

Review

Psychrophilic Extremophiles from Antarctica: Biodiversity and Biotechnological Potential

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Abstract : Recently there has been a rapid accumulation of knowledge of microbial life in cold and frozen ecosystems. This understanding has revealed the extensive diversity of psychrophilic prokaryotes. Cultivation-based and molecular-based surveys have been performed in Antarctic habitats ranging from glacial ice to continental shelf sediments. Results indicate that psychrophilic taxa permeate throughout the Bacteria while they represent a more mysterious element of diversity in the Archaea owing to a notable lack of cultured strains. In certain cold climate ecosystems the diversity of psychrophilic populations reach levels comparable to the richest temperate equivalents. Within these communities must exist tremendous genetic diversity that is potentially of fundamental and of practical value. So far this genetic pool has been hardly explored. Only recently have genomic data become available for various psychrophilic prokaryotes and more is required. This owes to the fact that psychrophilic microbes possess manifold mechanisms for cold adaptations, which not only provide enhanced survival and persistence but probably also contributes to niche specialisation. These mechanisms, including cold-active and ice-active proteins, polyunsaturated lipids and exopolysaccharides also have a great interest to biotechnologists.

Key words : Psychrophilic bacteria, psychrophily, Antarctica, 16S rRNA, Evolution

1. Biodiscovery and the Antarctic Treaty

The Antarctic Treaty was established in 1961 to protect Antarctica from uncontrolled commercial exploitation including mining, balkanization and militarization. Every 10 years or so the Antarctic Treaty is updated and in 1991 the Madrid Protocol was established which contains several annexes pertaining to protection of the Antarctic from environmental degradation through human activity. More recently, several Antarctic and Southern Ocean Coalition members have noticed emerging trends towards increased commercialization which were considered disturbing and express the belief that they threaten to “*overwhelm the Antarctic Treaty’s and the Protocol’s core values of science, peace and cooperation*” (ACTM XXII 1999). Indeed, some in the popular press end of the scientific and policy community see “*bio-piracy*” as a significant threat to Antarctica (Kirby 2004). The Antarctic Treaty advisory

body, the Scientific Committee on Antarctic Research (ACTM XXII 1999), have also raised recently several concerns about bioprospecting, quoting: “*While no current instance of harvesting for biotechnology is known, there are obvious environmental ramifications of the taking of animals and plants as a commercial venture*”...“*may develop into important pressures on Antarctic resources*”. Overall, popular press, sometimes with an hysterical and somewhat ignorant emphasis, suggests there is a new “*cold rush*” in the Antarctic (and perhaps more quietly in the Arctic) e.g. “*On Thin Ice: How the Quest for a Billion-Dollar Microbe is Running Out of Control in Earth’s Last Wilderness*” (Connor 2004). With these concerns a sensible, sensitive and highly justifiable approach must be conducted in the study of Antarctic microbial diversity for *any* form of commercial gain. Better understanding of microbial ecosystems and community members therein will provide more sensible outcomes for biotechnology that hopefully will be seen as useful and helpful not purely done for profit, exploitation and a need to justify science research dollars.

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In the case of the Antarctic continent and surrounding ocean, fundamental science should always take precedence since we still know so little about these places. Biotechnology (and commercialisation) can only benefit from a richer understanding of natural ecosystems and thus should be considered a goal of scientific labours, not the goal.

2. Polar Biodiscovery

Many polar ecosystems have been investigated in order to understand what types of microorganisms are present. In terms of prokaryotes this research has accelerated over the last 5 years. Both culture-dependent and culture-independent approaches have been used to explore polar ecosystems including sea-ice, seawater, marine aggregates, marine sediment, sponge microbiota, tundra cryopegs, soils (feldfield, ornithogenic etc.), ice (ice sheet, glaciers, cryconite holes), lakes and ponds (e.g. glacial, marine-derived). These studies have accumulated knowledge answering the question about what bacteria inhabit perpetually cold ecosystems. Cultivation studies are still very incomplete since growth conditions used for enrichment and isolation have been limited in scope. The use of novel approaches, selective techniques and more in depth studies should expand the current knowledge base for polar microbial biodiversity. High throughput multi-well and encapsulation techniques, filter-based isolation and other novel approaches to isolation offer the means to obtain a richer variety of bacteria, avoiding well known rapidly growing taxa which usually overwhelm conventional cultivation techniques (Connon and Giovannoni 2002; Kaeberlein *et al.* 2002; Hahn *et al.* 2004; Stevenson *et al.* 2004). Also the use of low nutrient agar media incorporating ecologically relevant and complex substrates and long incubation times have been used successfully to obtain novel taxa from seawater and soil. This includes strains from taxonomic groups previously only known from molecular sequence data (Joseph *et al.* 2003; Schoenborn *et al.* 2004). Direct molecular sampling of genetic diversity is also well established. Environmental genome libraries analysed by high-throughput technology now offer a database for basic knowledge as well as a source for the exploration of novel biotechnological possibilities (e.g. Venter *et al.* 2004).

3. Psychrophilic prokaryotes and biotechnology

Psychrophilic organisms are specialised for growth at relatively low temperatures, typically exhibiting optimal growth yields at 5-15°C and occupy one of the extreme

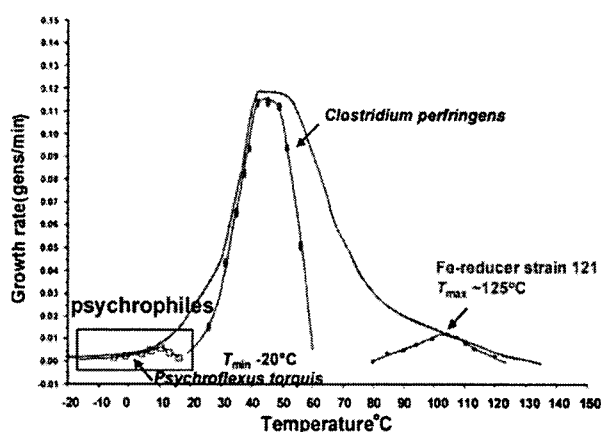


Fig. 1. Relationship between prokaryote growth rate and temperature. Growth kinetic curves are shown for a psychrophile (*Psychroflexus torquis*), a fast growing mesophile (*Clostridium perfringens*) and an so far unnamed hyperthermophile. T_{min} and T_{max} are mathematically estimated values of the lowest and highest temperatures for growth, respectively.

ends of the temperature spectrum for life as described in Fig. 1. At temperatures of 25°C or more they rapidly die and thus they represent an extremophile with specific molecular and biochemical adaptations, which are of interest to ecologists, molecular biologists, biochemists and biotechnologists. Though psychrophilic bacteria have been known to exist for several decades (Morita 1975) it is only in the last decade that a heightened interest in psychrophiles has occurred. This comes partly from the interest in extremophiles in general, particularly hyperthermophiles, in which various biotechnology (Table 1)- and astrobiology oriented initiatives have driven research and encouraged bioprospecting for more examples to investigate. Nichols and colleagues (1999) discuss at some length various aspects of Antarctic bacteria that have interesting possibilities for biotechnology outside of the pharmaceutical area. In the case of psychrophilic bacteria the research is also linked to studies of life in polar regions in which they proliferate.

Psychrophilic bacteria (as defined here) do not occur in environments affected by intermittent solar heating or in aquatic ecosystems where seasonal temperatures fluctuate above 4°C. Marine ecosystems have been found to harbour by far the highest diversity of psychrophiles, in particular sea-ice and sediment (Bowman *et al.* 1997, 2003). The biochemical and biophysical basis of adaptation mechanisms to cold are diverse and have undergone significant research and directly relate to biotechnological applications (Table 1).

Table 1. Antarctic and Southern Ocean ecosystems corresponding conditions and the possibilities for biotechnology.

Habitat	Defining condition	Bioprospecting opportunities
Seawater, maritime lakes, sea-ice	Low temperature	Cold-active enzymes/catalysts; bioremediation; surfactants; anti-freezes; polyunsaturated fatty acids; novel pigments
Southern Ocean seawater, Sea-ice	Low nutrient concentration	High affinity catalysts and ligands; food additives (pigments, emulsifiers)
Hypersaline lakes	High salinity	Halotolerant enzymes; novel metabolites; novel pigments
Marine/lake sediment	Anaerobic/low temperature	Anaerobic biotransformations; novel bioactives
Soil, lithic habitats	Cold, dry	Novel bioactives

Critical cold adaptations of greatest recent interest include the features given below and have been reviewed extensively in the literature.

Cold active enzymes

Provides resistance to cold denaturation and improved catalytic efficiency.

Thermal hysteresis (“anti-freeze”) proteins

A structurally diverse group of proteins, which bind to and inhibit growth of nascent ice crystals. Some anti-freeze proteins have been shown to have an ice-nucleating domain thus allowing control of ice crystal formation.

Fatty acid modulation in cell membranes

Improves membrane permeability and nutrient mass transport. This includes synthesis of omega-3 polyunsaturated fatty acids (PUFA) in some bacteria but also extends to monounsaturated and branched chain fatty acids. PUFA are of interest as they are an important nutrient for higher life forms including humans, needed for optimal nervous system and cardiovascular health (reviewed in Nichols *et al.* 1999).

Exopolysaccharides (EPS)

Possible role in cryoprotection and/or enhancement of nutrient acquisition; can be cold temperature stimulated.

The following sections are brief overviews of research carried out at the University of Tasmania investigating microbial diversity in key polar habitats and indicates some areas of interest in the realm of biotechnology.

4. Southern Ocean Sea-ice and related sea-water biodiversity

Sea-ice occurs as a spatially immense but highly transient ecosystem around Antarctica. In mid-winter the sea-ice extent is about 20×10^6 km²; this drops to 4×10^6 km² in summer. Sea-ice has a profound effect on the Southern

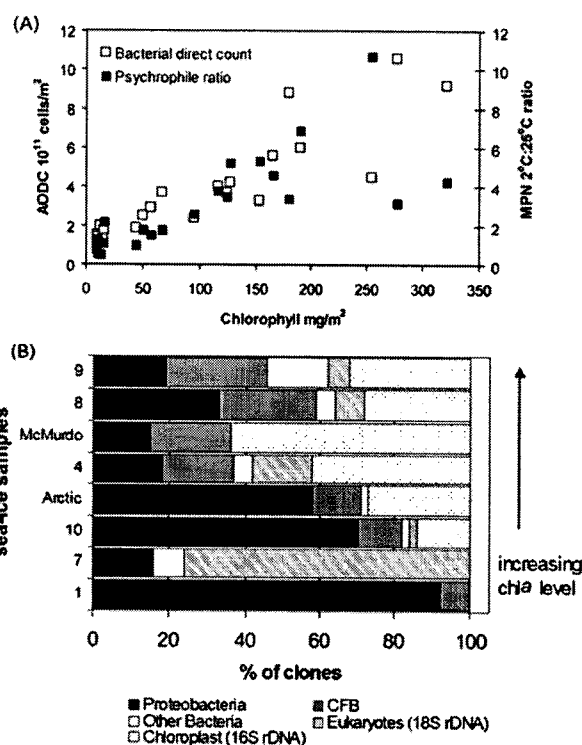


Fig. 2. (A) Psychrophilic bacterial most probable number (MPN) count and total direct count in relation to sea-ice chlorophyll *a* levels. (B) Proportions of different taxonomic groups based on rRNA gene sequence data derived from sea-ice samples (samples 1, 7, 10, 4, 8 and 9 are from different locations in the Southern Ocean ice pack; McMurdo-sample was from McMurdo Sound; Arctic-sample, was from the Arctic Ocean near Baffin Is. (Northern Open Water Polynya). Samples are arranged in order of chlorophyll content).

Ocean marine ecosystem as well as global climate (reviewed Brown and Bowman 2002). Sea-ice hosts large blooms of microalgae (distribution of which is somewhat patchy) representing about one-third of the Southern Ocean production. The sea-ice phytoplankton community also helps support a complex community incorporating small

metazoans, protists, bacteria and viruses. Psychrophilic bacteria have been shown to be most numerous and diverse in these sea-ice algal assemblages (Fig. 2A). Molecular studies based on 16S rRNA genes indicate that total biodiversity is also much higher in the assemblages compared to ice lacking assemblages, which tend to be dominated by class *Gammaproteobacteria* (taxonomic terminology is taken from Garrity *et al.* 2003). In complex algal assemblages dominant bacterial groups are essentially similar to those found in seawater particulates including mostly classes *Alphaproteobacteria* and *Gammaproteobacteria*, the phylum *Bacteroidetes* but also including smaller numbers of other phyla including *Verrucomicrobia* and *Actinobacteria* (Fig. 2B, from Brown and Bowman 2001). Increasing algal density (measured by chlorophyll a levels) appears

to be increasingly dominated by members of the class *Flavobacteria*, part of phylum *Bacteroidetes* (also called the CFB-*Cytophaga-Flavobacterium-Bacteroides* group) (Brown and Bowman 2001, Fig. 2B). Particulates in the Southern Ocean Antarctic Zone (59°S and higher latitudes) may derive partly from sea-ice assemblages (thawing and dispersal caused by feeding metazoans) as well as surface water production and are largely untapped for microbial diversity.

Recently we investigated seawater and seawater particulates for bacteria belonging to the class *Flavobacteria* (Abell and Bowman 2005). This was done since little was known about this group's diversity in the Southern Ocean but also it had been recognized that the group made up a large fraction of the bacterioplanktonic community, especially

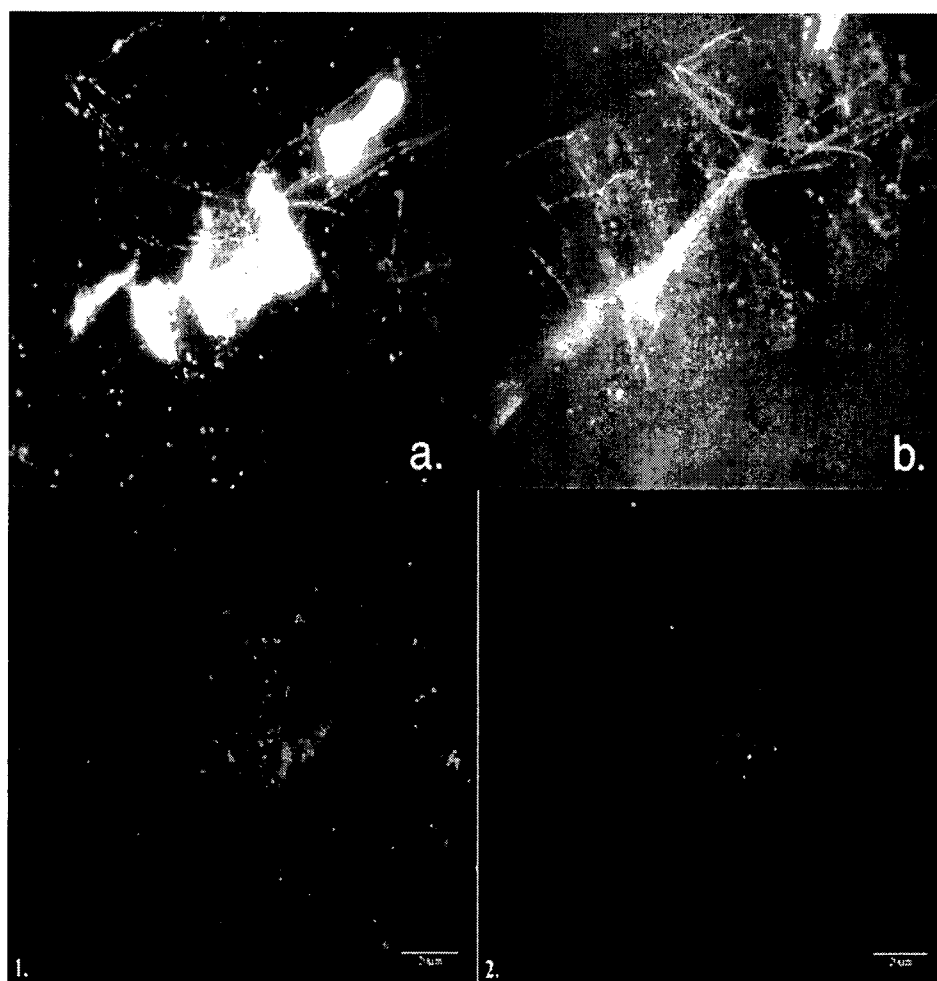


Fig. 3. (a) Bacteria colonizing Southern Ocean seawater particulates visualized by epifluorescence using DAPI. (b) The same image as (a) with cells detected with *cya3*-labelled probe FLA558, specific to members of class *Flavobacteria*. (1) DAPI stained bacteria colonizing a marine aggregate. (2) The same image as (1) with cells detected with *cya3*-labelled probe FLA558.

in Antarctic Zone waters and in algal blooms (Simon *et al.* 1999). The *Flavobacteria* are also recognized as being important sources of enzymes, which help degrade complex organic matter in the surface ocean (Kirchman 2002) and appear along with *Proteobacteria* critical for oceanic secondary production. We could easily visualise with fluorescent in situ hybridisation (FISH) the association of *Flavobacteria*, along with *Proteobacteria*, with diatom cells, fresh diatom detritus and settling marine aggregates

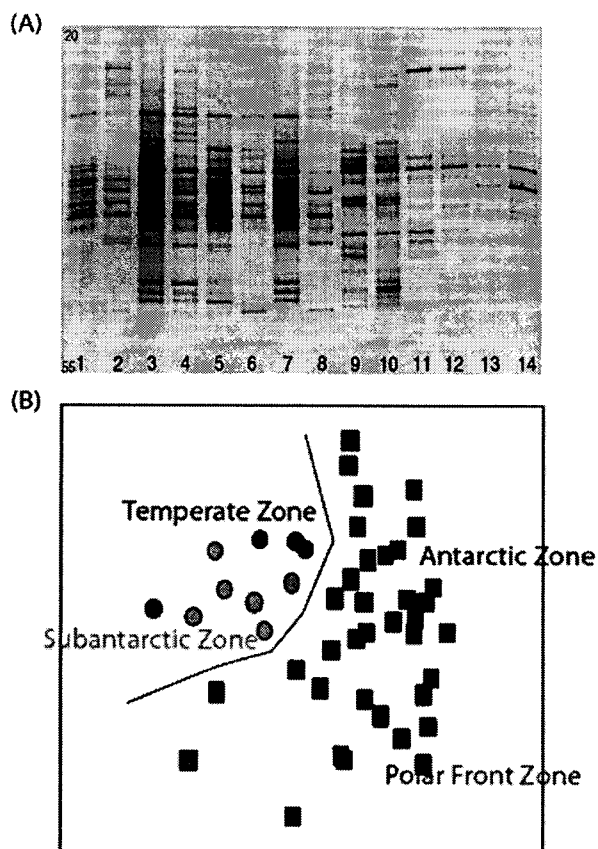


Fig. 4. (A) DGGE gel image showing banding patterns for class *Flavobacteria*-derived 16S rRNA gene fragments obtained from DNA extracted from filter fractionated Southern Ocean seawater. Odd numbered samples are from 0.8 micron filter samples (particulate fraction); even numbered samples are from 0.2 micron filter samples (planktonic fraction). (B) nMDS plot showing the relationship of *Flavobacteria* derived from Southern Ocean seawater (DGGE banding pattern data). Antarctic Zone and Polar Front Zone samples are significantly different from Temperate Zone and Subantarctic Zone samples ($p < 0.01$). No differences were found between particulate and planktonic filter fractions (Abell and Bowman 2005).

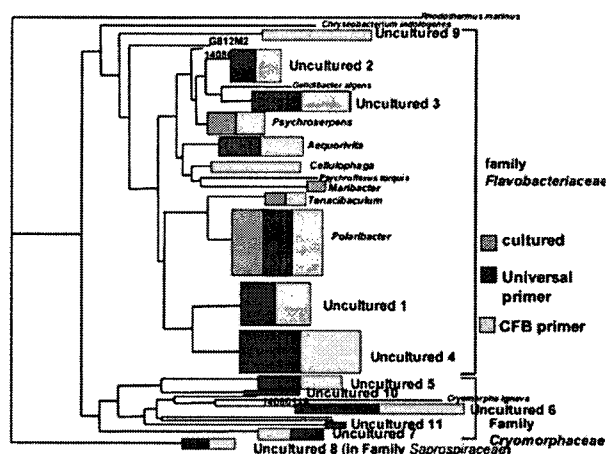


Fig. 5. 16S rRNA gene phylogenetic tree showing diversity of Southern Ocean seawater members of the CFB phylum. Cultured taxa were from a small survey of the same samples using conventional agar-based media.

(Fig. 3). The diversity of the *Flavobacteria* was assessed using denaturing gradient gel electrophoresis (DGGE) using Bacteria and class *Flavobacteria* specific primers (Fig. 4A). Comparison of DGGE band profiles using non-metric dimensional scaling ordination and analysis of similarity statistics showed that *Flavobacteria* communities differed north and south of the Polar Front (Fig. 4B). It was found that class *Flavobacteria* diversity, which incorporates the families *Flavobacteriaceae*, and *Cryomorpaceae* (Fig. 5), was richest in the Antarctic Zone waters. Indeed, this diversity was substantially higher than that found in waters samples collected north of the Polar Front. Higher abundance also occurred in the seawater particulate fraction (Abell and Bowman 2005). Many *Flavobacteria* appeared to represent an “endemic” psychrophilic community around the Antarctic. Sequence comparisons suggests however, a closely related set of taxa also exists in the Arctic Ocean (Bano and Hollibaugh, 2002; Brinkmeyer *et al.* 2003). Sequencing of DGGE bands from gels revealed 24 genus equivalent lineages, many of which are uncultivated (Fig. 5). Clearly much remains to be done for a fuller understanding of the ecology of this particular group of bacteria, their biological attributes and biotechnological applicability.

5. Antarctic marine sediment

In addition to exploring sea-ice and seawater we investigated marine sediment from the Antarctic region. We were especially interested in establishing what types of prokaryotes existed in polar marine sediments as well as

determine whether there was a predominance of psychrophiles. Marine sediment sites that have been studied included Antarctic lakes and some local fjord areas in the Vestfold Hills in Antarctica (Bowman *et al.* 2000), pristine and hydrocarbon and heavy metals polluted coastal sediments in the Windmill Island region (near Casey Station) (Powell *et al.* 2003) and finally continental shelf sediments in the Mertz Glacier Polynya (MGP) (Bowman *et al.* 2003; Bowman and McCuaig 2003). The latter site is explained here in more detail as it provided an excellent opportunity to explore a permanently cold ecosystem and compare it to other similar studies performed in the Arctic (Ravenschlag

et al. 1999) and in deep-sea sites (Li *et al.* 1999). Analyses performed included DGGE and clone library screening of 16S rRNA genes as well as rRNA hybridisation. The MGP samples investigated were a series of 20 cm cores collected over a 5000 km² area. Water temperature was -2°C and the site had been well-studied geologically (Harris *et al.* 2001). Using DGGE analysis we found the bacterial community in the top 4 cm layer was homogenous across the site (Fig. 6A), which likely experienced mixing by epibenthic fauna and as well as the oceanic current that swept the sampling area. Clone library analysis revealed very extensive diversity in three layers of a single sediment core investigated in detail (Bowman and McCuaig 2003). About 350 clones per library were examined and species richness estimation by the Chao model (Chao 1987) estimated >4300 prokaryote species were present in the core (Fig. 6B). The estimate was conservative as clone data was condensed into phylotypes which were defined as groups of clones which shared a sequence similarity of >0.98. Often pairs of prokaryote species will have 16S rRNA gene sequences that differ at less than this cut-off. *Proteobacteria* predominated, making up 50-70% of the community in the MGP sediment layers. Other major groups included the *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia*, *Actinobacteria* and *Chloroflexi*. Many other lineages containing no cultured representative were also found (e.g. candidate divisions OP8, OP11, etc.). Archaea detected included mostly Marine Group I crenarchaeota, however deeper in the cores a wide range of Euryarchaeota and Crenarchaeota, all representing uncultivated groups, were found (Fig. 7A). The overall diversity and clades resolved was found to be similar to other low temperature benthic sites, including Arctic fjord sediments and deep-sea for which less detailed analyses have been carried out (Ravenschlag *et al.* 1999; Li *et al.* 1999). Strong evidence now exists that marine sediment consists of a series of well-defined clades indigenous to the benthic environment across the world (Bowman 2004a; Bowman J.P., unpublished data). Cultivation studies of bacteria from Antarctic (and other) marine sediments have barely scratched the surface of this diversity. The microbial community in MGP sediments is clearly highly cold adapted (median optimal growth temperature 15°C). Much work is required to further understand marine sediment ecosystems, including functionality and environmental factors affecting community structure. Biotechnological potential appears immense as marine sediment has diversity equal to soil but presents an ecosystem mutually exclusive at the phylogenetic level. Environmental metagenomics may provide a way to access genetic information

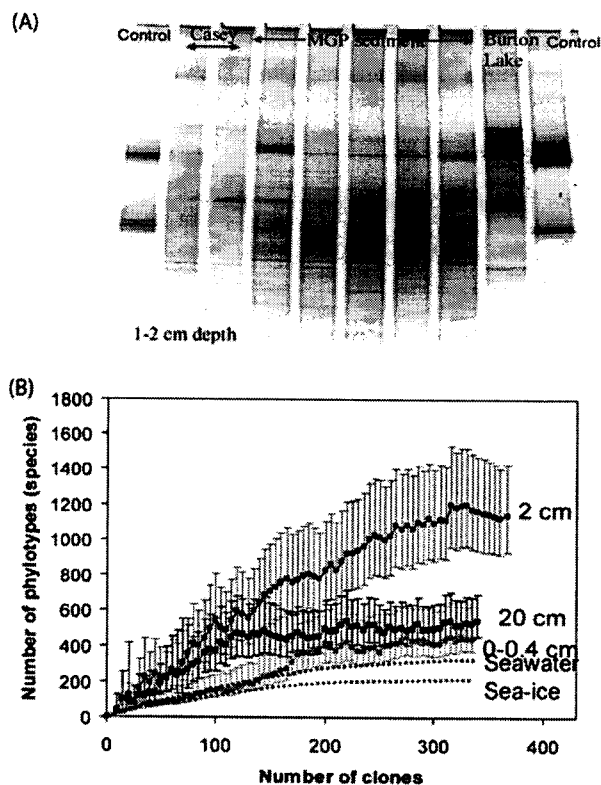


Fig. 6. (A) DGGE gel image showing bacterial 16S rRNA gene fragment bands from marine sediment samples (top 1-2 cm depth) from Antarctic areas, including MGP sediment (depth 700-940 m), coastal areas near Casey Station (depth 20-40 m) and Burton lake, a marine-derived lake located in the Vestfold Hills. Controls consist of bacterial pure cultures. (B) Chao-1 species richness for MGP sediment core samples (core depths indicated on graph) inferred from 16S rRNA gene clone libraries. Comparisons are made with data from Southern Ocean seawater and sea-ice (Data adapted from Bowman *et al.* 2003; Bowman and McCuaig 2003).

in sediment communities quickly but application of cultivation approaches (as mentioned above) will be necessary to yield dividends in the future.

6. Evolution of psychrophilic bacteria

From the above studies it is possible to determine how widespread psychrophily is amongst bacteria (Fig. 7B). Most major lineages contain psychrophiles except deep-branching thermophilic phyla such as the *Aquificales*, *Thermotageles* as well as some other terrestrial environment focussed lineages e.g. *Thermus-Deinococci*. The vast majority of cultured and a high proportion of uncultured cold-adapted microbial diversity resides in only a few phyla, namely

the *Proteobacteria* and the *Bacteroidetes*. In phylogenetic trees psychrophiles never form very deep lineages such as that found for thermophilic taxa. This has led to the theory that much psychrophilic diversity is likely evolutionarily recent in origin (Bowman 2004b).

7. Exopolysaccharides from Antarctic marine bacteria

EPS was explored in Antarctic marine bacteria as it offers an opportunity to develop insights into microbial ecology as well as potentially provide biotechnological outcomes. EPS is well known in having major ecological importance providing the basic structural material for biofilms and aggregates; as a carbon and energy source; and playing roles in ionic interactions between organic and inorganic molecules (Decho 1990). Recently, a collaboration was set up between the University of Tasmania and L'Institut Francais de Recherche pour l'Exploitation de la Mer (IFREMER) to explore EPS in Antarctic bacteria (Mancuso Nichols *et al.* 2004a, 2004b). In this study the EPS of isolates obtained from Southern Ocean sea-ice and seawater particulates were characterised chemically. Findings so far indicate that many Antarctic marine bacteria prolifically form EPS and that EPS chemistry varies considerably (Fig. 8). *Pseudoalteromonas* sp. CAM025 was found to produce a high molecular weight uronic acid rich EPS. The strain is typical of species inhabiting sea-ice including sea-ice brines, possessing an interesting combination of cold adaptation and halotolerance providing it the means to survive in this extreme environment. Its possible that its EPS may also play a protective role for this microorganism as briefly mentioned above though much work is needed to prove this. Also the role EPS may play in nutrient cycles, for example sorbing trace metals such as iron, vital for phytoplanktonic productivity is being investigated. Growth studies indicated CAM025 when growing at low temperatures (10 and -2°C) undergoes EPS production stimulation. At 10°C CAM025 synthesized 30-times as much EPS as at 20°C (Fig. 8) (Mancuso Nichols *et al.* 2004). Glucose conversion efficiency was also much higher even though growth yields were not significantly different between 10 and 20°C (Fig. 8) (Mancuso Nichols *et al.* 2005). Uronic acid content also increased when grown at low temperatures. Overall, the data begins to suggest that EPS production plays a physiological role in the survival of this microorganism and is likely crucial for its success and survival in sea-ice. Further studies will also be eventually conducted to determine the potential utility of the EPS of strain CAM025 and as

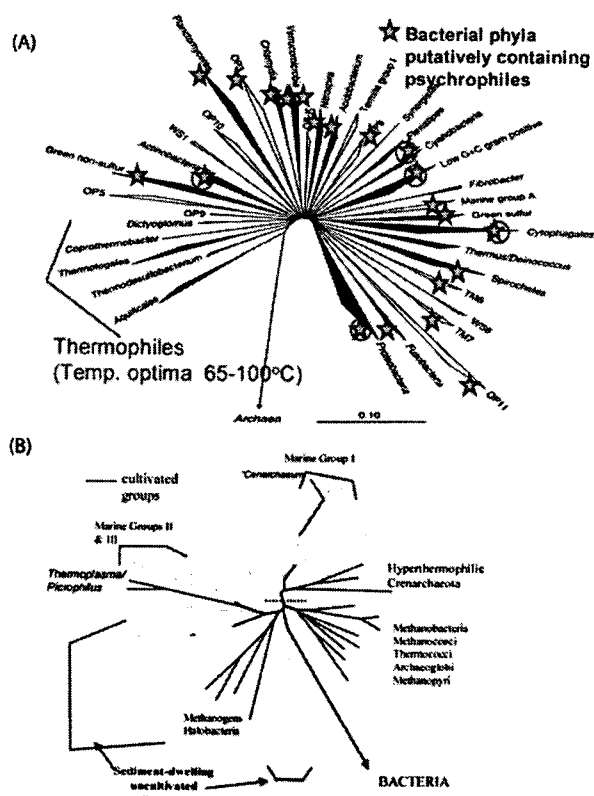


Fig. 7. (A) 16S rRNA gene phylogenetic tree showing putative associations of psychrophilic taxa with bacterial phyla. Phyla denoted by red rings are those which are most commonly encountered in natural samples, either marine or terrestrial in origin. *Cytophagales* is equivalent to the CFB phylum Figure was adapted from Hugenholtz *et al.* (1998). (B) 16S rRNA gene phylogenetic tree of Archaea from MGP sediments showing the position of MGP sediment lineages (yellow lines) to cultured lineages.

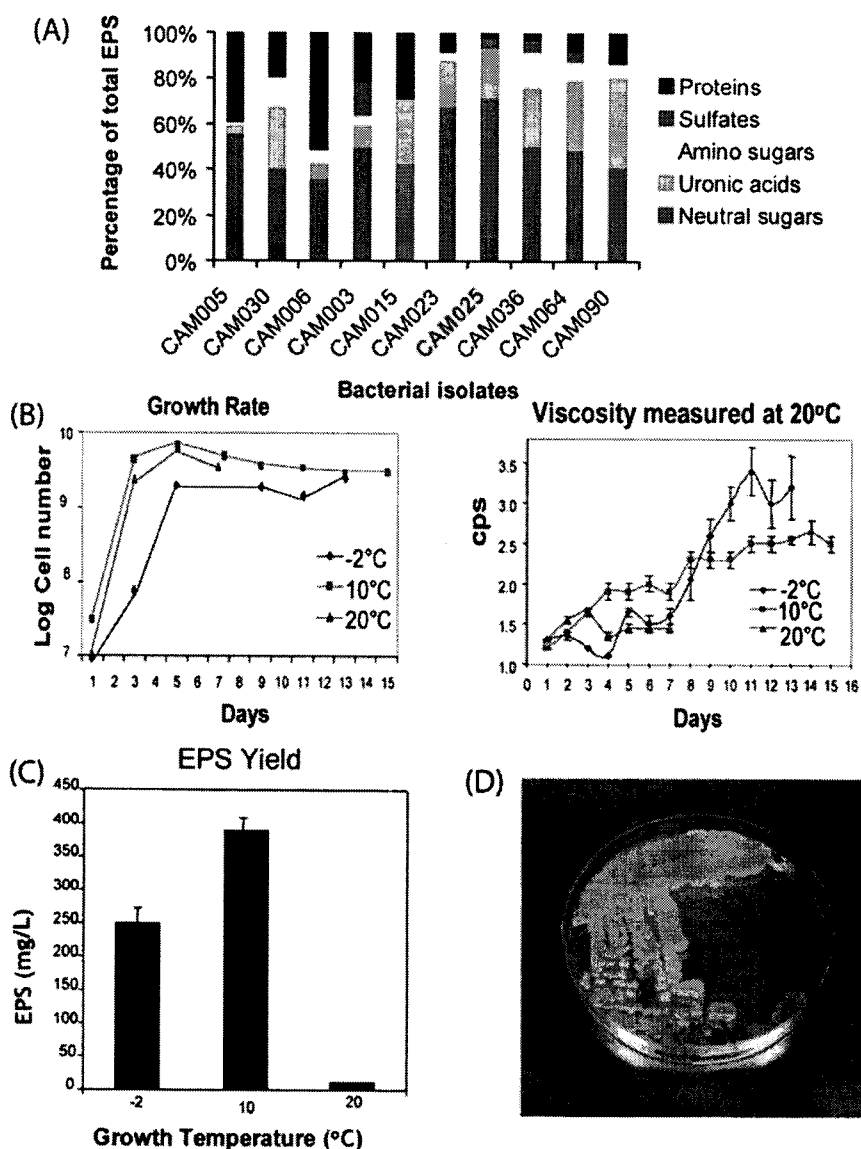


Fig. 8. (A) chemical composition of EPS produced by Antarctic bacteria. (B) Growth rate and culture viscosity (indicator of EPS production) for strain CAM025 grown in marine-glucose broth. (C) EPS yield of CAM025 is enhanced at -2°C and 10°C compared to 20°C . (D) Mucoid colonial morphology of CAM025 grown on marine-glucose agar.

well as other Antarctic strain in biotechnological applications.

8. Conclusions

Biodiscovery in polar regions has great potential with new molecular or cultivation approaches. For example, sea-ice and sediment offer opportunities for biodiscovery as they have largely untapped genetic pools. Psychrophilic bacteria are already known to be sources of biotechnologically useful enzymes and compounds such as PUFA and EPS.

Their pharmaceutical potentials are yet unrealised. New biodiscovery based research should further enhance basic knowledge of cold ecosystems and improve microbial ecological understanding. A sensitive approach to biodiscovery in the Antarctic should be thus compatible with the existing Antarctic Treaty system.

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