

Nitrate Assimilation Genes of the Marine Diazotrophic, Filamentous Cyanobacterium *Trichodesmium* sp. Strain WH9601

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A 4.0-kb DNA fragment of *Trichodesmium* sp. strain WH9601 contained gene sequences encoding the nitrate reduction enzymes, *nirA* and *narB*. A third gene positioned between *nirA* and *narB* encodes a putative membrane protein with similarity to the nitrate permeases of *Bacillus subtilis* (NasA) and *Emericella nidulans* (CrnA). The gene was shown to functionally complement a Δ *nasA* mutant of *B. subtilis* and was assigned the name *napA* (nitrate permease). NapA was involved in both nitrate and nitrite uptake by the complemented *B. subtilis* cells. *napA* is distinct from the *nrt* genes that encode the nitrate transporter of freshwater cyanobacteria.

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), *Plectonema boryanum* (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 DH5 α 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* σ_{70} . 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.

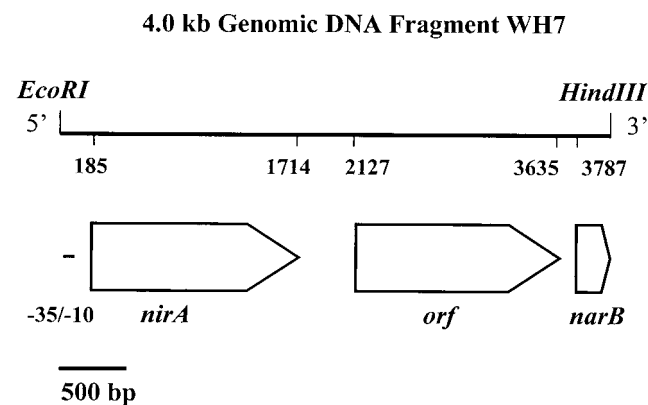


FIG. 1. Physical map of part of the *nir* operon of *Trichodesmium* strain WH9601. Numbers denote the positions of the first and last bases of coding regions, block arrows denote the ORFs with their direction of transcription. Double-stranded sequence was obtained by dye terminator cycle sequencing and primer hopping. Sequence reactions were analyzed on an ABI 377 Prism DNA sequencer (Perkin-Elmer Corp.) at the DNA Analysis Unit of the Life Sciences Institute, Hebrew University.

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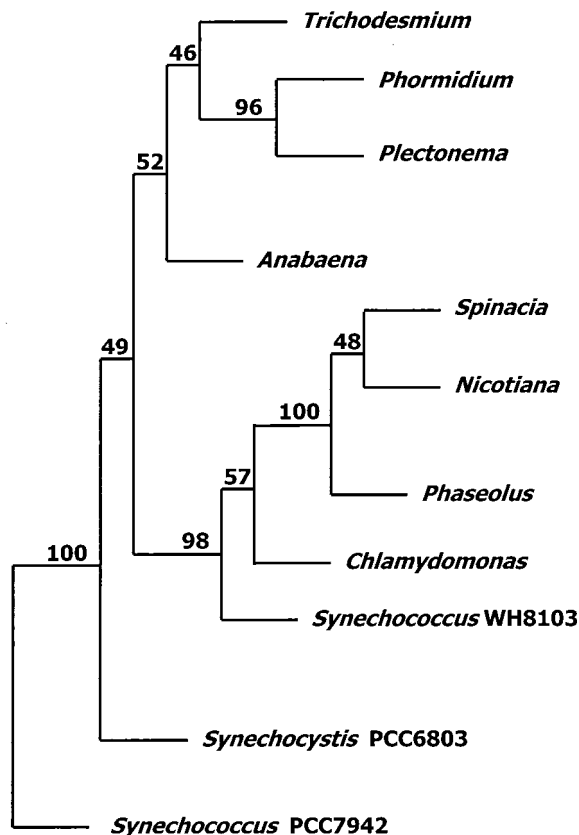


FIG. 2. Consensus tree based on random sampling of NirA amino acid sequences of 11 photosynthetic organisms. The organisms used (and their GenBank accession numbers) are *Trichodesmium* sp. (AF178846), *Phormidium laminosum* (Q51879), *Plectonema boryanum* (D31732), *Anabaena* sp. strain PCC7120 (U61496), *Synechococcus* sp. strain WH8103 (AF065403), *Synechococcus* strain PCC7942 (P39661), *Synechocystis* strain PCC6803 (D64003), *Chlamydomonas reinhardtii* (Y08937), *Spinacia oleracea* (P05314), *Nicotiana tabacum* (S23769), and *Phaseolus vulgaris* (S51945). Numbers at nodes indicate the bootstrap values from 100 trees constructed using the PHYLIP software.

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 the nitrite extrusion protein (NarK) of *B. subtilis* (Fig. 3).

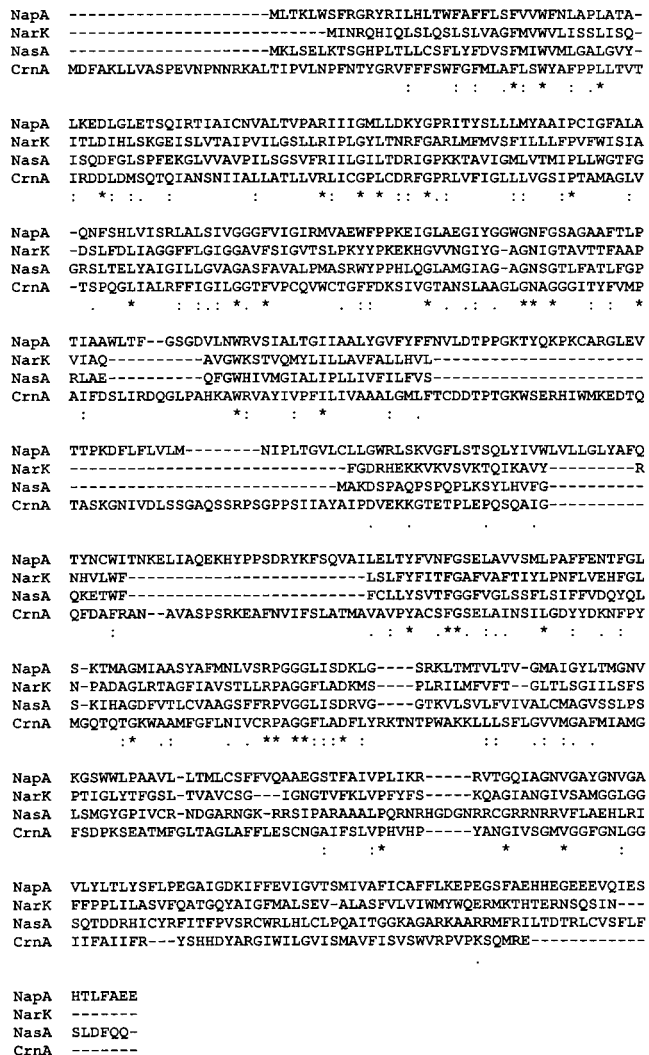


FIG. 3. ClustalW (version 1.74) alignment of the predicted NapA protein of *Trichodesmium* strain WH9601 with nitrate permeases of *B. subtilis* (NasA) and *E. nidulans* (CrnA), as well as the nitrite extrusion protein of *B. subtilis* (NarK). Asterisks indicate positions of identical amino acids; colons indicate positions with conserved replacements, and periods indicate semiconserved positions.

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*. Since a genetic manipulation system for *Trichodesmium* does not exist, we studied *napA* function by complementing Δ *nasA* strain LAB1798 of *B. subtilis*, which is impaired in nitrate uptake (12). The *napA* coding sequence was fused to the nitrate-specific *B. subtilis* promoter *PnasA*. Both the *PnasA* region and the *napA* region were obtained by PCR amplification using primers nas-f (5'-CCT CCT TTG GCG AAG CTT GTA AG-3'); nas-r (5'-CAG TTC CGA CAG CTT CAT AGA ATT CCC-3'), nap-f (5'-GGA CAA TTT CTG CAA TTA TCA AGA ATT CTA TGT TAAC-3'), and nap-r 5'-CCT CAA GGA TCC AGC ATA ACC GAG-3' (underlining denotes engineered restriction

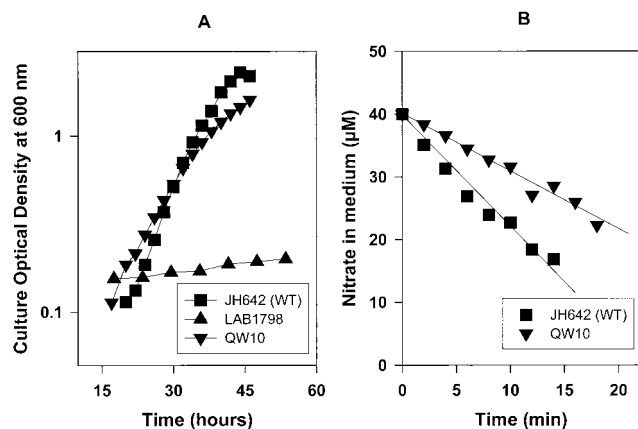


FIG. 4. Growth (A) and nitrate utilization (B) of *B. subtilis* wild-type (WT) strain JH642, $\Delta nasA$ strain LAB1798, and strain QW10 (LAB1798/*PnasA-napA*) at 37°C on minimal MMG medium with nitrate as the N source.

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 $100 \mu\text{g} \cdot \text{ml}^{-1}$ 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 6×10^8 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 (12) containing nitrate as the nitrogen source and erythromycin at $25 \mu\text{g} \cdot \text{ml}^{-1}$. 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 μM nitrate addition. QW10 cells assimilated nitrate at 57% of the rate of wild-type *B. subtilis* (Fig. 4B). LAB1798 cells did not take up any nitrate from the medium (data not shown). Figure 5A shows the growth on 20 mM nitrite of wild-type *B. subtilis* alongside mutant strain LAB1798 and strain QW10, *napA*-complemented LAB1798. All three strains grew equally well on nitrite, and apparently the presence of NapA is not required to meet the N demands of *B. subtilis* when it is growing on nitrite. Nitrite assimilation by *B. subtilis* was studied as described above for nitrate. Nitrite utilization by *B. subtilis* LAB1798 cells was undetectably low during the 20-min assay (Fig. 5B). These cells probably satisfy their N requirement from the slow diffusion of nondissociated HNO_2 over the cytoplasmic membrane at the elevated NO_2^- concentration in the growth medium. JH642 cells readily assimilated nitrite, albeit at a rate slower than that of nitrate assimilation ($0.53 \pm$

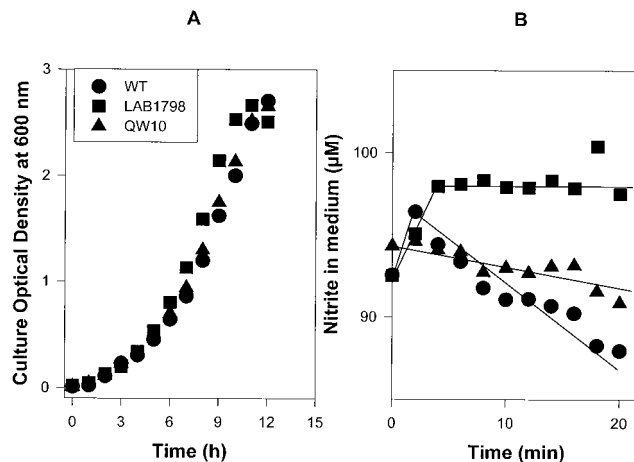


FIG. 5. Growth (A) and nitrite utilization (B) of *B. subtilis* wild-type (WT) strain JH642, $\Delta nasA$ strain LAB1798, and strain QW10 (LAB1798/*PnasA-napA*) at 37°C on minimal MMG medium with nitrite as the N source.

$0.05 \mu\text{mol of NO}_2^- \cdot \text{OD}_{600} \text{U}^{-1} \cdot \text{min}^{-1}$ versus $2.73 \pm 0.05 \mu\text{mol of NO}_3^- \cdot \text{OD}_{600} \text{U}^{-1} \cdot \text{min}^{-1}$). The *napA*-complemented QW10 cells removed nitrite from the medium at $0.20 \pm 0.04 \mu\text{mol of NO}_2^- \cdot \text{OD}_{600} \text{U}^{-1} \cdot \text{min}^{-1}$, approximately 50% of the rate found for 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 NrtABCD. 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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