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Culture-dependent and -independent molecular characterization of microbial communities sampled from beneath a high Arctic glacier (John Evans Glacier, Ellesmere Island, Canada)

by

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Abstract

High numbers of viable bacteria have been detected beneath John Evans Glacier (JEG), an environment that was once thought devoid of life. This study used culture-dependent and culture-independent amplified ribosomosal DNA restriction analysis (ARDRA) of 16S ribosomal RNA genes to characterize subglacial and proglacial microbial communities at JEG. ARDRA analysis revealed a high diversity of bacteria beneath JEG comprising members of the α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, *Cytophaga-Flavobacteria-Bacteriodes* , Actinobacteria and Firmicutes divisions. Many sequenced representatives from culture-dependent analysis were closely related to sequenced representatives from culture-independent analysis, showing reasonable correlation between the two methods. Theoretical 5' terminal fragments of 16S rRNA genes generated from sequenced representatives correlated with 11 terminal fragments detected through terminal restriction fragment length polymorphism analysis of JEG samples from a previous study. These results show that a combination of different characterization techniques is necessary to gain a more comprehensive understanding of environmental samples.

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List of Acronyms and Abbreviations

ARDRA	amplified ribosomal DNA restriction analysis
BC	basal ice within a basal ice cave
BF	basal ice at the junction of John Evans Glacier and Fox Glacier
CFB	Cytophaga-Flavobacteria-Bacteriodes
CFU	colony forming units
EDTA	ethylenediaminetetraacetic acid
JEG	John Evans Glacier
OTU	operational taxonomic unit
PSA	proglacial sediment near microbial mat A
SIB	subglacial initial burst
SOC	subglacial outburst channel
t-RFLP	terminal restriction fragment length polymorphism
t-RF	terminal restriction fragment

1.0 Introduction

1.1 Evidence of microbial life in polar and permanently cold environments

1.1.1 Life in cold environments

Microbes remarkably occupy air, water and soil and have adapted to live under conditions of high and low temperatures, pH, salinity and humidity (Staley and Gosink, 1999). Not surprisingly, microbial communities have recently been discovered in glacier ice, permafrost and other permanently cold (subzero) environments (Christner et al., 2000; Carpenter et al., 2000; Gilichinsky, 1995). Scientists have shown increasing interest in the ecological roles and biotechnological potential of these microbiota as they play an essential role in decomposition and mineralization processes, affecting nitrogen and carbon cycling (Vincent, 2000; Gilichinsky, 1995). As well, these microorganisms are integral members of most food chains, providing biomass for larger organisms. Cold-adapted enzymes produced by these bacteria can be marketed for biotechnological purposes (Nichols et al., 1999). Examination of potentially ancient biota found in geologically old soil and ice samples may help discover how life evolved and if life can exist elsewhere in our solar system (Miteva et al., 2004).

Characterization of microbial communities within these regions addresses several central questions (Vincent, 2000). Firstly, what strategies have microbes developed for living in these regions? Secondly, where do these microbes originate and how are they dispersed long range from the rest of the world and locally within polar regions? Thirdly, and most pertinent to this study, are there any microorganisms that are endemic to these regions? Baas-Becking (1934 cited by Staley and Gosink, 1999) suggested that "everything is everywhere, but the environment selects", meaning that microbes are already native to all environments on Earth, including glacier environments. However, a more widely accepted thought is that microbes can be dispersed from one environment to another, either by long range means or locally, and once seeded in a certain glacier environment, environmental pressures select for specific adaptive strategies over millions of years, allowing related bacteria to inhabit different environments (Vincent, 2000). Studies have shown that microbes can be transported long range to Antarctica by wind dispersal of pollen, fungal spores and bacterial spores (Poglazova et al., 2001). They may also be carried through fish, through bird and through human vectors, and even through

super-cooled cloud droplets (Saxena, 1983; Fuzzi et al., 1997). Then, local dispersion allows these microorganisms to be transported to other areas.

Once microorganisms have been inoculated into glacier environments, they may evolve or simply adapt to the glacier environment (Vincent, 2000). Psychrophilic and psychrotolerant organisms have adopted two different strategies for survival, which are often illustrated with respect to temperature (Gounot, 1991). Psychrophilic, or 'cold loving' bacteria, have an optimum growth temperature of $\leq$15°C and cannot withstand temperatures above 20°C. Psychrophiles occupy narrow niches and have evolved strategies such as high affinity nutrient transport, fast growth rate or altered proteins and lipids to have a competitive advantage in the environment (Vincent, 2000). Psychrotolerant, or 'cold tolerant' bacteria, conversely, grow optimally above 20°C, occupying a temperature range of about 5°C to 35°C (Morita, 1975). Psychrotolerant microorganisms occupy broad niches and grow suboptimally in glacier environments, tolerating extreme subzero temperatures (Gounot, 1991; Vincent, 2000). Psychrotolerant microorganisms are widespread in both cold and temperate regions because they adapt well to rapid environmental changes (Baross and Morita, 1978). Vincent (2000) suggested that common occurrences of psychrotolerant microorganisms are seen partly because insufficient time has been given for full evolutionary adaptation to occur. He proposed that selective pressures in glacier environments are relatively weak because biological interactions, such as competition, are less important than the ability to tolerate severe conditions.

Microbial communities must overcome several obstacles within glacier environments including freezing temperatures, high levels of UV irradiation, low nutrient availability and low water availability. Temperature has a direct influence on protein stability, which, in turn, affects microbial activity and viability in natural environments (Gilichinsky, 1995; Kennedy, 1993). The ability of microbes to live in these cold environments has been attributed to many structural and cellular adaptations. Under extremely low temperatures, bacteria incorporate unsaturated fatty acids into their lipid bilayer to maintain fluidity in the cell membrane (Gounot, 1991). Also, some microorganisms produce sheath pigments and carotenoids to protect themselves from high levels of UV radiation, which damage DNA (Vincent et al., 1993). It is widely

accepted that water availability plays the most influential role in determining the presence and abundance of life in cold regions and is necessary for the development of microbial life forms (Gilichinsky et al., 1993; Felip et al., 1995). Although most of the water in polar regions exists as ice, liquid water is available. Supraglacial environments, such as lakes, streams and cryoconite holes, are liquid-rich environments (Gordon et al., 2000; Säwström et al., 2002). Within glacier ice, water can be found in tiny veins of liquid water and within sea ice, water is trapped within a network of brine-filled channels (Price, 2000; Thomas and Dieckmann, 2002). Liquid water supplies important dissolved nutrients to microbial communities and also acts as a cryoprotective agent, preventing cellular damage from ice crystal formation (Gilichinsky, 1995).

Recently, diverse microbial communities have been discovered in the liquid- and sediment-rich environment beneath John Evans Glacier (JEG), a high Arctic glacier on Ellesmere Island, Nunavut, Canada (Skidmore et al., 2000). Little is known about the communities or geochemical processes beneath this polythermal glacier. However, previously characterized communities within other permanently cold environments will allow us to achieve a better understanding of communities beneath JEG. The following introduction will describe several polar and permanently cold environments where microbial communities have been found, present the major implications of these findings, review environmental techniques used to characterize microbial communities and outline the research objectives of the present study of samples from JEG.

1.1.2 Glacier environments

1.1.2.1 Glacial ice

Glacier ice sheets are formed by the deposition and the accumulation of snow at high elevations and in polar regions (Priscu and Christner, 2004). Glacier ice sheets formed at high elevations are termed valley or alpine glaciers, whereas glacier ice formed atop flat bedrock surfaces or inside volcanic craters are termed ice caps (Priscu and Christner, 2004). Ice cores obtained from these environments have become valuable sources of knowledge, archiving atmospheric conditions throughout history as well as microorganisms deposited over time (Priscu and Christner, 2004; Christner et al., 2000).

Common genera of bacteria, including *Sphingomonas, Acinetobacter, Arthrobacter* and endospore-forming *Bacillus*, have been found in ice cores from many

different glaciers (Handfield et al., 1992, Christner et al., 2000; Dancer et al., 1997; Priscu et al., 1999). Several of these bacteria form spores, have thick cell walls, have polysaccharide capsules and are pigmented. These features allow the bacteria to survive in low nutrient and unfavorable growth conditions (Priscu and Christner, 2004; Christner et al., 2000). Many of the recovered microbes are characterized by weakened cell membranes and decreased cell size due to freeze and thaw cycles, desiccation and UV radiation. Because of their weakened state, some researchers suggest that few microbes actually divide in situ (Priscu et al., 1999). Instead, these researchers propose that most merely survive, undergoing slow metabolic processes to repair cell damage (Priscu et al., 1999). Water, energy and carbon needed to facilitate these metabolic processes would be difficult to obtain through the solid ice matrix. However, evidence from chemistry experiments on the Antarctic ice cores drilled at the Vostok Station suggested that a system of liquid veins may exist at the junctions of solid ice crystals, providing a habitat conducive to active microbial living (Price, 2000). Through these thin veins, a supply of water and inorganic nutrients, such as sulfuric acid, nitric acid, formic acid, and mineral grains originating from the glacier ice surface, would be available to the resident microbes (Price, 2000).

Bacteria are transported by wind and snow to the glacier environment from nearby soil and rock. Several studies have shown that higher counts of viable bacteria (up to 180 CFU/mL in the Gulliya ice cap) are found in glaciers near areas of exposed rock and soil (Priscu and Christner, 2004; Christner et al., 2000; Handfield et al., 1992). Bacteria likely attach themselves to soil and rock for protection against harsh conditions, such as UV radiation and strong winds (Handfield et al., 1992). Because of this dispersal mechanism, high numbers of bacteria are directly correlated with climates where strong winds have occurred (Christner et al., 2000). Also, because psychrotolerant microorganisms can adapt more readily to new environments, they are more abundant in glacier environments than psychrophiles (Handfield et al., 1992).

1.1.2.2 Supraglacial environments

Wind-driven processes and solar radiation produce several liquid-rich supraglacial environments (those atop glacier surfaces). These environments have been compared to "oases", as each is a nutrient-rich ecosystem within a cold and arid glacier environment (Paerl and Priscu, 1998). Four of these supraglacial environments, glacier lakes, glacier lake ice cover, snow, and cryoconite holes, will be discussed in the following sections. Each of these environments contains unique microbial communities. Glacier lakes host simple food webs consisting of bacteria, plankton, protozoa and cyanobacteria (Alfreider et al., 1996; Battin et al., 2001; Felip et al., 1995). Permanent lake ice cover contains sediment-associated assemblages of phototrophic cyanobacteria and heterotrophic bacteria (Gordon et al., 2000). In areas where snowfall is abundant, snow and slush also accumulate on top of the lake ice cover, creating a distinct habitat of flagellates, ciliates and heterotrophic bacteria (Felip et al., 1995; Carpenter et al., 2000). And, finally, cryoconite holes provide a unique environment dominated by algae.

1.1.2.3 Glacier lakes

The most widely studied permanently cold lakes are located in the McMurdo Dry Valleys of Antarctica (Gordon et al., 2000). In the dry valleys, air temperatures average –20°C and high mountain ranges shield the valley from snow precipitation creating an ice-free polar desert (Gordon et al., 2000). The lakes found in the dry valleys are permanently covered with thick (3 to 6 m) layers of ice (Gordon et al., 2000). The lakes within Antarctica have had little anthropogenic influence and contain simple food webs without fish or macrozooplankton (Takacs and Priscu, 1998). For these reasons, they are good sites to study microbial processes within extreme and pristine environments. Takacs and Priscu (1998) have shown that Antarctic lakes can sustain high numbers of bacteria (10^3 to 10^7 cells/mL) that are comparable to other productive systems, such as the Amazon river (10^0 to 10^6 cells/mL). However, productivity, measured through thymidine uptake is much lower when compared to the Amazon River. Low productivity is likely due to cold temperatures, high salinity, and predation by carnivorous bacteria (Takacs and Priscu, 1998). Bacteria within the Antarctic lakes make up 30 to 60% of the microplankton biomass and are important to the food web, providing food for higher trophic levels (Takacs and Priscu, 1998). Non-polar lakes, such as Lake Gossenköllesee,

an oligotrophic, or nutrient-poor lake in the Tyrolean Alps, was mainly dominated by small aggregates of cocci and oval-shaped β-Proteobacteria (Alfreider et al., 1996). Bacterial assemblages in the lake, however, were quite different than those within the semi-permanent lake ice cover and the slushy snow above the lake ice. This discrepancy has also been found within Antarctic lakes and can be explained by the distinct way in which the ice cover and the microbial communities form (Alfreider et al., 1996).

1.1.2.4 Permanent lake ice cover

All of the lakes in the McMurdo Dry Valleys of Antarctica are permanently covered with ice (Priscu et al., 1998). Most studies have been conducted on Lake Bonney because unique cyanobacterial-heterotrophic bacterial assemblages have been discovered within its thick (4 m) lake ice cover. The ice surface atop Lake Bonney is uneven, containing numerous cracks and fissures caused by different ablation rates throughout the ice (Paerl and Priscu, 1998). Swift katabatic winds deposit soil into the ice fissures. During the summer, when solar light is continuous, this soil absorbs large quantities of light and melts the ice below it (Paerl and Priscu, 1998). Melting continues until the soil becomes embedded in the ice matrix and during the 150 day summer period, 40% of the ice cover interior can be filled with melt water (Paerl and Priscu, 1998). Numerous microorganisms are able to enter the ice cover attached to the soil particles. Gordon et al. (2000) showed that resident bacteria, actinomycetes, cyanobacteria, nematodes, and mosses originate from desiccated, and therefore inactive, microbial mats found in nearby sediment. Lake ice communities form quite differently than temperate lake ice communities because of different dispersion processes in these different areas (Priscu et al., 1998). Lake ice habitats arise from internal melting associated with aeolian deposited sediments (the biological seed), whereas microorganisms within the ice cover in temperate lakes originate from snow deposition and from the water column below (Priscu et al., 1998).

Unique sediment-associated assemblages of phototrophic cyanobacteria and heterotrophic bacteria form within the lake ice cover (Gordon et al., 2000). Cyanobacteria adhere to the sediment particles and ice-associated microflora, creating web-like structures that trap a variety of other microorganisms, amorphous minerals and organic particles (Paerl and Priscu, 1998). Together, the organisms within the

assemblages carry out two processes: physical aggregation and establishment of a nutrient-rich environment within the nutrient-poor ice cover. In this partnership, photosynthetic cyanobacteria become the primary producers, providing energy for heterotrophic bacteria. The heterotrophs in turn, decompose and recycle carbon to facilitate photosynthesis by the cyanobacteria (Gordon et al., 2000). Aeolian deposited soils provide the phosphorus and the organic carbon needed to fuel these processes. As well, water availability allows cyanobacteria to undergo photosynthesis (Paerl and Priscu, 1998). Many cyanobacteria are also diazotrophic, or nitrogen fixing, allowing photosynthesis to occur in nitrogen-limiting environments. The microbial species found in permanent lake ice have evolved to form highly cooperative and efficient partnerships with other species, transforming nutrient-depleted lake ice into a habitable environment (Paerl and Priscu, 1998).

1.1.2.5 Cryoconite holes

Cryoconite is fine-grained material that is transported by wind onto ice surfaces (Mueller et al., 2001). The Swedish explorer, Nordenskjöld, created the term cryoconite from the Latin words *kruos* (ice) and *konis* (wind) (Gajda, 1958, cited by Mueller et al., 2001). Aeolian sediments gather into small depressions in the ice. As in lake environments, these depressions absorb more radiation than the surrounding ice, causing vertical melting. Although these water-filled holes may refreeze at night or during the winter, the layer of black aeolian sediment absorbs heat and allows rapid melting during increased sunlight. Cryoconite holes are cylindrical in shape and range from 10 to 50 cm in depth and 5 to 55 cm in width (Mueller et al., 2001). Solar radiation alone may cause growth of cryoconite holes by penetrating through vertical ice walls and warming the water that fills the holes (Wharton et al., 1985). However, biothermal energy from solar radiation absorbed by algae also contributes to the uniform depth, distribution and growth pattern of the cryoconite holes (Mueller et al., 2001; McIntyre, 1984). Cryoconite holes have been studied in Arctic, Antarctic and temperate glaciers (Säwström et al., 2002; Wharton et al., 1985; Margesin et al., 2002). The microorganisms that inhabit cryoconite holes originate from nearby aquatic and terrestrial sources. In turn, organisms from cryoconite holes may inoculate other cryoconite holes or other lake environments (Mueller et al., 2001). Because microorganisms are commonly transferred between

environments, microbial communities within cryoconite holes have very similar populations to lake soil and lake communities (Wharton et al., 1985). These transient microbes must also adapt well to different cold environments. Margesin et al. (2002) showed that alpine glacier cryoconites were inhabited by cold-adapted bacteria from the genera *Pseudomonas* and *Sphingomonas*. These microorganisms have adapted to limited amounts of nutrients, numerous freeze-thaw cycles and prolonged UV radiation in four main ways (Mueller et al., 2001). First, motile organisms can maneuver to avoid UV irradiation. Second, pigments are produced to screen UV radiation. Third, carotenoids are produced to combat superoxides and other damaging free radicals (Mueller et al., 2001). Finally, DNA repair mechanisms help overcome damage from the UV radiation. In most Antarctic regions, the cryoconite holes are inhabited by the *Phormidum* and *Nostoc* genera of cyanobacteria (Säwström et al., 2002), which are common to cold, nutrient-limited areas (Vincent et al., 2000).

Each hole contains an ecosystem, capable of energy flow and nutrient cycling (Säwström et al., 2002; Wharton et al., 1985). Photosynthetic algae generally dominate cryoconite holes, but bacteria, rotifers, tartigrades, diatoms, and even insects have also been identified. The food webs seen in these cryoconite environments are often truncated and simplified versions of food webs seen in most Antarctic glacier lakes (Wharton et al., 1985). The cyanobacteria and algae initiate the food web cycle through photosynthesis and nitrogen fixation. Heterotrophic bacteria and fungi decompose organic matter to supply carbon for the algae and cyanobacteria (Mueller et al., 2001). The microorganisms are solely reliant on sediments as a source of nutrients, such as phosphorus, nitrate and ammonium. The sediments can originate from nearby moraines or nunataks, mountain peaks surrounded on all sides by glacial ice (Wharton et al., 1985). Also, because nutrient concentrations are so low, microorganisms must be closely associated to create a tight cycling of nutrients (Mueller et al., 2001).

The formation of cryoconites has larger implications and can change the glacier environment around them (Wharton et al., 1985). Cryoconites that are close in proximity may also combine to form small ponds or they may overflow to create small streams (Wharton et al., 1985). They also contribute to ice wastage by melting the ice and exposing underlying bedrock. As more bedrock is exposed, sediments can initiate

formation of additional cryoconite holes (Wharton et al., 1985). Finally, cryoconite holes contribute to the "nunatak hypothesis", which suggests that during previous ice ages certain species were able to survive by seeking shelter on nunataks or in refuges never completely covered with ice, such as cryoconite holes (Wharton et al., 1985).

1.1.2.6 Snow

Snow also provides a habitable environment for microorganisms. In South Pole snow, in situ hybridization revealed the presence of bacteria in snowmelt (Carpenter et al., 2000). Examination of snowmelt using scanning electron microscopy indicated the presence of coccoid and rod-shaped bacteria, some of which appeared to be dividing. Also, low levels of apparent DNA and protein synthesis were observed under near in situ conditions (Carpenter et al., 2000). Analysis of 16S rRNA genes revealed that sequences similar to several psychrophilic bacteria were found within these snow communities. Also, a bacterium was found that aligned closely with members of the genus, *Deinococcus*, an ionization-, radiation- and desiccation-resistant genus (Carpenter et al., 2000). Similarly, rich and active microbial communities were detected in the slush and snow layers covering Pyrenean and Alpine lakes in the Tyrolean Alps (Felip et al., 1995). Bacteria with filaments up to 100 μm long were detected, as well as flagellates, autotrophic and heterotrophic ciliates. Bacterial and algal activities were greater in the slush layers than the lake waters (Felip et al., 1995). Many species were typical of lake plankton communities however some predatory ciliates were restricted to the slush layers. This suggests that lake water flooding the ice and snow cover provides an inoculum for these environments (Felip et al., 1995).

1.1.3 Microbial transport through super-cooled cloud droplets

Bacteria and other microorganisms that inhabit surface glacier environments are transported mainly by snow and wind from nearby sources. For successful colonization to occur, these microorganisms must endure desiccation, UV radiation and nutrient deprivation during air transport. Early studies have reported that the atmosphere is un-favorable for bacterial reproduction and survival. However, several studies have shown that favorable conditions may sometimes exist for bacterial reproduction to occur (Saxena, 1983; Fuzzi et al., 1997). Microorganisms have been found within tiny super-cooled cloud droplets over both the northern and southern hemispheres (Saxena, 1983;

Jayaweera and Flanagan, 1982). Saxena (1983) detected growth of algae as well as proteinaceous matter. Jayaweera and Flanagan (1982) detected *Pseudomonas herbicola, Erwinia herbicola*, as well as five different fungal species in air samples collected over the Arctic Ocean. Fog droplets may act as a culture medium for bacteria, yeasts and molds (Fuzzi et al., 1997). Fuzzi et al. (1997) showed that fog clouds that form over the Po valley in Northern Italy contained substantial numbers of bacteria (100 to 500 CFU/m^3), yeast (100 to 200 CFU/m^3) and moulds (2000 to 3000 CFU/m^3). Gram positive and both non-sporulating and sporulating Gram negative bacteria, were detected. Yeasts with thin cell walls were also detected. Fog clouds at neutral pH and higher temperatures (10°C) showed microbial content and reproduction within the fog clouds. These findings indicate that cloud and fog droplets can be favorable environments for the growth and the reproduction of microorganisms. Furthermore, transfer of viable organisms through the atmosphere over long distances is possible.

1.1.4 Subglacial environments

Subglacial environments, or environments below the glacier ice, are very different from supraglacial environments. These areas are protected from harsh winds and UV radiation. Furthermore, because sunlight is absent from these areas, microbial communities within subglacial environments must find alternate sources of energy from chemoautotrophic processes or geothermal heat (Skidmore et al., 2000). Two subglacial environments, the subglacial ice below polythermal glaciers and the accreted ice above subglacial lake Vostok, will be discussed in the following sections. The microbes found within these areas are of major interest to scientists. Many of these microbes have had little contact with the surface. Because of this, the microorganisms may represent old biota that have not been affected by external sources (Priscu et al., 1999). Also, the subglacial environment may be similar to extraterrestrial environments on the planet Mars and on the Jovian moon, Europa (Priscu et al., 1999). For this reason, subglacial environments have been seen as attractive sampling analogs for extreme extraterrestrial environments (Vincent, 1999).

1.1.4.1 Subglacial ice

Subglacial basal ice, the sediment-rich ice layers beneath polythermal glaciers, provides another unique habitat for microbes, distinct from glacial ice. Unlike microbial communities in supraglacial environments, subglacial communities have access to sediment-associated nutrients and have protection from cold winds and UV radiation. These conditions allow them to actively divide (Skidmore et al., 2000). Also, the ability to thrive without access to light suggests that the microbial communities are chemoautotrophically driven, rather than photosynthetically driven (Sharp et al., 1999). Sampling of subglacial ice is challenging. There is limited access to these deep ice layers and sampling these environments aseptically is difficult (Foght et al., 2004). The subglacial environment becomes accessible for sampling when basal ice and sediment are transported to glacier margins and are exposed at these sites (Foght et al., 2004).

Foght et al. (2004) studied unfrozen subglacial sediments from Fox Glacier and Franz Joseph Glacier in the Southern Alps of New Zealand. Large numbers of viable bacteria were found (10^6 CFU/g dry weight sediment). Most of them were culturable aerobic heterotrophs (10^5 CFU/g dry weight sediment). Larger numbers of bacteria (3 to 4 orders of magnitude higher) were detected within the basal ice than within the overlying glacier ice. Further, nitrate-reducing and ferric iron-reducing bacteria were detected in the sediment of subglacial samples for both Fox and Franz Joseph glaciers, but not in the glacier ice. This indicates that the bacteria prefer environments rich in sediments, likely because they supply organic carbon and nutrients. These sediments may provide sufficient nutrients for substantial biological activity in situ (Foght et al., 2004).

Bacteria are equally abundant beneath polythermal glaciers at high latitudes, such as JEG, Canada and Bench Glacier, Alaska. Polythermal glaciers have cold, subfreezing ice at their surface, margins and termini, but a core zone of temperate ice at the pressure melting point (Sharp et al., 1999). At the glacier base, liquid water is supplied to subglacial microbial communities by melting of basal ice (from frictional and geothermal heat) and by meltwater percolating from the glacier surface through crevasses (Skidmore, 2001). From JEG, large numbers of bacteria were detected (10^5 to 10^6 cells/mL), including sulfate-reducers, nitrate-reducers and methanogens (Skidmore et al., 2000).

Both psychrophilic and psychrotolerant species, such as *Polaromonas vacuolata* and *Rhodoferax antarcticus*, have been found in subglacial ice. Many of these microorganisms have also been detected in Antarctic ice and in other permanently cold environments (Foght et al., 2004).

Scanning electron microscopy showed that the presence of bacteria correlates with the presence of sediment and debris (Skidmore et al., 2000). Also, the types of bacteria detected varied with the chemistry within the glacier site and the manner of glacier formation (Skidmore et al., 2005). Communities at the base of Bench Glacier, Alaska, a glacier with high sulfide oxidation, mostly comprised bacteria from the β-Proteobacteria division and contained microorganisms capable of sulfide and iron oxidation. Conversely, communities at JEG, Nunavut with high amounts of carbonate and gypsum were more diverse than those at Bench Glacier (Skidmore et al., 2005). These studies have shown that rich microbial communities, distinct from those in the glacier ice exist in the basal sediments.

1.1.4.2 Accreted ice above subglacial lakes

Another unique polar environment, that has been gaining recent attention is that of the subglacial lakes of Antarctica. Using airborne 60 Mhz radio echo sounding, over 70 subglacial lakes were discovered, lying 3 to 4 km beneath the East Antarctic ice sheet (Vincent, 1999; Seigert et al., 2001). These lakes are maintained in a liquid state by geothermal heat from the Earth's core and by over 350 atm of pressure from the overlying ice (Seigert et al., 2001). The largest of these subglacial lakes is Lake Vostok, having a maximum depth of 670 m and spanning 14 000 km^2 (Seigert et al., 2001). The mean age of the water is estimated to be 1 million years old and was formed under a climate warmer than that of the past 420 000 years (Jouzel et al., 1999). So far, 3359 m of ice above the southern region of Lake Vostok has been cored. Measurements of crystal size, electrical conductivity, total gas content and solid inclusions throughout the core revealed the presence of two distinct types of ice separated by a sharp transition (Priscu et al., 1999). The first 3310 m of the ice core represents the overlying glacier ice sheet. This core contains the longest record of past changes in climate and atmospheric conditions, information spanning four complete ice age interglacial-glacial cycles (Priscu et al., 1999). Between 3310 and 3359 m, the ice core comprises accreted ice, refrozen

from Lake Vostok. The microbiota detected described within the accreted ice may represent life in the ancient subglacial lake (Christner et al., 2001). Further drilling into Lake Vostok was stopped after concerns were raised about environmental contamination of the lake (Vincent, 1999). Aviation fluid and freons, used to keep the drill hole open, were present in such large amounts that their physical removal, recycling, or storage above ground would be impossible. Drilling into the lake would release these chemicals into the environment, contaminating the entire pristine lake within decades (Vincent, 1999). Non-contaminating methods are being devised to complete the drilling.

The accreted ice, formed from refrozen lake ice, gives the most reliable sample of Lake Vostok before its penetration (Karl et al., 1999). Within the accreted ice, low concentrations of growth nutrients were detected. Sodium and sulfate were found in amounts comparable to those in North American lakes, and carbon and nitrogen were found at concentrations five times lower than deep ocean environments (Karl et al., 1999). The accreted ice contained low numbers of viable microorganisms, 10^3 to 10^4 CFU/mL, similar to those in Lake Bonney. Also, the microorganisms were able to respire $^{14}CO_2$ at 3°C and 1 atm (Karl et al., 1999). Epifluorescence microscopy and scanning electron microscopy showed most of the bacteria were from the α– and β-Proteobacteria groups, and contained genera such as *Comamonas.* These genera were also found in the overlying glacial ice (Priscu et al., 1999). It is possible to conclude from these studies that Lake Vostok may contain relic populations adapted for oligotrophic (low nutrient, low biomass and low energy flux) habitats.

Poglazova et al. (2001) suggested that the microbes within the accreted ice originated from three sources: (1) the glacier ice, (2) Lake Vostok and (3) bedrock. Small cocci, rods, cyanobacteria, microalgae, yeast and plant pollens were found in both the accreted ice and the glacier ice. This suggests that those microorganisms, found in the accreted ice, originated from the glacier (Poglazova et al., 2001). Novel genera, such as *Caulobacter* and *Pleurocapsa*, were not found within the glacier ice or any Antarctic lake habitat. This suggests that these bacteria, found in the accreted ice, originated from Lake Vostok. Finally, the presence of genera, such as *Cytophaga*, associated with moraine material, suggests that the underlying bedrock is a third source for microorganisms (Poglazova et al., 2001).

1.2 Implications for microbial life in permanently cold environments

1.2.1 Origins of life: the snowball Earth

Polar environments that are able to sustain microbial communities may have served as refuges for life during major glaciations (Priscu and Christner, 2004). Because thermophilic bacteria and Archaea have deeply rooted phylogeny in the tree of life and are found in geothermal hot spots such as hydrothermal vents, it is generally thought that life on Earth evolved from a hot environment. However, the knowledge that microorganisms can exist on glacier surfaces (Gordon et al., 2000; Mueller, 2001), or beneath glaciers (Skidmore et al., 2000; Priscu et al., 1999), lends support to the hypothesis that life on Earth evolved from a cold environment. Recent geological evidence suggests that in the Neoproterozoic period, 580 to 700 million years ago, as many as four major global glaciation events occurred, freezing over the tropics and creating a "snowball Earth" (Hoffman and Schrag, 2000). Following deglaciation, the active and evolving communities of primitive prokaryotes and eukaryotes gave rise to most of the Earth's multicellular life.

In the snowball Earth hypothesis, global freezing was likely triggered when the equatorial supercontinent, Rodinia, fragmented into smaller landmasses because of plate tectonics (Hoffman and Schrag, 2000). Exposure of these land masses to the ocean induced rock weathering that led to the depletion of carbon dioxide, a heat trapping green house gas. Also, continents that spread to colder regions, more than 30° north or south of the equator, formed glaciers. Because glacier ice has a higher albedo than water, radiation from the sun would be reflected, creating cooler surface temperatures and allowing even more glaciers to form. This decrease in atmospheric CO_2, coupled with the ice albedo feedback, allowed the entire Earth surface to cool to a predicted -50°C and to become covered in ice (Hoffman and Schrag, 2000). Geothermal heat, however, was able to sustain a liquid ocean beneath an ice cover as thick as 1 km, creating another possible niche for microbial life (Hoffman and Schrag, 2000). At this time, all of the landmass rocks were trapped in glacier ice and abiotic geochemical weathering events as well as biological activity, were thought to have ceased. Deglaciation occurred as slow emissions of CO_2 from active volcanoes replenished the atmosphere. In a reversal of the ice albedo feedback, the lower albedo of newly melted water caused absorption of the

radiation from the sun and increased the melting of adjacent glaciers. Using the rate of CO_2 emission from volcanoes today, deglaciation of the Earth would have taken 100 000 years (Hoffman and Schrag, 2000). Rapid deglaciation and increases in CO_2 gases would also have led to a hothouse event, where the temperatures on Earth would have increased to a calculated 50°C for a short period before CO_2 emission could have been balanced with CO_2 consumption, and have allowed the temperature on Earth to be stabilized (Hoffman and Schrag, 2000).

Evidence of cryotolerant microbial mats would have provided a refuge for the survival, growth and evolution of organisms, including multicellular eukaryotes (Vincent et al., 1999). The snowball Earth hypothesis has been criticized on the basis that most or all surface life would have been extinguished by "freeze up" events (Vincent et al., 1999). However, evidence of Oscillitarion cyanobacteria, a group of cyanobacteria that are widely distributed in the Archaean and Proterozoic fossil records in ice shelf environments, show that life may have persisted during the Neoproterozoic period and through a global glaciation event. The need for microorganisms to form endosymbiotic relationships within cyanobacterial mats or communities would have caused an explosion in evolution after deglaciation (Vincent et al., 1999). Recent studies have shown that microbially mediated weathering and CO_2 generation occurs beneath polythermal glaciers (Skidmore et al., 2000). So far, the notion of microbial geochemical activity occurring during glaciation events has not been included in the snowball Earth model. Further study of communities and geochemical processes beneath polythermal glacier environments, such as JEG, may work to further fine-tune the snowball Earth hypothesis by including microbial contributions to carbon cycling calculations.

1.2.2 Carbon cycling models

The last glacial maximum event occurred 20 000 years ago. During that time, glaciers more than 2 km thick covered most of North America and Europe, reaching as far south as present-day New York City (Hoffman and Schrag, 2000). Glaciers covered approximately 20% of the northern hemisphere, including the boreal forest, the largest source of soil carbon (~400 Pg of C) (Skidmore et al., 2000). Evidence for a similar event during the last interglacial period, the Eemian, has been found in soil, peat and paleosols in unglaciated terrain beyond the ice margin (Skidmore et al., 2000). Models

have assumed that the carbon accumulated beneath the glaciers during the Eemian were returned to the atmosphere by the last glacial maximum, however, no explanations were given as to how this happened (Skidmore et al., 2000). It was assumed that because ice is an erosive agent, the organic carbon was likely moved into contact with the atmosphere through physical transport to ice sheet margins, deforming sediments or meltwater (Skidmore et al., 2000). These processes, however, cannot fully account for the recycling of the large amounts of organic carbon stored beneath the glaciers. Microbial communities are known to be involved in the chemical and physical modification of organic matter and nutrients in their environment and to influence a number of biologically-mediated geochemical processes (Paerl and Priscu, 1998). Evidence of microbial life in permanently cold environments today suggests that subglacial microbial environments could have existed during the Eemian and the last glacial maximum. Thus, contributions of microbially-mediated cycling of carbon may help to explain this phenomenon (Skidmore and Sharp, 1999).

Only a few studies to date have tested the basal ice layer of polythermal glaciers for evidence of microbial life and microbially mediated processes (Skidmore et al., 2000; Skidmore and Sharp, 1999; Souchez and Lemmens, 1995; Foght et al., 2004; Skidmore et al., 2005). The temperate ice, availability of liquid water and large stores of organic carbon beneath polythermal glaciers provides the perfect location for microbial production of CO_2 and CH_4 gases. The discovery of metabolically diverse microbes beneath JEG, including aerobic chemoheterotrophs, facultative anaerobic nitrate-reducers, sulfate-reducers and methanogens, suggests that such microorganisms may also exist beneath other polythermal glaciers. They may also have played an important role in cycling carbon before the last glacial maximum (Skidmore et al., 2000). Microbial populations have also been found within subzero marine and proglacial sediments, that provide necessary liquid and nutrients for microbial activity (Sahm et al., 1999; Sigler and Zeyer, 2002). It is likely, therefore, that microbial communities also existed during the last glaciation. Simple calculations suggest that microbial communities beneath JEG alone could convert 8.1 Pg of organic carbon to CO_2 through aerobic respiration over a glacial cycle (Skidmore et al., 2000). This calculation assumes that (1) O_2 is delivered to the bed at a rate determined by the basal ice melt rate and the O_2 content of the ice; (2)

that all of the O_2 is converted to CO_2 by microbial processes and; (3) that total CO_2 production is calculated by determining the integral over time of CO_2 production rates in the warm-based sectors of the ice sheets (Skidmore et al., 2000). It is important, therefore, to search for microbial life in glacier environments to calculate the effects of microbially-mediated processes in glacial-interglacial carbon cycling models.

1.2.3 Developments with Antarctic and Arctic microorganisms

Antarctic and Arctic microorganisms are being screened for the production of potentially marketable compounds and enzymes (Bowman *et al.*, 1997; Gounot, 1991). Newly described taxa within the genera of *Shewanella* and *Colwellia* produce polyunsaturated fatty acids (PUFAs), including eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). These compounds have traditionally been harvested from fish oils and some algae for aquacultural feeds; however, the rise in demand for such products has fueled recent research into the molecular biology of PUFA-production in prokaryotes (Nichols et al., 1999). EPA and DHA are essential for the normal growth and development of larvae in many aquacultural species, such as the Atlantic salmon. PUFA-producing prokaryotes and microalgae can be used directly to feed the larvae or indirectly, to enrich rotifers that in turn feed aquicultural larvae. PUFA-producing genera, such as *Dunaliella*, are also dried and marketed as nutritional supplements, primarily antioxidants (Rothschild and Mancinelli, 2001). Also, cold-adapted enzymes produced by microbes such as *Pseudomonas putida* and *Vespula maculata* have medical use. In order to prevent freezing under subzero temperatures, prokaryotes produce antifreeze and ice nucleation proteins (Xu et al., 1998). Antifreeze agents such as sorbitol or glycerol stabilize the liquid state of the organism, whereas ice nucleation proteins act to prevent intracellular freezing by seeding ice formation in the extracellular environment (Xu et al., 1998). These proteins show potential as cryoprotectants of frozen organs (Rothschild and Mancinelli, 2001.)

Fuel spills are common in polar regions and threaten these fragile ecosystems (Thomassin-Lacriox et al., 2001). Fortunately, hydrocarbon-degrading consortia, including diazotrophic microorganisms that can overcome nitrogen limitations, exist naturally in both Antarctic and Arctic soils, snow, sea ice and permanent lake ice (Thomassin-Lacroix et al., 2001; Eckford et al., 2002; Paerl and Priscu, 1998). These

cold-adapted microorganisms are ideal for bioremediation of polar regions (Whyte et al., 1996).

Public culture collections, such as Deutsche Collection Mikroorganisms und Zellkulturen and the American Type Culture Collection, contain only limited numbers of Antarctic microorganisms, 33 and 46 organisms, respectively (Nichols et al., 1999). It is clear from the applications listed that suitable culture collections need to be established for screening, propagation and study of these valuable organisms. It is possible that in the future, a public culture collection for the purpose of studying cold-adapted microorganisms may be established, or that private culture collections that have isolated several thousand Antarctic microorganisms will become accessible to the public (Nichols et al., 1998).

1.2.4 Life on Mars and on Europa

Polar environments may serve as analogues for viable extraterrestrial habitats (Skidmore et al., 2000). The presence of water on the planet Mars and on a satellite of Jupiter, Europa, presents the possibility that microorganisms may inhabit these environments. Water on Mars exists as ice in two polar ice caps (Skidmore et al., 2000). Surficial melting may have occurred at one time in the north polar ice cap. Polythermal characteristics may also be exhibited on these ice caps, such as basal melting and the production of sediment-rich basal ice. This suggests that water-rich environments may exist below the surface, providing enough nutrients and protection from temperature fluctuations and strong UV radiation at the planet surface for microorganisms to grow. An environment beneath the north polar ice cap might resemble subglacial environments beneath polythermal glaciers on Earth. For this reason, polythermal glaciers are good sites for studying microbial activity and survival. Also, these areas would be ideal sites to develop aseptique sampling devices, suitable to be used on Mars.

On Europa, an ocean twice the volume of the Earth's oceans is proposed to exist 50 to 100 km below the ice surface (Chyba, 2000; Priscu and Christner, 2004). Geothermal heat maintains this liquid ocean. Furthermore, radiogenic decay and tidal dissipation creates a heat flow within the interior of Europa, allowing liquid water to exist at shallow depths (Gaidos et al., 1999). This creates a zone of habitability below the thick (3 to 4 km) ice surface (Priscu and Christner, 2004). The possibility of life on

Europa is conceivable as our microorganisms on Earth have endured countless glaciation periods (Hoffman and Schrag, 2000). Models of metabolic processes on Europa have also been speculated upon, based on processes within cold environments on Earth (Gaidos et al., 1999; Chyba, 2000). Because metabolism on primitive Earth and in subglacial environments likely relied on methanogenesis and elemental sulfur reduction, these reactions may also be the main driving forces for life on Europa (Gaidos et al., 1999). Alternatively, Chyba (2000) proposed that particles accelerated into magnetosphere of Jupiter would produce enough organic carbon and oxidant molecules, such as formaldehyde, molecular oxygen, and peroxide to fuel a substantial Europan biosphere. By these calculations, a lifestyle completely independent of photosynthesis and chemoautotrophic metabolism was possible. Such microbial communities would be possible because some bacteria on Earth, such as methanogens and *Hyphomicrobium*, would be able to live in these Europan conditions (Chyba, 2000).

Even if these environments were conducive to growth, the source of these microorganisms and the mechanism of dispersal to these planets are still uncertain. It is possible that living organisms exist throughout the universe and develop wherever the environment is favorable, according to the theory of "panspermia" (Rothschild and Mancinelli, 2001). In flight experiments led by the European Space Agency, it was found that *Bacillus* spores were able to withstand the space environment for up to 2 years if they were mixed with glucose to protect them against high solar radiation (Rothschild and Mancinelli, 2001). Similarly, viruses such as bacteriophage T-1 and tobacco mosaic virus were able to survive space for up to 2 weeks. Polar and permanently cold environments are strikingly similar to the extraterrestrial environments of Mars and Europa (Priscu and Christner, 2004). For this reason, microbial communities found in these regions are the closest known models for life on other planets.

1.3 Methods for characterizing microbial communities

1.3.1 Culture-dependent techniques

Culture-dependent experiments involve the creation of pure cultures by inoculating colonies onto solid agar or into liquid medium (Rainey and Ward-Rainey, 2000). In most approaches, different prokaryotic cells can be separated from each other by direct plating of diluted samples followed by selection of single colonies for further propagation. The main advantage of the culture approach is that single strains are maintained and can be used for further characterization. As described by Rainey and Ward-Rainey (2000), our knowledge of the metabolic processes of prokaryotes and their functional roles in various ecosystems is almost entirely derived from studies of microorganisms in pure culture. Isolated colonies provide the base for other functional and metabolic tests, such as the BIOLOG® system (Biolog, Hayward, CA) which tests the ability of microbes to oxidize a range of carbon sources and has been used increasingly for characterizing microbial communities (Rainey and Ward-Rainey, 2000). Mixed liquid cultures enriched with radiolabeled carbon sources have been used to describe the metabolic capabilities of a microbial community. Skidmore et al. (2000) incubated glacier ice samples with ^{14}C-acetate, and monitored the production of $^{14}CO_2$. The detection of $^{14}CO_2$ indicated microbial activity was occurring at +0.3°C and suggested that these microorganisms were active in situ.

Because of inherent biases, however, culture-dependent experiments cannot be used on their own to completely characterize a microbial community. Enrichment and isolation of microbes onto nutrient medium impose many selective pressures that prevent some microorganisms from being cultured. Nutrient composition, incubation temperature, gaseous conditions, light, and even competition between neighboring organisms can select certain microbes over others. One of the main difficulties with culture-dependent techniques is that microorganisms can only be cultured after their physiological niche is perceived and reproduced (Ward et al., 1990). This inadvertent process of selection has been shown through reports of direct microscopic counts exceeding viable-cell counts (number of cultured isolates) and has been called the "great plate count anomaly" by Staley and Konokopa (cited by Amann et al., 1995). These biases exclude both known bacteria that are in an unculturable state and unknown

bacteria that have not yet been cultured due to unsuitable culture conditions (Amann et al., 1995). The latter case is often encountered when culturing psychrophilic microorganisms. With known psychrophiles, inoculation is often performed in a cold room because room temperature may be lethal. Also, simulating growth at subzero temperatures is difficult because agar media crystallize when frozen. Because of these biases, culture-independent techniques have been developed, capable of identifying bacteria without culture.

1.3.2 Culture-independent techniques

Culture-independent techniques identify bacteria based on their genetic sequences and as a result, offer a more sensitive, specific and rapid alternative to traditional culture-dependent techniques for community characterization (Louws et al., 1999). By analyzing genomic DNA from an organism without relying on cultivation: (1) novel taxa can be detected, including bacteria that have never been cultured; (2) the phylogenetic position of an organism can be determined; and (3) the physiological make-up of that organism can be obtained (Rainey and Ward-Rainey, 2000).

Although there are many different molecular techniques, most require two initial steps. The first step is the extraction of total DNA from a sample and the second step is the amplification of a molecular marker gene via polymerase chain reaction (PCR). DNA can be extracted from environmental samples and from cultured bacteria. Cell lysis is achieved through the application of chemicals (such as detergents, phenol, guanidine thiocyanate, EDTA), enzymes (such as lysozyme, pronase, proteinase K), mechanical disruption (such as sonication and bead-beating) or heat (Cullen and Hirsch, 1998). Often, a combination of these processes is used. PCR is a powerful method that can amplify a specific gene target over one million times (Maloy, 1994). The gene target usually used is the 16S rRNA gene, encoding the small ribosomal subunit RNA. The 16S rRNA gene is ubiquitous, because every bacterial cell needs it to replicate. Further, large 16S rRNA gene sequence databases have been established. Through amplifying the 16S rRNA gene, sufficient amounts can be obtained for sequencing. Alternatively, molecular fingerprinting techniques, such as amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (t-RFLP) analysis

(described below) can be applied to amplified DNA from a community without sequencing each PCR product.

Culture-independent methods have provided new ways to examine the microbial world, however, these methods are still quite limited in number and scope (Dunbar et al., 2001). von Wintzingerode et al. (1997) stated that "each physical, chemical and biological step involved in the molecular analysis of an environment is a source of bias which will lead to a distorted view of the real world". In order to accurately characterize a microbial community, DNA from each microorganism within an environmental sample must be extracted and amplified. Unfortunately, not all microorganisms lyse equally well (Amann et al., 1995). These problems result in under-representation of some microbes within the community. Also, extraction of DNA from soil samples is especially difficult because bacteria may adhere tightly to soil particles and resist lysis. The humic and fulvic acids, that are present in soil, contaminate the DNA product and affect subsequent PCR amplification, restriction endonuclease digestion and DNA–DNA hybridization steps (Cullen and Hirsch, 1998). Because of the sensitivity of this technique, PCR can become easily contaminated from tainted reagents, such as *Taq* polymerase or even epidermal skin cells (Amann et al., 1995; Cullen and Hirsch, 1998). Caution must be taken in nucleic acid extraction because fragmented single stranded DNA can reassociate and overlap, forming duplexes in conserved regions. This can lead to the formation of chimeras from hybrid molecules.

Another major problem in PCR is the preferential amplification of genes from one type of microorganism (Amann et al., 1995). Amann et al. (1995) reported that microorganisms that have more than one genome copy, such as the *Bacillus* species, may be over-represented in a community. The "universal" bacterial primers used to amplify the 16S rRNA gene are also questionable because they are derived from known bacterial isolates and have not been proven to amplify all bacteria domains (Liu et al., 1997). The results of these molecular identification techniques are often verified through DNA hybridization techniques (Rainey and Ward-Rainey, 2000). Hybridization can ensure that the amplified products do not originate from naked DNA (DNA not from within a viable bacterial cell) or contaminants. In these techniques, radioactively labeled PCR products or synthesized oligonucleotides are obtained and are hybridized to the DNA extracted

from the original sample, the library of clones or the PCR amplified sample. These techniques are advantageous because they do not rely on amplified genes.

1.3.2.1 PCR independent techniques: Fluoresence in situ hybridization

Fluorescence in situ hybridization (FISH) has become a powerful technique of analyzing community structure and has been widely used to characterize communities in many environments including high mountain lakes and lake ice (Alfreider et al., 1996; Hiorns et al., 1997; Gordon et al., 2000). FISH involves the detection of specific bacterial cells by in situ hybridization of oligonucleotide probes labeled with a fluorescent compound and subsequent enumeration with epifluoresence microscopes or confocal laser-scanning microscopes (Aoi, 2002). 16S rRNA gene-targeted oligonucleotide probes can be designed to detect large taxonomic groups of bacteria, such as Proteobacteria and CFB divisions, specific strains of bacteria within a single genus or bacteria possessing a specific gene. Through probing for a specific metabolic gene, metabolic function or activity can be inferred as long as there are multiple copies of this gene (Aoi, 2002). FISH allows the spatial organization to be known of unknown and unculturable bacteria within biofilms or when attached to solid surfaces (Aoi, 2002).

Several limitations have been attributed to the FISH technique. Firstly, oligonucleotide probes are designed based on the large number of sequences now entered in sequence databases (Aoi, 2002). Secondly, FISH requires multiple targets in a cell to give a detectable signal, thus low copy or single copy genes cannot be used. Thirdly, phylogenetic classification using FISH cannot be conclusively correlated with bacterial function and characteristics. However, FISH has also been coupled with microautoradiography to simultaneously determine the identities, activities and specific substrate uptake profiles of individual bacterial cells within complex microbial communities (Lee et al., 1999). This combination of microscopy techniques allows the spatial arrangement of cells to be seen, the identities of the microorganisms to be known, and the metabolic capabilities of the microorganisms to be determined (Lee et al., 1999). This technique overcomes culture biases in culture-dependent techniques as well as DNA extraction and PCR biases in molecular techniques.

1.3.2.2 rRNA gene fingerprinting techniques

1.3.2.2.1 Denaturing gradient gel electrophoresis (DGGE)

DGGE has also been widely used to characterize bacterial communities within environmental samples, such as snow and lake ice (Sigler and Zeyer, 2002). DGGE separates fragments with the same length, but different DNA sequences based on their decreased electrophoretic mobility within a polyacrylamide gel (Muyzer and Smalla, 1998). A gradient of denaturants, such as urea and formamide, is created in a polyacrylamide gel. This causes amplified double stranded DNA molecules of the same length to partially melt. Melting occurs within stretches of base pairs with the same melting temperature or 'melting domains' (Muyzer and Smalla, 1998). Once the melting domain with the lowest melting temperature reaches its melting temperature, a transition of a helical to partially melted molecule occurs and migration of that fragment will practically halt in the polyacrylamide gel. Sequence variation causes melting temperatures of melting domains to differ. Thus, molecules with different sequences will halt at different positions in the gel based on the melting temperatures of their melting domains. To further separate the DNA fragments, GC clamps, GC rich oligonucleotides, are added to the DNA fragments via PCR with tailored primers. The GC clamp acts as a high melting domain, preventing complete dissociation of the DNA into two strands and allowing greater separation between DNA fragments in the gel. This method creates a molecular community fingerprint where each band represents one organism within the environment. The color intensity of each band shows the abundance of that organism within the community.

DGGE profiles allow different microbial communities or changes within microbial communities to be compared visually and quite easily. DGGE has several limitations, however. The length of time needed to run the polyacrylamide gels, as well as the correct gel gradient, must be optimized for each environmental sample. Bands within DGGE fingerprints are often difficult to distinguish when DNA fragments are not clearly separated from each other. Individual DNA fragments may be removed from the polyacrylamide gel in plugs and used for sequencing, although, when bands are not clearly separated, the risk of sequencing contaminants is high, making analysis of individuals in the environment more difficult.

1.3.2.2.2 Terminal restriction fragment length polymorphism (t-RFLP)

t-RFLP is a relatively new technique that has become an increasingly popular method for the rapid comparison of microbial communities (Dunbar et al., 2001). This method has been successfully used for bacterial communities in various environments such as soil samples, activated sludge samples and termite guts (Osborn et al., 2000; Liu et al., 1997; Dollhopf et al., 2001). The speed of this method and high sample throughput enables large numbers of replicated experiments, using statistical analysis, to be conducted (Dunbar et al., 2001). In t-RFLP, a dye-labeled forward primer is used during PCR amplification. Upon digestion with restriction enzymes, 5' end labeled fragments of different lengths are obtained. When separated electrophoretically on a polyacrylamide gel, a fingerprint pattern that is unique to that community will be obtained, where each variable length terminal restriction fragment represents a distinct microorganism and the intensity of the radioactive signal represents its abundance in the community. Automated DNA sequencers will detect the 5' end labeled terminal restriction fragment and the intensity or abundance of each fragment can be quantified (Liu et al., 1997). Use of automated DNA sequencers with internal standards allows the length of each fragment to be determined with high accuracy and numerical data of exceptional resolution to be generated (Dunbar et al., 2001). By comparing community profiles, communities can be distinguished from each other. Also, communities that have similar profiles can be analyzed for a common source of origin. In this rapid technique, whole communities can be analyzed without the need to sequence or identify each every organism present (Louws et al., 1999).

Despite its ease of use, this method is still in a developmental stage and there are limitations to the technique (Dunbar et al., 2001). In addition to biases from nucleic acid extraction and PCR amplification, this technique often yields high sample variability and requires numerous replicates to be tested. Background noise can hinder the analysis of community composition (Dollhopf et al., 2001). With the use of restriction enzymes, incomplete digestion can also be a problem because it leads to the overestimation of certain species in the environment (Osborn et al., 2000). Suitable computer programs must be used to ensure that interpretation of data is accurate (Dollhopf et al., 2001). Osborne et al. (2000) suggested that t-RFLP analysis may permit at least a semi-

quantitative analysis of the relative proportions of dominant members and genotypes within a microbial community while cautioning that t-RFLP analysis is subject to all the biases inherent in any PCR amplification approach.

1.3.2.2.3. Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is a rapid and sensitive molecular technique that has been used successfully to characterize microbial communities within coal tar waste sites, insect guts, and foods (Bakerman and Madsen, 2001; Miteva et al., 2001; Stortichini et al., 2002). Like t-RFLP, it uses restriction enzymes to create DNA fingerprints for identifying microorganisms. The technique differs from t-RFLP, however, in that it requires the creation of a library of clones in order to selectively retrieve useful 16S rRNA gene sequence information (Amann et al., 1995). In the ARDRA method, after DNA is extracted from the mixed microbial community sample and the 16S rRNA genes are amplified, the amplified gene products are ligated and transformed into suitable bacterial hosts to produce a library of clones. The inserted PCR product is reamplified from each clone using primers specific to the cloning site (i.e. M13 primers) and finally digested using restriction enzymes to produce fragments of unique lengths. Separation of these fragments electrophoretically on an agarose gel yields a unique fingerprint for one microorganism. The profiles generated are reproducible and can distinguish subspecies of microorganisms (Louws et al., 1999; Miteva et al. 2001). A collection of many fingerprints will show the abundance and diversity of the organisms in each community (Appendix 1). Similar ARDRA patterns are grouped into operational taxonomic units (OTUs) with each OTU, in theory, comprising the same subspecies of bacteria. Representative clones from each OTU can be sequenced, alleviating the cost of sequencing every clone. ARDRA enables relatively fast strain analysis and is appropriate to obtain phylogenetic and taxonomic information. It also allows the construction of a database for identification purposes (Hendryckx et al., 1996).

ARDRA is also subject to many unavoidable methodological difficulties and genetic biases (Bakermans and Madsen, 2002). Cultivation, construction and screening of clone libraries are labor intensive (Lui et al., 1997). Furthermore, because not all of the PCR fragments will transform or ligate at the same efficiency, clones within the library may not accurately represent the community. Also, optimization of this procedure

is required with respect to using suitable restriction enzymes, for discriminating among genera. Some experiments must use up to five restriction enzymes in order to accurately distinguish among all of the microorganisms (Hendrickx et al., 1996).

Despite these limitations and biases, ARDRA has been used successfully and has been shown to be a superior method over culture techniques in detecting and identifying microorganisms in the environment. For this reason, ARDRA was used to characterize the communities within JEG. In this research, a bead-beating cell lysis technique was employed to extract nucleic acids. By using physical means to lyse bacteria, more complete lysis of the community is possible. Because this technique has PCR and cloning biases, the results were compared to, and supplemented with, results obtained from t-RFLP (Bhatia et al., 2004) as well as culture methods. Through combining results of culture-dependent and culture-independent approaches, a more accurate analysis of microbial communities within JEG can be acheived.

1.3.3 Phylogenetic analysis

With the development of new community characterization techniques, new methods were needed to name and relate these organisms to each other. In 1977, Carl Woese determined that organisms could be related to one another by comparing their rRNA gene sequences. Based on these data, he showed that there were three domains of evolutionary descent, Eukarya, Bacteria and Archaea (Pace, 1997). Woese created phylogenetic trees by aligning pairs of rRNA gene sequences. The calculated differences between the sequences gave a measure of the evolutionary distance between the organisms and these differences were used to infer a phylogenetic tree that shows the evolutionary relationship between the organisms (Pace, 1997). Phylogenetic trees can also be made by aligning other DNA sequences, however, these trees often differ from rRNA trees because of lateral gene transfer or genome intermixing events that occur throughout the course of evolution (Pace, 1997). Figure 1.1 shows a phylogenetic tree of 17 divisions within the domain Bacteria.

Distinctions between the genus, species and taxon of sequenced organisms can be loosely made by comparing the percent similarity of base pairs in the sequenced gene. Organisms that share greater than 97% similarity of their sequenced 16S rRNA genes are considered to be within the same species (Madigan et al., 2004). Two organisms with 93

to 95% identity are considered to be within the same genus and organisms with less than 93% identity are considered to be within different taxa (Madigan et al., 2004). Also, strict requirements were put into place for naming new bacterial species, including a description of metabolic properties, morphology, as well as 16S rRNA gene sequencing. Bernardet et al. (2002) proposed that the minimal standards for describing new genera in the family Flavobacteriaceae should be based on DNA-DNA hybridization data, 16S rRNA gene sequencing, phenotaxonomic and chemotaxonomic characterization.

1.4 John Evans Glacier (JEG)

1.4.1 General Description of JEG

JEG is a large valley glacier located 79° 40'N, 74° 00'W on Eastern Ellesmere Island, Nunavut, Canada. (Figure 1.2) The climate at JEG is that of a polar desert and the mean annual temperature from 1997-1999 at the glacier terminus was –14.3°C (Bhatia, 2004). Elevation ranges from 100 to 1500 m above sea level (Skidmore and Sharp, 1999). Data gathered from radio echo sounding showed a boundary between cold and temperate ice within JEG, indicating that it is a polythermal glacier (Skidmore, 2001). JEG contains cold-based ice at its surface, terminus and margins that are frozen to the bedrock. The cold ice surrounds a 400 m thick temperate ice core beginning 7 km upglacier from the snout. Basal melting occurs within the temperate core zone, as the ice is at its pressure melting point (Skidmore et al., 2000). The glacier ice and supraglacial meltwaters are characterized by low solute and sediment concentrations. In contrast, subglacial waters that interact with underlying rock and sediment, as well as the basal ice layer, exhibit high solute and suspended sediment concentrations. The 0.5 m thick basal ice layer forms at the glacier bed through relegation of ice and basal accretion of supercooled subglacial water, and is exposed to the atmosphere at the glacier margins (Figure 1.3) (Skidmore et al., 2000). The underlying bedrock is made primarily of carbonate but is also rich in the silicate, gypsum, anhydrite and calcite minerals (Skidmore and Sharp, 1999). The proglacial area in front of JEG is covered with a 400 m thick layer of unconsolidated sediment and contains a large network of streams and channels (Figure 1.4).

1.4.2 Previous studies of JEG

1.4.2.1 Hydrological and hydrochemical studies: evidence of a subglacial drainage system

During melt season (early June until early August), glacier runoff forms four main supraglacial streams: the supraglacial stream, proglacial stream, east marginal stream and west marginal stream. About 2 weeks after melting season commences, two subglacial outburst events can be observed (Skidmore, 2001). First, turbid water bursts through the glacier upper surface in the form of an artesian fountain as high as 2 m (fountain stream), followed by an upwelling event that occurs through the proglacial sediment in a 30 m wide area in front of the glacier terminus (upwelling stream). Second, two channelized outflows occur at the ice-bed interface at the glacier terminus (subglacial stream and east subglacial stream). These subglacial outburst events have been observed from aerial photographs taken in 1959 as well as during field observations in 1995, 1998 and 2002.

Water chemistry analysis of samples taken in 1994 and 1996 showed higher solute content in the subglacial streams in comparison to the supraglacial streams (Skidmore, 2001; Skidmore and Sharp, 1999). Subglacial water was dominated by $Ca^{2+}$and SO_4^{2-} species whereas Ca^{2+} and HCO_3^- dominated supraglacial water. Similar chemistry was observed in all three subglacial water samples (the fountain, upwelling and subglacial streams) indicating that all originated from a common source. Subglacial water, when compared to supraglacial water, contained higher concentrations of Na^+, K^+, and Si, products of silicate mineral weathering. These results were strong evidence that the subglacial stream samples were indeed routed subglacially, where the water would have prolonged contact with the silicate-rich bedrock material beneath JEG. Detection of these ionic species as well as Ca^{2+} and SO_4^{2-} in subglacial streams suggested that the solutes were derived from dissolution of gypsum, anhydrite and calcite. High SO_4^{2-} concentrations, unique to subglacial water, became indicators of subglacial derived water (Skidmore and Sharp, 1999).

Observations of the subglacial outburst events taken together with these preliminary water chemistry studies suggested the existence of a subglacial drainage system in JEG (Skidmore, 2001). Skidmore and Sharp (1999) devised the following model for a subglacial drainage system in JEG. Over winter, subglacial water is stored

unfrozen within the subglacial reservoir where it makes contact with the silicate-rich bedrock. When melt season begins, supraglacial meltwater drains into a large crevasse field and through it, enters the subglacial reservoir (Figure 1.5). A cold barrier of ice and frozen sediment prevents the subglacial reservoir from draining. However, when water pressure from the subglacial reservoir exceeds the pressure of the overlying ice, either melting or hydrofracturing of the cold ice barrier occurs, creating drainage outlets through which subglacial water can be released. Water release occurs through two observable outburst events: (1) the artesian fountain (Figure 1.6) followed by an upwelling stream (Figure 1.7) and (2) the subglacial stream event (Figure 1.8). Subglacial water within initial outburst events, the artesian fountain and the upwelling stream, contains water that has been in contact with bedrock for up to a year, whereas subglacial water within the subglacial outburst channel contains stored subglacial water diluted with fresh supraglacial meltwater that entered the reservoir through crevasses. After the outflow events, water accumulates again in the reservoir and will be released the following summer.

Skidmore and Sharp (1999) were the first to document a cyclic pattern of subglacial water release from a polythermal glacier. These findings are important because subglacial hydrology systems are key factors in controlling rock: water ratios, rock:water contact times, as well as access of the subglacial environment to atmospheric gases. It is possible that this model can also be applied to other polythermal glaciers where outburst events have been documented, such as White Glacier, North West Territories.

1.4.2.2 Culture studies: microbial life beneath JEG

Previously, it was thought that continental glaciation halted geochemical processes beneath the ice (Skidmore, 2001). Culture work by Skidmore et al. (2000), however, showed that a diverse group of microorganisms, capable of aerobic growth as well as nitrate and sulfate reduction, exists beneath JEG. Photographs taken using transmission electron microscopy showed microorganisms with typical prokaryotic cell morphologies, such as bacilli and cocci. Dividing cells were also observed, indicating that bacteria within these samples were active. The photographs also indicated that the microbes were closely associated with sediment particles. To further investigate these

microorganisms, supraglacial and subglacial meltwater and ice samples were collected over 3 years (1996, 1997 and 1998) and a series of culture experiments were conducted.

Preliminary qualitative experiments were conducted using samples collected in 1996. The meltwater samples were amended with one-tenth strength sterile R2A broth, a standard liquid growth medium used to enumerate bacteria from aquatic environments, and incubated in the dark at $\leq 4°C$ and 22°C. After 7 weeks, oxygen depletion, decreased pH, and presence of H_2S were observed in the growth medium, indicating that active microbes were present. Unamended samples (without the addition of R2A medium) were slower to show these positive results and sterile controls did not indicate changes within the growth medium. Culturing of amended subglacial samples yielded higher CFU/mL (10^6 CFU/mL at both 4°C and 22°C) than unamended subglacial samples (10^3 CFU/mL at 4°C and 10^2 CFU/mL at 22°C). Many of the colonies were pigmented yellow or orange, characteristic of cold-adapted microorganisms. To test for anaerobes, R2A-amended samples were incubated in sealed Hungate tubes supplemented with nitrate medium and quarter-strength sulfate medium and pregassed with sterile CO_2 plus N_2. Turbidity and browning or blackening of the media demonstrated that anaerobes were present in these environments. These initial qualitative experiments clearly showed that viable microorganisms, respiring aerobically and anaerobically, were present in supraglacial and subglacial environments (Skidmore et al., 2000). It was also observed that an increase in turbidity was seen in samples containing higher amounts of sediment.

Examination of glacier ice and basal ice samples collected in 1997 confirmed that the subglacial environment harbored both aerobes and anaerobes. Microbial activity was detected in thawed basal ice samples that were incubated both aerobically and anaerobically in nitrate- and sulfate-amended R2A medium. Under anaerobic incubation, nitrate and sulfate were significantly depleted. As well, CO_2 and CH_4 were produced at concentrations 10^1 and 10^4 times higher than glacier ice samples, respectively. Analysis of the δ^{13} C-CH_4 levels produced by the thawed basal ice samples clearly demonstrated that they were of microbial origin. No reduction of nitrate or sulfate was detected in the thawed glacier ice samples, supporting previous observations that microbial activity is associated with the presence of sediment within the ice. These results showed that heterotrophs, nitrate-reducing bacteria, sulfate-reducing bacteria and

methanogens were all present in the basal ice samples. The presence of methanogens was interesting, because they are strict anaerobes and support the idea that anaerobic microenvironments exist beneath the glacier (Skidmore et al., 2000).

Further experiments were conducted with ice samples collected in 1998 to determine whether or not subglacial microbes were capable of respiration at near in situ conditions (incubation at +0.3°C without R2A amendment) (Skidmore et al., 2000). Radioactively labeled acetate (^{14}C-acetate) was added to both ice and thawed ice samples and mineralization of ^{14}C-acetate to $^{14}CO_2$ signified microbial activity. Mineralization was observed in both glacial ice and basal ice samples, but only after they were thawed (in liquid form). Higher amounts of $^{14}CO_2$ were detected in basal ice samples than in glacial ice samples. These results indicate that aerobic growth occurred at near in situ conditions. Upon amendment of the thawed glacier ice samples with R2A medium, mineralization significantly increased, indicating that the microbes within the glacier ice were nutrient-deprived. In contrast, R2A-amended basal ice samples did not show an increase in mineralization of ^{14}C-acetate. Dissolved organic carbon (DOC) was measured to confirm this inference and indeed, the basal ice samples had higher DOC content (100 μM C) than glacier ice samples (24 μM C). These results indicate that enough organic carbon is present in the subglacial environment to sustain microbial life (Skidmore et al., 2000).

Traditional taxonomic tests were performed to classify aerobically cultured organisms from basal ice samples collected in 1997 and 1998 (Balakrishnan, 2000). Samples were thawed, serially diluted and then incubated on half-strength R2A agar and incubated at 4°C or 22°C in the dark. It was found that the number of bacteria (~30 colonies) in either sample did not vary, despite the observation of higher sediment content in the 1997 sample. Samples from 1997, however, did show a greater number of pigmented colonies, suggesting that a more diverse community could be correlated with sediment content. Preliminary taxonomic tests on the isolates revealed that most were Gram positive, catalase positive and oxidase negative. Secondary taxonomic tests revealed that most were unable to utilize glucose, and grew in the presence of azide. Some microorganisms were also motile and had acid-fast cell walls. From these taxonomic tests, the bacteria were tentatively assigned to the genera *Planococcus,*

Oreskovia, Nocardia, Bacillus and *Mycobacterium.* Fermenters and anaerobic nitrogen fixers were detected in the samples collected in 1998 and 1999. Also, temperature growth experiments showed that most microorganisms were restricted to growth between 4°C and 22°C, but that some grew at 37°C. Furthermore, 70% of the isolates were psychrotolerant and 30% were psychrophilic. These results confirmed that numerous and diverse microbes were present beneath JEG.

1.4.3 Molecular characterization of JEG communities

Skidmore et al. (2005) characterized microbial communities in subglacial ice samples at JEG using 16S rRNA gene sequencing, dotblot hybridization and ARDRA analysis. Analysis of 133 clones revealed that β-Proteobacteria comprised 56% of the total bacterial community. Bacteria from CFB (24%), α-Proteobacteria (7%), Actinobacteria (5%) and γ-Proteobacteria (1.5%) comprised the remaining clone library. Sulfate-reducing bacteria were not detected at JEG even though high sulfate concentrations were present in the subglacial sample and culture-based studies detected sulfate-reducing activity.

Bhatia (2004) used t-RFLP analysis to determine the source, diversity and distribution of supraglacial, subglacial and proglacial microbial communities at JEG. t-RFLP analysis of 141 samples digested with *Hae*III and 126 samples digested with *Hha*I yielded a total of 142 and 102 terminal restriction fragments, respectively. Bacterial communities beneath, on, or adjacent to JEG appeared to be unique to each environment. t-RFLP analysis showed that bacterial communities were heterogeneously distributed and that differences in community composition was likely caused by physical and chemical factors, such as energy sources, nutrient availability and environmental stresses.

1.5 Thesis objectives and hypothesis

Only two molecular studies have characterized microbial communities beneath JEG. The majority of experiments performed with samples from JEG have been cultural studies. The dominant microorganisms within one subglacial ice sample have been characterized at JEG by Skidmore et al. (2005) using ARDRA analysis, however, t-RFLP experiments have shown that diverse microbial communities exist at different areas within the subglacial environment. Thus, microbial characterization and comparisons of

different samples within the subglacial environment would add to our knowledge of microbes beneath JEG.

This study characterized a wide variety of bacterial communities in subglacial and proglacial environments using a combination of culture-dependent and culture-independent ARDRA analysis methods. ARDRA analysis revealed community diversity and identified dominant organisms within each sample. Culture-dependent and culture-independent techniques were compared and ARDRA analysis was compared to previous t-RFLP analysis (Bhatia, 2004) on the same samples. This project is the first to study the microbial diversity within JEG using both culture-based and non-culture based fingerprinting methods. This study tested the hypotheses that (1) the subglacial environment at JEG is diverse and that (2) the use of different analysis techniques would partially overcome biases inherent in those methods, resulting in a more complete view of microbial communities at JEG.

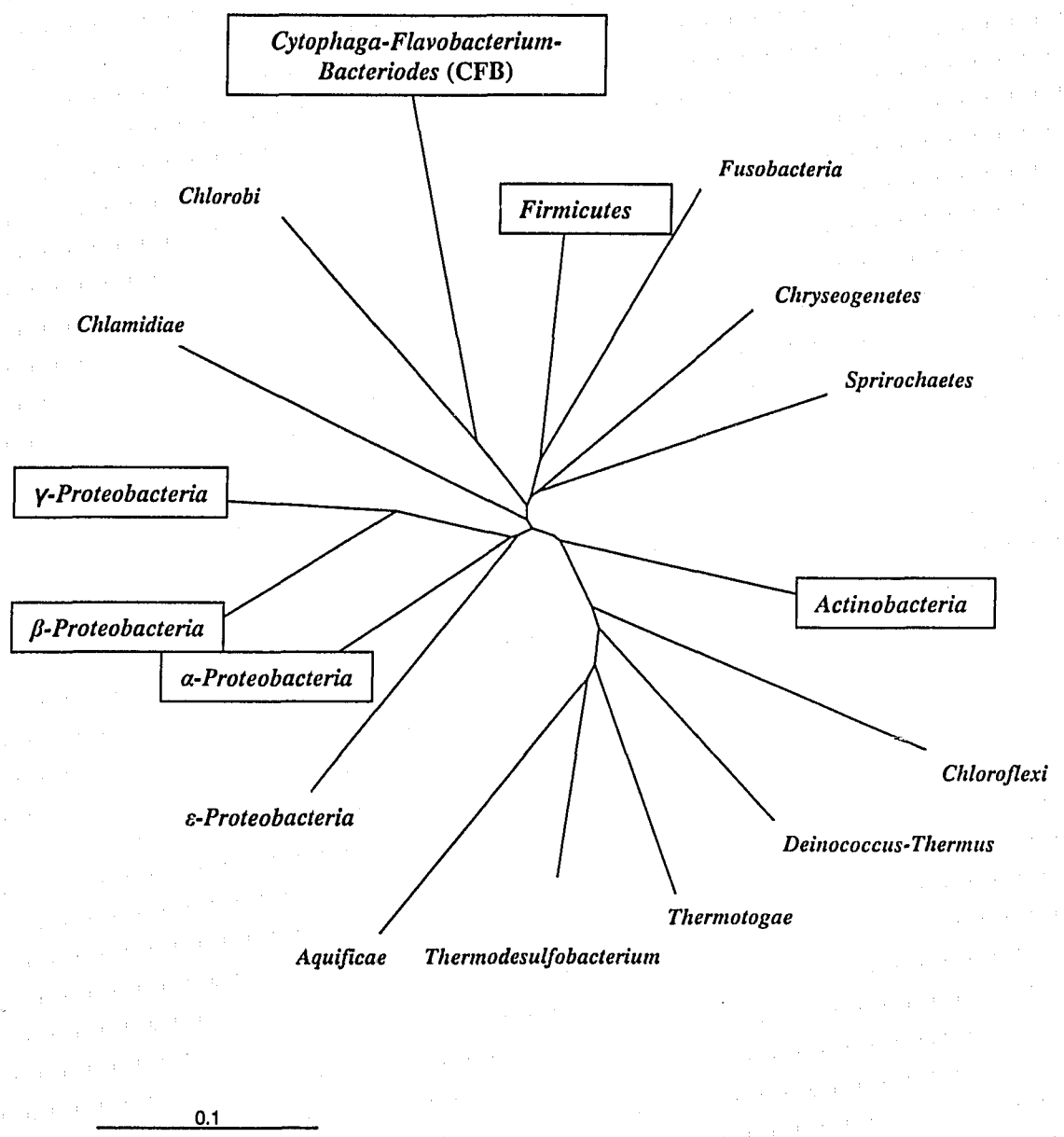

Figure 1.1. Phylogenetic tree showing 17 divisions within the domain Bacteria. Bacterial divisions found in this study are shown in boxes.

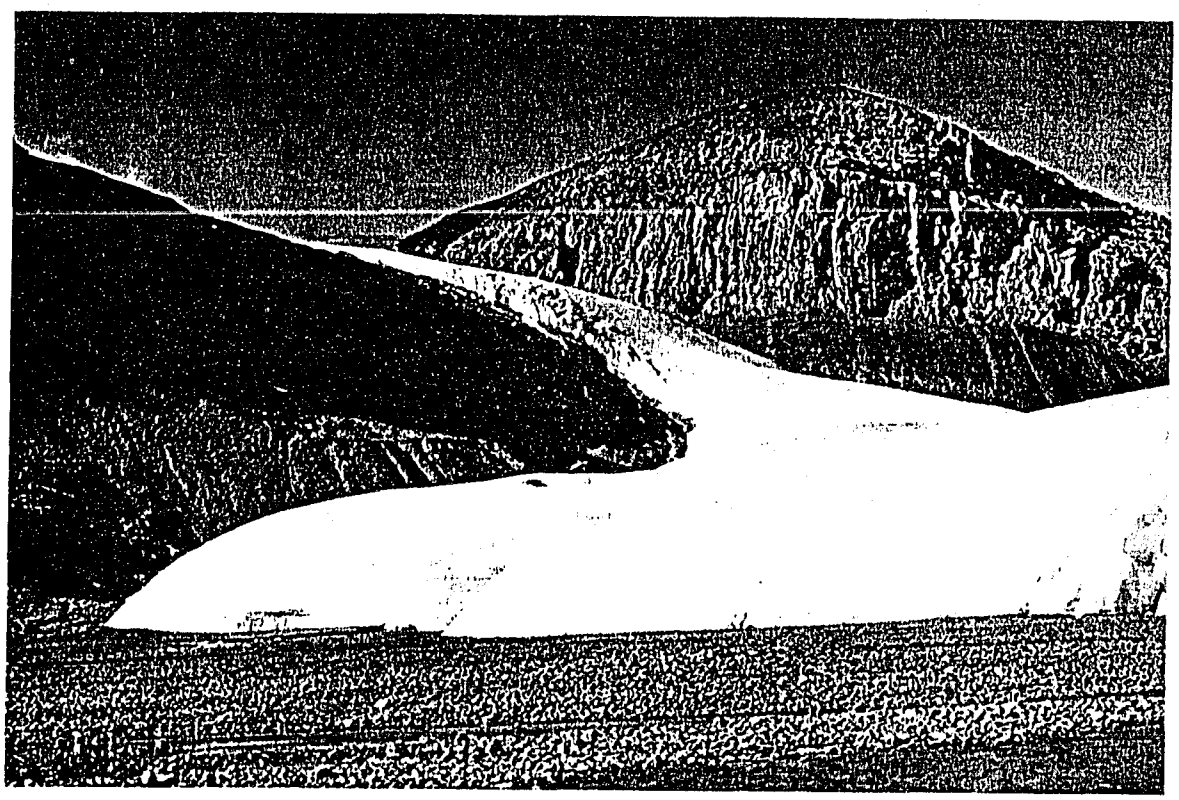

Figure 1.2. An image of the terminus of JEG, Ellesmere Island, Nunavut, Canada (Photo courtesy of J. Foght).

Figure 1.3. An image of sediment-rich basal ice layers found within a cave at the snout of JEG, Ellesmere Island, Nunavut (Photo courtesy of M. Bhatia).

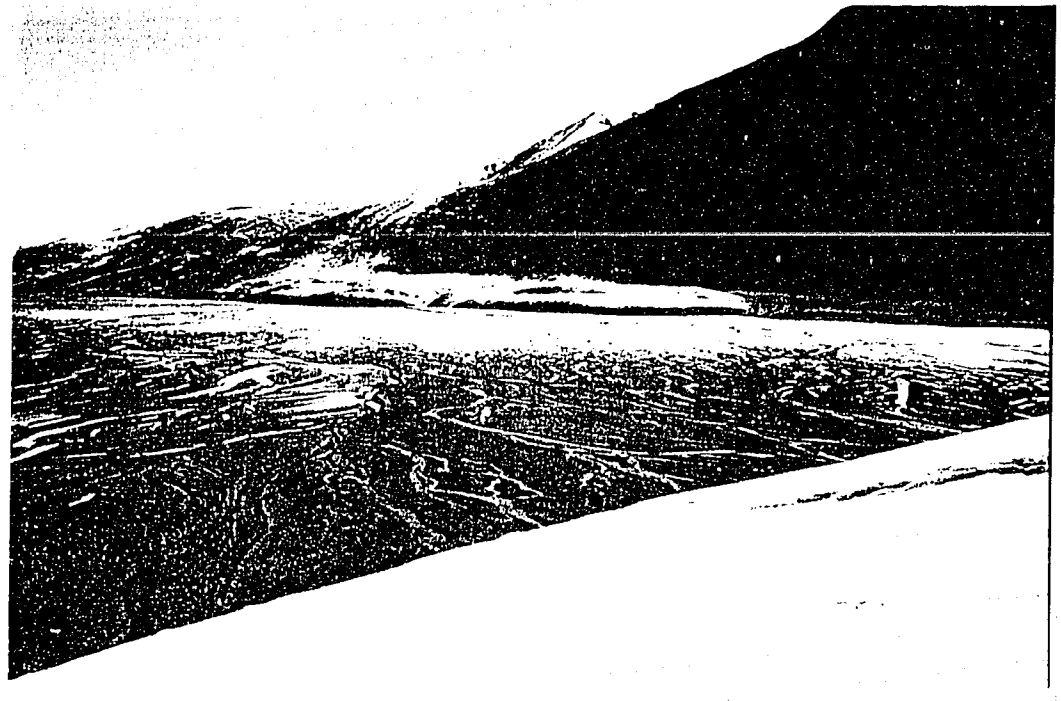

Figure 1.4. An image of the large network of proglacial streams in front of JEG, Ellesmere Island, Nunavut (Photo courtesy of M. Bhatia).

Figure 1.5. An image of a supraglacial stream that originated from big Moulin lake and is feeding into the subglacial reservoir at JEG, Ellesmere Island, Nunavut (Photo courtesy of M. Bhatia).

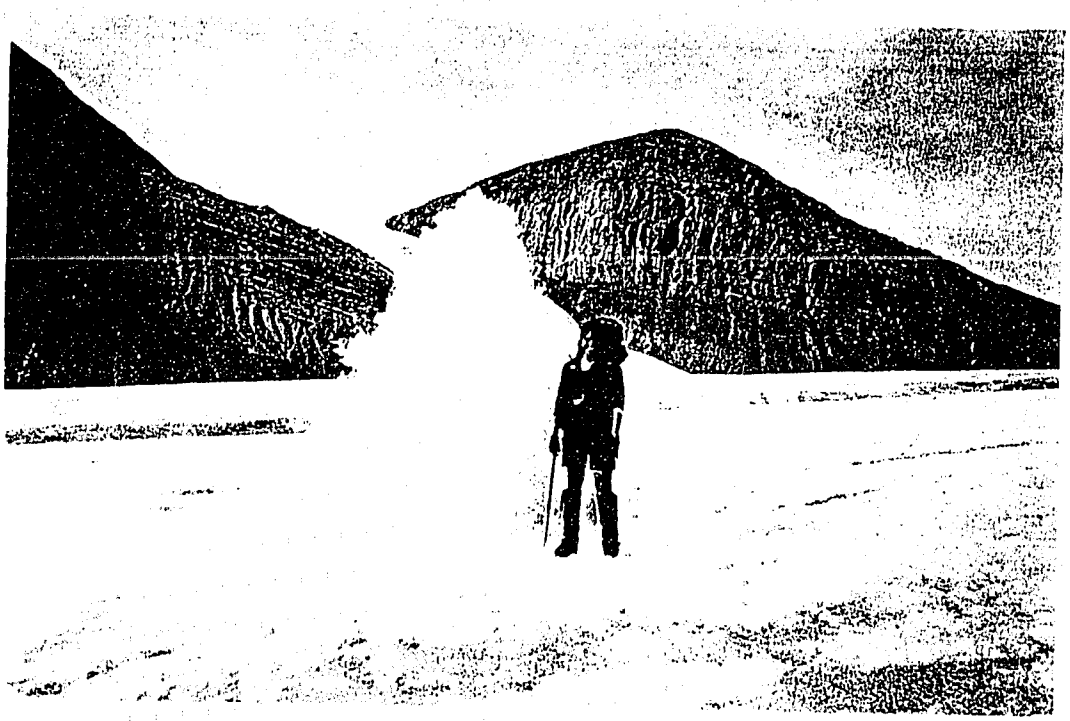

Figure 1.6. An image of a subglacial outburst in the form of an artesian fountain from the glacier surface at JEG, Ellesmere Island, Nunavut (Photo courtesy of M. Bhatia).

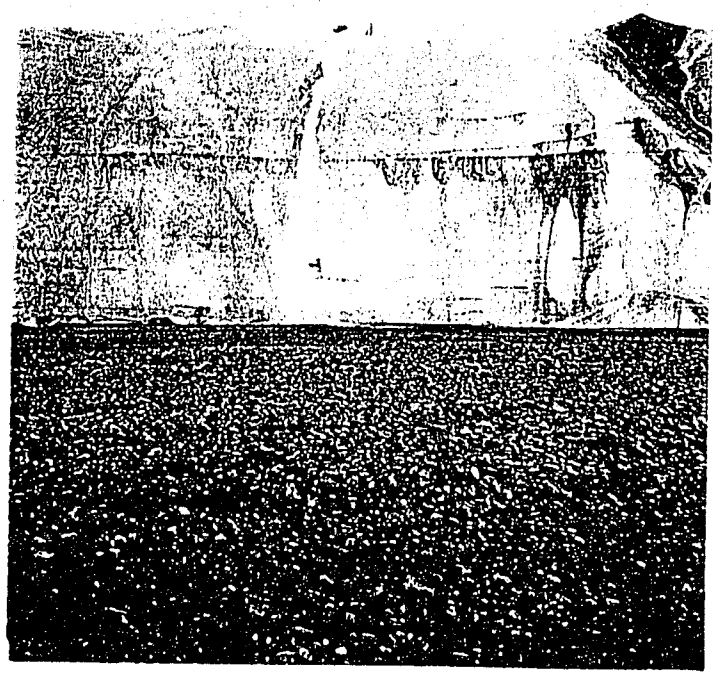

Figure 1.7. An image of a subglacial outburst in the form of an upwelling stream from the glacier surface at JEG, Ellesmere Island, Nunavut (Photo courtesy of M. Bhatia).

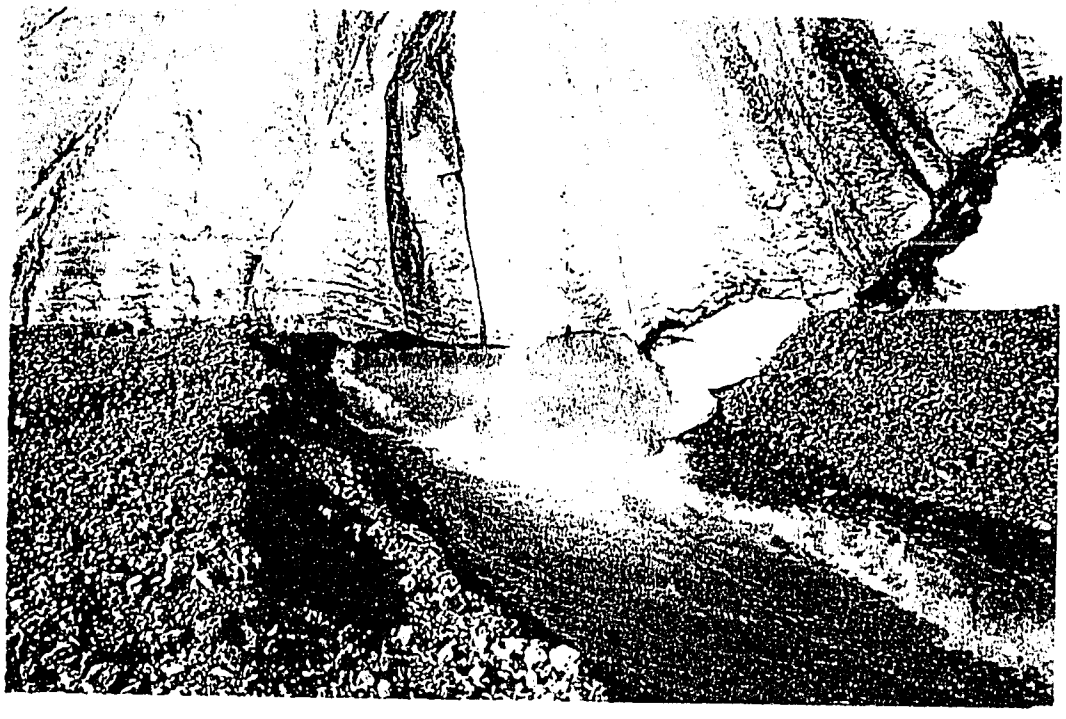

Figure 1.8. An image of a subglacial outburst in the form of a subglacial stream from the glacier surface at JEG, Ellesmere Island, Nunavut (Photo courtesy of M. Bhatia).

2.0 Materials and Methods

2.1 Sampling of glacier environments

Sampling of JEG was conducted throughout summer melt season, May 21 to August 1, 2002 by M. Bhatia and M. Sharp. The pressure created by melt water entering the subglacial system through cracks and moulins caused subglacial water that had been stored under the ice over winter to burst forth at three locations. A subglacial initial burst event occurred where subglacial water burst at the glacier front from the ice in a pressurized horizontal flow. After ~20 h, the subglacial flow shifted from the glacier front to an artesian fountain (1.4 to 2.0 m high). The artesian fountain lasted for 1 week, during which subglacial outflow shifted to a channelized flow (outburst channel) at the base of the glacier front. Samples from the subglacial initial burst (SIB) and the subglacial outburst channel (SOC) events were chosen for culture-dependent and culture-independent analyses in this study. The subglacial water from SIB represents water that has been stored at the base of JEG over-winter and the subglacial water from SOC represents a mix of stored subglacial water and supraglacial influx. Subglacial (basal) ice was collected from two locations: at a junction between JEG and an adjacent glacier, Fox glacier (BF), as well as inside an ice cave near the west marginal stream (BC) (Figure 2.1). Proglacial sediment was collected from the sediment directly in front of the glacier terminus (PSA). For a complete description of sample sites and conditions, see Bhatia (2004).

2.1.1 Subglacial water samples

Five 1-L water samples from the SIB event were collected June 30, 2002 at 19:00, using sterilized screw closure Nalgene bottles (11-825-B Nalgene, Rochester, New York). Prior to use, the brand new bottles had been washed with soap, rinsed with doubly distilled water, rinsed in ion exchange milliQ water (Millipore) and autoclaved on 2 successive days to eliminate both vegetative cells and spores. The samples were stored on ice (<4°C) in coolers for 8 days in the field, transported to a laboratory (72 h) at <4°C and frozen at –20°C until processed.

The third bottle, of five bottles, was chosen and processed November 29, 2002 for culture-dependent and culture-independent analyses. The 1-L sample of subglacial water was thawed at 4°C for approximately 72 h and then passed through a disposable, sterile

analytical filter unit containing a removable 0.2 μm cellulose nitrate membrane (09-740-21A Nalgene, Rochester, New York) in 150-mL increments. The filter unit was disassembled and the filter was aseptically divided into quarters and added to sterile bead beating tubes using a sterile scalpel blade. One quarter of the filter, representing 250 mL of water, was used for genomic DNA extraction and clone library construction. One half of the filter, representing 500 mL, was used to obtain pure isolates. The remaining filter sections were placed into empty sterile bead beating tubes (FSSP9700936, Fisher Scientific Co., Ottawa, Canada) and stored at -80°C for future analysis. The filtration process was conducted aseptically in a UV-sterilized biohazard hood. The entire process was conducted using sterile instruments that had been autoclaved on two consecutive days, sterilized with bleach and then rinsed in twice autoclaved milliQ water (Millipore).

Three 1-L water samples from the subglacial outburst channel (SOC) were collected early in the melt season, July 4, 2002 at 12:45. The first of the three bottles was processed on May 23, 2003 and used for molecular- and culture-based community analyses. The water was passed through two filter units in a similar fashion as the SIB sample. Using the first filter unit, 450 mL of water was passed through the filter. The filter unit was disassembled and the membrane was sliced aseptically into sections. One half of the filter, representing 225 mL of water, was used to obtain pure culture isolates. The remaining filter sections were placed into empty sterile bead beating tubes and stored at –80°C for future analysis. Using the second filter unit, the remaining 550 mL of water was passed through the filter in 150-mL increments. The membrane filter was sliced into sections in the same fashion as the first filter and 3/8 of the membrane, representing 207 mL of water, was used for genomic DNA extraction.

2.1.2 Basal ice samples

Five bags of basal ice were collected July 31, 2002 at a junction between JEG and a tributary glacier, Fox glacier, where sediment-rich basal ice had been exposed. Blocks of the basal ice were collected aseptically using an ethanol flame-sterilized ice axe. The first few centimeters of the ice surface was chipped away to ensure that the basal ice sampled was not contaminated by surface ice. The freshly exposed basal ice was chipped into flame-sterilized collection trays and transferred without handling into sterile 69 oz, 3.0-mm thick plastic bags (Whirl-Pak, Nasco Products, New Hambury, Ontario, Canada).

The basal ice samples were stored in ice coolers (≤0°C) for 30 days, transported frozen to a laboratory (72 h) and stored at –20°C. The condition of the samples upon arrival at the laboratory indicated that the samples had not melted during storage and transport.

The first of the five bags was processed September 24, 2002. The ice blocks were carefully transferred from the original bag into autoclaved 2-L and 3-L beakers using large sterile, DNA-free forceps, being careful not to touch the bag to the sterile beaker. All of this work was done in a UV-sterilized biohazard hood. The ice blocks were placed at 4°C and melted over three days, stationary. The resulting melted sample separated into a layer of clear supernatant and a thick underlying layer of muddy sediment. The clear supernatants were removed without disturbing the sediment and passed through two Nalgene filter membranes in 150 mL increments. The contents of the first beaker, containing 400 mL of liquid supernatant, was passed through one filter and the contents of the second beaker, containing 230 mL of supernatant, was passed through a second filter. The filters were sectioned and placed into sterile bead beater tubes as described for the subglacial water samples. Three- quarters of the first filter, representing 100 mL of supernatant, was used for genomic DNA extraction and clone library construction. A 1-g sample of the muddy sediment was used to obtain pure culture isolates.

Three sterile bags of basal ice were collected June 15, 2002 at 5:30 pm from an ice cave located at the west margin of JEG, where basal ice had been exposed by a stream cutting into the glacier bed. The first of the three bags was processed September 25, 2002. The ice was transferred to a sterile beaker, covered with autoclaved tinfoil, placed at 4°C and melted over 3 days as described above. The resulting melted sample separated into a layer of clear supernatant and a thick underlying layer of muddy sediment. A 1-g sample of the muddy sediment was used to obtain pure culture isolates.

2.1.3 Proglacial sediment samples

Proglacial sediment samples were collected July 19, 2002 from gravel that had been exposed by streams banks cutting directly in front of the glacier terminus. Samples were collected late in the melt season to ensure that the sediment was unfrozen and exposed. Sampling was performed aseptically using an ethanol flame-sterilized trowel. Subsurface samples (20 to 40 cm depth) were collected to avoid surface contamination. The sediment was stored in coolers (<4°C) for 16 days, transported frozen to a laboratory

(72 h) and stored at -20°C in the original sterile bags. Portions of sediment, ranging from 0.1-0.5 g, were transferred into bead beating tubes using autoclaved scoopulas that had been bleach sterilized twice and rinsed in autoclaved milliQ water. One sediment sample containing 0.23 g of sediment was used for genomic DNA extraction and clone library construction. A 1-g sample of proglacial sediment was used to obtain pure culture isolates.

2.2 Culture-based ARDRA analysis

2.2.1 Pure culture isolates

To obtain pure isolates from subglacial water filters, the half-filters were placed into 9.0 mL of cold (4°C), sterile 3 mM potassium phosphate buffer (pH 7.0) in a test tube containing approx. 0.5 g of 3-mm diameter glass beads. The tube was mixed vigorously by vortex at high speed for 1 min, after which no sediment remained on the membrane. Five ten-fold serial dilutions were performed immediately using the 3 mM phosphate buffer blanks with mixing by vortex. Triplicate plates of cold R2A agar (Difco) were immediately inoculated with 0.1 mL dilution per plate, spread with chilled, sterile glass spreaders. Dilution blanks were mixed by vortex before each series of plates was inoculated. These plates were incubated at 4°C in the dark immediately after the inoculation. Subsequent series of plates for incubation at room temperature (22°C) were inoculated after the 4°C plates. A total of three parallel series of cold R2A agar plates was inoculated and incubated in the dark (Table 2.1). Microaerobic incubation conditions were created by placing the R2A plates in sealed jars containing Anaerocult sachets (Merck, Darmstadt, Germany) that decreased oxygen content while releasing 5 to 7% CO_2. Each time the jars were opened, the sachets were replaced with new ones.

To obtain pure cultures from proglacial sediment and subglacial basal ice sediment, 1 g of wet weight sediment was diluted into 9.0 mL of cold (4°C), 0.1% sodium pyrophosphate buffer (pH 7.0) in a sterile test tube containing approx. 0.5 g of 3-mm diameter glass beads. The tube was mixed vigorously by vortex at a high speed for 1 min. Five ten-fold serial dilutions were performed immediately using 0.1% sodium pyrophosphate buffer with mixing by vortex. Triplicate plates of cold R2A agar were immediately inoculated with 0.1 mL dilution per plate and spread with chilled glass spreaders. R2A agar plates inoculated with proglacial sediment were incubated in the

dark under the three incubation conditions described for subglacial water (Table 2.1). R2A plates inoculated with subglacial sediment were only incubated aerobically at 4°C and aerobically at 20°C to reduce workload.

2.2.2 PCR amplification of 16S rRNA genes

Two methods were used to amplify 16S rRNA from pure culture isolates: colony PCR and PCR amplification from genomic DNA. In the first method, a suspension of bacterial colonies from agar plates was directly added as the template in the colony PCR reaction. In some cases, when colony PCR was unsuccessful, genomic DNA was extracted from the pure culture and subsequently used as template for 16S rRNA amplification. Bacterial 16S rRNA from both cultured isolates and extracted genomic DNA was amplified by PCR using the oligonucleotide primers PB36 (5'-AGRGTTTGATCMTGGCTCAG-3') and PB38 (5'- GKTACCTTGTTACGACTT-3') (Foght et al., 2005) corresponding to *E. coli* positions 8 to 27 and 1492 to 1509, respectively (Brosius et al., 1981).

When amplifying from cultured isolates (colony PCR), a suspension of bacterial colonies in TE buffer was used as the DNA template. The TE buffer, consisting of 10 mM, Tris HCl (pH 9.0), and 1 mM EDTA (pH 8.0), was made using autoclaved stock reagents and dispensed with filtered pipette tips. The TE buffer was passed through a 0.2 μm filter to remove any contaminating organisms and stored in a sterile Falcon polyethylene tube (BD Biosciences, Oakville, Ontario). One to five single colonies (depending on the colony size) were scraped from R2A plates using a flamed metal pick and suspended in 100 μL of TE buffer in autoclaved 1.5 mL microfuge tubes (Rose Scientific Ltd., Edmonton, Alberta). As a negative amplification control, the flamed metal pick was swirled into TE buffer without being inoculated and 5 μL portions of this buffer were added to the PCR reaction.

When amplifying using genomic DNA, 5 μL portions of the extracted DNA (described in section 2.3.3) were added directly to the PCR reaction. The PCR reactions were carried out in a final volume of 50 μL using 5 μL of bacterial suspension or genomic DNA, 0.5 μM of each forward and reverse primer, 50 mM Tris HCl (pH 9.0), 1.5 mM magnesium chloride, 0.4 mM β-mercaptoethanol (BDH Laboratory Supplies, Poole, England), 5 μg nuclease-free bovine serum albumin (Roche Diagnostics GmbH,

Manheim, Germany), 10 mM ammonium sulfate, 0.2 mM each of dGTP, dCTP, dATP and dTTP, 5% dimethyl sulfoxide (DMSO) and 0.5 U *Taq* (Roche Diagnostics GmbH, Manheim, Germany). Reactions were performed using either a Techne or Eppendorf thermocycler for 30 cycles (39 s at 93°C, 60 s at 54°C, 120 s at 73°C) after an initial denaturation of 4 min at 95°C, followed by a final extension at 73°C for 10 min.

2.2.3 Genomic DNA extraction

A direct, physical lysis method, employing 10% sodium dodecyl sulfate (SDS) and chloroform was used to extract genomic DNA from whole cells. This physical method of extraction was chosen to extract genomic DNA from a wide range of organisms that may not lyse when exposed to detergents alone. To a 2-mL screw cap tube, 0.5 g each of 2.5 mm and 0.1 mm diameter zirconium-silica beads (Fisher Scientific Co., Ottawa, Canada) were added in a UV-sterilized biosafety hood. All zirconium beads were handled with sterile, DNA-free metal spatulas. The bead filled tubes were autoclaved twice on successive days for 20 min. Next, 300 μL each of 100 mM phosphate buffer (pH 8.0), 10% SDS lysis buffer (100 mM NaCl, 500 mM Tris pH 8.0), and chloroform-isoamyl alcohol (24:1) were added to the tubes. Half a loopful of bacterial cells was picked from R2A plates using a flamed inoculating loop and suspended in the contents of the bead beating tubes. The bacterial samples were homogenized in a Model B110BX mini bead-beater (BioSpec Products, Bartlesville, OK) for 40 s at 5000 reciprocations per min. Next, cellular debris was removed by microcentrifugation for 5 min at 13 000 rpm. The DNA-containing supernatant (about 650 μL) was transferred into a new autoclaved 1.5 mL microfuge tube and gently mixed with 7 M ammonium acetate (pH 8.0) to achieve a final concentration of 2.5 M. The coagulated protein was removed by microcentrifugation for 5 min at 13 000 rpm. The supernatant was again transferred to a new autoclaved microfuge tube and an equal volume of isopropanol was added. DNA was precipitated by incubating the mixture for 30 min at -20°C and recovered by spinning at 14 000 rpm for 10 min. The pellet was air dried, then dissolved in 40 μL of autoclaved milliQ water. This genomic DNA was then stored at –20°C for later analysis. The entire procedure was performed aseptically at a bench designated for PCR work, using autoclaved or filter-sterilized reagents, micropipettes with filtered tips and while wearing gloves. The amplified 16S rRNA

fragments from each pure culture were digested with restriction endonucleases to create ARDRA patterns as described in section 2.4.

2.2.4 Minimum PCR detection limit

A culture of *E. coli* cells was created by inoculating a test tube containing 10 mL of Luria-Bertani (LB) broth with one loopful of *E. coli* cells and incubating the test tube at 37°C over night while shaking. Serial dilutions of the *E. coli* culture (10^0 to 10^{-9}) were made by transferring 1 mL of culture to 9 mL of 100 mM phosphate buffer (pH 8.0). Aliquots (300 µL) of these dilutions were bead beated using the genomic DNA extraction protocol as described in section 2.2.3. The resulting genomic DNA was used for PCR amplification as described in section 2.2.2. PCR product was run out on a 2% agarose gel and the presence of a PCR product band was recorded for all *E. coli* culture dilutions. Aliquots (100 µL) of the *E. coli* culture dilutions were also spread in duplicate onto plate count agar (PCA) and incubated at 37°C over night. The number of colonies on each plate were counted and used to determine the number of cells bead beated in genomic DNA extraction. The minimum PCR detection limit is the minimum number of *E. coli* cells that after genomic DNA extraction and amplification yields a detectable PCR product band on a 2% agarose gel.

2.2.5 Biochemical tests for pure culture isolates

Catalase test. Isolates were tested for the ability to produce catalase. A loopful of cells was smeared onto a glass slide containing a drop of 1.5% H_2O_2. The presence of bubbles from the breakdown of H_2O_2 and production of O_2 indicated the presence of catalase. *Escherichia coli* was used as a positive control.

Oxidase test. Isolates were tested for the presence of cytochrome C. A loopful of cells was smeared onto filter paper containing a few drops of a 1% N,N,N′,N′ tetramethyl-p-phenylenediamine and 0.05% ascorbic acid solution. A rapid color change from colorless to blue-purple within 10 s indicated the presence of cytochrome C. *Pseudomonas aeruginosa* was used as a positive control.

Nitrate test. Isolates were tested for the ability to reduce nitrate to nitrite or dinitrogen gas. A loopful of cells was inoculated into test tubes containing 5 mL of nitrate broth (0.8 g nutrient broth [Difco], 1.0 g KNO_3 and 1.7 g agar per litre). After incubation in the dark at either 4°C or 20°C for 2 to 3 weeks, 1 mL of sulfanilic acid and

1 mL of alpha-naphthylamine were added to each test tube. The production of a red color in the presence of nitrite indicates the ability of the organism to reduce nitrate to nitrite. To broths showing a negative reaction, a few particles of zinc were added. The appearance of a red color change indicates that the nitrate has not been reduced by the organism. A lack of color change indicates that the organism has reduced the nitrate through nitrite to nitrogen gas. *E. coli* was used as a positive control for production of nitrite.

Motility test and microbial growth. Isolates were tested for motility in semisolid agar. A loopful of cells was stabbed into a test tube containing 5 mL of semisolid TSA agar (3.0 g trypticase soy broth [Difco]; 1.7 g agar [Difco] per litre). After incubation in the dark at either 4°C or 20°C for 2 to 3 weeks, the test tubes were observed for growth along the stab line. Motile isolates were able to migrate into the agar away from the stab line, whereas non-motile isolates grew only along the stab line. *Proteus vulgaris* incubated at 22°C was used as a positive control. Oxygen requirements were also noted based on the depth of growth in the TSA agar. Strict aerobes grew only at the agar surface. Microaerophiles grew in a band below the agar surface. Facultative anaerobes grew throughout the agar.

Gelatinase test. Isolates were tested for the ability to produce gelatinase. A loopful of cells was stabbed into a test tube containing 5 mL of semisolid gelatin medium (0.8 g nutrient broth (Difco), 50 g gelatin per litre). The cultures were incubated in the dark at either 4°C or 20°C for 2 to 3 weeks. The test tubes were then cooled on ice for 30 min. Organisms that produced gelatinase would degrade the gelatin agar and the medium would remain liquid at 4°C. *Erwinia carotovora* incubated at 20°C was used as a positive control.

2.3. Molecular-based ARDRA analysis

2.3.1 Genomic DNA extraction

Total community genomic DNA was extracted from the sections of filtered glacier ice samples described in Section 2.1.1. Genomic DNA extraction was performed as described in section 2.3.3, using the direct, physical lysis method, employing 10% SDS and chloroform with the following amendments: before isopropanol was added to precipitate DNA, trace soil contaminants were removed using a GENECLEAN® kit (Bio

101, Carlsbad, CA) according to the manufacturer's directions. The purified DNA was recovered from the GLASSMILK® in 40 μL of milliQ water.

2.3.2 Clone library construction

Total community 16S rRNA genes were amplified from the extracted community genomic DNA with Bacterial PB36 and PB38 primers as described in section 2.2.2. PCR product (100 μL) was purified using a High Pure PCR Product Purification Kit (Roche, Laval, Quebec). Amplified products were cloned into *E. coli* using a pGEM-T Easy Vector cloning kit (Promega) and transformed into JM109 cells according to the manufacturer's instructions with selection on Luria-Bertani (LB) agar with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg/mL 5-bromo-4-chloro-3-indolyl-β -galactopyranoside (X-gal). White colonies were picked using sterile toothpicks into 96- well plates, each well containing 100 μL of sterile LB broth and 100 μg/mL of ampicillin. Five microliter portions of the LB suspension were used directly for 16S PCR amplification of 16S rRNA gene as described below. Later, 50 μL of 50% glycerol was added to each well and the plates were stored at -80°C for future use.

2.3.3 16S rRNA gene PCR amplification with M13 primers

The cloned 16S rRNA gene fragments were amplified for restriction enzyme digestion using M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAGGAAACAGCTATGACC3') primers. The PCR reactions were carried out in a final volume of 50 μL using 5 μL of the LB-bacterial suspension, 1.2 μM of each forward and reverse primer, 50 mM Tris HCl (pH 9.0), 1.5 mM magnesium chloride, 0.4 mM β-mercaptoethanol (BDH Laboratory Supplies, Poole, England), 5 μg nuclease free bovine serum albumin (Roche Diagnostics GmbH, Manheim, Germany), 10 mM ammonium sulfate, 0.2 mM each of dGTP, dCTP, dATP and dTTP, 5% dimethyl sulfoxide (DMSO) and 0.5 U *Taq* (Roche Diagnostics GmbH, Manheim, Germany). Reactions were performed using either a Techne or Eppendorf thermocycler for 2 cycles (90 s at 94°C, 45 s at 56°C, 90 s at 72°C), followed by 22 cycles (30 s at 90°C, 30 s at 56°C, 60 s at 72°C) and a final extension at 72°C for 10 min.

2.4. Restriction enzyme digestion and ARDRA gels

Amplified 16S rRNA PCR product was digested using either *Hae*III or *Cfo*I (Roche Diagnostics GmbH, Manheim, Germany) as suggested by the supplier. In a 20-

μL reaction, 16.8 μL of PCR product was digested with 1 U of restriction enzyme and 0.2 μL of bovine serum albumin (Roche Diagnostics GmbH, Manheim, Germany) in either autoclaved microfuge tubes (Rose) or autoclaved 8 chain PCR tubes (Axygen Scientific, Union City, CA). On some occasions, the DNA was pre-cleaned by a simple ethanol precipitation to prevent smearing on the gel. The entire digested DNA sample was separated on a 2% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer for 3 h at 96 V. The gels were stained for 30 min in ethidium bromide (Sigma) and viewed using a UV transilluminator. Each gel contained two or three lanes of 100 base pair molecular weight standard marker (Roche) to compare band sizes. The resulting banding patterns were measured against the 100 base pair markers manually. ARDRA patterns were compared by visual inspection and categorized into operational taxonomic units (OTUs) based on both *Hae*III and *Cfo*I ARDRA patterns. OTUs generated from the culture collections were numbered separately from OTUs generated from the clone libraries (i.e. OTU 1 in the culture collections is different from OTU 1 in the clone libraries). The M13 priming site was positioned on the cloning vector such that 200 bp of cloning vector was also amplified with the insert. The addition of cloning vector sequence created small differences in the ARDRA patterns generated, preventing comparisons between patterns generated from the culture collections and the clone libraries.

2.5 Sequencing

Representatives from OTUs containing two or more culture isolates or clones were chosen for sequence analysis. 16S rRNA was amplified from culture isolates or clones through PCR amplification, as described in sections 2.2.2 and 2.2.3. The PCR product (100 μL) was cleaned according to the directions of the manufacturer using either the Qiaquick PCR purification kit (Qiagen, Misissauga, ON) or the Roche High Pure PCR purification kit (Roche diagnostics GmbH, Manheim, Germany). Sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit. The sequencing PCR reactions were carried out in a final volume of 20 μL using 90 ng of purified PCR product, 0.25 μM of each sequencing primer, 1X sequencing dilution buffer (200 mM Tris HCl [pH 9.0], 5 mM magnesium chloride) and 2 μL of Big Dye terminator sequencing premix. A final volume of 20 μL was achieved by adding appropriate amounts of sterile milliQ water.

Nearly full-length bi-directional 16S rRNA gene sequences were obtained using seven sequencing oligonucleotide primers (Table 2.2). Sequencing reactions were performed using either a Techne or Eppendorf thermocycler for 25 cycles (20 s at 95°C, 15 s at 58°C, 60 s at 68°C). PCR product was cleaned up via ethanol precipitation. Polyacrylamide electrophoresis was performed at the Molecular Biology Service Unit (Department of Biological Sciences, University of Alberta) using an ABI 3100 genetic analyzer (Applied Biosystems Instruments (ABI), Foster City, Calif.).

2.6 Phylogenetic analysis

Sequences were aligned using the Pregap and Gap4 v3.0 programs from the Staden Package software. Sequence data processing and analysis was conducted using the EMBOSS suite, and PHYLIP software package v 3.6a3 (Felenstein, 1989). Bioinformatics tools were accessed through the Canadian Bioinformatics Resource (http://cbr-rbc.nrc-cnrc.gc.ca.login.ezproxy.library.ualberta.ca). Sequences were compared to known sequences in the GenBank database using a BLAST search (Atschul et al., 1990). The assembled clone sequences were tested for chimeric origin with the program CHIMERA_CHECK (http://rdp.cme.msu.edu/). Phylogenetic trees were constructed by the neighbor joining method with Kimura 2-parameter model for nucleotide change (Kimura, 1980). Bootstrap values (100 replications) generated by the neighbor joining method are shown above relevant nodes. Bootstrap values above 50 are shown.

2.7 Calculating theoretical ARDRA patterns and theoretical 5' terminal restriction fragments (t-RFs)

Theoretical ARDRA patterns were created by simulating digestion of 16S rRNA gene sequences with *Hae*III restriction enzyme using the restrict tool from the EMBOSS suite, accessed through the Canadian Bioinformatics Resource (http://cbr-rbc.nrc-cnrc.gc.ca.login.ezproxy.library.ualberta.ca). For 16S rRNA gene sequences of cultured isolates, the length of the 5' fragment from the simulated ARDRA pattern was the length of the terminal restriction fragment (t-RF). Manual deletion of the vector sequence from 16S rRNA gene sequences of clones was required to obtain the t-RF for clone sequences. Simulated digestion of the altered sequence with *Hae*III restriction enzyme was performed using the restrict tool to give the length of the 5' t-RF.

Table 2.1. Incubation conditions and temperatures for R2A plates inoculated with subglacial water.

Incubation condition	Incubation temperature	Sample Designation
Aerobic	4°C	A4
Aerobic	20°C	A20
Microaerobic	20°C	M20

Table 2.2. Primers used to obtain near full-length 16S rRNA sequences (Foght et al., 2005).

Primer	Sequence	*E. coli* Position (Brosius et al., 1981)
PB36	5'-AGR GTT TGA TCM TGG CTC AG-3'	8-27
16S.1	5'-ACT CCT ACG GGA GGC AGC AG-3'	360-379
16S.2	5'-GTA TTA CCG CGG CTG CTG GCA-3'	559-539
16S.3	5'-GGA TTA GAT ACC CKG GTA GTC C-3'	808-829
16S.4	5'-GGT TAA GTC CCG CAA CGA GC-3'	1125-1144
16S.5	5'-GCT CGT TGC GGG ACT TAA C-3'	1144-1125
PB38	5'-GKT ACC TTG TTA CGA CTT-3'	1509-1492

Figure 2.1: Aerial photo of JEG illustrating the subglacial and proglacial sites from which samples were taken for ARDRA analysis. The BF basal ice sample site at the junction with Fox glacier is indicated BF. The BC basal ice sample site in an ice cave at the west margin is indicated BC. The SIB subglacial initial burst occurred at the glacier front and is indicated SIB. The SOC subglacial outburst channel emptied from the glacier front into the proglacial area and is indicated SOC. The PSA proglacial sample site, located in stream bank cuts directly in front of the glacier terminus, is indicated as PSA. (Photo courtesy of M. Sharp)

3.0 Results and Discussion

3.1 Enumeration of viable heterotrophic isolates

The number of culturable microorganisms were determined after 27 days of incubation under different temperature (4°C or 20°C) and different oxygen (aerobic or microaerobic) incubation conditions on full strength R2A medium (Table 3.1). Mean counts ranged from 10^1 to 10^7 CFU/g dry weight or per mL. The number of culturable microorganisms were highest in the sediment-rich BF, BC and PSA samples, with counts ranging from 10^6 to 10^7 CFU/g dry weight or per mL. In contrast, the number of culturable microorganisms in the SOC and SIB water samples were ~500 times lower than the other three samples, ranging from 10^1 to 10^3 CFU/mL, with the exception of SIB isolates cultured at 20°C, which yielded an unexpectedly high value of 4.20×10^7 CFU/mL. Although higher numbers of microorganisms were cultured from the BF, BC and PSA samples compared to SIB and SOC, the colony morphologies were less diverse and fewer were pigmented. Most of the pigmented isolates were yellow, orange and red in color. Skidmore et al. (2000) also made similar observations that many colonies in the subglacial environment at JEG were highly pigmented and were mostly yellow and orange colored. Pigmentation has been widely reported in heterotrophic isolates of glacier and polar environments (Foght et al., 2004) and is considered an adaptation for surviving in such extreme conditions (Zhu et al., 2003; Van Trappen et al., 2005). Synthesizing certain types of carotenoids allows psychrophilic and psychrotolerant bacteria to modulate membrane fluidity and increase cell survival at low temperatures (Chintalapati et al., 2005). Bacteria may also modulate membrane fluidity by altering their fatty acid composition or chain length, lipid head groups or protein content in the membrane (Chintalapati et al., 2004).

Plate counts of SOC incubated aerobically at 20°C (2×10^2 CFU/mL,) agreed with previous estimates of viable counts of subglacial water at JEG: aerobic plate counts of unamended JEG subglacial melt water incubated aerobically at 22°C on quarter strength plate count agar yielded 10^2 CFU/mL (Skidmore et al., 1999). High microbial counts have also been reported in subglacial ice and sediment beneath the polythermal Franz Josef Glacier, where culturable aerobic heterotrophs ranged from 6.0×10^5 to 9.0×10^5 CFU/g dry weight (Foght et al., 2004).

The number of culturable microorganisms were highest in samples rich in debris and sediment; mainly the basal ice samples (BF and BC) and proglacial sediment near microbial mats (PSA). Higher counts in these samples support previous observations that microbial populations beneath JEG associate with particles (Skidmore et al., 1999; Sharp et al., 1999). Similar culturable heterotrophic numbers have been reported in glacier cryoconite sediment (8.6×10^4 CFU/mL)(Christner et al., 2003) and Antarctic microbial mat communities (10^5 to 10^{10} CFU/g dry weight)(Van Trappen et al., 2005), suggesting that particle association and microbe interaction within mats increase microbial populations in other glacier environments also.

Furthermore, high degrees of culturability, 3 to 82% of total direct counts, have been reported in subglacial sediment and ice beneath Franz Joseph and Fox glacier (Foght et al., 2004), contradicting the commonly cited value that less than 1% of total microbial counts can be cultured (Amann et al., 1995). Foght et al. (2004) suggested that high culturability may be a result of the simplicity of microbial communities within cold environments relative to those in temperate environments. Though total microbial counts were not performed to determine the degree of culturability in our samples, the high numbers of culturable microorganisms observed in the PSA, BF and BC samples were similar to those beneath Franz Joseph and Fox glaciers, suggesting comparable levels of culturability at JEG.

As a consequence of numerous culture transfers during isolation, many isolates of axenic cultures from the SIB, BF, BC and PSA environments were lost, limiting culture-dependent ARDRA analysis of these culture collections. Regardless, all culturable organisms with morphologically distinct colonies were tested in order to make rough comparisons with culture-independent ARDRA analysis results. However, conclusions (Section 3.2.1.1) about microbial community composition could be made only for the SOC sample, as it represented the largest and most complete culture collection.

3.2 Cultured-based ARDRA analysis

Culture-based ARDRA analysis was performed on a total of 192 pure isolates obtained from SOC, SIB, BF, BC and PSA samples. Because these microorganisms were grouped based on restriction enzyme digestion patterns, and not by DNA sequences, each group of isolates represented by a pair of unique ARDRA patterns was referred to as an OTU (Appendices 2 and 3). Each OTU was presumed to contain the same microorganism. A total of 59 OTUs was defined for all five culture collections (Figure 3.1). Seventeen OTUs contained two or more isolates and potentially represent the main cultivable microbial species within the subglacial environment at JEG. Five OTUs comprised isolates from two or more different environments (Table 3.2), with the other 54 OTUs found exclusively in one environment.

3.2.1 Subglacial water

3.2.1.1 Subglacial Outburst Channel (SOC)

ARDRA analysis was performed on morphologically distinct isolates incubated aerobically at 4°C, aerobically at 20°C and microaerobically at 20°C. One hundred and thirty-four morphologically distinct pure isolates from the SOC sample were analyzed. Seventy-two isolates were incubated aerobically at 20°C, 23 were incubated microaerobically at 20°C and 39 were cultured aerobically at 4°C. ARDRA analysis detected a total of 39 different OTUs from all three incubation conditions (Figure 3.2). Eleven OTUs (OTU 1, 3, 4, 5, 7, 8, 9, 13, 15, 16, and 17) contained two or more microorganisms. Because these microorganisms were abundant, they potentially represent the main cultivable microbial species within the community and were chosen for sequence analysis. Representatives from each of the 11 OTUs were sequenced and compared to other 16S rRNA gene sequences from the GenBank database as of April 2005 (Table 3.3). All representatives had at least 97% homology to other 16S rRNA gene sequences submitted to GenBank.

A rarefaction curve was created to determine whether the sampling of cultured isolates was a good estimate of the diversity of the microbial community within the SOC sample (Figure 3.3). The asymptotic curve in Figure 3.3 shows a steep "linear" portion, indicating that initially each isolate tested represented a new OTU, followed by a plateau, showing that fewer new OTUs were detected as more isolates were sampled. This figure

shows that a sufficient sampling size was tested and that analyzing additional isolates should not significantly alter the number of new organisms detected or the proportion of the organisms within each OTU in the environment.

3.2.1.1.1 Abundant phylogenetic divisions and source of nearest neighbors

Sequence analysis revealed that this collection of pure isolates comprised microorganisms belonging to CFB (46%), β-Proteobacteria (17%), α-Proteobacteria (11%), Actinobacteria (3%), and γ-Proteobacteria (2%) divisions (Table 3.3). Phylogenetic positioning of the SOC representatives is shown in Figures 3.4 through 3.9. The 11 most abundant OTUs comprised 79% (106/134) of the entire SOC culture collection. The two largest OTUs in the SOC culture collection, OTU 1 and OTU 3, that together comprised almost half of the culture collection, were both represented by members of the *Flavobacteriaceae* family in the CFB division. Isolate SOCA20(40), representing OTU 1, had 99% homology to *Chryseobacterium* sp. TB4-8-II [AY599655], a bacterium isolated from the asocarp of ectomycorrhizal fungus (Barbieri et al., unpublished). GenBank BLAST searches showed it also had 98% homology to two Antarctic organisms: a psychrophilic *Flavobacterium* sp. AT1042 [AY581114] and Antarctic clone R- 7933 [AJ440987], a heterotrophic microorganism found in Lake Fryxell (Yi and Chun, unpublished; Van Trappen et al., 2002). Isolate SOCA4(12), representing OTU 3, had 97% homology to its closest neighbor, *Flavobacterium* sp. EP28 [AF493663], isolated from river epilithon or rock biofilm (O'Sullivan et al., 2002). Isolate SOCA20(36), representing OTU 7, was also closely related to a glacier bacterium, Glacier bacterium FJS5 [AY315161], which was isolated from subglacial ice in New Zealand (Foght et al., 2004). Members of the Genus *Flavobacterium* have been shown to be highly adapted to low nutrient and low temperature environments. Noble et al. (1990) showed that *Flavobacterium* isolates from oligotrophic environments were more efficient and versatile at taking up substrates at lower temperatures and in low nutrient media than in rich media. Members of the CFB division are widely found in freshwater environments and often create biofilms, as they are gliding organisms that produce large amounts of extracellular polymeric substances (O'Sullivan et al., 2002). Because of their ability to create biofilms, CFB contribute to the majority of heterotrophic activity in glacier stream ecosystems (Battin et al., 2001).

The second most abundant group of microorganisms, making up OTUs 4, 8 and 9, were members of the *Comamonadaceae* family of β-Proteobacteria (Figure 3.5). Interestingly, all three representative isolates, SOCA20(17), SOCA4(5) and SOCA20(82), were most closely related to microorganisms found in groundwater. Isolate SOCA20(17) had 99% homology to β-Proteobacterium Wuba 72 [AF336361], a microorganism found within a karstic aquifer. Isolate SOCA4(5) had 99% homology to bacterium clone RA13C6, isolated from monochlorobenzene-contaminated groundwater (Alfreider et al., 2002), and isolate SOCA20(82) was closely related to β-Proteobacterium JS666 [AF038497], which was isolated from *cis*-dichloroethene-contaminated ground water (Coleman et al., 2002). Close homology to isolates from within freshwater environments suggests that many organisms beneath JEG are able to survive in diverse dark and oligotrophic environments. Also, close relatedness to temperate, freshwater organisms may suggest that these organisms were transported to the JEG via long range dispersal from temperate regions.

The rest of the sequenced SOC representatives belonged to the α-Proteobacteria, γ-Proteobacteria and Actinobacteria divisions in genera such as *Brevundimonas* and *Arthrobacter,* which are also ubiquitous in aquatic environments (Abraham et al., 1999; Glöckner et al., 2000) (Figures 3.6, 3.7 and 3.8). Isolate SOCM20(30) had 98% homology to *Brevundimonas* strain FWC04 [AJ227793], isolated from a freshwater slough (Abraham et al., 1999). Isolate SOCA20(63) had 99% homology to Actinobacteria strain PB90-5 [AJ229241], an *Arthrobacter* sp. isolated from anoxic bulk soil. Representatives of these OTUs were closely related to other microorganisms isolated in polar or oligotrophic environments. Isolates SOCA20(60) and SOCM20(20) had 99% homology to microorganisms found in Antarctic microbial mats and Siberian permafrost, respectively.

The abundance of microorganisms well adapted and ubiquitous to groundwater, freshwater and microbial mat environments within the SOC environment suggests that low nutrient availability is a limiting factor beneath JEG, rather than low temperature. The SOC environment may select for those microorganisms that are best adapted to obtaining nutrients in oligotrophic environments, either through adaptations in metabolism, as seen in members of the *Flavobacterium* genus, or through associating

with sediments or with other organisms, as seen in microbial mat- and biofilm-forming microorganisms. In river environments, it was shown that bacteria that formed aggregates with particles were 100 times more active than free-living bacteria and were responsible for 90% of bacterial heterotrophic activity in the river community (O'Sullivan et al., 2002). Biofilms are also an important strategy to increase productivity. O'Sullivan et al. (2002) analyzed bacterial communities on epilithon, or biofilms formed on river stones, in the United Kingdom and found they were also mainly comprised of CFB and, α-, β-, and γ-Proteobacteria. Biofilms within glacial streams were also dominated by CFB, α-, and β-Proteobacteria (Battin et al., 2001).

CFB and β-Proteobacteria isolates were the abundant members of the SOC culture collection and are likely important contributors to heterotrophic activity beneath JEG. These heterotrophs are highly adapted to forming associations with sediment particles and other microorganisms. This enables a wide variety of microbes to efficiently utilize the organic carbon obtained from the subglacial sediment. So far, only speculations can be made as to how each microorganism contributes to the microbial community beneath JEG. Further studies to test carbon utilization can be determined using BIOLOG® plate analysis. In BIOLOG® analysis, isolates are inoculated into 96-well plates with each well containing a different carbon source. Oxidation of the carbon source causes a color change allowing visual indication of the organisms' metabolism. The color pattern produced by all 96 wells can also be compared to a small database (1, 900 bacterial species) for identification. In the future, this study would be pertinent to further understanding the roles of each microorganism in the subglacial environment.

3.2.1.1.2 Colony morphology

No direct correlation was found between pigmentation and the assigned OTU, an observation also made by Foght et al. (2004) in analysis of subglacial isolates at Fox and Franz Joseph Glaciers. In fact, some isolates grouped into the same OTU had very different colony morphologies (Table 3.4). Further testing of four differently pigmented isolates within OTU 3, isolates SOCA4(12), SOCA4(40), SOCA4(44), and SOCA4(51), yielded identical ARDRA patterns after single digestions with *Cfo*I or *Hae*III and double digestions with both *Cfo*I and *Hae*III. All four, were shown to be closely related members of the *Flavobacterium* genus after 16S rRNA gene sequencing (Table 3.4).

Also, all four isolates clustered quite closely within the *Flavobacterium* clade (Figure 3.4). These data show that different colony morphology is not necessarily an indicator of phylogenetic distance and reinforces the danger of assigning taxonomy based upon morphology (Abraham et al., 1999). This contradicts a study done by Christner et al. (2003), who found that all nine of the visibly different colonies isolated from cryoconite sediment, when sequenced, belonged to different genera. These results expose a flaw in the original experimental design of selecting isolates with different colony morphologies in order to analyze different bacterial species within JEG. In hindsight, random selection and testing of isolates would have yielded less biased results and may have shown a different community composition of SOC than the community composition determined here.

3.2.1.1.3 Biochemical Tests

Biochemical tests of the sequenced representatives gave variable results when compared with similar tests performed on their close phylogenetic neighbors. Isolate SOCA20(36), a Gram negative rod having red-orange pigmented colonies matched closely to its relative, Glacier bacterium FJS5, also a Gram negative rod with red pigmented colonies (Foght et al., 2004). However, isolate SOCA4(4) with bright medium yellow, circular, convex, entire colonies and catalase positive and oxidase negative tests, matched only partially with its neighbor *Flavobacterium omnivorum,* a Gram negative rod, producing circular, convex, entire orange pigmented colonies with oxidase positive tests (Zhu et al., 2003). Isolate SOCA20(82), a beige-colored, Gram negative coccus with catalase and oxidase negative tests differed from its phylogenetically close relative, β-Proteobacterium JS666, a yellow-pigmented, Gram negative rod with oxidase-positive and catalase-negative tests. Because the biochemical test results were variable, they did not prove to be very useful for distinguishing different bacterial species.

3.2.1.1.4 Culture conditions

In general, different OTUs were detected for each incubation condition (Figure 3.10). Only four OTUs (OTU 1, 4, 5 and 9) contained microorganisms found in two or more incubation conditions. OTU 1 and 4, together comprising 40% of the SOC culture collection, contained microorganisms found in all three incubation conditions; OTU 5

contained microorganisms incubated aerobically and microaerobically at 20°C; and OTU 9 contained microorganisms incubated aerobically at 4°C and 20°C. These OTUs potentially comprise psychrotolerant microorganisms capable of growth over a wide range of temperature and oxygen conditions. They also show that the large diversity of psychrotolerant microorganisms in the SOC belongs to the CFB, α-Proteobacteria and β-Proteobacteria divisions. In a study on Antarctic microbial mat communities, Van Trappen et al. (2002) attributed high diversity among bacterial divisions in Antarctic microbial mat samples to similar fatty acid structures within multiple taxa. It would be interesting to the study fatty acid composition of JEG isolates to determine if high diversity is also a result of a common adaptation to the subglacial environment.

In contrast, OTU 3, the second most abundant group of bacteria comprising 16% of the cultured SOC isolates, contained isolates that only grew at 4°C and may presumably be psychrophiles. Isolate SOCA4(12), SOCA4(40), SOCA4(44) and SOCA4(51) were closely related (Figure 3.4) to the psychrophilic microorganisms *Flavobacterium psychrophilum* [AY662493], *Flavobacterium hibernum* [L39067] and *Flavobacterium xinjiangense* [AF433173] (Zhu et al., 2003). Christner et al. (2003), however, found that large number bacterial colonies, presumed to be psychrophiles, were in fact psychrotolerant after further temperature tests were performed. Although initial culture growth conditions and close phylogenetic relationships to psychrophiles are seen in the current study, actual temperature growth assays need to be performed on the OTU 3 isolates in order to conclude that they are true psychrophiles.

3.2.1.2 Subglacial Initial Burst (SIB)

ARDRA analysis was performed on 20 morphologically distinct pure isolates from SIB. Eight isolates were cultured aerobically at 20°C, six were cultured microaerobically at 20°C and six were cultured aerobically at 4°C. ARDRA analysis detected a total of 11 different OTUs with all three incubation conditions (Figure 3.11). Four OTUs (OTU 2, 6, 12, and 18) contained two or more microorganisms. Representatives from each of the four OTUs were sequenced and compared to other 16S rRNA gene sequences from the GenBank database as of April 2005 (Table 3.5). All representatives had at least 98% homology to other 16S rRNA gene sequences submitted

to GenBank. Representatives of these four OTUs (OTU 2, 6, 12, and 18) belonged to the CFB, Actinobacteria, Firmicutes and α-Proteobacteria divisions, respectively.

The largest OTU in the SIB culture collection, OTU 6, contained five isolates that were all isolated aerobically at 4°C. Isolate SIBA4(8), the representative for OTU 6, had 98% homology to *Flavobacterium* sp. R-7550 [AJ440979] from a microbial mat in lake Fryxell, Antarctica [AJ440979] (Van Trappen et al., 2002). This microorganism also clustered with *Flavobacterium* spp. detected in the SOC sample, suggesting that these SIB isolates are also uniquely suited to survive in subzero temperatures (Figure 3.4). The second largest OTU in the SIB culture collection, OTU 2, contained four isolates, which were all isolated microaerobically at 20°C. The representative of OTU 2, isolate SIBM20(5), had 99% homology to *Arthrobacter* sp. An34 [AJ551172], an Antarctic isolate (Wang et al., unpublished). Preliminary characterization of this representative, a Gram positive, catalase positive, motile aerobe, agrees with characteristics of the Arthrobacter genus (Wauters et al., 2000; Dworkin, 2005). Members of the *Arthrobacter* genus are found widespread in the soil (Dworkin, 2005). It is likely that these *Arthrobacter* species also associate with sediment particles beneath JEG.

Isolate SIBA20(24), representing OTU 12, had 99% homology to *Staphylococcus cohnii* [AJ717378], a member of the Firmicutes or low G+C Gram positive division (Figure 3.9). *Staphylococcus cohnii* [AJ717378] was isolated from an alkaline groundwater environment in Portugal, having a pH of 11.4 (Tiago et al., 2004). Interestingly, oligotrophic alkaline lake environments, though uncommon on Earth, are also presumed habitat analogs of some environments found on Mars (Tiago et al., 2004). Most members of the *Staphylococcus* genus are human microbiota. However, their isolation from a wide variety of environmental sources such as soil, beach sand, seawater, freshwater and plant surfaces make it unlikely that isolate SIBA20(24) is a human contaminant (Dworkin, 2005).

Isolate SIBM20(6), representing OTU 16, had 99% homology to *Caulobacter* sp., strain FWC20 [AJ227766] (Figure 3.6). This α-Proteobacterium was isolated from activated sludge in British Columbia, Canada and clustered with a group of *Caulobacter* spp. found in freshwater environments (Abraham et al., 1999). Abraham et al. (1999) found that *Caulobacter* sp. strain FWC20 formed a distinct phylogenetic branch with

Caulobacter henricii [AJ227758], a yellow pigmented aerobe. This colony morphology description agrees with the light orange pigmentation of SIBM20(6) colonies.

Detection of *Caulobacter* sp. is not surprising because members of the genus *Caulobacter* are ubiquitous in water and have low nutrient requirements. *Caulobacter* species have unique reproductive cycles that are specialized for oligotrophic environments and would be advantageous for living beneath JEG. These dimorphic, prosthecate bacteria produce two daughter cells that are morphologically and behaviorally different from each other (Abraham et al., 1999). One daughter cell is non-motile, affixing to surfaces via adhesive material, and also prosthecate, possessing at least one elongated, cyndrical appendage that is an outgrowth of the cell envelope. The other daughter cell is motile, having one polar flagellum. This mode of replication ensures the dispersion of its population at each generation and reduces competition for resources in environments where nutrients are limiting (Abraham et al., 1999).

Much like the SOC, the SIB also contains microorganisms that are found in microbial mats, soil environments and oligotrophic freshwater. The presence of these types of organisms beneath JEG again suggests that their ability to extract nutrients from sediment or low-nutrient waters and their ability to form associations with other microorganisms is beneficial for living in the subglacial water environment.

3.2.2 Subglacial ice

3.2.2.1 Junction at Fox Glacier (BF)

ARDRA analysis was performed on four morphologically distinct pure isolates from the BF environment. Three isolates were incubated aerobically at 20°C and one was incubated microaerobically at 20°C. All isolates fell within four different OTUs OTU 2, 4, 29 and 58. Though only four isolates were cultured, each was sequenced in order to compare them with the most abundant organisms determined by culture-independent ARDRA analysis in the BF environment. OTU representatives were compared to other 16S rRNA gene sequences from the GenBank database (Table 3.6). All representatives had 99% or 100% homology to other 16S rRNA gene sequences submitted to GenBank.

Sequence results revealed that isolates BFA20(22) and BFA20(13) were members of the Actinobacteria division (Figure 3.8). Isolate BFA20(22) was identical to Gram

positive bacterium Wuba45 [AF336354], a member of the *Kocuria* genus that was obtained from a karstic aquifer. Isolate BFA20(22) was also 99% similar to *Kocuria palustris* sp. nov. TAGA27 [Y16263] isolated from the rhizoplane of a narrow-leaved cattail in the Danube river, Hungary (Kovács et al., 1999). *Kocuria palustris* sp. nov. TAGA27 was described as a non-motile, Gram positive coccus, having pale yellow, opaque and smooth colonies with irregular edges. It was also described as being an aerobic, catalase positive, oxidase negative and gelatinase negative chemoorganotroph that did not exhibit growth above 30°C. The results reported by Kovács et al. (1999) mostly agree with the biochemical tests, with differences only in motility and gelatinase test results.

Isolate BFA20(13) was 99% similar to *Arthrobacter ramosus* [X80742] and *Arthrobacter pascens* [AJ576068], which are both Gram positive spherical aerobes. Biochemical tests describing BFA20(13) as a Gram positive coccus agrees with the description of *Arthrobacter ramosus*. The 16S rRNA gene sequences of *Arthrobacter ramosus* and *Arthrobacter pascens* are identical, despite occupying well-defined distinct species and showing differences in their peptidoglycan composition (Koch et al., 1994). These results reinforce the idea that neither morphology nor 16S rRNA gene sequencing alone can be the sole measures to identify bacteria. Physiological characterization, chemotaxonomic characterization, % G+C content and whole DNA-DNA hybridization are required when proposing new bacterial species (Kovács et al., 1999; Abraham et al., 1999). Unfortunately, the *Micrococcus* and *Arthrobacter* genera have traditionally been assigned by colony morphology and phylogenetic intermixing of these species is widely seen (Koch et al., 1994).

The final two isolates analyzed were isolate BFM20(8), which grouped closely to the β-Proteobacteria detected in the SOC clone library, and isolate BFA20(17), which was the only *Rhodobacter* species detected in both the culture-independent and culture-dependent ARDRA studies (Figure 3.5 and 3.6). Isolate BFA20(17) had 99% homology to uncultured bacterium clone methyl chloride 40 [AY439191], a methylotrophic microorganism capable of degrading methyl chloride and methyl bromide, which is toxic to most microorganisms and is used as a preplant soil fumigant (Miller et al., 2004).

3.2.2.2 Basal Ice Cave (BC)

ARDRA analysis was performed on five morphologically distinct pure isolates from the BC environment. All five isolates were cultured aerobically at 20°C and fell into four different OTUs (OTU 2, 19, 41 and 42), with OTU 2 containing two isolates. Sequencing was not performed because all representatives of OTU 2 were previously shown to be members of the *Micrococcaceae* family (Sections 3.2.1.2 and 3.2.2.1). Representatives within the SIB and PSA environments had 99% homology to *Arthrobacter* sp. An34 and a representative of OTU 2 in the BF culture collection had 100% homology to Gram positive bacterium Wuba 45 from the genus *Kocuria* (Figure 3.8)

3.2.3 Proglacial Sediment (PSA)

ARDRA analysis was performed on 29 morphologically distinct pure isolates from the PSA sample. Fourteen isolates were cultured aerobically at 20°C, eight were cultured microaerobically at 20°C and seven were cultured aerobically at 4°C. ARDRA analysis detected a total of eight different OTUs from all three incubation conditions (Figure 3.12). Three OTUs, (OTU 2, 10 and 11), contained two or more microorganisms. Because these microorganisms were abundant, they potentially represent the main cultivable microbial species within the proglacial sediment community and were chosen for sequence analysis. Representatives from each of the three OTUs were sequenced and compared to other 16S rRNA gene sequences from the GenBank database as of April 2005 (Table 3.7). All representatives had 99% homology to other 16S rRNA gene sequences submitted to GenBank.

Twenty-two of the 29 PSA isolates, represented by OTU 2 and 10, belonged to the Actinobacteria division. Isolates PSAM20(9) and PSAA20(6) were both 99% similar to the Antarctic microorganism *Arthrobacter* sp. An34 and clustered closely with isolates from the BF and SIB environments (Figure 3.8). To date, no information has been published on *Arthrobacter sp.* An34, however, it is a close relative to *Arthrobacter oxidans,* a catalase positive aerobe (Wauters et al., 2000). These results agree with the biochemical tests. Members of the Actinobacteria division traditionally have been associated with soil, however, many have since been isolated from aquatic environments (Warnecke et al., 2004; Glöckner et al., 2000). Warnecke et al. (2004) suggested that

Actinobacteria are one of the most abundant groups of freshwater bacterioplankton. Persistence in the environment can be attributed to their small cell size and Gram positive cell wall, which helps resist predation. Though they are common in lakes, this group of microorganisms is largely underrepresented in environmental clone libraries generated with universal Bacterial primers (Warnecke et al., 2004).

Isolate PSAA4(4) was 99% similar to Antarctic bacterium R-9113 [AJ441004], which was isolated from a microbial mat community in Antarctica, and grouped closely with the *Pseudomonas* genus (VanTrappen et al., 2002) (Figure 3.7). The presence of *Pseudomonas* species within the PSA environment was expected because they commonly inhabit soil environments (Dworkin, 2005).

3.2.4 Comparisons between cultured isolates at JEG

Five OTUs were detected in two or more JEG environments (Table 3.2). OTU 1, comprising *Flavobacterium* spp. was detected in both the SOC and PSA environments. OTU 2, comprising *Arthrobacter* and *Kocuria* species, was by far the most widely distributed OTU. It was represented by isolates in all environments except SOC. OTU 2 was most commonly detected in the PSA environment, which is characteristic of Actinobacteria, as they are widely found in the soil environment (Dworkin, 2005). The *Arthrobacter* and *Kocuria* species may represent the most versatile and adaptable organisms within JEG, inhabiting proglacial sediment, subglacial water and subglacial ice samples. OTU 6 was also made up of *Arthrobacter*-related spp. and found in the PSA and SIB samples. OTU 4, made up of *Comamonas*-related spp. was detected in both the SOC and BF samples. Finally, OTU 10, comprising *Flavobacterium*-related species was detected in the PSA and SIB samples. Overall, the SIB and PSA environments shared the highest number of related species. This result was surprising because both environments differ greatly in terms of water and nutrient availability and exposure to air. Also, t-RFLP analysis of the same JEG samples showed most samples from either the proglacial or subglacial environment were dominated by terminal restriction fragments only found in that environment (Bhatia, 2004). This observation may be due to the fact that only a small number of isolates was analyzed from the SIB and PSA samples, which may skew the results. Additional isolates within these samples should be cultured to determine the relative abundances of these microorganisms.

Because the SIB and SOC water samples originated from the same water source (i.e., the subglacial reservoir beneath JEG) both samples were predicted to contain similar key microorganisms found in that subglacial environment. The microorganisms shared between these samples would potentially reveal a group of microbes uniquely suited to the JEG subglacial environment. However, only one related group of *Flavobacterium* spp. was found in both environments, OTU 3 (Figure 3.4). The group of *Flavobacterium* spp. was represented by SOCA4(12), SOCA4(40), SOC A4(44) and SOCA4(51) in the SOC environment and SIBA4(8) in the SIB environment (Tables 3.3 and 3.4). These *Flavobacterium* spp. were cultured only aerobically at 4°C in both environments and were also related to other psychrophilic microorganisms from polar environments, such as *Flavobacterium fryxellicola* and *Flavobacterium xinjiangense*, suggesting that they are psychrophiles and are unique to subglacial water beneath JEG (Van Trappen et al., 2005; Zhu et al., 2003). Because these microorganisms have been found in other environments, it is possible that they were brought to JEG in the past through long-range transport and over time developed a niche within the subglacial reservoir beneath JEG.

Flavobacterium spp. were also found in the proglacial sediment and may have been introduced to this environment from exposure to the subglacial outburst water, which flood the proglacial environment each year, creating a network of streams. They also may have been remnant populations of the subglacial sediment environment that became exposed as the glacier receded.

Arthrobacter- and *Kocuria*-related isolates were found in proglacial sediment, subglacial water and subglacial ice. Isolates BFA20(13), SIBM20(5), BFA20(22), PSAA20(9) and PSAA20(6) also fell within the same clade in the *Micrococcaceae* family (Figure 3.8). These microorganisms may have originated from subglacial soil and ice and may have populated the proglacial environment after subglacial burst waters flood adjacent plains. Finally, isolates related to members of the *Comamonadaceae* family were shared between subglacial water and subglacial ice environments. Isolate SOCA20(17) and SOCA4(5) clustered with BFM20(8), near β-Proteobacterium Wuba72 [AF336361] and *Comamonas naphthalovorans* [AY166684] (Figure 3.5).

3.2.5 Comparison of culture-based ARDRA analysis with t-RFLP analysis

t-RFLP analyses were performed on portions of extracted genomic DNA from the same four SOC, SIB, BF and PSA samples used for ARDRA analysis in this study (Bhatia, 2004), allowing direct comparison of results. The 16S rRNA genes from the community genomic DNA were amplified using dye-labeled forward primers and digested with *Hae*III to obtain a collection of labeled 5' terminal restriction fragments (t-RFs). Each fragment potentially represents a different microorganism in the sample. Seventy-one t-RFs were generated after t-RFLP analyses of the four samples (Table 3.8). ARDRA analysis of isolates detected a higher number of unique microorganisms (39 OTUs) than t-RFLP analysis (27 t-RFs) in the SOC sample. However, the number of unique microorganisms detected in the SIB, BF and PSA samples using ARDRA was expectedly lower than with using t-RFLP because only a few isolates were tested from each sample using ARDRA.

Theoretical 5' terminal fragments after digestion with *Hae*III were generated by computer analysis of the 16S rRNA gene sequences of all sequenced isolates. Theoretical t-RFs from cultured isolates were compared to t-RFs generated through t-RFLP analysis. The JEG environment from which the isolates were cultured using ARDRA was also compared to the JEG environment from which t-RFs were generated using t-RFLP. Only five theoretical t-RFs from eleven cultured isolates were also generated in t-RFLP analysis (Table 3.9). These isolates were members of the Actinobacteria, β-Proteobacteria and CFB divisions. Some correlation was seen between the JEG sample from which isolates were cultured and the JEG sample in which overlapping t-RFs were found using t-RFLP. Taken together, these results show that the culture-based ARDRA technique can detect a larger number of unique microorganisms than the t-RFLP technique, when complete culture collections are tested. Only a small portion of the microorganisms detected through ARDRA was also detected in t-RFLP, showing that both techniques have different sensitivities to different microorganisms. However, ~20% of the isolates in the culture collection were not sequenced. Because the abundance of each t-RF was not calculated, it is possible that the t-RFs that were not detected in ARDRA analyses were less abundant microorganisms in the JEG sample.

3.3 Culture-independent ARDRA analysis

Culture-independent ARDRA analysis was performed on a total of 341 clones obtained from the SOC, SIB, and BF environments. Though analysis was also performed on 143 clones from the PSA environment, 92% of the clone library comprised OTUs with only one clone, indicating that hundreds of clones would need to be further sampled from PSA to obtain complete community characterization in that sample. Therefore, ARDRA analysis from the PSA sample is not included in this report.

Clones were placed into OTUs based on the pair of ARDRA patterns resulting from digestion with *Cfo*I and *Hae*III (Appendices 4, 5 and 6). A total of 153 OTUs was defined for the SOC, SIB and BF clone libraries (Figure 3.13). Thirty-two OTUs contained two or more clones and were considered the main detectable microbial species within the subglacial environment at JEG. Eight OTUs comprised isolates from two or more different environments (Table 3.10). Rarefaction curves were made for all three clone libraries to determine the thoroughness of clone library sampling (Figures 3.14, 3.15 and 3.16). All three curves were similar. The trend of each curve, gradually reaching an asymptotic line, indicates that the total number of OTUs found in each clone library is a good estimate of the actual number of OTUs in the clone library and that each clone library has been sampled thoroughly, contrasting with the PSA clone library noted above.

3.3.1 The SOC clone library

ARDRA analysis of 159 clones from the SOC environment yielded 73 distinct OTUs (Figure 3.17). Representatives of 13 OTUs that contained two or more clones were chosen for 16S rRNA gene sequencing (Table 3.11). All but two OTU representatives showed pairwise sequence similarities of 98% or greater to their nearest neighbors. Clone SOC1 10F and clone SOC 1 8E showed 96% and 93% sequence similarities to their nearest neighbor, respectively, indicating that they may represent as yet unnamed new taxa or belong to species for which no sequences are yet available (Van Trappen *et al.*, 2002). The sequence results give an estimate of the identities of 62% of the clone library. The SOC clone library was dominated by members of the β-Proteobacteria, CFB and Actinobacteria phylogenetic divisions, comprising 26%, 22% and 15% of the clone library, respectively. Several representative clones were closely

related, despite occupying different OTUs, showing that slight differences in the 16S rRNA gene sequences of close relatives were readily detected using *Hae*III and *Cfo*I restriction enzymes (Figures 3.5 and 3.8).

Clones SOC1 1C, SOC1 3G, SOC1 1B and SOC1 6F all belonged to the *Comamonadaceae* family within the β-Proteobacteria division, and were related to other psychrotolerant members of this family, such as glacier bacterium FXS1 [AY315177], isolated from sediment in Fox Glacier, New Zealand (Foght et al., 2004) and uncultured clone GKS16 [AJ224987], detected from a high mountain lake, lake Gossenköllesee, in the central Alps of Austria (Glöckner et al., 2000). These isolates were also closely related to the recently discovered microorganism, *Polaromonas naphthalenovorans*, a psychrotolerant, naphthalene-degrading bacterium isolated from a coal tar waste site (Jeon et al., 2004). This microorganism, though having similar colony morphology, carbon substrate utilization, and naphthalene degrading abilities as many *Pseudomonas* sp., was phylogenetically related to members of the *Polaromonas* genera by 16S rRNA gene sequencing (Figure 3.5). These results agree with a study by Skidmore et al. (2005) who found that 56% of clones detected from subglacial samples at JEG composed of β-Proteobacteria.

Several clones (SOC1 2B, SOC1 4G, SOC1 7F and SOC1 10F) represented members of the *Sphingobacteriales* family. Clone SOC1 2B and SOC1 4G, representing OTU 90 and 92, respectively, grouped closely with the *Flectobacillus* and *Aligoriphagus* genera (Figure 3.4). These clones had 99% homology to uncultured clone 207ds20 [AY212653] from a ground water microbial community (Simpson et al., 2004). SOC1 7F was also 98% similar to uncultured clone PRDO1a001B [AF289149] found in freshwater rivers and lakes, and grouped within the *Sphingobacterium* genus (Zwart et al., 2002). Clone SOC1 10F was only 96% similar to isolate *Sphingobacterium sp.* AC74 [AJ717393] from a nonsaline alkaline lake environment, that clustered with the *Aligoriphagus* genus.

Clone SOC1 8C, the only representative that grouped within the *Flavobacterium* genus, was 98% similar to *Flavobacterium omnivorum sp.* nov., a psychrophilic microorganism, which was isolated from China No. 1 glacier (Zhu et al., 2003). It was also 97% similar to *Flavobacterium hibernum* sp. nov., a psychrotolerant microorganism,

which was isolated from Crooked Lake in Antarctica. This microorganism is bacteriovorous, having the ability to lyze Gram negative cells and is considered an important organism for nutrient recycling. Also, it can produce exopolysaccharides to resist desiccation and when grown at 4°C produces a thick capsule (McCammon, 1998). It is thought that through creating biofilms, these organisms maximize nutrient uptake and protection in the water community.

Members of the Actinobacteria division are frequently detected by molecular techniques, which has resulted in large clusters of unnamed and uncultured Actinobacterium clones. Three clones from the SOC library fell within this uncultured Actinobacterium cluster. Clones SOC1 1D and SOC1 6H had 99% homology to uncultured Actinobacterium clone S7 [AC575508], found in Lake Schöhsee, Northern Germany (Warnecke et al., 2004) (Figure 3.8). These clones fell within a monophyletic Actinobacterial clade, ac1, that almost exclusively harbored 16S rRNA genes from freshwater and estuaries (Warneck et al., 2004). Warnecke et al. (2004) believe that this group of Actinobacteria is autochthonous component of lake or freshwater microbial assemblages. Clone SOC1 8E was 99% similar to uncultured Actinobacterium clone S4 [AJ575506], also from Lake Schöhsee (Warnecke et al., 2004). This clone fell within a monophyletic lineage that had sequence types from freshwater, marine sediments and soils. The SOC clone library also showed that this environment contained Actinobacteria found widely in other freshwater and soil environments. Actinobacteria are important members of freshwater bacterioplankton communities, making up 63% of the bacterioplankton biomass in lakes of different trophic status and geographical locations (Pernthaler et al., 2001; Eiler et al., 2004). Their small cell size helps them resist flagellate grazing and their ability to grow on organic substrates allows them to accumulate to high biomass (Pernthaler et al., 2001; Eiler et al., 2004).

The presence of clones that are closely related to freshwater microorganisms detected through culture-dependent ARDRA analyses reinforces the idea that the subglacial microbes beneath JEG microbes are suited for water environments and tend to associate with sediment particles or form associations with other microorganisms (Skidmore et al., 2000). These results are supported by Reardon et al. (2004) who showed that hematite and quartz rock promote close physical and temporally stable

cooperative associations between members of bacterial communities in freshwater environments. In a study on attached microbes in a contaminated aquifer, Reardon et al. (2004) found that the majority of biomass and activity was attributed to attached microbes in the contaminated aquifer, instead of planktonic microbes.

3.3.2 The SIB clone library

ARDRA analysis of 92 clones from the SIB environment yielded 39 distinct OTUs (Figure 3.18). Representatives of eight OTUs that comprised three or more clones were chosen for 16S rRNA gene sequencing (Table 3.12). All OTU representatives showed pairwise sequence similarities of 97% or greater to their nearest neighbors. The sequence results give an estimate of the identities of 57% of the clone library. The SIB clone library was clearly dominated by members of the β-Proteobacteria, comprising 52% of the clone library. The only other division represented in the clone library was the CFB division, comprising the remaining 5% of the OTUs represented in the clone library. Several representative clones were close phylogenetic relatives to representative clones in the SOC and BF clone libraries (Figures 3.4 and 3.5).

The majority of clone representatives matched closely with other uncultured clone sequences submitted to the GenBank database, with the exception of clone SIB2 1C and SIB2 1G, which matched closely with Antarctic and New Zealand cultured isolates (Table 3.12). All of the β-Proteobacteria clone representatives, despite showing different ARDRA patterns, clustered closely within the *Comamonadaceae* family. Both clones SIB2 1E and SIB2 4D had 99% homology to unnamed clones from river biofilms in Germany. Clone SIB2 1B matched 99% to β-Proteobacterium Wuba 72 [AF336361] and was also a close neighbor to representatives from both the SIB and SOC culture collections. Clones SIB1 2B, SIB2 1D and SIB1 10G were closely related to uncultured clones from contaminated groundwater sites. Clone SIB1 2B had 97% homology to an uncultured bacterium clone 160ds20 [AY212612] taken from a groundwater community, which was also identified in the SOC clone library (Simpson et al., 2004). Clone SIB2 1D was closest to a clone from a low-temperature, biodegraded Canadian oil reservoir. Clone SIB1 10G was closest to a clone from a uranium-contaminated aquifer (Reardon et al., 2004). The only clone falling within the CFB division was SIB2 1C, which had 99%

homology to *Flavobacterium psychrolimnae* type strain LMG 22018 [AJ585428], a cultured isolate from an Antarctic microbial mat.

Whereas the SIB clone library was mostly made up of β-Proteobacteria, they were not present in the SIB culture collection. Actinobacteria was not detected in the clone library. This may reflect certain biases in clone library construction that favor Gram negative cells. Eiler et al. (2004) found that Actinobacteria were underrepresented in the literature due to preferential annealing of universal Bacterial primers to microorganisms other than Actinobacteria. Construction of Actinobacterial-specific primers and the use of FISH and probe hybridization techniques have helped identify more microorganisms within this division (Eiler et al., 2004; Glöckner et al., 2004). FISH methods have been used to show Actinobacterial counts of 1.7 x 10^5 cells/mL in lake Gössenkollesee, Austria (Glöckner et al., 2000). Also, because Gram positive bacteria have a thicker cell wall, the physical lysis method of beadbeating may preferentially lyse Gram negative bacteria prior to PCR amplification of the SIB community genomic DNA. However, 43% of the clone library (39 clones), showing unique ARDRA patterns, have not been sequenced and may comprise microorganisms within the Actinobacteria and CFB divisions.

3.3.3 The BF clone library

ARDRA analysis of 90 clones from the BF environment yielded 50 distinct OTUs (Figure 3.19). Representatives of nine OTUs that comprised of two or more clones were chosen for 16S rRNA gene sequencing (Table 3.13). The sequence results give an estimate of the identities of 54% of the clone library. All but one OTU representative showed pairwise sequence similarities of 98% or greater to their nearest neighbors. Clone BFM 4C showed only 96% sequence similarities to its nearest neighbor, showing that it represents an as yet unnamed new taxon or belongs to species for which no sequences are yet available (Van Trappen *et al.*, 2002). Similar to the SIB clone library, only members of the CFB and β-Proteobacteria divisions, comprising 38% and 17% of the clone library, respectively, were detected in the BF clone library.

Several clones in the BF library showed homology to phylogenetic neighbors of other isolates and clones from the BF, SIB and SOC samples (Figures 3.4 and 3.5). Clone BFA 6E had 99% identity to *Flavobacterium xinjiangense* AS1.2749 [AF433173],

a psychrophilic isolate from frozen soil at China No. 1 glacier, Northwest China, which was also a close relative of isolate SOCA4(44) (Zhu et al., 2003). Clones BFA 1H and BFM 6B had 99% identity to β-Proteobacterium Wuba72 from a karstic aquifer. This microorganism was also a close relative of isolate BFM20(8). BFM 2C had 98% identity to *Flavobacterium psychrolimnae* type strain LMG 22018 [AJ585428], a psychrotroph isolated from microbial mats in both Lake Fryxell and Lake Hoare in Antarctica (VanTrappen et al., 2005). Clone BFM 4C had 99% identity to uncultured β-Proteobacteria clone LiUU-9-233 [AY509483] from a freshwater lake. Eiler et al. (2004) found that 30% of the clones from three freshwater lake environments were affiliated with β-Proteobacteria, which persist in freshwater lakes because of their ability to degrade complex organic macromolecules. Clones BFM 4E and BFA 8A both had 98% identity to Glacial ice bacterium CanDirty89 [AF179326], a psychrotolerant microorganism isolated from cryoconite sediment on Canada Glacier, Antarctica (Christner et al., 2003).

3.3.4 The PSA clone library

ARDRA analysis of 143 clones from the PSA environment yielded 132 different OTUs. Only five clone patterns were detected twice in the clone library. Almost every ARDRA pattern produced was unique. A rarefaction curve was created from the results generated within the PSA sample (Figure 3.20) The rarefaction curve in Figure 3.20 yielded a steep "linear" line. No indication of a plateau was given, showing that the sample size was insufficient. Because this environmental sample was clearly more diverse than the other three tested, additional clone libraries would need to be created to more fully characterize the communities in this environment. Therefore, these data were not included in the results presented here.

The detection of high diversity in the proglacial environment was not surprising. The PSA sample was collected near a microbial mat, which likely comprised a wider variety of bacteria. The availability of sunlight, exposure to oxygen and access to soil nutrients would add new dynamics to the environment, enabling a whole host of phototrophs to grow and support a variety of heterotrophs inhabiting this area. Cyanobacteria have been shown to increase heterotrophic bacterial counts in freshwater lake environments by providing organic carbon (Eiler et al., 2004). Increased numbers of

heterotrophs allow new community dynamics to occur, such as competition for nutrients through chemical warfare with alleopathic substances (Eiler et al., 2004). Often, *Cytophaga* species become abundant because of their ability to lyze cyanobacterial cells. Probe hybridization techniques, such as FISH, using specific primers that represent the main phylogenetic divisions to probe the PSA library, would be a more efficient method of analyzing the PSA population.

3.3.5 Comparison of SOC, SIB and BF clone libraries

The SOC library was the most diverse library of the three fully analyzed clone libraries (SOC, SIB and BF clone libraries), containing microorganisms from the CFB, β-Proteobacteria and Actinobacteria divisions (Figure 3.21). High diversity in the SOC sample may be attributed to favorable physical and chemical conditions in that environment. The SOC subglacial waters were enriched with high concentrations of dissolved solutes and sulfate but had depleted nitrate concentrations (Bhatia, 2004). This environment may have been more favorable for a wider distribution of microorganisms than the SIB subglacial water, which was rich in both sulfate and nitrate concentrations or the BF subglacial ice, which had lower sulfate and low nitrate concentrations (Bhatia, 2004). t-RFLP analysis of subglacial, proglacial and supraglacial JEG samples showed that bacterial communities are distributed heterogeneously at JEG resulting from varied distributions of energy sources, nutrients and environmental stresses (Bhatia, 2004).

Both BF and SIB clone libraries comprised only CFB and β-Proteobacteria divisions. These data, interpreted in the absence of culture studies, would have suggested that the Actinobacteria found in the SOC library might not be native to the subglacial environment, but introduced from either supraglacial or proglacial environments. However, members of the Actinobacteria, Firmicutes and α–Proteobacteria were cultured from the SIB and BF environments, showing that they can be found there. The ability to clone a wide variety of isolates undetected by molecular techniques supports the notion that both culture and molecular techniques are necessary for characterizing microbial communities. The SIB clone library was dominated by β-Proteobacteria, whereas members of the CFB division mainly dominated the BF clone library. The SOC library did not seem to be dominated by one division, but was mainly composed of β-Proteobacteria, Actinobacteria and CFB divisions. Further studies and comparison of the

supraglacial community to the SOC and SIB clone libraries would assist in inferring the origin of the most abundant organisms in each clone library.

Members of the β-Proteobacteria and CFB divisions were detected in all three libraries, showing the importance of these microorganisms in subglacial environments (Table 3.10). OTU 1 was found in all three clone libraries and clustered with a group of psychrophilic *Flavobacterium* spp., including *Flavobacterium omnivorum* (Figure 3.4). The genus *Flavobacterium* consists of aerobic bacteria, which predominantly contain meaquinone-6 as the primary respiratory quinone (Bernardet et al., 2002). The organisms' requirement for O_2 as well as the large number of aerobes cultured using culture-based ARDRA analysis suggests that sufficient oxygen is available to sustain aerobic communities beneath JEG. Oxygen may be introduced into the subglacial environment through release of dissolved oxygen from melting glacier ice, through input of oxygen saturated supraglacial waters or through chemical weathering reactions (Bhatia, 2004).

Several microorganisms were detected in two of the three libraries. The representative of OTU 60, a *Sphingobacterium*, was found in both the BF and SOC clone libraries (Figure 3.4). O'Sullivan et al. (2002) suggested that bacteria within the CFB division exhibit biofilm-forming behavior and that their lack of flagella may indicate that they adhere or aggregate to enable survival. Isolates within OTUs 5 and 11 were detected in both SIB and SOC clone libraries (Table 3.10). These β-Proteobacteria were also closely related to members of OTU 47, which were detected in both SIB and BF clone libraries and also OTUs 52 and 56, which were detected in both SOC and BF clone libraries. The high abundance of β-Proteobacteria within the three clone libraries suggests that they are strong competitors that are suited to the subglacial JEG environment. Ice core samples from the accreted ice above lake Vostok (3.6 km depth, > 400 000 years old) that are presumed to contain organisms from the subglacial lake were also dominated by the genus *Comamonas* (Priscu et al., 1999). However, other genera that dominated in the accreted ice environment, such as *Actinomyces, Acidovorax* and *Afipia*, were not detected beneath JEG. Because these *Comamonas* species have been found in other temperate environments also, it suggests that JEG has been has been open

to microbial colonization through long range transport for a long period of time (Vincent et al., 2000).

Despite having different ARDRA patterns, clones from different OTUs clustered closely together. For example, clones BFM 2C and BFA 6E, from OTU 6 and 3, respectively, appear to be nearly identical organisms by their tight clustering within the *Flavobacterium* clade (Figure 3.4). Also, good correlation was found between representatives of the same OTU. Clones SIB2 1G and SOC1 1C, both representing OTU 56 from different clone libraries, clustered quite closely together (Figure 3.5). This reaffirms that assigning organisms to an OTU based on restriction digestions with *HaeIII* and *CfoI* is a good method of distinguishing and grouping organisms within the JEG subglacial environment.

3.3.6 Comparison of culture-independent ARDRA analysis with t-RFLP analysis

Culture-independent ARDRA analysis detected over twice as many unique microorganisms in the SOC and BF clone libraries than t-RFLP analysis (Table 3.8). ARDRA analysis of the SIB clone library, however, detected nearly the same number of organisms (39 OTUs) as t-RFLP analysis (35 t-RFs). Little overlap, however, was seen between culture-independent analysis and t-RFLP analysis. Only six theoretical t-RFs generated from computer-simulating digestion of sequenced clone representatives with *Hae*III matched with t-RFs generated during t-RFLP analysis (Table 3.9). Sequencing of all OTU representatives within the clone libraries was not done however, and it is possible that theoretical t-RFs generated from unsequenced representatives may resolve some discrepancies between the two techniques. Interestingly, almost all theoretical t-RFs generated from 16S rRNA gene sequences in the ARDRA culture collections were different from in the ARDRA clone libraries.

3.4 Comparison of clone libraries to culture collections

Community composition determined by ARDRA analysis of the SOC culture collection differed from that determined with the SOC clone library (Figure 3.22). The culture collection was more diverse, detecting members of the CFB, β-Proteobacteria, α-Proteobacteria, γ-Proteobacteria and Actinobacteria divisions, whereas the clone library detected representatives from only the CFB, β-Proteobacteria and Actinobacteria

divisions. The relative abundance of each of the divisions was quite different as well. In the culture collection, over 40% of all isolates were members of the CFB division, namely *Flavobacterium*-related species. β-Proteobacteria and α-Proteobacteria each made up over 10% of the cultured isolates. Finally, Actinobacteria and γ-Proteobacteria were the smallest group of microorganisms, each making up less than 5% of the culture collection. In the clone library, however, the CFB and β-Proteobacteria each represented 20% of the clone library and Actinobacteria made up the remaining 11% of the identified clone library. No and α- or γ-Proteobacteria were detected. Further, the Actinobacteria detected in the clone library clustered with uncultured Actinobacterium clones, whereas Actinobacteria isolates from the culture collection grouped with named isolates from the *Cryobacterium* and *Frigoribacterium* genera (Figure 3.8).

The differences in culture-dependent ARDRA analysis and culture-independent ARDRA analysis show that biases are inherent to both techniques. The initial culturing of isolates selects for microorganisms within the subglacial and proglacial environments that are heterotrophic and are capable of growth aerobically and microaerobically on R2A agar at 4°C or 20°C. Further, all microorganisms would have been exposed to oxygen either during the thawing process, in the case of microorganisms from basal ice samples, or during the filtering process, in the case of microorganisms from subglacial water samples. This method of culturing biases the results in favor of aerobic or aerotolerant microorganisms most adapted to growth in R2A medium. The molecular ARDRA technique, however, selects for those organisms whose cell wall can be more easily lyzed and whose genomic DNA can most easily anneal to the universal Bacteria primers we used in the study. The results of these biases were that the culture-dependent technique was able to detect more diverse Gram positive organisms belonging to the Actinobacteria and γ-Proteobacteria divisions. The clone library, alternatively, did not detect any members of the γ-Proteobacteria and the Actinobacteria detected fell into a separate clade of uncultured Actinobacterium clones (Figure 3.8). Over 40% of the clone library comprised OTUs with only one clone and because representatives of these OTUs were not sequenced, a large part of the community composition remains to be determined. Sequencing of OTU representatives in the entire clone library may alter the clone library profile to appear more like the culture collection.

Despite certain biases in the abundance of taxonomic groups, there was fairly reasonable overlap between the SOC clone library and culture collections. In the CFB division, clone SOC1 8C clustered very closely with isolate SOCA4(40) in the *Flavobacterium* clade and clone SOC1 7F clustered closely with isolate SOCA20(36) in the *Sphingobacterium* clade (Figure 3.4). In the β-Proteobacteria division, SOC isolates SOCA20(82) and SOCA20(17) also fell within the same *Comamonadaceae* clade as clones SOC1 1B and SOC1 1C (Figure 3.5).

The SIB culture collection differed quite noticeably from the SIB clone library. The culture collection consisted of members of the Actinobacteria, CFB, Firmicutes and α-Proteobacterium divisions. However, the SIB clone library was heavily dominated by β-Proteobacteria. Only one cultured SIB representative microorganism was also detected in the SIB clone library. Clone SIB2 1C appeared to be nearly identical to isolate SIBA4(8) (Figure 3.4). The BF culture collection also detected microorganisms not found within the BF clone library. The culture collection was made up of Actinobacteria, α-Proteobacteria and β-Proteobacteria, whereas the BF clone library was dominated by CFB and β-Proteobacteria. Only one cultured BF representative, BFM20(8), grouped closely with a clone BF representative, BFM 6B within the *Comamonadaceae* clade (Figure 3.5). Complete overlap was not expected, however, between culture and clone libraries for the SIB and BF environments because so few isolates were cultured. If a larger number of both isolates was analyzed, greater overlap between the two results would have been expected. Also, about 40% of the clone libraries were made up of individual OTUs and had not been sequenced. Sequencing of the other clones may reveal the less abundant microorganisms found in the culture technique.

Literature describing community characterization has differed in opinion about on the correlation between culture-dependent and culture-independent techniques. Testing of an adequate sample size is crucial to obtaining parallel results. Christner et al. (2003) cultured nine isolates and sequenced 18 clones from a cryoconite hole in Canada. Though significant numbers of psychrophilic microorganisms were detected belonging to the CFB, Actinobacteria and β-Proteobacteria divisions, they found no overlap between clone and culture results. Huber et al. (2004), however, found that there was a good correlation between nonculturable and culturable biota within the rainbow trout intestine.

In this study, 146 cultured isolates were studied and results complemented the community structure detected by FISH. Our results have shown that fairly reasonable correlation exists between culture-independent and culture-dependent ARDRA analysis. These results contradict the generalization that <1% of the species in an environment can be cultured (Amann et al., 1995).

3.5 The possible role of abundant microorganisms beneath JEG

Because the subglacial environment beneath JEG is dark, chemoheterotrophs and autotrophs, such as chemolithotrophs would likely be present in this environment. The presence of autotrophs may also help sustain the activity of heterotrophs beneath JEG. No chemolithotrophs were found, however, through culture-independent ARDRA analysis. It is also possible that heterotrophs rely solely on influxes of organic carbon from the supraglacial environment or obtain it from the bedrock beneath the glacier.

Skidmore et al. (1999) detected nitrate-reducing, nitrogen-fixing and sulfate-reducing activity in the subglacial sediment beneath JEG. Chemical analysis revealed that sufficient amounts of sulfate (1000 to10 000 μeq/L) and nitrate concentrations (up to 3.5 μeq/L) were present in subglacial water samples to support these subglacial communities. The BC and BF basal ice had high dissolved organic carbon concentrations (~90 ppm), which could support active heterotrophic microbial communities beneath JEG (Bhatia, 2004). Unfortunately, clone analysis did not show that sulfate-reducers such as *Desulfovibrio* spp., which belong to the δ-Proteobacteria division, exist beneath JEG. Culturing samples from each environment under anaerobic conditions and using the MPN tube assay method may result in the ability to culture these anaerobes. It is also possible that the number of sulfate-reducing bacteria was too low for detection using the ARDRA method. It was determined that the minimum PCR detection limit was 5700 *E. coli* CFU per sample. Thus, if a low number of sulfate-reducing cells were present in these JEG samples, they would not be observed. The presence of nitrate-reducers was detected, however. Isolate PSAA4(4) and SIBA20(24) were related to *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively, that both can reduce nitrate (Dworkin, 2005).

It is impossible to correctly deduce the role and contributions of each bacterial community member from phylogenetic positioning without performing carbon utilization tests, such as BIOLOG® assays or mineralization assays. However, the role of several

close phylogenetic neighbors in other environments that display unique nutrient degrading capabilities may give some clues as to the role of each isolate.

Many bacteria within the *Comamonadaceae* family of the β-Proteobacteria division have been found in other polar environments, suggesting that they are important members of glacial microbial communities. Similar to results found by Foght et al. (2004), clones SIB2 1D, SIB1 2B and SOC1 3G clustered closely to *Rhodoferax* species within the β-Proteobacteria division (Figure 3.5). *Rhodoferax antarcticus* and *R.x fermentans* are facultative anoxygenic phototrophs that respire aerobically in the dark. Further, *R. fermentans* is a facultative phototroph that can also reduce nitrate and *Rhodoferax ferrireducens* is a dissimilatory ferric iron reducer (Foght et al., 2004). Because these clones group very closely to the Fe(III)-reducing β-Proteobacterium *R. ferrireducens*, they may also possess Fe(III)-reducing abilities.

Actinobacteria have also been shown to occupy different niches. Glöckner et al. (2000) observed seasonal fluctuations of Actinobacteria and β-Proteobacteria biomass by monitoring populations over a 1 year period in a high mountain lake in Austria. Their results showed that β-Proteobacteria biomass increased during August after the lake ice covering the lake melted and broke open, providing an allochthonous input of nutrients from melted ice above the ice cover. Actinobacteria biomass increased during April, after a phytoplankton bloom. The authors suggested that these fluctuations show the distinct temporal and functional niches of β-Proteobacteria and Actinobacteria. β-Proteobacteria use the main annual inputs of nutrients, whereas Actinobacteria rely on their nutritional efficiency to consume lower concentrations of organic carbon at low temperatures.

3.6 The possible origin of microbes beneath JEG

Many of the microorganisms found beneath JEG were closely related to other microorganisms from mesophilic and polar environments (Figures 3.4 through 3.9). Two schools of thought exist on how these polar microorganisms can be found widespread among different environments (Vincent, 2000). Baas-Becking (1934, cited by Staley and Gosink 1999) suggested that "everything is everywhere, but the environment selects", meaning that microbes are already native to all environments on Earth but certain environmental factors are favorable for some microorganisms over others. However, a

more widely accepted thought is that microbes can be dispersed from one environment to another, either by long range means or locally, and once seeded in a certain glacier environment, environmental pressures select for specific adaptive strategies over millions of years, allowing related bacteria to inhabit different environments (Vincent, 2000). Several long range means of dispersal have been previously discussed, such as through atmospheric circulation in the air or through fish and human vectors. Interestingly, clones BFM 2C and BFA 6E and isolate SOCA4(51) clustered closely with rainbow trout intestinal bacterium B76 (Figure 3.4), suggesting that these microorganisms may have been introduced to the Arctic environment through fish and carried to JEG by atmospheric dispersal.

Once microorganisms have been introduced into glacier environments, they may evolve or adapt to the glacier environment by using different strategies (Vincent, 2000). As previously discussed, psychrophiles occupy narrow niches and can evolve strategies such as high affinity nutrient transport, fast growth rate or altered proteins and lipids to have a competitive advantage in the environment. Psychrotolerant microbes, however, occupy broad niches and grow suboptimally in glacier environments, tolerating extreme subzero temperatures (Vincent, 2000). In the JEG subglacial environment, the majority of cultured microbes have been psychrotolerant isolates related to those that are mostly found in mesophilic freshwater environments, such as the Actinobacteria, α-Proteobacteria and β-Proteobacteria that are efficient at obtaining nutrients from oligotrophic environments. These microorganisms are likely widely found in supraglacial streams and lakes at JEG. Bacteria such as *Rhodoferax* are facultative phototrophs and were likely brought to the subglacial environment from surface water but actively respire in the dark in situ. Vincent (2000) suggested that common occurrences of psychrotolerant microbes are seen partly because insufficient time has been given for full evolutionary adaptation to occur, but also that selective pressures in glacier environments are relatively weak. Biological interactions such as competition are less important than the ability to tolerate severe conditions. The microorganisms found within the *Flavobacterium* clade of the CFB division, however, are most likely true psychrophiles (Figure 3.4). All isolates that fell within this cluster were cultured only at 4°C and most of the isolates within this group were isolated from polar environments.

This study has shown that a rich community of microbes exists beneath JEG. Although ecological theory holds that the selective pressures of limited nutrients, limited light, limited oxygen and near freezing temperatures should decrease microbial abundance and diversity (Reardon et al., 2004), I have shown that the subglacial environment at JEG holds surprising diversity. Previous studies by Skidmore et al. (2000) have shown that these populations are metabolically active at near in situ temperatures. These microbial communities are important because they influence geochemistry by producing CO_2, organic acids, methane, and reduced or oxidized forms of sulfur, nitrogen and iron (Foght et al., 2004). Further, many related microorganisms in this study have been isolated from contaminated freshwater environments or activated sewage sludge and are capable of degrading compounds such as, *cis*-dichloroethene, monochlorobenzene, methyl chloride and methyl bromide (Alfreider et al., 2002; Miller et al., 2004). Because these psychrotolerant microorganisms are able to degrade toxic waste compounds, they may be possible candidates for low-cost bioremediation of spills in polar environments (Abraham et al., 1999).

3.7 Challenges and limitations to the ARDRA method

3.7.1 Measurement of base pairs

Many challenges were encountered in performing ARDRA analysis on the JEG culture collections and clone libraries, revealing several weaknesses in this method. All ARDRA patterns were grouped visually, using the measured base pairs of each fragment as a guide. Initial experiments using the computer program Gelcompar II (Applied Maths Inc., Austin, TX, USA) to align ARDRA patterns could not allow for inter-gel differences in ARDRA patterns. Slight inconsistencies in ARDRA patterns run on different gels were seen due to using different power sources, slight differences in gel consistency and also in some cases distortions known as gel "smiling". Although DNA molecular weight ladders and positive controls were included as references in each gel, the computer program made distinctions between ARDRA patterns that were obviously similar. Examining ARDRA patterns by eye was ultimately the easier method of analysis but tedious and prone to human error.

Because of inter-gel differences, the calculated base pair sizes of similar ARDRA patterns differed sometimes by as much as 20 to 30 bases. ARDRA patterns that were

visually the same but gave slightly different base pair measurements due to inter-gel differences could be easily confused with different ARDRA patterns on the same gel that had only slightly different base pair sizes. ARDRA analysis was done very carefully, however. In the end, ARDRA patterns that fell within the same OTU had an average standard deviation of about 20 bases for each fragment.

Fragments below 100 base pairs in size were often too faint to see on the 2% agarose gels. For this reason, the sum of ARDRA fragments did not always add up to 1500 bp, the size of the amplified 16S rRNA gene PCR fragment (Table 3.14). Also, doublet and triplet bands were difficult to differentiate when measuring fragment size. When calculated base pair sizes were compared to theoretical base pair sizes determined from sequenced representatives, these discrepancies were apparent (Table 3.14). Although calculated base pairs matched quite closely with theoretical ARDRA patterns, fragments with sizes below 100 base pairs as well as doublets were often not detected.

3.7.2 Limitations of clone library construction

To create the clone libraries, community genomic DNA was extracted from environmental samples using a bead beating method. Next, the 16S rRNA gene was amplified from the extracted genomic DNA. Finally, the amplified genomic DNA was cloned into a pGEM vector and transformed into *E. coli* cells. Before creating the clone libraries, preliminary genomic DNA extraction controls were performed to investigate the parameters of the bead beating method. First, the optimal length of time for bead beating was determined. Pure isolates were bead beaten for 40 s, 90 s, 120 s, and 180 s to determine at what time the greatest amount of genomic DNA was extracted without excessive shearing, which would reduce amplification efficiency. It was found that equal amounts of DNA could be amplified from genomic DNA extracted at each time period (Appendix 7). So, a bead beating period of 40 s was chosen to reduce shearing of genomic DNA.

Because Gram positive cells have thicker cell walls than Gram negative cells, it was thought that the bead beating protocol would preferentially lyze Gram negative cells. To determine if the bead beating method was sufficient to lyse Gram positive cell walls, genomic DNA from a pure of *Lactobacillus plantarum* was extracted using the bead beating method. Four different bead beating times (40 s, 90 s, 120 s and 180 s) were used

for each method. Similar results were seen for all bead beating times. Genomic DNA from the *Lactobacillus* sp. was also extracted in the presence of sediment, showing that the basal sediment did not prevent DNA extraction and amplification. Samples of 0.2 to 0.4 g of mud were added to pure cultures to simulate extraction from environmental samples as they would contain mud and fine particles. Results showed that *Lactobacillus* sp. genomic DNA was successfully extracted from the mud. Despite this optimization procedure, Gram negative bacteria from β-Proteobacteria and CFB divisions still heavily dominated the BF and SIB clone libraries. Both clone libraries showed a bias toward Gram negative organisms, even though Gram positive organisms were present and detected in the SIB and BF environments through culture techniques.

Another potential weakness in the bead beating method was the ability to lyze all of the cells present in the sample and in doing so, amplify representative amounts of 16S rRNA genes from the microbial community. Three consecutive extractions were performed on the same sample to determine whether a single genomic DNA extraction was sufficient to recover the majority of genomic DNA. Three Antarctic pure isolates that had been used successfully in previous DNA extraction experiments were used as positive extraction controls for this experiment. Water was used as the negative control. The results of this experiment showed that genomic DNA was still present, as determined by PCR, after the second and third bead beating event. The amount of PCR product generated after the second extraction was equivalent to the amount of PCR product generated from the first extraction. Considerably less PCR product was produced from genomic DNA in the third extraction, when compared to the amount of PCR product after the first extraction. Negative controls in each of the extraction and in the PCR reactions showed no contamination. These observations showed that a single extraction was not sufficient to recover all the genomic DNA from a pure isolate and that at least three extraction are needed to recover most of the genomic DNA. Only one bead beating step was used, however, to create the clone libraries. This done was because genomic DNA was relatively difficult to amplify from the filtered samples and once a PCR product was detected from the genomic DNA, a clone library was constructed. In retrospect, at least three extractions should have been performed on each environmental sample. Replicate PCR reactions should have been done on the extracted genomic DNA and then pooled to

obtain a complete collection of the amplified 16S rRNA genes. Also, through minimum PCR detection limit experiments, it was determined that a high threshold of at least 5700 CFU per sample, was needed for detection of DNA by PCR. This shows that the PCR protocol was not very efficient at amplifying 16S rRNA genes from small numbers of microbes. These limitations in the bead beating procedure may have also been the cause of the discrepancies seen between the profiles of the culture collection and clone libraries in the BF, SIB and SOC environments.

Table 3.1. Total number of culturable microorganisms for subglacial water (SOC and SIB), subglacial ice (BF and BC) and proglacial sediment samples (PSA), determined on R2A plates incubated 27 days.

Sample source and incubation condition	Mean CFU/g dry weight or CFU/per mL (±1 SD) [1]	Number of colonies with distinct morphology (Number of colonies analyzed using ARDRA)	Proportion of pigmented colonies (%)
SOC A20[2]	2.0 (±0.46) x 10^2	84 (72)	95%
SOC A4	2.1 (±0.38) x 10^3	51 (39)	95%
SOC M20	1.6 (±0.20) x 10^1	35 (24)	95%
SIB A20	4.2 (±1.05) x 10^7	28 (8)	90%
SIB A4	1.2 (±0.30) x 10^3	15 (6)	90%
SIB M20	3.3 (±0.83) x 10^3	24 (6)	90%
BF A20	1.1 (±0.28) x 10^7	23 (3)	11%
BF M20	9.7 (±2.4) x 10^6	11 (1)	11%
BC A20	1.8 (±0.46) x 10^7	13 (5)	11%
PSA A20	3.5 (±0.88) x 10^6	15 (14)	9%
PSA A4	1.7 (±0.43) x 10^6	9 (7)	9%
PSA M20	1.6 (±0.40) x 10^6	14 (8)	9%

[1] SD , standard deviation.
[2]Three culture conditions were used: aerobic at 20°C (A20), aerobic at 4°C (A4) and microaerobic at 20°C (M20). Total viable microbial counts were performed by E. Marques and J. Foght.

Table 3.2. The number of isolates, related genera, and source of isolates found within five OTUs. All five dominant OTUs contained isolates from two or more JEG environments.

OTU #	Related genera	Total number of isolates in OTU	Environment from which isolates were obtained and number of isolates cultured				
			SOC	SIB	BF	BC	PSA
1	*Flavobacterium*	37	36				1
2	*Arthrobacter* and *Kocuria*	27		4	1	2	20
4	*Comamonas*	17	16		1		
6	*Flavobacterium*	6		5			1
10	*Arthrobacter*	3		1			2

Table 3.3. Representative cultured isolates from the 11 most abundant OTUs from the SOC sample. 16S rRNA gene sequence results, colony morphology, Gram stain, and biochemical test results for each representative are shown. The number of isolates analyzed from each incubation condition and within each OTU is also shown.

	Incubation condition[1]							Biochemical tests[3]						
OTU #	A20	A4	M20	% Abundance	Representative isolate name	Colony Morphology	Gram Stain[4]	Catalase	Oxidase	Motility	Gelatinase	Nearest neighbor (Source) (Accession #)	% identity[4]	Division[5]
1	28	4	4	27	SOCA20(40)	Bright yellow, transparent in center, granules surrounding transparent center and dispersed throughout colony, irregular, raised, entire	Gram – small rods	-	-	-	NG	Chryseobacterium sp. TB4-8-II (ascocarp of ectomycorrhizal fungus) (AY599655)	99%	CFB
3	0	21	0	16	SOCA4(12)	Dark orange in center surrounded by a lighter translucent orange border, circular, concentric, pulvinate, entire	Gram – cocci in Chains	+	-	NG	NG	Flavobacterium sp. EP28 (River Taff epilithon) (AF493663)	97%	CFB
4	13	2	1	12	SOCA20(17)	Beige with thin transparent border, circular, concentric, raised, entire	Gram – cocci in chains	+	+	-	NG	β-Proteobacterium Wuba72 (karstic aquifer) (AF336361)	99%	β-Proteobacteria
5	6	0	7	10	SOCA20(60)	Dark orange in center fading to medium orange with thin transparent border, irregular, pulvinate, entire	Gram – small rods	-	-	-	-	Antarctic Bacterium R-8358 (Antarctic lakes microbial mat) (AJ440993)	99%	α-Proteobacteria
7	5	0	0	4	SOCA20(36)	Dark orange with red tinge, transparent thin border, pulvinate, smooth, entire	Gram – thin rods	+	-	-	-	Glacier bacterium FJS5 (New Zealand subglacial sediment) (AY315161)	99%	CFB
8	0	4	0	3	SOCA4(5)	White, pinpoint colonies, circular, convex, smooth, entire	Gram – cocci in pairs	+	-	+	+	Bacterium clone RA13C6 (monochlorobenzene contaminated groundwater) (AF407405)	99%	β-Proteobacteria
9	2	1	0	2	SOCA20(82)	Transparent beige, transparent colorless thin border, circular, smooth, raised, entire	Gram – cocci	+	-	NG	NG	β-Proteobacterium JS666 (contaminated groundwater) (AF408397)	98%	β-Proteobacteria
13	2	0	0	2	SOCA20(46)	Bright yellow, thin transparent border, circular, convex, entire	Gram – rods	-	-	NG	NG	γ-Proteobacterium Gitt-GS-126 (heavy metal contaminated environment) (AJ582198)	98%	γ-Proteobacteria

CONTINUED

15	2	0	0	2	SOCA20(63)	Yellow pinpoint colonies, circular, convex, smooth, entire	Gram + cocci	+	-	NG	NG	Actinobacteria strain PB90-5 (anoxic bulk soil) (AJ229241)	98%	Actinobacteria
16	0	0	2	2	SOCM20(20)	Translucent bright yellow, slightly darker in center, transparent border, irregular, contoured, raised, entire	Gram + cocci	+	+	+	NG	Actinobacterium clone 45B137 (ancient Siberian permafrost) (AY539810)	99%	Actinobacteria
17	0	0	2	2	SOCM20(30)	Brown-orange with colorless transparent border, circular, raised, smooth, entire	Gram – small rods	+	-	NG	-	Brevundimonas strain FWC04 (fresh water slough) (AJ227793)	98%	α-Proteobacteria

[1], number of isolates in OTU.
[2], Cell morphology is likely coccobacilli rather than cocci.
[3], + indicates positive test for characteristic; - indicates negative test; NG indicates no growth on medium.
[4], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).
[5], CFB, Cytophaga-Flavobacterium-Bacteriodes.

Table 3.4. Biochemical test and 16S rRNA gene sequence analysis results for four morphologically distinct SOC isolates from OTU 3 incubated aerobically at 4°C.

Isolate name	Colony Morphology	Gram Stain	Catalase	Oxidase	Nearest neighbor (Accession #)(Source)	% identity[2]	Division
SOCA4(12)	Dark orange in center, light translucent orange ring surrounded by a thin transparent colorless border, circular, concentric, pulvinate, entire	Gram – cocci in chains	+[1]	-	*Flavobacterium* sp. EP28 (AF493663) (River Taff Epilithon)	97%	CFB
SOCA4(40)	Bright medium yellow with thin transparent border, small, circular, convex, entire	Gram – cocci in chains	+	-	*Flavobacterium psychrolimnae* LMG 221018 (AJ585428) (Antarctic microbial mats)	98%	CFB
SOCA4(44)	Dark translucent orange fading to medium orange at edges, light orange textured border, circular, raised, entire, smooth, radiate	Gram – cocci in chains	+	-	*Flavobacterium xinjiangense* As1.2749 (AF433173) (Glacier ice)	98%	CFB
SOCA4(51)	Red-orange translucent, flat, thick transparent border, circular, smooth	Gram – cocci in chains	+	-	*Flavobacterium omnivorum* As1.2747 (AF433174) (Glacier ice)	98%	CFB

[1], + indicates positive test for characteristic; - indicates negative test.
[2], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).

Table 3.5. Representative cultured isolates from the four most abundant OTUs from the SIB. 16S rRNA gene sequence results, colony morphology, Gram stain, and biochemical test results of each representative is shown. The number of isolates analyzed from each incubation condition and within each OTU is also shown.

	Incubation condition[1]						Biochemical tests						
OTU #	A20	A4	M20	Representative isolate name	Colony Morphology	Gram Stain[2]	Catalase	Oxidase	Motility	Gelatinase	Nearest neighbor (Accession #) (Source)	% identity[4]	Division[5]
2	0	0	4	SIB2M0 (5)	White, granular, flat, translucent, serrate	Gram + cocci in clusters	+[3]	+	-	+	*Arthrobacter* sp. An34 (AJ551172) (Antarctic lake sediment)	99%	Actinobacteria
6	0	5	0	SIBA4(8)	Yellow with translucent border, circular, entire, raised	Gram – cocci	+	-	NG	+	Antarctic bacterium R-7550 (AJ440979) (Antarctic microbial mat)	98%	CFB
12	2	0	0	SIBA20(24)	White with translucent edges, circular, concentric, raised	Gram – rods	+	+	NG	NG	*Staphylococcus cohnii* (AJ717378) (nonsaline alkaline lake)	99%	Firmicutes
18	0	1	1	SIBM20(6)	Light orange, entire, convex	Gram – thin rods	+	-	+	+	*Caulobacter* sp. strain FWC20 (AJ227766) (fresh water slough)	99%	α-Proteobacteria

[1], number of isolates in OTU.
[2], Cell morphology is likely coccobacilli rather than cocci.
[3], + indicates positive test for characteristic; - indicates negative test; NG indicates no growth on medium.
[4], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).
[5], CFB, Cytophaga-Flavobacterium-Bacteriodes.

Table 3.6. Representative cultured isolates from the four most abundant OTUs from the BF sample. 16S rRNA gene sequence results, colony morphology, Gram stain, and biochemical test results for each representative are shown. The number of isolates analyzed from each incubation condition and within each OTU is also shown.

	Incubation condition[1]						Biochemical tests[2]						
OTU #	A20	A4	M20	Representative isolate name	Colony Morphology	Gram Stain	Catalase	Oxidase	Motility	Gelatinase	Nearest neighbor (Accession #) (Source)	% identity[2]	Division[4]
2	1	0	0	BFA20(22)	Light orange, irregular, serrate, flat	Gram+ cocci in pairs	+	-	+	+	Gram-positive bacterium Wuba45 (AF336354) (karstic aquifer)	100%	Actinobacteria
4	0	0	1	BFM20(8)	White, circular, entire, flat	Gram – cocci	+	+	+	NG	β-Proteobacterium Wuba72 (AF336361) (karstic aquifer)	99%	β-Proteobacteria
29	1	0	0	BFA20(17)	White with darker center concentric, irregular, serrate	Gram – cocci in chains	+	+	NG	NG	Uncultured bacterium clone MeCl 40 (AY439191) (contaminated soil)	99%	α-Proteobacteria
58	1	0	0	BFA20(13)	Yellow, concentric, translucent border with granules inside	Gram – cocci in chains	+	-	+	NG	*Arthrobacter ramosus* (X80742) (forest soil)	99%	Actinobacteria

[1], number of isolates in OTU.
[2], + indicates positive test for characteristic; - indicates negative test; NG indicates no growth on medium.
[3], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).
[4], CFB, Cytophaga-Flavobacterium-Bacteriodes.

Table 3.7. Representative cultured isolates from the three most abundant OTUs from the PSA. 16S rRNA sequence results, colony morphology, Gram stain, and biochemical test results of each representative is shown. The number of isolates analyzed from each incubation condition and within each OTU is also shown.

	Incubation condition [1]						Biochemical tests [2]					
OTU #	A20	A4	M20	Representative isolate name	Colony Morphology	Gram Stain	Catalase	Oxidase	Motility	Nearest neighbor (Accession #)	% identity [3]	Division
2	9	4	7	PSAM20(9)	Irregular, lobate, with, small, convex	Gram+ cocci in pairs	+	+	+	*Arthrobacter* sp. An34 (AJ551172) (Antarctic lake sediment)	99%	Actinobacteria
10	2	0	0	PSAA20(6)	Irregular, granular, concentric, yellowish center with translucent border	Gram+ cocci in pairs	+	+	+	*Arthrobacter* sp. An34 (AJ551172) (Antarctic lake sediment)	99%	Actinobacteria
11	0	2	0	PSA A4(4)	Irregular, concentric, translucent border with white center and dark inner center	Gram + cocci	+	+	NG	Antarctic bacterium R-9113 (AJ441004) (Antarctic microbial mat)	99%	γ-Proteobacterium

[1], number of isolates in OTU.
[2], + indicates positive test for characteristic; NG indicates no growth on medium.
[3], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).

Table 3.8. Comparison of number of unique organisms detected in four JEG samples using culture-dependent ARDRA analysis, culture-independent ARDRA analysis and t-RFLP analysis.

Analysis technique	Number of OTUs or t-RFs detected in sample (number of replicate subsamples analyzed)			
	SOC	SIB	BF	PSA
Culture-dependent ARDRA analysis	39	11	4	8
Culture-independent ARDRA analysis	73	39	50	-[2]
t-RFLP analysis[1]	27 (1)	35 (21)	22 (7)	35 (5)

[1] t-RFLP analysis was performed by M. Bhatia.
[2] -, not analyzed with culture-independent ARDRA analysis.

Table 3.9. Comparison of 5' terminal fragments detected in JEG samples through t-RFLP analysis, culture-based ARDRA analysis and non-culture-based ARDRA analysis.

	t-RFLP analysis					ARDRA analysis				
5' terminal fragment length (base pairs) [1]	Presence in samples tested with ARDRA [2]	Presence in samples tested with t-RFLP [3]	% occurrence in subglacial samples [4]	% occurrence in supraglacial samples [5]	% occurrence in proglacial samples [6]	JEG sample [7]		Clone name [8]	Isolate name [9]	Division [10]
						Detection through non-culture-based ARDRA analysis	Detection through culture-based ARDRA analysis			
218-219	SOC, SIB, BF, PSA	All environments	48	4	82	SIB, BF		BFA 8A BFM 4E SIB1 2B		β-Proteobacteria
220-221	SIB, BF	All environments	44	4	41		SOC		SOCM20(20)	Actinobacteria
227-228	SOC, BF, PSA	All environments	25	13	6		SOC, PSA		SOCA20(63) PSAM20(9) PSAA20(6)	Actinobacteria
199-200	SOC, BF, PSA	All environments	16	17	6		BF		BFM20(8)	β-Proteobacteria
325	BF	Subglacial and Supraglacial	17	42	0	BF	SOC	BFA 1H BFM 6B	SOCA20(82)	β-Proteobacteria
225-226	SOC, SIB	Subglacial and Supraglacial	8	4	0	SOC		SOC1 8B SOC1 1D SOC1 6H		Actinobacteria
230-231	BF, PSA	Subglacial and Proglacial	12	0	6		SIB		SIBM20(5)	Actinobacteria

CONTINUED

612-614	SOC, SIB, BF	Subglacial only	58	0	0	SOC, BF	SOC	BFA 6E BFM 2E SOC1 8C	SOCA4(51) SOCA4(40) SOCA4(12) SOCA4(44)	CFB
203	SIB, BF	Subglacial only	38	0	0	SOC, SIB, BF		BFM 9H SOC1 6F SOC1 3G SIB2 1E SIB2 4D SIB2 10G		β-Proteobacteria
204-205	SOC	Subglacial only	10	0	0		SOC		SOCA20(17)	β-Proteobacteria
217	PSA	Proglacial only	0	0	6	BF		BFM 4C		β-Proteobacteria

[1], Length of 5' terminal fragment found in both ARDRA analysis, through computer analysis of amplified 16S rRNA gene sequences, and t-RFLP analysis. Data obtained from M. Bhatia (2004) and this study

[2], Presence of 5' terminal fragment in the four samples (SOC, BF, SIB and PSA samples) analyzed in both culture-dependent and culture-independent ARDRA analysis. Data obtained from M. Bhatia (2004)

[3], Presence of 5' terminal fragment in all 143 JEG samples (subglacial, supraglacial and proglacial samples) analyzed using t-RFLP analysis. Data obtained from M. Bhatia (2004)

[4], Percent occurrence of 5' terminal fragment in all 104 subglacial JEG samples analyzed using t-RFLP analysis. Data obtained from M. Bhatia (2004)

[5], Percent occurrence of 5' terminal fragment in all 43 supraglacial JEG samples analyzed using t-RFLP analysis. Data obtained from M. Bhatia (2004)

[6], Percent occurrence of 5' terminal fragment in all 50 proglacial JEG samples analyzed using t-RFLP analysis. Data obtained from M. Bhatia (2004)

[7], Presence of organisms with 5' terminal fragment in either clone libraries or culture collections made from the four JEG samples (SOC, BF, SIB and PSA samples) using culture-dependent and culture-independent ARDRA analysis. Data obtained from this study

[8], Name of sequenced clone(s) possessing the 5' terminal fragment. Data obtained from this study

[9], Name of sequenced isolate(s) possessing the 5' terminal fragment. Data obtained from this study

[10], Bacterial division in which sequenced clones or isolates belong. Data obtained from this study

Table 3.10. The number of clones, genus of related organisms, and source of clones within eight OTUs. Each OTU contains clones from two or more JEG environments.

OTU #	Genus of related organisms	Total number of clones in OTU	Number of clones from each sample in OTU		
			SOC	SIB	BF
1	*Flavobacterium*	34	6	5	23
5	*Comamonas*	8	5	3	
11	*Comamonas*	7	6	1	
17	*Comamonas*	2	1	1	
47	*Comamonas*	11		10	1
52	*Comamonas*	33	15		18
56	*Comamonas*	19	15		4
60	*Sphingobacterium*	6	4		2

Table 3.11. Representative clones from the 13 most abundant OTUs from the SOC clone library.

OTU #	% Abundance [1]	Representative Clone name	Nearest neighbor (Source)	Accession no.	% Identity [2]	Division
56**	9	SOC1 1C	Glacier bacterium FXS1 (New Zealand Subglacial sediment)	AY315177	99%	β-Proteobacteria
90	9	SOC1 2B	Uncultured bacterium clone 207ds20 (contaminated water sample)	AY212653	99%	CFB
52**	9	SOC1 3G	Uncultured Bacterium clone 50 (contaminated sediment)	AY250098	99%	β-Proteobacteria
91	8	SOC1 1D	Uncultured Actinobacteria clone S7 (fresh water lake)	AJ575508	99%	Actinobacteria
11*	4	SOC1 1B	Unidentified eubacterium clone GKS16 (mountain lake)	AJ224987	99%	β-Proteobacteria
92	4	SOC1 4G	Uncultured Cytophagales clone PRD01a001B (fresh water lake)	AF289149	98%	CFB
1***	4	SOC1 8C	*Flavobacterium omnivorum* As1.2747 (frozen glacier soil)	AF433174	98%	CFB
5*	3	SOC1 6F	Uncultured bacterium clone B-Y34 (contaminated aquifer)	AY622248	99%	β-Proteobacteria
93	3	SOC1 6H	Uncultured Actinobacterium clone S7 (fresh water lake)	AC575508	99%	Actinobacteria
60**	3	SOC1 7F	Uncultured *Sphingobacteriaceae* bacterium clone LiUU-5-303 (fresh water lake)	AY509378	98%	CFB
94	3	SOC1 10F	*Sphingobacterium* sp. AC74 isolate (ground water)	AJ717393	96%	CFB
95	2	SOC1 8E	Uncultured Actinobacterium clone S4 (fresh water lake)	AJ575506	93%	Actinobacteria
97	2	SOC1 8B	Uncultured Actinobacterium clone N3	AJ575530	99%	Actinobacteria

*, Also found in the BF clone library
**, Also found in the SIB clone library
***, Found in all three clone libraries
[1], Percent abundance of one OTU in the SOC clone library.
[2], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).

Table 3.12. Representative clones from the eight most abundant OTUs from the SIB clone library.

OTU #	% Abundance [1]	Clone name	Nearest neighbor (Source)	Accession no.	% Identity	Division
52**	20	SIB2 1E	Uncultured β-Proteobacterium (river biofilm)	AJ421938	99%	β-Proteobacteria
47*	9	SIB2 1B	β-Proteobacterium Wuba 72 (karstic aquifer)	AF336361	99%	β-Proteobacteria
54	7	SIB2 4D	Uncultured β-proteobacterium clone Spb98 (river biofilm)	AJ422160	99%	β-Proteobacteria
1***	5	SIB2 1C	*Flavobacterium psychrolimnae* type strain LMG 22018 (Antarctic microbial mat)	AJ585428	98%	CFB
55	4	SIB1 2B	Uncultured bacterium clone 160ds20 (contaminated groundwater)	AY212612	97%	β-Proteobacteria
56**	4	SIB2 1G	Glacier bacterium FXS1 (New Zealand subglacial ice sediment)	AY315177	99%	β-Proteobacteria
57	4	SIB2 1D	Uncultured bacterium clone PL-34B2 (oil reservoir)	AY570588	98%	β-Proteobacteria
58	3	SIB1 10G	Uncultured bacterium clone B-Y34 (contaminated aquifer)	AY622248	99%	β-Proteobacteria

*, Also found in the BF clone library
**, Also found in the SOC clone library
***, Found in all three clone libraries
[1], Percent abundance of one OTU in the SIB clone library.
[2], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).

Table 3.13. Representative clones from the nine most abundant OTUs from the BF clone library.

OTU #	% Abundance [1]	Clone name	Nearest neighbor (Source)	Accession no.	% Identity[2]	Division
1***	26	BFA 7C	*Flavobacterium xinjiangense* AS1.2749 (Glacier soil)	AF433173	99%	CFB
3	9	BFA 6E	*Flavobacterium omnivorum* sp. nov. isolate (Glacier soil)	AY599655	98%	CFB
4	4	BFA 1H	β-Proteobacterium Wuba 72 (karstic aquifer)	AF336361	99%	β-Proteobacteria
5**	3	BFA 9H	Uncultured bacterium clone B-Y34(contaminated aquifer)	AY622248	99%	β-Proteobacteria
6	3	BFM 2C	*Flavobacterium psychrolimnae* type strain LMG 22018 (Antarctic microbial mat)	AJ585428	98%	CFB
7	2	BFM 4C	Uncultured β-Proteobacterium clone LiUU-9-233 (freshwater lake)	AY509483	96%	β-Proteobacteria
8	2	BFM 4E	Glacial ice bacterium CanDirty89 isolate (cryoconite sediment, Antarctica)	AF479326	98%	β-Proteobacteria
9	2	BFM 6B	β-Proteobacterium Wuba 72 (karstic aquifer)	AF336361	99%	β-Proteobacteria
10	2	BFA 8A	Glacial ice bacterium CanDirty89 isolate (cryoconite sediment, Antarctica)	AF479326	98%	β-Proteobacteria

**, Also found in the SOC clone library

***, Found in all three clone libraries

[1], Percent abundance of one OTU in the BF clone library.

[2], based on near-full-length bi-directional 16S rRNA gene sequence (approx. 1500 bp).

Table 3.14. Measured ARDRA patterns from three pure isolates digested with either *Hae*III or *Cfo*I restriction enzymes compared to calculated ARDRA patterns from 16S rRNA gene sequences.

Isolate name	OTU	*Cfo*I Pattern	Measured base pairs cut with *Cfo*I	Calculated base pairs cut with *Cfo*I	*Hae*III Pattern	Measured base pairs cut with *Hae*III	Calculated base pairs cut with *Hae*III
SOCA20(17)	4	B	510	528	B	405	419
			390	399*		300	312
			350	361		195	190, 204*
			205	195		112	119
							85
							58
Total bases (Number of Fragments)			1455 (4 fragments)	1483 (4 fragments)		1012 (4 fragments)	1483 (7 fragments)
SOCA20(60)	5	G	580	576	K	560	571
			330, 330	331*		270	277
				312		150	158, 154
			132	135		113	121
			84	65		58	100
							39*
Total bases (Number of Fragments)			1456, (5 fragments)	1419 (5 fragments)		1151 (5 fragments)	1419 (fragments)
SOCA20(36)	7	T	1000	994	E	370	366*
			400	399*		350	338
			110	51		290	277
						180	181
							166
						118	116
Total bases (Number of Fragments)			1510 (3 fragments)	1444 (3 fragments)		1308 (5 fragments)	1444 (6 fragments)

*, 5' fragment

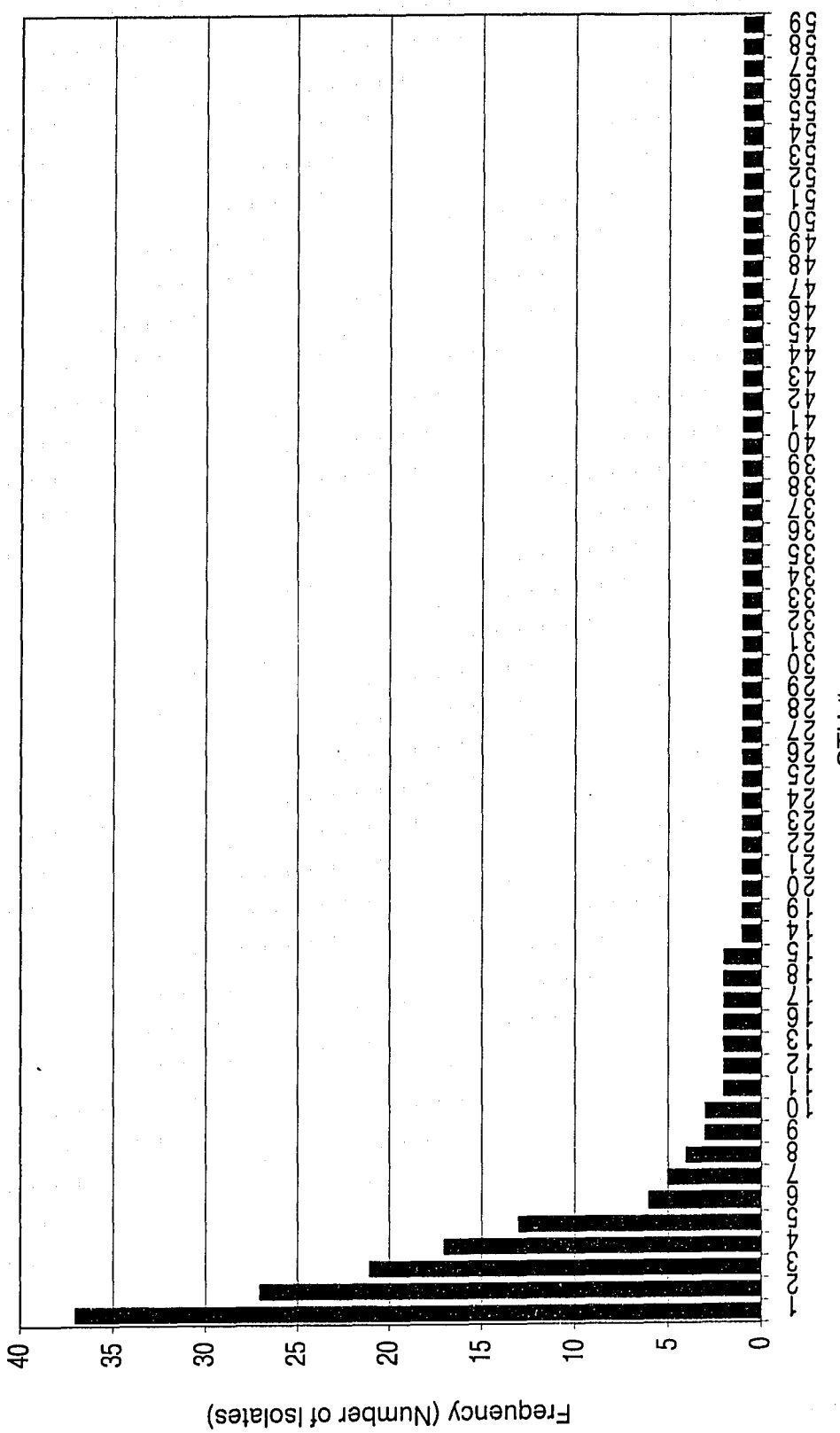

Figure 3.1. Histogram showing the frequency of occurrence of 59 OTUs arising from ARDRA analysis of 192 morphologically distinct pure isolates from all five JEG samples (SOC, SIB, BF, BC and PSA).

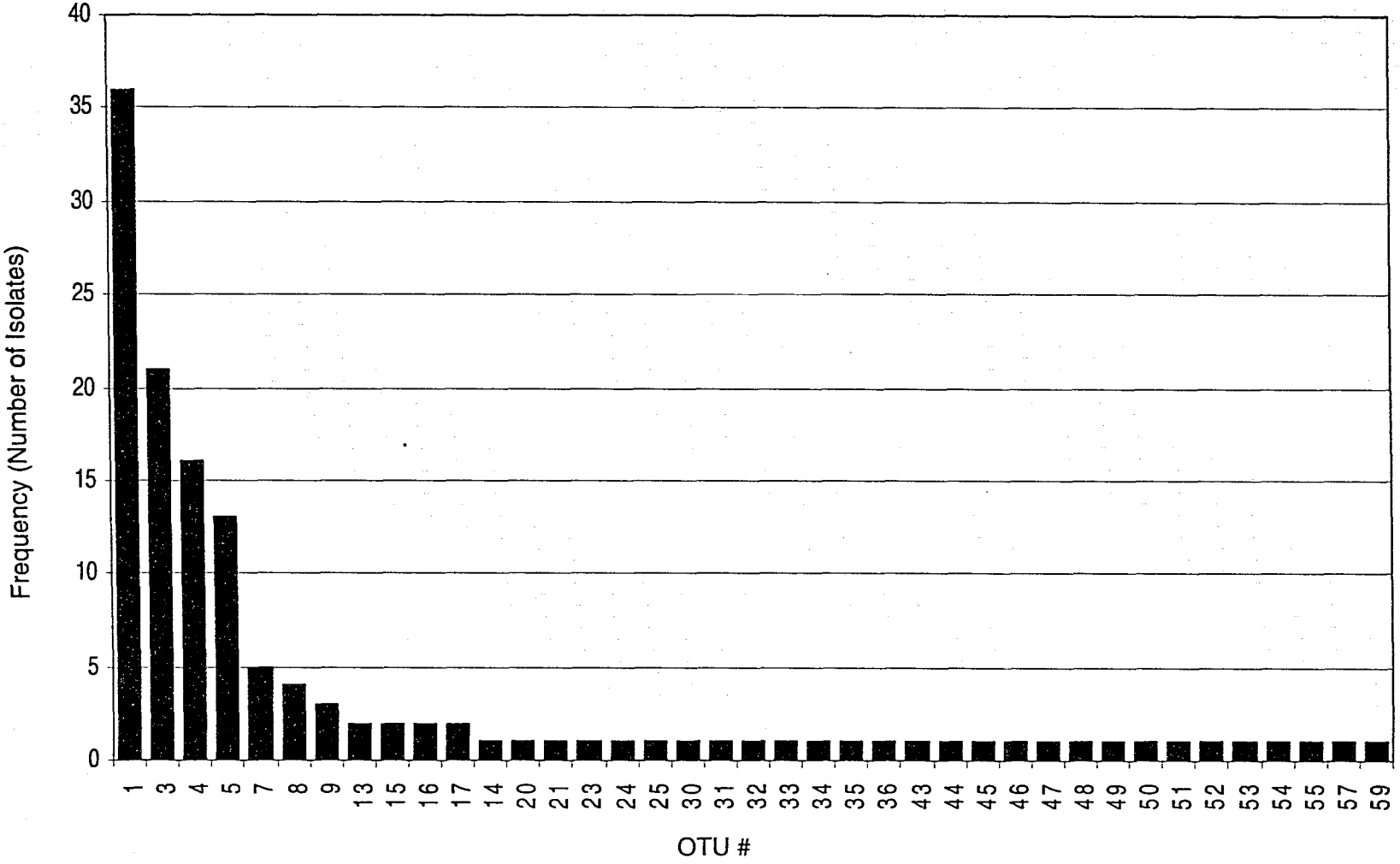

Figure 3.2. Histogram showing the frequency of occurrence of 39 OTUs arising from ARDRA analysis of 134 morphologically distinct pure isolates from the SOC sample.

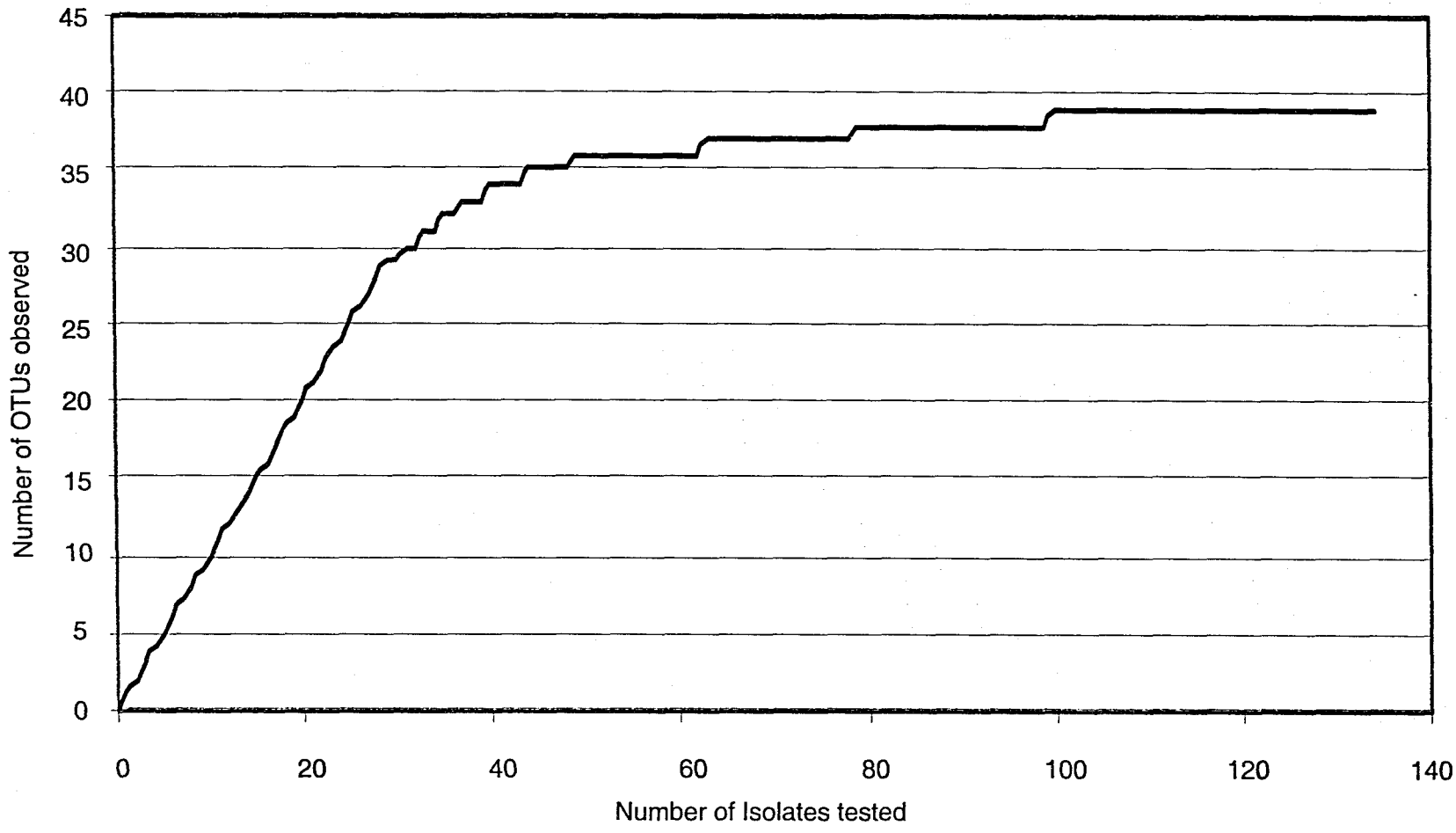

Figure 3.3. Rarefaction curve showing the number of new OTUs observed with an increasing number of isolates analyzed from the SOC sample. If every unique organism within a microbial environment is detected, an increase in the number of isolated analyzed will not affect the number of OTUs observed in a sample, causing the curve to eventually reach a plateau.

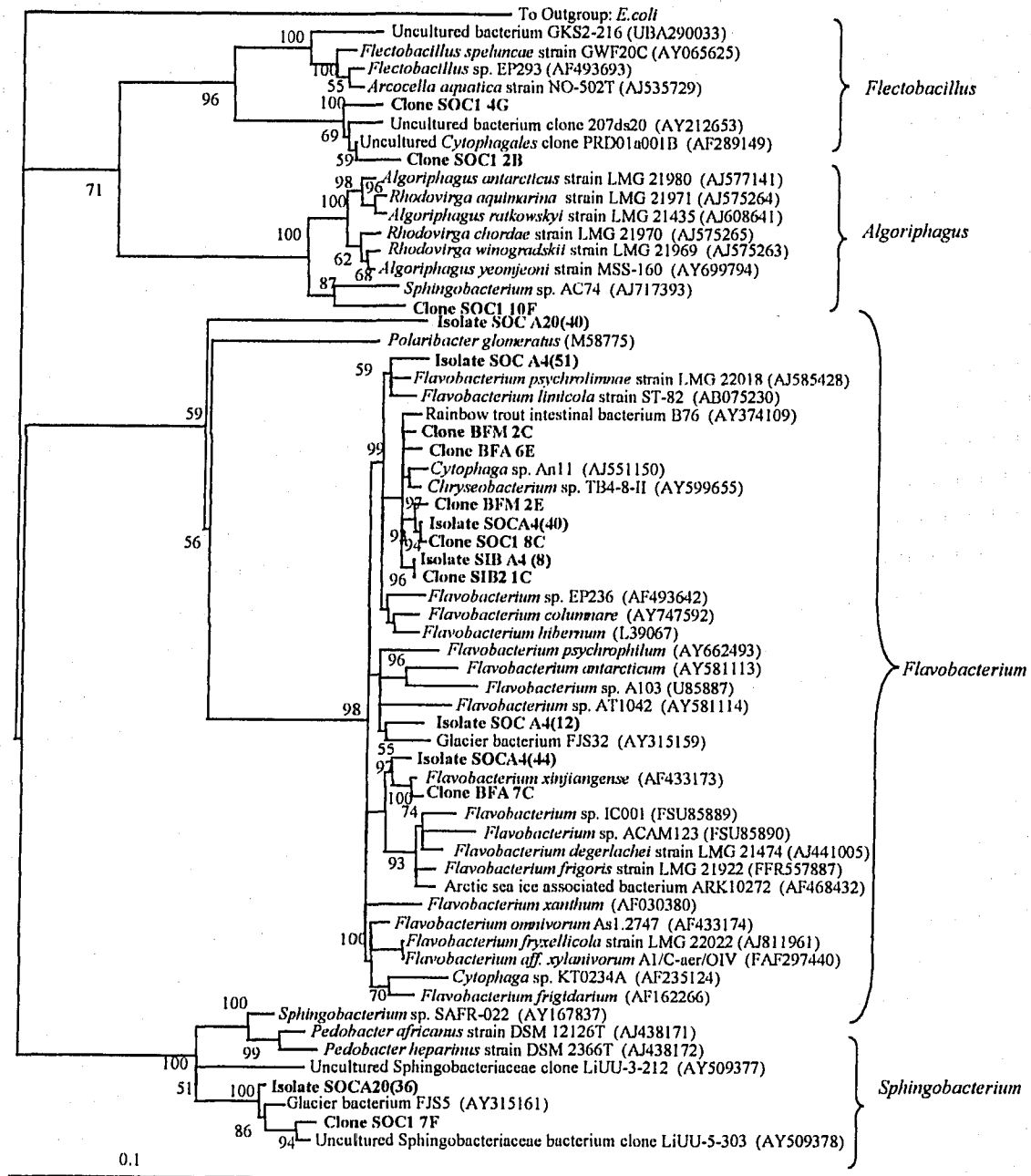

Figure 3.4. Rooted phylogenetic consensus trees of 16S rRNA gene sequences showing affiliation of SOC and SIB isolates and SOC, SIB and BF clones within the CFB division. The scale bar corresponds to a 10% difference in nucleotide sequence. *E. coli* was used as an outgroup.

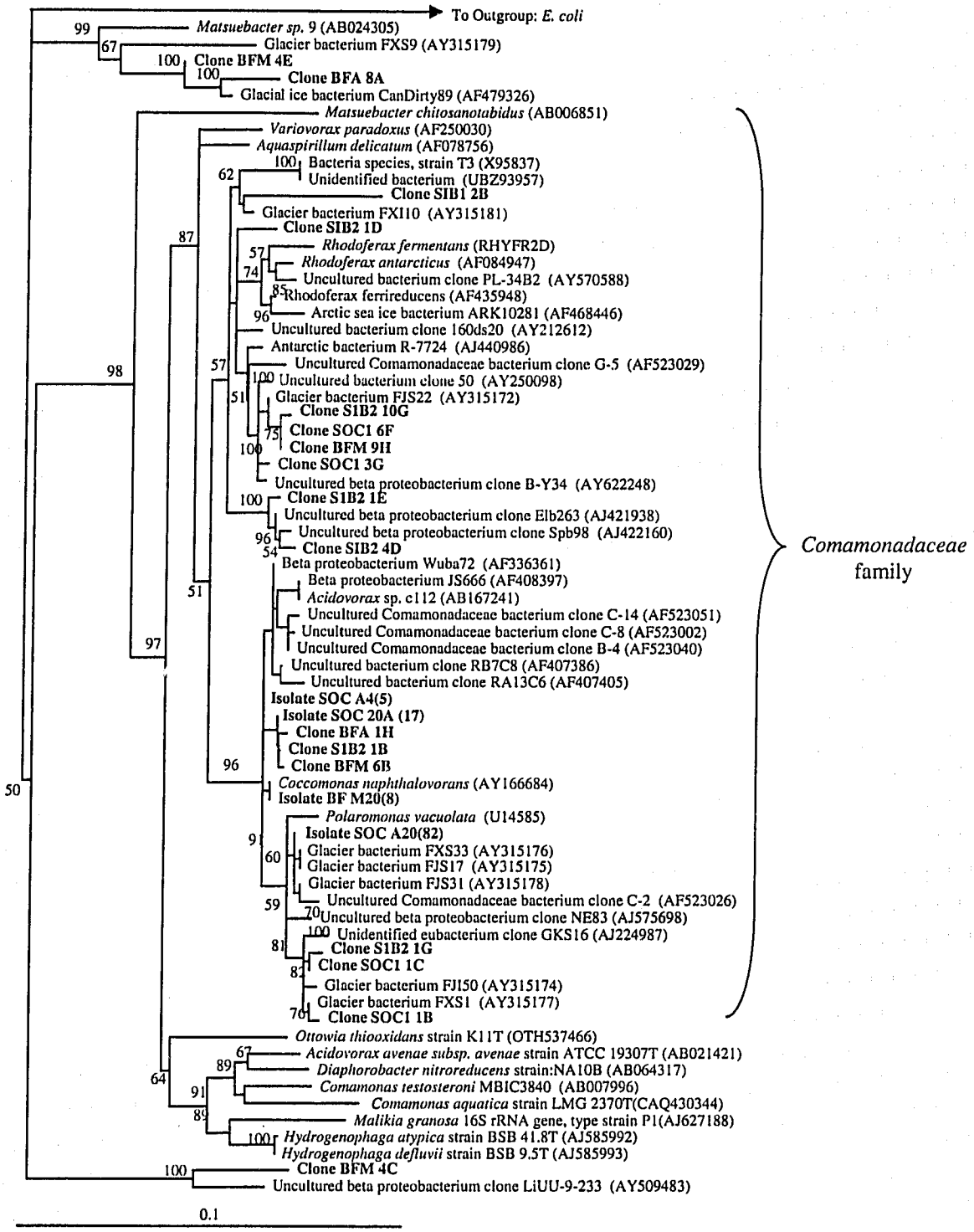

Figure 3.5. Rooted phylogenetic consensus tree of 16S rRNA gene sequences showing affiliation of SOC, SIB and BF isolates within the β-Proteobacteria division. The scale bar corresponds to a 10% difference in nucleotide sequence. *E. coli* was used as an outgroup

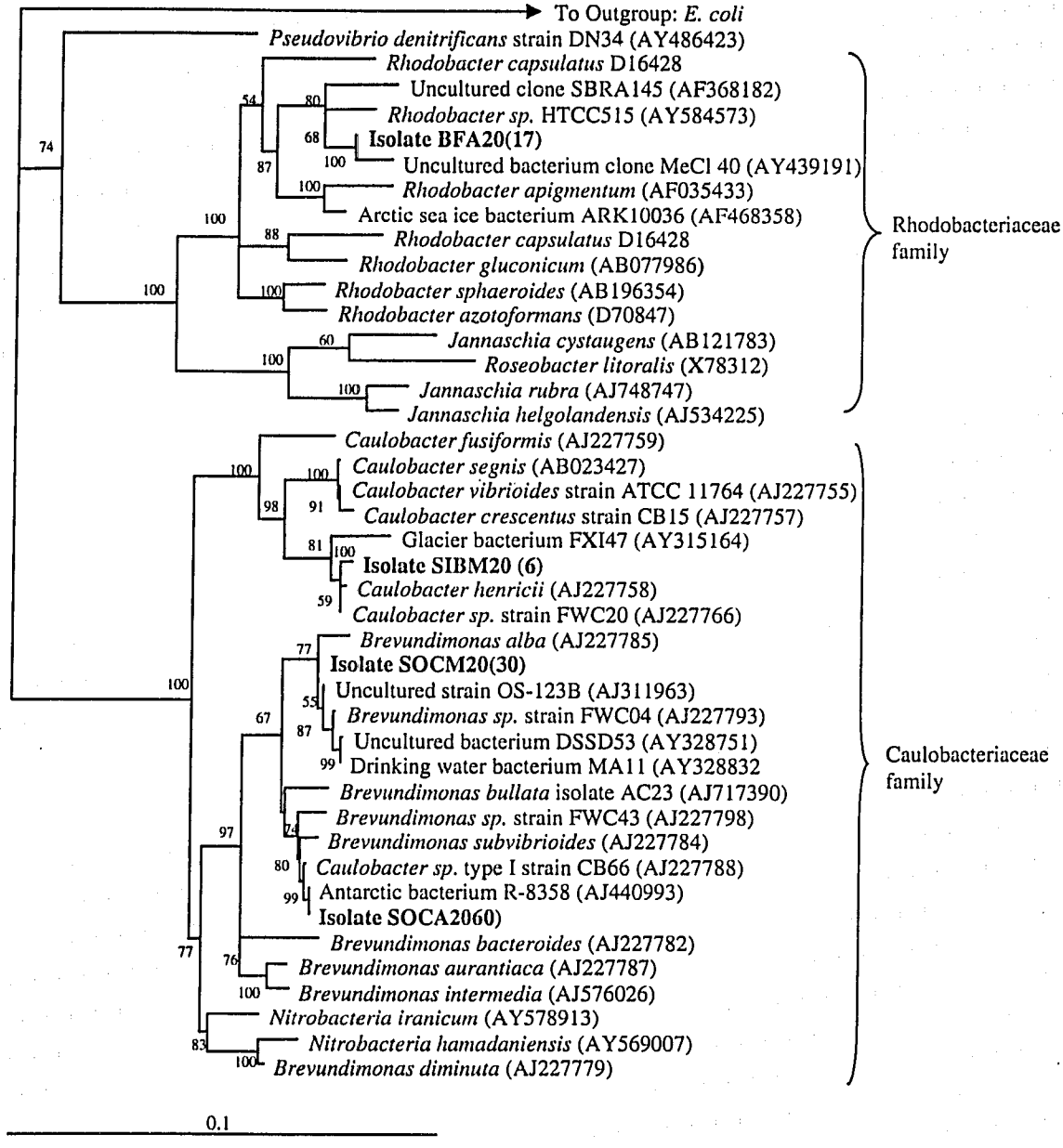

Figure 3.6. Rooted phylogenetic consensus tree of 16S rRNA gene sequences showing affiliation of SOC, SIB and BF isolates within the α-Proteobacteria division. The scale bar corresponds to a 10% difference in nucleotide sequence. *E. coli* was used as an outgroup

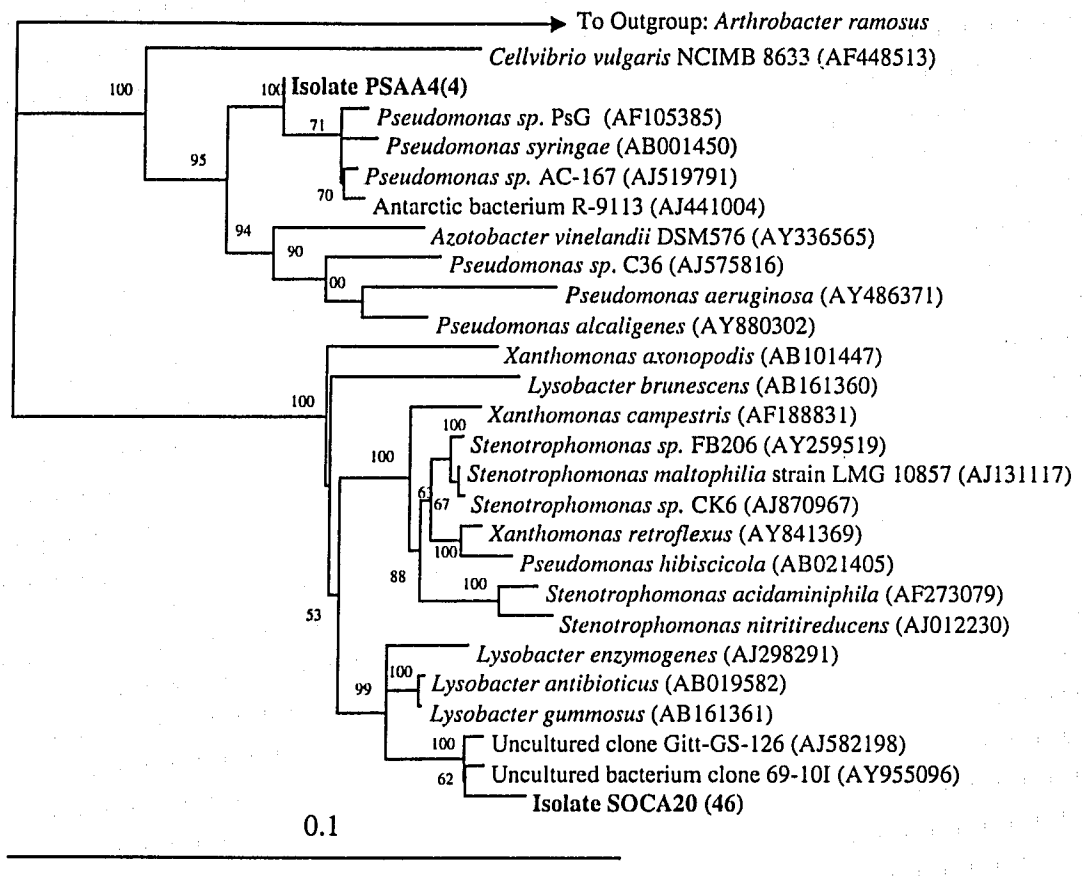

Figure 3.7. Rooted phylogenetic consensus tree of 16S rRNA gene sequences showing affiliation of SOC and PSA isolates within the γ-Proteobacteria division. The scale bar corresponds to a 10% difference in nucleotide sequence. *E. coli* was used as an outgroup

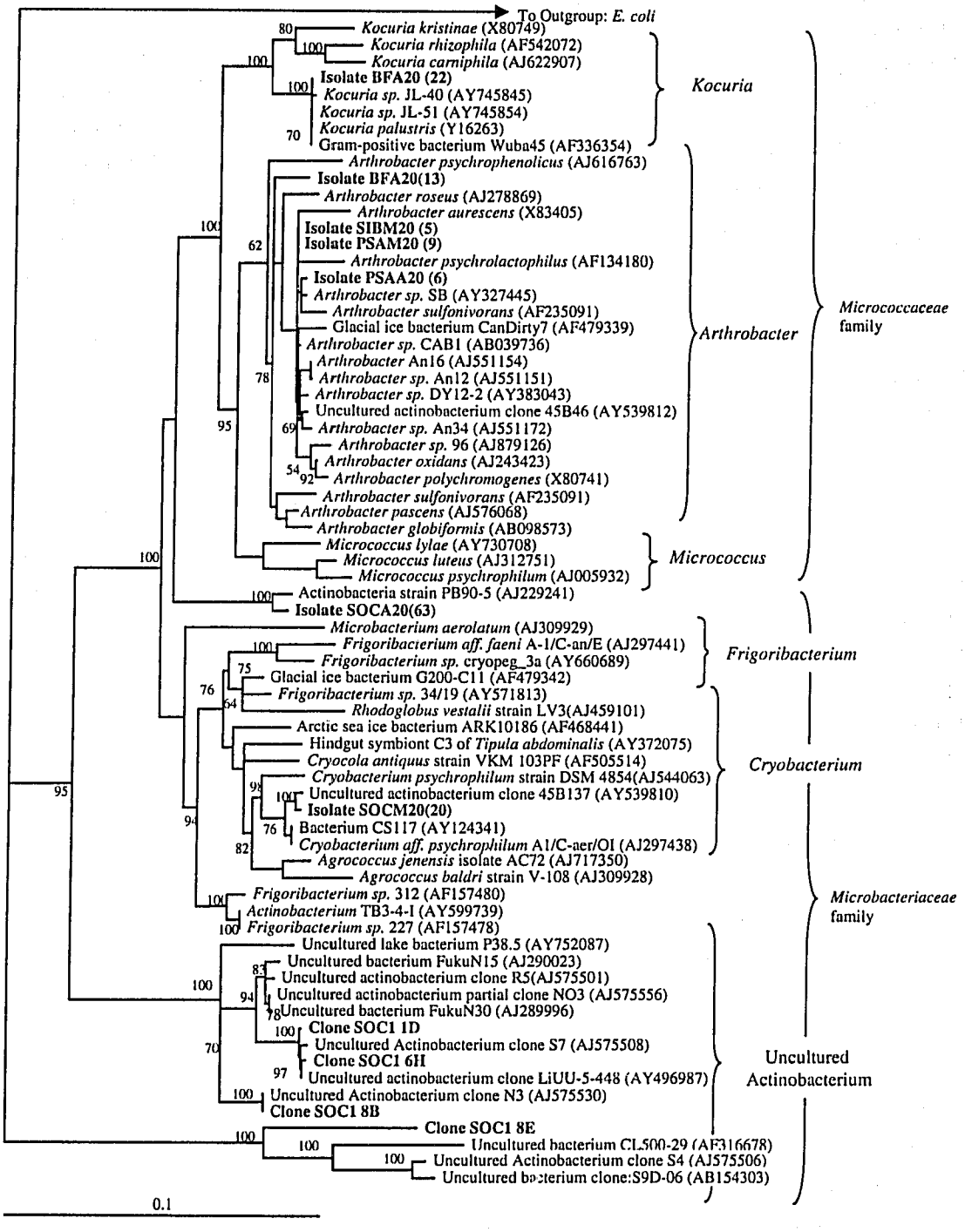

Figure 3.8. Rooted phylogenetic consensus tree of 16S rRNA gene sequences showing affiliation of SOC, SIB, BF and PSA isolates and SOC clones within the Actinobacteria division. The scale bar corresponds to a 10% difference in nucleotide sequence. *E. coli* was used as an outgroup

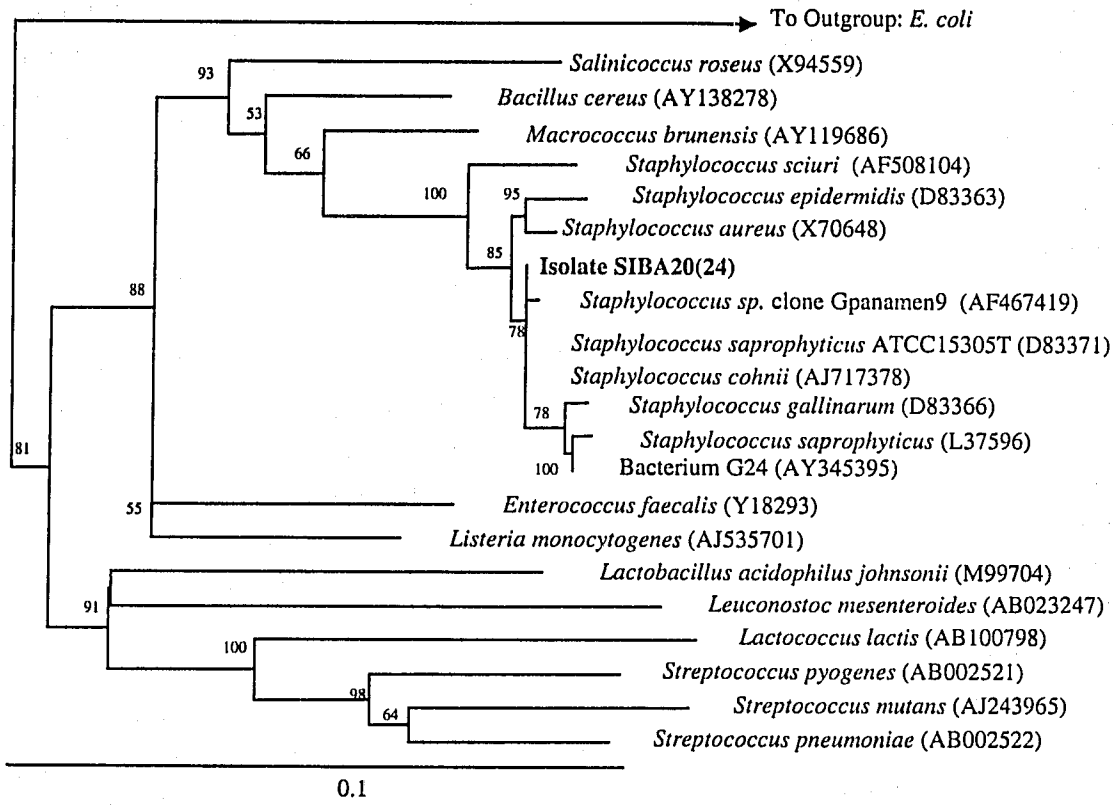

Figure 3.9. Rooted phylogenetic consensus tree of 16S rRNA gene sequences showing affiliation of an SIB isolate within the Firmicutes division. The scale bar corresponds to a 10% difference in nucleotide sequence. *E. coli* was used as an outgroup

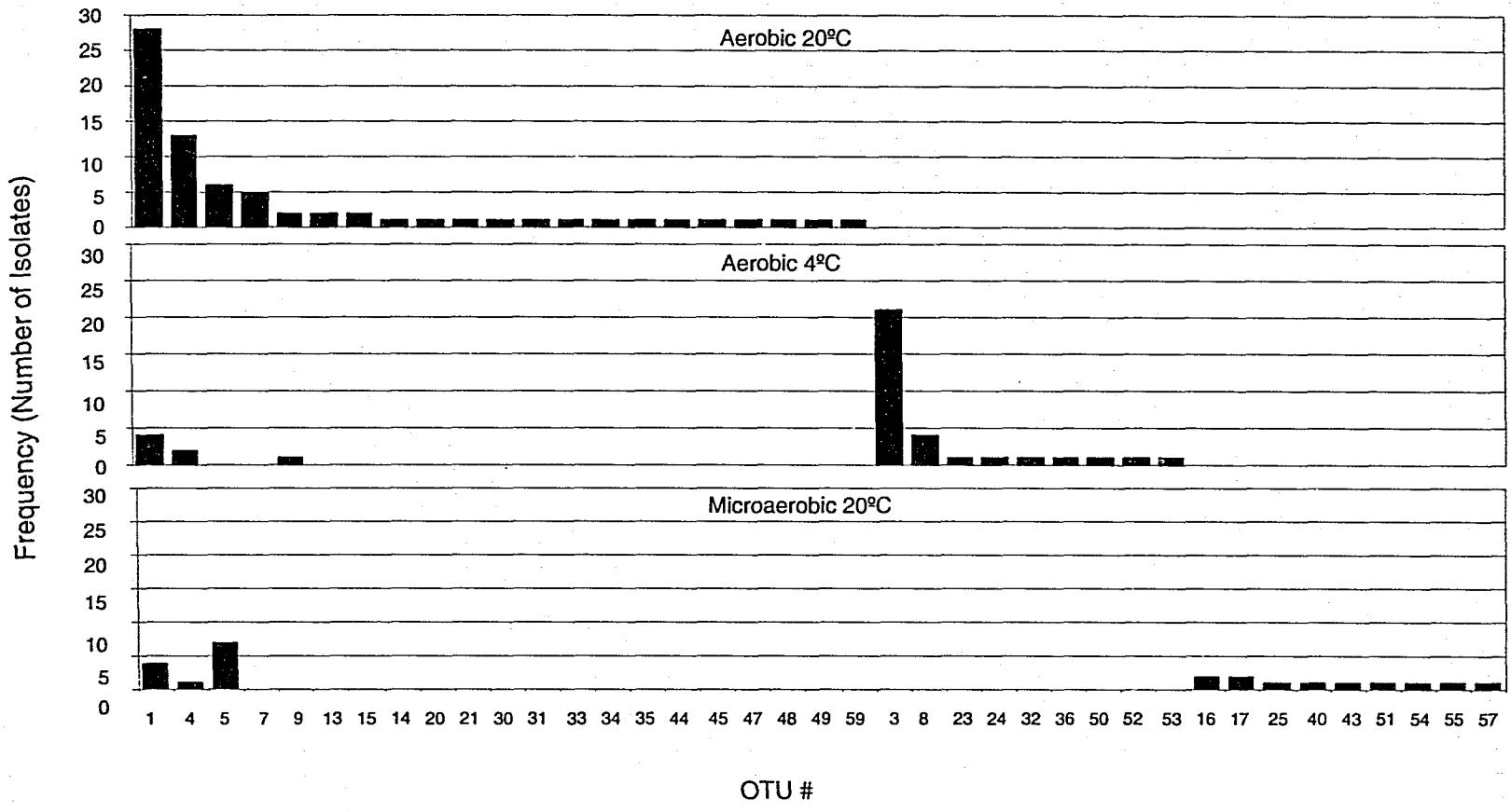

Figure 3.10. Distribution and frequency of 39 OTUs representing 134 morphologically distinct pure cultures isolated from SOC under three different incubation conditions.

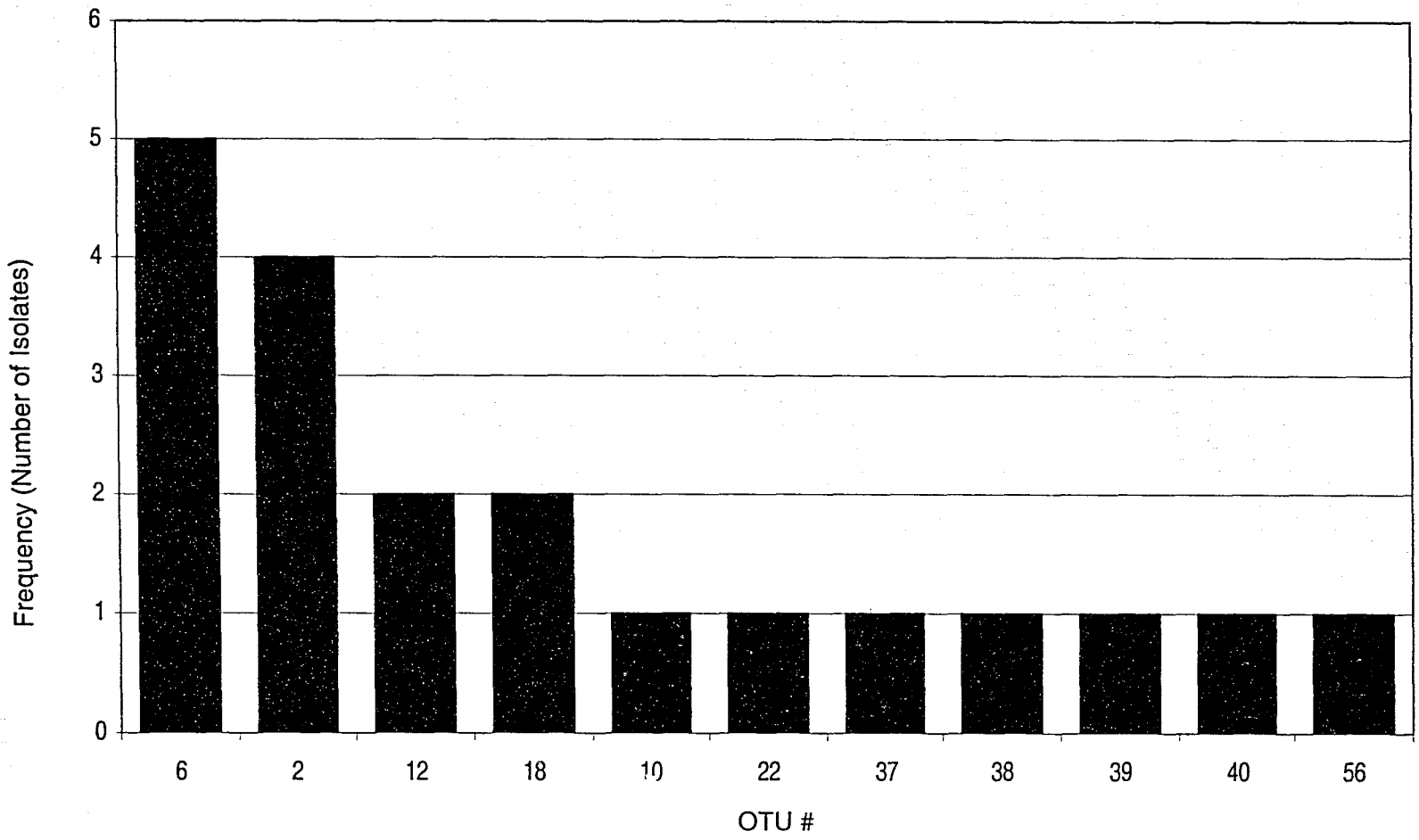

Figure 3.11. Histogram showing the frequency of occurrence of the 11 OTUs arising from ARDRA analysis of 20 morphologically distinct pure isolates from the SIB sample.

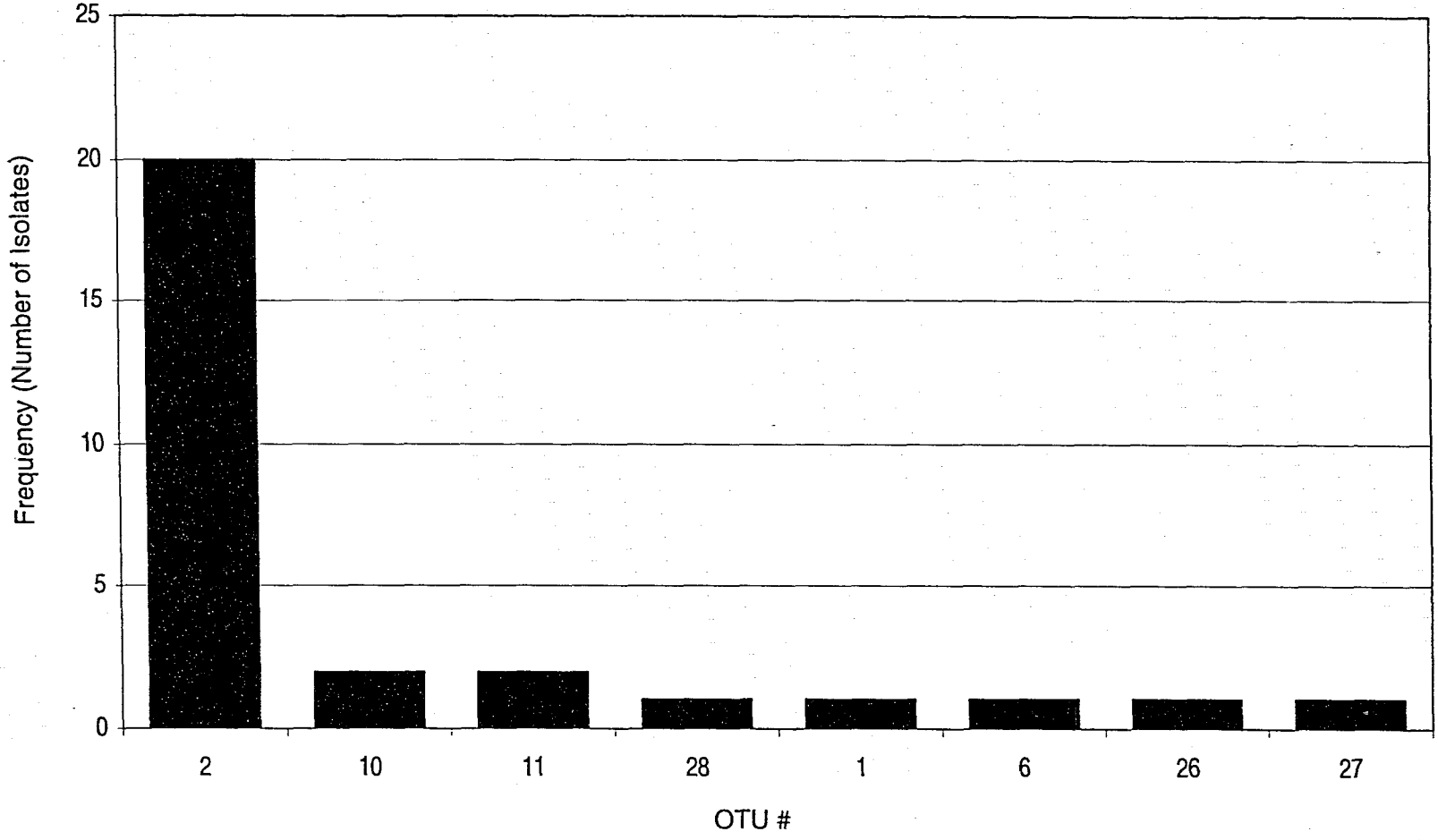

Figure 3.12. Histogram showing the frequency of occurrence of the eight OTUs arising from ARDRA analysis of 29 morphologically distinct pure isolates from the PSA sample.

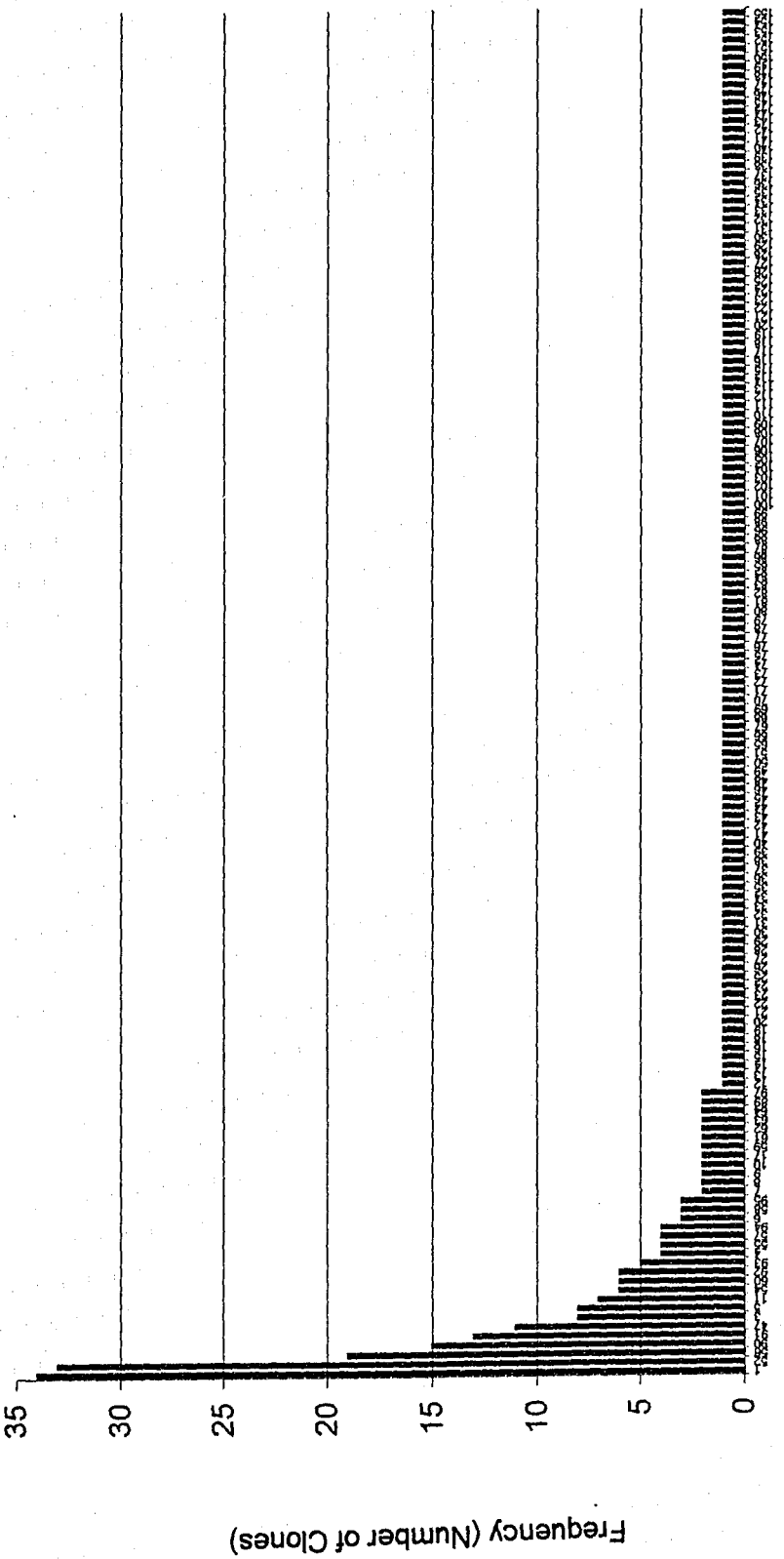

Figure 3.13. Histogram showing the frequency of occurrence of 153 OTUs arising from ARDRA analysis of 341 clones from JEG.

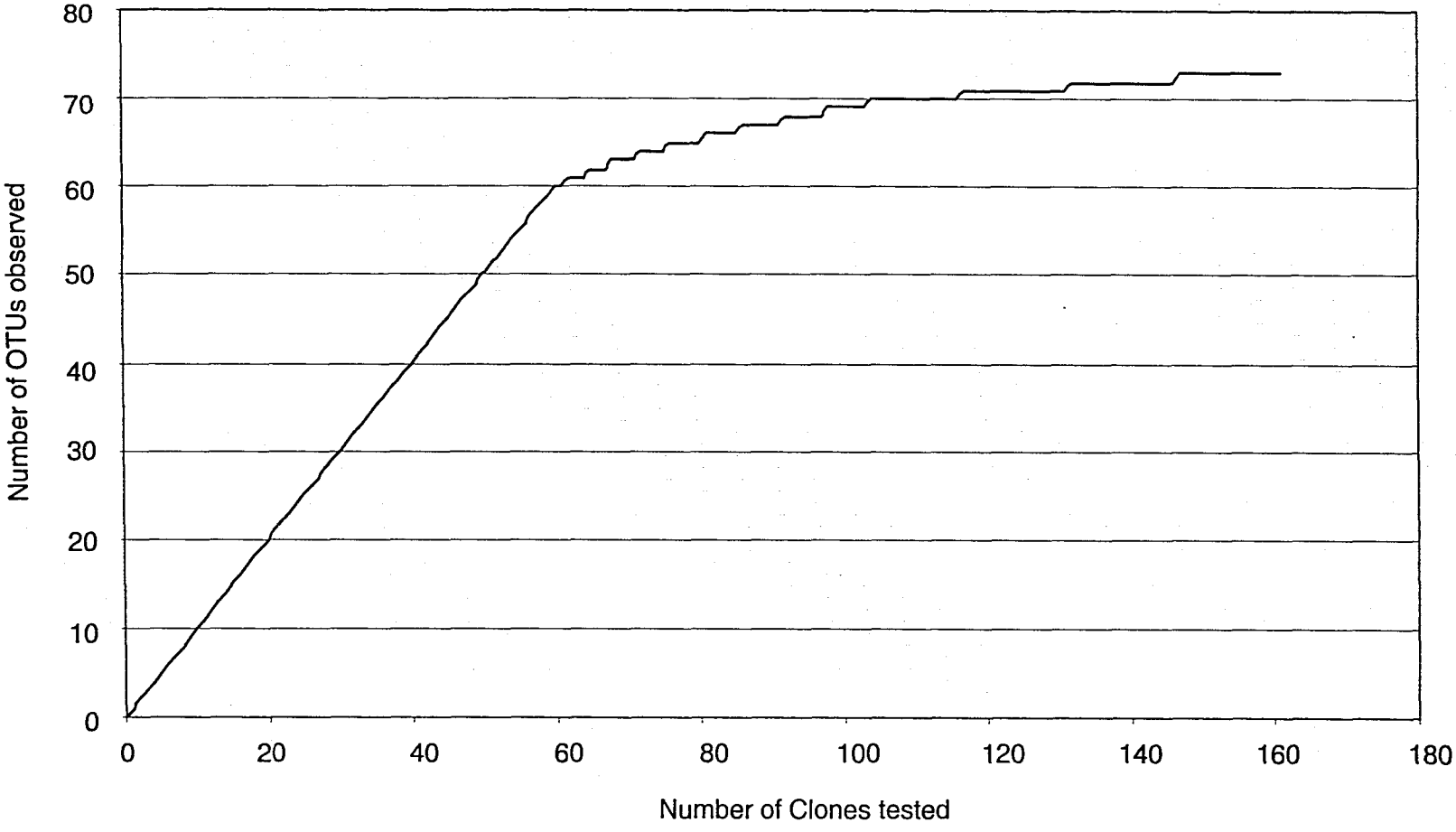

Figure 3.14. Rarefaction curve showing the number of new OTUs observed with an increasing number of clones analyzed from the SOC clone library. The rarefaction curve shown above appears to be nearing a plateau after 100 clones. This shows that the majority of the organisms in the SOC environment have been identified after analysis of 159 clones.

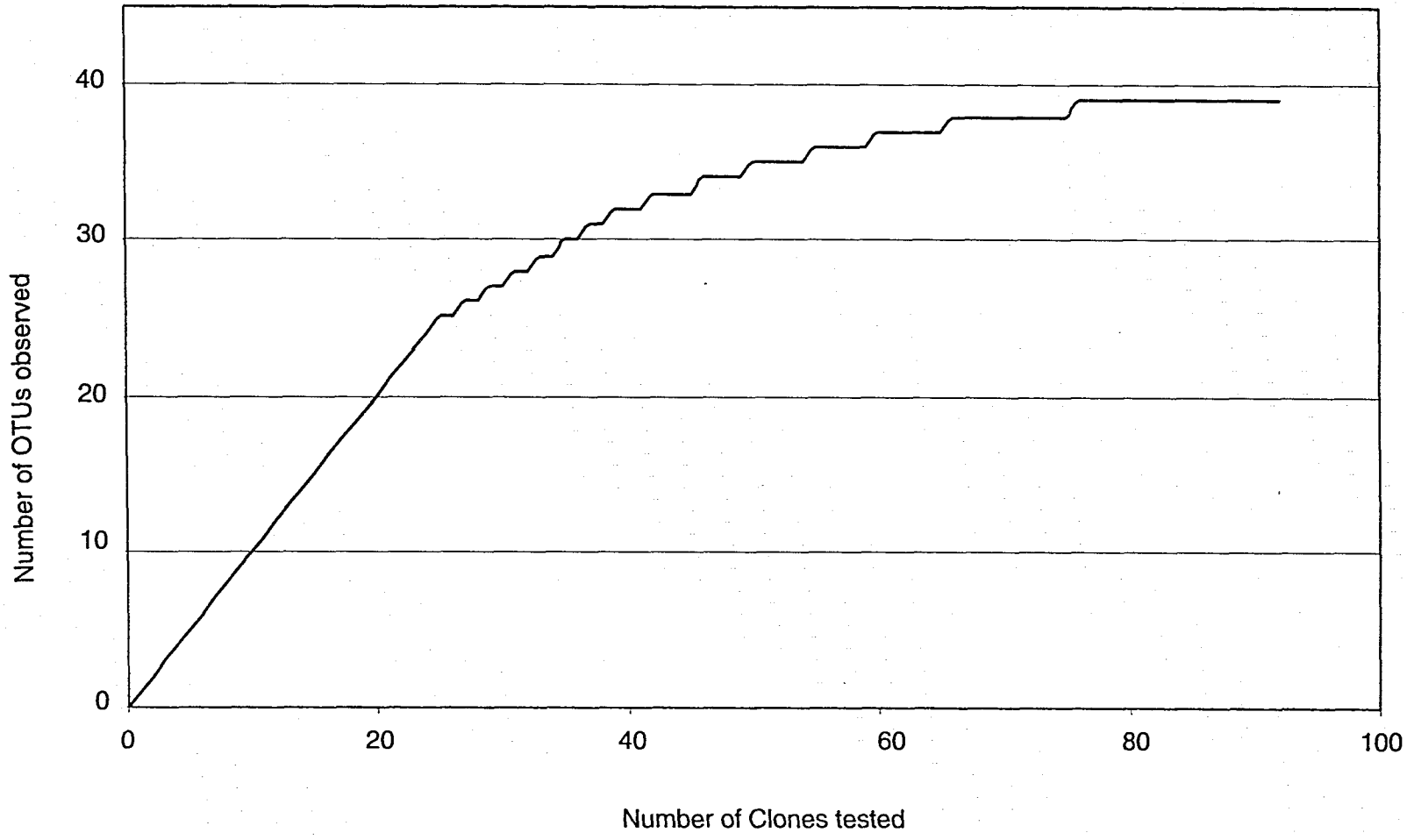

Figure 3.15. Rarefaction curve showing the number of new OTUs observed with an increasing number of clones analyzed from the SIB clone library. The rarefaction curve shown above appears to be nearing a plateau after analysis of 92 clones.

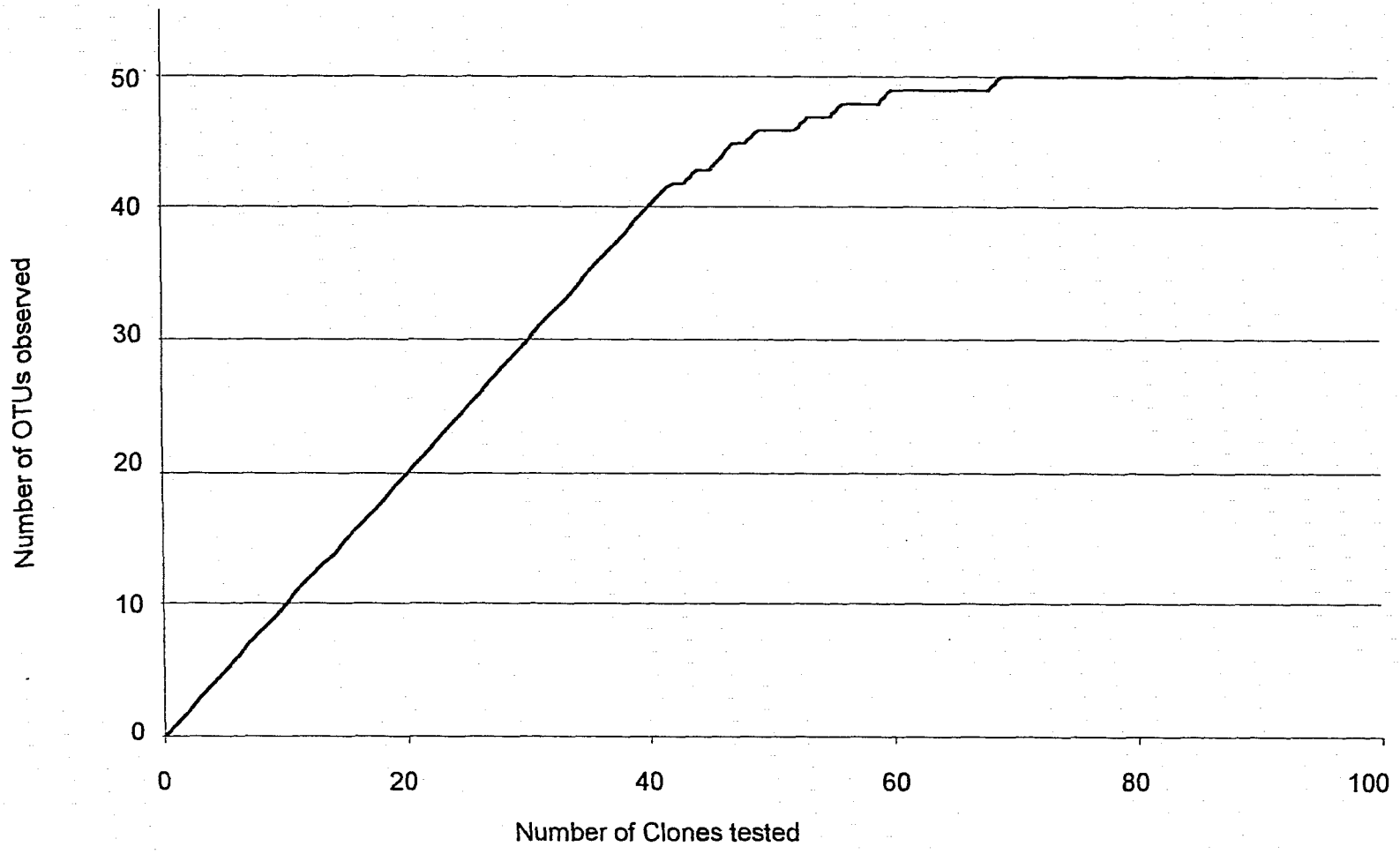

Figure 3.16. Rarefaction curve showing the number of new OTUs observed with an increasing number of clones analyzed from the BF clone library. The rarefaction curve shown above appears to be nearing a plateau after analysis of 90 clones.

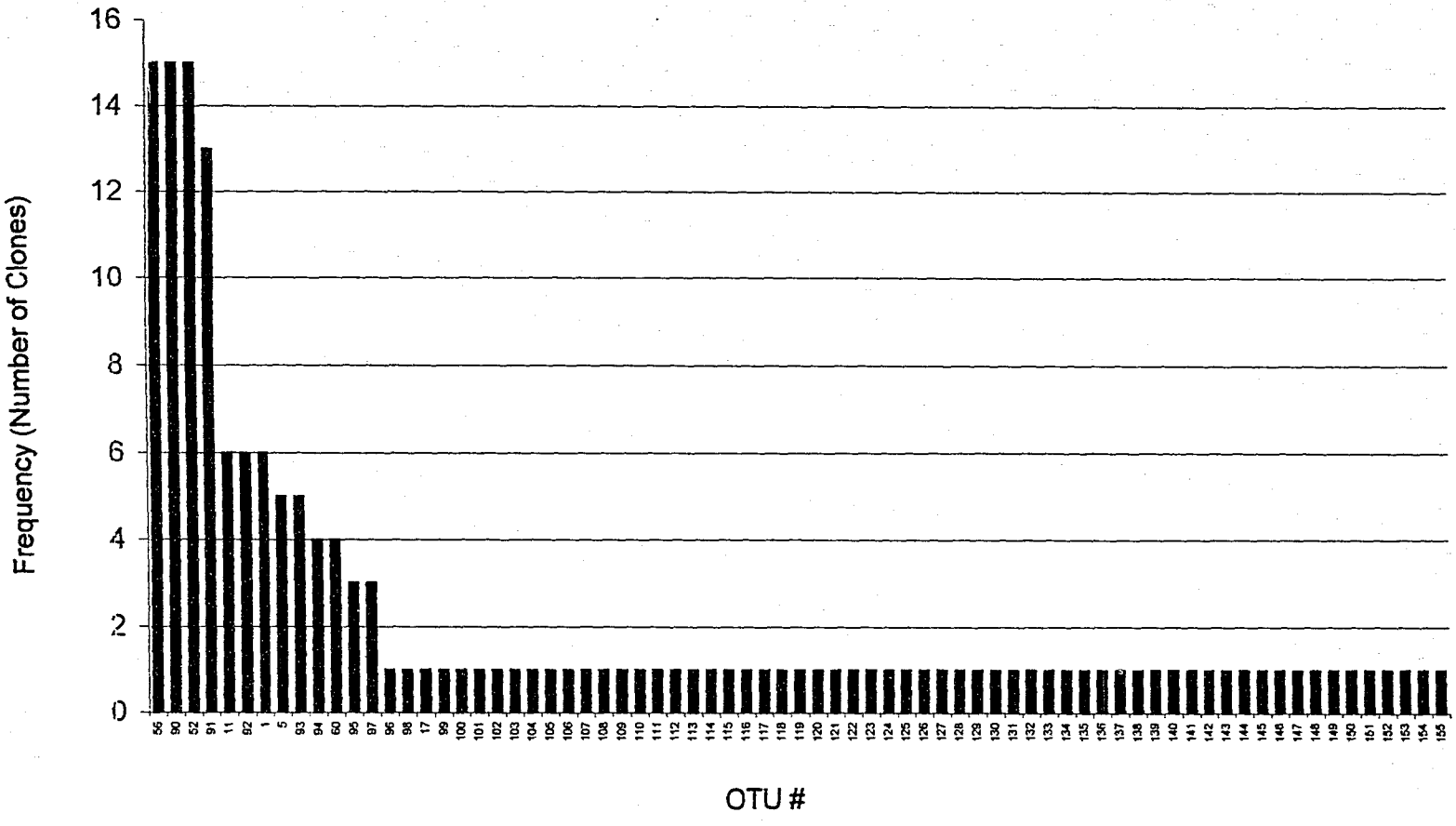

Figure 3.17. Histogram showing the frequency of occurrence of 73 OTUs arising from culture-independent ARDRA analysis of 159 clones from the SOC clone library.

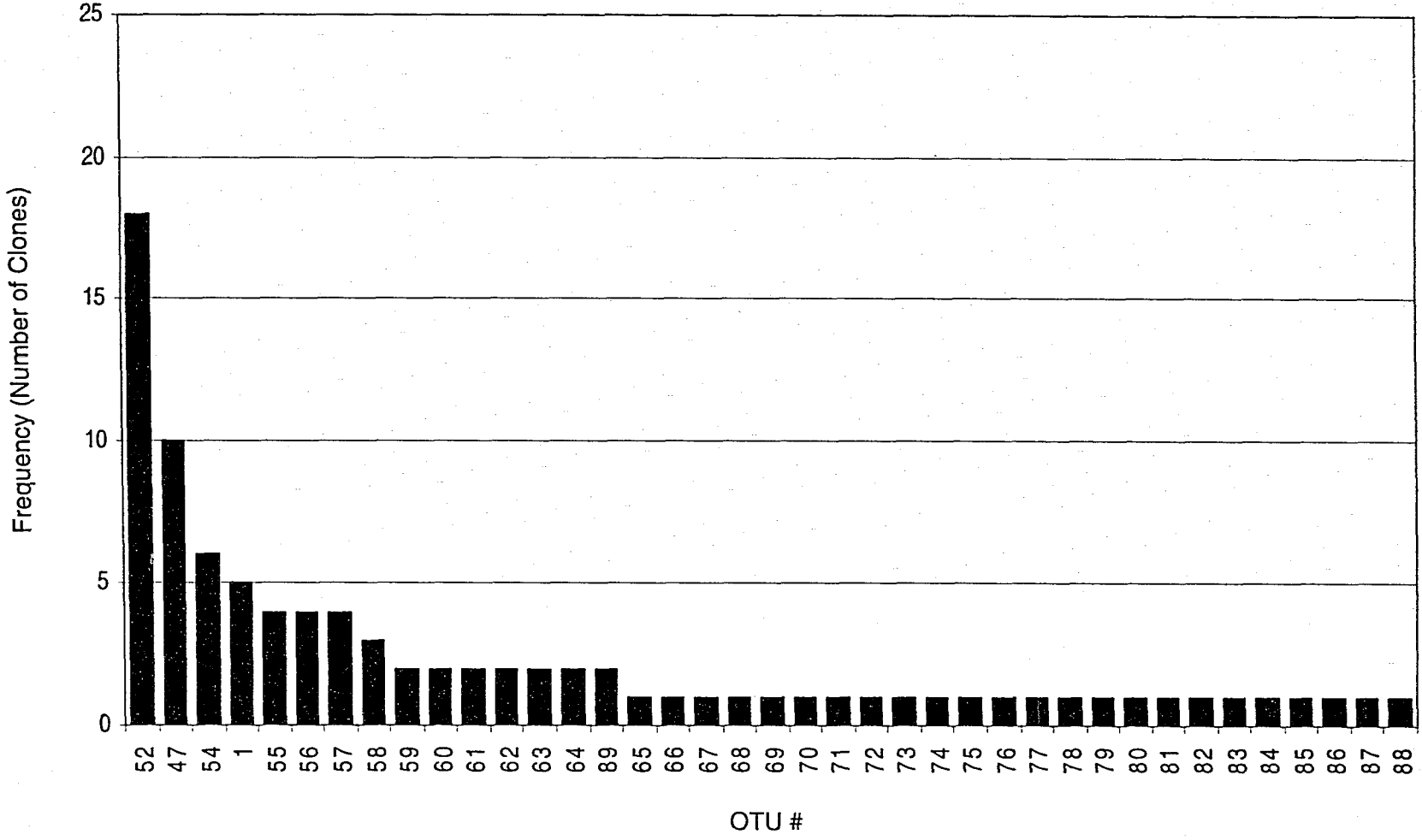

Figure 3.18. Histogram showing the frequency of occurrence of 39 OTUs arising from ARDRA analysis of 92 clones from the SIB clone library.

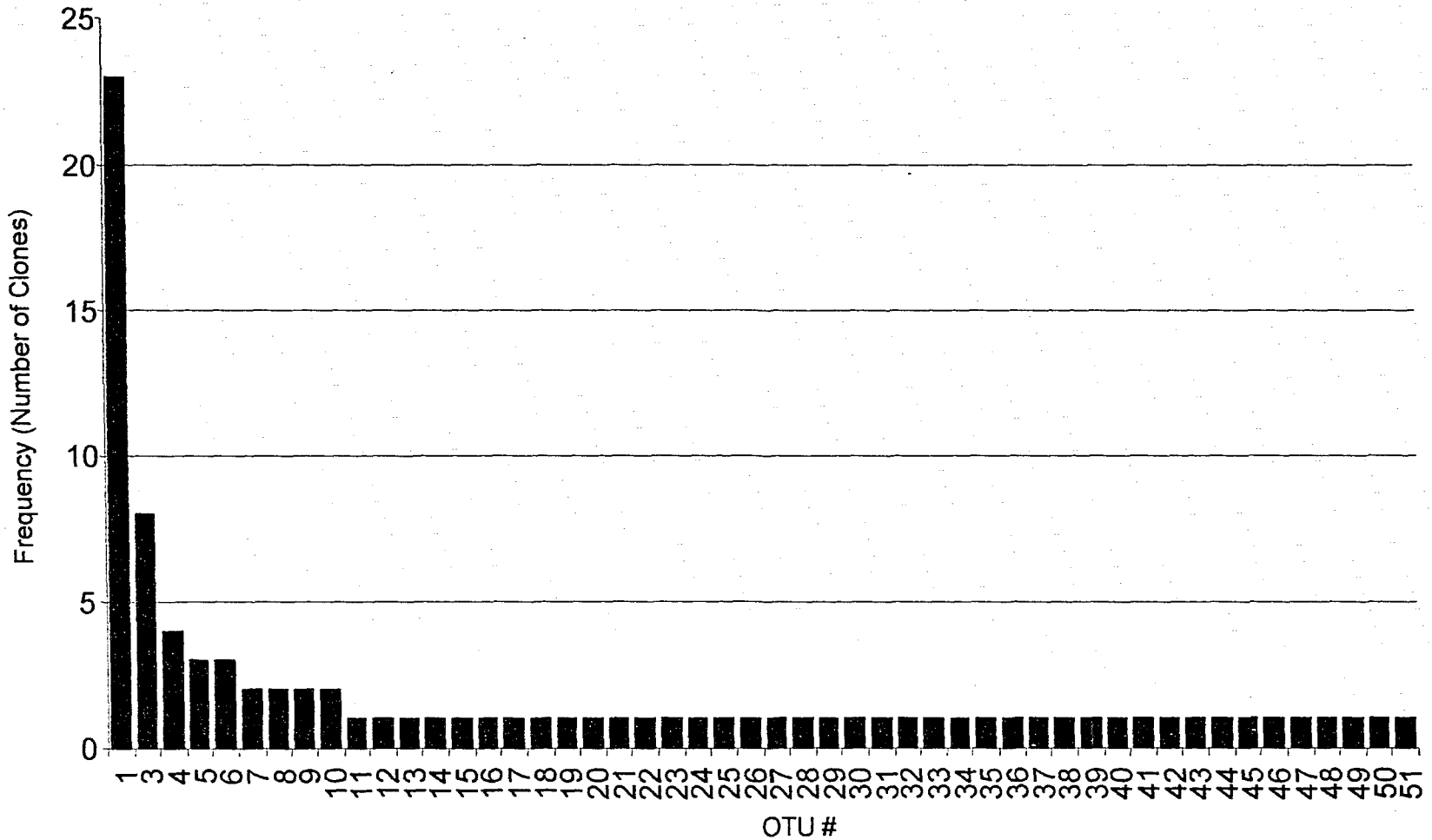

Figure 3.19. Histogram showing the frequency of occurrence of 50 OTUs arising from culture-independent ARDRA analysis of 90 clones from the BF clone library.

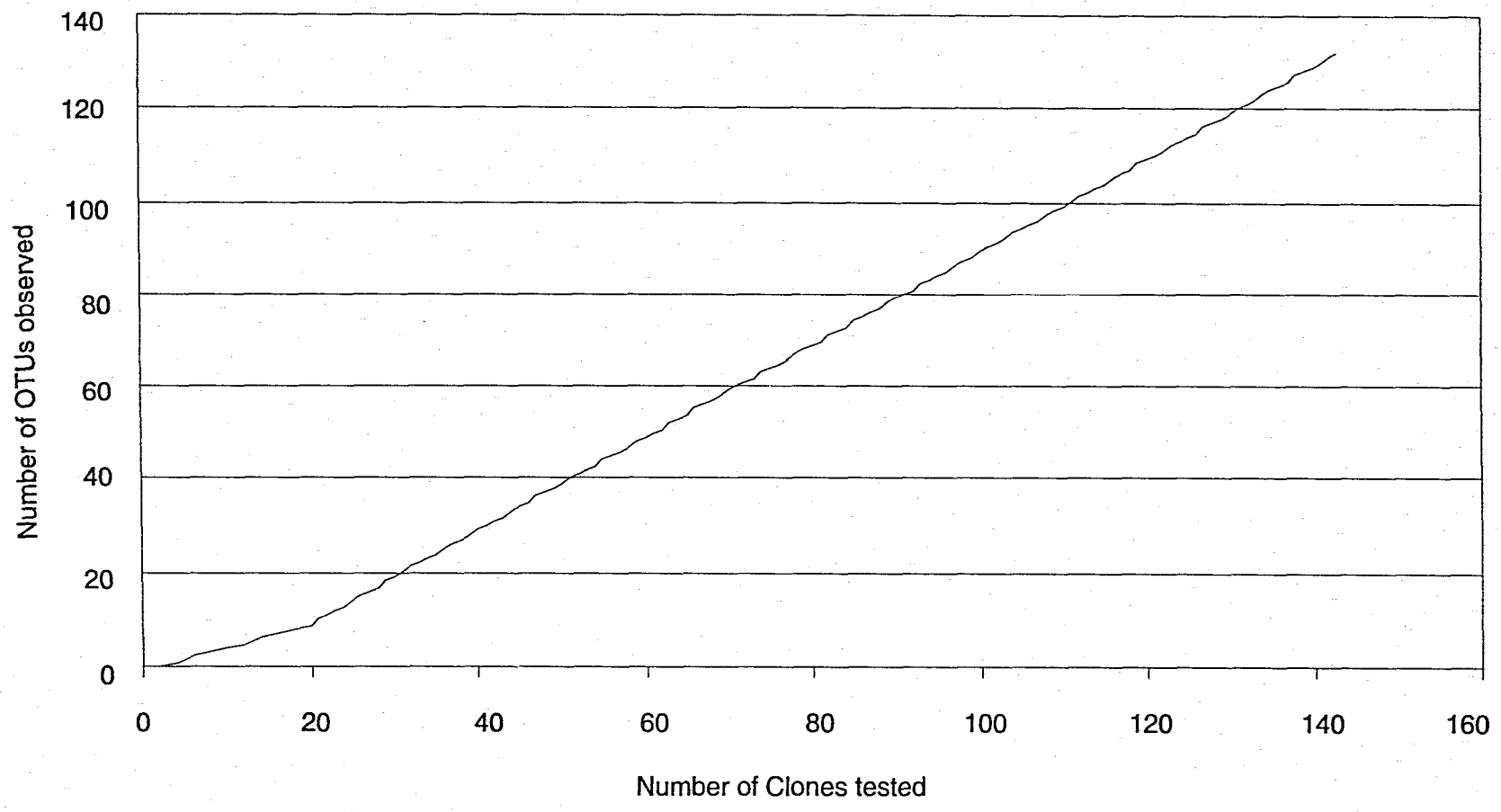

Figure 3.20. Rarefaction curve showing the number of new OTUs observed with an increasing number of clones analyzed from the PSA clone library. The rarefaction curve shown above is a steep linear line that does not appear to be nearing a plateau after clone library analysis.

Figure 3.21. Comparison of the community composition of SIB, SOC and BF clone libraries. Community composition was estimated by near full-length, bi-directional sequencing of 13 clones in the SOC clone library to represent 100 of 161 (62% total) SOC clones, near full-length, bi-directional sequencing of 8 clones in the SIB clone library to represent 52 of 92 (57% total) SIB clones, and near full-length, bi-directional of 9 clones in the BF clone library to represent 49 of 90 (54% total) BF clones.

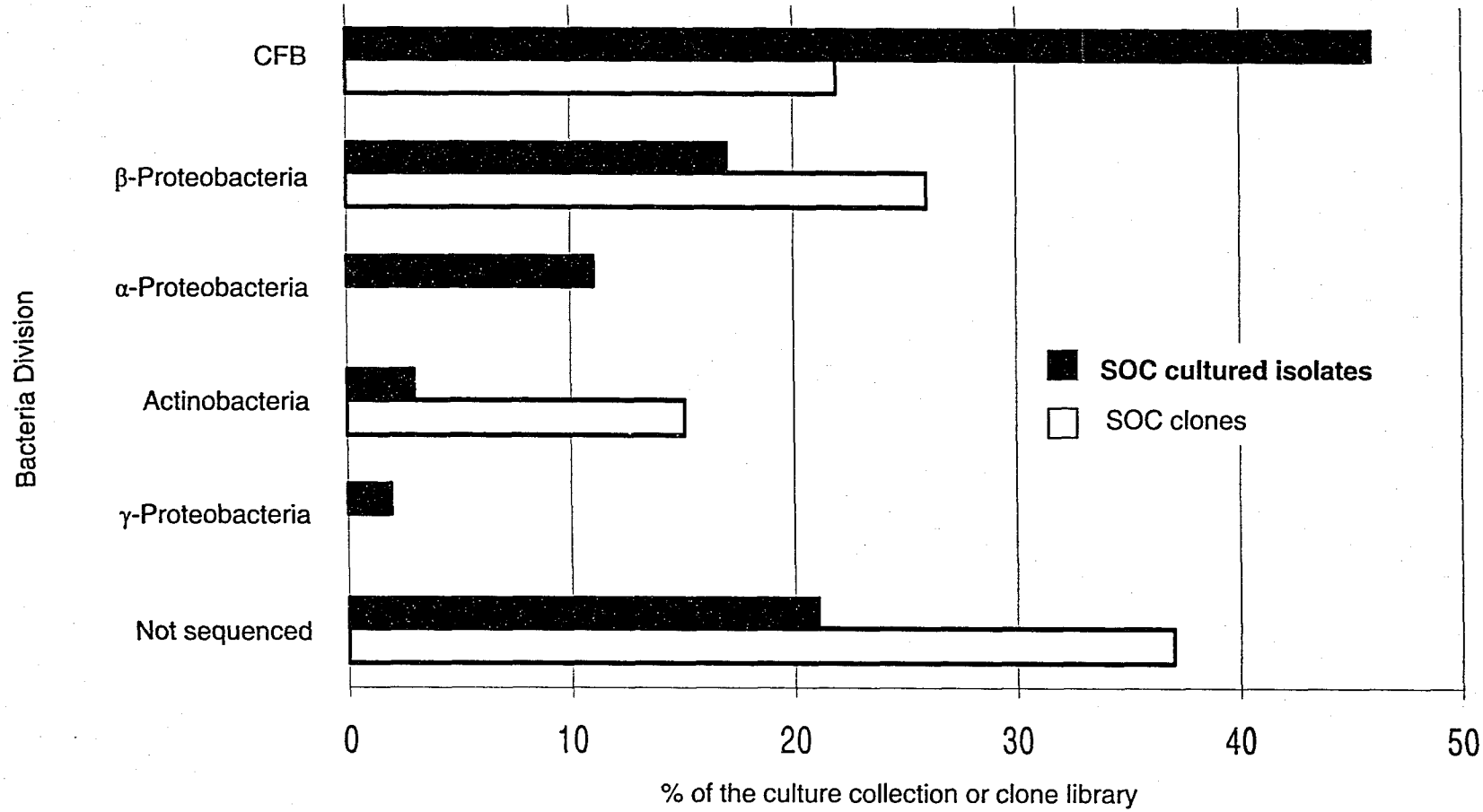

Figure 3.22. Comparison of composition of the SOC clone library and culture collection. The clone library composition was estimated by near full-length, bi-directional sequencing of 13 clones to represent 100 of 161 (62%) SOC clones. The SOC 16S rRNA gene culture collection was estimated by near full-length, bi-directional 16S rRNA gene sequencing of 11 cultured isolates to represent 106 of 134 (79%) isolates.

4.0 Summary and future research

4.1 Summary

In this study, culture-dependent ARDRA analysis was used to reveal the diversity of heterotrophic bacteria cultured from the SOC environment and to identify the dominant species within this sample. One hundred and thirty-four morphologically distinct pure-cultured isolates yielded 39 different OTUs in the SOC sample. The culture collection was dominated by a unique group of *Flavobacterium* spp. that was only cultured at 4°C. Several culturable heterotrophs were identified from subglacial water samples (SIB), subglacial ice samples (BF and BC) and proglacial samples (PSA), including organisms from the *Arthrobacter* and *Comamonas* genera.

Culture-independent ARDRA analysis was used to reveal the microbial diversity and identify dominant members of SOC, SIB and BF samples. The SOC community was the most diverse of the three samples, whereas the SIB and BF communities were less diverse and comprised only two bacterial divisions: the CFB and β-Proteobacteria. Differences in subglacial microbial communities can be attributed to differences in nutrient availability, energy sources and environmental stresses.

Comparisons of sequenced representatives from cultured-dependent and culture-independent ARDRA analysis showed reasonable overlap between the two techniques. Related bacteria from the Flavobacteriaceae family within the CFB division and Comamonadaceae family within the β-Proteobacteria division were detected in both techniques. Comparison of ARDRA analysis with t-RFLP analysis of the same JEG samples showed little overlap despite the fact that each method detected a large number of unique organisms. Only 11 terminal fragments found using t-RFLP were detected using ARDRA. These results show that combining culture-dependent and culture-independent characterization techniques allow a more comprehensive understanding of environmental samples.

4.2 Future research

Culture-dependent ARDRA analysis in this study focused largely on characterization of aerobic heterotrophic bacteria beneath JEG. Differences in temperature and oxygen availability during initial isolation of these organisms resulted in considerably different community compositions. Culture-dependent ARDRA analysis should be conducted under a wider range of incubation conditions, including under anaerobic conditions, so that more diverse organisms can be detected. Also, Archaea and Eukaryotes have been found glacier environments. ARDRA analysis should be performed using Archaea- and Eukarya-specific primers. Alternatively, PCR-independent molecular techniques, such as FISH, might be used as an easier method of detecting these specific organisms.

A group of potentially psychrophilic *Flavobacterium* spp. was dominant in the subglacial samples. This group of bacteria was also detected by t-RFLP analysis and shown to be unique to the subglacial community. Growth temperature ranges need to be performed on these isolates to confirm their psychrophilic nature. It would also be interesting to analyze the carotenoids produced by this group of microorganisms because it was noticed that several isolates within this group of microorganisms were differently pigmented. Production of carotenoids might be one method these microorganisms use to maintain membrane fluidity under subzero temperatures.

t-RFLP analysis revealed that bacterial communities beneath, on and adjacent to JEG appeared to be unique to each environment. Also, bacterial communities were heterogeneously distributed because of physical and chemical factors, such as energy sources, nutrient availability and environmental stresses. ARDRA analysis could be performed on additional samples that potentially comprise different microbial communities within the subglacial environment, proglacial and supraglacial environments. The large number of ARDRA patterns generated could then be used to create a database of ARDRA patterns so that comparisons of glacier bacteria can be made based on restriction patterns, instead of 16S rRNA gene sequencing, which can be quite costly.

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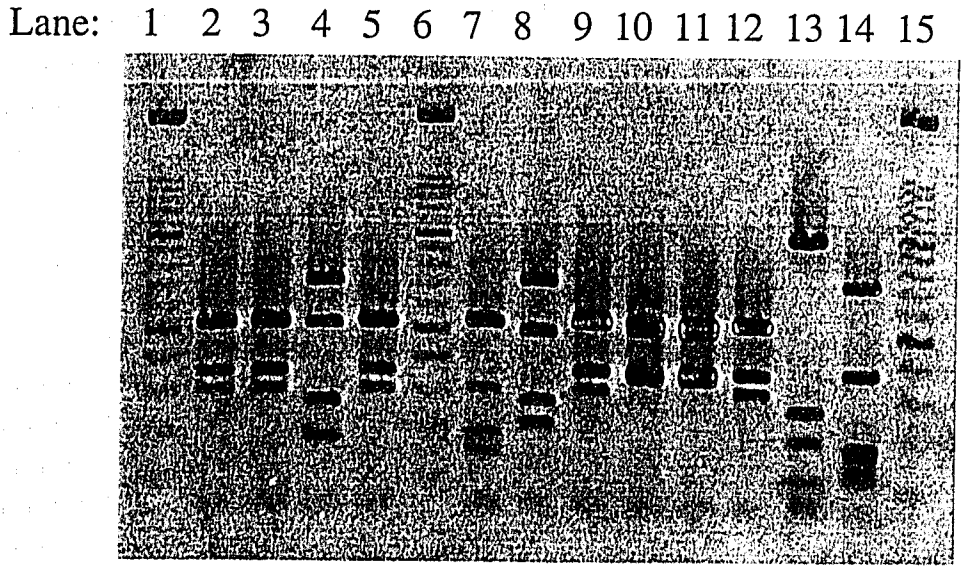

Appendix 1. A collection of 12 ARDRA patterns generated with *Cfo*I restriction enzyme. Lanes 1, 6 and 15 contain 100 bp molecular weight marker. Lanes 2, 3, 5, 9 and 10 contain the same ARDRA patterns and would be grouped into the same OTU.

Appendix 2. OTU number assigned to ARDRA patterns generated using *Cfo*I and *Hae*III restriction endonucleases on pure isolates. The number of isolates assigned to each OTU: in total, within each culture collection from PSA, SIB, BF, BC and SOC environments, and within each incubation condition (aerobic at 20°C, A20; aerobic at 4°C, A4; and microaerobic at 20°C, M20) are shown.

*Cfo*I ARDRA pattern	*Hae*III ARDRA pattern	OTU No.	Total No. of Isolates	PSA Isolates				SIB Isolates				BF Isolates			BC Isolates		SOC Isolates			
				A20	A4	M20	Total	A20	A4	M20	Total	A20	M20	Total	A20	Total	A20	A4	M20	Total
I	J	1	37		1		1				0			0		0	28	4	4	36
A	A	2	27	9	4	7	20			4	4	1		1	2	2				0
H	Dd	3	22				0				0			0		0		21		21
B	B	4	17				0				0		1	1		0	13	2	1	16
G	K	5	13				0				0			0		0	6		7	13
H	H	6	6	1			1		5		5			0		0				0
T	E	7	5				0				0			0		0	5			5
B	Ee	8	4				0				0			0		0		4		4
B	V	9	3				0				0			0		0	2	1		3
L	A	10	3	2			2			1	1			0		0				0
C	P	11	2		2		2				0			0		0				0
P	N	12	2				0	2			2			0		0				0
R	J	13	2				0				0			0		0	2			2
S	S	14	1				0				0			0		0	1			1
V	J	15	2				0				0			0		0	2			2
Bb	J	16	2				0				0			0		0			2	2
Gg	K	17	2				0				0			0		0			2	2
Hh	G	18	2				0		1	1	2			0		0				0
A	Ee	19	1				0				0			0	1	1				0
B	D	20	1				0				0			0		0	1			1
B	K	21	1				0				0			0		0	1			1
B	L	22	1				0	1			1			0		0				0
B	Ff	23	1				0				0			0		0		1		1
B	J	24	1				0				0			0		0		1		1
B	Bb	25	1				0				0			0		0			1	1
C	C	26	1	1			1				0			0		0				0
D	D	27	1	1			1				0			0		0				0
E	J	28	1			1	1				0			0		0				0
F	F	29	1				0				0	1		1		0				0
G	O	30	1				0				0			0		0	1			1
G	J	31	1				0				0			0		0	1			1
H	J	32	1				0				0			0		0		1		1
I	E	33	1				0				0			0		0	1			1

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I	B	34	1	0		0		0		0	1			1
I	X	35	1	0		0		0		0	1			1
I	Z	36	1	0		0		0		0		1		1
L	L	37	1	0	1	1		0		0				0
M	M	38	1	0	1	1		0		0				0
N	N	39	1	0	1	1		0		0				0
O	Q	40	1	0	1	1		0		0				0
Q	Ee	41	1	0		0		0	1	1				0
Q	A	42	1	0		0		0	1	1				0
S	J	43	1	0		0		0		0			1	1
T	Y	44	1	0		0		0		0	1			1
U	T	45	1	0		0		0		0	1			1
U	W	46	1	0		0		0		0			1	1
W	T	47	1	0		0		0		0	1			1
X	E	48	1	0		0		0		0	1			1
Y	J	49	1	0		0		0		0	1			1
Z	Cc	50	1	0		0		0		0		1		1
Aa	Aa	51	1	0		0		0		0			1	1
Cc	Gg	52	1	0		0		0		0		1		1
Dd	J	53	1	0		0		0		0		1		1
Ee	V	54	1	0		0		0		0			1	1
Ee	B	55	1	0		0		0		0			1	1
Ff	P	56	1	0	1	1		0		0				0
Gg	Aa	57	1	0		0		0		0			1	1
Ii	A	58	1	0		0	1	1		0				0
I	W	59	1	0		0		0		0	1			1

Appendix 3. ARDRA patterns using *Cfo*I and *Hae*III restriction endonucleases assigned to each isolate within SOC, SIB, BF, BC and PSA culture collections. Isolates are named based on JEG source, incubation condition (aerobic at 20°C, A20; aerobic at 4°C, A4; and microaerobic at 20°C, M20) and isolate number.

PSA A20 isolates			SOC isolates														
Isolate name	CfoI pattern	HaeIII pattern	Isolate number	CfoI pattern	HaeIII pattern	Isolate number	CfoI pattern	HaeIII pattern	Isolate number	CfoI pattern	HaeIII pattern	Isolate number	CfoI pattern	HaeIII pattern	Isolate number	CfoI pattern	HaeIII pattern
A20 2A	C	C	A20 1	B	K	A20 51	I	J	A4 2	H	Dd	M20 1	I	J			
A20 2B	D	D	A20 4	B	B	A20 52	I	J	A4 3	B	Ee	M20 3	S	J			
A20 3	A	A	A20 5	T	E	A20 53	I	J	A4 4	H	Dd	M20 5	G	K			
A20 4	L	A	A20 6	G	K	A20 54	I	J	A4 5	B	Ee	M20 6	B	B			
A20 6	L	A	A20 7	W	T	A20 55	I	J	A4 6	Z	Cc	M20 8	G	K			
A20 7	A	A	A20 8	I	J	A20 56	B	B	A4 7	B	Ff	M20 9	I	J			
A20 8	H	H	A20 9	I	J	A20 57	I	J	A4 10	Dd	J	M20 10	G	K			
A20 9	A	A	A20 10	I	J	A20 58	I	J	A4 14	H	Dd	M20 11	G	K			
A20 10	A	A	A20 11	I	E	A20 59	B	B	A4 15	H	Dd	M20 12	Aa	Aa			
A20 11	A	A	A20 12	T	Y	A20 60	G	K	A4 16	H	Dd	M20 14	S	S			
A20 12	A	A	A20 13	X	E	A20 61	I	J	A4 17	H	Dd	M20 16	G	K			
A20 13	A	A	A20 14	G	O	A20 62	I	J	A4 18	H	Dd	M20 18	U	W			
A20 14	A	A	A20 16	B	B	A20 63	V	J	A4 19	H	Dd	M20 19	B	Bb			
A20 15	A	A	A20 17	B	B	A20 64	I	J	A4 20	H	Dd	M20 20	Bb	J			
A4 2	A	A	A20 19	B	B	A20 65	G	K	A4 21	H	Dd	M20 23	G	K			
A4 3	A	A	A20 20	B	D	A20 66	G	J	A4 22	I	J	M20 24	I	J			
A4 4	C	P	A20 21	B	B	A20 67	I	J	A4 23	H	Dd	M20 26	Gg	K			
A4 5	A	A	A20 24	B	B	A20 68	I	X	A4 24	H	Dd	M20 27	G	K			
A4 6	A	A	A20 27	U	T	A20 69	I	J	A4 25	Cc	Gg	M20 28	Ee	V			
A4 7	I	J	A20 29	S	S	A20 71	Y	J	A4 26	I	Z	M20 29	Bb	J			
A4 8	C	P	A20 30A	B	B	A20 72	I	J	A4 27	H	J	M20 30	Gg	K			
M20 1	A	A	A20 30B	B	B	A20 73	I	J	A4 28	I	J	M20 33	Gg	Aa			
M20 5	A	A	A20 31	B	V	A20 74	I	J	A4 31	H	Dd	M20 34	I	J			
M20 1	A	A	A20 32	B	B	A20 75	G	K	A4 32	B	Ee	M20 35	Ee	B			
M20 5	A	A	A20 34	T	E	A20 76	I	J	A4 35	B	B						
M20 6	A	A	A20 35	T	E	A20 77	V	J	A4 36	B	J						
M20 7	E	J	A20 36	T	E	A20 78	I	B	A4 35	B	B						
M20 8	A	A	A20 37	I	J	A20 81	B	B	A4 36	B	J						
M20 9	A	A	A20 38	G	K	A20 82	B	V	A4 37	B	B						
M20 10	A	A	A20 39	I	J	A20 83	B	B	A4 39	B	Ee						
M20 14	A	A	A20 40A	I	J	A20 84	I	J	A4 40	H	Dd						
			A20 40B	I	J				A4 41	B	V						
			A20 41	G	K				A4 42	H	Dd						
			A20 42	I	J				A4 43	I	J						
			A20 44	I	J				A4 44	H	Dd						
			A20 45	I	J				A4 45	H	Dd						
			A20 46A	R	J				A4 46	H	Dd						
			A20 46B	R	J				A4 47	I	J						
			A20 47	T	E				A4 48	H	Dd						
			A20 48	I	W				A4 50	H	Dd						
			A20 50	I	J				AS 51	H	Dd						

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SIB isolates			BF isolates			BC isolates		
Isolate name	CfoI pattern	HaeIII pattern	Isolate name	CfoI pattern	HaeIII pattern	Isolate name	CfoI pattern	HaeIII pattern
A20 6	M	M	A20 17	F	F	A20 1	A	Ee
A20 13	L	L	A20 22	A	A	A20 6	Q	Ee
A20 14	Ff	P	A20 13	ii	A	A20 8	A	A
A20 22	N	N	M20 8	B	B	A20 10	Q	A
A20 23	B	L				A20 13	A	A
A20 24B1	P	N						
A20 24B2	P	N						
A20 27	O	Q						
A4 4	H	H						
A4 5	H	H						
A4 8	H	H						
A4 9	Hh	G						
A4 12	H	H						
A4 15	H	H						
M20 3	L	A						
M20 4	A	A						
M20 5	A	A						
M20 6	Hh	G						
M20 7	A	A						
M20 15	A	A						

Appendix 4. Number of clones from individual BF, SIB and SOC clone libraries and total number of clones comprising each OTU.

	number of clones					number of clones					number of clones					number of clones			
OTU #	BF	SIB	SOC	Total	OTU #	BF	SIB	SOC	Total	OTU #	BF	SIB	SOC	Total	OTU #	BF	SIB	SOC	Total
1	23	5	6	34	23	1	0	0	1	87	0	1	0	1	138	0	0	1	1
52	0	18	15	33	24	1	0	0	1	88	0	1	0	1	139	0	0	1	1
56	0	4	15	19	25	1	0	0	1	96	0	0	1	1	140	0	0	1	1
90	0	0	15	15	26	1	0	0	1	98	0	0	1	1	141	0	0	1	1
91	0	0	13	13	27	1	0	0	1	99	0	0	1	1	142	0	0	1	1
47	1	10	0	11	28	1	0	0	1	100	0	0	1	1	143	0	0	1	1
3	8	0	0	8	29	1	0	0	1	101	0	0	1	1	144	0	0	1	1
5	3	0	5	8	30	1	0	0	1	102	0	0	1	1	145	0	0	1	1
11	1	0	6	7	31	1	0	0	1	103	0	0	1	1	146	0	0	1	1
54	0	6	0	6	32	1	0	0	1	104	0	0	1	1	147	0	0	1	1
60	0	2	4	6	33	1	0	0	1	105	0	0	1	1	148	0	0	1	1
92	0	0	6	6	34	1	0	0	1	106	0	0	1	1	149	0	0	1	1
93	0	0	5	5	35	1	0	0	1	107	0	0	1	1	150	0	0	1	1
4	4	0	0	4	36	1	0	0	1	108	0	0	1	1	151	0	0	1	1
55	0	4	0	4	37	1	0	0	1	109	0	0	1	1	152	0	0	1	1
57	0	4	0	4	38	1	0	0	1	110	0	0	1	1	153	0	0	1	1
94	0	0	4	4	39	1	0	0	1	111	0	0	1	1	154	0	0	1	1
6	3	0	0	3	40	1	0	0	1	112	0	0	1	1	155	0	0	1	1
58	0	3	0	3	41	1	0	0	1	113	0	0	1	1					
95	0	0	3	3	42	1	0	0	1	114	0	0	1	1					
7	2	0	0	2	43	1	0	0	1	115	0	0	1	1					
8	2	0	0	2	44	1	0	0	1	116	0	0	1	1					
9	2	0	0	2	45	1	0	0	1	117	0	0	1	1					
10	2	0	0	2	46	1	0	0	1	118	0	0	1	1					
17	1	0	1	2	48	1	0	0	1	119	0	0	1	1					
59	0	2	0	2	49	1	0	0	1	120	0	0	1	1					
61	0	2	0	2	50	1	0	0	1	121	0	0	1	1					
62	0	2	0	2	51	1	0	0	1	122	0	0	1	1					
63	0	2	0	2	65	0	1	0	1	123	0	0	1	1					
64	0	2	0	2	66	0	1	0	1	124	0	0	1	1					
89	0	2	0	2	67	0	1	0	1	125	0	0	1	1					
97	0	0	2	2	68	0	1	0	1	126	0	0	1	1					
12	1	0	0	1	69	0	1	0	1	127	0	0	1	1					
13	1	0	0	1	70	0	1	0	1	128	0	0	1	1					
14	1	0	0	1	71	0	1	0	1	129	0	0	1	1					
15	1	0	0	1	72	0	1	0	1	130	0	0	1	1					
16	1	0	0	1	73	0	1	0	1	131	0	0	1	1					
18	1	0	0	1	74	0	1	0	1	132	0	0	1	1					
19	1	0	0	1	75	0	1	0	1	133	0	0	1	1					
20	1	0	0	1	76	0	1	0	1	134	0	0	1	1					
21	1	0	0	1	77	0	1	0	1	135	0	0	1	1					
22	1	0	0	1	78	0	1	0	1	136	0	0	1	1					

Appendix 5. ARDRA patterns generated using *Cfo*I and *Hae*III restriction enzymes assigned to OTUs within SOC, SIB, and BF clone libraries.

*Cfo*I pattern	*Hae*III patter	OTU #	*Cfo*I pattern	*Hae*III patter	OTU #	*Cfo*I pattern	*Hae*III patter	OTU #	*Cfo*I pattern	*Hae*III patter	OTU #	*Cfo*I pattern	*Hae*III patter	OTU #
BD	BE	1	BS	BQ	43	IB	IE	52	OC	OB	56	OA	Oaa	124
BE	BE	3	BT	BR	44	IB	IB	47	OF	OE	90	Oaa	Obb	125
BA	BA	4	BU	BI	45	IE	IE	54	OC	OF	52	OH	OE	126
BA	BL	5	BV	BH	46	IC	IC	1	OA	OA	91	Obb	OW	127
BY	BV	6	BV	BU	47	IB	ID	55	OB	OB	11	Occ	Occ	128
Bb	BY	7	BX	BP	48	IB	IG	56	ON	OE	92	OF	OB	129
Bc	BF	8	BX	BB	49	ID	ID	57	OI	OI	1	Odd	Od	130
BG	BW	9	BX	BV	50	IE	IO	58	OB	OF	5	Off	Oee	131
BI	BF	10	BZ	BF	51	IE	IB	59	OJ	OA	93	OE	Off	132
BA	BK	11				IF	IH	60	OR	OT	94	OL	Oaa	133
Ba	BX	12				IG	II	61	OS	OQ	60	Ogg	OM	134
Ba	BW	13				II	IJ	62	OL	OM	95	ON	Off	135
BB	BA	14				IN	IL	63	OB	ON	96	Ojj	OD	136
BB	BE	15				II	IZ	64	OD	OA	97	OC	OK	137
Bb	BF	16				IX	IY	89	OD	OC	98	Odd	Ogg	138
Bb	Be	17				IA	IA	65	OE	OD	17	OF	Ohh	139
BC	BB	18				IC	ID	66	OF	OH	99	OE	Oii	140
Bc	BZ	19				IE	IN	67	OG	OI	100	OK	OH	141
BD	BC	20				IG	IQ	68	OH	OA	101	OJ	Ojj	142
Bd	BZ	21				ID	IP	69	OI	OB	102	OJ	Okk	143
Bd	Bd	22				IH	IB	70	OC	OI	103	Okk	OE	144
BE	BD	23				IJ	IR	71	OJ	OJ	104	OE	OM	145
Be	Bb	24				IK	IC	72	OI	OK	105	Oll	OF	146
BF	BD	25				IL	IR	73	OK	OE	106	OY	Oll	147
BF	BS	26				IM	IK	74	OC	OL	107	Ohh	OB	148
Bf	Bf	27				IO	IM	75	OM	OD	108	Odd	OB	149
BG	BA	28				IP	IO	76	OP	OO	109	Oee	OA	150
BG	BT	29				IQ	IL	77	OE	OR	110	Omm	Oll	151
Bg	Bc	30				IR	IS	78	OO	OS	111	Ooo	OK	152
BH	BD	31				IS	IQ	79	OQ	OF	112	Opp	OB	153
BI	BA	32				IE	IP	80	OI	OU	113	OG	OK	154
BJ	BI	33				IC	IS	81	Ohh	OP	114	Odd	Omm	155
BK	BJ	34				IT	IT	82	OU	OV	115			
BK	BA	35				IE	IU	83	OV	OW	116			
BL	BC	36				IE	IV	84	OW	OJ	117			
BM	BM	37				IU	IW	85	OX	OX	118			
BN	BN	38				IV	IA	86	OY	OP	119			
BO	BO	39				IW	IX	87	OZ	OG	120			
BP	BE	40				IY	IL	88	OK	OY	121			
BQ	BP	41							OC	OY	122			
BR	BE	42							OR	OZ	123			

Appendix 6. *Cfo*I and *Hae*III ARDRA patterns for BF, SIB and SOC clones.

BF Clone Library

Clone name	*Cfo*I pattern	*Hae*III patter	Clone name	*Cfo*I pattern	*Hae*III patter	Clone name	*Cfo*I pattern	*Hae*III patter
M 1C	BV	BU	A 1H	BA	BA	A 12B	BQ	BP
M 1D	BW	BV	A 2A	BB	BA	A 12C	BR	BE
M 1E	BX	BP	A 2E	BC	BB	A 12D	BS	BQ
M 1F	BG	BW	A 3C	BD	BC	A 12E	BT	BR
M 2C	BY	BV	A 3D	BA	BA	A 12F	BF	BS
M 2D	BZ	BF	A 3E	BA	BA	A 12G	BE	BE
M 2E	BW	BV	A 4E	BF	BD	A 9E	BD	BE
M 3C	BW	BV	A 5D	BE	BE	A 4H	BU	BI
M 3D	BX	BB	A 6E	BG	BA	A 12H	BG	BT
M 3E	Ba	BX	A 6G	BE	BE			
M 4B	BX	BV	A 7B	BD	BE			
M 4C	Bb	BY	A 7C	BD	BE			
M 4D	BY	BV	A 7D	BD	BE			
M 4E	Bc	BF	A 7E	BD	BE			
M 5C	BW	BV	A 7F	BH	BD			
M 5D	Bc	BZ	A 7G	BE	BE			
M 5E	BW	BV	A 7H	BD	BE			
M 5G	BK	Ba	A 8A	BI	BF			
M 6B	BG	BW	A 8B	BA	BL			
M 6C	Be	Bb	A 8C	BV	BH			
M 6D	Bd	BZ	A 8D	BB	BE			
M 6H	Bc	BF	A 8E	BD	BE			
M 7B	BW	BV	A 8F	BE	BE			
M 7C	BW	BV	A 8H	BD	BE			
M 9A	Bb	BF	A 9A	BJ	BI			
M 9B	BW	BV	A 9B	BK	BJ			
M 9C	BW	BV	A 9D	BA	BK			
M 8B	Ba	BW	A 9F	BE	BD			
M 8C	BW	BV	A 9G	BL	BC			
M 8D	Bf	Bf	A 9H	BA	BL			
M 10B	BW	BV	A 10B	BA	BL			
M 10C	Bg	Bc	A 10C	BE	BE			
M 10D	BW	BV	A 10D	BE	BE			
M 11B	BW	BV	A 10E	BM	BM			
M 11C	BI	BF	A 10F	BD	BE			
M 11D	Bd	Bd	A 11A	BN	BN			
M 12A	Bb	Be	A 11B	BO	BO			
M 12B	BY	BV	A 11C	BD	BE			
M 12C	BW	BV	A 11D	BA	BA			
M 12D	BW	BV	A 11E	BP	BE			
			A 11F	BI	BA			
			A 12A	BE	BE			

SIB Clone Library

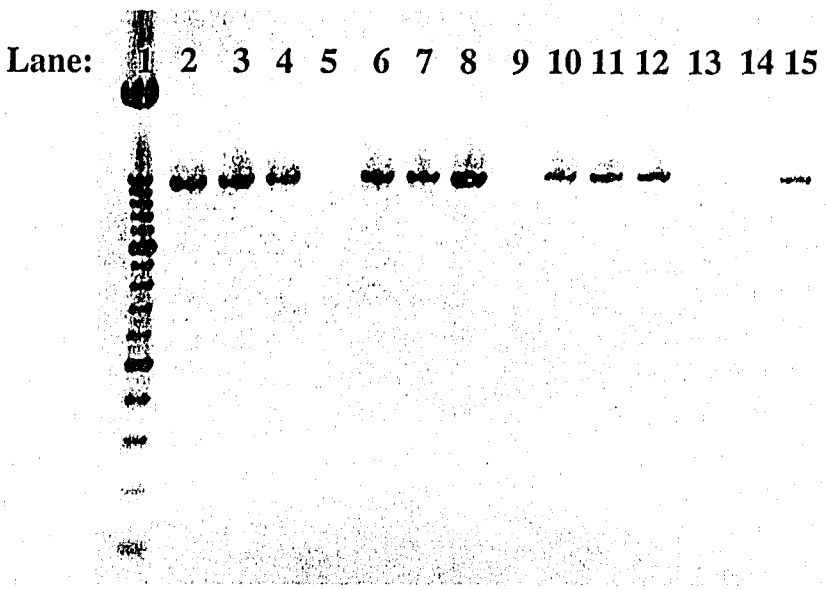
Clone name	*Cfo*I pattern	*Hae*III patter	Clone name	*Cfo*I pattern	*Hae*III patter
1 1A	IS	II	2 1A	IA	IA
1 1B	IB	IB	2 1B	IB	IB
1 1C	IB	IE	2 1C	IC	IC
1 1G	IE	IP	2 1D	ID	ID
1 1H	IB	IE	2 1E	IB	IE
1 2A	IC	IS	2 1G	IB	IG
1 2B	IB	ID	2 4A	ID	ID
1 2C	IB	ID	2 4C	IB	IE
1 2D	IT	IT	2 4D	IE	IE
1 2H	IB	IE	2 4E	IC	ID
1 3B	IE	IU	2 4F	IF	IH
1 4A	IE	IV	2 4G	IE	IE
1 4B	IB	IE	2 4H	ID	ID
1 4C	IU	IW	2 5A	IC	IC
1 5A	IV	IA	2 5B	IB	IE
1 5C	IB	IE	2 5C	IB	IE
1 5D	IW	IX	2 5D	IB	IE
1 5H	IB	IE	2 5E	IC	IC
1 6B	IC	IC	2 5H	IB	IE
1 6C	IB	IB	2 6C	IE	IB
1 7B	IX	IY	2 6D	IE	IB
1 7G	IB	IE	2 6G	IG	II
1 8A	IB	IG	2 6H	IH	IB
1 8B	IY	IL	2 7B	II	IJ
1 9A	IB	IB	2 7D	IC	IC
1 9E	IE	IO	2 7E	IJ	IR
1 9F	II	IZ	2 7H	IB	IB
1 9G	II	IZ	2 8E	IK	IC
1 10A	IF	IH	2 9A	IG	II
1 10B	IX	IY	2 10G	IM	IK
1 10D	IE	IO	2 10H	IN	IL
1 10G	IE	IO	2 11A	IN	IL
1 10H	IB	IE	2 11C	IO	IM
1 11A	IB	IE	2 11D	IE	IN
1 11B	IE	IE	2 11G	IB	IE
1 11D	ID	ID	2 12C	IB	IG
1 12A	IE	IE	2 12D	IP	IO
1 12B	IB	IB	2 12E	IB	IE
1 12C	IB	IB	2 12F	IB	IG
		II	2 12G	IE	IE
			2 12H	IB	ID
			2 2A	IB	IE

SOC Clone Library

Clone name	*Cfo*I pattern	*Hae*III patter	Clone name	*Cfo*I pattern	*Hae*III patter
1 1A	OA	OA	1 6B	OA	OA
1 1B	OB	OB	1 6C	OO	OS
1 1C	OC	OB	1 6E	OC	OF
1 1D	OA	OA	1 6F	OB	OF
1 1F	OD	OC	1 6G	OB	OF
1 1G	OA	OA	1 6H	OJ	OA
1 2A	OE	OD	1 7A	OQ	OF
1 2B	OF	OE	1 7C	OR	OT
1 2C	OF	OH	1 7D	OC	OB
1 2D	OG	OI	1 7E	OC	OF
1 2E	OH	OA	1 7F	OS	OQ
1 2F	OI	OB	1 7G	ON	OE
1 3A	OC	OI	1 7H	OI	OU
1 3B	OJ	OJ	1 8A	OA	OA
1 3C	OI	OK	1 8B	OD	OA
1 3D	OK	OE	1 8C	OI	OI
1 3E	OC	OL	1 8D	OA	OA
1 3F	OA	OA	1 8E	OL	OM
1 3G	OC	OF	1 8F	OC	OF
1 3H	OC	OF	1 8G	OS	OQ
1 4A	OC	OB	1 9A	OB	OB
1 4B	OL	OM	1 9B	OB	OB
1 4C	OF	OE	1 9C	OI	OI
1 4D	OM	OD	1 9D	OA	OA
1 4E	OC	OF	1 9E	OC	OB
1 4F	OB	ON	1 9F	OR	OT
1 4G	ON	OE	1 9G	OU	OV
1 4H	OB	OF	1 10A	ON	OE
1 5A	OF	OE	1 10B	OJ	OA
1 5B	OP	OO	1 10D	OV	OW
1 5C	OF	OE	1 10E	OA	OA
1 5D	OC	OF	1 10F	OR	OT
1 5E	OA	OA	1 10G	OA	OA
1 5F	Ohh	OP	1 10H	OC	OB
1 5G	OS	OQ	1 11A	OW	OJ
1 5H	ON	OE	1 11B	OX	OX
1 6A	OE	OR	1 11C	OF	OE
			1 11D	OC	OB
			1 11E	OY	OP
			1 11F	OC	OF
			1 11G	OB	ON
			1 11H	OI	OI

CONTINUED

SOC Clone Library							
Clone name	*Cfo*I pattern	*Hae*III patter	Clone name	*Cfo*I pattern	*Hae*III patter
1 12B	OF	OE	2 5F	OA	OA
1 12C	OF	OE	2 5G	Ogg	OM
1 12D	OC	OB	2 5H	ON	Off
1 12E	OZ	OG	2 6A	OC	OF
1 12F	OF	OE	2 6D	OC	OB
2 1B	OC	OB	2 6E	OS	OQ
2 1C	OK	OY	2 6F	Oij	OD
2 1D	OF	OE	2 6G	OB	OB
2 1E	OC	OY	2 6H	OC	OK
2 1F	OR	OZ	2 7A	Odd	Ogg
2 1G	OA	Oaa	2 7B	OF	OE
2 2A	OI	OI	2 7C	OJ	OA
2 2B	ON	OE	2 7D	OJ	OA
2 2C	Oaa	Obb	2 7E	OF	Ohh
2 2D	OC	OF	2 7F	OC	OB
2 2E	OH	OE	2 7G	OC	OB
2 2F	OB	ON	2 7H	OE	Oii
2 2G	OC	OF	2 8A	OA	OA
2 2H	Obb	OW	2 8B	OK	OH
2 3A	OB	OB	2 8C	OJ	Oij
2 3B	Occ	Occ	2 8D	OJ	Okk
2 3C	OR	OT	2 8E	OC	OF
2 3D	OD	OA	2 8F	OF	OE
2 3E	OL	OM	2 8G	Okk	OE
2 3G	OF	OB	2 8H	OE	OM
2 3H	Occ	OB	2 9H	OF	OE
2 4B	OD	Odd	2 10A	OC	OB
2 4C	Off	Oee	2 10B	Oll	OF
2 4D	ON	OE	2 10C	OI	OI
2 4E	OF	OE	2 10D	OB	OF
2 4F	OC	OB	2 10E	OY	Oll
2 4G	OC	OF	2 10F	OF	OE
2 5A	OB	OB	2 10G	Ohh	OB
2 5B	OC	OF	2 10H	Odd	OM
2 5C	OE	Off	2 11A	Oee	OA
2 5D	OF	OE	2 11B	Omm	Oll
2 5E	OL	Oaa	2 11C	OI	OI
2 5F	OA	OA	2 11D	OC	OF
2 5G	Ogg	OM	2 11E	Onn	OM
2 5H	ON	Off	2 11F	Ooo	OK
2 6A	OC	OF	2 11G	OJ	OA
2 6D	OC	OB	2 11H	Opp	OB
2 5A	OB	OB	2 12D	OC	OB
2 5B	OC	OF	2 12E	OG	OK
2 5C	OE	Off	2 12F	Odd	OMm
2 5D	OF	OE	2 12G	OB	OF
2 5E	OL	Oaa			



Appendix 7. 16S rRNA gene amplification after three consecutive genomic DNA extractions from three pure isolates. Lane 1 contains 100 bp molecular weight marker. Lanes 2, 3 and 4 show amplification of the 16S rRNA gene from three pure isolates after the first genomic DNA extraction. Lane 5 shows the negative extraction control in the first extraction. Lanes 6, 7 and 8 show amplification of the 16S rRNA gene from the same three pure isolates after a second genomic DNA extraction. Lane 9 shows the negative extraction control in the second extraction. Lanes 10, 11 and 12 show amplification of the 16S rRNA gene from the same three pure isolates after a third genomic DNA extraction. Lane 13 shows the negative extraction control in the third extraction. Lane 14 and 15 show negative and positive PCR amplification controls, respectively.