2.0	0.5
283_2100	PTS system, N-acetylglucosamine-specific IIBC component		1.0	1.4	-0.6
283_2101	Phosphotransferase system IIC components glucose/maltose/N-acetylglucosamine-specific		0.7	3.8	0.2
284_2172	Sugar-specific permease, EIIA 1 domain		3.0	-0.9	0.2
284_2210	Alpha amylase, catalytic subdomain	AmyA	0.6	-1.6	0.3
285_2848	Alpha amylase, catalytic subdomain	AmyA	1.5	2.6	1.0
285_2893	Alpha amylase, catalytic subdomain	AmyA	0.0	3.0	-0.3
283_2005	Pyruvate-formate lyase	PflD	0.9	1.9	1.7
266_510	pyruvate ferredoxin oxidoreductase, alpha subunit	PorA	-1.2	-2.4	0.1
Amino acid metabolism					
249_105	3-dehydroquinate synthase	AroB	-1.3	-1.9	-0.3
250_112	phosphoglycerate mutase	GmpB	-0.3	0.9	0.2
250_114	Nicotinate phosphoribosyltransferase related	PncB	0.9	1.0	0.4
250_121	Alanine racemase region	Alr	-0.5	-1.0	0.2
257_273	cystathionine gamma-synthase	MetC	-2.6	1.1	-0.4
257_274	cysteine synthase	CystK	-3.1	0.3	-0.7
260_328	probable amino-acid transporter transmembrane protein		0.5	-1.6	0.8
262_366	Amino acid/peptide transporter		-1.1	-1.4	-0.3
264_434	Histidinol phosphate phosphatase	HisJ	0.4	1.5	-0.3
264_435	ATP phosphoribosyltransferase involved in histidine biosynthesis	HisZ	-0.5	1.3	0.0
264_436	ATP phosphoribosyltransferase	HisG	-0.7	1.4	0.5
264_437	Histidinol dehydrogenase	HisD	-0.9	1.1	0.1
264_438	histidinol-phosphate aminotransferase	HisC	-0.6	1.2	0.3

Table 5.2 (cont'd)

264_439	imidazoleglycerol-phosphate dehydratase	HisB	-0.6	1.5	0.3
264_440	Imidazole glycerol phosphate synthase, glutamine amidotransferase subunit	HisH	-0.5	0.9	0.3
264_441	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	HisA	-0.6	1.4	0.2
264_442	Histidine biosynthesis protein	HisF	-0.6	1.8	0.4
264_443	phosphoribosyl-ATP pyrophosphatase/phosphoribosyl-AMP cyclohydrolase	HisI	-0.6	1.6	0.3
264_444	phosphoribosyl-ATP pyrophosphatase		-0.7	1.0	-0.2
264_447	prolyne dehydrogenase		0.7	1.1	-0.8
266_508	L-threonine 3-dehydrogenase	Tdh	-1.1	-1.8	0.0
272_784	Amino acid ABC transporter, permease protein, 3-TM region, His/Glu/Gln/Arg/opine	HisM	2.0	1.7	-0.4
272_785	ABC-type amino acid transport	HisJ	1.0	2.6	0.1
270_612	Choline-glycine betaine transporter	BetT	1.3	-1.2	-0.7
279_1267	Threonyl-tRNA synthetase, class IIa	ThrS	0.1	-1.8	-0.8
279_1276	RNA methyltransferase, TrmH family	PheS	-0.2	-1.6	-0.8
279_1277	Phenylalanyl-tRNA synthetase, alpha subunit	PheT	-0.3	-1.5	-0.2
281_1604	Glycyl-tRNA synthetase, alpha subunit	GlyQ	0.0	-2.1	-0.6
283_1956	Gutamate-1-semialdehyde aminotransfer	HemL	0.5	2.8	-0.8
283_2041	Arginine biosynthesis	ArgJ	-0.1	3.2	1.7
283_2089	D-alanyl-D-alanine carboxypeptidase	VanY	0.1	5.2	0.8
283_2102	Ornithine aminotransferase	PtsG	0.7	2.7	-0.7
283_2104	arginine ornithine antiporter		1.3	1.4	-0.7
283_2111	Glycine betaine/L-proline transport ATP-binding subunit	OpuBC	1.4	2.9	0.0
283_2112	Peptidoglycan-binding LysM	OpuBA	1.8	2.5	-0.6

Table 5.2 (cont'd)

283_2117	Dihydrodipicolinate reductase	DapD	0.3	1.0	-0.3
283_2118	Dihydrodipicolinate synthase subfamily	DapB	1.4	1.6	-0.1
283_2119	Aspartate-semialdehyde dehydrogenase, USG-1 related	DapA	1.9	1.7	0.1
285_2845	Phosphoserine aminotransferase	SerC	1.7	2.2	-1.0
285_2846	D-3-phosphoglycerate dehydrogenase	SerA	0.9	2.4	-0.3
285_2878	Glutamate synthase, NADH/NADPH, small subunit 1	GltD	0.6	4.9	1.1
285_2879	glutamate synthase (NADPH) large chain		1.5	4.5	1.0
259_295	Lysine/ornithine N-monooxygenase	IucD	0.2	2.0	0.8
DNA replication, transcription and translation					
251_136	Alanyl-tRNA synthetase, class IIc	AlaS	-0.2	-1.5	-0.4
268_568	Tyrosyl-tRNA synthetase, class Ib	TyrS	-0.9	-2.5	-0.4
262_353	Helicase, C-terminal:DEAD/DEAH box helicase, N-terminal	SrmB	1.5	0.9	-0.7
271_642	Ribosome-binding factor A	RbfA	0.4	0.9	-0.6
271_643	Initiation factor 2:Small GTP-binding protein domain	InfB	0.5	1.1	-0.8
271_644	ribosomal protein, L7Ae family (50S ribosomal protein)	Rpl8A	0.9	1.5	-0.4
271_645	Predicted nucleic-acid-binding protein implicated in transcription termination		0.6	1.0	-0.6
271_646	Transcription termination factor NusA	NusaA	0.7	1.2	-0.6
271_656	Elongation factor Ts	Tsf	-0.9	-1.9	-0.5
271_694	Bacterial DNA topoisomerase I	TopA	-0.1	-1.7	-0.9
272_724	Inosine guanosine and xanthosine phosphorylase	DeoA	1.6	2.7	1.4
272_723	pyrimidine-nucleoside phosphorylase	Pnp	0.7	2.3	1.0
272_765	Bacterial DNA topoisomerase III	TopA	0.6	1.6	0.2
277_1053	DnaB helicase	DnaB	0.0	2.3	1.4
278_1201	Isoleucyl-tRNA synthetase, class Ia	IleS	1.0	-1.9	-1.0
278_1221	Ribosomal protein S32, bacterial and organelle form	RpmF	0.2	-1.7	-0.1

Table 5.2 (cont'd)

279_1285	Ribonuclease HIII	RnhC	-0.3	2.1	0.2
279_1325	Glutamate-1-semialdehyde-2,1-aminomutase	HemL	0.5	1.7	0.6
279_1326	Valyl-tRNA synthetase, class Ia	ValS	0.6	-1.3	-0.4
279_1322	Glutamyl-tRNA reductase	HemA	0.1	-1.0	-0.3
280_1438	similar to Oligoendopeptidase F		-1.8	2.2	0.1
280_1496	DNA topoisomerase VI subunit A		-0.8	3.7	1.4
281_1533	NusB antitermination factor	NusB	-0.6	-1.9	-0.4
281_1593	Helicase, C-terminal:DEAD/DEAH box helicase, N-terminal	SrmB	0.9	1.6	-0.6
284_2171	Oligoendopeptidase F		1.9	4.4	0.0
284_2386	Transcription termination factor Rho	Rho	0.1	1.4	-0.1
285_2751	Ribosomal protein L25	RplY	0.4	6.2	2.5
285_2752	Helicase, C-terminal:DEAD/DEAH box helicase, N-terminal	HepA	1.1	0.7	2.0
285_2754	Gram positive topoisomerase IV, subunit B	GyrB	-0.1	4.4	2.6
285_2756	Gram positive topoisomerase IV, subunit A	GyrA	0.0	1.1	0.5
248_82	ATP-dependent DNA helicase RecQ	RecQ	-1.0	-1.9	0.2
261_350	RecF protein	RecF	0.1	1.4	-0.8
266_514	RecA bacterial DNA recombination protein	RecA	0.3	1.3	0.4
273_836	Exonuclease	DnaQ	-0.5	-1.2	-0.6
278_1162	RecG-like helicase	RecG	0.4	1.5	-0.3
281_1531	Exonuclease VII, small subunit	XseB	-0.5	-1.9	-0.1
281_1532	Exonuclease VII, large subunit	XseA	-0.5	-1.4	-0.1
242_17	Bacterial regulatory protein, MarR	MarR	8.9	2.2	0.7
248_81	Bacterial regulatory protein, MerR	MerR	-0.4	-0.6	0.8
248_89	Bacterial regulatory protein GntR, HTH	GntR	-0.4	-1.8	-1.1
255_236	RNA polymerase sigma-54 factor	RpoN	0.4	2.2	1.4
271_659	Sigma-70 factor		0.0	-2.2	0.1

Table 5.2 (cont'd)

285_2541	Bacterial regulatory protein, MarR	MarR	0.0	1.5	-0.2
285_2542	Transcriptional regulator	MarR	-0.1	-2.8	-0.8
285_2572	Transcriptional regulator	GntR	0.4	-3.1	1.3
285_2919	Helicase, C-terminal:DEAD/DEAH box helicase, N-terminal		0.3	1.5	0.5
Miscellaneous observations					
271_660	methylation of methyl-accepting chemotaxis protein KO: K03411 chemotaxis protein CheD	CheD	0.0	-2.1	0.3
271_661	chemotactic methyltransferase inhibitor KO: K03410 chemotaxis protein CheC	CheC	-1.0	-3.1	-0.1
271_662	CheW-like protein	CheW	-0.5	-3.2	0.1
271_663	CheW-like protein:ATP-binding region, ATPase-like:Hpt	CheA	-0.5	-2.9	0.2
271_664	putative flagellar biosynthesis protein FlhG KO: K04562 flagellar biosynthesis protein FlhG	FlhG	-0.3	-3.0	0.3
271_665	ATPas	FlhF	-0.8	-3.7	0.0
271_666	Flagellar biosynthesis protein FlhA	FlhA	-0.8	-3.2	0.3
271_667	Flagellar biosynthetic protein FlhB	FlhB	-1.0	-3.7	0.3
271_668	Flagellar biosynthesis protein FliR	FliR	-0.8	-3.5	0.1
271_669	Flagellar biosynthesis protein FliQ:Type III secretion protein HrpO	FliQ	-0.1	-3.8	0.7
271_670	Flagellar transport protein FliP	FliP	-1.0	-3.3	0.3
271_671	Flagellar biogenesis protein	FliO	-0.8	-4.4	0.1
271_672	Response regulator receiver	CheY	-0.6	-3.3	0.4
271_673	Surface presentation of antigens (SPOA) protein	CheC	-0.4	-3.8	0.4
271_674	Flagellar motor switch protein FliM	FliM	-1.2	-3.7	0.0
271_675	flagellar FliL protein KO	FliL	-0.5	-3.1	0.5
271_679	Flagellar hook-length control protein	FliK	-0.6	-2.9	0.5
271_682	ATPase FliI/YscN	FliI	-0.8	-2.9	0.4

Table 5.2 (cont'd)

271_683	Flagellar biosynthesis/type III secretory pathway protein	FliH	-0.5	-2.2	0.5
271_684	Flagellar motor switch protein FliG	FliG	-0.3	-3.0	1.1
271_685	Flagellar FliF M-ring protein	FliF	-0.9	-3.6	0.3
271_686	Flagellar hook-basal body complex protein FliE	FliE	-0.4	-1.4	0.0
271_688	Flagellar basal-body rod protein FlgB		-0.1	-3.1	1.0
275_944	Flagellar protein FliS	FliS	-0.2	0.7	-0.1
275_945	flagellar hook-associated protein 2	FliD	0.4	1.4	0.1
275_946	flagellar protein	FlaG	0.4	1.4	0.5
284_2328	flagellar hook-basal body protein	FlaG	0.6	-2.2	-1.1
284_2329	flagellar basal-body rod protein KO	FlaG	-0.1	-2.0	-1.1
273_798	Response regulator receiver	OmpR	0.5	1.2	0.4
279_1330	Type II secretory pathway component PulJ	PulJ	-0.2	-1.4	-0.8
279_1331	Tfp pilus assembly protein	PilW	-0.3	-0.4	-0.3
279_1335	Type II secretory pathway ATPase	PulE	-0.4	-1.6	-1.2
279_1336	Pilus retraction protein PilT	PilT	-0.5	-2.0	-1.5
279_1337	type IV pilus assembly protein PilC	PilC	-0.8	-1.9	-1.1
279_1338	Tfp pilus assembly protein	PilE	-0.1	-2.5	-1.3
279_1339	signal peptidase	PulO	-0.1	-1.5	-1.3
ID	**Gene Product**	**Gene**	**40°C**		
Phosphate starvation			**28°C**	**10 °C**	**-2.5°C**
262_382	low-affinity inorganic phosphate transporter	PiT	-0.5	-1	-1.9
281_1571	PhoU	PhoU	2.3	2.9	1.8
281_1572	Phosphate transport system permease protein 1	PstB	2.0	2.4	1.8
281_1573	Phosphate transport system permease protein 2	PstA	2.6	2.8	1.6
281_1574	Phosphate ABC transporter, permease protein PstC	PstC	1.5	1.8	1.7
281_1575	Phosphate binding protein	PstS	2.9	3.1	1.7
heat shock proteins					
281_1649	Heat shock protein DnaJ, N-terminal	DnaJ	-0.3	0.6	0.9

Table 5.2 (cont'd)

281_1650	molecular chaperone DnaK (Hsp70)	DnaK	0.4	2.0	0.5
281_1651	molecular chaperone GrpE	GrpE	0.8	1.9	1.5
281_1652	Negative regulator of class I heat shock protein	HrcA	1.4	2.0	1.1
283_2134	ATPases with chaperone activity ATP-binding subunit	ClpA	1.3	1.9	0.9
284_2427	Chaperonin Cpn60/TCP-1	GroL	1.7	3.3	2.4
284_2428	chaperonin GroES (Hsp10)	GroS	2.0	3.8	3.5

The results from the transcriptome analysis confirmed the projections from the genome analysis and from previous physiological studies done with this microorganism (29, 35). The general trend of *E. sibiricum* metabolism seen as temperature decreases is that changes in expression of functions that are associated with the reduction of growth rate (Figure 5.5 and Figure 5.8). This reduction in metabolic rate is certainly in part conferred by the gradual reduction in expressed levels of pyrimidine, purine and several other metabolic processes. A similar observation was reported by Budde and collaborators with *B. subtilis* growing at low temperature (2).

Changes in gene expression commonly observed in cells enduring heat and cold shock were also observed during growth at the upper temperature limit of *E. sibiricum* 255-15 and at subzero temperature, respectively. These changes were in transcripts associated with carbohydrate metabolism, energy metabolism, amino acid biosynthesis and catabolism, membrane and cell wall adaptation as well as DNA replication, transcription and translation (Figure 5.9).

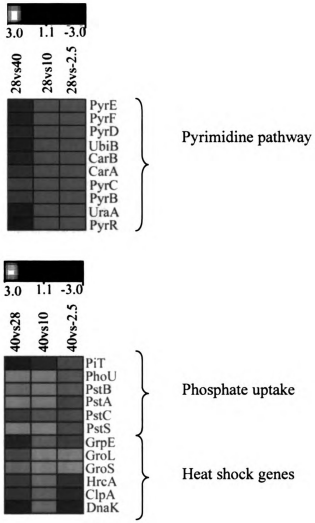

Figure 5.8. Heat maps of *E. sibiricum* strain 255-15 showing gradient of gene expression with increase or decrease of temperature growth. Image in this thesis is presented in color.

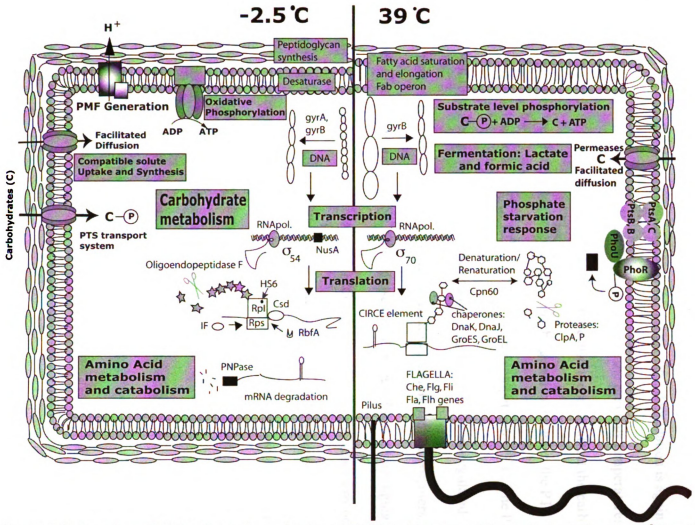

Figure 5.9. Summary of *Exiguobacterium sibiricum* 255-15 architecture of thermal adaptation at -2.5°C and 39°C

Carbohydrate metabolism. There seemed to be some differences in preference for carbon source utilization due to temperature (Table 5.2). For instance, glycerol can be used as a carbon source at 24°C but not at 4°C in the physiology study (29) and in the transcriptome the genes from the glycerol metabolism such as one of the genes for glycerol 3-phosphate dehydrogenase (glpA) was up regulated at 39°C, but down regulated at 10°C. At -2.5°C, however, the pathway for glycerol degradation is again up regulated and its differential expression is higher than at all the previous temperatures (*glp* K, F, A) as well as D-galactose genes. Temperature also seems to affect the carbon source take up, since we observed up-regulation of all the genes involved in the PTS transport system of glucose at -2.5°C, 10°C and 28°C when compared to 39°C. The PTS system is down regulated at 39°C but glucose is still being taken up by up-regulated glucose permeases, as well as other sugars such as maltose, melobiose and polymers, since maltose-binding proteins and Na^+/ melobiose and alpha-amylases are up regulated at 39°C. The fact that enzyme structure and function can be affected by, either low or higher temperatures, suggests that cells will increase the synthesis of proteins to compensate for a decrease in activity and stability (22), but in this case the cell is coping with the different temperatures by changing its carbon source utilization or uptake mechanism. Another interesting observation is that at -2.5°C several glucosidases (alpha-amylases) have different gene expressions at different temperatures; some are highly expressed at -2.5°C and others are down regulated when compared to the other temperatures. In a few cases, we observed the gene expression for this type of enzyme to gradually change expression with the temperature. Several others have described

different physiological efficiencies of mesophilic and psychrophilic alpha-amylases (5, 39). Maybe, *E. sibiricum* has alleles for both mesophilic and psychrophilic alpha-amylases, which would explain the preferential gene expression at certain temperatures.

E. sibiricum can certainly produce intra and extracellular polymers that are probably carbohydrate, since storage granules were observed previously in transmission electron microscopy (35), as well as external slime capsule (Figure 5.3), additionally several genes related to capsular polysaccharide biosynthesis were also expressed. The diverse genes for exopolysaccaride synthesis were downregulated at -2.5°C when compared to 10°C. In microscopic observations, the exopolysaccharide was present at all temperatures (Figure 5.3), but probably since the metabolism of *E. sibiricum* is reduced at -2.5°C, this is also reflected in the gene expression level, suggesting that it is still producing the exopolysaccharide but in smaller quantities.

Energy metabolism. *E. sibiricum* is certainly able to grow aerobically as well as anaerobically. This was observed not only experimentally by growing the cells under anaerobic conditions (data not shown) but also by the results obtained in the transcriptome analysis at 39°C (Table 5.2). At this temperature several genes from different pyruvate fermentation pathways were expressed, such as pyruvate:ferrodoxin oxidoreductase, pyruvate-formate lyase and L-lactate dehydrogenase. This transcriptome result is not surprising since oxygen solubility is significantly reduced at 39°C in water, about 6.1 mg l^{-1} (3, 51). This amount of oxygen is considered to be close to the limit for survival of most vertebrates (51). Additionally, other factors such as higher culture medium viscosity than water and the microorganism's high metabolic rate at this temperature would contribute to the decrease of oxygen availability to *E. sibiricum*,

generating a microaerophilic or even anaerobic environment that would trigger the expression of fermentation pathways. Like oxygen, CO_2 availability also seems to be an issue at 39°C, since the gene for carbonic anhydrase synthesis is induced in *E. sibiricum* as it is for *E. coli* under heat stress (25). This enzyme interconverts CO_2 and bicarbonate. Various essential metabolic processes require either CO_2 or bicarbonate. Although carbon dioxide and bicarbonate spontaneously equilibrate in solution, the low concentration of CO_2 in air and its rapid diffusion from the cell mean that insufficient bicarbonate is spontaneously made in vivo to meet metabolic and biosynthetic needs, so the cell needs to express this gene to overcome this problem (25). The gene for this enzyme is also up regulated at -2.5°C when compared to 28°C, but down regulated when compared to 39°C; this probably indicates that CO_2 is also limiting for the cell at -2.5°C. Even though at -2.5°C the solubility of gases increases considerably in the medium, the amount of CO_2/bicarbonate produced by the cell is probably not sufficient due to the low metabolism of the cell, leading to the expression of this gene.

Another indication that *E. sibiricum* is changing its cell bionergetics at the temperature extremes is the fact that ATPase sythase and cytochrome synthesis (*cyo* C, B, A, E) operons are down regulated at -2.5°C and 39°C. These two operons are used for ATP synthesis and generation of proton motive force during aerobic respiration, respectively. However, the reasons for down regulating these genes are certainly different for these extreme temperatures. In the case of -2.5°C, the cell is still respiring O_2 due to the high solubility of this gas at this temperature but also, since several electron carrier genes are up regulated when compared to all the other temperatures, namely, the genes for FAD biosynthesis (Rib H, B, D) and Rieske Fe/S. The better explanation for reducing

the expression of these genes at -2.5°C is the low metabolic rate of the microorganism. On the other hand, the low expression of ATP synthase and cytochrome c genes at 39°C is probably due to decreasing respiration, i.e. proton motor force (PMF), caused by low O_2 concentration in the medium. This is consistent into a switch from oxidative phosphorylation to substrate-level phosphorylation indicated by the expression change of two genes, known to be expressed in microorganisms under anaerobic conditions (50), pyruvate-formate lyase (PfyD) and pyruvate-ferrodoxin oxidorectase (PorA).

Energy metabolism also requires phosphate for the synthesis of ATP. At -2.5°C, 10°C and 28°C, *E. sibiricum* seems to have enough phosphate available in the medium; however at 39°C the cell seems to undergo phosphate starvation, since all the genes for inorganic phosphate starvation response are up regulated (PstABC and PhoU). This response suggests that somehow this high temperature affects the availability of phosphate to the cell.

Amino acid biosynthesis and catabolism. Amino acids in the cells are not only important as building blocks and energy sources but also as osmoprotectants (44). At -2.5°C and 10°C the transport of osmoprotectants for glycine betaine, carnitine, choline system permease is up regulated and completely down regulated at 39°C (Table 5.2). Additionally, proline dehydrogenase, which is responsible for the first step in the conversion of proline to glutamate, is progressively up regulated with the decrease of temperature, reaching a high gene expression at -2.5°C. In *E. coli* the shift to higher osmolarity triggers the accumulation of glutamate as an osmoprotectant (8). In *E. sibiricum* at -2.5°C the media becomes supercooled (24), the supercooled water generates a water flow out of the cell as if it was under salt stress. So the decrease of temperature is

sensed by the cell due to the differential chemical potential that probably triggers the production of glutamate, from proline degradation, as an osmoprotectant as well as the uptake of other osmoprotectants. In addition, genes for carnitine degradation that generate several osmoprotectans are also upregulated at -2.5°C. Therefore, besides the cold stress *per se*, cold temperatures also seem to affect the osmotic homeostasis of the cell.

E. sibiricum also seems to be changing its amino acids metabolism, especially at -2.5°C. Histidine, serine, arginine and lysine biosynthesis genes are up regulated at -2.5°C when compared to the other temperatures. Hence, cold stress seems to lead to flux and pool size redistribution throughout the entire network of amino acid metabolism. A similar effect was observed in cowpea cells under heat shock, which modified the metabolism and concentration of diverse amino acids in the cells (23). The reason for a change in cell metabolism is uncertain, but it maybe to synthesize more stable proteins at lower temperatures.

Cell membrane and cell wall adaptation. Cell membrane is important for the transmembrane transfer of molecules and other functions of the membrane-bound protein. After cold shock, bacterial cells experience membrane modifications, such as to increase unsaturation in the membrane phospholipids and decrease in chain length of fatty acids (11, 27, 34, 38) to help maintain the homeoviscosity and hence function (11, 38).

This phenomenon has been extensively studied in cyanobacteria, *Anabaena variabilis* and *Synechocystis* PCC 6803. Mutants defective in the desaturation of fatty acids (gene *des*A) that have a lower growth rate at low temperature (47). It has also been

found that some bacteria such as *Vibrio sp.* and *Micrococcus cryophilus* adapted to low temperature by decreasing the chain length of the fatty acids (34, 38). Further studies of cold-shock showed an increased degree of unsaturation in the cold-adapted lipid A of *E. coli* (4) and changes in the branched fatty acid profile of *B. subtilis* (19).

E. sibiricum also seems to change its saturation and chain length under different temperature conditions. Ponder and collaborators (29) demonstrated that *E. sibiricum* 255-15 shifted the fatty acids from saturated to unsaturated at 4°C, consistent with my finding of an increase in fatty acid desaturase gene expression at 10°C and -2.5°C. The fatty acid C16:0 was the predominant fatty acid in *E. sibiricum* 255-15 at mesophilic but at 4°C a shift occurred to iso C17:0 (29). Therefore, *E. sibiricum* seems to be keeping its homeoviscous adaptation by increasing the membrane fatty acid saturation.

Another important change in *E. sibiricum* 255-15 with cold temperature is the increase of gene expression of peptidoglycan biosynthesis genes (MurA, D, E, I) as well as the lysine biosynthesis (DapA, B, D), which is one of the main amino acids in the *E. sibiricum* peptidoglycan structure (35) only observed at -2.5°C (Table 5.2). The thickening of the cell wall at -2.5°C was noticed as well by the increased difficulty to lyse the cell with lyzozyme during the RNA extraction (data not shown). This thickening of the cell wall at subzero temperature is probably for protection against cell disruption by ice formation and/or osmotic pressure that can be generated at subzero temperatures.

DNA replication, transcription and translation dynamics. The DNA becomes more negatively supercoiled (11), while under heat stress, the DNA becomes less negative supercoiled (21). In both cases the DNA must be stabilized in a more functional conformation. In the case of cold stress, nucleoid-associated proteins such as Gyrase A,

IHF and H-NS are suggested to be necessary for its relaxation (13, 40, 48); while for heat stress only gyrase A has been described as being important. In *E. sibiricum* only at -2.5°C and 39°C DNA topoisomerases are up-regulated (gyrase B for both temperatures and gyrase A only at -2.5°C DNA), suggesting that *E. sibiricum* DNA topology at -2.5°C and 39°C has to constantly adapt its supercoiling by expressing the DNA topoisomerase genes (Table 5.2).

At the transcriptional level, we observed several genes enconding transcriptional regulators in *E. sibiricum*, including ARO8, MarR and GntR family proteins, which showed significant expression changes at 39°C. The MarR family transcriptional regulators were also induced in *B. subtilis* and *Termothoga maritima* during heat stress response (16, 30). In the case of *T. maritima* (30), the expression change of MarR family protein was dramatically higher during a long-term heat adaptation experiment, which was similar to the response observed in *E. sibiricum* at 39°C (Table 5.2). This suggests that this gene is playing a role in the cell adaptation under high temperatures.

The transcription system can also be affected by cold temperatures, for instance, the transcription factor NusA is known to be involved in both termination and antitermination of transcripts in *E. coli,* and it is highly expressed under lower temperatures (10, 34). This gene is also up regulated at -2.5°C and 10°C in *E. sibiricum.* Additionally, the RNA polymerase sigma 54 (rpoN) is up regulated and the RNA polymerase sigma 70 (housekeeping sigma) is down regulated only at -2.5°C, suggesting that sigma 54 may be responsible for the transcription of genes under cold or subzero conditions.

Besides the transcription, the cells need to cope with the translation of all transcripts produced, and for that the cells need to assure that all ribosome-associated proteins required for the formation of the translation initiation complex are produced. During cold-shock, several studies demonstrated that different genes are needed for this initiation complex to be fully functional, such as DeaD-box RNA helicase, ribosome binding factor A (RbfA), initiation factor 2 (IF-2) (33, 34) among several other ribosomal proteins. *E. sibiricum* does up regulated RNA helicases at -2.5°C and 10°C that may help unwind the RNA secondary structure for efficient translation at low temperature (9, 34). However, it is only at -2.5°C that IF-2, IF-3 and RbfA are significantly up regulated in *E. sibiricum* (Table 5.2). Different temperatures, especially at -2.5°C and 39°C, also affect the transcription of ribosomal proteins. The transcripts of ribosomal proteins L25 (RplY) are up regulated at 39°C and -2.5°C; while Rpl8A (L7Ae family) is up regulated only at -2.5°C. On the other hand, the ribosomal protein transcript of Rpm F is down regulated at -2.5°C (Table 5.2). This change in ribosomal protein expression has been observed as well in several other microorganisms, such as *Lactobacillus plantarum* (6), *Halobacterium* sp. NRC-1 (43) and *B. subtilis* (42), among others. These proteins are supposed to be the binding site for several of the factors involved in protein synthesis and appeared to be essential for accurate translation (45).

After the mRNA is transcribed and translated it needs to be degraded. It has been shown that during cold-shock, the RNA produced is degraded by a 'cold-adapted' RNA degradosome that contains PNPase (13). *E. sibiricum* also increases its Pnp gene expression with the decrease of temperature with a higher expression at -2.5°C, which

suggests that *E. sibiricum* may need the expression of this gene for growth in cold temperature.

The proteins that come out of the translational machinery also needs to be folded properly in order to be fully functional, and this can be an issue, especially under high temperatures that can cause protein denaturation. *E. sibiricum* seems to overcome this problem by inducing genes, at 39°C, from diverse heat shock proteins. These proteins are homologous to *B. subtilis* and *T. maritima* class I heat-shock genes hrcA-gprE-dnaJ-dnaK and GroEL-groES. In *E. sibiricum* 255-15, two operons are observed, the first contains the genes Dnak, GrpE and HrcA, respectively, that have identical expression levels and are in the reverse strand. The other contains Adk (Cpn60) and GroL also in the reverse strand and presents a similar expression level to the first operon at 39°C. The gene for DnaJ is also highly expressed at 39°C, but it is not part of the same operon that contains DnaK, as seen in *B. subtilis* (41). Additionally, some genes encoding ATP-dependent proteases ClpA and ClpP are also up regulated in *E. coli* and *B. subtilis,* under heat stress (7, 30, 52), as well as in *E. sibiricum* at 39°C. Futhermore, the genes Dna K, GroEs, Cpn60 are downregulated at -2.5°C and start to gradually increase expression with the increase in temperature and become highly expressed at 39°C.

Miscellaneous observations. Even thought at 10°C, *E. sibiricum* is fully motile (Figure 5.10), at -2.5°C, *E. sibiricum* appears to not be able to move by flagellar movement, since all the Che genes in the operon, as well as all the genes involved in flagella synthesis (all the genes from FLh, Fli and Flg operons) are down regulated (Table 5.2). The absence of flagella was also observed by TEM (Figure 5.3). The subzero temperature makes the media more viscous and it becomes close to freezing temperature, so the cell at this

temperature seems to be preparing for a frozen environment where flagella is no longer necessary. Additionally, several genes for pilus assembly proteins (Pil genes) and type II secretory pathway (Pul genes) are also down regulated at -2.5°C.

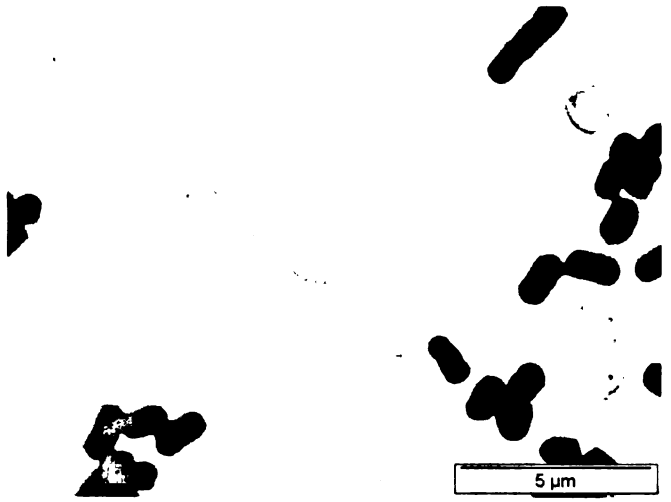

Figure 5.10. Negatively stained electron micrograph of *E. sibiricum* strain 255-15. Cell with flagella at 10°C.

In conclusion, not too many differences in gene expression related to cold adaptation are observed in *E. sibiricum* at the temperatures 10°C, 28°C and even at 4°C, previously analyzed by proteomics (31). This work demonstrates that this microorganism is constitutively adapted to cold temperatures since at stressful temperatures for mesophiles, such as 4°C and 10°C, no genes related to temperature stress are significantly expressed. Even though the growth rates change between 10°C and 28°C, it was surprising to see very little difference in gene expression and no expression of genes related to stress, especially because these two temperatures belong to two different phases of the biphasic Arrhenius plot (Figure 5.5). It seems that the growth rate shift in the Arrhenius plot does not have a strong significance on the microorganism physiology, at least observable at the transcriptome level. On the other hand, at the extremities of the Arrhenius profile, *E. sibiricum* undergoes several physiological adaptations very similar

to cold and heat shock responses previously studied in other organisms. Previous studies suggested that these physiological or gene/protein expression changes were only temporary during the initial shock (11, 44), while this study demonstrates that some of the changes in gene expression are not transient but necessary in the longer term for the cell survival and growth at these more extreme growth temperatures.

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EPILOGUE

RESEARCH SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The aim of the present work was to explore the ecology, physiology and metabolism of representative cold-adapted genera from Siberian Permafrost, to try to understand the important traits that make microorganisms successful in a cold environment and hence to disperse to other habitats than the Siberian permafrost. The results of this work showed that *Exiguobacterium* spp. and *Psychrobacter* spp. had a patchy distribution in temperate and tropical environments, but were ubiquitous in polar regions. The diversity of these microorganisms seems to have a latitudinal diversity gradient at the species level and their distribution in the environment seems to be influenced by environmental physico-chemical factors, such as pH, salinity, potassium (K^+), cation exchange capacity (CEC) and the micronutrient copper (Cu). The metabolism of these microorganisms correlates very well with physico-chemical factors of the environment, which shows the value of understanding the physiology to interpret the ecology of the microorganism.

The genome of *E. sibiricum* 255-15 was sequenced to better understand its general metabolism and thermal adaptation to a wide range of growth temperatures. At temperatures between 28°C to 4°C the cells do not show any significant physiological change, but at the extremities of its growth range, i.e., 39°C and -2.5°C, the microorganism undergoes several physiological changes to grow. Several physiological changes previously believed to occur under shock (cold or heat) appear necessary for sustained growth at subzero and high temperatures. Hence this present study provided insights about ecology, physiology and metabolism of psychrotrophic microorganisms.

FUTURE DIRECTIONS

Exiguobacterium sibiricum has been shown to be a very useful model for thermal-adaption, however no genetic system is available for this microorganism to be able to test the importance of several genes expressed under the differenttemperatures on the growth of this microbe. Hence, developing a genetic system for *Exiguobacterium* would be of great value for directly determining the role of some of the genes identified by differential expression in temperature adaptation.

Secondly, recently we obtained approval for the genome sequencing of a moderate thermophilic *Exiguobacterium* from a Yellowstone National Park thermal pool, that will provide an excellent resource for comparative genomics and proteomics under different temperatures.The genome sequence of both microorganisms will answer questions, at the molecular level, about how the enzymes of these microorganisms differ from one another.

A third question is whether *Exiguobacterium* spp. are present in older soils from tropical and temperate regions. If present, how do they diverge from the ones from the poles, as well as how diverse are the different species compared to the ones found in the poles?

Finally, defining the best carbon sources and growth conditions for these microorganisms will help understand of the role of these species in the microbial community.