The utilization of nitrate as an essential nitrogen source for growth is a property common to many heterotrophic and photosynthetic organisms. Following the transport of nitrate into the cell, its assimilation requires the reducing activity of two enzymes, nitrate reductase and nitrite reductase. The genes required for the assimilation of nitrate in the cyanobacterium *Synechococcus* sp. strain PCC7942 are organized in the so-called *nir* operon (14). *nirA*, the gene encoding nitrite reductase, is the first gene of the operon, followed by the genes encoding the nitrate transporter (*nrtABCD*) and finally the nitrate reductase gene *narB*. A similar gene organization has been reported for other freshwater species: *Synechocystis* sp. strain PCC6803 (8), *Anabaena* sp. strain PCC7120 (4, 6), *Plecostomonas boranum* (21), and *Phormidium laminosum* (11). Nitrate is an important N source in the ocean, and it can be readily assimilated by marine *Synechococcus* spp. in the absence of ammonium (7). Other marine cyanobacteria, including species of the diazotrophic, colony-forming genus *Trichodesmium*, grow well on nitrate as the sole N source (13). *Trichodesmium* filaments are incapable of N₂ fixation when growing on ammonium but maintain this capacity in the presence of nitrate (13). Here we report on the identification and partial characterization of nitrate assimilation genes from axenic *Trichodesmium* sp. strain WH9601 (derived from original strain IMS101).

Using degenerate primers to conserved *nirA* regions (21), we PCR amplified a 507-bp *nirA* fragment using *Trichodesmium* sp. strain WH9601 genomic DNA as a template (PCR conditions: denaturation at 95°C for 1 min, annealing at 45°C for 1 min, and elongation at 72°C for 1 min; 40 cycles). The PCR product was used to probe EcoRI-HindIII-digested genomic DNA of *Trichodesmium* strain WH9601. A positive 4.0-kb fragment designated WH7 was cloned into plasmid pUC19 and transformed into *Escherichia coli* host strain DH5α using standard protocols (19). Sequence analysis of the WH7 fragment showed the presence of three open reading frames (ORFs) in the same orientation (Fig. 1). They are identified below as genes encoding nitrite reductase (*nirA*), a nitrate permease (*napA*), and nitrate reductase (*narB*). The orientation of the three genes would allow them to form part of a polycistronic message. Putative promoter elements were found at positions 27 to 32 (TTGATA) and 49 to 54 (TAAAAT). These elements show a high level of similarity to the −35 and −10 sequences for *E. coli* σ₇₀. The DNA fragment WH7 contained no recognizable promoter elements further downstream.

**Nitrate and nitrite reductase genes.** The deduced amino acid sequence of the first ORF identified it as NirA on the basis of its strong sequence identity (1) of >63% with NirA of other cyanobacterial species (4, 6, 8, 10, 11, 21) and >44% identity with eukaryotic algae and higher plants (3, 9, 17, 20). Since *nirA* was the only nitrate assimilation gene of *Trichodesmium* sp. strain WH9601 which was obtained in a full-length sequence and identified unambiguously, it was used to determine the phylogenetic relationship among the various groups of organisms. Figure 2 shows the consensus tree deduced from a parsimony analysis of available NirA sequences using the PHYLIP phylogeny package (5). The analysis was based on an aligned stretch of 376 amino acids from position 66 to position 442 in the deduced *Trichodesmium* NirA sequence. Our analysis places the unicellular cyanobacteria near the base of the unrooted tree with two major branches: a branch containing the sequences of filamentous, mainly N₂-fixing cyanobacteria and a branch made up of higher plant and algal sequences.
Interestingly, marine Synechococcus sp. strain WH8103 NirA was grouped near the eukaryotic sequences rather than with Synechococcus sp. strain PCC7942 and Synechocystis sp. strain PCC6803. This grouping occurred irrespective of the choice of parameters for construction of the tree. The narB sequence encodes an 82-amino-acid peptide with strong similarity (55% identity) to the N terminus of cyanobacterial nitrate reductase genes. As for nirA, the highest similarity was observed for narB of filamentous cyanobacteria (4, 6, 22).

Nitrate and nitrite transporter gene. The nrtABCD genes located between nirA and narB in both unicellular and filamentous freshwater cyanobacteria are known to encode nitrate and nitrite uptake proteins which together form an ABC-type transport system (15). The nitrogen-fixing, filamentous cyanobacteria that together with Trichodesmium formed a related group based on nirA and narB sequences all possess such a nitrate transporter (4, 6, 8, 10, 11, 21). However, in Trichodesmium strain WH9601, a single ORF was found between nirA and narB. The ORF’s deduced amino acid sequence predicts a 55-kDa membrane protein with 12 potential α-helices and a pI of 8.1. A similarity search indicated affinity with a family of microbial permeases, especially with the nitrate permeases of Bacillus subtilis (NasA) and Emericella nidulans (CrnA), as well as with the nitrite extrusion protein (NarK) of B. subtilis (Fig. 3).

The similarity to each of the three proteins is relatively low (22% identity) and based mostly on conserved amino acid stretches in the N-terminal half of the protein. Based on the ORF’s position between nirA and narB, its orientation, the characteristics of the predicted protein, and the sequence similarity to known nitrate permeases, we hypothesized that it encodes the nitrate transporter in Trichodesmium sp. strain WH9601 and tentatively named it napA.

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sites, and boldface denotes base replacements). Primers were designed such that the products ligated at an engineered EcoRI site 3 bases upstream of the ATG start codon. The modification involved a minimal number of bases and did not affect obvious regulatory sequence or alter the length of the upstream sequence. The PnasA-napA construct was introduced into plasmid pHT304, a suitable shuttle vector for Bacillus spp. (2), to yield plasmid pQW10. In order to obtain a nonmethylated plasmid, pQW10 was amplified in dam dcm mutant E. coli strain JM110 using ampicillin at 100 µg · ml⁻¹ as a selective marker. Competent B. subtilis LAB1798 cells were then transformed by electroporation (two pulses at 200 Ω, 1.25 kV, and 25 µF [GenePulse II; Bio-Rad Inc.] for 6 × 10⁸ cells with 0.5 µg of plasmid). Transformants were allowed to recover in Luria-Bertani medium at 37°C for 3 h and subsequently transferred to solid MMG medium containing nitrate as the nitrogen source and erythromycin at 25 µg · ml⁻¹. Of 25 erythromycin-resistant colonies, 7 were capable of sustained growth on liquid minimal medium with nitrate. One of these transformants, QW10, was chosen for further analysis. QW10 attained growth rates that approached those of wild-type B. subtilis JH642 grown in minimal MMG medium with 20 mM nitrate (Fig. 4A). Nitrate removal was studied in nitrate-grown B. subtilis strains that were collected by centrifugation for 5 min at 8,000 rpm and resuspended in MMG medium lacking combined nitrogen. Nitrate was measured as described in reference 16 after Cd-catalyzed reduction of nitrate to nitrite. Following 5 min of acclimation, cells received a 40 min addition. QW10 cells assimilated nitrate at 57% of the rate of wild-type JH642. It was concluded that napA encodes an essential component of the nitrate transport system in Trichodesmium strain WH9601 and that this component also facilitates uptake of nitrite. Both functions showed an apparent efficiency of 50% compared to wild-type B. subtilis.

The napA gene encountered in marine Trichodesmium strain WH9601 has not been described as part of the nir operon in any cyanobacterium. Genes with high similarity to napA have recently been found in euryhaline Synechococcus strain PCC7002 (18) and in oceanic Synechococcus sp. strain WH7803 (23). These observations suggest that nitrate utilization by cyanobacteria in saline environments is facilitated by NapA rather than by NrABC. The two transporters have not been found to coexist in cyanobacteria. The delineation of the two nitrate transport systems along low-salt and high-salt cyanobacteria is interesting, especially considering that (i) NirA and NarB sequences of both marine and freshwater filamentous cyanobacteria group together in phylogenetic analyses and (ii) the delineation occurs within marine and freshwater representatives of the genus Synechococcus. These observations suggest that both environments exert considerable selective pressure on cyanobacteria with respect to their nitrate and nitrite acquisition systems.

**Nucleotide sequence accession number.** The sequence of the WH7 fragment was deposited in the GenBank database and assigned accession no. AF178846.

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