Demonstration of antifreeze protein activity in Antarctic lake bacteria

Jack A. Gilbert,¹,²† Philip J. Hill,¹ Christine E. R. Dodd¹ and Johanna Laybourn-Parry²

¹Division of Food Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK
²School of Life and Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Antifreeze proteins (AFPs) are a structurally diverse group of proteins that have the ability to modify ice crystal structure and inhibit recrystallization of ice. AFPs are well characterized in fish and insects, but very few bacterial species have been shown to have AFP activity to date. Thirty eight freshwater to hypersaline lakes in the Vestfold Hills and Larsemann Hills of Eastern Antarctica were sampled for AFPs during 2000. Eight hundred and sixty six bacterial isolates were cultivated. A novel AFP assay, designed for high-throughput analysis in Antarctica, demonstrated putative activity in 187 of the cultures. Subsequent analysis of the putative positive isolates showed 19 isolates with significant recrystallization inhibition (RI) activity. The 19 RI active isolates were characterized using ARDRA (amplified rDNA restriction analysis) and 16S rDNA sequencing. They belong to genera from the z- and γ-Proteobacteria, with genera from the γ-subdivision being predominant. The 19 AFP-active isolates were isolated from four physico-chemically diverse lakes. Ace Lake and Oval Lake were both meromictic with correspondingly characteristic chemically stratified water columns. Pendant Lake was a saline holomictic lake with different chemical properties to the two meromictic lakes. Triple Lake was a hypersaline lake rich in dissolved organic carbon and inorganic nutrients. The environments from which the AFP-active isolates were isolated are remarkably diverse. It will be of interest, therefore, to elucidate the evolutionary forces that have led to the acquisition of functional AFP activity in microbes of the Vestfold Hills lakes and to discover the role the antifreezes play in these organisms.

INTRODUCTION

Antifreeze proteins (AFPs) are a structurally diverse group of proteins that have the ability to modify ice crystal structure (Raymond & De Vries, 1977) and inhibit recrystallization of ice, which is the growth of ice at high subzero temperatures (Knight et al., 1988). They have been extensively described in polar fish, some insects, plants and fungi. Although they have not been well characterized in prokaryotes, AFPs and antifreeze lipoproteins (AFLPs) have been found in some bacteria (Duman & Olsen, 1993; Sun et al., 1995; Xu et al., 1998; Mills, 1999; Kawahara et al., 2001; Raymond & Fritsen, 2001; Yamashita et al., 2002). To date, five types of fish AFP (types I–IV and antifreeze glycoprotein) (Fletcher et al., 2001), two types of insect AFP (Duman, 2001) and six types of plant AFP (Griffith et al., 1992; Duman, 1994; Worrall et al., 1998; Meyer et al., 1999; Pudney et al., 2003) have been described. Despite this diversity there is little structural relatedness between the proteins. One current hypothesis argues that AFPs bind to ice because of intimate surface-to-surface complementarity between ice and protein, and that diversity in AFP types can partly be explained by there being different planes of ice to which AFPs can bind (Jia & Davies, 2002).

To our knowledge only five bacteria have been shown to possess AFP activity, Pseudomonas putida (Sun et al., 1995; Xu et al., 1998; Kawahara et al., 2001), Micrococcus cryophilus, Rhodococcus erythropolis (Duman & Olsen, 1993), Marinomonas protea (Mills, 1999) and a Moraxella species (Yamashita et al., 2002). P. putida was isolated from plants in the high Arctic, R. erythropolis was isolated from the mid-gut of beetle larvae from an unknown location, Mic.
cryophilus was purported to have been isolated from chilled fresh pork sausages, Mar. protea was isolated from the ice–water interface of Ace Lake in the Vestfold Hills of Antarctica and Moraxella sp. was isolated from the McMurdo Dry Valleys region of Antarctica. AFPs are likely to have evolved in organisms that experience regular phases of low temperature and freezing. Polar regions, with their continuous low temperatures, are environments that may be expected to force the evolution of AFPs within their populations of extremophile bacteria.

The availability of free water controls the biological activity of Antarctic organisms. The Antarctic continent has only small areas of ice-free land. These so-called Antarctic oases carry a surprising diversity of lake types. One such area is the Vestfold Hills of Eastern Antarctica, which contains over 150 lakes ranging from freshwater to hypersaline. Most of these lakes were formed by a process of isostatic uplift following the last major glaciation some 10,000 years ago (Adamson & Pickard, 1986). During uplift fjords were cut off and seawater was trapped in hollows. Those lakes in closed basins evolved into saline lacustrine systems. During evolution they suffered sublimation and ablation that altered the anion and cation ratios (Burton, 1981). Some of the lakes underwent episodes of meromixis. Simultaneously, some freshwater lakes were formed at the ice cap edge as it retreated.

The lakes of Antarctica are dominated by microbial communities. They have few or no zooplankton, and no fish (Laybourn-Parry et al., 2002). Despite the harsh environmental conditions experienced in these lacustrine ecosystems throughout the year, their microbial communities continue to function, even during winter (e.g. Henshaw & Laybourn-Parry, 2002). Physiological plasticity is the key to survival and is likely to involve novel biochemistries such as low-temperature enzymes, AFPs and modified phospholipids. As yet there is limited published information on this important aspect of polar microbial ecophysiology (Herbert, 1986; Whyte & Innis, 1992; Rotert et al., 1993; Ray et al., 1994; Russell & Hamamoto, 1998; Barrett, 2001).

The current study was undertaken to assess the occurrence of AFP-active Antarctic lake bacteria across a salinity spectrum from freshwater to hypersaline (240‰) and to determine their taxonomy by molecular techniques. We were particularly interested in relating the occurrence of AFP activity in bacterial strains to the physico-chemical conditions in which they evolved. This article provides information on a wide range of Antarctic lake environments and the occurrence of bacterial AFPs therein.

Five lakes were also chosen for detailed analysis on three sampling dates during the winter in July, September, and November (Ace Lake, Pendant Lake, Triple Lake, Deep Lake and Club Lake). The lakes had a wide range of salinities from freshwater (Crooked Lake) to hypersaline (240‰, Deep Lake). Several lakes were subject to non-lake-derived nutrient input from penguin faecal matter (Rookery Lake) and occasional marine influx (Cemetery Lake, Angel ‘2’ Lake and Lake Lateralula). The lakes showed a range of stratification types, including monomictic (Deep Lake), holomictic (Triple Lake) and meromictic (Ace Lake).

Sampling from lakes. Water samples were obtained with a 2 l Kemnener bottle through a hole drilled in the ice with a jiffy drill (AAA International). When the lake had ice cover capable of supporting personnel and equipment, it was possible to collect samples at different depths. Water was collected from 0, 2, 4, 8 and 10 m, where the depth of oxygenated waters or the depth of the lake dictated the depth of sampling. Zero metre depth was taken as the base of the ice cover and all other depths were taken from that point. In the absence of ice cover capable of supporting personnel and equipment, only surface water samples were taken. For each sample, 4 l water was taken and stored in acid-washed (10% HCl) polypropylene containers for subsequent inorganic chemical analysis and dissolved organic carbon (DOC) analysis. A further 100 ml water was taken into a sterilized glass Duran bottle (for microbiological analysis). Ice cores were also taken from some lakes (e.g. Ace Lake, Pendant Lake) using a Sipre corer, sliced in the laboratory and the slices thawed in sterilized lake water to prevent osmotic shock. The sampling equipment was soaked in 10% HCl and then rinsed in deionized distilled water prior to sampling at a new site.

Bacterial culturing. Lake water (100 ml) and melted ice cores (100 ml) were spread-plated onto Tryptic Soy Agar (TSA; Sigma), yeast agar (YSA) [all components from Sigma except where indicated (1 L): 3 g yeast extract, 3 g malt, 5 g peptone, 10 g glucose, 20 g agar (Difco)], seawater agar (SWA) [3-8% salinity; all components from Sigma except where indicated (1 L): 1 g yeast extract, 1 g peptone, 38 g salt (Coral Life; Aquatics Online), 15 g agar (Difco)] and 1/2 seawater agar (1/2 SWA; 1-9% salinity: same as SWA but only 19 g salt). Initially all plates were grown for 1 week at three different temperatures, 5, 15 and 25°C. All cultures grew better at 15°C, hence all subcultures were incubated at 15°C for 1 week. All bacterial isolates were Gram-stained using a standard method (Rodina, 1972).

Water chemistry analysis. Samples were stored at 1°C until all initial processing was completed. First, 500 ml aliquots were taken for inorganic nutrient analysis, filtered through GF/F (Whatman) glass microfibre filters and analysed colorimetrically for soluble reactive phosphate (SRP, PO4-P), ammonium (NH4-N), nitrate (NO3-N) and nitrite (NO2-N) according to the method of Mackereth et al. (1988). The remaining water was filtered through a 47 mm GF/F (Whatman) microfibre filter. The filtrate was decanted into a 2.1 polypropylene bottle (acid-washed, 10% HCl), 25 ml of which was dispensed into a sterile universal tube and frozen at −20°C for DOC analysis, which was performed using a Shimadzu TOC 5000 carbon analyser (Duisburg).

Chromosomal DNA extraction. The CTAB method of chromosomal DNA extraction (Ausubel et al., 1999) was used on cultures grown for 1 week at 15°C in liquid media that matched the solid media upon which they were isolated.

Amplified rDNA restriction analysis (ARDRA). The ARDRA procedure was adapted from Vaneechoutte et al. (1995). Chromosomal DNA (1 μl) was added to a 49 μl PCR reaction mixture consisting of 21 μl sterile reverse osmosis (RO) H2O, 16 μl dNTP mixture (Abbene) (6-26 μl each dNTP made up to 1 ml in sterile H2O), 5 μl Buffer IV (Abbene), 4 μl 25 mM MgCl2 (Abbene), 1 μl universal

**METHODS**

**Study sites.** Thirty eight lakes in the Vestfold Hills (68°S, 78°E) and two lakes from the Larsenmills (69°S, 76°E), both in Eastern Antarctica, were sampled between January and March 2000.
primer 1.5 F (5’-TGGCTCAGATTGACGTGGCG-3’) (Sigma-Genosys), 1 µl universal primer 1.5 R (5’-TACTTGGTAACGACT-TCACCCCCA-3’) (Sigma-Genosys) and 1 µl Taq DNA Polymerase (ABgene). The PCR reaction was run on a thermal cycler (Technne; Progene) using the following programme: 10 min at 95 °C followed by the addition of ice-cold Taq polymerase (1 µl per reaction); then 30 cycles of 0·5 min at 95 °C, 1 min at 55 °C, 1·5 min at 72 °C, after which there was a single cycle of 72 °C for 8 min. The PCR reaction amplified a 1500 bp 16S rDNA amplicon.

PCR products were digested overnight with AluI or HpaII (BRL Gibco–Invitrogen). The reaction conditions comprised 12 µl sterile H2O, 1 µl HpaII or 1 µl AluI, 2·0 µl buffer (10× React 1; BRL Gibco) and 5·0 µl PCR product, incubated overnight at 37 °C. The digests were separated by electrophoresis on a 2% agarose gel (3:1 Nu-Sieve) in 1× TAE buffer (Sambrook et al., 1989) at 20 V.

The The restriction digest images were imported into the ImageMaster 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech and Non-Linear Dynamics) and patterns were compared using the Dice similarity coefficient and UPGMA cluster analysis (Michener & Sokal, 1957).

16S rDNA sequencing. The 16S rRNA gene was amplified using PCR (see ARDRA section), purified using the QIAquick PCR purification kit (Qiagen) and the nucleotide sequence of the gene was determined by the Biopolymer Synthesis and Analysis Unit, Nottingham University. Where satisfactory results were not obtained from the purified PCR product, it was cloned using the TOPO TA Cloning kit (Invitrogen) prior to sequencing.

Crude cellular lysate extraction protocol. The protocol for preparing crude cellular lysates was adapted from Johnston et al. (1982). Isolates were incubated at 15 °C for 1 week in 250 ml broth cultures (based on original isolation medium) and then cold-acclimatized at 4 °C for 1 week. The cultures were then centrifuged (15000 g, 4 °C, 10 min; Beckman JA-14 fixed angle rotor) and the cell pellet was resuspended in 1 ml ice-cold protease inhibitor buffer (25 mM Tris/HCl, pH 7·0, 1 mM EDTA, 1 mM PMSF in ethanol, 2 µg Pepstatin A ml−1 in methanol), to which was added 500 mg 106 µm or finer glass beads (Sigma). To break the cells, this suspension was shaken using a Mini Beadbeater (Biospec Products) for 1 min at 5000 r.p.m. and then chilled on ice for 1 min (repeated five times). After mixing the lysate with 500 µl ice-cold protease inhibitor buffer the liquid layer (supernatant) was transferred to a 16 mm diameter, numbered coverslips, which were blotted dry to remove any excess fluid. The coverslips were flash-frozen in 2,2,4-trimethylpentane pre-cooled to −70 °C (using dry ice) for 2 min, then placed in a −6 °C solvent bath [2,2,4-trimethylpentane pre-chilled to −6 °C using a Haake C water bath circulator and two Haake temperature control units (PG40 and F4)] and held for 1 h to allow recrystallization. The coverslips were viewed in the bath using an EF L 20/0.30 160/0-2 objective on a Leitz Dialux 20 EB stage. The observed crystal shape, size and density were recorded and related to the level of AFP activity using the Splat scoring system (see Table 1).

RESULTS

Physico-chemical properties of lakes

Data for Ace Lake, Pendant Lake and Triple Lake were recorded during four sampling trips throughout 2000. For Oval Lake, data were only recorded during the summer of 1999/2000 and no depth recordings were performed due to insufficient ice cover. For all physico-chemical results refer to Table 2.

Ice cover on Ace Lake and Pendant Lake thickened during winter, with some fluctuation. This continued into November, with no observed melt. Triple Lake showed total melt-out during the summer months and the early winter months, but ice thickness increased to 0·4 m during the winter, followed by a rapid thinning in November. Oval Lake showed a 20% loss of ice cover, but it is known that there was approximately 80% melt before refreezing. None of the lakes studied showed complete freezing of the entire water body.

Water temperature in Ace Lake and Pendant Lake varied only slightly throughout the year, with surface water temperature fluctuating between −2 and +1 °C. Temperature was shown to steadily increase with depth throughout the year. A temperature anomaly was noted in both lakes during September with a relatively high temperature of 14–15 °C at ~10 m (data not shown). In Triple Lake, the depth profile for temperature was virtually uniform, but the temperatures were relatively warm in summer (~3 °C) and extremely cold in winter (~14 °C). Oval Lake had the coldest water temperature recorded during the summer, −1·9 °C.

Ace Lake, a meromictic lake, showed a distinct salinity profile in the mixolimnion (the monimolimnion was not sampled during this study) throughout the year with an increase in salinity with increasing depth, with the exception of September and November when the water column was virtually isohaline. Pendant Lake also showed an increase in salinity with depth on all sampling dates except November when a slight decrease in salinity with increasing depth was recorded. The mean salinity was shown to increase from January to November in both lakes.
Table 1. Gram-negative isolates showing some level of RI activity

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Splat score*</th>
<th>Total cellular protein (mg ml(^{-1}))</th>
<th>Lake of isolation</th>
<th>Depth of isolation</th>
<th>Date of isolation</th>
<th>Cell morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>213</td>
<td>++++</td>
<td>2.03</td>
<td>Triple</td>
<td>4 m</td>
<td>07/09/00</td>
<td>Long rods</td>
</tr>
<tr>
<td>492</td>
<td>++++</td>
<td>2.34</td>
<td>Ace</td>
<td>0 m</td>
<td>15/07/00</td>
<td>Rods</td>
</tr>
<tr>
<td>53</td>
<td>++++</td>
<td>1.32</td>
<td>Triple</td>
<td>8 m</td>
<td>07/09/00</td>
<td>Small rods</td>
</tr>
<tr>
<td>732</td>
<td>++++</td>
<td>2.532</td>
<td>Ace</td>
<td>2 m</td>
<td>04/03/00</td>
<td>Rods</td>
</tr>
<tr>
<td>302</td>
<td>++++</td>
<td>2.069</td>
<td>Ace</td>
<td>0 m</td>
<td>23/01/00</td>
<td>Rods</td>
</tr>
<tr>
<td>154</td>
<td>++ +/+ + + +</td>
<td>2.17</td>
<td>Ace</td>
<td>4 m</td>
<td>13/11/00</td>
<td>Rods</td>
</tr>
<tr>
<td>54</td>
<td>++ +/+ + + +</td>
<td>3.29</td>
<td>Pendant</td>
<td>4 m</td>
<td>13/11/00</td>
<td>Rods</td>
</tr>
<tr>
<td>744</td>
<td>++++</td>
<td>2.6</td>
<td>Pendant ice core</td>
<td>50 cm</td>
<td>13/11/00</td>
<td>Rods</td>
</tr>
<tr>
<td>494</td>
<td>++++ +/+ + + + + +</td>
<td>2.39</td>
<td>Pendant</td>
<td>0 m</td>
<td>13/11/00</td>
<td>Rods</td>
</tr>
<tr>
<td>794</td>
<td>+++ +/+ + + + + +</td>
<td>3.33</td>
<td>Pendant ice core</td>
<td>Ice–water interface</td>
<td>13/11/00</td>
<td>Rods</td>
</tr>
<tr>
<td>466</td>
<td>+++</td>
<td>1.539</td>
<td>Oval</td>
<td>0 m</td>
<td>04/02/00</td>
<td>Rods</td>
</tr>
<tr>
<td>47</td>
<td>+++</td>
<td>2.49</td>
<td>Ace</td>
<td>6 m</td>
<td>14/01/00</td>
<td>Rods</td>
</tr>
<tr>
<td>86</td>
<td>+++</td>
<td>2.02</td>
<td>Triple</td>
<td>2 m</td>
<td>17/07/00</td>
<td>Rods</td>
</tr>
<tr>
<td>39</td>
<td>+++</td>
<td>1.889</td>
<td>Ace</td>
<td>4 m</td>
<td>14/01/00</td>
<td>Rods</td>
</tr>
<tr>
<td>583</td>
<td>+++</td>
<td>1.535</td>
<td>Triple</td>
<td>4 m</td>
<td>07/09/00</td>
<td>Rods</td>
</tr>
<tr>
<td>51cE3</td>
<td>+++</td>
<td>1.977</td>
<td>Pendant</td>
<td>50 cm</td>
<td>07/09/00</td>
<td>Thin rods</td>
</tr>
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<td>124</td>
<td>+++</td>
<td>2.55</td>
<td>Ace</td>
<td>8 m</td>
<td>13/11/00</td>
<td>Rods</td>
</tr>
<tr>
<td>214</td>
<td>+++</td>
<td>2.79</td>
<td>Ace</td>
<td>2 m</td>
<td>13/11/00</td>
<td>Rods</td>
</tr>
</tbody>
</table>

*All Splat scores are results of triplicate testing. +++++, Very small/dense crystals (e.g. fish AFP III); ++++, small not dense or quite small dense with medium crystals; ++ +, small not dense with medium large or small medium not dense crystals; ++, medium or large with some small crystals; +, large round discrete crystals (e.g. 30% sucrose).

Table 2. Physico-chemical data for Antarctic lakes from which AFP-active bacterial strains were isolated

All data are results of duplicate analyses. All data are averaged over the depth of sampling. Ace Lake and Pendant Lake data were averaged over 10 m depth, Triple Lake data were averaged over 8 m depth during July, September and November, and single-surface water measurement was performed during summer. Only a single measurement was taken for Oval Lake during the summer and ice depth could not be recorded as the ice was too thin to support personnel and equipment. BLD, Below level of detection.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Ice cover (m)</th>
<th>Water temperature (°C)</th>
<th>Salinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace Lake</td>
<td>1.2</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Pendant Lake</td>
<td>1.5</td>
<td>2</td>
<td>2.75</td>
</tr>
<tr>
<td>Triple Lake</td>
<td>0</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Pendant Lake</td>
<td>85%</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrate concentration (µg l(^{-1}))</th>
<th>Nitrite concentration (µg l(^{-1}))</th>
<th>Ammonium ion concentration (µg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace Lake</td>
<td>BLD</td>
<td>12.05</td>
</tr>
<tr>
<td>Pendant Lake</td>
<td>BLD</td>
<td>30.9</td>
</tr>
<tr>
<td>Triple Lake</td>
<td>BLD</td>
<td>49.9</td>
</tr>
<tr>
<td>Pendant Lake</td>
<td>BLD</td>
<td>40.87</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SRP concentration (µg l(^{-1}))</th>
<th>DOC concentration (mg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace Lake</td>
<td>54.7</td>
</tr>
<tr>
<td>Pendant Lake</td>
<td>23.2</td>
</tr>
<tr>
<td>Triple Lake</td>
<td>12.17</td>
</tr>
<tr>
<td>Pendant Lake</td>
<td>52.45</td>
</tr>
</tbody>
</table>
The salinity profile for Triple Lake was virtually isohaline; however, there was generally a small increase in salinity with depth. Oval Lake had a surface salinity of 34%.

Inorganic nutrients and DOC concentrations are crucial to controlling heterotrophic bacterial growth. Ace Lake and Pendant Lake both had nitrate concentrations below the level of detection during the 1999/2000 summer and very low or below the level of detection during July, which increased by September. During November, Pendant Lake nitrate concentration increased again but the concentration in Ace Lake decreased to below the detection threshold. Triple Lake showed an increase in concentration from January to July. Nitrate was below the level of detection in Oval Lake during the summer. Nitrite concentrations in all four lakes were high during the 1999/2000 summer, but decreased considerably through the winter and remained low through to November. Ammonium ion concentration profiles were similar in Ace, Pendant and Triple Lakes, with low concentrations during the first summer followed by a peak in July. The concentration then decreased to near the level of detection in September and remained low in November, except in Ace Lake which had its highest concentrations during September. Oval Lake had a similar ammonium ion concentration to the other lakes during the summer.

SRP showed a similar profile to ammonium ion with concentrations higher in January to March than September to November, and peaking in July. This was indicative of all four lakes.

Concentration profiles for DOC were similar for both Ace Lake and Pendant Lake, with a relatively constant level throughout the sampling season. However, concentrations were lower in July than September, and November had the lowest concentration for the year. Triple Lake concentrations were highest in January, lowest in July and all showed a steady increase in concentration from July to November. Oval Lake showed a higher DOC concentration than either Ace or Pendant Lake, yet a lower concentration than Triple Lake, indicating a salinity dependent scale for DOC concentration.

**Bacterial isolation and characterization**

Eight hundred and sixty six bacterial cultures were isolated from the 38 lakes sampled. All bacterial isolates grew more densely at 15 °C, than at 5 or 25 °C; this putatively suggests a psychrophilic classification (based on Herbert, 1986). Approximately 87% of bacterial isolates were Gram-negative, of which ~98% had rod morphologies that varied in length and type, from straight to club-shaped to long, thin curved rods. The remaining bacterial isolates were coccoid; these included all Gram-positive isolates.

**HTAP development**

The assay was based on the principle that a snap-frozen (rapidly frozen at −70 °C) sample is made up entirely of very small ice crystals. When a non-AFP solution is recrystallized, the lack of ice-growth inhibition means that surviving crystals increase in size at the expense of the others, whereas solutions containing AFPS show a limited increase in crystal size (Knight et al., 1988). The highly dense, small crystals resulting from AFP activity cause extensive light refraction so that the solution appears opaque as opposed to the transparent non-AFP solutions. Fish AFP III (1 mg ml⁻¹) was used as a positive control. Crude cellular lysate extract from E. coli strain JM109 and sterile RO H₂O were used as negative controls. It was found that 100 µl solutions (50 µL LE + 50 µL 60% sucrose) in 96-well microtitre plates provided the most effective, practical guide for rapid visual identification of AFP activity.

Serial dilutions of fish AFP III (1:10, 1:100 and 1:1000) were compared against the standard 1 mg fish AFP III ml⁻¹ solution. A decrease in AFP concentration produced a decrease in RI activity (indicated by a decrease in opacity of solution), confirming a quantitative relationship between degree of opacity and AFP concentration, and that the minimum concentration tested, 1 µg ml⁻¹, was detectable when visualized against a negative control.

The assay was applied to all 866 isolates from the Antarctic lake environments. Of these, 187 (21.6%) proved to be positive for AFP activity. The level of activity could not be quantified for the samples as with a Splat assay, but were recorded as either active or non-active. Those with reduced transparency (increased opacity) or colouration which was difficult to confirm were classed as positive, and those extracts with refraction equal to or less than the negative control (30% sucrose) were classed as negative. Of the 187 bacteria identified as AFP-active using the HTAP assay, only 12 were Gram-positive.

**Splat assessment of the HTAP positive isolates**

The 187 presumptive AFP producers were analysed using the Splat assay to confirm AFP activity. Nineteen of the bacteria were confirmed to have some level of AFP activity (an activity of 3 or more) (Table 1), all of which were Gram-negative. Ten of these 19 isolates showed a Splat score of 4 or 5 (Table 1), indicating high AFP activity, comparable with fish AFP III (1 mg ml⁻¹). These strains were isolated from Ace Lake, Triple Lake, Pendant Lake, Pendant Ice cores and Oval Lake. The greatest number of AFP-active isolates was cultured from Ace Lake, possibly due to a higher sampling frequency in this lake compared with the others (Table 1).

Those extracts which proved negative using the HTAP assay were assessed using the Splat assay to demonstrate that there were no false negative results. One hundred of the HTAP negative isolates were also shown to be negative for AFP activity using the Splat assay (results not shown). This indicated that the HTAP assay did not produce false negative results.
There was no direct relationship between concentration of protein in the crude cellular extracts, prepared from each putative AFP-active isolate, and the Splat score (Table 1). This might indicate that the specific activity of the AFPs varied between bacterial strains, and that high total cellular protein concentrations did not necessarily correspond to high AFP activity. The conditioned growth media for two of the isolates (302 and 124) demonstrated no activity with either the HTAP or Splat assays, suggesting that the putative AFP is not expressed extracellularly. However, the conditioned growth media for the remaining isolates was not assessed in this investigation.

**ARDRA of AFP-active bacterial strains**

A comparison was made of the *Hpa*II and *Alu*I digests of the 1500 bp 16S rDNA amplicons for the 19 AFP-active isolates (Table 1) and *Mar. protea* (Mills, 1999), an AFP-active γ-proteobacterium which was isolated from Ace Lake during 1996/1997 (Fig. 1a, b). The latter was included because eight AFP-active isolates were cultured from Ace Lake. Analysis of the *Hpa*II ARDRA patterns (Fig. 1a) showed that five of the isolates formed a 100 % similarity group with the *Mar. protea* isolate, indicating that this was the likely identity of these strains. Strains 302 and 33, 39 and 86, and 47 and 492 also showed 100 % similarity, suggesting that these were identical species. The remaining strains showed a much lower association, suggesting a more distant relationship.

Using the restriction enzyme *Alu*I (Fig. 1b), the same five Ace Lake isolates produced a cluster with the *Mar. protea* isolate, supporting the *Hpa*II analysis that these were identical species; this was also the situation with strains 39 and 86. Strains 47 and 492 formed a cluster of much lower similarity, suggesting a close relationship but not a species identity; the same applied to strains 302 and 33. Of the remaining strains only 583 and 213 maintained a degree of association, suggesting a true relatedness. The remainder changed association and therefore no firm conclusions could be made as to their relationships to the other strains.

**16S rDNA sequencing**

Sequence data for the 16S rDNA genes were entered into the GenBank database and each sequence assigned an accession number (Table 3). The closest published relative for each isolate was determined by comparison with the database using the FASTA and BLAST search programs (Altschul et al., 1990).

Isolates 124, 154, 214, 5ice3, 54, 744 and 794 were all 100 % identical to *Mar. protea* (AJ238597), confirming the ARDRA findings that these are all one species. Isolates 86 and 39 were 99-4 and 99-6 % related, respectively, to an unspeciated *Pseudoalteromonas* isolate (U85856); therefore it is probable that these two isolates were of the same species. Isolates 33 and 302 were identified as an Antarctic seawater bacterium (AJ293825) and *Pseudomonas fluorescens* (AF228367), respectively. However, isolate 33 was also 97-8 % related to an unspeciated *Pseudomonas* (AB021318) and therefore showed a close relationship with isolate 302 as suggested by ARDRA. Isolate 302 was further confirmed as *P. fluorescens* by production of fluorescein on *Pseudomonas* agar F (fluorescence agar) and CFC agar (Bridson, 1998). Isolate 33 was confirmed as a pseudomonad by culturing on the same medium but did not produce fluorescein.

Isolates 492 and 47 showed more than 99 % similarity to *Stenotrophomonas maltophilia* (AJ293464). Their similarity shown by ARDRA also supported their being related species. However, the sequence data for isolate 492 only comprised 408 bp and so were not sufficient to make a definitive identification. Identification of isolate 47 was based on 1182 bp and was therefore sufficient for putative affiliation.

Isolate 494 was identified as a *Sphingomonas* strain. However, this identification was based on 153 bp which, as with isolate 492, was considered too small an amplicon.
DISCUSSION

The goal of the present study was to examine the AFP activity of isolates from Antarctic environments and, integral to this, to evaluate an alternative field-based method for measuring AFP activity. Both the HTAP assay and the Splat assay are based on the RI activity of a sample. Whereas the Splat assay uses crossed polarized microscopy to assess protein samples using the HTAP assay was sometimes hindered by colouration, caused by pigment–protein complexes, such as cytochromes or carotenoids (found extensively in Antarctic bacteria as protection against UV damage) (George et al., 2001), which prevented the level of refraction from being observed. Degradation of these pigmented proteins may have also caused AFP inactivation, although this was not determined here. This artefact meant that samples with refraction inhibitory colouration were not recorded as positive for AFP activity and then assessed using the Splat assay at a later date. Due to the large sample size and uncertainty of location of pigment within the cell, fractionation of the solution to remove pigmentation was not performed. The HTAP assay has a long recrystallization time (5 days) compared with the Splat assay (1 hour), which provided the optimal level of contrast between microtitre plates, etc.) and the equipment involved in the protein extraction methodology, there are no further costs. Certain steps in the Splat assay require rapid freezing at −70 °C in a dry-ice cooled solvent, which for field conditions such as Antarctica where the dry-ice has to be made in situ, means the transportation of CO₂ cylinders, which is both dangerous and expensive. Expensive solvents (2,2,4-trimethylpentane) and microscope objectives (EF L 20/0.30) are also required. Hence for large-scale screening studies under field conditions where limited facilities are available, the HTAP assay represents a major advantage.

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positive and negative samples. For an experienced observer this time can be reduced to 24 hours. At 5 days, there was no variation between the scores of an experienced observer and a novice.

Assessment of AFP activity in the HTAP-positive isolates using the Splat assay suggested that only 2.1 % of the cultured bacteria were AFP-active; however, the cultured biota undoubtedly does not reflect the true bacterial diversity. This would indicate that AFP activity is a rare phenomenon in culturable Antarctic lake bacteria; however, as the 866 bacterial isolates have not yet been characterized, replication of species within the collection could lead to underestimation of species richness. It is also widely accepted that the majority of bacterial diversity is not presently amenable to culture. The presence of other cold adaptation techniques, which mainly involve structural adaptations in proteins and membrane lipids to maintain functionality at cold temperatures (Fields, 2001), indicates other strategies could have been implemented for growth and multiplication in the extreme cold. It is also possible that some bacteria may be genotypically AFP-positive, but phenotypically negative because of inappropriate induction conditions or protein degradation/inactivation during extraction. If this were the case, then there could be a very high proportion of false negatives within the 866 bacteria cultivated. Knowledge of the genes which encode AFPs should provide alternative strategies for investigation. However, to date, bacterial AFP genes remain uncharacterized. Another potential difficulty is that AFP proteins, and hence the genes from which they are derived, are often non-homologous (Jia & Davies, 2002). Thus such an approach could still lead to an underestimation of the presence of AFP genes in a bacterial community.

The evolution of the brackish and saline lacustrine systems drives the evolution of the microbial communities within the lakes. Changes in salinity, stratification of the water column, natural eutrophication and the geological context of the lake all influence the species composition and trophic structure of the microbial community. The saline lakes of the Vestfold Hills are marine-derived. Through time the original marine bacterial communities have undergone significant changes. The invasion and establishment of aerobiological propagules (Marshall, 1996) and periodic marine incursions (Burton, 1981) have probably acted to a small degree to change the microbial community composition during its evolution; however, changes in the salinity of the lake and accompanying changes in temperature regimen and stratification patterns probably imposed the greatest impact.

During the current study it was noted that the saline lakes usually had higher inorganic nutrient and DOC concentrations than the freshwater lakes (Table 2). The high concentrations of DOC in the extremely hypersaline lakes is thought to be due to a slow turnover of the DOC pool, in the absence of significant microbial decomposition resulting from reduced bacterial abundance (Hand, 1980). As one moves along the salinity continuum of the Vestfold Hills lakes the number of eukaryotic species (algae, protozoa) in the community declines (Perriss & Laybourn-Parry, 1997). Consequently, the grazing pressure on bacteria also diminishes.

AFP-active bacteria were demonstrated in isolates from Ace Lake, Pendant Lake, Triple Lake and Oval Lake, with Ace Lake and Pendant Lake containing the greatest number. The lakes share a similar salinity (Table 2), but Ace Lake is meromictic. In contrast Oval Lake (34 %), which is also meromictic, provided only one AFP strain. Triple Lake is a hypersaline system (170 %) and 5 of the total 19 AFP-active bacteria were isolated from this lake. Thus bacterial AFPs appear to have evolved under or been selected by quite different lake conditions. It has been suggested that AFPs might arise within drastically different ecosystems (Davies & Sykes, 1997; Barrett, 2001). The bacteria in the lakes of the Vestfold Hills are all exposed to some form of cold stress throughout the year, and in the saline lakes they suffer severe stress in winter. While temperatures are low in the freshwater lakes and the range is small, usually only a few degrees over the year, but the temperature range in the saline lakes can be very significant (Table 2). For example, in Deep Lake the annual temperature can range from 11 °C in summer to −18 °C in winter (Kerry et al., 1977). No AFPs were found in the freshwater isolates. Bacteria in the saline lakes face much greater cold and freezing stress and our data indicate that AFPs appear to be limited to isolates from these ecosystems. Fish AFPs have so far only been discovered in marine fish (Fletcher et al., 2001), presumably because they are exposed to temperatures below the freezing point of their cellular fluid due to the higher solute concentration of their environment. Freshwater fish will never experience such temperatures and hence never require a thermal hysteresis active protein. This could explain why no freshwater AFP-active bacteria were isolated.

Due to the extremely low temperatures experienced by these bacteria it is not considered likely that the AFP would act as a freezing point depressant. We therefore suggest that RI activity plays a more crucial role in protecting the bacteria from recrystallization damage. This is also observed in plant AFPs where low thermal hysteresis activity was demonstrated, suggesting RI activity is important in protecting the plants during freeze/thaw cycles (Meyer et al., 1999; Pudney et al., 2003). This is also shown by Raymond & Fritsen (2001) in Antarctic cyanobacterial mats, which have negligible thermal hysteresis activity but demonstrate RI activity. An absence of activity in assayed conditioned growth medium suggests that the AFPs from at least two of the bacterial strains are expressed either intracellularly or in the periplasmic space. This is contrary to previous reports of most bacterial AFPs, which suggest extracellular secretion (Sun et al., 1995; Kawahara et al., 2001; Yamashita et al., 2002).

Relatedness between AFP-active isolates was determined using ARDRA with two restriction enzymes (Fig. 1). Whilst
The 19 bacterial isolates that demonstrated AFP activity used to analyse 32 strains of variation in clustering occurred when five enzymes were noted previously by Jawad et al. (1998) who showed that variation in clustering occurred when five enzymes were used to analyse 32 strains of Acinetobacter spp. It has been recommended that additional phenotypic tests be used to reconcile such variations (Vaneechoutte et al., 1995).

The 19 bacterial isolates that demonstrated AFP activity were found predominantly within the \( \gamma \)-Proteobacteria, with one isolate from the \( \alpha \)-Proteobacteria. All the closest relatives of these isolates have been shown to be common in marine and/or Antarctic ecosystems. They have also all been shown to have demonstrated some level of psychrophily, with the exception of Idiomarina loihiensis, which to date has only been isolated from the hydrothermal fluids of a submarine volcano in Hawaii (Ivanova et al., 2000). The extreme differences in the respective environments of isolate 53 and I. loihiensis suggest that they are separate species, as to date no single species is known to survive across the entire temperature range found in the biosphere (Fields, 2001). It is possible that many of the non-culturable bacteria in the lakes also have AFP proteins. As molecular biology technology progresses with respect to the isolation of environmental DNA and ability to screen it for genes of interest, we may be able to develop a much more accurate picture of AFP distribution among polar saline lake bacterial communities.

To date, all characterized AFP-active bacteria have been shown to belong to the \( \alpha \)- and \( \gamma \)-Proteobacteria (Duman & Olsen, 1993; Sun et al., 1995; Xu et al., 1998; Mills, 1999; Kawahara et al., 2001; Yamashita et al., 2002, ibid.), except Rhodococcus erythropolis which belongs to the Actinobacteria (high G+C Gram-positive class of the phylum Firmicutes) (Duman & Olsen, 1993) and the cyanobacteria Nostoc and Phormidium from Antarctic cyanobacterial mats (Raymond & Fritsen, 2001). Thus whilst AFP activity is predominantly found among the Proteobacteria, which is the dominant group in marine/marine-derived ecosystems, it is also found outside this group, and hence is potentially widespread amongst Domain Bacteria taxonomic groups. Based on the data presented here, it is, at present, difficult to unravel the evolutionary forces that have lead to the acquisition of functional AFP activity in microbes of the Vestfold Hills lakes. Characterization and purification of the AFPs from these strains is currently being undertaken.

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