Latent Nitrate Transport Activity of a Novel Sulfate Permease-like Protein of the Cyanobacterium Synechococcus elongatus*

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The Synechococcus elongatus mutant lacking the nrtABCD gene cluster (NA3) is defective in active nitrate transport and requires high nitrate concentrations (>30 mM) for sustained growth. Prolonged incubation of NA3 in medium containing 2 mM nitrate led to isolation of a pseudorevertant (NA3R) capable of transport of millimolar concentrations of nitrate, from which three mutants with improved affinity for nitrate were obtained. We identified three genes responsible for the latent transport activity for nitrate: ltnA, which encodes a response regulator with no effector domain; ltnB, which encodes a hybrid histidine kinase with two receiver domains; and ltnT, which encodes a sulfite permease-like protein with a putative cyclic nucleoside monophosphate (cNMP)-binding domain. Missense mutations of the high affinity derivatives of NA3R were found in ltnT, verifying that LtnT acts as the transporter. Overexpression of truncated LtnT lacking the cNMP-binding domain (but not full-length LtnT) conferred nitrate transport activity on NA3, suggesting that the cNMP-binding domain inhibits transport under normal conditions. A nonsense mutation in ltnB that resulted in elimination of the receiver domains of the encoded protein was responsible for expression of nitrate transport activity in NA3R. Expression of LtnB derivatives lacking the receiver domains also conferred low affinity nitrate transport activity on NA3. The phosphoryl group of the histidine kinase domain of LtnB was transferred to Asp 52 of LtnA in vitro. Overexpression of LtnA (but not LtnA(D52E)) led to manifestation of the latent nitrate transport activity in NA3, indicating involvement of phosphorylated LtnA in activation of the novel transporter.

Nitrate is a major source of nitrogen for plants, algae, fungi, and many species of bacteria (1, 2). It is transported into the cells by an active nitrate transport system (NRT) 3 and reduced to nitrite by nitrate reductase. Nitrite is further reduced to ammonium by nitrite reductase, and the resulting ammonium is fixed as the amide group of Gln by glutamine synthetase. Three distinct types of nitrate transport systems have been identified among the large variety of organisms that assimilate nitrogen, viz. NRT1, NRT2, and ATP-binding cassette (ABC)-type NRT. NRT1 and NRT2 possess 12 membrane-spanning regions and belong to the major facilitator superfamily of secondary active transport systems (3). ABC-type NRT is a primary active transport system consisting of a substrate-binding protein and a membrane transporter complex (4). Although NRT2 is widespread and found in plants, algae, fungi, the marine strains of cyanobacteria, and some species of heterotrophic bacteria, NRT1 has been found only in vascular plants. ABC-type NRT is present only in prokaryotic organisms, including the freshwater strains of cyanobacteria and some heterotrophic bacteria.

The cyanobacterium Synechococcus elongatus has an ABC-type NRT encoded by four genes: nrtA encodes the substrate-binding protein; nrtB encodes the integral membrane component of the transporter; and nrtC and nrtD encode the ABC proteins (5–7). The genes encoding NRT, nitrate reductase (nirB) (8, 9), and nitrite reductase (nirA) (10, 11) form an operon, nirA-nrtABCD-narB (nirA operon) (11). The NA3 mutant, which was constructed by deleting the nrtABCD genes from the nirA operon, lacks NRT activity and requires high concentrations of nitrate (>30 mM) for sustained growth (12). In this study, we isolated and characterized a pseudorevertant of NA3 (NA3R) that is capable of uptake of low concentrations of nitrate. Genetic analysis of NA3R and its derivatives revealed that a novel sulfate permease-like protein of unknown substrate specificity shows low affinity nitrate transport activity. We also showed that a two-component regulatory system involving a hybrid histidine kinase and a response regulator with no effector domain is required for activation of the transporter. Possible roles of the phosphotransfer reaction via the two-component system in the regulation of the activity of the novel transport system are discussed.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—A derivative of S. elongatus that was cured of the resident small plasmid pH24 (R2–SpC (13); hereafter designated as the wild-type strain) and the mutant strains derived from it were grown photoautotrophically at 30 °C under continuous illumination provided by fluorescent lamps (70 microeinsteins m−2 s−1). The basal medium used was a nitrogen-free medium obtained by modification of the BG11 medium (14) as described previously (15). Ammonium-containing medium and nitrite-containing medium were prepared by the addition of 3.75 mM (NH4)2SO4 and 5 mM NaNO3, respectively to the basal medium. Nitrate-containing medium was prepared by the addition of KNO3 at the indicated concentrations to the basal medium. Solid medium was prepared by adding 1.5% Bacto agar (Difco) to the liquid medium. All media were buffered with 20 mM HEPES-KOH (pH 8.2). When appropriate, kanamycin and/or chloramphenicol was added to the media at 25 and 10 μg/ml, respectively. The Synechococcus strains and plasmids used in this study are listed in Table 1.

Isolation of a Pseudorevertant of NA3 and Its Derivatives—Cells of the NA3 mutant of S. elongatus strain PCC 7942 (lacking the nitrate/nitrite transporter genes nrtABCD) were inoculated into liquid medium containing 2 mM nitrate and cultivated with occasional dilution with fresh...
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TABLE 1
Cyanobacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Ref. or source |
|-------------------|--------------------------|---------------|
| **Strains**       |                          |               |
| Spc               |                          |               |
| NA3               | S. elongatus strain PCC 7942 cured of the pUH24 plasmid, wild-type | 13            |
| NA3R              | NA3 pseudorevertant      | This study    |
| HAR1              | NA3R derivative with improved affinity for nitrate | This study |
| HAR2              | NA3R derivative with improved affinity for nitrate | This study |
| HAR3              | NA3R derivative with improved affinity for nitrate | This study |
| NA3Rlux           | NA3R NpIra::luxAB        | This study    |
| **Plasmids**      |                          |               |
| pSE1              | Km′, Synechococcus shuttle expression vector | 12            |
| pLTnA             | pSE1 derivative encoding LtnA | This study |
| pLtnA(D52E)       | pSE1 derivative encoding LtnA(D52E) | This study |
| pLtnB             | pSE1 derivative encoding LtnB | This study |
| pLtnB-(1–854)     | pSE1 derivative encoding LtnB lacking C-terminal receiver domain | This study |
| pLtnB-(1–735)     | pSE1 derivative encoding LtnB lacking both receiver domains | This study |
| pLtnB-(108–977)   | pSE1 derivative encoding LtnB lacking N-terminal region | This study |
| pLtnB-(808–735)   | pSE1 derivative encoding LtnB lacking N-terminal region and both receiver domains | This study |
| pLtnT             | pSE1 derivative encoding LtnT | This study |
| pLtnT/LC          | pSE1 derivative encoding LtnT lacking C-terminal cNMP-binding domain | This study |
| pLtnT(L246I)      | pSE1 derivative encoding LtnT(L246I) | This study |
| pLtnT(L246I)/C    | pSE1 derivative encoding LtnT(L246I) lacking C-terminal cNMP-binding domain | This study |
| pQE30, pQE31      | Amp′, E. coli expression vector | Qiagen Inc. |
| pQE-LtnA          | pQE30 derivative encoding LtnA | This study |
| pQE-LtnA(D52E)    | pQE30 derivative encoding LtnA(D52E) | This study |
| pQE-LtnB-(408–977)| pQE31 derivative encoding LtnB lacking N-terminal region | This study |
| pQE-LtnB-(408–735)| pQE31 derivative encoding LtnB lacking N-terminal region and both receiver domains | This study |

Nitrate (2 mM)-containing medium. After 4 months of cultivation, the color of the culture turned yellow green, indicating growth of cells. Straking of the culture onto a plate containing 5 mM nitrate allowed isolation of a colony of a pseudorevertant (designated NA3R) that grew well on 5 mM nitrate, but failed to grow on 0.5 mM nitrate. For selection of NA3R derivatives with high affinities for nitrate, NA3R cells were incubated under low nitrate conditions that did not support growth of the pseudorevertant. Thus, after incubation for 3 months on solid medium containing 0.3 mM nitrate, one colony arose and was isolated as a high affinity derivative of the pseudorevertant (designated HAR1). Two independent NA3R derivatives capable of growth on solid medium containing 0.3 mM nitrate were isolated from liquid cultures containing 1 mM and 0.5 mM nitrate and designated HAR2 and HAR3, respectively.

Loss-of-Function Analysis of the Genes Required by NA3R for Nitrate Transport—A transcriptional fusion of the nitrogen-responsive promoter of nirA (nucleotides −275 to −15 with respect to the nirA translation start site) and the coding sequences of luxA and luxB (PnIra::luxAB) (16) was integrated into the cmpB-cmpC region of the Synechococcus genome as described previously (16) to construct a reporter strain (NA3Rlux) from NA3R. For random tagging mutagenesis of NA3Rlux, genomic DNA isolated from wild-type Synechococcus strain PCC 7942 was used. Therefore, the lesions in the NA3Rlux derivatives unable to grow on 5 mM nitrate.

Construction of the Insertion Mutants—Site-directed insertion mutants were constructed as described previously by Williams and Szalay (17). DNA fragments that contained complete or partial sequences of the target open reading frames (ORFs) of Synechococcus were amplified by PCR and cloned into the pGEM-T vector (Promega Corp.). A kanamycin-resistant (18) or chloramphenicol-resistant (19) marker was subsequently inserted at a suitable restriction site in each of the ORFs in the same orientation as the ORFs. The resulting plasmids were used to transform Synechococcus to kanamycin or chloramphenicol resistance through homologous recombination. The transformants were allowed to grow on solid medium supplemented with kanamycin or chloramphenicol. After serial streak purifications to promote segregation of alleles and to isolate homozygous mutants, genomic DNA from selected clones was analyzed by PCR to confirm the presence and position of the kanamycin- or chloramphenicol-resistant marker.

Expression of Plasmid-encoded Proteins in Synechococcus—A shuttle expression vector (pSE1) (12) was used for expression of cloned genes in Synechococcus. The coding regions of ltnA, ltnB, and ltnT were amplified from the Synechococcus chromosomal DNA by PCR. Sense primers carried mismatches with the genomic sequence that created an NcoI or a BspHI recognition site immediately downstream of the termination codon. The ltnA sequence with a T-to-A base substitution at the nucleotide 156 was generated by overlap extension PCR (20). For expression of LtnB derivatives lacking the N-terminal signal input domain, an internal ATG codon located at position 1222 of the ORF was used as the initiation codon. For expression of LtnB derivatives lacking one or two of its receiver domains, antisense primers were used to introduce stop codons at nucleotides 2563 and 2206, respectively. For expression of an LtnT derivative lacking the C-terminal cyclic nucleotide monophosphate (cNMP)-binding domain, an antisense primer was

Cyanobacterial strains and plasmids used in this study

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| HAR3              | NA3R derivative with improved affinity for nitrate | This study |
| NA3Rlux           | NA3R NpIra::luxAB        | This study    |

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designed to introduce a stop codon at position 1756 of the coding region. The PCR products were digested with a combination of Ncol and BamHI, Ncol and XbaI, BspHI and BamHI, or BspHI and XbaI and cloned between the Ncol and BamHI sites or the Ncol and XbaI sites of pSE1. The resulting plasmids were introduced into the NA3 cells after verification of the nucleotide sequence.

Preparation of Recombinant Proteins in E. coli—The expression vectors pQE30 and pQE31 (Qiagen Inc.) were used for overexpression of LtnA, LtnB, and their derivatives as His-tagged proteins. The coding sequences of the ltnA and ltnA(T156A) genes were prepared essentially as described above, except that the sense primer used for PCR carried mismatches with the genomic sequence to create a BamHI recognition site nine nucleotides upstream of the translation start site and that the antisense primer carried a BamHI recognition sequence immediately downstream of the termination codon. The PCR products were digested with BamHI and cloned into the BamHI site of pQE30. The truncated ltnB genes were excised from the pSE1 derivatives used for expression of the genes in Synechococcus (see above) by digestion with Ball and XbaI and cloned between the Smal and Nhel site of pQE31. After verification of the nucleotide sequences, the resulting plasmids were transformed into E. coli M15 (pREP4) (Qiagen Inc.), and the His-tagged proteins were purified on nickel-nitrilotriacetic acid resin.

Phosphotransfer Experiment—Purified proteins were incubated in 15 μl of buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 2 mM dithiothreitol, and 10% glycerol supplemented with 0.05 mM [γ-32P]ATP (200 TBq/mmol), 50 mM KCl, and 5 mM MgCl2 according to the protocol of Aiba et al. (21). After incubation for 20 min at 30 °C, the reaction was stopped by the addition of 4 μl of SDS buffer containing 250 mM Tris-HCl (pH 6.8), 4% SDS, 8% 2-mercaptoethanol, 60% glycerol, and 0.02% bromphenol blue. The samples were subjected to 10−20% gradient SDS-PAGE, and the 32P-labeled signals were detected using a bio-imaging analyzer (Fuji Photo Film).

Measurement of Nitrate Uptake—Cells grown in medium containing 60 mM nitrate were washed with the basal medium supplemented with 5 mM NaHCO3 and 20 mM HEPES-KOH (pH 8.2) and suspended in the same medium at a chlorophyll concentration of 5 or 10% gradient SDS-PAGE, and the 32P-labeled signals were detected.

RESULTS

Nitrate Uptake Capacity of the Mutant Strains—Fig. 1 compares the growth curves of the wild-type strain, the NRT-deficient mutant NA3, and the pseudorevertant of NA3 (designated NA3R). The wild-type strain grew equally well in ammonium- or nitrate-containing medium with a generation time of 6.4 h (Fig. 1A). The NA3 mutant grew as fast as the wild-type strain in ammonium-containing medium with a generation time of 6.8 h, but hardly grew in medium containing 2 mM nitrate; the generation time was 90 h, and the culture showed a yellow color due to reduced pigmentation (Fig. 1B) as described previously (12). Growth of NA3 was improved with increasing concentrations of nitrate, but even at a nitrate concentration of 60 mM, growth was slower than in ammonium-containing medium, with a generation time of 10 h. NA3R cells grew with a generation time of 11 h in medium containing 2 mM nitrate. At ≥6 mM nitrate, growth of NA3R was as fast as in ammonium-containing medium, with a generation time of 7.0−7.3 h (Fig. 1C). The results indicate that NA3R has a higher capacity for utilization of low concentrations of nitrate compared with NA3.

Fig. 2 compares the dependence of nitrate uptake rate on nitrate concentration in the medium. Wild-type cells took up nitrate at a rate of 40 μmol/mg of chlorophyll/h in an external nitrate concentration range of 0.1–6 mM, but NA3 cells were unable to take up nitrate in this concentration range as described previously (12). NA3R did not exhibit nitrate uptake at external concentrations of 0.1 mM or lower, but the nitrate uptake rate increased as the external nitrate concentration was...
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![Graph showing nitrate uptake rate vs. nitrate concentration](image)

**Figure 2.** Dependence of the nitrate uptake rate on the nitrate concentration of the medium. Cells grown in nitrate (60 mM)-containing medium were used for the assay. Wild-type strain (WT), □ NA3; ▲, NA3R; ○, HAR1; △, HAR2; □, HAR3. CHL, chlorophyll.

raised, reaching a rate of 38 μmol/mg of chlorophyll/h at 6 mM nitrate. The results indicate that a low affinity nitrate transport system is present in NA3R.

NA3R was virtually unable to grow on solid medium containing 0.3 mM nitrate, but prolonged incubation of the cells at the low nitrate concentration led to isolation of three independent NA3R derivatives that could grow on 0.3 mM nitrate (designated HAR1, HAR2, and HAR3). The maximum nitrate uptake rates of the HAR mutants were lower than that of the parental NA3R strain, but the mutants showed much higher rates of nitrate uptake compared with NA3R at external nitrate concentrations of 1 mM or lower (Fig. 2), indicating that the affinity of the nitrate transport system for nitrate had been improved in the HAR mutants.

**Genes Required for the Low Affinity Nitrate Transport Activity of NA3R**—For genetic identification of the genes involved in the low affinity nitrate transport of the NA3R strain, we first introduced a PtirA::luxAB fusion into the genome of NA3R to construct a reporter strain (NA3Rlux) and then mutagenized this strain by random tagging. The PtirA promoter is responsive to nitrogen and is most active in nitrate-limited cells (16). When NA3Rlux was grown on 60 mM nitrate, the cells were nitrate-replete, and the luciferase expression level was 0.01 of that observed in the PtirA::luxAB strain constructed from NA3 (data not shown). After random tagging mutagenesis of NA3Rlux, we selected strongly luminescent clones in the presence of 60 mM nitrate to isolate mutants with impaired nitrate assimilation. Of the nine mutants thus isolated, three mutants that showed very strong bioluminescence grew normally in ammonium-containing agar plates, but grew very slowly with significant loss of pigmentation on plates containing 5 mM nitrate or 60 mM nitrate, indicating that they were defective in assimilation of nitrate. These mutants carried the pHSG298 vector inserted at different positions in the nirA gene for nitrite reductase. The other six mutants were similar to NA3 in that they grew normally on ammonium-containing agar plates, at an appreciable rate on plates containing 60 mM nitrate, and only poorly on plates containing 5 mM nitrate, indicating that they had lost the low affinity nitrate transport activity. In five of the six mutants, pHSG298 was found to replace a 4.5-kilobase pair genomic region carrying the six ORFs from syc2273_c to syc2278_d (Fig. 3A). The other nitrate transport mutant was shown to have pHSG298 inserted between syc1141_c and syc1148_c, replacing the six ORFs from syc1142_c to syc1147_c (Fig. 3B). To identify the genes required for the low affinity nitrate transport activity, we insertion-inactivated each of the 12 ORFs in NA3Rlux. Interruption of syc2277_d, syc2278_d, and syc1147_c (but none of the other ORFs) abolished the ability to grow on plates containing 5 mM nitrate and caused high level expression of the PtirA::luxAB fusion when grown on plates containing 60 mM nitrate (data not shown), indicating that the three genes are essential to the low affinity nitrate transport activity. syc2277_d encodes a response regulator of 121 amino acids that has no effector domain and that belongs to the CheY subfamily of response regulators. syc2278_d encodes a hybrid histidine kinase of 977 amino acids that has a predicted signal peptide at the N terminus, a membrane-spanning region, a histidine kinase domain, and two receiver domains (Fig. 4A). syc1147_c encodes a novel sulfite permease-like protein of 727 amino acids that consists of two domains: an N-terminal “sulfite permease (SuIP)” domain of 554 amino acids (NCBI accession no. COG0659) and a C-terminal cNMP-binding domain of 115 amino acids (NCBI accession no. cd00038) (Fig. 4B). The three ORFs required for low affinity nitrate transport, viz. syc2277_d, syc2278_d, and syc1147_c, were named genes ltnA, ltnB, and ltnT, respectively, where “ltn” stands for latent transport activity for nitrate (LTN).

**Mutation Responsible for Activation of LTN in NA3R**—Comparison of the nucleotide sequences of the ltnA, ltnB, and ltnT genes of NA3 and NA3R revealed a mutation in ltnB, but not in ltnA or ltnT. The ltnB mutation of NA3R was a base substitution from C to T at position 2362, yielding an early termination codon (Fig. 4A). The truncated ltnB gene encodes a histidine kinase lacking the C-terminal 190 amino acids comprising the two receiver domains. These results suggest that altered signal transmission via LtnB is responsible for activation of LTN.

**Structure-Function Relationship of LtnT**—Nucleotide sequence analysis revealed that the HAR mutants, having higher affinity for nitrate compared with the parental NA3R strain, have missense mutations in ltnT (Fig. 4B); HAR1 has a base substitution from T to A at position 256 of ltnT, yielding an amino acid substitution from Tyr to Asn at position 86 of the sulfate permease-like protein. HAR2 has a C-to-T base substitution at position 736, yielding a Leu-to-Ile amino acid substitution at position 246 of the encoded protein. In addition to the mutation found in HAR2, HAR3 carries a second base substitution from C to T at position 733, resulting in an additional amino acid substitution from Pro to Ala at position 245. The amino acid replacements critical for improving the affinity of the LTN system are found at relatively hydrophilic positions in the membrane-spanning regions of the SuIP domain. These results confirm that LtnT acts as the permease of the LTN system. When introduced into NA3 cells using the shuttle expression vector pSE1, LtnT did not support growth of the cells on plates containing 5 mM nitrate (Fig. 5, row e), indicating that expression of the gene is not sufficient for expression of nitrate transport activity. Expression of ltnT(C736A), which has the same nucleotide substitution as HAR2, did not confer nitrate transport activity on NA3 either (Fig. 5, row g). Surprisingly, expression of the truncated forms of ltnT and ltnT(C736A) lacking the 3’-terminal part that encodes the cNMP-binding domain supported growth of the cells on 5 mM nitrate (Fig. 5, rows f and h). This finding indicates that the cNMP-binding domain inhibits the nitrate transport activity of the permease domain in NA3. Expression of the truncated form of ltnT(C736A) (but not ltnT) supported growth of the cells on 0.5 mM nitrate (Fig. 5, rows f and h), confirming that the C736A nucleotide substitution in LtnT is responsible for the improved affinity of nitrate uptake by HAR2.

**Structure-Function Relationships of LtnA and LtnB**—To investigate the roles of ltnA and ltnB in expression of LTN, the ltnA and ltnB ORFs and their derivatives were fused to the Ptrc promoter and introduced into the NA3 mutant using the pSE1 shuttle expression vector. The ability of the transformants to grow on nitrate was examined (Fig. 5).
Although neither of the genes encoding full-length LtnB nor the LtnB derivative lacking the C-terminal receiver domain (LtnB-(1–854)) conferred on NA3 the ability to grow on agar plates containing 5 mM nitrate (Fig. 5, rows i and j), introduction of the truncated ltnB gene encoding the LtnB derivative lacking the two receiver domains (LtnB-(1–735)) supported cell growth on 5 mM nitrate (row k). These results confirm that expression of the LtnB protein without a receiver domain results in expression of LTN. When isopropyl-β-D-thiogalactopyranoside (IPTG) was added, the Synechococcus transformants lost all pigmentation and died, showing that overexpression of LtnB, LtnB-(1–735), and LtnB-(1–854) is lethal. Because the LtnB derivatives lacking the signal input domain (amino acids 1–407) did not kill the cell even when overexpressed, these results indicate that the C-terminal receiver domain is required for expression of the latent nitrate transport gene in Synechococcus.
pressed (Fig. 5, rows l and m), we deduced that overexpression of the signal input domain of LtnB is toxic to the cell. Although the plasmid encoding LtnB-(408–735) supported nitrate utilization in the absence and presence of IPTG, the plasmid encoding LtnB-(408–977) failed to do so even in the presence of IPTG (Fig. 5, rows l and m). These results confirm the inhibitory effect of the receiver domains of LtnB on expression of LTN.

The NA3 derivative carrying a Prc::ltnA fusion did not grow on plates containing 5 mM nitrate in the absence of IPTG. However, in the presence of IPTG, the cells grew on nitrate (Fig. 5, row n), indicating that overexpression of the response regulator protein can activate LTN in the wild-type ltnB background. IPTG-induced overexpression of a modified ltnA gene that encodes an LtnA derivative with its putative phosphorylation site (Asp52) replaced with Glu did not support cell growth on 5 mM nitrate (Fig. 5, row o). These results indicate that phosphorylation of LtnA is essential for expression of the low affinity nitrate transport activity.

**Phosphotransfer Reaction from LtnB to LtnA**—To examine whether phosphotransfer occurs from LtnB to LtnA, LtnA and LtnA(D52E) were expressed in *E. coli* as His-tagged proteins and purified to near homogeneity (Fig. 6A, lanes 1 and 2). The soluble derivatives of LtnB lacking the N-terminal 407 amino acid residues, viz. LtnB-(408–977) and LtnB-(408–735), were also expressed in *E. coli* as His-tagged proteins and purified to near homogeneity (Fig. 6A, lanes 3 and 4). When incubated for 20 min at 30 °C with 0.05 mM [*γ-32P*]-ATP, LtnB-(408–977) and LtnB-(408–735) were radiolabeled (Fig. 6B, lanes 2 and 7, respectively), whereas LtnA was not (lane 1), demonstrating the capacity of the LtnB derivatives for autophosphorylation. When LtnA was incubated with LtnB-(408–977) or LtnB-(408–735), the radiolabel in the LtnB derivatives was decreased with concomitant labeling of LtnA (Fig. 6B, lanes 3–5 and 8–10, respectively), indicating that the phosphoryl group was transferred to LtnA. When LtnA(D52E) was subjected to the same analysis, there was no transfer of the phosphoryl group to the LtnA derivative (Fig. 6B, lanes 6 and 11, respectively), indicating that Asp52 is the phosphoacceptor site of LtnA.

**DISCUSSION**

The NA3 mutant of *Synechococcus elongatus* lacks the four genes (*nrtABCD*) encoding the ABC-type nitrate/nitrite transporter and is unable to assimilate low concentrations of nitrate (12). A pseudorevertant (NA3R) capable of sustained growth in medium containing 5 mM nitrate was obtained from NA3 and shown to have low affinity nitrate transport activity (Fig. 2). Of the three genes required for nitrate transport by NA3R (Fig. 3), *ltnA* and *ltnB* encode a CheY-type response regulator and a hybrid histidine kinase consisting of a signal input domain, a histidine kinase domain, and two receiver domains, respectively (Fig. 4A). NA3R was found to have a nonsense mutation in *ltnB* resulting in elimination of the receiver domains from the encoded protein, suggesting that altered signal transfer in a His-Asp phosphorelay system had caused manifestation of LTN. Expression of various truncated forms of *ltnB* in NA3 confirmed that the LtnB derivatives with no receiver domains support nitrate transport (Fig. 5). Because LtnA (but not LtnA(D52E)) receives the phosphoryl group from His-tagged LtnB-(408–735) (Fig. 6), we deduced that the phosphoryl group is transferred from the histidine kinase domain of LtnB (amino acids 408–735) to Asp52 of LtnA. Overexpression of LtnA (but not LtnA(D52E)) leads to expression of nitrate transport activity in NA3 (Fig. 5), indicating that phosphorylation of LtnA is essential for manifestation of LTN. Although His-tagged LtnB-(408–977), which retains the two receiver domains, can transfer the phosphoryl group to LtnA in *vitro* (Fig. 6),

**FIGURE 5.** Growth test showing the effects of expression of the Ltn proteins and their derivatives on expression of nitrate transport activity in NA3. *Synechococcus* cells (*n = 10⁶*) were spotted on solid medium containing ammonium (7.5 mM) or nitrate (0.5 or 5 mM) as nitrogen sources and incubated under illumination for 4 days. Where indicated, IPTG (1 mM) was added to overexpress the proteins from the plasmid-borne genes. WT, wild-type; *ltnB*::pSE1, gene that encodes an LtnA derivative with its putative phosphoacceptor aspartate residue; WT*, glutamate residue replaced with the phosphoacceptor aspartate residue.

### Table

| Host Strains | Plasmids | Structure of the Encoded Proteins | Growth Test |
|--------------|----------|----------------------------------|-------------|
| (a) WT       | –        | [Sulfate permease](#)            | +IPTG (1 mM) |
| (b) NA3      | –        | [Sulfate permease](#)           | 7.5 mM 5 mM |
| (c) NA3R     | –        | [Sulfate permease](#)           | 0.5 mM 3 mM |
| (d) NA3      | pSE1     |                                  | +IPTG (1 mM) |
| (e) NA3      | pLtnT    | [Sulfate permease](#)           | 7.5 mM 5 mM |
| (f) NA3      | pLtnTAC  | [Sulfate permease](#)           | 0.5 mM 3 mM |
| (g) NA3      | pLtnT(L246I) | [Sulfate permease](#)   | 7.5 mM 5 mM |
| (h) NA3      | pLtnT(L246I)AC | [Sulfate permease](#)  | 0.5 mM 3 mM |
| (i) NA3      | pLtnB    | [Sulfate permease](#)           | +IPTG (1 mM) |
| (j) NA3      | pLmb[1–854] | [Sulfate permease](#)          | 7.5 mM 5 mM |
| (k) NA3      | pLmb[1–735] | [Sulfate permease](#)          | 0.5 mM 3 mM |
| (l) NA3      | pLmb[408–977] | [Sulfate permease](#)         | +IPTG (1 mM) |
| (m) NA3      | pLmb[408–735] | [Sulfate permease](#)         | 7.5 mM 5 mM |
| (n) NA3      | pLtnA    | D                                | +IPTG (1 mM) |
| (o) NA3      | pLtnA(D52E) | E                               | 7.5 mM 5 mM |
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FIGURE 6. In vitro phosphoryl transfer experiment using LtnA, LtnB, and their derivatives. A, SDS-PAGE of the purified proteins. Purified Histagged proteins (3 μg) were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, LtnA; lane 2, LtnA(D52E); lane 3, LtnB-(408–977); lane 4, LtnB-(408–735). Molecular mass markers are indicated in kilodaltons. B, labeling experiments with the LtnA and LtnB proteins. Purified proteins were added to the reaction mixtures to give the indicated concentrations and incubated with 50 μM [γ-32P]ATP for 20 min. The proteins were subsequently fractionated on a 10–20% gradient SDS-polyacrylamide gel, and 32P-labeled signals were detected using a bio-imaging analyzer.

wild-type LtnB or its derivatives with one or two receiver domains cannot support nitrate transport when expressed in NA3 (Fig. 5), showing that the receiver domains interfere with phosphorylation of LtnA in vivo. This suggests that the receiver domains compete with LtnA for the phosphoryl group by continuously shifting the phosphoryl group to a phosphoreceptor in the cell. Alternatively, a phosphatase may dephosphorylate the receiver domains to allow continued transfer of the phosphoryl group to the receiver domains.

The other gene essential to nitrate transport by NA3R (ltnT) encodes a protein belonging to the SulP family of the major facilitator superfamily. LtnT is unique among the SulP family proteins in having a C-terminal extension (Fig. 4B), which negatively regulates the activity of the permease domain (Fig. 5). Because no mutation was found in the ltnT gene of NA3R, we deduced that phosphorylation of LtnA had alleviated the inhibitory effect of the C-terminal domain of LtnT on permease activity. Although it is similar to cNMP-binding proteins, the C-terminal domain of LtnT lacks one of the two amino acids residues involved in binding cNMP (Fig. 4C) (25), making it questionable whether cNMP has a role in the regulation of LTN. No interaction was detected between LtnA and the C-terminal domain of LtnT by yeast two-hybrid analysis of the proteins. The molecular mechanism responsible for the regulation of LtnT activity thus remains unknown. Response regulators of the CheY family, which consist of only the receiver domain, have been shown to be involved in the regulation of chemotaxis of bacteria (26) and phototaxis of cyanobacteria (27). LtnA appears to be the first example of a CheY family protein involved in the regulation of a transport process. Much more work is required to elucidate the mechanism responsible for this unique form of regulation.

Fig. 7 summarizes the roles of LtnA, LtnB, and LtnT elucidated in this study. The histidine kinase domain of LtnB is autophosphorylated, and the phosphoryl group is normally transferred to the receiver domains, leaving LtnA in the unphosphorylated state. Under these conditions, the C-terminal domain of LtnT inhibits the permease activity of the protein. Because of the nonsense mutation in ltnB, NA3R expresses the truncated LtnB protein lacking the receiver domains, allowing phosphotransfer from the histidine kinase domain of LtnB to LtnA. The phosphorylated LtnA protein somehow alleviates the inhibitory effect of the LtnT C-terminal domain on permease activity. It is unknown whether LtnA interacts directly with LtnT or whether other signal-transducing proteins are involved in the regulation. Additionally, the circumstances under which the phosphoryl group of the wild-type LtnB protein is transferred to LtnA remain to be determined.

Eighteen cyanobacterial genomes have been sequenced to date, but apart from those of S. elongatus strains PCC 6301 and 7942, only the genome of Crocosphaera watsonii WH 8501 carries a gene encoding an LtnT-like protein. LtnT-like genes are found in the genomes of the bacteria Bradyrhizobium japonicum, Bradyrhizobium sp. BT4ii, Desulfovibrio vulgaris, Legionella pneumophila, Magnetococcus sp. MC-1, and Magnetospirillum magnetotacticum, but not in other genomes. The LtnT-like protein is thus limited to a few species of bacteria and cyanobacteria. The SulP domains of LtnT and its homologs are 27–31% identical to one another and are more closely related to one another than to the other cyanobacterial SulP family proteins (Fig. 4D). This suggests that the LtnT gene was acquired by cyanobacteria by horizontal gene transfer.

In the NA3R strain, LtnT supports growth of the cells at 5 mM nitrate, but not at 0.5 mM nitrate (Fig. 5). The low affinity of LtnT for nitrate suggests that the substrate for LtnT may not be nitrate. LtnT clearly does not transport sulfate because overexpression of LtnT-(1–585) (the SulP domain of LtnT) or LtnB-(408–735) (the histidine kinase domain of LtnB) in an S. elongatus cysA deletion mutant that lacks the ABC-type sulfite transporter and that is unable to grow on low concentrations of sulfite (28) did not confer on the mutant the ability to grow on 30 mM sulfate. SulP family proteins involved in active transport of bicarbonate (BiCA) were recently identified in the cyanobacteria Synchococcus sp. strain PCC 7002, Synechocystis sp. strain PCC 6803, and Synechococcus sp. WH 8102 (29), but these proteins are only 18–21% identical to the SulP domain of LtnT and are distantly related to LtnT (Fig. 4D), suggesting that LtnT is unlikely to function as a bicarbonate transporter. An ltnT single mutant of S. elongatus grew normally under standard labo-

S.-i. Maeda, unpublished data.
Latent Nitrate Transport Activity of a Novel Permease

It is important to note that S. elongatus has made use of a normally unused permease for the purpose of nitrate transport by redirecting the phosphorylation signal of LtnB toward LtnA. This indicates that genetic modification of a regulatory network can play a role in adaptation of an organism to unfavorable conditions. Furthermore, secondary mutations in ltnT enabled the cells to grow on lower concentrations of nitrate compared with NA3R (Figs. 2 and Fig. 4B). Spontaneous mutations and selection thus allowed S. elongatus to develop a better permease from the normally unused LtnT permease. Bacterial and cyanobacterial genomes contain large numbers of genes with no known functions, which presumably include many unused genes. Although the physiological significance of the presence of these genes is unclear, we infer from the present results that they enhance the potential abilities of the cells to survive various adverse conditions.

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