Diversity within cyanobacterial mat communities in variable salinity meltwater ponds of McMurdo Ice Shelf, Antarctica

Anne-Dorothee Jungblut,[1] Ian Hawes,[2]
Doug Mountfort,[3] Bettina Hitzfeld,[4] Daniel R. Dietrich,[5]
Brendan P. Burns[1] and Brett A. Neilan[1]*
[1]*University of New South Wales, Australia.*
[2]*National Institute of Water and Atmospheric Research, New Zealand.*
[3]*Cawthron Institute, New Zealand.*
[4]*Swiss Agency for the Environment, Forests and Landscape, Switzerland.*
[5]*University of Konstanz, Germany.*

Summary

This study investigated the diversity of cyanobacterial mat communities of three meltwater ponds – Fresh, Orange and Salt Ponds, south of Bratina Island, McMurdo Ice Shelf, Antarctica. A combined morphological and genetic approach using clone libraries was used to investigate the influence of salinity on cyanobacterial diversity within these ecosystems without prior cultivation or isolation of cyanobacteria. We were able to identify 22 phylotypes belonging to *Phormidium* sp., *Oscillatoria* sp. and *Lyngbya* sp. In addition, we identified Antarctic *Nostoc* sp., *Nodularia* sp. and *Anabaena* sp. from the clone libraries. Fresh (17 phylotypes) and Orange (nine phylotypes) Ponds showed a similar diversity in contrast to that of the hypersaline Salt Pond (five phylotypes), where the diversity within cyanobacterial mats was reduced. Using the comparison of identified phylotypes with existing Antarctic sequence data, it was possible to gain further insight into the different levels of distribution of phylotypes identified in the investigated cyanobacterial mat communities of McMurdo Ice Shelf.

Introduction

Cyanobacteria are the dominant primary producers in extreme permafrost environments of the aquatic systems on McMurdo Ice Shelf, Antarctica. They are also one of the oldest groups of organisms and are thought to be the first oxygenically active organisms with fossil findings confirming the antiquity (Castenholz and Waterbury, 1989). Cyanobacteria are of key interest for a better understanding of the evolution of their present diversity and mechanisms of adaptation, which are essential for their survival in cold environments.

The McMurdo Ice Shelf comprises an area of about 1500 km^2 with a thickness from 10 to 50 m (Swithnbank, 1970; Howard-Williams *et al.*, 1990). During the summer, the area is covered with a network of meltwater ponds that vary in size, shape and physicochemical conditions, even though some of them are only several meters apart (De Mora *et al.*, 1991). Three of these ponds, Fresh, Orange and Salt Ponds, have been intensively studied with regard to their physicochemical parameters as well as the morphological diversity of cyanobacteria, protozoa, rotifers, tardigrades and nematodes present (Howard-Williams *et al.*, 1990). High salinities in these ponds originated from diluted seawater or chemical weathering of sedimentary material such as the dissolution of mirabilite ($Na_2NO_3SO_4H_2$) and thernadite (Na_2SO_4), in combination with small amounts of gypsum ($CaSO_4H_2O$) (Keys and Williams, 1981; De Mora *et al.*, 1994).

In these hypersaline environments, cyanobacteria need to maintain their intracellular osmotic equilibrium and cell turgor to ensure survival through mechanisms such as the production of organic osmolytes (Oren, 2000). High UV radiation is another major stress factor for cyanobacteria in Antarctic aquatic ecosystems on McMurdo Ice Shelf (Ross and Vincent, 1998), which can lead to the photoinhibition of photosynthesis, mutagenesis, or chlorophyll-bleaching (Castenholz, 1992; Ehling-Schulz and Scherer, 1999).

Cyanobacteria are often found in the form of thick microbial benthic mats, having a characteristic vertical stratification of different functional microbial groups (Howard-Williams *et al.*, 1989). Cyanobacteria are most dominant in the top layer of these mats (Stal *et al.*, 1985) and motile species such as Oscillatoriales, as well as the nitrogen-fixing Nostocales, are very prominent in these communities (Howard-Williams *et al.*, 1990).

Previous attempts to classify species in these ponds have relied on traditional microscopic studies (Howard-Williams *et al.*, 1990; Broady and Weinstein, 1998) and

Table 1. Physicochemical conditions of the meltwater ponds – Fresh, Orange and Salt Ponds, McMurdo Ice Shelf, Antarctica.

Site	Conductivity (μS/cm)	DRP[a] (mg/m^3)	NH$_4$-N[b] (mg/m^3)	NO$_3$-N[c] (mg/m^3)	DOP[d] (mg/m^3)	DON[e] (mg/m^3)
Fresh Pond	158	5.5	18	2	34	1 131.5
Orange Pond	937	39	18.5	2.5	99	2 080.5
Salt Pond	52 900	29	50.5	4	565.5	13 356.5

a. Dissolved reactive phosphorous.
b. Ammonium.
c. Nitrate.
d. Dissolved organic phosphorous.
e. Dissolved organic nitrogen.

culturing of morphotypes (Nadeau *et al.*, 2001). Because of the variable morphological appearance of cyanobacteria depending on the prevailing environmental conditions, this traditional approach of characterizing cyanobacteria is very difficult. Additionally, culturing only provides limited information on the composition of communities because of selectivity. In the present study, benthic cyanobacterial mat communities of three meltwater ponds on McMurdo Ice Shelf were investigated for the first time via a polyphasic morphological and genotypic approach. These meltwater ponds are Fresh, Orange and Salt Ponds (unofficial names), and they are situated south of Bratina Island on McMurdo Ice Shelf. The ponds were chosen because of their significant differences in salinities and physicochemical parameters described above.

Cyanobacterial mats are not only a dominant feature of Antarctic ecosystems, but also in Arctic as well as other extreme environments such as hot springs and desert crusts (Rudi *et al.*, 1997; Ward *et al.*, 1998; Vincent, 2000a; Wynn-Williams, 2000). Therefore, it is of interest to compare the diversity of these mats on McMurdo Ice Shelf in the context of speciation and physiological adap-

tation and distribution, using a polyphasic characterization. Finally, this study will also address the question of the role of the McMurdo meltwater ponds as biotic pools for the aquatic systems in the ice-free regions of Victoria Land during interglacial periods (Howard-Williams *et al.*, 1990; Vincent, 2000a).

Results

Conductivity

The investigated McMurdo meltwater ponds Fresh, Orange and Salt Ponds had very different conductivities (Table 1). The highest conductivity was measured in Salt Pond with 52 900 μS/cm. In Orange Pond the conductivity was 937 μS/cm and the lowest conductivity was found in Fresh Pond with only 158 μS/cm.

Morphological diversity

The cyanobacterial mat communities in the three meltwater ponds investigated were dominated by cyanobacteria belonging to the filamentous orders Oscillatoriales and Nostocales (Table 2). *Nostoc* sp. and *Nodularia* sp. were

Table 2. Identified morphogroups and species of the cyanobacterial mat communities in the meltwater ponds – Fresh, Orange and Salt Ponds, McMurdo Ice Shelf, Antarctica.

Order	Genus	Morphogroups (tichome-width)	Species	Fresh pond	Orange pond	Salt pond
Oscillatoriales	*Oscillatoria*					
	Phormidium					
	Lyngbya	A: <3 μm		+	+	+
			P. cf. *deflexum* (Anagnostidis and Komarek)	+	+	+
			O. cf. *fragile* (Gom)		+	
			P. cf. *Priestleyi* (Fritsch)			+
			Lyngbya cf. *limnetica*		+	
		B: 3–5 μm		+	+	+
		C: 5–9 μm		+	+	+
			Osc cf. *priestleyii* (West and West)	+		+
			P. cf. *autumnale* (Anagnostidis and Komarek)	+	+	
		D: 9–12 μm			+	
			Oscillatoria cf. *limosa* (Anagnostidis and Komarek)		+	
Nostocales	*Nostoc*			+	+	
	Nodularia			+	+	+
			N. cf. *harveyana* (Thuret)	+	+	+

observed from the order Nostocales. The morphotypes belonging to Oscillatoriales were identified as *Phormidium* sp., *Oscillatoria* sp. and *Lyngbya* sp. Four morphogroups were defined for morphotypes belonging to Oscillatoriales but did not agree clearly with the oscillatorian identification keys. These morphogroups were distinguished on the basis of the width of the trichome such as trichome width <3 μm (morphogroup A), 3–5 μm (morphogroup B), 5–9 μm (morphogroup C) and 9–12 μm (morphogroup D). Following this approach, Orange Pond exhibited the largest diversity of morphotypes. The number of identified morphotypes in Fresh Pond was slightly lower and the lowest morphological diversity was observed in Salt Pond.

Nodularia cf. *harveyana* (Kuetz) was present in all three ponds. However, it appeared that these heterocystous cyanobacteria were more prominent in Fresh and Orange Ponds than in Salt Pond. *Nostoc* sp. was only observed in Orange and Fresh Ponds. In Orange Pond the oscillatorian species *P.* cf. *deflexum*, *Lyngbya* cf. *limnetica*, *Oscillatoria priestley*, *P. autumnale*, *O.* cf. *fragile* and *Oscillatoria limosa* were identified. In Fresh and Salt Ponds, the morphogroups A, B and C were abundant as were *P.* cf. *deflexum* and *O. priestley*. Additionally, *P. autumnale* was identified in Fresh and Orange Ponds.

Phylogenetic diversity

One hundred and two clones from the cyanobacterial mat community of Orange Pond, 100 from Salt Pond and 115 from Fresh Pond were obtained. These 16S rDNA clones from the three communities were screened with the two restriction enzymes, *Alu*I and *Scr*FI (data not shown). Two clones were chosen from each novel restriction enzyme pattern for sequencing to evaluate the levels of discrimination of the amplified restriction digest rDNA analysis (ARDRA), and to determine the 16S rDNA sequence of each restriction pattern type.

Phylogenetic analysis was performed using reference sequence data from the orders Oscillatoriales and Nostocales. Sequence data of the orders Pleurocapsales, Chroococcales and Prochloron were excluded, because the clones did not show a high similarity to these orders in the GenBank (NCBI) BLAST searches. Sequence data from other Antarctic environments and various Arctic strains were also used in the analysis to assess the geographical distribution of the uncultured cyanobacterial clones from the three ponds (Table 3). The Antarctic references originated from Lake Fryxell and Lake Bonney in the Dry Valleys, Vestfold Hills (East Antarctica) and the meltwater system south of Bratina Island (McMurdo). We were able to obtain sequence data of isolates from several meltwater ponds south of Bratina Island, as well as sequence data from Salt and Orange Ponds. Sequences were included when they were the closest match in the GenBank (NCBI) BLAST to sequences obtained in the present study, and when that species was morphologically identified in this study.

The sequences from the three clone libraries grouped into 12 different clusters. Four of these clusters were formed in the order Nostocales, comprising seven different phylotypes, whereas eight clusters belonged to the Oscillatoriales containing 19 different phylotypes (Table 4).

Table 3. Antarctic and Arctic reference sequences.

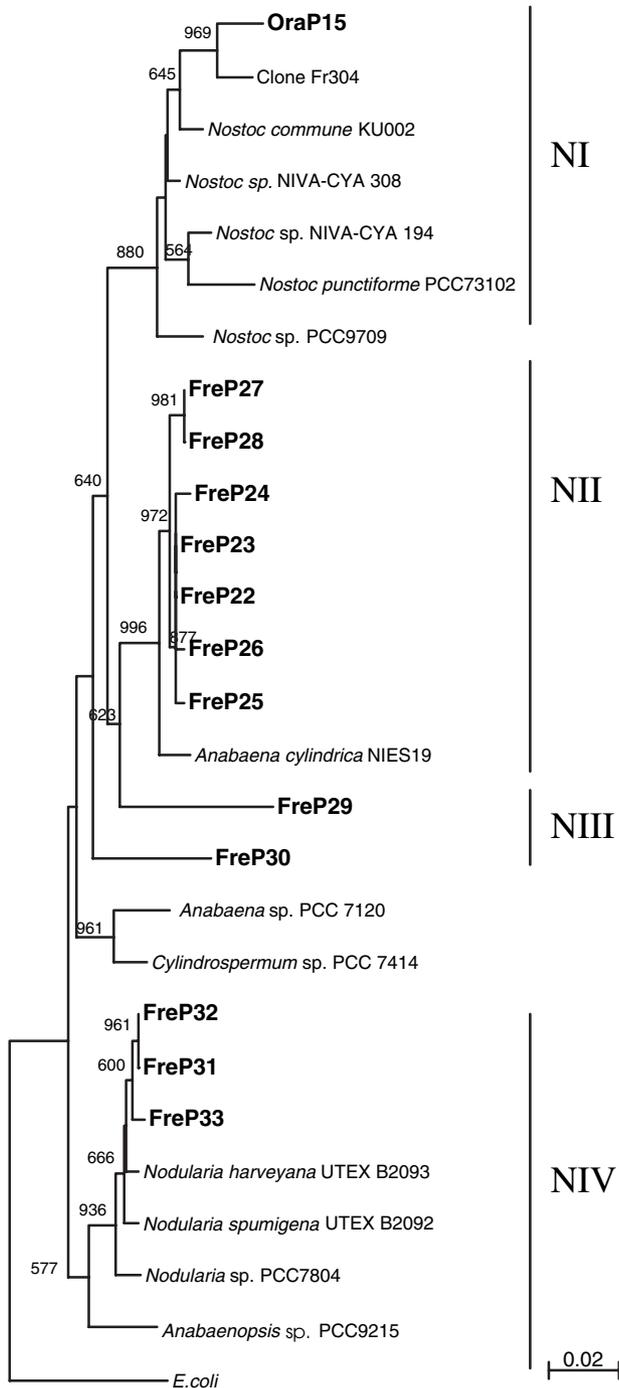| Location	Sequence(s)	Reference
Antarctica		
Meltwater ponds south of Bratina Island, McMurdo Ice Shelf	*Oscillatoria* sp. Ant-G16, Ant-G17, Ant-Pancreas, Ant-Salt, Ant-SOS *Phormidium* sp. Ant-Brack2, Ant Brack3, Ant-Lunch, Ant-Orange, Ant-Skua	Nadeau *et al.* (2001)
Lake Fryxell, McMurdo Taylor Valley	Uncultured clones BGC-Fr080, FR132, Fr054, FR147, Fr391, Fr239, FrE313, FR048, FR005, FR032, FR304, FR304, FR397, FR239, FR094, FR127, BGC-FR056, BGC-Fr032, Fr121	Taton *et al.* (2003)
Lake Bonney, McMurdo Dry Valley	Uncultured clones LB3-7, LB3-46, LB3-75, LB3-76	Priscu *et al.* (1998)
Pond, McMurdo Ice Shelf	*P. murrayii* UTCC475	D.A. Casamatta and M.L. Vis, unpubl. data
Pond, McMurdo Ice Shelf	*Phormidium subfuscum* UTCC474	D.A. Casamatta and M.L. Vis, unpubl. data
Pond, McMurdo Ice Shelf	*O. priestleyii* UTCC476	D.A. Casamatta and M.L. Vis, unpubl. data
Pond, McMurdo Ice Shelf	*Phormidium* sp. NIVA-CYA177	Rudi *et al.* (1997)
Quartz stones, Vestfold hills	Uncultured clones QSSC9L-3, QSSC8L-5	Smith *et al.* (2000)
Beacon sandstone, McMurdo Dry Valley	Uncultured clones FBP 403	de la Torre *et al.* (2003)
Arctic		
Pond, Canadian Arctic	*Oscillatoria* sp. strain E17	Nadeau *et al.* (2001)
Pond, Canadian Arctic	*Phormidium* sp. strain E18	Nadeau *et al.* (2001)
Norwegian Arctic	*Phormidium* sp. strain NIVA-CYA 202	Rudi *et al.* (1997)
Norwegian Arctic	*Phormidium* sp. strain NIVA-CYA 203	Rudi *et al.* (1997)
Norwegian Arctic	*Nostoc* sp. strain NIVA-CYA 308	Rudi *et al.* (1997)

Fig. 1. Phylogenetic analysis of 16S rDNA clones with a relationship to the order Nostocales. Sequences were identified through the construction of clone libraries from the cyanobacterial mat communities of the three meltwater ponds Fresh, Orange and Salt Ponds, McMurdo Ice Shelf. The phylogenetic relationship was constructed using the Neighbour-Joining Method. Bootstrap values ≥500 (after 1000 data re-sampling events) are shown. The scale is 0.02 mutations per nucleotide positions. Sequence data obtained in this study are shown in bold letters. FreP, Fresh Pond; OraP, Orange Pond.

sequences from Lake Fryxell. Cluster OCI also contained reference sequences from the meltwater ponds investigated, including Orange Pond, other locations in Antarctica, and Arctic reference sequences. Cluster OCIV contained phylotypes that grouped with uncultured clones from Lake Bonney, Dry Valleys.

In contrast to Fresh and Orange Ponds, the clones from Salt Pond grouped with only two of the oscillatorian clusters, OCII and OCVII. These clones were delineated from 16S rDNA sequences from Fresh and Orange Ponds, Lake Fryxell or Lake Bonney. Cluster OCII comprised the majority of the Salt Pond clones. The sequences showed high similarity (99%) to two reference sequences, namely *O. priestley*, which was isolated from a pond on the McMurdo Ice Shelf, and *Oscillatoria* sp. Ant-Salt (99%) which originated from Salt Pond. The other Salt Pond cluster, OCVII, contained representative clones of *Oscillatoria* sp. Ant-SOS and *Leptolyngbya* sp.

The Shannon-Wiener index was applied for the coverage (H) of diversity (Good, 1953). The index showed that almost all the diversity in Salt Pond clone library was detected with H = 96. The coverage was also high for Fresh (H = 86.956) and Orange Ponds (H = 90.196) (Good, 1953).

Discussion

Morphological characterization of cyanobacterial diversity

Microscopic observations agreed with previous morphological studies on the three ponds Fresh, Orange and Salt (Hitzfeld *et al.*, 2000; Nadeau and Castenholz, 2000). The cyanobacterial mat communities were dominated by oscillatorian species as well as species belonging to the order Nostocales. No unicellular cyanobacteria were identified in the morphological or genotypic analysis. This agrees with studies on Antarctic microbial communities by Stal (2000) and Taton and colleagues (2003), whereas other studies such as Olson (Olson *et al.*, 1998) have observed coccoid cyanobacteria in Lake Bonney, McMurdo Dry Valleys. The broad distribution of *P.* cf. *deflexum* (morphogroup A) and *P. autumnale* (morphogroup B) has also been recorded in previous studies (Nadeau and Castenholz, 2000). It was suggested that the oscillatorian species are likely to be distributed based on the different conductivities of meltwater ponds (Broady and Kibble-white, 1991). *Oscillatoria priestley*, for example, is preferentially found in hypersaline environments such as Salt Pond; however, in this study it was also present in Fresh Pond. The absence of *Nostoc* sp. in Salt Pond is also considered to be due to its conductivity (Howard-Williams *et al.*, 1990). In contrast to *Nostoc* sp., the *Nodularia* sp. morphotype was present in all three ponds and therefore its abundance seems to be independent of conductivity.
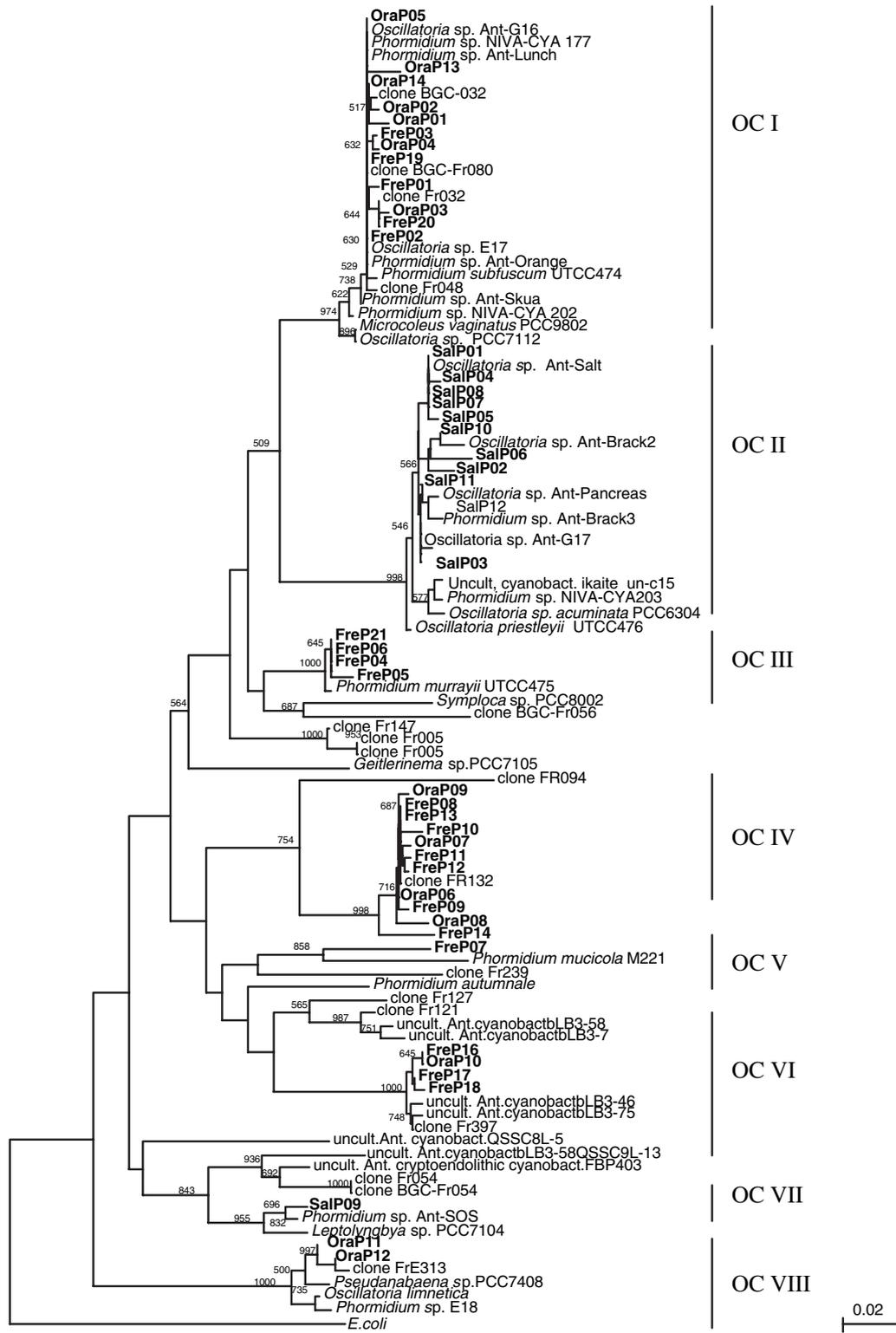
Fig. 2. Phylogenetic analysis of 16S rDNA clones with a relationship to the order Oscillatoriales. Sequences were identified through the construction of clone libraries from the cyanobacterial mat communities of the three meltwater ponds Fresh, Orange and Salt Ponds, McMurdo Ice Shelf. The phylogenetic relationship was constructed using the Neighbour-Joining Method. Bootstrap values ≥500 (after 1000 data re-sampling events) are shown. The scale is 0.02 mutations per nucleotide positions. Sequence data obtained in this study are shown in bold letters. FreP, Fresh Pond; OraP, Orange Pond; SalP, Salt Pond.

Even though this morphological study was limited by the difficult taxonomy and the plastic morphology of cyanobacteria, it revealed that cyanobacterial communities are relatively consistent in their morphological diversity over long time periods (Howard-Williams *et al.*, 1990).

Because of its immobility and fragile vegetative cell-heterocyst structure, the genus *Nodularia* sp. is generally not considered to be well adapted to conditions in microbial mats. However, it has frequently been found in mat-forming communities on the McMurdo Ice Shelf (Stal, 2000; Taton *et al.*, 2003), potentially because their ability to fix nitrogen is advantageous in these highly oxic environments, as shown for *Nostoc* sp. in microbial communities of lakes in the McMurdo Dry Valleys (Olson *et al.*, 1998). The three investigated ponds were shown to have different levels of dissolved organic nitrogen (Table 1), which negatively correlates with the abundance of morphotypes belonging to the nitrogen-fixing order Nostocales.

Phylogenetic characterization of cyanobacterial diversity

For the first time it was possible to obtain phylotypes of *Nodularia* sp., *Nostoc* sp. and *Anabaena* sp. from Antarctic environmental samples via the construction of a clone library. However, it was only possible to obtain phylotypes belonging to *Nodularia* sp. from Fresh Pond even though *Nodularia* sp. was observed microscopically in all three ponds. This discrepancy was also encountered during the analysis of a clone library from a cyanobacterial mat community from Lake Fryxell (Taton *et al.*, 2003). These apparent contradictions might be related to the particular species present in Antarctica and could suggest the presence of a novel Antarctic *Nodularia* phylotype, possessing regions of the 16S rDNA not recognized by the polymerase chain reaction (PCR) primers employed. Morphological features of the species, however, suggest the classification of the identified phenotype as *Nodularia* cf. *harveyana*, which is a cosmopolitan species (Moffitt *et al.*, 2001). This is also supported by a 16S rDNA sequence similarity of 98% to the reference strains *N. harveyana* and *N. spumigena*. Sequence analysis of single trichomes might be a useful approach for a more conclusive genotypic classification of *Nodularia* sp. present in Antarctica.

Clones with a high similarity to *Nostoc* sp. were only obtained from Orange Pond, although the phenotype *Nostoc* sp. was also observed in Fresh Pond. However in Fresh Pond, clones of the *Anabaena* sp. phylotype were identified, even though the phenotype *Anabaena* sp. was not observed microscopically. Previous morphological studies have revealed the presence of *Anabaena* sp. in these ponds (Howard-Williams *et al.*, 1990; Hitzfeld *et al.*, 2000). Possible explanations for this disagreement between genotypic and phenotypic characterization may

be due to this low abundance of *Anabaena* sp. or ambiguous morphotypes. Furthermore, a phylotype was identified with only 93% similarity (*Anabaena* sp. NIES 19) to any 16S rDNA database entry in GenBank, which suggests the potential presence of a novel lineage of heterocystous cyanobacteria in Antarctica.

The cyanobacterial community in Salt Pond was shown to contain less diversity either than in Fresh or Orange Ponds. The clone library data suggested that *O. priestley*, and the psychrotolerant *Oscillatoria* sp. Ant-Salt are the dominant genotypes, even though the clone library data can only semiquantify species abundance. This phylogenetic analysis supported the present morphological investigations as well as those of previous studies. Salt Pond clones formed a lineage with *Oscillatoria* sp. Ant-SOS and *Leptolyngbya* sp. *Oscillatoria* sp. Ant-SOS was isolated from 'Son of Salt' Pond (SOS Pond, unofficial name) (Nadeau and Castenholz, 2000), which is situated in very close proximity to Salt Pond. Interestingly, Fresh Pond lays *c.* 300 m from Orange and 50 m from Salt Pond. However, the cyanobacterial communities of Fresh and Orange Ponds are more similar to each other than to Salt Pond. The phylogenetic approach used revealed here that there is greater diversity present in Fresh and Orange Ponds than the initial morphological characterization suggested. Furthermore, the number of phylotypes identified is likely to be an underestimate because of the selectivity of the method and the sample size. The morphological diversity observed in Salt Pond, however, was not indicated by the phylogenetic characterization. Possible explanations for these differences included the variable morphology of certain species or the inherent selectivity of the molecular method. Isolation of specific morphotypes and the sequencing of 16S rDNA obtained from single trichomes may overcome these problems.

Antarctic hypersaline environments

As shown in Table 1, the major defining characteristic of Salt Pond is its high conductivity (52 900 µS/cm). In this habitat therefore, cyanobacteria not only have to survive the metabolic stress of low temperature, desiccation and high UV irradiation, but they also have to maintain their intracellular osmotic equilibrium against extreme conductivities. Cyanobacteria belonging to the order Oscillatoriales, such as *Oscillatoria* sp., *Microcoleus* sp. and *Phormidium* sp., are often found in hypersaline environments (Oren, 2000), in agreement with the results of this study. Studies on *O. priestley* also demonstrated a higher UV sensitivity than *P. murrayi*. However, *O. priestley* was able to utilize its motility more effectively to retreat into mat layers with lower UV radiation (Quesada and Vincent, 1997). *Microcoleus chthonoplates* has been found to produce trehalose and glycosylglycerol as organic

compatible solutes in order to maintain intracellular salt concentrations. Further, *M. chthonoplates* was shown to interact with bacteria and archaea to reduce sulfide levels which can inhibit oxygenic photosynthesis (Oren, 2000; Stal, 2000). However, to our knowledge, little is known about the specific mechanisms involved in the regulation of intracellular salt concentrations in cyanobacteria inhabiting the hypersaline meltwater ponds of Antarctica.

Low temperatures also increase stress with psychrotolerant cyanobacteria having to perform photosynthesis and other housekeeping functions under suboptimal conditions. It is likely that only a few cyanobacterial species are able to adapt to and grow under these extreme conditions over a long period, which results in the low levels of diversity and speciation observed. Another possible explanation for the low biodiversity of Salt Pond might be that the total area of hypersaline ponds is smaller than that of other aquatic systems with low or medium conductivities such as Fresh and Orange Ponds, and therefore only a smaller number of species can be supported. The clones identified formed a shared lineage with Arctic reference strains and several uncultured clones from ikaite tuffa columns (Greenland) (Stougaard *et al.*, 2002), suggesting a cosmopolitan distribution of these halotolerant taxa.

Distribution of cyanobacteria

To date, morphological studies have concluded that cyanobacteria originated in temperate climates and therefore polar cyanobacterial species were not assumed to be endemic in Antarctica (Vincent, 2000b). These studies were supported by the assumption that their dispersal was too slow to evolve into such a large range of species and speciation in the ice-free periods of polar regions (Castenholz, 1992). The present investigation is in concordance with Taton and colleagues (2003), where different levels of distribution were postulated for Antarctic cyanobacterial phylotypes. In our study the Antarctic phylotypes of the order Oscillatoriales comprised phylotypes which have only thus far been identified in Antarctic environments (lineages OCIII, OCIV and OCVI) or only in polar environments (lineages OCI, OCII), as well as those that had high similarities to temperate cyanobacterial phylotypes (lineages OCV, OCVII and OCVIII). The *Nostoc* sp. phylotypes identified clustered with the *Nostoc* sp. of Lake Fryxell; however, the similarity between the sequences did not exceed 95%. These results concur with the phylogenetic analysis of the cyanobacterial mat communities of Lake Fryxell (Dry Valleys) (Taton *et al.*, 2003). Some of the clusters which were defined by Taton and colleagues (2003) as novel and new evolutionary branches from Lake Fryxell, were also present in Fresh and Orange Ponds. The combined results of these studies also indicated that psychrotolerant and psychrophilic cyanobacterial species

are present in the Antarctic communities. It has been suggested that psychrotolerant rather than psychrophilic characteristics would be more beneficial for survival in freshwater systems, including meltwater ponds, because of the rapid diurnal changes of temperatures during the summer (Tang *et al.*, 1997). Finally, the phylotypes obtained in this study did not cluster with any reference sequences obtained from Vestfold Hills, East Antarctica, which might be due to the limited available data on oscillatorian phylotypes.

The meltwater system south of Bratina Island is part of a region on the McMurdo Ice Shelf which has the most extensive microbial growth and largest non-marine biota in southern Victoria Land (Howard-Williams and Vincent, 1989; Broady and Weinstein, 1998). Therefore, it was suggested that microbiota, such as dried cyanobacterial mat material, play an important role as inocula through the dispersal by the strong Antarctic winds for freshwater and saline habitats in the ice-free regions of Victoria Land (Saxena, 1982). Recent studies also showed that cyanobacterial material was found in a 500 km^2 radius surrounding the meltwater systems of McMurdo Ice Shelf (Vincent, 2000a). The present study also supported this hypothesis of a 'biogenetic nuclei' (Saxena, 1982), as most of the genotypes obtained from Fresh Pond and Orange Pond have a very high similarity to those obtained from Lake Bonney and Lake Fryxell which are situated in the arid Dry Valleys of Victoria Land.

In summary, this study has revealed that even though the ponds investigated are in close proximity, their phylogenetic communities are not similar. However, known polar strains clustered together with clones obtained from all the three ponds. These data have provided a better understanding of the complex diversity and distribution of Antarctic cyanobacterial mat communities inhabiting meltwater ponds of different conductivities on the McMurdo Ice Shelf. Further genotypic as well as physiological data are needed to describe the continental distribution in Antarctica as well as globally, and the metabolic processes involved in their survival in these extreme environments. This will enhance our understanding on the role of cyanobacteria as the dominant primary-producing organisms in permafrost environments and their role in the evolution of life on earth.

Experimental procedures

Sampling and sample sites

Benthic microbial mat material was collected from the meltwater ponds – Salt, Orange and Fresh Ponds (unofficial names) on the McMurdo Ice Shelf, located south of Bratina Island (78.00′S, 165.30′E) in January 2002. Water chemistry parameters such as conductivity were obtained as described (Hitzfeld *et al.*, 2000).

Cyanobacterial diversity in variable salinity ponds 527

Microscopic observation

The morphological characterization of the samples was performed with a Zeiss light microscope (Axio Star plus, Jena, Germany). The morphological characterization was based on the literature by Geitler (1932), Broady and Kibblewhite (1991), and Anagnostidis and Komarek (Anagnostidis, 1985; Anagnostidis and Komarek, 1988, 1990). Photo documentation was carried out with a digital camera and 100× magnification (Axio, Cam HR, Zeiss, Jena, Germany).

Determination of the organic partition

An aliquot of each sample was lyophilized and the organic content was determined by furnace incineration at 600°C for 14 h (Hitzfeld *et al.*, 2000).

DNA isolation

Freeze-dried material was used for DNA isolation. An aliquot of 20 mg of the organic partition of freeze-dried material was suspended and ground with a sterile micropestle in 900 µl of SLP-buffer [50 mM Tris-HCl (pH 8.3), 40 mM EDTA, 0.75 mM sucrose]. After re-suspension, 50 µl of proteinase K (10 mg ml^{-1} SLP) and 50 µl 20% w/v SDS was added. After each addition, the extract was thoroughly vortexed. The mixture was incubated and shaken for 1–2 h at 55°C. The extract was then centrifuged at 3000 g for 3 min to remove the cell debris. The DNA was extracted with an equal volume of phenol chloroform-isoamyl alcohol (25:24:1), mixed and centrifuged at 3000 g for 3 min. An equal volume of chloroform-Isoamyl alcohol (25:24:1) was then added to the removed aqueous phase and centrifuged at 3000 g for 3 min. The two steps were repeated twice. The extracted DNA was precipitated with a standard NaCl-ethanol precipitation. The DNA was re-suspended in 100 µl of sterile water.

Polymerase chain reaction and cloning

All PCR reactions were performed using 0.5 unit *Taq* polymerase (Fischer Biotech, Perth, Australia) in a 25-µl reaction mix containing 2.5 mM MgCl$_2$, 1× Taq-Polymerase buffer (Fischer Biotech), 0.2 mM dNTPs (Fischer Biotech, Australia) and 0.2 pM forward primer. Thermal cycling was performed in a GeneAmp PCR System 2400 Thermocycler (Perkin Elmer, Norwalk, USA). Polymerase chain reaction amplification of the cyanobacterial 16S rDNA was performed using the cyanobacterial specific primer set 27F1 (5′-AGAGTTT GATCCTGGCTCAG-′3) and 809R (5′-GCTTCGGCACG GCTCGGGTCGATA-′3) (T.P. Salmon and B.A. Neilan, unpublished data). The initial denaturation step at 92°C for 2 min was followed by 30 cycles of DNA denaturation at 92°C for 20 s, primer annealing at 50°C for 30 s, strand extension at 72°C for 1 min and a final extension step at 72°C for 7 min. For cloning of the PCR product the p-Gem (R) T Easy Vector System (Promega, Madison, USA) was used. The ligation and transformation were performed according to the manufacturer. For each sample at least 100 positive (insert containing) clones were used for further analysis.

Amplified restriction digest rDNA analysis

Clones of interest were amplified using vector specific primers and subjected to ARDRA. Amplified restriction digest rDNA analysis was performed on at least 100 positive clones from each sample. Each clone was tested in a separate incubation with two restriction enzymes. Approximately 200 ng of PCR product was digested with 1.5 U of *Alu*I and *Scri*F1 (Fermentas, Hanover, USA) in a 10-µl reaction as described by the supplier. The generated ARDRA patterns were analysed on 2% and 3% agarose gels with 1× TAE-buffer. The ARDRA fragments were stained with ethidium bromide (1 µg ml^{-1}) for 10 min. For photo documentation a Gel-DOC Bio-RAD System with Quantity One 4.1R software (BIO-RAD, USA) was used.

Sequencing

Sequencing of at least two clones was performed for each combination of ARDRA patterns. The primer pair 27F1/809R was also used for sequencing. Automated sequencing was performed using the PRISM Big Dye cycle sequencing system and ABI 3730 Capillary Applied Biosystem (Foster City, CA) using Polymer 7.

Phylogenetic sequence analysis

Sequence data were analysed using the Applied Biosystem Auto-Assembler computer programme. The sequences were compared in a multiple alignment using the package Clustral x (1.8) (Thompson *et al.*, 1994). Phylogenetic trees were obtained using the Neighbour-Joining Methods, confidence levels were calculated via the Bootstrap-NJ-Methods (Felsenstein, 1978) and the bootstrap re-sampling number was 1000. The reference strains were obtained via BLAST searches on GenBank.

Nucleotide sequence accession numbers

16S rRNA gene sequences are available under GenBank accession numbers AY541527–AY541584.

Acknowledgements

We would like to thank Antarctica New Zealand for providing logistical support during the sampling. Many thanks to Paul Broady (University of Canterbury, New Zealand) and Steve Skinner (Sydney Royal Botanical Garden, Australia) for helping with the morphological analysis of the samples. We would also like to acknowledge the anonymous reviewers for their helpful comments. A.-D.J. is the recipient of research scholarships from the University of New South Wales and the School of Biotechnology and Biomolecular Sciences.

References

Anagnostidis, K. (1985) Modern approach to the classification system of cyanophytes; 1. Introduction. *Arch Hydrobiol Suppl* **71:** 291–302.

© 2005 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*, **7**, 519–529

Anagnostidis, K., and Komarek, J. (1988) Modern approach to the classification system of cyanophytes; 3. Oscillatoriales. *Arch Hydrobiol Suppl* **50:** 327–472.

Anagnostidis, K., and Komarek, J. (1990) Modern approach to the classification system of cyanophytes: 5. Stigonematales. *Arch Hydrobiol Suppl* **86:** 1–73.

Broady, P., and Kibblewhite, A. (1991) Morphological characterization of Oscillatoria (Cyanobacteria) from Ross Island and southern Victoria Land, Antarctica. *Antarct Sci* **3:** 35–45.

Broady, P., and Weinstein, R. (1998) Algae, lichens and fungi in La Gorce Mountains, Antarctica. *Antarct Sci* **10:** 376–385.

Castenholz, R.W. (1992) Species usage, concept, and evolution in the cyanobacteria (blue-green algae). *J Phycol* **28:** 737–745.

Castenholz, R.W., and Waterbury, J.B. (1989) Oxygenic photosynthetic bacteria. Group 1. Cyanobacteria. In *Bergey's Manual of Systematic Bacteriology.* Stanley, J.T., Bryant, M.P., Pfennig, N., and Holt, J.G. (eds). Baltimore, USA: Lippincott Williams and Wilkins, pp. 1710–1806.

De Mora, S.J., Whitehead, R.F., and Gregory, M. (1991) Aqueous geochemistry of major constituents in the Alph River and tributaries in Walcott Bay, Victoria Land, Antarctica. *Antarct Sci* **3:** 73–86.

De Mora, S.J., Whitehead, R.F., and Gregory, M. (1994) The chemical composition of glacial melt water ponds and streams on the McMurdo Ice Shelf, Antarctica. *Antarct Sci* **6:** 17–27.

Ehling-Schulz, M., and Scherer, S. (1999) UV protection in cyanobacteria. *Eur J Phycol* **34:** 329–338.

Felsenstein, J. (1978) Cases in which parsimony or compatibility methods will be positive misleading. *Syst Zool* **40:** 366–375.

Geitler, L. (1932) *Cyanophyceae.* Rabenhorst's Kryptogamen-Flora von Deutschland, Österreich und der Schweiz. Leipzig: Akademische Verlagsgesellschaft.

Good, I.J. (1953) The population frequencies of species and the estimation to the population parameters. *Biometrika* **40:** 237–264.

Hitzfeld, B., Lampert, C., Späth, N., Mountfort, D., Kaspar, H., and Dietrich, D. (2000) Toxin production in cyanobacterial mats from ponds on the McMurdo Ice Shelf, Antarctica. *Toxicon* **38:** 1731–1748.

Howard-Williams, C., and Vincent, W.F. (1989) Microbial communities in southern Victoria Land streams (Antarctica). I. photosynthesis. *Hydrobiology* **172:** 27–38.

Howard-Williams, C., Pridmore, R., Downes, M., and Vincent, W. (1989) Microbial biomass, photosynthesis and chlorophyll *a* related pigments in the ponds of the McMurdo Ice Shelf, Antarctica. *Antarct Sci* **1:** 125–131.

Howard-Williams, C., Pridmore, R., Broady, P., and Vincent, W. (1990) Environmental and biological variability in the McMurdo Ice Shelf ecosystem. In *Antarctic Ecosystems. Ecological Change and Conservation.* Kerry, K., and Hempel, G. (eds). Berlin, Heidelberg: Springer Verlag, pp. 23–31.

Keys, J.R., and Williams, K. (1981) Origin of crystalline, cold desert salts in the McMurdo region, Antarctica. *Geochimica Cosmochimica Acta* **2309:** 1981.

Moffitt, M.C., Blackburn, S.I., and Neilan, B.A. (2001) rRNA sequences reflect the ecophysiology and define the toxic cyanobacteria of the genus *Nodularia*. *Int J Syst Evol Microbiol* **51:** 505–512.

Nadeau, T.L., and Castenholz, R.W. (2000) Characterization of psychrophilic Oscillatorians (Cyannobacteria) from Antarctic Meltwater Ponds. *J Phycol* **36:** 914–923.

Nadeau, T.L., Milbrandt, E.C., and Castenholz, R.W. (2001) Evolutionary relationships of cultivated Antarctic Oscillatorians (Cyanobacteria). *J Phycol* **37:** 650–654.

Olson, J.B., Steppe, T.F., Litaker, R.W., and Paerl, H.W. (1998) N_2-fixing microbial consortia associated with the ice cover of Lake Bonney, Antarctica. *Microb Ecol* **36:** 231–238.

Oren, A. (2000) Salt and brines. In *The Ecology of Cyanobacteria*. Whitton, B.A., and Potts, M. (eds). the Netherlands: Kluwer Academic Publisher, pp. 281–306.

Priscu, J., Fritzen, C., Adam, S., Giovannoni, S., Paerl, H., McKay, P., *et al.* (1998) Perennial Antarctic lake ice: an oasis for life in a polar desert. *Science* **280:** 2095–2098.

Quesada, A., and Vincent, W.F. (1997) Strategies of adaptation by Antarctic cyanobacteria to ultraviolet radiation. *Eur J Phycol* **32:** 335–342.

Ross, J.C., and Vincent, W.F. (1998) Temperature dependence of UV radiation effects on Antarctic Cyanobacteria. *J Physiol* **34:** 118–125.

Rudi, K., Skulberg, O.M., Larsen, F., and Jakobsen, K.S. (1997) Strain characterization and classification of oxyphotobacteria in clone cultures on the basis of 16S rRNA sequences from the variable regions V6, V7, and V8. *Appl Environ Microbiol* **63:** 2593–2599.

Saxena, V.K. (1982) Biogenic nuclei involvement in clouds over the Ross Ice Shelf. *Antarct J US* **17:** 212–214.

Smith, M.C., Bowman, A.P., Scott, A.P., and Line, M.A. (2000) Sublithic bacteria associated with Antarctic quartz. *Antarct Appl Environ Microbiol* **12:** 469–472.

Stal, L.J. (2000) Cyanobacterial mats and stromatolites. In *The Ecology of Cyanobacteria*. Whitton, B.A., and Potts, M. (eds). the Netherlands: Kluwen Academic Publisher, pp. 61–120.

Stal, L.J., Van Gemerden, H., and Krumbein, W.E. (1985) Structure and development of a benthic marine microbial mat. *FEMS Microbiol* **45:** 111–125.

Stougaard, P., Jorgensen, F., Johnsen, M.G., and Hansen, O.C. (2002) Microbial diversity in ikaite tuffa columns, an alkaline, cold ecological niche in Greenland. *Environ Microbiol* **4:** 487–493.

Swithnbank, C.W.M. (1970) Ice movement in the McMurdo sound area of Antarctica. In *International Symposium on Antarctic Glaciological Exploration*. Crary, A.P. (ed.). Hanover, New Hamphire: International Association of the Science of Hydrology, pp. 472–487.

Tang, E.P.Y., Tremblay, R., and Vincent, W.F. (1997) Cyanobacterial dominance of polar freshwater ecosystems: are high-latitude mat-formers adapted to low temperatures? *J Phycol* **33:** 171–181.

Taton, A., Grubisic, S., Brambilla, E., de Wit, R., and Wilmotte, A. (2003) Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): a morphological and molecular approach. *Appl Environ Microbiol* **69:** 5157–5169.