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Lichen-symbiotic cyanobacteria associated with *Peltigera* have an alternative vanadium-dependent nitrogen fixation system

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In past decades, environmental nitrogen fixation has been attributed almost exclusively to the action of enzymes in the well-studied molybdenum-dependent nitrogen fixation system. However, recent evidence has shown that nitrogen fixation by alternative pathways may be more frequent than previously suspected. In this study, the nitrogen fixation systems employed by lichen-symbiotic cyanobacteria were examined to determine whether their diazotrophy can be attributed, in part, to an alternative pathway. The mining of metagenomic data (generated through pyrosequencing) and PCR assays were used to determine which nitrogen-fixation systems are present in cyanobacteria from the genus *Nostoc* associated with four samples from different geographical regions, representing different lichen-forming fungal species in the genus *Peltigera*. A metatranscriptomic sequence library from an additional specimen was examined to determine which genes associated with N_2 fixation are transcriptionally expressed. Results indicated that both the standard molybdenum-dependent system and an alternative vanadium-dependent system are present and actively transcribed in the lichen symbiosis. This study shows for the first time that an alternative system is utilized by cyanobacteria associated with fungi. The ability of lichen-associated cyanobacteria to switch between pathways could allow them to colonize a wider array of environments, including habitats characterized by low temperature and trace metal (e.g. molybdenum) availability. We discuss the implications of these findings for environmental studies that incorporate acetylene-reduction assay data.

Key words: alternative nitrogenase, cyanobacteria, dinitrogen fixation, lichen, molybdenum, vanadium

Introduction

A lichen has traditionally been defined as a specific structure formed by a fungus (mycobiont) that develops a symbiotic relationship with a green alga and/or a cyanobacterium (photobiont). More recently, lichens have come to be viewed as complex ecosystems, hosting diverse microbial lineages from across the tree of life (Arnold *et al.*, 2009; Hodkinson & Lutzoni, 2009; Bates *et al.*, 2011; Cardinale *et al.*, 2012; Hodkinson *et al.*, 2012*b*). While evidence suggests that certain bacterial and fungal lineages may have strong preferences for living in association with lichens, as members of the lichen microbiome (Arnold *et al.*, 2009; Hodkinson & Lutzoni, 2009; U'Ren *et al.*, 2010, 2012; Bates *et al.*, 2011; Schneider *et al.*, 2011; Hodkinson *et al.*, 2012*b*), the relationship between the two main associates (mycobiont and photobiont) is usually considered to be unique because the partners appear to be adapted to the symbiotic lifestyle (Ahmadjian, 1993) and comprise the bulk of the lichen biomass. Cyanolichens – those lichens with heterocystous cyanobacterial photobionts – are special among lichen symbioses because the photobiont fixes nitrogen (N_2). Cyanolichens contribute significant levels of fixed nitrogen to ecosystems worldwide (Crittenden & Kershaw 1978; Gunther, 1989; Nash,

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Fig. 1. A cyanolichen thallus formed by the lichenized fungus (mycobiont) *Peltigera rufescens* with a cyanobacterial photobiont from the genus *Nostoc*.

2008*a*), making them key for understanding global nutrient cycling and terrestrial ecology.

The most common type of cyanobacterial lichen photobiont is *Nostoc* (Nostocaceae) (Antoine, 2004; Nash, 2008*a*), which is the symbiont associated with members of the lichen-forming fungal genus *Peltigera* (Tschermak-Woess, 1988; Miadlikowska & Lutzoni, 2004; O'Brien *et al.*, 2005; Hodkinson *et al.*, 2012*a*), the group that is the focus of our research (Fig. 1). Nitrogen fixation by lichen-associated *Nostoc* has been attributed solely to the enzyme complex encoded by the *nifHDK* operon (Brigle *et al.*, 1985; Thiel *et al.*, 2002). The two components of this enzyme complex are an iron (Fe) protein (dinitrogenase reductase 1, encoded by *nifH*) and a molybdenum–iron (Mo–Fe) protein (dinitrogenase 1, for which *nifD* and *nifK* encode the α- and β-subunits, respectively) (Gillum *et al.*, 1977; Hausinger & Howard, 1983; Brigle *et al.*, 1985; Georgiadis *et al.*, 1992).

Studies of the cyanobacterial genus *Anabaena* (a member of the same family as *Nostoc*, Nostocaceae) revealed that certain strains, all of which appear to be either obligate or facultative symbionts with plants, have an additional nitrogenase complex that is encoded by the *vnf* gene cluster (Thiel, 1993; Zehr *et al.*, 2003; Raymond *et al.*, 2004; Boison *et al.*, 2006). The presence of the latter has also been inferred in a single strain of the cyanobacterial genus *Nostoc* (PCC 7422, isolated from plant tissue of *Cycas* sp.; Nostocaceae) through Southern hybridization, growth on Mo-free media, and ethane formation from acetylene (Masukawa *et al.*, 2009); however, neither the presence of *vnf* genes in the strain nor the phylogenetic placement of the strain within *Nostoc* has been confirmed using sequence data. One of the two components of the *vnf*-encoded complex is an iron (Fe) protein (dinitrogenase reductase 2, encoded by *vnfH*) that is essentially identical in sequence to the product of *nifH*, and is cross-functional with it as an electron donor in *Anabaena variabilis* (Pratte *et al.*, 2006). The other component is a vanadium–iron (V–Fe) protein (dinitrogenase 2, for which *vnfDG* encodes the fused α-/δ-subunit and *vnfK* encodes the β-subunit) (Robson *et al.*, 1986, 1989; Joerger *et al.*, 1990; Zehr *et al.*, 2003; Raymond *et al.*, 2004; Boison *et al.*, 2006). In *A. variabilis*, the *vnfEN* genes are also essential for N_2 fixation (functioning as a scaffold for catalytic cluster formation), although these genes are apparently absent in many strains of bacteria that utilize this system (e.g. *Azotobacter* spp.) (Thiel, 1996). A third system encoded by the *anfHDGK* operon (which is dependent upon Fe only and expressed under both Mo- and V-limited conditions) is known from lineages outside of the phylum Cyanobacteria (Bishop & Joerger, 1990).

The three major enzyme complexes for N_2 fixation can be viewed in terms of a cascade of efficiency, with the Mo-dependent (*nif*-encoded) complex being most efficient, followed by the V-dependent (*vnf*-encoded) complex, and finally the Fe-dependent (*anf*-encoded) complex (Robson *et al.*, 1986, 1989; Hales *et al.*, 1986*a*, 1986*b*; Chisnell *et al.*, 1988; Eady, 1989, 2003; Eady *et al.*, 1988; Joerger *et al.*, 1990; Walmsley & Kennedy, 1991; Raina *et al.*, 1993; Bellenger *et al.*, 2011). Paradoxically, Mo is the least abundant of the three crucial biometals in the continent crust, whereas V is approximately two orders of magnitude more abundant, and Fe is by far the most abundant of the three (Erickson, 1973; Wedepohl, 1995). Under special conditions, such as when Mo is limiting in particular microenvironments, N_2 fixation via the alternative pathways may be favoured (Silvester, 1989; Barron *et al.*, 2009; Boyd *et al.* 2011; Wurzburger *et al.*, 2012). It is also known that the V-dependent nitrogenase is more efficient than the Mo-dependent nitrogenase at low temperatures (Miller & Eady, 1988), and that, while the V-dependent nitrogenase is typically considered to be repressed by Mo (based primarily on studies performed at room temperature), this enzyme is not documented to be regulated by Mo at any temperature under 14°C (Walmsley & Kennedy, 1991).

While alternative N_2 fixation has been considered rare, Betancourt *et al.* (2008) demonstrated that diazotrophs using these systems can be isolated from diverse natural environments. The purpose of this study was to determine whether the V-dependent N_2 fixation pathway is used by strains of *Nostoc* associated with cyanolichens from the genus *Peltigera*. To accomplish this, we used a sequence-based approach: (1) probing metagenomic libraries from three

Peltigera samples for sequences representing *vnf* genes, (2) using PCR and Sanger sequencing both to supplement the lowest coverage metagenomic library and to determine the presence/absence of *vnf* genes in an additional sample, and (3) probing a metatranscriptomic sequence library from an additional sample to examine whether RNA is transcribed from the *vnf* operon.

Materials and methods

Metagenomic analyses

To determine the presence or absence of cyanobacterial *vnf* genes in *Peltigera* lichen thalli, metagenomes from three different samples were examined (Table 1). A metagenomic sequence library was constructed from a *P. dolichorhiza* thallus (extract: DNA-2289) using Roche 454 sequencing as described by Magain *et al.* (2010). *Peltigera malacea* and *P. membranacea* metagenomic assemblies were constructed from Roche 454 sequence reads as described by Xavier *et al.* (2012). The nucleotide sequence of a full-length cyanobacterial *vnf* operon was retrieved from the NCBI nr/nt collection (*A. variabilis* ATCC 29413; GenBank accession: CP000117, GI: 75699950; bases 5009717 to 5019000 were extracted in FASTA format) and a BLASTn search was conducted using BLAST+ 2.2.24 against the metagenomic sequence libraries (Altschul *et al.*, 1997), which had been formatted as BLAST databases using the mpiformatdb command in MPIBLAST 1.6.0. Sequences in the metagenomes that returned results with bitscores above 65 and E-values below 1.00E-07 were identified as putative *vnf* sequences (see sequences archived in GenBank for final bitscores and E-values through online BLAST).

PCR for vnf *genes*

PCR and Sanger sequencing were performed on two *Peltigera* samples (1) to obtain longer sequences from the *vnf* operon in the sample of a *P. dolichorhiza* thallus discussed above, and (2) to determine whether sequences encoding *vnf* genes were present in one additional *Peltigera* sample representing *P. neopolydactyla* (and, if so, to obtain representative sequences of *vnfDG* and *vnfN* from this sample). To design primers for the amplification of cyanobacterial *vnf* genes, *Anabaena vnf* operon sequences from GenBank (GI: 75699950, 37694441, 83034994 and 37694450) were aligned with fragments that showed high sequence similarity (determined using the BLASTn criteria defined above under 'Metagenomic analyses') from both the *P. dolichorhiza* metagenome discussed above and a *P. praetextata* metatranscriptome (Hodkinson, 2011; Table 1; see below, 'Metatranscriptomic analyses'). Regions in which sequences were conserved between the GenBank reference sequences and at least one metagenomic or metatranscriptomic sequence fragment were targeted for primer design.

For PCR reactions to amplify fragments of *vnfDG*, primers vnfDG1F, 2F and 3F were combined with all six reverse *vnfDG* primers (Fig. 2; Table 2); additionally, vnfDG4F and 5F were combined with vnfDG9R (giving a total of 20 primer combinations for the *vnfDG* region). For *vnfN* amplification, all forward primers were combined with all reverse primers (a total of 12 possible combinations for the *vnfN* region). PCR conditions and thermocycler settings followed Hodkinson & Lutzoni (2009). Two *Peltigera* DNA extracts were analysed: DNA-2289, also used for generating the *P. dolichorhiza* metagenomic dataset described in detail by Magain *et al.* (2010), and DNA-2308, extracted from a sample of *P. neopolydactyla* using a modified CTAB extraction protocol (Table 1). Sequencing reactions were carried out with ABI Big Dye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) diluted to 1/64th reactions using BDX64 BigDye enhancing buffer (MCLAB, South San Francisco, California, USA) according to standard BDX64 protocols with the same primers used for amplification; electrophoresis was performed using an ABI 3130xl Genetic Analyzer (Applied Biosystems) and sequence reads were assembled using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Table 1. *Nostoc*-containing cyanolichen samples analysed as part of this study. 'Data-type' gives the type of sequence data generated: MG = Roche 454-based shotgun metagenome; MT = Roche 454-based metatranscriptome; PCR = PCR-amplified, Sanger-sequenced set of *vnf* gene fragments.

Mycobiont	Geographical region	Data type	Voucher Identifier(s)	Herbarium
Peltigera dolichorhiza	La Réunion, Indian Ocean	MG+PCR	*Magain & Sérusiaux 11-09* / DNA-2289	LG
Peltigera malacea	British Columbia, Canada	MG	Accession: DB3992	UBC
Peltigera membranacea	Reykjavik, Iceland	MG	LA-31632 / Accession: XBB013	IINH
Peltigera neopolydactyla	Alaska, USA	PCR	*Goffinet 9381* / DNA-2308	CONN
Peltigera praetextata	North Carolina, USA	MT	*Hodkinson 11804* / Barcode: 3133	DUKE

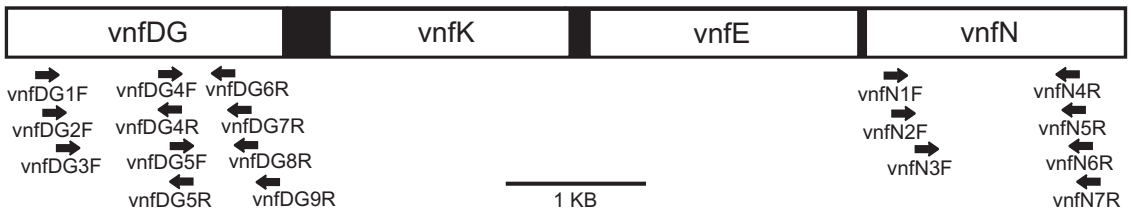

Fig. 2. Primer map of the *vnf* gene cluster in cyanobacteria (see Table 2 for individual primer sequences).

Table 2. Cyanobacterial *vnf* primers designed for this study.

Primer	Sequence (5'–3')
vnfDG1F	TATTAAAGTGCGACGAAAC
vnfDG2F	ATCAAAGAAAARGGSGAAGA
vnfDG3F	GAAGAYACAACCCAATTTCT
vnfDG4F	GGCAAAAAAGTCTGTAT
vnfDG5F	TGGCACTGGACMAAAGC
vnfDG4R	ATACAGACTTTTTTGCC
vnfDG5R	GCTTTKGTCCAGTGCCA
vnfDG6R	CCATCATCAATATAGAT
vnfDG7R	TCAAAGCCCATRTATGG
vnfDG8R	AGGMGAATARATGGCATT
vnfDG9R	AAACAMCGYTCTTGAAT
vnfN1F	AAAGACCCAATYGTIGGCTGTGC
vnfN2F	AAAGATGTCAGTATTGT
vnfN3F	ATGTTTGTCGTTGGYACTTGCGC
vnfN4R	GCCATGTATTTTTCCCA
vnfN5R	GGACTAAATACATCAAAA
vnfN6R	CCTTCATAGCCAAAATA
vnfN7R	CTCTWTCCCAATCGTTAGCAA

Metatranscriptomic analyses

To determine whether transcription of genes from the *vnf* cluster could be detected in a sample of *Peltigera*, we probed a metatranscriptomic sequence library of a single sample from a lichen thallus dominated by the fungus *P. praetextata* and the cyanobacterium *Nostoc* sp. (Table 1). Sample collection, permanent voucher storage, nucleic acid extraction, rRNA subtraction, cDNA preparation and Roche 454 GS FLX sequencing were performed as outlined by Hodkinson (2011). Sequence data were uploaded for overall assessment of metatranscriptome content to the MG-RAST server (http://metagenomics.nmpdr.org/; Meyer *et al.*, 2008) and the dataset was made public under project 3216, metagenome 4453629.3. Transcripts identified by MG-RAST as being derived from genes involved in N_2 fixation were downloaded and MegaBLAST searches were performed against the NCBI nr/nt database to confirm their taxonomic affinities (Zhang *et al.*, 2000). Additionally, BLASTn searches for N-fixation genes were conducted on the entire metatranscriptomic dataset as previously outlined above (in 'Metagenomic analyses') using the metatranscriptomic sequence file as a BLAST database to identify transcripts potentially derived from N_2 fixation genes that were not detected by MG-RAST.

Phylogenetic inference

To determine the affinities of the *vnf* sequences found in lichen thalli, a phylogeny was inferred using a *vnfD* sequence dataset assembled from the following: (1) composite sequences made of sequence fragments from PCR and metagenomic libraries that showed high sequence similarity to the *vnfD*-like portion of the *vnfDG*-encoding sequence in *A. variabilis* (determined using BLASTn criteria defined above); (2) the top BLASTn hits for the *P. membranacea vnfD*-like portion of the *vnfDG*-encoding sequence in the NCBI nr/nt database ('maximum bitscore' of 100+); and (3) the list of sequences found by searching for 'vnfD' in the NCBI nucleotide database in September 2012. Sequences from the second and third categories were downloaded from GenBank in FASTA format and were processed using a PERL script written to rename the sequences for easy downstream processing ('genbank_fasta_renamer.pl,' available here as Supplementary file 3). When multiple GenBank records existed for the same strain, only the most complete sequence was retained; sequences with significant unalignable portions within the *vnfD* region were likewise removed. Sequences were aligned by hand and ambiguously aligned regions were designated as exclusion sets using Mesquite 2.74 (Maddison & Maddison, 2010). The full sequence *vnfD* alignment file is available in NEXUS format in Supplementary File 1. To prepare the alignment for analyses with RAxML 7.2.6 (Stamatakis, 2006), characters in exclusion sets were manually deleted and the alignment was exported in extended PHYLIP format. Maximum likelihood phylogenetic analyses were performed with three partitions for the three different codon positions. A set of 500 bootstrap replicates was run (Pattengale *et al.*, 2009), followed by a thorough topology search with the '-f a' function in RAxML, using a GTRGAMMA model. A general-time-reversible (GTR) model was selected because its use was not prohibited by time or computing resources. Simplified models (that can be viewed as special cases of GTR) have often been favoured in cases where it can be demonstrated that inferences would not suffer significantly from their use (based on LRT or AIC) (Posada & Crandall, 1998); however, given the speed provided by RAxML, there was no need in this case to simplify the model. Since the use of a gamma distribution is the best way to account for among-site rate heterogeneity (Mayrose *et al.*, 2005), GTRGAMMA was the logical choice for a model. While some researchers have attempted to take into account the proportion of invariant sites while using gamma, we did not consider it appropriate, as it creates a strong correlation created between I and α, which could bias results (Yang, 1993; Gu *et al.*, 1995; Sullivan *et al.*, 1999; Minin *et al.*, 2003; Mayrose *et al.*, 2005; Ren *et al.*, 2005; Yang, 2006). The procedures outlined above for *vnfD* were used to analyse sequences from the *vnfN* region (the alignment is given in Supplementary File 2), with the following differences in dataset assembly: (1) in addition to sequence data from PCR and metagenomic libraries, data from the *P. praetextata* metatranscriptomic sequence library were also included (this consisted of a single *vnfN* sequence fragment); (2) the *P. membranacea vnfN* sequence fragment was used for obtaining top BLASTn hits from the NCBI nr/nt database (as above, a 'maximum bitscore' of 100+ was used); and (3) the term 'vnfN' was used to query the NCBI nucleotide database in September 2012.

Sequences from the *vnf* gene cluster in *Nostoc* were deposited in GenBank under accession numbers KF662359–KF662370 (see Table S1).

Results

For the *Peltigera dolichorhiza* sample, DNA-2289, PCR with Sanger sequencing and Roche 454-based shotgun metagenomic sequencing produced *vnf* sequences showing high similarity to *Anabaena vnf* sequences as determined by BLAST searches (GenBank accessions KF662362 and KF662369). Sequences for the cyanobacterial *vnf* gene cluster were found by probing the metagenomes of *P. malacea* (GenBank accessions KF662359 and KF662365)

and *P. membranacea* (GenBank accessions KF662360, KF662364, KF662366 and KF662370). Portions of cyanobacterial *vnfDG* and *vnfN* genes were successfully amplified through PCR and sequenced from *Peltigera neopolydactyla* (DNA-2308; GenBank accessions KF662361 and KF662368). Cyanobacterial genes from the *nif* and *vnf* clusters were found through MG-RAST analyses of the metatranscriptomic dataset generated from the single sample of *P. praetextata* (MG-RAST project 3216, metagenome 4453629.3). All *nif* and *vnf* fragments from the metatranscriptome were identified as belonging to the Cyanobacteria. For the single *P. praetextata* sample, two transcripts were found from the *vnf* cluster, one representing the *vnfN* gene (GenBank accession KF662367), and one representing a part of the *vnfDG* gene that is partially at the interface between the *vnfD*-like and the *vnfG*-like portions and partially within the *vnfG*-like portion (GenBank accession KF662363). The results from MG-RAST were corroborated by BLAST analyses of the entire metatranscriptomic dataset against the *Anabaena vnf* cluster.

Alignment and phylogenetic analyses of *vnfD* sequences (and sequences representing the *vnfD*-like portion of *vnfDG*) demonstrated the relationship of sequences derived from the *Peltigera* thalli to *Anabaena* sequence fragments, which is best characterized as close but distinct, since all *Nostoc*-derived (lichen-associated) sequences formed a clade separate from the *Anabaena* clade (Fig. 3). The *vnfN*-encoding sequence detected in the metatranscriptome

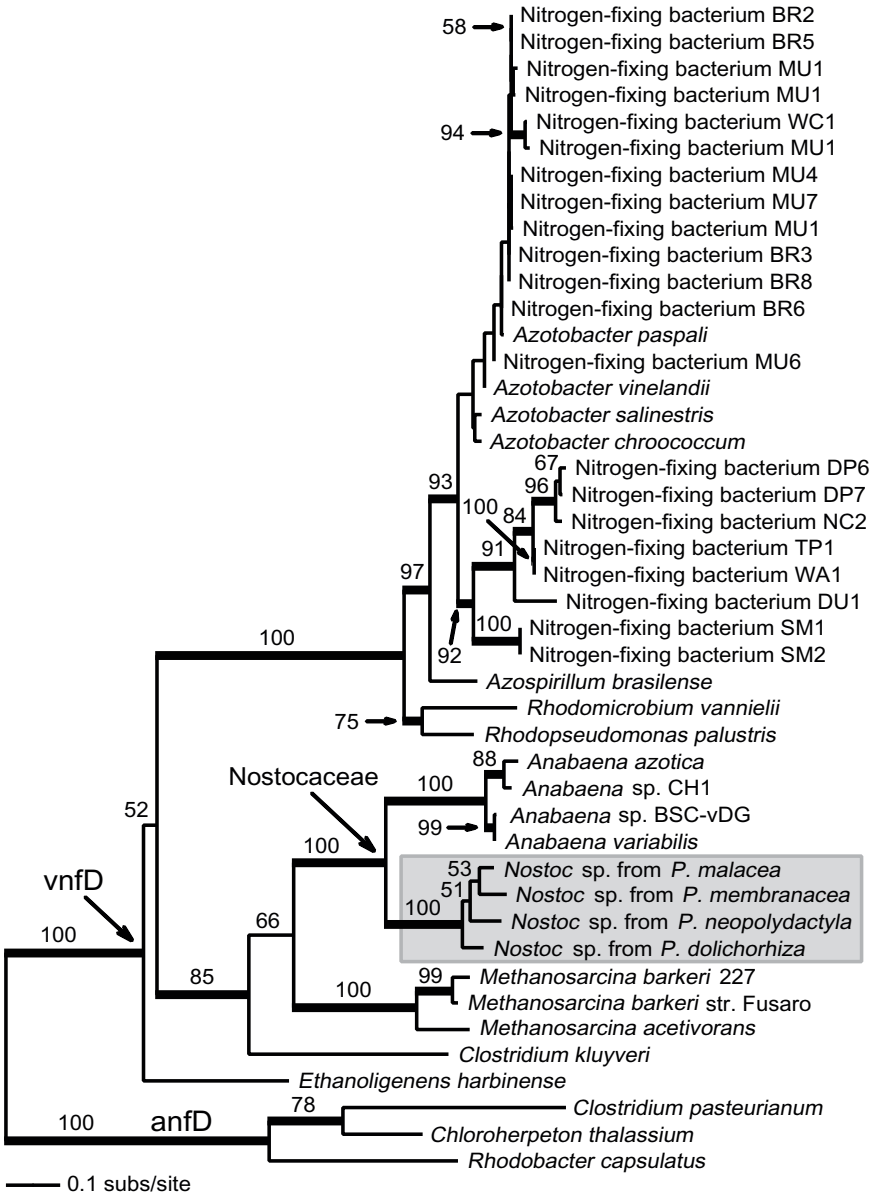

Fig. 3. Maximum likelihood (ML) phylogeny of *vnfD* (including the *vnfD*-like portion of *vnfDG* for strains in which a fusion of adjacent genes has occurred) demonstrating the placement of lichen-associated sequences as a clade sister to *Anabaena* (Nostocaceae). Values above branches represent ML bootstrap proportions (BP) ≥ 50%; branches in bold indicate ML-BP support ≥ 70%.

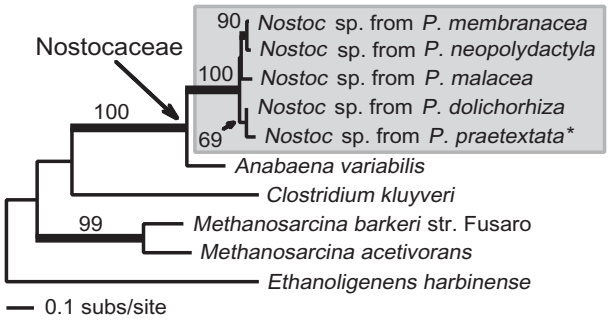

Fig. 4. Maximum likelihood (ML) phylogeny of *vnfN*, demonstrating the placement of the *Peltigera praetextata vnfN* transcript (*). Values above branches represent ML bootstrap proportions (BP) ≥ 50%; branches in bold indicate ML-BP support ≥ 70%.

(generated from a single *P. praetextata* thallus) was inferred to be part of the *Nostoc* clade, which contained all other *vnfN*-encoding sequences derived from lichen thalli (Fig. 4).

Discussion

The evidence presented here demonstrates the presence and transcription of genes encoding enzymes for two N_2 fixation pathways in lichen-associated *Nostoc*. The alternative (V-dependent) system, which has not previously been detected in this type of cyanobacterium, is shown to be present in all five *Peltigera*-dominated cyanolichens examined, which represent different fungal species from different geographical regions, and is demonstrated to be transcriptionally expressed in the one sample examined in this manner (Table 1).

The presence of two N_2 fixation pathways presumably gives the cyanobacteria in this association the ability to use either one pathway or another when different trace elements become scarce or unavailable (Kutsche *et al.*, 1996; Premakumar *et al.*, 1998). For this reason, they will have a greater ability to colonize a wide array of environments, particularly those with habitats characterized by low temperature and metal availability. The V-dependent pathway is known to be less efficient for N_2 fixation under typical circumstances (Eady, 1996, 2003; McKinlay & Harwood, 2011). However, if Mo is not present in a high enough concentration, or temperatures are too low for the Mo-dependent pathway to operate effectively, the V-dependent pathway can allow N_2 fixation to continue (Miller & Eady, 1988; Young, 2005). Given that vanadium is more abundant on the Earth's continental surface than molybdenum (Erickson, 1973; Wedepohl 1995), and that Mo has been shown to limit N_2 fixation in certain ecosystems, this flexibility could be quite significant for lichens, which often live in nutrient-poor habitats and acquire much of their mineral nutritional requirements simply through aerial deposition (Nash, 2008*b*).

It is noteworthy that all transcripts representing N_2 fixation genes were identified as belonging to the Cyanobacteria in the single sample for which transcripts were examined. These results perhaps suggest that the various non-cyanobacterial inhabitants of cyanolichen thalli that are potential nitrogen fixers (e.g. diverse members of Rhizobiales from families or genera containing known nitrogen fixers: Hodkinson *et al.*, 2012*b*) do not fix a significant amount of nitrogen on the ecosystem level. However, one cannot discount the possibility that these results could stem from a more punctuated, less continuous strategy of N_2 fixation in the majority of bacteria, compared to heterocystous cyanobacteria. Additional transcriptional observations are required at various time-points (e.g. under different conditions or at different developmental stages) to understand the relative fixed nitrogen contributions of cyanobacteria and the myriad non-photoautotrophic potential nitrogen fixers found in association with cyanolichen thalli.

Our findings demonstrate that N_2 fixation by lichen-symbiotic cyanobacteria is more complex than previously suspected, and that the possession of different nitrogenases could potentially 'buffer' cyanolichens against environmental stresses, such as Mo availability and variation in abiotic factors (e.g. temperature). The fact that the V-dependent nitrogenase is found in a number of symbiotic strains of heterocystous cyanobacteria, while it is not known from any exclusively free-living cultured strains of the same genera, suggests a possible link with symbiosis. However, further speculation regarding the possibility of a greater evolutionary advantage of such a dual system of N_2 fixation in symbiotic vs. free-living cyanobacteria will require more research into the fine-scale movements of micronutrients in various symbiotic and non-symbiotic microcosms.

The new findings presented here can also help scientists to gain a better understanding of N_2 fixation in lichen-rich environments because they inform the interpretation of results from the acetylene reduction assay (ARA), the most common method used for examining amounts of environmental N_2 fixation (Koch & Evans, 1966; Schollhorn & Burris, 1966; Stewart *et al.*, 1967; Hardy *et al.*, 1968; Silvester, 1989; Matzek & Vitousek, 2003; Pinto-Tomás *et al.*, 2009; Davis *et al.*, 2010; Cassar *et al.*, 2012). ARA results can be misleading when N_2 fixation is accomplished through alternative means because alternative pathways do not produce the same amounts of ethylene (Dilworth *et al.*, 1988), which is the final product measured to estimate amounts of N_2 fixation (note that alternative pathways produce ethane in addition to ethylene). In all cases, an ARA/N_2 conversion factor, which can be determined experimentally using ^{15}N enrichment experiments, must be applied to properly estimate dinitrogen fixation (Kurina & Vitousek, 2001). However, due mostly to the

destructive, time-intensive nature of the enrichment, it is often assumed that N_2 fixation through alternative means is negligible and the ARA/N_2 conversion factor for the Mo-dependent nitrogenase is applied (Hällbom & Bergman, 1979; Blacklock *et al.*, 1980; Cusack *et al.*, 2009), despite the current controversy regarding the conversion of ARA values to N_2 fixation rates (Belnap, 2001). Since cyanobacteria from lichens represent a major source of fixed nitrogen in a number of ecosystems, our findings could be quite significant for a proper understanding of global nutrient cycling and require scientists to re-evaluate many ecosystem-wide studies of nitrogen cycling. As highlighted in a recent review by Reed *et al.* (2011), determining which nitrogenases are responsible for N_2 fixation in the environment is a critical matter; demonstrating the importance of alternative means of N_2 fixation would significantly impact current conceptual models relating N_2 fixation to trace element control. Most studies on alternative N_2 fixation are the result of lab research on very specific culturable N_2 fixers. With the exception of the work by Betancourt *et al.* (2008), the presence of alternative nitrogenases has been poorly studied in the natural environment and has never been demonstrated in cyanolichens. Thus, our results invite reconsideration of current conceptual models for N_2 fixation in cyanolichens and open new avenues of research such as vanadium dynamics and homeostasis in cyanolichens and surrounding environments.

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Supplemental data

Supplemental data for this article can be accessed here http://dx.doi.org/10.1080/09670262.2013.873143

Supplementary Table S1. Sequences used in phylogenetic analyses.

Supplementary File 1. *VnfD* alignment (nexus).

Supplementary File 2. *VnfN* alignment (nexus).

Supplementary File 3. PERL script for renaming the GenBank sequences.

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