*Antarctic Science 22(2), 117–122 (2010)* © Antarctic Science Ltd 2009 doi:10.1017/S0954102009990514

# *NifH* gene diversity and expression in a microbial mat community on the McMurdo Ice Shelf, Antarctica

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**Abstract:** $N_2$-fixation is an important mechanism in microbial mats of the McMurdo Ice Shelf as nitrogen sources are limited. Here we applied molecular analyses of the $N_2$-fixing diversity in cyanobacterial dominated microbial mats in a meltwater pond, known as Orange Pond, on the McMurdo Ice Shelf. Phylogenetic analyses of *nifH* genes and *nifH* gene transcripts were performed in association with acetylene reduction assay measurements. Eighteen phylotypes with the highest similarities to cyanobacteria, firmicutes, beta-, gamma- and deltaproteobacteria, spirochaetes and verrumicrobia were identified. All cyanobacterial *nifH* phylotypes grouped solely in the genus *Nostoc* spp. Clone-library analysis of *nifH* gene transcripts only identified sequences with a highest match to *Nostoc* spp. and acetylene reduction activity was identified in the presence of light and absence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea. These molecular results indicate that a variety of bacterial phyla possess the ability to fix nitrogen. However, under the tested conditions the only organisms actively transcribing *nifH* genes were *Nostoc* spp. This underlines the importance of *Nostoc* for the nitrogen budget on the McMurdo Ice Shelf.

Received 9 April 2009, accepted 28 July 2009

**Key words:** cyanobacteria, dinitrogen reductase, meltwater pond, *Nostoc*

## Introduction

The ability of microorganisms to fix dinitrogen ($N_2$) is of particular interest in meltwater ponds on the McMurdo Ice Shelf, where microbial mats form thick accumulations in ponds and lakes within an area of up to $10^4$ m$^2$ during the summer months (Howard-Williams 1990), as nitrogen sources are limited due to the prevalent low ratios of dissolved inorganic nitrogen and phosphorous. The fixed nitrogen input into these ponds is limited to recycled ammonium from sediments and snow/ice melt (Hawes *et al*. 1993, Howard-Williams & Hawes 2007).

Nitrogen concentrations in the interstitial fluids of the mats are much higher than in the water column (Howard-Williams *et al*. 1990, Howard-Williams & Hawes 2007) due to $N_2$-fixation by the phototrophic microbial community. Although the phototrophic community is dominated by oscillatoriales cyanobacteria, most of the determined $N_2$-fixation activity was attributed to *Nostoc* spp., (Howard-Williams *et al*. 1989, Vincent *et al*. 1993, Fernández-Valiente *et al*. 2001) and was suggested to satisfy 30% of the annual fixed nitrogen requirement of the ecosystem.

However, molecular data confirming these findings and further elucidation of the $N_2$-fixing diversity are lacking. Therefore, we characterized the dinitrogenase reductase (*nifH*) gene and *nifH* transcript diversity of microbial mat communities in association with acetylene reduction assay measurements from Orange Pond, located within the network of meltwater ponds on the McMurdo Ice Shelf.

## Material and methods

### *Sampling for DNA and RNA analysis and water chemistry*

Orange Pond (unofficial name, 78.00'S, 165.30'E) is located on the McMurdo Ice Shelf south of Bratina Island, and it is part of a network of meltwater ponds on the ice shelf during the summer. Orange Pond has a maximum dimension of *c.* 5 m x 2 m, and was ice free at the time of sampling. The microbial mats were thin (*c.* 5 mm) and submerged in the sediments. Microbial mat material collected in January 2005 was lyophilised and stored at -20°C until further use. Material collected in January 2006 was immediately transferred into RNALater buffer (Ambion) and stored at -80°C. It was not possible to obtain material for RNA analysis in January 2005. Water chemistry parameters and chlorophyll *a* (chl *a*) were determined as described by Hawes *et al*. (1993).

### *DNA extraction*

Freeze-dried mat material was suspended in 600 µl XS-buffer (1% potassium-methyl-xanthogenate, 800 mM ammonium acetate, 20 mM EDTA, 1% SDS, 100 mM Tris-HCl, pH 7.4, (Tillett & Neilan 2000). The mixture was vortex-mixed and incubated at 65°C for 6 h and cooled overnight at -20°C. Cell debris was removed by centrifugation at 12 000 *g* for 10 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was then

added to the removed aqueous phase and centrifuged at 12 000 *g* for 5 min. The two steps were repeated twice. DNA was precipitated by the addition of 1 volume of isopropanol and 1/10 volume of 4 M ammonium acetate overnight at -20°C. Precipitated DNA was concentrated by centrifugation at 12 000 *g* for 10 min and washed with 70% ethanol. The extracted DNA was resuspended in 100 µl of sterile water. The DNA concentration was measured using a ND-1000 Spectrophotometer (Wilmington, DE).

### *RNA extraction and reverse transcription polymerase chain reaction* (*RT-PCR*)

Total RNA was extracted as described by Schmidt-Goff & Federspiel (1993) and Summers *et al*. (1995). Briefly, microbial mat material was lysed in 0.58 g silicate beads (5 mm), 33.3 µl SDS (20%), 167 µl celite (3%) and 583 µl Tris-buffered phenol by bead beating. Cell debris was removed by centrifugation at 14 000 *g* for 15 min at 4°C. The extracted RNA was combined with an equal volume of chloroform, mixed and centrifuged at 14 000 *g* for 10 min at 4°C. The RNA was precipitated with 4 M LiCl, 20 mM Tris-HCl buffer (pH 7.4) and 10 mM EDTA (pH 8) overnight at -20°C. The RNA pellet was rinsed with 2 M LiCl and precipitated again for 20 min at -80°C. Extracted RNA was washed in 75% ethanol and resuspended in DEPC-treated nuclease and stored at -80°C.

Residual DNA was removed by four DNase treatments (Promega, Madison, USA), purified with Trizol and chloroform, precipitated with isopropanol and two 75% ethanol wash steps. The air dried RNA was resuspended in 20 µl DEPC-treated nuclease free water and stored at -80°C. Reverse transcription (RT)-PCR was performed using random hexamers according to the protocol provided by Marligen Biosciences (Hanover, USA) first-strand cDNA synthesis system kit (20 µl reaction using 150 ng $\mu l^{-1}$ RNA).

### *PCR, cloning, RFLP* (*Restriction Fragment Length Polymorphism*) *analysis and sequencing*

A nested PCR was used to amplify the dinitrogen reductase *nifH* (Zani *et al*. 2000). All PCR reactions were performed on 25 ng of template DNA. The first PCR of the nested approach was performed using NIFH4 (5'-TTYTAYGGNAARGGNGG-3') and NIFH3 (5'-ATRTTRTTNGCNGCRTA-3'; Zani *et al*. 2000) using 0.3 U *Taq* DNA polymerase (Fischer Biotech, Perth, Australia) in a 20 µl reaction mix containing 2.5 mM MgCl2, 1x *Taq*-Polymerase buffer (Fischer Biotech), 0.5 mM dNTPs (Fischer Biotech) and 2 pmol each of forward and reverse primers. The initial denaturation step at 94°C for 4 min was followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, strand extension at 72°C for 1 min and a final extension step at 72°C for 7 min. The second amplification round was performed using 2 µl of initial PCR-product and the primers NIFH1 (5'-TGYGAYCCNAARGCNGA-3') and NIFH2 (5'-ANDGCCATCATYTCNCC-3'; Zehr & McReynolds 1989). The reaction mix and amplification protocol were used as described above except that the annealing temperature was increased to 57°C. PCR-products (350 bp) were analysed on 4% agarose gels, excised and gel-purified using a Promega Wizard SV Gel and PCR clean-up system (Promega).

For cloning, the pGem-T Easy Vector System (Promega) was used as described in Jungblut *et al*. (2005). Clones of interest were amplified using the vector specific primer MPF (5'-CCCAGTCACGACGTTGTAAAAC-3'). RFLP were performed in separate reactions using 2 units of the restriction enzymes *Alu*I or *ScriF*1 (Fermentas, Hanover, MA) in a 10 µl reaction, and RFLP patterns were analysed on 3% agarose gels with TAE-buffer. At least 100 positive clones were screened from each clone-library. Sequencing of at least one clone per unique RFLP pattern was carried out for the clone-libraries based on DNA samples as described (Jungblut *et al*. 2005). Due to the low number of different RFLP patterns obtained from the cDNA clone-library at least 4 clones were sequenced for each unique pattern. More than one pattern was obtained for each phylotype as PCR-products ligate in the forward and reverse direction into the cloning vector.

The sequence data has been submitted to the GenBank database under the accession number EU915049-EU915068.

### *Phylogenetic sequence analysis*

Translated sequences were aligned using ClustalX (1.8) (Thompson *et al*. 1994) and manually corrected. A phylogenetic tree was obtained using the PHYLIP programme version 3.67 (Felsenstein 1989), and constructed using neighbor-joining with the Dayhoff PAM Matrix (Prodist, Neighbor). The confidence levels were calculated via bootstrapping using 1000 resampling events (Seqboot, Consense, Felsenstein 1989).

### *Acetylene reduction assay*

Nitrogenase activity of the Orange Pond microbial mats was measured by the *in situ* acetylene reduction assay (ARA) following the procedures of Stewart *et al*. (1967) and Fernández-Valiente *et al*. (2001). Experiments were carried out under natural light irradiance (no shading) from 13h00–15h00 in a water bath at 0.7°C in January 2005. Fresh microbial mat material was collected and transferred into reaction vessels within thirty minutes of collection. One mat core of 20 mm diameter was placed into each reaction vessel with the surface layer facing upwards. Each treatment was incubated in duplicate in 30 ml serum bottles with 15 ml of pond water from the respective pond added. The serum bottles were sealed, and 2 ml of the gas phase was removed and 5 ml of acetylene (BOC, Australia) added

**Table I.** Water chemistry, organic content and chlorophyll *a* (chl *a*).

| | Sample 2005 | Sample 2006 |
|---|---|---|
| Chl *a* ($\mu g\,g^{-1}$ total dry weight) | 27.9 | - |
| Organic partition of total material (%) | 6.5 | - |
| $NH_4$-N[1] ($mg\,m^{-3}$) | 21 | 11 |
| $NO_3$-N[2] ($mg\,m^{-3}$) | 21 | 1 |
| TDP[3] ($mg\,m^{-3}$) | 89 | 64 |
| pH | 9.9 | 9.9 |
| Conductivity ($\mu S\,cm^{-1}$) | 3469 | 1640 |
| T (°C) | 3 | 5 |

Notes: [1] ammonium-N, [2] nitrate-N (2), [3] dissolved reactive phosphorus.

to the gas phase. Two ml of the gas phase was transferred into pre-vacuated 4 ml vacutainers (Greiner Bio-One, Australia) 2 h after the start of the experiment. Controls comprised 15 ml of pond water without a mat core. Fixation rates determined for the controls were subtracted from the treatments to account for any potential nitrogen-fixation activity in the water. Duplicate samples with 0.01 mM final concentration of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) were also prepared. The samples were carefully shaken without disturbing the mat matrix. Ethylene concentrations were determined using a gas chromatography flame ionisation detector (Shimazu model GC-8A-FID), and a Poropak T column (Alltech, Australia) at 130°C, with $N_2$ (BOC, Australia) as the carrier gas and a 0.5 ml injection volume. The acetylene reduction activity was based on a calibrated ethylene (BOC) standard curve.

## Results and discussion

Molecular analysis of functional gene diversity such as the *nifH* gene, coding for the dinitrogen reductase in the dinitrogen fixation gene cluster (Zehr *et al*. 2003), and

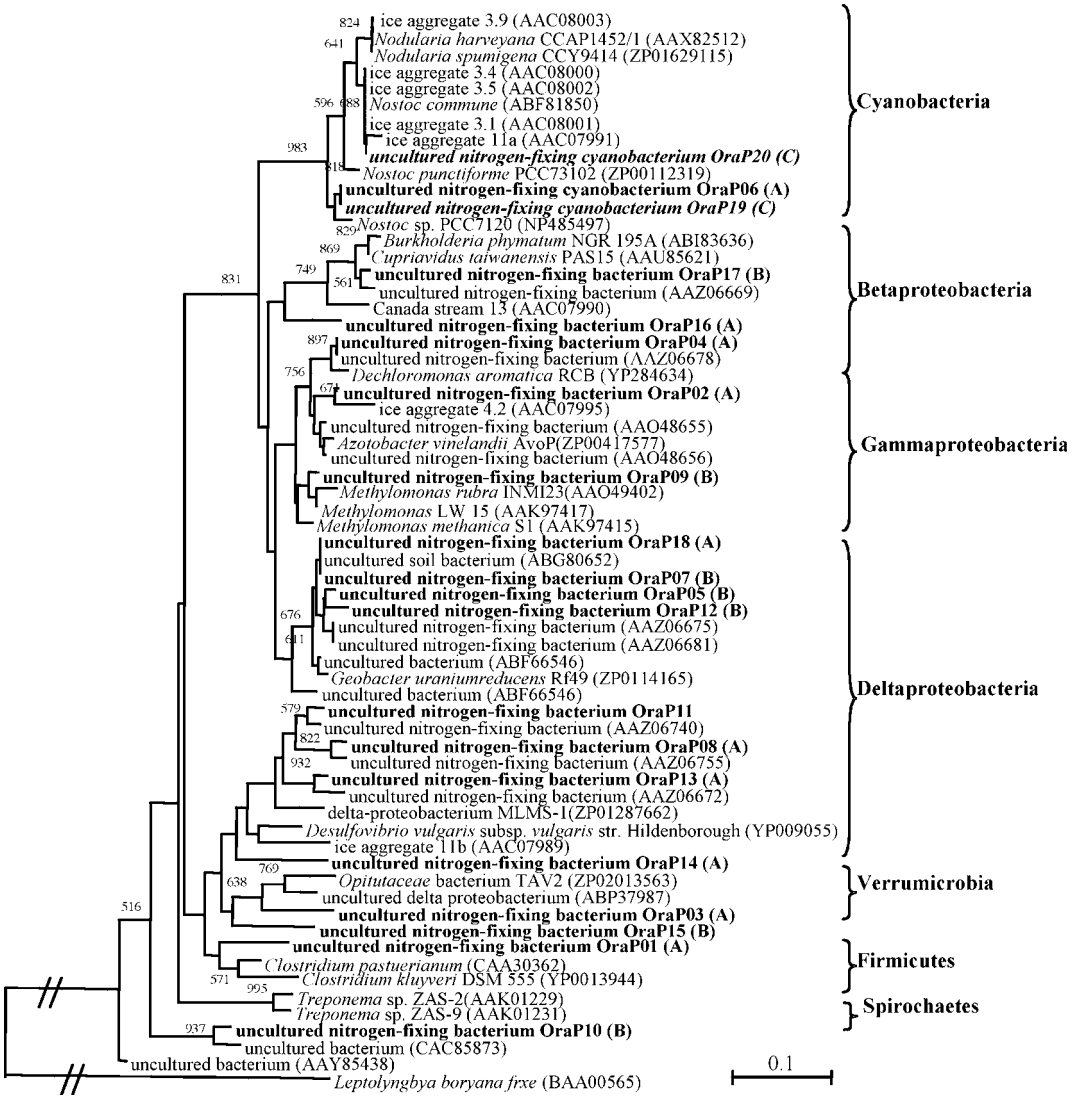


**Fig. 1.** Phylogenetic analysis with *nifH* sequence types identified from Orange Pond. A indicates sequences obtained from DNA 2005. B indicates sequences obtained from DNA 2006. C indicates sequences obtained from 2006 RNA are in italics.

https://doi.org/10.1017/S0954102009990514 Published online by Cambridge University Press

**Table II.** Summary of representative *nifH*-gene sequences recovered from Orange Pond microbial mats.

| Representative sequences | No. of clones | Habitat of highest blast match to uncultured bacterium | Accession number | Identity of sequences (%) | Highest BLAST match of cultivated bacteria | Accession number | Identity of sequences (%) | Phylogenetic affiliation based on BLAST search |
|---|---|---|---|---|---|---|---|---|
| DNA 2005 | | | | | | | | |
| Orange clone 1 | 9 | termite gut[1] | BAF52274 | 90 | *Clostridium kluyveri* DSM 555 | YP_001394423 | 88 | firmicutes |
| Orange clone 2 | 25 | - | - | - | *Azotobacter vinelandii* AvOP | ZP_00417577 | 95–97 | gamma-proteobacteria |
| Orange clone 3 | 1 | seawater[2] | ABP37987 | 91 | *Opitutaceae bacterium* TAV2 | ZP_02013563 | 88 | verrucomicrobia |
| Orange clone 4 | 4 | seawater[3] | AAZ06678 | 99–100 | *Dechloromonas aromatica* RCB | YP_284634 | 95–97 | beta-proteobacteria |
| Orange clone 5 | 4 | rhizosphere, soil[4] | ABG80652 | 100 | *Geobacter uraniumreducens* Rf4 | ZP_01141655 | 95 | delta-proteobacteria |
| Orange clone 6 | 10 | - | - | - | *Nostoc* sp. PCC 7120 | NP_485497 | 94–95 | cyanobacteria |
| DNA 2006 | | | | | | | | |
| Orange clone 7 | 8 | - | - | - | *Methylomonas methanica* S1 | AF378717 | 95, 96 | gamma-proteobacteria |
| Orange clone 8 | 1 | seawater[3] | AAZ06755 | 97 | delta-proteobacterium MLMS-1 | ZP_01287662 | 87 | delta-proteobacteria |
| Orange clone 9 | 5 | seawater[3] | AAZ06675 | 97–98 | *Geobacter uraniumreducens* Rf4 | ZP_01141655 | 94–95 | delta-proteobacteria |
| Orange clone 10 | 9 | marine sediment (oil)[5] | AAY85438 | 88–90 | *Treptonema* sp. ZAS-9 | AAK01231 | 82–84 | spirochaetes |
| Orange clone 11 | 6 | sea water[3] | AAZ06740 | 95–97 | delta-proteobacterium MLMS-1 | ZP_01287662 | 90 | delta-proteobacteria |
| Orange clone 12 | 4 | rhizosphere, soil[3] | ABG80652 | 98–100 | *Geobacter uraniumreducens* Rf4 | ZP_01141655 | 95–96 | delta-proteobacteria |
| Orange clone 13 | 2 | seawater[3] | AAZ06672 | 96, 97 | delta proteobacterium MLMS-1 | ZP_01287662 | | delta-proteobacteria |
| Orange clone 14 | 1 | termite gut[1] | BAF52274 | 90 | *Clostridium kluyveri* DSM 555 | YP_001394423 | 88 | firmicutes |
| Orange clone 15 | 1 | soil[6] | ABF66546 | 90 | *Methylomonas* sp. LW13 | AF378715 | 85 | gamma-proteobacteria |
| Orange clone 16 | 1 | mangrove[7] | ABM66820 | 89 | *Desulfovibrio vulgaris* (*vulgaris* H.) | YP_009055 | 85 | delta-proteobacteria |
| Orange clone 17 | 4 | seawater[3] | AAZ06669 | 100 | *Burkholderia* sp. PTK47 | AAU85620 | 96 | beta-proteobacteria |
| Orange clone 18 | 2 | seawater[3] | AAZ06681 | 96 | *Geobacter uraniumreducens* Rf4 | ZP_01141655 | 95 | delta-proteobacteria |
| RNA 2006 | | | | | | | | |
| Orange clone 19 | 15 | - | - | - | *Nostoc* sp. PCC 7120 | NP_485497 | 94–96 | cyanobacteria |
| Orange clone 20 | 1 | - | - | - | *N. punctiforme* PCC73102 | ZP_00109382 | 99 | cyanobacteria |

Habitat notes: 1) *Cryptocercus punctulatus*, termite gut, 2) western English Channel, 3) USA: Chesapeake Bay, seawater, 4) white spruce (*Picea glauca*) rhizosphere soil, 5) oil-contaminated marine sediment, 6) long-term tilled soil, macroaggregate, 7) mangrove.

characterization of its RNA transcript diversity, allows us to link community diversity with physiological activities (Omoregie *et al*. 2004). In the study presented here, molecular analysis enabled us to gain further insights in to the $N_2$-fixing diversity of Orange Pond microbial mats on the McMurdo Ice Shelf.

Orange Pond had a conductivity of 3469 $\mu S\,cm^{-1}$ and therefore represents a medium conductivity pond within the network of McMurdo Ice Shelf meltwater ponds (Howard-Williams *et al*. 1989). The pH values recorded were 9.9 for Orange Pond water in both sampling years. The organic content of the total mat material was 6.5%, with a chl *a* concentration of 27.9 $\mu g\,g^{-1}$ of the total dry weight in the sampling year 2005. Ammonium-N and nitrate-N concentrations were 21 $mg\,m^{-3}$ or lower with total dissolved phosphorus of 89 $mg\,m^{-3}$ or less (Table I).

The RNA yield was 17.78 $ng\,mg^{-1}$ of Orange Pond microbial mat wet weight. The DNA yields were at least 150 $ng\,mg^{-1}$ of Orange Pond microbial mat dry weight (2005) and wet weight (2006), respectively. PCR-products were successfully amplified and cloned from DNA and cDNA templates using the nested amplification approach. Based on the RFLP analyses of at least 100 clones for each clone-library, a total of 113 clones were sequenced, 43 from the 2005 DNA sample, 53 from the 2006 DNA sample, and 16 from the 2006 cDNA clone library.

Phylogenetic analysis of the *nif*H gene sequences was performed in relation to reference sequence data obtained from NCBI BLAST searches (Altschul *et al*. 1990). For each new phylotype, reference sequences were included when they were the closest match out of all GenBank (NCBI) sequences. Other reference sequences were included when they were the closest related taxonomically characterized isolate. *nifH* sequence data from Lake Bonney, Canada Stream and Dry Pond (McMurdo Dry Valleys) (Olson *et al*. 1998) were also included in the phylogenetic analysis to compare the overall distribution of the Antarctic *nifH* phylotype diversity.

Using *nifH* transcript analysis by reverse transcription-PCR, we only identified sequences with highest similarity to *Nostoc* spp. (Table I) based on more than 100 analysed RFLP patterns. All sequences of *nifH* transcripts had an identity of at least 94% to *Nostoc* sp. PCC7120 (NP_485497) and 99% to *Nostoc* sp. PCC73102 (ZP_00109382) (Fig. 1, Table I).

In contrast, we identified 18 *nifH* gene phylotypes from the DNA samples with highest similarities ranging from 82–97% to cyanobacteria, firmicutes, gamma-, beta- and deltaproteobacteria, spirochaetes and verrumicrobia (Table II, Fig. 1). Differences in *nifH* gene diversity between the two sampling years could be due to PCR-bias, storage or small-scale spatial variations in microbial diversity within the pond. The meltwater ponds and mat communities are generally considered to be stable due to the short annual growth periods, constant nutrient and physico-chemical condition (Vincent *et al*. 1993, Hawes *et al*. 1999, Jungblut *et al*. 2005).

**Table III.** Acetylene reduction assay measurements.

| ARA | Sample 2005 |
|---|---|
| ARA (pmol $C_2H_4$ $\mu g$ chl $a^{-1}\,h^{-1}$) | 540 ± 194 |
| ARA ($\mu mol$ $C_2H_4$ $cm^{-2}\,h^{-1}$) | 3.4 ± 1.2 |
| ARA + DCMU (pmol $C_2H_4$ $\mu g$ chl $a^{-1}\,h^{-1}$) | 0 |

Interestingly, no sequences within cyanobacteria other than *Nostoc* sp. were obtained in the analysis of the *nifH* gene diversity, even though Oscillatoriales comprises at least 70% of the cyanobacterial assemblage (Vincent *et al*. 1993). This suggests that $N_2$-fixation genes are not present in the Oscillatoriales assemblage. This agrees with previous $N_2$-fixation measurements on microbial mats on the McMurdo Ice Shelf, where the acetylene reduction activity was mainly attributed to heterocystous cyanobacteria and no activity was observed in absence of light (Fernández-Valiente *et al*. 2001). This is in contrast to microbial mat from Guerrero Negro, Baja California (Mexico) where typically cyanobacterial *nifH* phylotypes are Oscillatoriales groups, including *Phormidium* and *Plectonema,* as well as unicellular *Halothece* and *Synechocystis* (Omoregie *et al*. 2004).

Although, the diversity of non-cyanobacterial *nifH* phylotypes matches previous 16S rRNA gene, culturing and signature lipid studies of bacterial diversity of the McMurdo Ice Shelf (Mountfort *et al*. 1997, Sjöling & Cowan 2003, Jungblut *et al*. 2009), the present molecular data suggest that *nifH* transcription was absent or under the detection limit with the tested conditions based on the screening of 100 clones. This stands also in contrast to microbial mats from Guerrero Negro, Baja California (Mexico) where *nifH* transcripts were recovered from cyanobacteria and heterotrophic bacteria (Omoregie *et al*. 2004). Therefore, additional analysis of clones from the cDNA-library could give further clarification to the here identified *nifH* transcript diversity. However, the absence of *nifH* transcripts related to heterotrophic bacteria is consistent with previous studies by Fernández-Valiente *et al*. (2001), where contributions to the total nitrogen budget by heterotrophic bacteria were suggested to be very low. This could be potentially due to unfavourable conditions such as micronutrient limitations and the absence of anoxia due to high photosynthesis rates with 24-h light during the summer.

Furthermore, mean nitrogenase activity was similar to previous measurements by Fernández-Valiente *et al*. (2001) with 540 ± 194 pmol ethylene $\mu g$ chl $a^{-1}\,h^{-1}$ ($n = 2$), (3.4 ± 1.2 $\mu mol$ ethylene $cm^{-2}\,h^{-1}$) in the presence of light and absence of DCMU, whereas no activity was observed in the presence of DCMU (Table III). This $N_2$-fixation activity is indicative of phototrophic activity such as potentially heterocyst-forming cyanobacteria (*Nostoc* sp.) and therefore agrees with the molecular findings. However, additional combined physiological and molecular analyses would provide a more detailed understanding of

https://doi.org/10.1017/S0954102009990514 Published online by Cambridge University Press

non-cyanobacterial $N_2$-fixation in McMurdo Ice Shelf mat systems.

Comparisons with other Antarctic freshwater ecosystems suggested the presence of similar *nifH* phylotype diversity, although Orange Pond mats contained greater diversity than reported for theMcMurdo Dry Valley communities. Phylotypes from Dry Valley mats with highest similarity to *Nostoc* sp. were also the only cyanobacterial related sequences that were recovered. Our results here were also characterized by a large number of clone sequences that had relatively low similarity to other known species, a finding that is in common with many other environmental studies (Zehr *et al*. 2003).

In summary, additional combined physiological and molecular analyses are needed to fully understand the functional diversity behind $N_2$-fixation in Antarctic microbial mats. However, these first molecular results elucidate the *nifH* phylotype diversity and confirm the importance of *Nostoc* spp. for the nitrogen budget on the McMurdo Ice Shelf, Antarctica.

## Acknowledgements

We would like to thank Donna Sutherland and Ian Hawes for their help, and Antarctica New Zealand for providing logistical support during the field event of 2005, as well as for financial support from the New Zealand Foundation for Research, Science and Technology. Thanks to Reut Sorek Abramovich for assistance and Michelle Gehringer and Brendan Burns for revisions. The field event of 2005 was supported by a Boehringer-Ingelheim Funds Travel Allowance to A.D.J. Antonio Quesada is thanked for his assistance with the 2006 sampling which was made possible in part by the US National Science Foundation-sponsored International Graduate Training Course in Antarctic Biology (NSF grant No. 0504072 to D.T. Manahan). B.A.N was supported by a grant and fellowship from the Australian Research Council.

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