ELSEVIER

FEMS Microbiology Ecology 21 (1996) 149–156

FEMS MICROBIOLOGY ECOLOGY

Consortial N_2 fixation: a strategy for meeting nitrogen requirements of marine and terrestrial cyanobacterial mats

T.F. Steppe [a], J.B. Olson [a], H.W. Paerl [a,*], R.W. Litaker [b], J. Belnap [c]

[a] *Institute of Marine Sciences, University of North Carolina-Chapel Hill, Morehead City, NC 28557, USA*
[b] *Program in Molecular Biology and Biotechnology, University of North Carolina-Chapel Hill, Chapel Hill, NC 27599, USA*
[c] *Canyonlands National Park, National Park Service, Moab, UT 84532, USA*

Received 27 April 1996; revised 20 June 1996; accepted 20 June 1996

Abstract

Four microbial mat-forming, non-axenic, strains of the non-heterocystous, filamentous, cyanobacterial genus *Microcoleus* were maintained in culture and examined for the ability to fix atmospheric nitrogen (N_2). Each was tested for nitrogenase activity using the acetylene reduction assay (ARA) and for the presence of the dinitrogenase reductase gene (*nifH*), an essential gene for N_2 fixation, using the polymerase chain reaction (PCR). The *Microcoleus* spp. cultures were incapable of growth without an exogenous nitrogen source and never exhibited nitrogenase activity. Attempts to amplify a 360-bp segment of the *nifH* gene using DNA purified from the cyanobacterial cultures did not produce any cyanobacteria-specific *nifH* sequences. However, several non-cyanobacterial homologous *nifH* sequences were obtained. Phylogenetic analysis showed these sequences to be most similar to sequences from heterotrophic bacteria isolated from a marine microbial mat in Tomales Bay (California, USA), and bulk DNA extracted from a cryptobiotic soil crust in Moab (Utah, USA). *Microcoleus* spp. dominated the biomass of both systems. Cyanobacteria-specific 16S rDNA sequences obtained from the cultured cyanobacterial strains demonstrate that the lack of cyanobacteria-specific *nifH* sequences was not due to inefficiency of extracting *Microcoleus* DNA. Hence, both the growth and genetic data indicate that, contrary to earlier reports, *Microcoleus* spp. appear incapable of fixing N_2 because they lack at least one of the requisite genes for this process. Furthermore, our study suggests epiphytic N_2-fixing bacteria form a diazotrophic consortium with these *Microcoleus* spp. and are likely key sources of fixed N_2 generated within soil crusts and marine microbial mats.

Keywords: Cryptobiotic soil crust; Microbial consortia; Marine microbial mat; N_2 fixation; *nifH* Gene; Polymerase Chain Reaction

1. Introduction

A common and distinguishing feature of laminated microbial ecosystems such as cryptobiotic soil crusts and marine cyanobacterial mats is their ability to grow and proliferate in nitrogen (N) deficient environments. Mats and crusts thrive because their N requirements are supplemented through N_2 fixation [1,2]. The conversion of atmospheric N_2 to biologically utilizable NH_4^+ (N_2 fixation) within cryptobiotic soil crusts and marine cyanobacterial mats yields a significant amount of N needed to sustain the productivity and fertility of surrounding soils [1] and waters [3]. N_2 fixation is an energy intensive enzymatic process carried out by the nitrogenase system which is only found in a limited number of prokary-

* Corresponding author. E-mail: Hans_Paerl@unc.edu

PII S0168-6496(96)00047-5

otic species [4]. Because of its large energy requirements, N_2 fixation in mats and crusts has been attributed to the highly visible standing stocks of oxygenic, phototrophic cyanobacteria that dominate the primary productivity and biomass of these systems [2,5]. Ubiquitous, filamentous, non-heterocystous cyanobacteria often identified as *Microcoleus* spp. are commonly found to be the dominant structural component of laminated microbial ecosystems inhabiting vastly different environments [1,6–10] and are widely assumed to be a primary N_2 fixer in these systems [11–13].

In particular, previous studies suggested two cyanobacteria frequently identified as being present in mats and crusts, *Microcoleus vaginatus* and *M. chthonoplastes*, have the ability to fix N_2 [1,14]. However, cultured *Microcoleus* strains PCC7420 (*M. chthonoplastes*) and PCC6304 (*M. vaginatus*) were found incapable of fixing N_2 even under anaerobic conditions [15]. In this paper we more closely evaluate the role *Microcoleus* spp. play in the N_2-fixing dynamics of microbial mats and soil crusts by assessing the capability and genetic potential of three cyanobacterial strains identified as *Microcoleus chthonoplastes* and one strain identified as *Microcoleus vaginatus* for N_2 fixation.

2. Materials and methods

2.1. Culture studies

Cultures designated as PCC7420 and WH8701 were kindly provided by J. Waterbury (Woods Hole Oceanographic Institute, Woods Hole, MA, USA). NCR, a *Microcoleus* strain isolated from the Rachel Carson National Estuarine Research Reserve (RCNERR, Beaufort, NC, USA), and SCHZ, a '*Schizothrix*' strain, were provided by L. Prufert-Bebout. IMS9401 was isolated from a cryptobiotic crust of the Colorado Plateau, (Moab, UT, USA). PCC7420, WH8701, SCHZ, and NCR, were grown in seawater-based F/2 media [16] while IMS9401 was grown in Chu-10 media [17], both at a final concentration of 0.88 mM $NaNO_3$. Growth conditions were set at 25°C with a 14:10 h light:dark cycle. Isolates were initially grown on plates containing 1% agar with individual trichomes being transferred every 2–3 days to clean cultures. Heterotrophic microaerophilic bacteria were obtained by inoculating 0.1% agarose tubes of an N-free artificial seawater medium [18] with stabs from *Microcoleus* sp. dominated microbial mats. Isolates were obtained by the repeated streaking of single colonies onto 1.0% agar plates (8.0 g mannitol, 10.0 g agar, 0.05 g yeast extract, 1.0 ml of 5.0 g/l NaH_2PO_4 solution, and 0.6 ml F/2 trace metal solution per 1 l filtered seawater). The isolates were transferred back into the semi-solid media described above to screen for nitrogenase activity using the acetylene reduction assay.

2.2. Chlorophyll a and acetylene reduction

Three replicates of each *Microcoleus* strain were grown in liquid N-free media (F/2 for *M. chthonoplastes* strains and Chu-10 for IMS9401). After 7, 14, and 21 days, 4.0 ml aliquots were taken from each replicate culture for chlorophyll *a* measurements [19]. Samples were spun down in 15 ml centrifuge tubes, resuspended in 4.0 ml acetone, sonicated, and filtered through a 0.45 μm syringe tip filter (Gelman) prior to analysis on a Turner Fluorometer model 110. After the 21st day, a 5 ml aliquot was removed from each culture for acetylene reduction assays [20]. Samples were placed into 15 ml glass test tubes and sealed with a rubber stopper. 2.0 ml of the headspace was removed, replaced with 2.0 ml of acetylene, and incubated for 24 h as above. At the end of the 24 h, 0.225 ml of gas was removed for analysis using a Shimadzu GC-9A gas chromatograph.

2.3. DNA extraction, PCR and sequencing

The QIAamp Tissue Kit (Qiagen, Chatsworth, CA, USA) was used for extraction of DNA from all samples. An initial crude extract was obtained for the soil crust sample based upon the method in Giovannoni et al. [21]. Amplification of the *nifH* segment was performed using the parameters and primers outlined in Zehr and McReynolds [22]. 16S rDNA was amplified with primers corresponding to *E. coli* bp positions 8–27 [23] and 1492–1510 [24]. PCR products were gel purified and cloned into pCR® 2.1 Vector (Invitrogen, San Diego, CA, USA). Sequencing was done by the UNC-CH Automated DNA

Sequencing Facility on a Model 373A DNA Sequencer (Applied Biosystems) using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems).

2.4. Phylogenetic analysis

NifH sequences were aligned and translated using GCG software [25] prior to being checked manually. The Protein Distance (Dayhoff PAM Matrix) and Neighbor (Neighbor-Joining) programs in Phylip [26] were used to construct phylogenetic trees. 16S sequence comparisons were made using BLASTA and FASTA functions in GCG.

2.5. Immunofluorescence

Monospecific, polyclonal antibodies to the Fe-S protein (NifH) were used to detect the presence of the nitrogenase enzymes following the protocol of Currin et al. [27]. *Microcoleus* sp. filaments were obtained by scraping the surface of the RCNERR mat with a sterile inoculating loop then grown overnight in semi-solid, seawater-based, N-free media.

3. Results and discussion

Four strains of *Microcoleus* spp., NCR, WH8701, PCC7420, and IMS9401 and one marine strain identified as a *Schizothrix* sp., were obtained and maintained in culture. NCR, WH8701, and PCC7420 are marine strains identified as *M. chthonoplastes* and IMS9401 is a terrestrial isolate identified as *M. vaginatus*, all of which possess sheathed trichomes with tapered terminal cells indicative of *Microcoleus* spp. [28]. Stringent isolation and transfer techniques

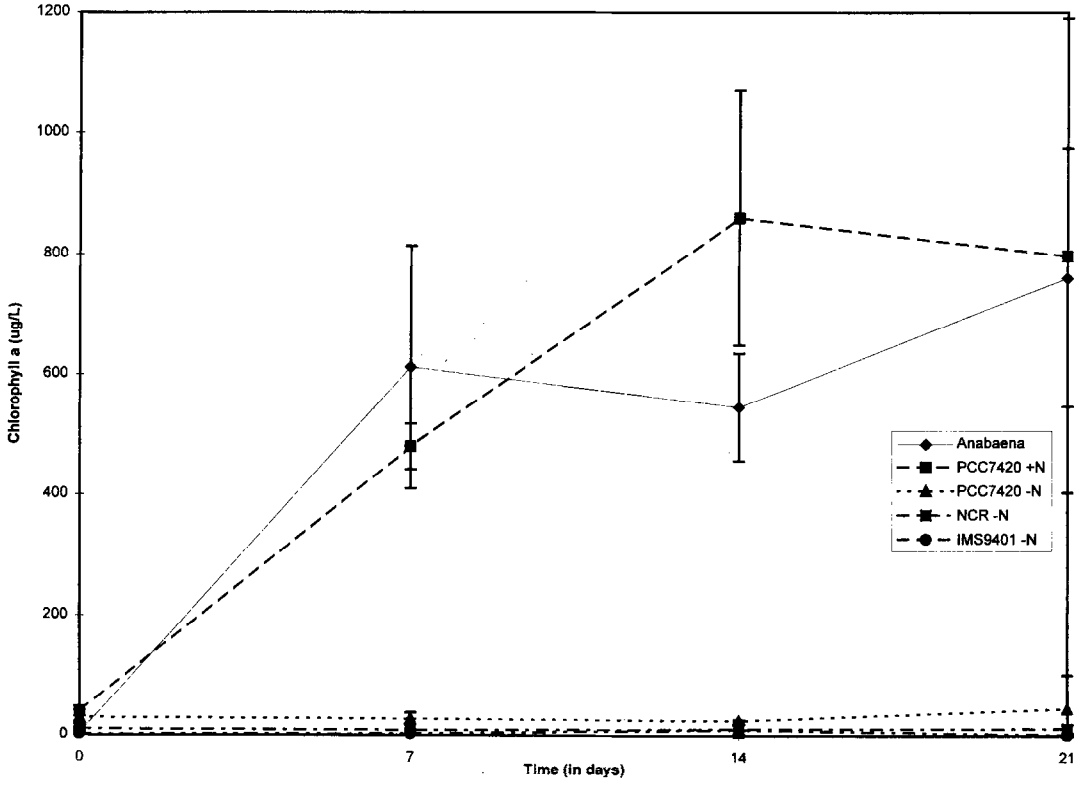
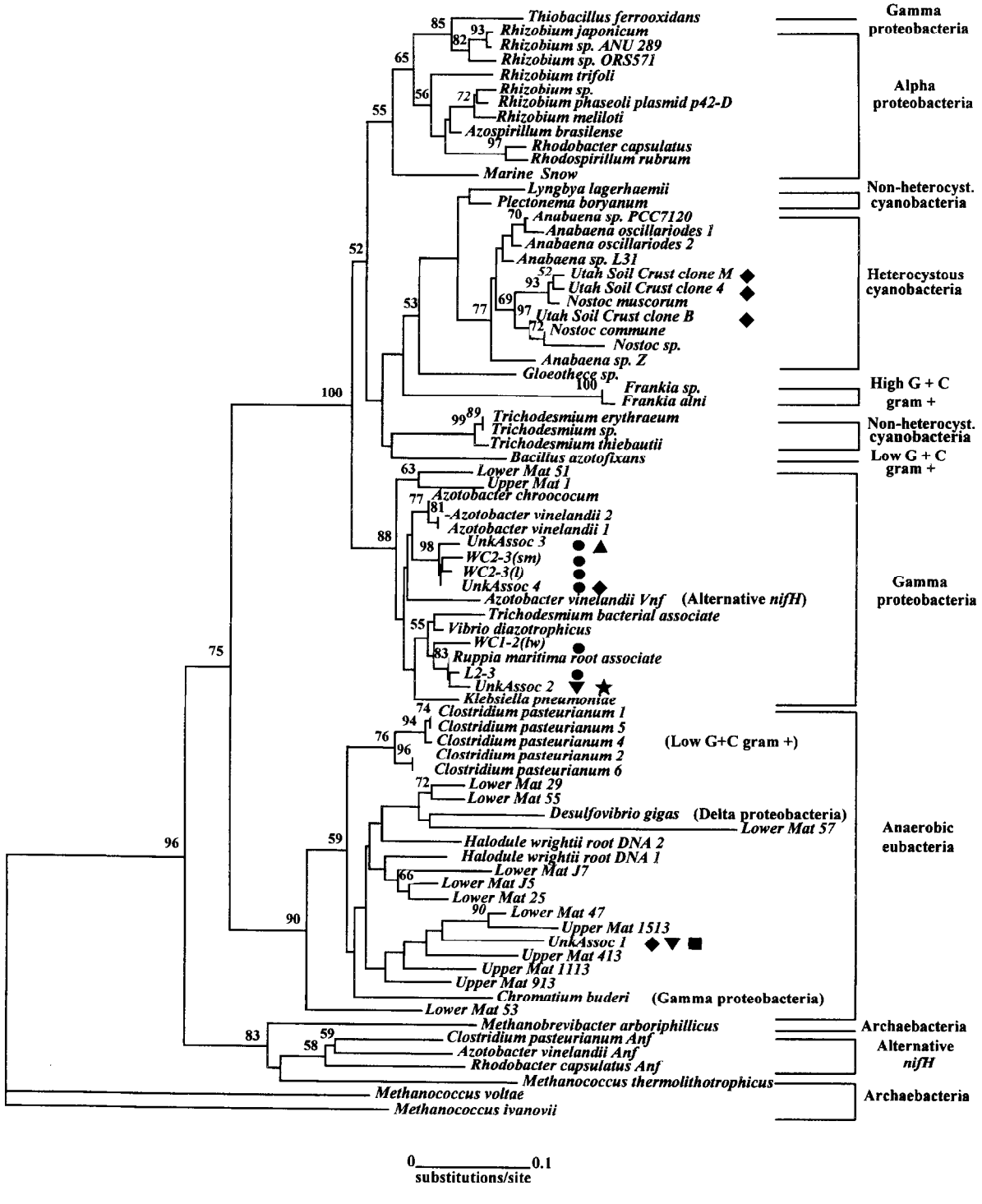


Fig. 1. Graph demonstrating the inability of *Microcoleus* spp. PCC7420, NCR, and IMS9401 to grow without N. Also shown are PCC7420 grown with N and an *Anabaena* sp., a heterocystous cyanobacteria, grown without N.


Thiobacillus ferrooxidans
Rhizobium japonicum
Rhizobium sp. ANU 289
Rhizobium sp. ORS571
Rhizobium trifoli
Rhizobium sp.
Rhizobium phaseoli plasmid p42-D
Rhizobium meliloti
Azospirillum brasilense
Rhodobacter capsulatus
Rhodospirillum rubrum
Marine Snow
Lyngbya lagerhaemii
Plectonema boryanum
Anabaena sp. PCC7120
Anabaena oscillariodes 1
Anabaena oscillariodes 2
Anabaena sp. L31
Utah Soil Crust clone M
Utah Soil Crust clone 4
Nostoc muscorum
Utah Soil Crust clone B
Nostoc commune
Nostoc sp.
Anabaena sp. Z
Gloeothece sp.
Frankia sp.
Frankia alni
Trichodesmium erythraeum
Trichodesmium sp.
Trichodesmium thiebautii
Bacillus azotofixans
Lower Mat 51
Upper Mat 1
Azotobacter chroococum
Azotobacter vinelandii 2
Azotobacter vinelandii 1
UnkAssoc 3
WC2-3(sm)
WC2-3(l)
UnkAssoc 4
Azotobacter vinelandii Vnf (Alternative nifH)
Trichodesmium bacterial associate
Vibrio diazotrophicus
WC1-2(tw)
Ruppia maritima root associate
L2-3
UnkAssoc 2
Klebsiella pneumoniae
Clostridium pasteurianum 1
Clostridium pasteurianum 5
Clostridium pasteurianum 4 (Low G+C gram +)
Clostridium pasteurianum 2
Clostridium pasteurianum 6
Lower Mat 29
Lower Mat 55
Desulfovibrio gigas (Delta proteobacteria)
Lower Mat 57
Halodule wrightii root DNA 2
Halodule wrightii root DNA 1
Lower Mat J7
Lower Mat J5
Lower Mat 25
Lower Mat 47
Upper Mat 1513
UnkAssoc 1
Upper Mat 413
Upper Mat 1113
Upper Mat 913
Chromatium buderi (Gamma proteobacteria)
Lower Mat 53
Methanobrevibacter arboriphillicus
Clostridium pasteurianum Anf
Azotobacter vinelandii Anf
Rhodobacter capsulatus Anf
Methanococcus thermolithotrophicus
Methanococcus voltae
Methanococcus ivanovii
Gamma proteobacteria
Alpha proteobacteria
Non-heterocyst. cyanobacteria
Heterocystous cyanobacteria
High G + C gram +
Non-heterocyst. cyanobacteria
Low G + C gram +
Gamma proteobacteria
Anaerobic eubacteria
Archaebacteria
Alternative nifH
Archaebacteria
0 0.1
substitutions/site

were employed in an attempt to render the cyanobacterial cultures axenic. The number of bacterial associates could be greatly reduced, but axenic cultures were not generated despite numerous transfers of each strain. Although microscopic inspection of the cyanobacterial cultures revealed that they were unialgal, a small number of bacteria remained associated with the cyanobacterial filaments. The inability to obtain axenic cultures is a common problem with *Microcoleus* spp. [28] and may potentially lead to a misinterpretation of these species' physiological capabilities and limitations. The environmental samples from which NCR and IMS9401 were obtained have exhibited substantial rates of N_2 fixation as measured by the acetylene reduction assay [1,12]. However, the cleaned *Microcoleus* strains, including NCR and IMS9401, were unable to grow without nitrogen and repeatedly failed to exhibit nitrogenase activity when grown in N-free media. Fig. 1 shows the growth response of the *Microcoleus* strains in cultures with and without N. The lack of detectable nitrogenase activity could be the result of unfavorable conditions or physiological changes occurring within the *Microcoleus* spp. Yet, when *Lyngbya aestuarii*, also a mat-forming filamentous cyanobacteria, was grown under similar conditions considerable nitrogenase expression and activity were demonstrated [28].

The *nifH* gene is the most evolutionarily conserved within the nitrogenase gene system [4] and the phylogenetic trees generated by *nifH* sequence data are largely in agreement with those generated by 16S rRNA sequence data [29]. The degenerate primers developed from the analysis of *nifH* gene alignments have been shown to be effective at amplifying the desired product from a large number of diverse organisms [30] and from DNA obtained from small numbers of diazotrophic cells ($\leq 100/l$) [31].

A positive PCR signal (360 bp band) was detected for all cultures except IMS9401. The amplified products were cloned and three or more clones from each culture were sequenced. No cyanobacteria-specific *nifH* sequences were obtained. Instead, the *nifH* sequences obtained were most closely related to γ-proteobacteria and anaerobes such as *Clostridium pasteurianum* and *Desulfovibrio gigas* (Fig. 2). Also, these culture-derived sequences were either identical or highly similar at the amino acid and nucleotide level to sequences obtained from a soil crust sample dominated by *M. vaginatus* from the Colorado Plateau (Moab, UT) and several bacterial isolates derived from a *Microcoleus* spp. dominated mat in Tomales Bay [32]. A Tomales Bay mat heterotrophic isolate which exhibited substantial rates of N_2 fixation (Fig. 2) differed by only one base pair at the nucleic acid level and was identical at the amino acid level to the culture-derived sequence obtained from WH8701.

To eliminate the possibility that the lack of cyanobacteria-specific *nifH* sequences resulted from the insufficient lysis of cyanobacterial cells and/or the presence of greater numbers of non-cyanobacterial cells, 16S rDNA specific primers were used to amplify DNA from the purified cyanobacterial cultures. At least two partial sequences were obtained (approximately 500 bp) for each culture and compared to known sequences. Except for NCR, all 16S sequences obtained were most similar to cyanobacteria-specific 16S sequences.

NifH sequences obtained from amplifying the DNA extracted from N_2-fixing natural mat and crust samples further indicate that when populations of N_2-fixing cyanobacteria exist, PCR techniques are capable of detecting their presence [30]. For example, the DNA extracted from a Colorado Plateau soil crust sample yielded three unique cyanobacterial sequences that were most similar to the heterocystous cyanobacteria *Nostoc* spp. Microscopic inspection of the assayed sample revealed small, but detectable numbers of a filamentous, heterocystous cyanobacteria possessing terminal heterocysts. In addition, a previous study utilizing the same *nifH* specific

Fig. 2. Phylogenetic tree showing the genetic relationship of 78 (67 from Genbank) deduced *nifH* amino acid sequences corresponding to PCC7120 amino acid residues 48–155. *Methanoccocus ivanovii* was used as an outgroup. Bootstrap values greater than 50% for neighbor-joining are given above the branches. Those sequences with symbols beside them were obtained during this study. Multiple symbols denote identical translated amino acid sequences obtained from different DNA samples. Tomales Bay bacterial isolates, ●; Utah Soil Crust (UtSC), ◆; NCR, ▼; PCC7420, ■; SCHZ, ★; and WH8701, ▲. Sequence accession numbers: U41507 and U43436–U43445.

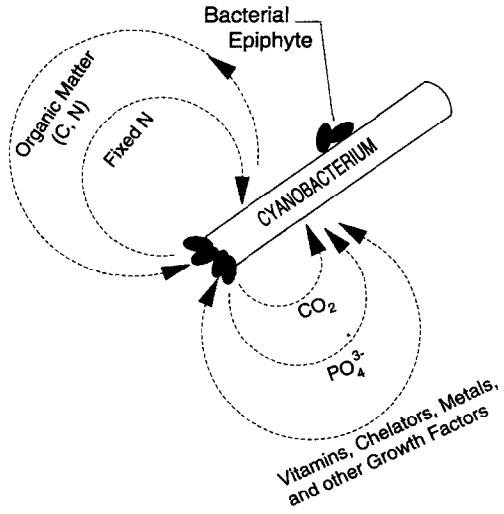


Fig. 3. Conceptual model depicting consortial interactions between cyanobacteria and associated bacteria. Arrows indicate the proposed flow of carbon, nitrogen, and other metabolites.

primers to analyze a *Microcoleus* spp. dominated cyanobacterial mat from the RCNERR similarly failed to detect any cyanobacteria-specific *nifH* sequences, yet revealed a surprisingly diverse population of non-cyanobacterial diazotrophs [30]. However, when the same mat was assayed once substantial numbers of the known N_2-fixing cyanobacterial genus *Lyngbya* inhabited the mat, *L. lagerhaemii* cyanobacteria-specific *nifH* sequences were detected [30].

The inability of the purified *Microcoleus* cultures to grow without nitrogen or exhibit nitrogenase activity combined with the *nifH* and 16S rDNA sequence data indicates these cyanobacteria are not capable of N_2 fixation. Our findings suggest these ubiquitous *Microcoleus* spp. and perhaps other filamentous non-heterocystous cyanobacteria found in mats and crusts obtain the N necessary to sustain their growth from alternative sources. The results indicate that the *nifH* sequences detected were most likely from bacteria growing in association with the cyanobacteria. Using an antibody specific to NifH, *Microcoleus* sp. associated bacteria can be seen expressing nitrogenase enzymes (Fig. 4C). We propose that in conjunction with the cyanobacteria, epiphytic diazotrophic bacteria form a mutualistic N_2-fixing consortium. In this consortium *Microcoleus* spp. provide a habitat for growth and a readily available source of organic carbon for energy, while the bacteria provide the fixed N_2 and perhaps other requisite factors for the 'host' cyanobacterium's growth [33] (Fig. 3).

An essential element provided by the physiological interaction of the consortial members are anaerobic microzones within and around the mucilaginous sheaths that *Microcoleus* spp. typically exude (Fig. 4A,B). Anaerobic microzones provide a

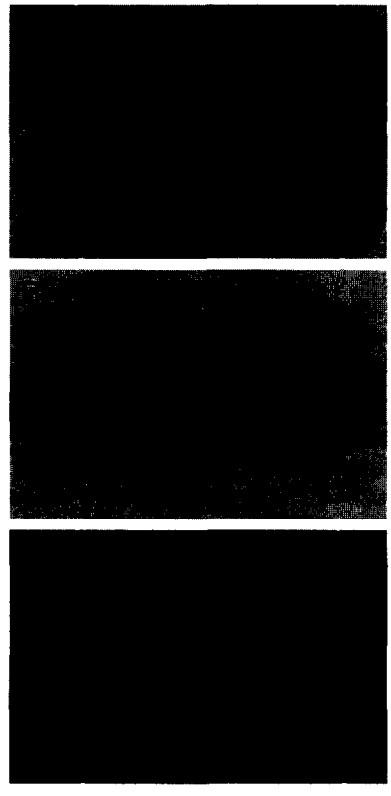


Fig. 4. (**A**) Phase-contrast light microscopy picture of *Microcoleus* sp. bundle from a Tomales Bay, CA, cyanobacterial mat showing the mucilaginous sheath and bacterial associates surrounding the bundle. (**B**) Bundles of *Microcoleus* sp. from the RCNERR incubated with the tetrazolium salt TTC (see [34]). Red areas indicate O_2-depleted microzones. (**C**) Epifluorescence photomicrograph showing *Microcoleus* sp. filament (orange) and epiphytic bacteria incubated with a polyclonal antibody specific for NifH. Green fluorescence from the associated bacteria indicates presence of NifH within these cells. Orange fluorescence of the *Microcoleus* sp. filament is due to autofluorescence of chlorophyll *a*.

locally reduced region where associative diazotrophic bacteria can escape the potential inhibitory effects of O_2 on nitrogenase activity [4]. Localized reduction of the tetrazolium salt 2,4,5-triphenyltetrazolium chloride (TTC) has demonstrated the presence of anaerobic microzones around the *Microcoleus* spp. growing in the RCNERR mat [33,34] (Fig. 4B) and the Utah soil crust (not shown). In addition, the *nifH* sequences obtained from the oxic zone of the RCNERR (cyanobacterial layer) revealed anaerobic diazotrophs to be present within this layer [30].

The evolution of consortial N_2 fixation relationships would offer several advantages for the cyanobacteria and the N limited mat and crust systems they pervade. Physiological and genetic data from mats and crusts discussed here suggest a variety of functionally and taxonomically distinct prokaryotes may form the N_2-fixing consortia [30,35]. Within the mats and crusts there exist well-defined, highly dynamic spatial gradients and temporal fluctuations in factors controlling N_2 fixation, including O_2 and NH_4^+ concentrations, pH, temperature, and available labile organic carbon [1,35–37]. Diverse autotrophic and heterotrophic diazotrophs populating the mats and crusts ensure a broad spectrum of physiological responses to these dynamic environmental constraints. Thus, a high diversity of diazotrophs would allow N_2-fixing consortia to optimize N_2 fixation when confronted with short term (diel) and long term (seasonal) shifts in microscale and mesoscale environmental factors.

The phenomenon of cyanobacteria participating in consortial associations with epiphytic bacteria appears to be a common strategy for optimizing N_2 fixation in terrestrial and aquatic ecosystems [29,38]. Previously, it has been assumed that the cyanobacteria were the exclusive sites of N_2 fixation and that the epiphytic bacteria were the beneficiaries of the new N supplied by cyanobacteria. Evidence presented here, however, points to an additional, thus far overlooked, source of N_2 fixation in the form of epiphytic diazotrophy. This mechanism may be of critical importance in providing host cyanobacteria with new N and therefore play a crucial role in satisfying N requirements of mats and cryptobiotic crusts.

Acknowledgements

We thank D. Barr for technical assistance, J. Pinckney for discussions, and F. Lutzoni for help with PHYLIP. This work was supported by grants from the U.S. Army Corps of Engineers and the National Science Foundation, projects DEB 9408471, OCE 9415985, and DEB 9220886.

References

[1] Belnap, J. (1995) Surface Disturbances: Their role in accelerating desertification. J. Environ. Monitor. Assess. 37, 1–19.
[2] Stal, L.J., Grossberger, S. and Krumbein, W.E. (1984) Nitrogen fixation associated with the cyanobacterial mat of a marine laminated microbial ecosystem. Mar. Biol. 82, 217–224.
[3] Capone, D.G. (1983) Benthic nitrogen fixation. In: Nitrogen in the Marine Environment (Carpenter, E. and Capone, D.G., Eds.), pp. 105–137. Academic Press, New York, NY.
[4] Postgate, J. (1982) The Fundamentals of Nitrogen Fixation. Cambridge, UK.
[5] Belnap, J. and Gardner J.S. (1993) Soil microstructure of the Colorado Plateau: The role of the cyanobacterium *Microcoleus vaginatus*. Great Basin Naturalist 53, 40–47.
[6] Bauld, J. (1984) Microbial mats in marginal marine environments: Shark Bay, Western Australia, and Spencer Gulf, South Australia. In: Microbial Mats: Stromatolites (Cohen, Y., Castenhholz, R.W., and Halverson, H.O., Eds.), pp. 39–58. Alan R. Liss, New York, NY.
[7] Cohen, Y. (1984) The Solar Lake cyanobacterial mats: Strategies of photosynthetic life under sulfide. In: Microbial Mats: Stromatolites (Cohen, Y., Castenhholz, R.W., and Halverson, H.O., Eds.), pp. 133–148. Alan R. Liss, New York, NY.
[8] Guerrero, R., Urmenta, J. and Rampone, G. (1993) Distribution and types of microbial mats at the Ebro Delta, Spain. BioSystems 31, 135–144.
[9] Javor, B.J. and Castenholz, R.W. (1984) Productivity studies of microbial mats, Laguna Guerrero Negro, Mexico. In: Microbial Mats: Stromatolites (Cohen, Y., Castenhholz, R.W. and Halverson, H.O., Eds.), pp. 149–170. Alan R. Liss, New York, NY.
[10] Zhang, Y. and Hoffmann, L. (1992) Blue-green algal mats of the salinas in San-ya, Hai-nan Island (China): Structure, taxonomic composition, and implications for the interpretation of Precambrian stromatolites. Precambrian Res. 56, 275–290.
[11] Paling, E.I., McComb, A.J. and Pate, J. (1989) Nitrogen fixation (Acetylene Reduction) in nonheterocystous cyanobacterial mats from the Dampier, Archipelago, Western Australia. Aust. J. Mar. Freshwater Res. 40, 147–53.
[12] Paerl, H.W. (1990) Physiological ecology and regulation of

N_2 fixation in natural waters. In: Advances in Microbial Ecology, II (Marshall, K.C., Ed.), pp. 305–343. Plenum Press, New York, NY.

[13] Potts, M., Krumbein, W.E. and Metzger, J. (1978) Nitrogen fixation in anaerobic sediments determined by acetylene reduction, a new ^{15}N field assay, and simultaneous total ^{15}N determination. In: Environmental Biogeochemistry and Geomicrobiology: Methods, Metals, and Assessment (Krumbein, W.E., Ed.), 753–769. Ann Arbor Science, Ann Arbor, MI.

[14] Pearson, H.W., Malin, G. and Howsley, R. (1981) Physiological studies on in vivo nitrogenase activity by axenic cultures of the blue-green alga *Microcoleus chthonoplastes*. Br. Phycol. J. 16, 139.

[15] Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979) Generic assignments, strain histories, and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1–61.

[16] Guillard, R.D.C. and Ryther, J.H. (1962) Studies of marine planktonic diatoms 1. *Cyclolella nana* Husedt and *Detonula confervacea* (Cleve). Gran. Can. J. Microb. 8, 229–339.

[17] Chu, S.D. (1942) Influence of mineral composition of the medium of the growth of planktonic algae. 1. Methods and culture media. J. Ecol. 31, 109–148.

[18] Tibbles, B.J. and Rawlings, D.E. (1994) Characterization of N_2-fixing organisms from a temperate salt marsh lagoon including isolates that produce ethane from acetylene. Microb. Ecol. 27, 65–80.

[19] Strickland, J.D.H. and Parsons, T.R. (1968) A practical handbook of seawater analysis. In: Bulletin of the Fisheries Research Board of Canada, 167.

[20] Hardy, R.W.F., Holsten, F., Jackson, R.D. and Burns, E.K. (1968) The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Physiol. 43, 1185–1207.

[21] Giovannoni, S.J., DeLong, E.J., Schmidt, T.M. and Pace, N.R. (1990) Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl. Environ. Microbiol. 56, 2572–2575.

[22] Zehr, J.P. and McReynolds, L.A. (1989) Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. 55, 2522–2526.

[23] Reysenbach, A.L., Wickham, G.S. and Pace, N.R. (1994) Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ Microbiol. 60, 2113–2119.

[24] Reysenbach, A.L., Giver, L.J., Wickham, G.S. and Pace, N.R. (1992) Differential amplification of rDNA genes by Polymerase Chain Reaction. Appl. Environ. Microbiol. 58, 3417–3418.

[25] Program Manual for the Wisconsin Package (1994) (8c), Genetics Computer Group, Madison, WI.

[26] Felsenstein, J. PHYLIP (Phylogeny Inference Package) (1995) (3.57c), University of Washington.

[27] Currin, C.A.H., Paerl, H.W., Suba, G. and Alberte, R.S. (1991) Immunofluorescence detection and characterization of N_2-fixing microorganisms from aquatic environments. Limnol. Oceanogr. 35, 59–71.

[28] Prufert-Bebout, L. and Garcia-Pinchel, F. (1994) Field cultivated *Microcoleus chthonopastes*: The search for clues to its prevalence in marine microbial mats. In: Microbial Mats: Structure, Development, and Environmental Significance (Stal, L. and Caumette, P., Eds.), pp. 111–116. Springer-Verlag, Berlin.

[29] Young, J.P.W. (1992) Phylogenetic classification of nitrogen-fixing organisms. In: Biological Nitrogen Fixation (Stacey, G. B. Burris, R.H., Evans, H.J., Eds.), pp. 43–86. Chapman and Hall, London.

[30] Zehr, J.P., Mellon, M., Braun, S., Litaker, W., Steppe, T. and Paerl, H.W. (1995) Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. Appl. Environ. Microbiol. 61, 2527–2532.

[31] Kirshtein, J.D., Zehr, J.P. and Paerl, H.W. (1993) Determination of N_2 fixation potential in the marine environment: Application of the Polymerase Chain Reaction. Mar. Ecol. Prog. Ser. 95, 305–309.

[32] Joye, S. and Paerl, H.W. (1993) Contemporaneous nitrogen fixation and denitrification in the intertidal and subtidal environments of Tomales Bay, California. Mar. Ecol. Prog. Ser. 94, 267–274.

[33] Paerl, H.W. and Pinckney, J.L. (1996) Microbial consortia: Their roles in aquatic production and biogeochemical cycling. Microb. Ecol., in press.

[34] Paerl, H.W. and Bland, P.T. (1982) Localized tetrazolium reduction in relation to N_2 fixation, CO_2 fixation, and H_2 uptake in aquatic filamentous cyanobacteria. Appl. Environ. Microbiol. 43, 218–226.

[35] Bebout, B., Fitzpatrick, M. and Paerl, H.W. (1993) Identification of the sources of energy for nitrogen fixation and physiological characterization of nitrogen-fixing members of a marine microbial mat community. Appl. Environ. Microbiol. 59, 1495–1503.

[36] Paerl, H.W. and Carlton, R.G. (1988) Control of N_2 Fixation by oxygen depletion in surface associated microzones. Nature 332, 260–262.

[37] Bebout, B.M., Paerl, H.W., Crocker, K.M. and Prufert, L. (1987) Diel interactions of oxygenic photosynthesis and N_2 fixation (acetylene reduction) in a marine microbial mat community. Appl. Environ. Microbiol. 53, 2353–2362.

[38] Paerl, H.W. and Galluci, K.K. (1985) Role of chemotaxis in establishing a specific nitrogen-fixing cyanobacterial association. Science 227, 647–649.