STUDIES ON THE REGULATION OF THE ASSIMILATORY NITRATE REDUCTASE OPERON IN *AZOTOBACTER VINELANDII*

By

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PLANT SCIENCES
DIVISION OF PLANT PATHOLOGY & MICROBIOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN PLANT PATHOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2009

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation

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entitled "Studies on the Regulation of the Assimilatory Nitrate Reductase Operon in *Azotobacter vinelandii*"

and recommend that it be accepted as fulfilling the dissertation requirement for the

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ACKNOWLEDGEMENTS

It would have been impossible to finish my Ph.D studies without the support and assistance of numerous colleagues and the Department of Plant Sciences at the University of Arizona.

First and foremost, I would like to thank Dr. Christina Kennedy, my mentor, for providing me a friendly academic environment in which I can perform research. I also thank her for consistent encouragement during my study and research progress, and for trust on my capability and potential in biological research.

I am grateful to my committee members: Dr. Sandy Pierson, Bentley Fane, Chris Rensing, and Marc Orbach. They were immensely helpful in revising my manuscripts and provided me valuable suggestions about scientific writing. Dr. Pierson took responsibility for directing my Ph.D completion due to the tragic loss of Dr. Kennedy.

I thank Drs. Sandy Pierson, Martha Hawes, and Hans VanEtten for their generous sharing of lab equipments. I thank Drs. Robert Leonard, Rachel Pfister, and Donna-Rae Marquez for generous support from the department. I am grateful to Dr.Gerard White, Michele Hoffman, and Dr. Jeffrey Coleman for proofreading. I thank my previous lab members Mali Gunatilaka and Paul Rudnick for providing daily research support and help. I would like to acknowledge the help and support from Drs. Zhongguo Xiong and from Fushi Wen for numerous insights.

Finally, I would like to thank my wife, Hui, and my son, Leo, who have accompanied me and have weathered all the tough times that we have experienced.

DEDICATION

To

my beloved parents

Jinhan Wang and Ailan Wang

without whose sacrifices and inspiration this is impossible

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ABSTRACT

Azotobacter vinelandii is a free-living diazotroph. This bacterium fixes atmospheric nitrogen in different environments using three genetically distinct nitrogenases. *A. vinelandii* is also capable of utilizing nitrate and nitrite from the environment. Nitrate is reduced sequentially into nitrite and ammonia. The assimilatory nitrate reductase and nitrite reductase are encoded by the *nasAB* operon. Previous genetic studies identified a number of factors that influence *nasAB* expression. However, the molecular mechanisms controlling the expression of *nasAB* are unclear.

The current study was initiated to characterize the region preceding the *nasAB* operon which was previously implicated in its regulation and to further study the molecular mechanisms of *nasAB* regulation. The results confirm that *nasAB* is subject to multiple layers of regulation. The operon is under the control of an NtrC-dependent promoter; nitrate/nitrite induction occurs at the post-transcriptional level via antitermination within the *nasAB* leader region; and nitrate/nitrite induction is mediated by NasS/NasT, a sensor-antiterminator two-component regulatory system.

Together, these data suggest a model for the regulation of the assimilatory nitrate reductase operon in *A. vinelandii*.

1. INTRODUCTION

1.1 Introduction to Nitrogen and Nitrate Reduction

Nitrogen is an essential element in all organisms as both amino acids and nucleic acids contain nitrogen. In the biosphere, nitrogen exists in several oxidation states, from +5 to -3 (NO_3^- to NH_3, respectively). The conversions of nitrogen among different oxidation statues constitute the biogeochemical nitrogen cycle (Figure 1).

As part of the biological nitrogen cycle, nitrate reduction occurs in plants, fungi, archaea, and bacteria. Various bacteria can perform three forms of nitrate reductions for different physiological purposes. For example, nitrate is used as the nitrogen source for many molecular biosynthetic reactions. Nitrate can not be incorporated directly into organic molecules by organisms; it has to be reduced into ammonia via nitrate reductase and nitrite reductase. Ammonia can be either incorporated directly into 2-oxoglutarate to form glutamate via glutamate dehydrogenase (GDH), or first be incorporated into glutamate to form glutamine via glutamine synthetase (GS) and then transferred to 2-oxoglutarate via glutamate synthase (GOGAT). This nitrate reduction pathway is also known as assimilatory nitrate reduction. The nitrate reductase and nitrite reductase genes are correspondingly named *nas* (nitrate assimilation).

Additionally, many facultative bacteria can use nitrate as a terminal electron acceptor during anaerobic respiration. During this process, nitrate is reduced sequentially into nitrite, nitrite oxide (NO), nitrous oxide (N_2O), and finally into free nitrogen (N_2),

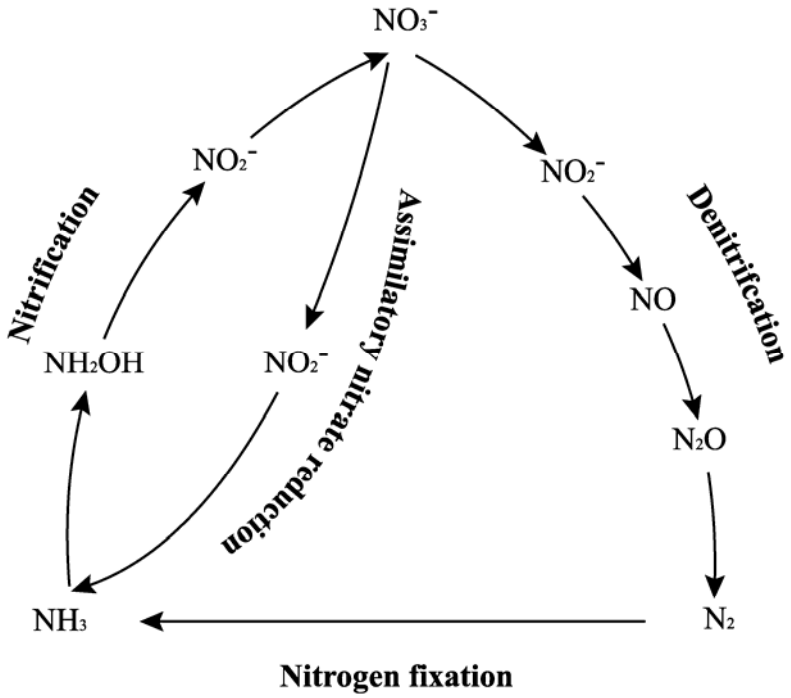


Figure 1. Biological nitrogen cycle. (Adapted from Richardson, 2001)

which is released into the atmosphere (Figure 1). This process is called denitrification. The genes for the reductases involved in this pathway are called *nar* (nitrate respiration) genes.

Finally, some bacteria use nitrate reduction as a tool to balance cellular redox levels under certain physiological conditions. This pathway is also called dissimilatory nitrate reductase. The dissimilatory nitrate reductase resides in the periplasmic region and named *nap* (periplasmic nitrate reductases).

1.2 *Azotobacter vinelandii*

A. vinelandii is a free-living diazotroph, belonging to the *Pseudomonadaceae* family of Gammaproteobacteria (Bergey's Manual of Systematic Bacteriology). The genome of *A. vinelandii* AvOP has been sequenced and annotated (http://genome.jgi-psf.org/draft_microbes/azovi/azovi.home.html). The *A. vinelandii* genome is composed of a single circular chromosome of 5.4 Mb with a high GC content (65.7%).

An intriguing feature of *A. vinelandii* is that its genome can be present in multiple copies: the number of *A. vinelandii* genome copies varies from 2 to 80 depending on growth conditions (36). Correspondingly, *A. vinelandii* has a relatively larger size as compared with other bacteria. As indicated in the photo shown on Figure 2, *A. vinelandii* cells are more than 16 fold larger than *E. coli* cells in volume (16).

A. vinelandii has three genetically distinct nitrogenases that utilize different metal cofactors: molybdenum (Mo); vanadium (V); or iron (Fe) (48). The presence of multiple nitrogenases provides the bacterium with survival advantages under different

environments that vary in metal availability. Expression of these nitrogenases is highly coordinated, preventing metabolic energy waste.

The genetic regulation of nitrogen fixation (*nif*) in *A. vinelandii* has been studied for more than 30 years, and the thorough understanding of the *nif* regulation in *A. vinelandii* makes it an excellent model organism.

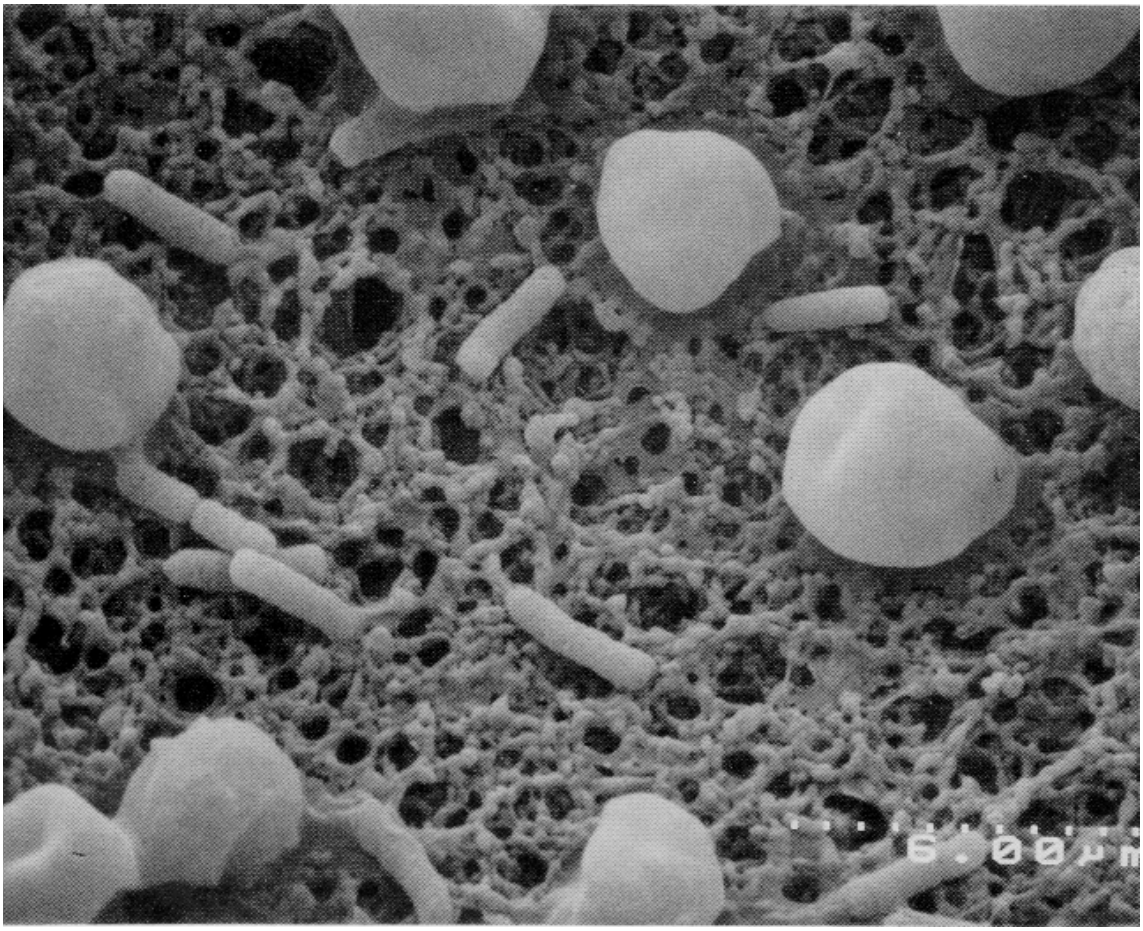


Figure 2. Scanning electron micrograph of *A. vinelandii* and *E. coli.* (From Efuet et al 1996). Note the large spherical *A. vinelandii* cells as compared to the small rod-shaped *E. coli* cells.

1.3. Protein-mediated Antitermination in Bacteria

Bacteria are unicellular organisms. They exchange energy and materials with the environment through the cellular membranes. Since biochemical and physical parameters of the environment are not stable, bacteria have to adjust their metabolisms dynamically and rapidly to meet external changes. To adapt to this lifestyle, bacteria evolved complex and sophisticated mechanisms to regulate gene expression

The most common type of gene regulatory mechanism occurs at the initiation of transcription. Activators, repressors, and alternative sigma factors coordinate physiological signals and determine the frequency of transcription initiations. In addition to transcription initiation, gene regulation may occur at the stages of transcription termination or translation, known as post-transcriptional regulation. Post-transcriptional regulation is a type of short-term regulation, a fine-tuning regulation of initiated transcription. One type of post-transcriptional regulation involves premature termination/antitermination of transcription near the 5' end of nascent mRNAs. The following sections describe several well studied paradigms of post-transcriptional control.

1.3.1. *trp* operon regulation in *E. coli*

Many amino acid biosynthetic operons in bacteria use antitermination to fine-tune the regulation of gene expression. The paradigm of this type of regulation is the *trp* operon in *E. coli*, first indentified by Yanofsky et al. (67). The *trp* operon contains five

structural genes encoding enzymes responsible for the synthesis of tryptophan. The *trp* transcript has a 5' end leader region before the first structural gene. The leader region is 162 nucleotides in length and has the following characteristics (Figure 3). First, four sequences in this leader region (designated 1, 2, 3, and 4), can form mutually exclusive alternative hairpin structures: either 2:3 or 1:2 and 3:4. The 2:3 hairpin is also called the antiterminator. Second, it contains a small ORF encoding a 14 amino acid polypeptide TrpL that contains two tandem Trp residues. In addition, the 3'end of *trpL* overlaps with sequence 1. Three, the 3:4 hairpin is GC-rich and followed immediately by a downstream poly(U) region, constituting an intrinsic factor-independent transcription terminator.

During transcription, formation of the 2:3 hairpin or the alternate 1:2 and 3:4 hairpins depends on the relative rates of TrpL translation and the transcription elongation complex (EC) movement. The rate of TrpL translation is determined by the availability of charged $tRNA^{Trp}$ in the cell. Limitation of cytoplasmic $tRNA^{Trp}$ causes the ribosome to stall in the tandem Trp codons, leaving sequence 2 to form a stemloop with sequence 3. As a result, limitation of cytoplasmic $tRNA^{Trp}$ leads to the formation of the antitermination structure and subsequent transcription of the *trp* biosynthetic operon genes. In contrast, sufficient levels of cellular $tRNA^{Trp}$ lead to unrestricted translation of *trpL*, and ribosome reaches the stop codon and dissociates before the emerging sequence 3 becomes available to form a hairpin with sequence 2. In this situation, sequence 2 forms hairpin with sequence 1, leaving sequence 3 to form the terminator hairpin with sequence 4. So, in this mechanism, the rate of ribosome movement acts as the sensor for the cellular levels of charged $tRNA^{Trp}$ (66).

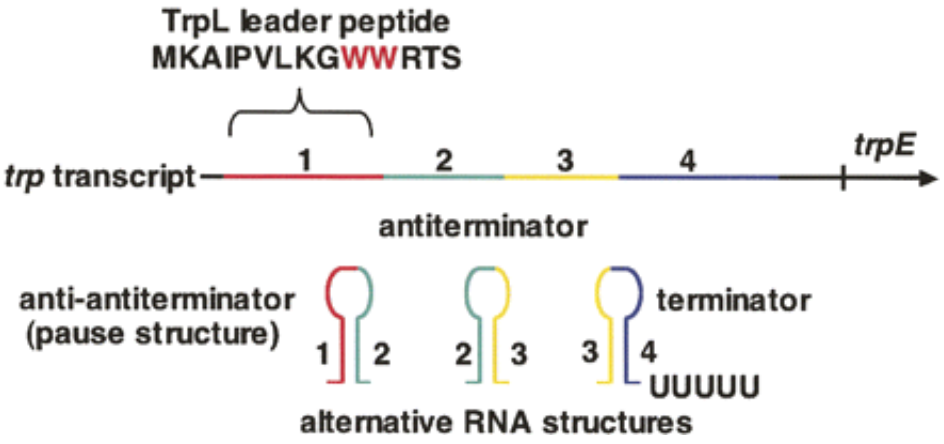


Figure 3. Schematic representation of *trp* leader in *E .coli*. (From Yanofsky, 2007)

1.3.2 *trp* operon regulation in *Bacillus subtilis*

Regulation of the *trp* operon in the Gram positive bacterium *B. subtilis* also involves antitermination. However, the molecular mechanism of *trp* antitermination in *B. subtilis* is different than in *E. coli*. No small ORF translation occurs within the *B. subtilis trp* leader region, antitermination is mediated by a protein regulator TRAP (*trp* RNA-binding attenuation protein).

The *B. subtilis trp* leader region has an intrinsic terminator and an antiterminator hairpin. Formation of these two structures is mutual exclusive due to an overlap sequence ACCC (Figure 4) (29). In addition, the *B. subtilis trp* leader contains 11 (G/U)AG triple nucleotides separated by two or three nucleotides, and six (G/U)AG at the 3' side reside with the antiterminator sequence (7, 29).

TRAP is an oligomer of 11 identical subunits which forms a symmetrical ring shape (1, 14). Each subunit is a short polypeptide of 75 amino acids. Between the adjacent subunits of TRAP exist hydrophobic pockets that can each accommodate a Trp amino acid (2, 38). Under cellular conditions with sufficient tryptophan, 11 Trp amino acids bind the 11 pockets of a TRAP. This binding of Trp amino acids triggers a TRAP conformational change and activates its regulatory function (6, 38). The activated TRAP binds to the (G/C)AG at the 5' end of nascent mRNA and rolls along the emerging mRNA in 5' $\rightarrow$ 3' direction, wrapping the mRNA around the ring structure (8). The

TRAP-RNA interaction blocks the formation of the antiterminator hairpin and leads to the formation of the transcription terminator.

When the cellular Trp concentration is low, TRAP does not interact with the amino acid Trp and is inactive. Without TRAP disruption, the antiterminator structure forms in the leader region of nascent mRNA, blocking the terminator from formation.

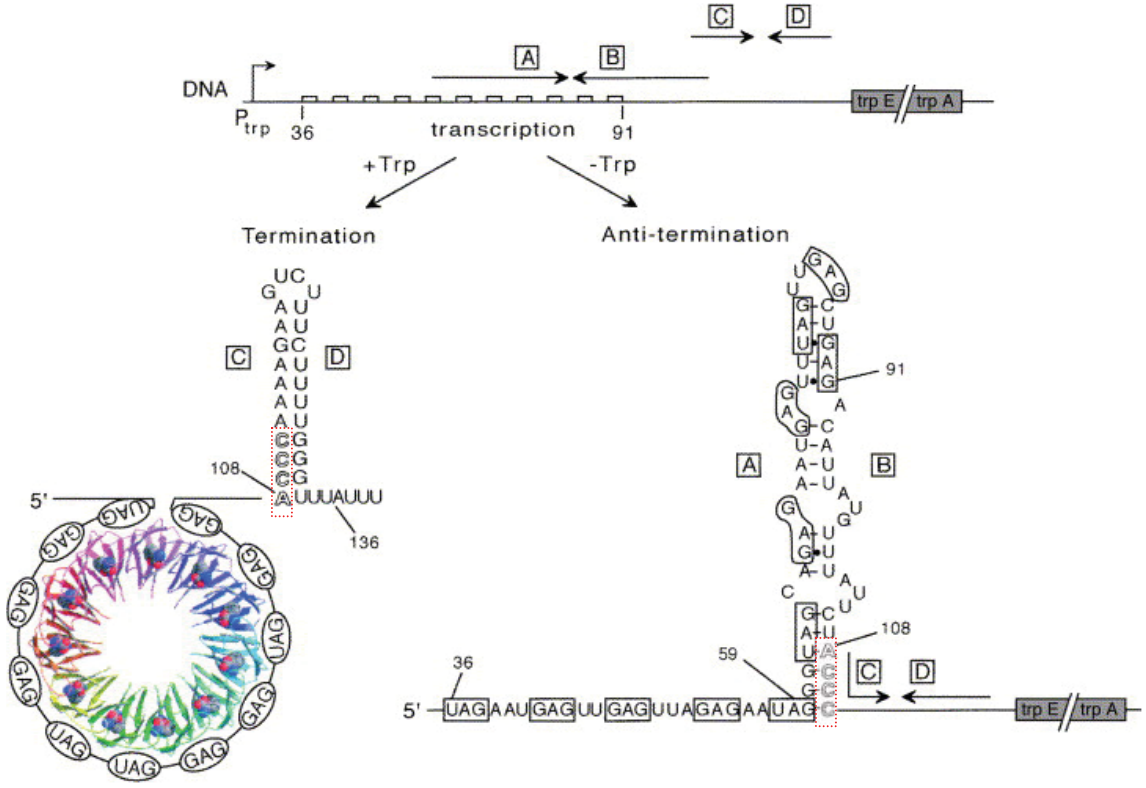


Figure 4. Model of transcription attenuation of the *B. subtilis trp* operon. (From Barbolina et al, 2005)

1.3.3. Carbohydrate catabolic operon antitermination in *E. coli* and *B. subtilis*

The *E. coli bgl* operon and *B. subtilis sac* operon represent a large group of carbohydrate operons in bacteria that are regulated by antitermination. The 5' mRNA leader regions of these operons have conserved intrinsic terminators and antiterminator hairpin structures named RAT (ribonucleic antiterminator). The two structures overlap by six nucleotides and are mutually exclusive (Figure 5). Since the terminator structure is more energetically favored, the terminator is a dominant structure that causes premature termination of initiated transcription of these carbohydrate operons.

The antiterminator hairpin is approximately 30 nucleotides in length and can be stabilized with the help of a regulatory protein antiterminator (AT). The antitermination proteins are classified as members of the BglG/SacY family of antiterminators that contain more than 50 members (65). The activities of these antiterminators are regulated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) system (15).

BglG/SacY family antiterminators feature an N-terminal antitermination domain named CAT (co-antiterminator) and two tandem PTS (phosphoenolpyruvate:sugar phosphotransferase system) regulatory domains PRD1 and PRD2 (15, 61). Each of the PRD domains contains a conserved histidine residue, which can be phosphorylated or dephosphorylated by PTS in response to substrate uptake. The phosphorylation of the PRD domains determines whether the protein exists as the inactive monomer or the active dimer (15, 33).

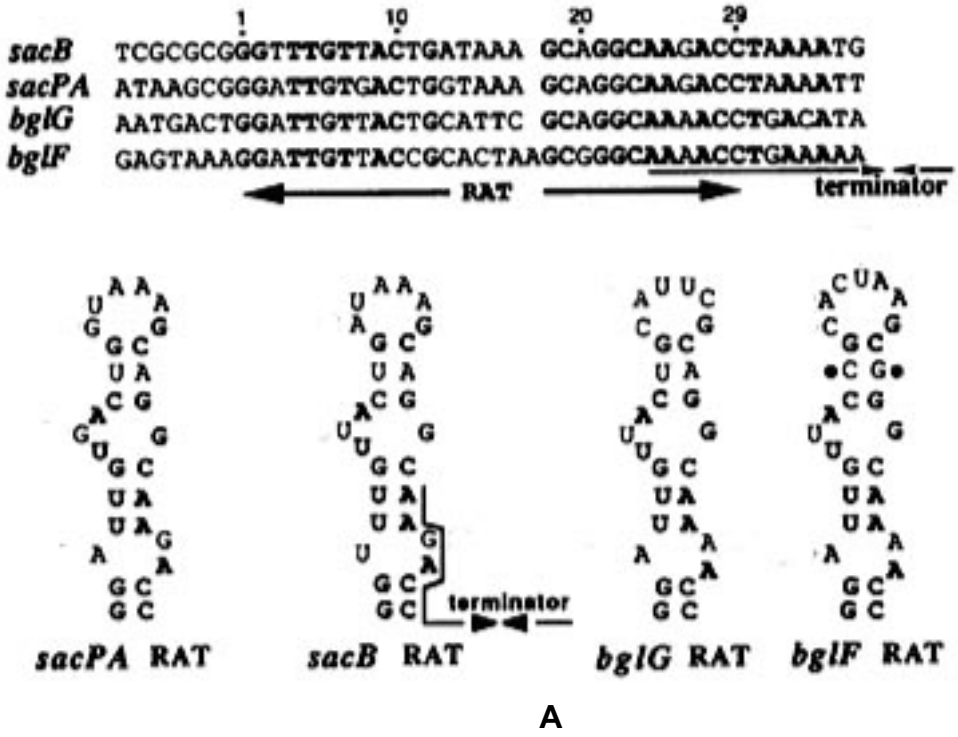


Figure 5. Sequences and secondary structures of RATs (ribonucleic antiterminator) of *sacB*, *sacPA* from *B. subtilis* and *bglG* and *bglF* from *E. coli*. (From Aymerich and Steinmetz, 1992)

Some bacteria, such as *B. subtilis*, have multiple BglG/SacY family antiterminator-mediated antitermination (5, 54). High conservation of protein regulator sequences and their target mRNA secondary structures suggest that they evolved from a common ancestral system and diverged to meet the specific requirements of various carbohydrate operons (5).

1.3.4 *hut* operon regulation in *B. subtilis*

The *hutPHUIGM* operon in *B. subtilis* is responsible for histidine catabolism. The genes *hutHUIGM* encode proteins involved in histidine importation and degradation, while the promoter proximal *hutP* gene encodes an autoregulator that controls transcription termination between *hutP* and *hutH* (Figure 6A). The intergenic region of *hutP* and *hutH* contains an intrinsic transcription terminator, which is characterized by two XAG (UAGNNNNUAGNNNNUAG) regions. One motif is located inside the terminator while the other is in the single-stranded region preceding the 5' end stem.

Functional HutP is a hexamer with three fold symmetry, composed of three homodimers (28). In the center of each dimer exists a hydrophobic pocket that can bind an L-histidine. The binding of this amino acid leads to a structural reorientation and activates the protein's regulatory function (26, 27).

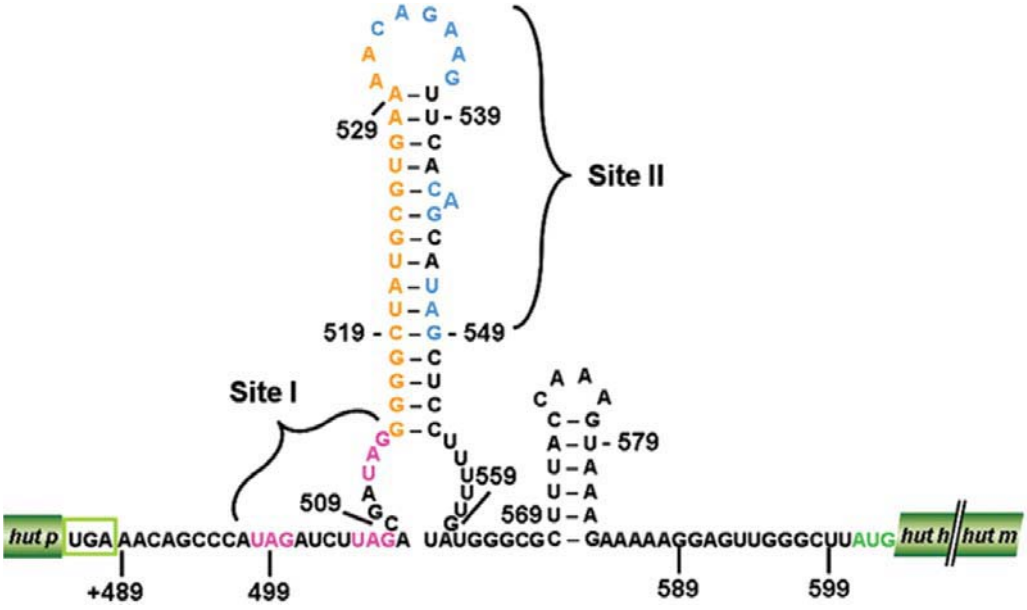
Activated HutP recognizes and binds the XAG rich region on the 5' end of terminator hairpin. It was shown that the substitution of the first XAG in the region abolished HutP-RNA interaction. After binding the first XAG, the HutP rolls along the RNA in 5' → 3' direction and wraps the RNA around itself, resulting the melting of the

hairpin, exposing the second XAG rich region (20). The second exposed XAG rich motif binds another HutP, preventing the formation of the transcription terminator hairpin ahead of a poly(U) sequence (Figure 6B) (20).

1.3.5 *nasF* operon regulation in *Klebsiella oxytoca* M5al

The assimilatory nitrate reductase operon *nasFEDCBA* in *K. oxytoca* M5al is induced by nitrate/nitrite in the absence of ammonia (12, 31). The 5' end of the *nasFEDCBA* transcript has a 119 nucleotide leader sequence containing an intrinsic terminator and an independent hairpin structure preceding the terminator (Figure 7). The initiated transcript prematurely terminates at the terminator region unless nitrate/nitrite is present in the medium.

Nitrate induced antitermination is mediated by the protein regulator NasR, which is encoded by a single gene operon located immediately upstream of *nasFEDCBA* (19). NasR is composed of two domains, the N-terminus is a nitrate/nitrite sensing domain (NIT) only found in signal transduction proteins in prokaryotes (57). The C-terminus of NasR is an RNA binding domain, which binds the independent hairpin preceding the terminator (12, 13). Site-directed mutagenesis analyses of the RNA indicated that the promoter-proximal hairpin was critical for NasR binding and



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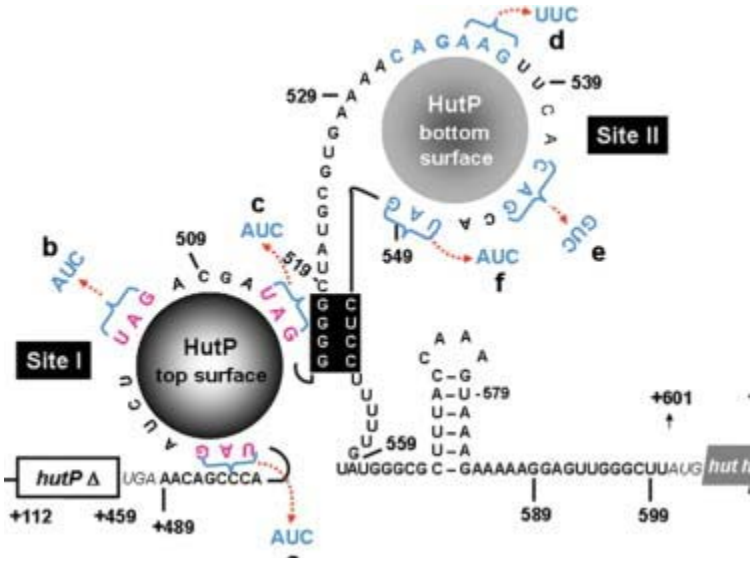


B

Figure 6. Model of transcription attenuation of the *B. subtilis hut* operon. A) Secondary structure of *hut* operon leader region. B) Interaction of HutP with target mRNA. (From Subash et al, 2008)

antitermination function (12). Although NasR can bind to the leader RNA, the activation of NasR requires the binding of nitrate/nitrite at the NIT domain (13). The exact molecular mechanism of NasR mediated antitermination is not clear, available evidence suggests that the formation of alternative hairpin structures is not involved (13).

1.3.6 AmiR-directed antitermination in *Pseudomonas aeruginosa*

P. aeruginosa PAC1 can grow on short-chain aliphatic amides by virtue of an amidase encoded by the *amiEBCRS* operon (62, 63). The protein products of *amiCR* are not involved in amide catabolism, but constitute an amide sensor-antiterminator two-component regulatory system that autoregulates *ami* operon expression.

The *ami* transcript leader region contains a 5' end hairpin and a 3'end intrinsic terminator (Figure 8) (63). Nascent mRNA elongation aborts at the terminator region when there is no amide source in the medium. Premature transcription termination is prevented by the protein regulator AmiR when amide is supplied. AmiR consists of a C-terminal RNA-binding domain and an N-terminal domain homologous to receiver domains of the response regulator of two-component regulatory systems (46). The results of titration assays suggest that AmiR binds at 35-74 residues of the *ami* leader region (Figure 8) (64). Whether AmiR-mediated antitermination involves alternative secondary structure formation is unknown.

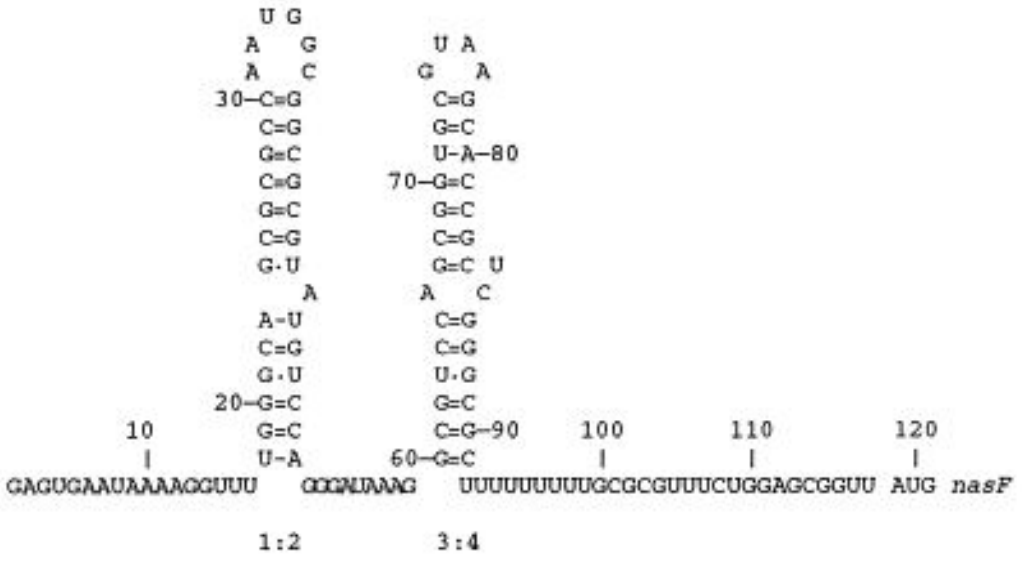
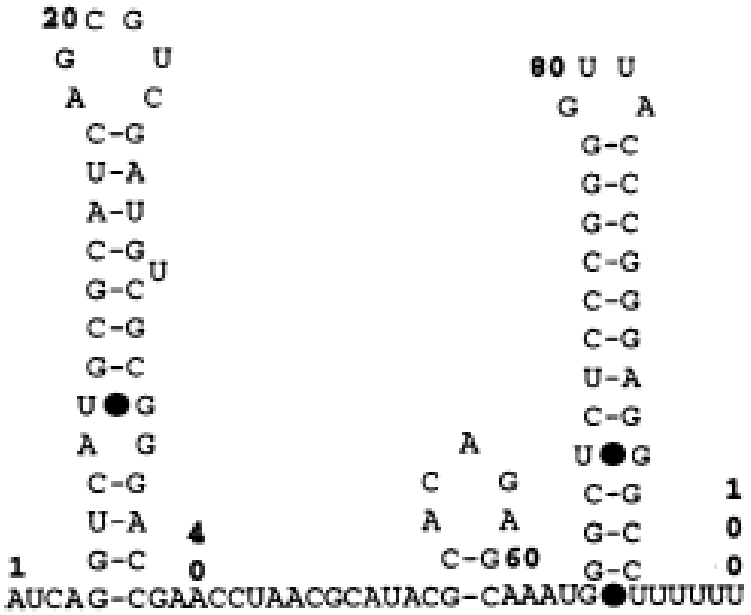


Figure 7. The secondary structure of *nasF* operon leader region in *K. oxytoca* M5al. (From Chai et al,1998)

Signal transduction between AmiC and AmiR occurs through steric hindrance (44). In the absence of amide, AmiC and AmiR form a tetrameric complex composed of an AmiC dimer and an AmiR dimer. AmiR in the complex has no regulatory function. When amide is available, AmiC binds to the amide and dissociates from the AmiC·AmiR complex. The released AmiR dimer acts within the *ami* leader region to help the elongation polymerase read through the intrinsic terminator structure (44).



A

Figure 8. The secondary structure of *ami* operon leader region in *P. aeruginosa*. (From Wilson et al, 1996)

1. 4. Global nitrogen regulation in proteobacteria

In proteobacteria, the global nitrogen regulation (Ntr) system coordinates cellular nitrogen metabolism (39). The Ntr system is composed of a P_{II} signal transduction protein (GlnB), an uridylyltransferase/UMP-removing enzyme (GlnD), and a two-component regulatory system NtrB/NtrC. NtrB is a sensor kinase protein, and NtrC is a σ^{54}-dependent transcription activator. NtrB regulates NtrC activity in response to a signal from the P_{II} protein.

P_{II} protein regulates NtrB activity through protein-protein interaction (50). P_{II} itself is regulated by GlnD through covalent modification (23). GlnD is an intracellular nitrogen sensor that senses glutamine levels. When nitrogen is depleted, cellular glutamine levels become low, and P_{II} is uridylylated by GlnD. P_{II}-UMP is not able to bind to NtrB. Under such conditions, NtrB phosphorylates NtrC and activates its regulatory function. Phosphorylated NtrC (NrC-P) activates transcription initiation of its target operons, including those related to nitrate assimilation. When nitrogen is sufficient, glutamine binds GlnD and triggers its UMP-removing activity, leading to P_{II}-UMP deuridylylation. P_{II} interacts with NtrB and promotes NtrB to dephosphorylate NtrC-P. NtrC without phosphate modification does not have activator function.

NtrC recognizes a conserved dyad symmetry sequence 5'-TGCACCA$(N)_3$TGGTGCA-3' sequence 100 to 120 nucleotides upstream of the regulated promoter (31). NtrC-mediated activation, however, needs direct interaction between NtrC and σ^{54}-RNA polymerase holoenzyme. NtrC is brought to the holoenzyme

through a DNA-loop structure (59). When in contact, NtrC converts the DNA-RNA polymerase complex from a closed state to an open state in which DNA strands at the transcription initiation region melt, allowing transcription to initiate (4).

NtrC is composed of three domains (52). The N-terminus is a signal receiver domain, which has a set of conserved amino acids involved signal transduction and phosphorylation. The central domain is an AAA+ domain involved in ATP binding, hydrolysis, and interaction with RNA polymerase. The C-terminus is a typical helix-turn-helix DNA-binding domain.

A. vinelandii harbors *glnD*; *ntrB* and *ntrC*; and *glnK*, a homolog of *glnB* (25). Our understanding of the functions of GlnK and GlnD comes from studies of nitrogen fixation regulation. GlnK is de-uridylylated by GlnD when nitrogen is sufficient, and uridylylated when nitrogen is depleted. Although the signal transduction from GlnK to NtrB/NtrC has not been studied thoroughly, it is assumed that NtrC in *A. vinelandii* is regulated in an analogous fashion to its regulation in enteric bacteria (21).

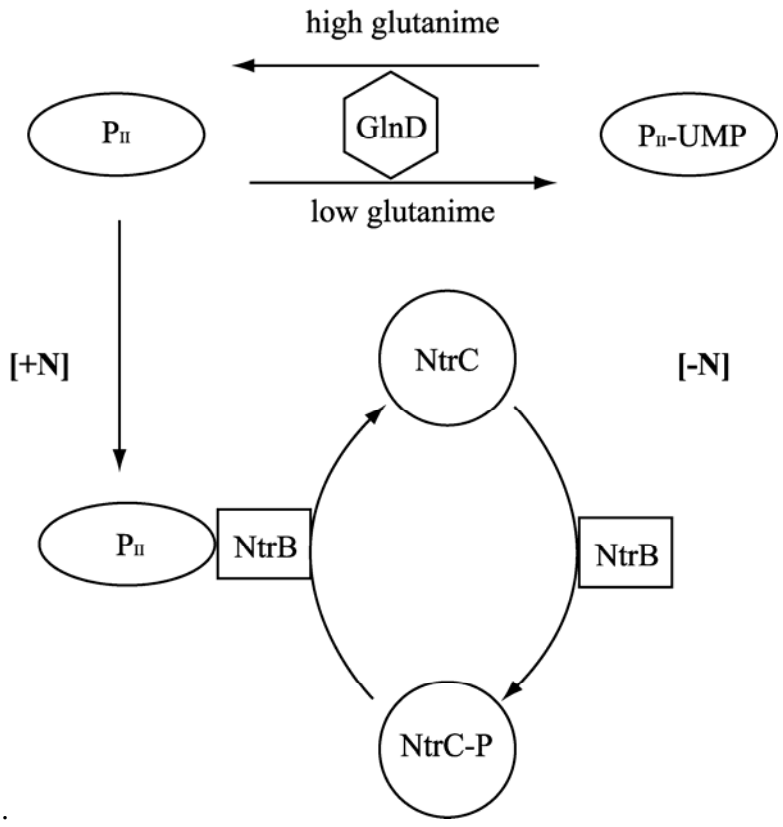


Figure 9. Ntr system in enteric bacteria. (Adapted from Masepohl and Forchhammer, 2007)

1. 5 NifL and NifA in *A. vinelandii*

The nitrogen fixation (*nif*) operons in *A. vinelandii* are regulated by a non-typical two-component regulatory system, NifL/NifA. The NifL/NifA system regulates *nif* transcription in response to cellular redox, nitrogen, and carbohydrate status (55). Both NifL and NifA have modular structures with domains sensing specific signals (Figure 10) (37).

NifL has an N-terminal PAS (period circadian protein, Ah receptor nuclear translocator protein, single-minded protein) domain with FAD as the prosthetic group that senses cellular redox status (58, 68). The NifL C-terminal domain is homologous to the autophosphorylation domain of sensor kinases of two-component regulatory system with the conserved histidine residue where phosphate is attached. In contrast with other sensor kinases, substitution mutation of the histidine residue does not affect its regulatory function. This domain can bind ADP or ATP, the affinity for ADP is 10-fold higher than for ATP, suggesting that this domain senses cellular ADP: ATP ratios (58).

NifA has a central AAA+ domain and C-terminal helix-turn-helix DNA binding domain, similar to the corresponding parts of NtrC (11, 40). The N-terminus of NifA is a GAF domain (cGMP-specific and –stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *E. coli* FhlA) which can bind 2-oxoglutarate, sensing cellular carbohydrate status (3, 55).

NifA is a σ^{54}-dependent transcription activator, and recognizes a consensus sequence 5'-TCT-N_{10}-AGA-3' located 100 to 200 nucleotides upstream of NifA-

regulated promoters (9). Like NtrC, NifA contacts σ^{54}-RNA polymerase holoenzyme through a DNA-loop (10).

NifL regulates NifA activity through protein-protein interactions (4). When bound by NifL, NifA has no activator function. In *A. vinelandii*, NifL-NifA interactions are promoted by GlnK (34) . Thus, the Ntr global nitrogen system regulates *nif* expression through the NifL/NifA system (Figure 10)

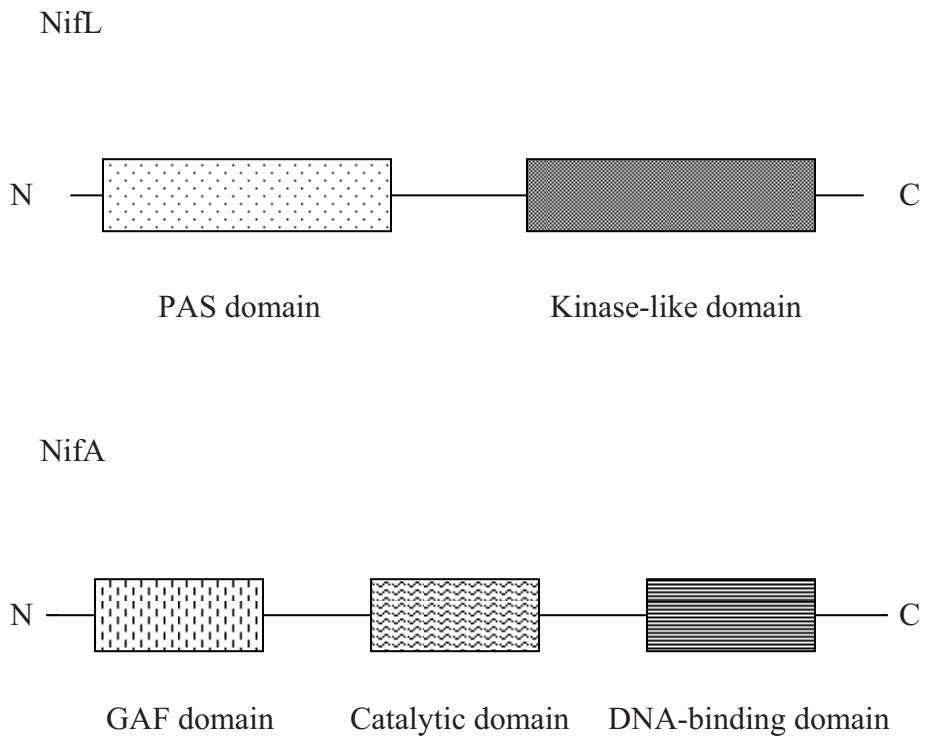


Figure 10. Modular structures of NifL and NifA. (Adapted from Dixon, 2002)

1.6 Nitrate transporters and the regulation of assimilatory nitrate reduction in bacteria

Nitrate needs to be transported into the cell as assimilatory nitrate reduction occurs in the cytoplasm. There are two types of nitrate transporters in bacteria (41). The first type is the ABC-type nitrate transporters, which import nitrate at the expense of ATP hydrolysis. This type of nitrate transporter has been identified in *Klebsiella* (32), and the cyanobacteria *Synechococcus* (47), *Synechocystis* (24), and *Anabaena* (17, 18). The second type of nitrate transporters is permeases belonging to the major facilitator superfamily (MFS). These MFS nitrate permeases rely on transmembrane proton motive force (PMF) for their activity. One example of this type transporter is the *Bacillus subtilis* nitrate transporter (45).

Genes related to nitrate transport, reduction, and their regulations are clustered on the bacterial genome (Figure 11) (42). Expression of assimilatory nitrate reductases are subject to global nitrogen regulation (NH_3 repression) and operon-specific induction (nitrate/nitrite induction) (30). For each operon, the regulatory proteins and molecular mechanisms involved in its regulation may be unique.

In the enteric bacterium *K. oxytoca* 51, the assimilatory nitrate transporter genes, nitrate reductase, and nitrite reductase genes are encoded by the operon *nasFEDCBA* (32). The *nasFED* genes encode an ABC-type nitrate transporter, while *nasC* and *nasA* encode nitrate reductase, and *nasB* encodes nitrate reductase.

The *nasFEDCBA* operon is subject to two levels of control. The general nitrogen regulation system regulates operon transcription initiation through NtrC. NtrC regulates operon transcription in response to intracellular nitrogen status. In the presence of ammonia, the promoter of *nasFEDCBA* is inhibited due to the inactivation of NtrC; in the absence of ammonia, NtrC is phosphorylated by NtrB and activates transcription initiation of the operon. Expression of *nasFEDCBA* requires the presence of nitrate or nitrite in the medium. Nitrate/nitrite induction on *nasFEDCBA* is mediated by an antiterminator protein NasR which acts positively within the operon leader region (The details of nitrate/nitrite induction are discussed at section 1.3.5).

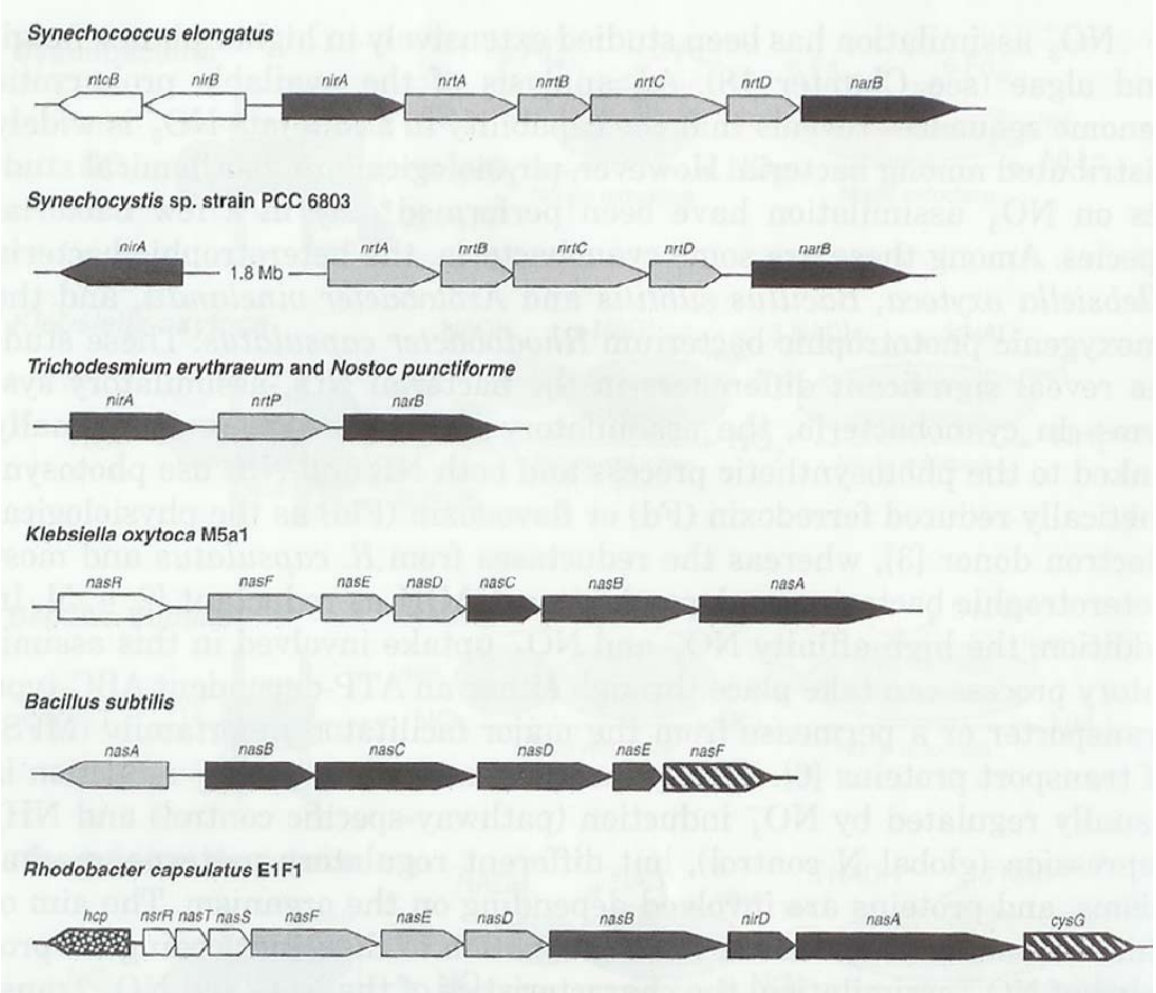


Figure 11. Organization of the operon related NO_3^- assimilation in bacteria. Genes with similar function are marked with same colors and patterns. Light gray, nitrate transporter; black, nitrate reductase; dark gray, nitrite reductase; white, regulatory gene; strip, siroheme cofactor biosynthesis. (From Moreno-Vivan et al, 2007)

1.7 Regulation of the *A. vinelandii* Assimilatory Nitrate Reductase Operon *nasAB*

In addition to atmospheric nitrogen (N_2), *A. vinelandii* can use ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-) as nitrogen sources. Nitrate is converted into nitrite, and then into ammonia before being incorporated into glutamate by glutamine synthetase (GS). Assimilatory nitrate reduction is the only nitrate reduction pathway in *A. vinelandii*. The assimilatory nitrate reductase and nitrite reductase are encoded by the *nasB* and *nasA* genes of the *nasAB* operon, respectively (51). Expression of *nasAB* is affected by multiple factors. Ammonia was shown to repress *nasAB* expression (35). It is assumed that ammonia inhibition of *nasAB* expression was a type of global nitrogen regulation, mediated by NtrC. This assumption is based on the observation that mutation of either *ntrC* or the alternate sigma factor gene *ntrA* (σ^{54}) abolished *nasAB* expression (51, 53, 60), suggesting that *nasAB* may be driven by an NtrC-dependent promoter.

Nitrate or nitrite induces *nasAB* expression in the absence of ammonia (35). Nitrate and nitrite induction on *nasAB* expression also involve the products of the *nasST* operon (22). The *nasST* operon is located 12,130 bp upstream of *nasAB*. The deduced protein NasS shares sequence homology with nitrate transporters in *Klebsiella* spp. and *Synechococcus* spp. (22). However, deletion of *nasS* had no effect on nitrate uptake by *A. vinelandii*. Mutation of *nasS* without affecting the expression of downstream *nasT* led to constitutive expression of the *nasAB* operon independent of nitrogen source, suggesting that NasS negatively affected *nasAB* expression.

NasT is a 192-amino-acid polypeptide consisting of two domains (22, 56). The C-terminal domain of NasT is highly homologous to an RNA-binding domain, first identified in the C-termini of AmiR and NasR, antitermination regulators present in *Pseudomonas aeruginosa* and *Klebsiella* spp. respectively (56). The NasT N-terminal domain is homologous to the receiver domain of the response regulator of classic two-component regulatory systems (22, 43). Mutation of *nasT* abolished *nasAB* expression even in the presence of nitrate and nitrite, indicating that a functional NasT was required for *nasAB* expression (22). Possible regulatory roles for NasT in *nasAB* expression and the role of the N-terminal domain in NasT activation have been hypothesized. However, these hypotheses have not been examined in detail and the regulation of *nasAB* is unclear (30, 43, 49). One of the goals of this research was to shed further insight into this regulation.

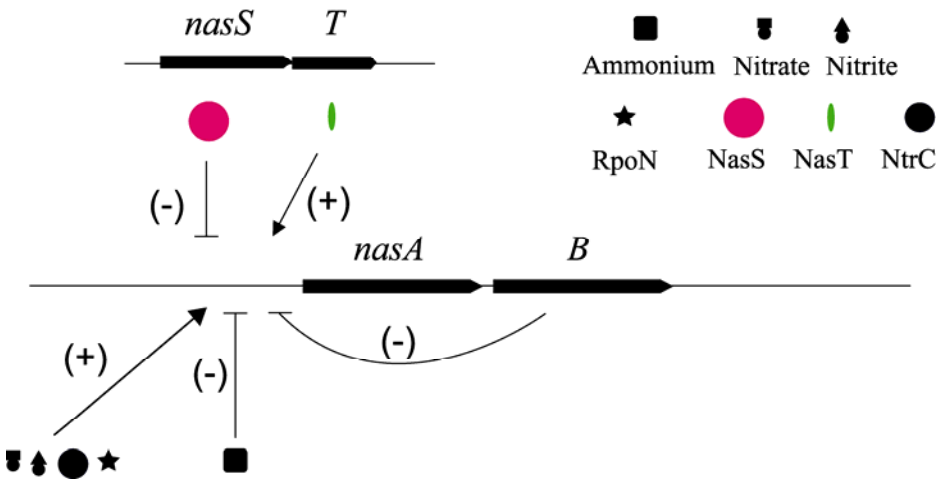


Figure 12. Features known or hypothesized to be involved in the regulation of *nasAB* operon in *A. vinelandii*.

```
                   *        20         *        40         *        60
AmiR : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :   -
nasT : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :   -
nasR : MNNMAGNTPEVVDWFARARRLQKQQLHQLAQQGTLAGQISALVHMLQCERGASNIWLCSG :  60

                   *        80         *       100         *       120
AmiR : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :   -
nasT : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :   -
nasR : GRLYAAECRAGAALVDEQLTRFYAALEPARDAASSALCWRIACAVWYLPQLAALRKRVRD : 120

                   *       140         *       160         *       180
AmiR : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :   -
nasT : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :   -
nasR : REIAAEEATGQFSRIIRHLLNIVPQLNDSIDDPQIAGRMVALYSFMQGKELAGQERALGA : 180

                   *       200         *       220         *       240
AmiR : ~~~~~~~~~~~~~~~~~MSANSLLGSLRELQVLVLNPPGEVSDALVLQLIRIGCSVRQCW :  43
nasT : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~MLRILLINDTPKKVGRLKSALVEAGFEVVD-- :  30
nasR : LGFARGQFSDELRQQLVDRIDGQQPCFDSFQALA--QPPQT--ALFAEQCQASLEIEQLR : 236

                   *       260         *       280         *       300
AmiR : PPPESFDVPV---------DVVFTSI-FQNRHHDEIAALLAAGTPRTTLVALVEYESPAV :  93
nasT : ESGLTIDLPV--RVEAVRPDVILIDTESPGRDVMEQVVLVSRDRPR-PIVMFTDEHDPQV :  87
nasR : RVACTRQPPADEGETALRWFCAQTQRLEQLRGVEELLIVDLLNAAD----ALLEGEEPEA : 292

                   *       320         *       340         *       360
AmiR : LSQIIELECHGVITQPLDAHRVLPVL-VSARRISEEMAKLKQKTEQLQERIAGQARINQA : 152
nasT : MRQAIQAGVSAYIVEGIQAQRLQPILDVAMARFESDQA-LRAQLQAREAQLAERKRVELA : 146
nasR : QLPPADWQEDSIALR-LDKQ-LLPLV----RQQAHELQQLSGQLASLKDALEERKLIEKA : 346

                   *       380         *       400         *
AmiR : KALLMQRHGWDEREAHQYLSREAMKRREPILKIAQELLGNEPSA~~~~~~ : 196
nasT : KGLLMKMKNCSEEEAYTLMRRQAMSRQQKLIQVAEQVIAMHDMLGS*~~~ : 192
nasR : KSVLMTYQGMQEEQAWQALRKMAMDKNQRMVEIARALLTVKALWRVTPKE : 396
```

Figure 13. Alignment of NasT, AmiR, and NasR

1.8 Dissertation Format

All of the research pertaining to this dissertation is presented in two manuscripts submitted for publication. The goal of the research was to elucidate the molecular mechanisms controlling the *A. vinelandii* assimilatory nitrate reductase operon *nasAB*.

1.8.1 Antitermination Regulation of the *Azotobacter vinelandii* Assimilatory Nitrate Reductase Operon *nasAB* (Appendix A)

This first manuscript focuses on the identification of the *nasAB* promoter and testing whether antitermination is involved in *nasAB* regulation. The results of bioinformatic analysis suggest a putative σ^{54} recognition site and two NtrC binding sites are separated from the structural gene *nasA* by a potential intrinsic terminator structure. To dissect the roles of these sequences in *nasAB* regulation, I constructed a transcriptional *lacZ* fusion vector and a translational *lacZ* fusion vector. The translational probe vector was used to test the effects of sequence mutations on *nasAB* expression; the transcriptional probe vector was used to test *nasAB* promoter activity altered in aspects of nitrogen regulation.

1.8.2 NasS/NasT, a non-canonical two-component regulatory system regulates the assimilatory nitrate reductase operon (*nasAB*) in *Azotobacter vinelandii* via antitermination (Appendix B)

The second manuscript focuses on the regulatory role of NasT and signal transduction between NasT and its cognate partner NasS. NasT is homologous to AmiR, an experimentally confirmed RNA-binding antiterminator. I constructed a bacterial two-vector system to test whether NasT regulates within the *nasAB* leader region, and used a yeast two-hybrid system to test whether NasT forms a dimer, analogous to AmiR. At the same time, I analyzed the effect of NasS: NasT ratio alterations on *nasAB* expression.

2. PRESENT STUDY

The methods, results, and conclusions of this dissertation are presented in the attached appendices. The following sections summarize the important findings of each appendix.

2.1 Antitermination Regulation of the *Azotobacter vinelandii* Assimilatory Nitrate Reductase Operon *nasAB* (Appendix A).

1. A potential σ^{54} recognition site, two potential NtrC recognition sites, and a putative Rho-independent terminator structure were identified upstream of the *nasA* gene.

2. Secondary structure analysis of the *nasAB* leader sequence suggests an intrinsic transcriptional terminator structure upstream of *nasA*.

3. The potential σ^{54} recognition site is essential for *nasAB* expression.

4. Both NtrC recognition sites are essential for *nasAB* expression.

5. The putative terminator structure upstream of *nasA* plays a negative role in *nasAB* expression.

6. The promoter proximal hairpin upstream of the terminator plays a positive role in *nasAB* expression.

7. *nasAB* regulation is subject to global nitrogen regulation.

8. Mutation of *nifA* affects *nasAB* transcription.

2.2 NasS/NasT, a non-canonical two-component regulatory system regulates nitrate reductase operon (*nasAB*) in *Azotobacter vinelandii* via antitermination

1. Mutation of *nasST* in *A. vinelandii* is not lethal.

2. NasT acts within the *nasAB* leader region as an antiterminator.

3. NasT exists as a dimer or oligomer.

4**.** Proper stoichiometry of NasS and NasT is essential for *nasAB* regulation.

5. *nasS* and *nasT* are translationally coupled.

6. The assimilatory nitrate reductase operons in *A. vinelandii* and *K. oxytoca* (*pneumomiae*) M5al use different signal circuits to regulate antitermination.

2.3 Concluding Remarks and Future Directions

A. vinelandii has served as a paradigm for the genetic study of nitrogen fixation in free-living bacteria for more than thirty years. The molecular mechanism that *A. vinelandii* uses to sense the intracellular nitrogen status has been characterized; the signal cascades that the bacterium uses to regulate *nif* gene expression in response to nitrogen status change have been elucidated. Despite these achievements, N-regulation other than the nitrogen fixation pathway is largely unknown.

Results from this work confirmed that regulation of the assimilatory nitrate reductase operon *nasAB* in *A. vinelandii* is subject to both general nitrogen regulation and nitrate/nitrite induction (Figure 14). The global nitrogen regulatory system regulates

nasAB transcription via NtrC, a σ^{54}-dependent activator, in response to the availability of ammonia in the medium. This regulation was observed in the Nif$^-$ strain UW1. In the Nif$^+$ strain UW136, the activity of the *nasAB* promoter, however, is inhibited even in the absence of ammonia, suggesting that the activity of NtrC is modulated in UW136 or NtrC may not be the only regulator acting at the *nasAB* promoter region.

Strain UW1 differs from wild type strain UW136 in that it harbors a mutation in the *nifA* structural gene, indicating that NifA may be involved in *nasAB* repression. However, the conserved NifA recognition sequence (TGT-N_{10}-ACA) was not identified within the *nasAB* promoter region. One question still to be addressed is whether NifA acts directly within the *nasAB* promoter region. An approach to test this could use an *E. coli* (*lacZ*$^-$) two-vector system: one vector contains an inducible *nifA* gene, and the other vector has the *nasAB* promoter–*lacZ* transcriptional fusion.

Further elucidation of the role of NtrC in *A. vinelandii* nitrogen regulation would clarify the role of the general nitrogen regulatory system in nitrate assimilation. NtrC is known to play an essential role in nitrogen regulation in enteric bacteria. Although NtrC is not involved in nitrogen fixation regulation, it is assumed that *A. vinelandii* NtrC plays an essential role in nitrogen metabolisms other than nitrogen fixation. Since atmospheric nitrogen represents a more accessible and sustainable nitrogen source than other nitrogen sources for *A. vinelandii*, the activity of NtrC may be modulated in response to the status of nitrogen fixation. The phosphorylation/dephosphorylation of NtrC under different cellular metabolism conditions can be identified using two-dimensional gel

electrophoresis. The results of works may shine light on the still unknown signal transduction cascades in *A. vinelandii*.

Nitrate/nitrite regulation involves antitermination within the *nasAB* leader region. The leader terminator structure regulates initiated transcripts negatively in the absence of nitrate/nitrite. Results from my research (Appendix B) confirmed that NasT is required to overcome the negative role of the leader terminator. My results and the modular structure of NasT suggest that it may act as an RNA-binding antiterminator. However, the interaction of NasT with the *nasAB* leader mRNA needs to be demonstrated directly using RNA electrophoretic mobility shift assays.

The results of secondary structure analysis suggest that antitermination may not involve the formation of alternative secondary structures at the *nasAB* leader region (Appendix A). One possibility is that NasT interacts with hairpins I and II within the leader region and modulates the activity of RNA polymerase to overcome the negative role of the terminator hairpin structure. A strategy to test this hypothesis is to analyze whether hairpins I and II cause RNA polymerase to read through heterologous terminator structures fused on the same sequence.

NasS appears to regulate the activity of NasT in response to the availability of nitrate or nitrite. The structures of the two proteins suggest that signal transduction between them is not via phosphorylation. The requirement of conserved stoichiometry of the two proteins for *nasAB* regulation supports a model that NasS regulates NasT activity through protein-protein interactions. However, a direct demonstration of this interaction

is still needed. Co-immunoprecipitation assays of the two proteins or a yeast two-hybrid system approach would provide direct evidence of this interaction.

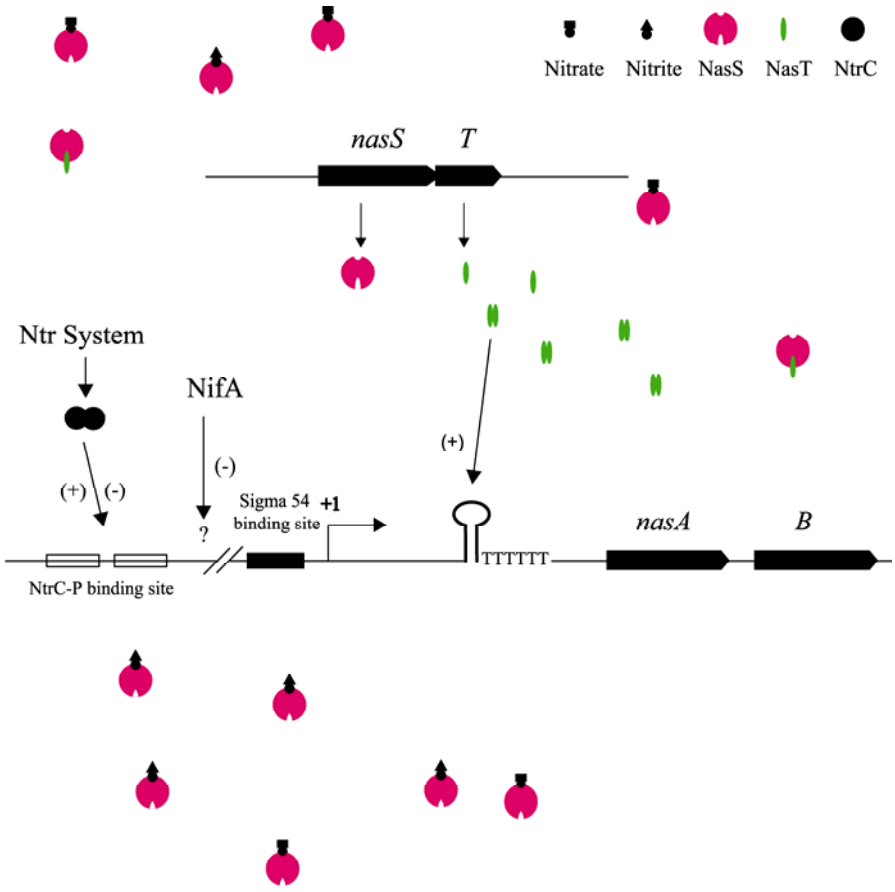


Figure 14. Proposed model for *nasAB* regulation in *A. vinelandii*.

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APPENDIX A. ANTITERMINATION REGULATION OF THE *AZOTOBACTER VINELANDII* ASSIMILATORY NITRATE REDUCTASE OPERON *NASAB*

Antitermination regulation of the *Azotobacter vinelandii* assimilatory nitrate reductase operon *nasAB*

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Dedication (in memoriam): This manuscript is dedicated to the memory of Christina Kennedy.

ABSTRACT

Azotobacter vinelandii can use nitrate or nitrite as its sole nitrogen source. Nitrate is reduced into nitrite by nitrate reductase and subsequently into ammonia by nitrite reductase. The assimilatory nitrate and nitrite reductases are encoded by the *nasAB* operon. Previous genetic studies showed that *nasAB* expression is subject to multiple regulation factors. However, the molecular details of this regulation are largely unknown. Several sequences identified upstream of the NasA open reading frame may play roles in *nasAB* operon expression. These sequences include a potential σ^{54} binding site, two putative NtrC binding sites, and several potential hairpin structures, one of which is located adjacent to a poly (U) region which resembles an intrinsic mRNA terminator. To further determine the role of these sequences, two *lacZ* fusion probe vectors for *A. vinelandii* were developed. The vectors can be stably maintained in *A. vinelandii* by integration into the *vnf* locus. Site-directed and deletion mutational analyses confirmed that *nasAB* contains a σ^{54}-dependent promoter that requires NtrC for activation. The *nasAB* promoter is repressed to basal levels in wild-type *A. vinelandii* regardless of the presence of ammonia. In addition, analyses of a series of deletion mutations in the *nasAB* leader region confirm that the putative transcription terminator plays a negative role in operon expression. A model for *nasAB* regulation is proposed based on the data presented here and the current understanding of NasS/NasT two component regulation.

INTRODUCTION

Azotobacter vinelandii is a gram-negative, aerobic diazotroph with multiple nitrogen fixation systems. *A. vinelandii* harbors three genetically distinct but related nitrogenase enzymes that utilize different metal cofactors – molybdenum, vanadium, or iron (19). . Multiple nitrogenases provide *A. vinelandii* with the advantage of using atmospheric nitrogen as a nitrogen source under different environmental conditions.

In the absence of atmospheric nitrogen, *A. vinelandii* can use nitrate or nitrite as sole nitrogen sources. Nitrate is reduced sequentially into nitrite and ammonium, which is incorporated into glutamate by glutamine synthetase (41). The enzymes responsible for *A. vinelandii* assimilatory nitrate reduction are encoded by the *nasAB* operon (31). The *nasB* gene encodes a nitrate reductase and *nasA* encodes a nitrite reductase.

Expression of *nasAB* requires multiple factors. Transcription of *nasAB* requires functional *ntrA* and *ntrC* genes. Inactivation of either *ntrA* or *ntrC* abolished *nasAB* expression (24, 31, 34, 40). The *ntrA* gene encodes the alternate sigma factor σ^{54} that recognizes promoter regions characterized by conserved GG/GC dinucleotide pairs located at positions -24/-12 (2). NtrC is a σ^{54}-dependent transcriptional activator and binds to a consensus sequence -100 to -120 nucleotides (nt) upstream of the promoter (33). The regulatory role of NtrC in enteric bacteria has been well studied. NtrC tightly regulates the transcription of nitrogen operons in response to intracellular nitrogen levels

(29). Although not studied directly, it was assumed that NtrC in *A. vinelandii* plays a similar regulatory role as in enteric bacteria (15).

The *nasAB* operon is induced by the presence of nitrate or nitrite (24). The molecular mechanisms of nitrate and nitrite induction in *A. vinelandii* are not clear, and are further complicated by the finding that nitrate and nitrite induction require the products of the *nasST* operon, located 12-kb upstream of *nasAB* (16). NasS is homologous to the substrate-binding component of nitrate ABC-type transporters. The results of mutational analyses, however, suggest that NasS is not required for nitrate import, but instead plays a negative role in *nasAB* expression. NasT is a two-domain positive regulator and is homologous to the RNA-binding antiterminator AmiR of *Pseudomonas aeruginosa* (16, 36).

Inspection of the *A. vinelandii* genome sequence identified a putative σ^{54} recognition sequence, two putative NtrC binding sites, and several potential hairpin structures, one of which is located adjacent to a poly(T) sequence preceding the *nasA* structural gene. In the mRNA, the hairpin and poly(U) structure resembles a Rho-independent transcription terminator, suggesting that attenuation may be involved in *nasAB* operon regulation. In this study, we constructed *lacZ* translational and *lacZ* transcriptional fusion probe plasmids to test the regulatory role of the sequences upstream of *nasA*. The results of mutation analysis confirmed that *nasAB* has an NtrC-dependent promoter that is subject to nitrogen regulation. Activation of the *nasAB* promoter is repressed in wild-type *A.*

vinelandii regardless of the nitrogen source. The expression profile of *lacZ* fusions with mutations in the *nasAB* leader region suggests that the putative transcription terminator plays a negative role in *nasAB* regulation. In addition, the results of deletion assays show that a promoter-proximal hairpin is required for induction of *nasAB* by nitrate/nitrite. Based on available data, a model for *nasAB* regulation is proposed.

MATERIALS AND METHODS

Bioinformatics. DNA secondary structure analysis was performed using Mfold (46); the putative σ^{54} binding site and NtrC binding site were detected by PromScan (http://www.promscan.uklinux.net). The sequence of the *A. vinelandii* genome is available at the Department of Energy Joint Genome Institute (JGI) website (http://genome.jgi-psf.org/draft_microbes/azovi/azovi.home.html).

Strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *A. vinelandii* UW136 and its derivatives were grown at 30°C in modified Burk's nitrogen-free salts (BS) medium supplemented with 1% sucrose (39). When needed, BS was supplemented with the following fixed nitrogen sources: ammonium acetate, 15 mM; urea, 10 mM; $NaNO_2$, 5 mM; KNO_3 10 mM; or $NaNO_3$, 10 mM. *E. coli* DH5α was grown on Luria-Bertani agar medium or in LB liquid at 37°C. Media were supplemented with antibiotics where appropriate: for *A. vinelandii*, carbenicillin (20 μg/ml) and gentamicin (0.05 μg/ml); for *E. coli*, carbenicillin (50 μg/ml) and gentamicin (15 μg/ml).

Oligonucleotides. Oligonucleotides used in this study were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 2).

DNA manipulation. Plasmid isolations were carried out using the GeneJET Plasmid miniprep kit (Fermentas, Glen Burnie, MD). Restriction enzyme digestions, ligations, cloning, and DNA electrophoresis were conducted following standard protocols (25). DNA fragments were purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA)**.**

Transformations. For general cloning, DNAs were transformed into chemically competent *E. coli* DH5α (25). *A. vinelandii* transformations were performed on competence medium as described previously (1).

Cloning of sequences around the promoter of nitrate reductase gene. *A. vinelandii* UW136 genomic DNA was isolated and purified as described previously (32). Purified DNA was digested with *Xho*I and separated by agarose gel electrophoresis. DNA fragments of approximately 7.8-kb were excised from the gel, purified, and integrated into the *Xho*I site of pBluescript II KS(+). After transformation into *E. coli* DH5α, positive clones were screened by PCR using primers WB30screenf and WB30screenr. The plasmid pWB30 from one positive colony was sequenced using primers M13f and M13R to confirm the cloned sequence.

Construction of a *ntrC* deletion mutant. A 2.4-kb region of the *A. vinelandii* chromosome carrying *ntrC* was amplified using primers 103NtrCf1 and 106NtrCr1 and cloned into the pGEM-T vector (Promega, Madison, WI). The resulting plasmid was linearized with *Sac*I, blunt-ended using T4 DNA polymerase, and self-ligated to yield pWB680. Using pWB680, PCR was performed using primer pair 107NtrCSacIf and 108NtrCSacIr. The PCR product was digested with *Sac*I and ligated with the *Sac*I-Gm-*Sac*I cassette from pTn*Mod*-OGm, giving rise to pWB685. Sequence analysis (LMSE sequencing facility, University of Arizona) confirmed that the Gm^r cassette was correctly inserted in the middle region of *ntrC* in pWB685.

The plasmid pWB685 was transformed into *A. vinelandii*, and a carbenicillin sensitive and gentamicin resistance (15 μg/ml) transformant was selected on BSN medium. The *ΔntrC*::Gm allelic replacement mutation in the transformant was confirmed by colony PCR using the primer pair 103NtrCf1and 106NtrCr1.

Construction of *lacZ* fusion vectors. A transcriptional and a translational *lacZ* fusion vector for *A. vinelandii* were constructed. The two plasmids differ mainly in the multiple cloning sites (MCSs) preceding the *lacZ* start codon (Figure 2).

The β-lactamase (*bla*) gene and the pMB1 replicon were amplified from the plasmid pBluescript II KS(+) using the primer pair 73pblursalIhindIIIrf and 74bpluesacIr. The amplicon was gel purified and self-ligated, giving rise to the plasmid pWhite.

A 2.8-kb *vnf* sequence was amplified from the plasmid pJW1 using primer pair vnff and vnfr. The amplicon was digested with *Sac*I and *Xho*I and cloned into the *Sac*I and *Sal*I sites of pWhite, giving rise to the plasmid pWvnf.

A fragment containing four tandem copies of the *E. coli* terminator *rrnB1* was amplified from pPROBE-NT using primer 54T4f and 55T4r and cloned into the *Sph*I site of pIC20H using blunt end ligation. The resulting plasmid was designated pICT4.

Plasmid pSUP102::Tn5-B21 was digested with *Bam*HI and *Xho*I and the 3.0 kb *Bam*HI-*lacZ-Xho*I was cloned into the *Bam*HI-*Xho*I sites of pBluescript II KS(+), yielding the plasmid pBlue-lacZ. The *XbaI-lacZ-Xho*I fragment was then removed from pBlue-lacZ and cloned into the *XbaI-Xho*I sites of pICT4, resulting in pIC-lacZ. pIC-lacZ was digested by *Hin*dIII and a ~4-kb *Hind*III-*rrnB1-lac*Z-*Hin*dIII fragment was cloned into the *Hin*dIII site of pWvnf, leading to the probe plasmid pVnflacZa.

Plasmid pKT2lacZ was digested with *Bam*HI and *Sal*I and the *Bam*HI-*lacZ-Sal*I fragment was cloned into the *Bam*HI-*Xho*I site of pVnflacZa, leading to the probe plasmid pVnflacZb,

The insertion of a foreign sequence in both probe plasmids can be verified by PCR using primers 65Indenf and lacZr. 65Indenf recognizes the sequence immediately downstream

of the *rrnB1* and upstream of the *Xba*I site, while lacZr recognizes the sequence close to the 70th bp in the *lac*Z open reading frame (ORF) and reads towards the *lacZ* start codon.

Construction of site-directed and deletion mutations within the DNA sequences. Site specific substitution and deletion mutations were constructed using the overlap extension method based on two rounds of PCR (11). During the first round, two fragments were amplified from the wild-type sequence using two pairs of primers: one of each primer pair contained the desired mutation. The two mutagenizing primers were complementary. In the second round of PCR, the two products of the first reaction were used as templates. The amplification was performed with two outsider primers. The resulting PCR product is the merger of two fragments from the first round of PCR containing the desired mutation.

β-galactosidase assay. A 10 ml overnight culture of *A. vinel*andii was concentrated by centrifugation. The pellet was rinsed twice with 5 ml BS solution, diluted in BS solution and distributed into 10 ml fresh medium for growth and β-galactosidase activity was assayed as described previously and reported as Miller Units (42).

RESULTS

The *nasAB* promoter region. NtrC and NtrA (σ^{54}) are required for *nasAB* expression in *A. vinelandii* (24, 35, 40), suggesting that the *nasAB* operon may be driven by a σ^{54}-dependent promoter that requires NtrC for activation. σ^{54}-dependent promoters are unique in containing a consensus GG/GC pattern, which is located 24/12 base pairs upstream of

the transcript initiation site (28). Sequence inspection identified a GG/GC pattern (5'-CTGGCACAGCCCCTGCA-3') 146 nucleotides upstream from the *nasA* start codon. In addition, there are two regions of dyad symmetry homologous to the consensus NtrC binding sequence 5'-TGCACCNNNNNTGGTGCA-3' (12, 22) located approximately 80 nucleotides upstream of the putative σ^{54} binding site (Figure 1A). The arrangements of the putative NtrC and σ^{54} binding sites resemble a typical NtrC-regulated promoter (44). Attempts to identify the transcription initiation nucleotide using primer extension were unsuccessful. We tentatively designated the G, 12 nucleotides downstream of the GC, as +1 (Figure 1A).

The putative mRNA sequence between the putative σ^{54} recognition sequence and the *nasA* start codon was subject to secondary structure analysis. Three potential hairpin structures were identified using Mfold. The promoter proximal hairpin (I) is located within the +16 to +41 region, with a 10 base-pair stem and a hexanucleotide AACGTG loop (ΔG=-13.5 kcal/mol). The second hairpin (II) is separated from hairpin I by a single T, comprising a 6 base-pair stem and a hexanucleotide ACAGAA loop (ΔG=-8.5 kcal/mol). The third hairpin (III) has a high GC content and contains a conserved GNRA (N is any nucleotide; R is a purine) tetraloop (ΔG=-28 kcal/mol) (18). This hairpin is separated from the hairpin II by six nucleotides and followed by a poly(T) sequence (Figure 1B). The GC-rich hairpin in combination with the poly(U) sequence in the mRNA resembles an intrinsic transcription terminator structure. In addition to the hairpin structures, a small potential ORF, which might encode an eight amino acid polypeptide,

was identified. The small ORF starts at the last base of the middle stem and ends within the third stemloop (Figure 1B).

***A. vinelandii nasAB* expression probes**. To elucidate functions of the sequences upstream of the *nasA* ORF in operon expression, two *lacZ* fusion plasmids were constructed. These two plasmids have similar vector backbones. They contain a pMB1 replicon for propagation in *E. coli*, a *bla* gene encoding β-lactamase for ampicillin or carbenicillin resistance, a tandem terminator *rrnB1* sequence $T1_{(4)}$ upstream of the MCSs to eliminate external transcription effects.

Both plasmids cannot replicate in *A. vinelandii*, but can be integrated into the *vnf* region of the genome through a single-crossover event (Figure 2). The *vnf* locus is not essential for bacterial viability and its expression is inhibited in the presence of molybdenum (5). Both plasmids are highly stable after integration into the *A. vinelandii* genome and are maintained without selection for more than 30 generations (data not shown).

The two probe plasmids differ mainly in the MCS and translation initiation region of *lacZ*. The plasmid pVnflacZa, a translational fusion probe, has the *lacZ* gene beginning within the *Bam*HI of the MCS. The tested DNA sequences with a Shine-Dalgarno (SD) sequence can be cloned into the MCS to drive *lacZ* expression. The plasmid pVnflacZb, a transcriptional fusion probe, has a SD sequence eight nucleotides upstream of the *lacZ* start codon. Promoter sequences can be cloned into the MCS for *lacZ* expression assays.

To test whether the tandem terminator *rrnB1* sequence $T1_{(4)}$ upstream of the MCS can block expression from external promoter, a DNA sequence containing the *nasA* 5' end 162 nucleotides and its preceding SD sequence was cloned into the *Xba*I-*Bam*HI region of pVnflacZa, giving rise to the plasmid pWB674. Transformant AVW674 with pWB674 integrated into the *A. vinelandii vnf* region gave insignificant levels of β-galactosidase activity seven hours after growth in BS media supplemented with ammonium, dinitrogen, nitrite, or nitrate (Table 3), suggesting that $T1_{(4)}$ in probe vectors efficiently insulated the downstream *lacZ* sequence from external transcription.

Time course assay of Φ(*nasA-lacZ*) fusion in *A. vinelandii*. A DNA sequence covering the -316 to +297 region of *nasAB* operon was fused to *lacZ* in pVnflacZa, giving rise to the plasmid pWB552. The transformant AVWB552 with an integrated copy of pWB552 showed increasing β-galactosidase activity as a function of time when supplemented with nitrate or nitrite and reached a plateau at six hr. When the nitrogen source was replaced with ammonia, the β-galactosidase activity showed a slight increase during the first hour but declined afterwards (Figure 3). These results are consistent with previous observations that nitrate and nitrite induce, and ammonia represses nitrate reductase expression in *A. vinelandii* (24, 31, 40). They also indicate that the reporter plasmids are suitable for further regulatory analyses.

The roles of *nasAB* leader sequences in nitrate and nitrite induction. To dissect the regulatory role of the *nasAB* leader hairpin structures, three mutations with deletions of individual hairpins were constructed. One mutation lacked the 5' side of the hairpin I; the second one lacked the 3' side stem of the hairpin II; and the third one has the hairpin III of the putative transcriptional terminator deleted (Figure 4). Bioinformatic analysis indicated that each of the individual hairpin deletions would not significantly affect the adjacent hairpin structures (data not shown).

The mutated DNA fragments were translationally fused with *lacZ* in pVnflacZa and transformed in to *A. vinelandii* for β-galactosidase assays (Figure 4). The deletion of the putative terminator hairpin led to constitutive Φ(*nasA-lacZ*) expression under all nitrogen conditions tested, confirming that the structure plays a negative role in *nasAB* expression. High expression of Φ(*nasA-lacZ*) in the presence of ammonia, however, was unexpected. It was assumed that *nasAB* transcription is subject to general nitrogen regulation mediated by NtrC and repressed when ammonia is supplied in the medium.

Disruption of hairpin I reduced Φ(*nasA-lacZ*) expression to background levels, indicating that this hairpin is required for *nasAB* expression. Disruption of hairpin II did not appear to affect Φ(*nasA-lacZ*) expression. Thus, a regulatory role for the middle hairpin was not apparent.

For many amino acid biosynthetic operons in bacteria, small ORFs located in the 5' end leader region of the mRNAs play a pivotal role in antitermination (14, 17). To test whether the putative *nasAB* leader ORF behaved in an analogous manner, the ORF start codon ATG was changed to a TAG stop codon. The mutant fusion had an expression profile similar to the wild-type control (Figure 4), suggesting that the putative small ORF has no role in *nasAB* regulation.

The *nasAB* promoter is subject to nitrogen regulation. To analyze the effect of nitrogen sources on *nasAB* promoter activity, a transcriptional fusion of the *nasAB* promoter (P_{nasA}) and *lacZ* was constructed. The region from -316 to +16 containing the *nasAB* promoter was cloned into the *Spe*I-*Bam*HI region of pVnflacZb. Since *lacZ* in pVnflacZb has its own SD sequence, β-galactosidase activity reflects directly the activity of P_{nasA}. The Φ(P_{nasA}-*lacZ*) fusion was expressed constitutively under the nitrogen conditions tested: ammonia, dinitrogen, urea, and nitrate. This is in consistent with the expression profile of Φ(*nasA*-*lacZ*) translational fusion derivative in which the leader terminator hairpin III deleted.

In strain UW1, a Nif⁻ derivative of UW136, the Φ(P_{nasA}-*lacZ*) fusion showed a different expression pattern. In the presence of ammonia, β-galactosidase activity was similar to the wild-type strain. In contrast, β-galactosidase activity increased four fold or more in the mutant as compared to the wild type strain when ammonia was replaced with urea,

nitrate, or nitrate. These observations suggest that the *nasAB* promoter is subject to nitrogen regulation (29).

Integrity of σ^{54} and NtrC binding sites are essential for *nasAB* expression. To determine if the presumptive σ^{54}-binding site is required for *nasAB* operon expression, the dinucleotide pairs GG/GC in the promoter region were substituted with AA/AT. The mutation reduced translational Φ(*nasA-lacZ*) expression to background levels under all nitrogen conditions tested (Table 3), indicating that the GG/GC motif was required for *nasAB* expression.

Two putative NtrC binding sites were identified upstream of the σ^{54} binding site, centering at -100 and -118 nt regions (Figure 1A). To verify the roles of these sequences, a series of 5' deletions in the *nasAB* promoter region were constructed (Figure 5A). These constructs contain the 5' end of *nasA* to nucleotide 162 and lack the leader terminator hairpin III.

The constructs deleted to bases –316, –168, and –131, which contained the intact putative NtrC binding sites exhibited levels of β-galactosidase activity comparable to the intact control (Figure 5). The construct deleted to base -108 exhibited a 10-fold reduction in β-galactosidase activity while the construct deleted to base -43 exhibited a further reduction. These results indicate that the putative NtrC binding sites are required for *nasAB* expression.

DISCUSSION

The *nasAB* operon in *A. vinelandii* encodes assimilatory nitrate reductase and nitrite reductase. The results of previous studies showed that the synthesis of nitrate reductase requires both NtrA and NtrC. The results presented here confirm that the *nasAB* operon is driven by a σ^{54}-dependent promoter that requires NtrC for activation.

The regulatory role of NtrC in enteric bacteria has been well studied (29). The transcriptional activator NtrC and its cognate sensor NtrB constitute a two-component regulatory system, regulating the expression of nitrogen-related operons. NtrB and NtrC are components of the global nitrogen regulatory system (Ntr), which coordinates cellular nitrogen metabolism in response to the intracellular nitrogen status. In nitrogen-limited environments, NtrC is phosphorylated by its cognate sensor partner NtrB. Phosphorylated NtrC (NtrC-P) activates transcription of target operons to synthesize enzymes that assimilate alternative nitrogen sources, including nitrate and nitrite. In ammonia-sufficient environments, NtrC-P is dephosphorylated by NtrB, and genes under its control are not activated.

Although the regulatory role of NtrC in *A. vinelandii* had not been well studied, it was assumed that the *A. vinelandii* NtrC functions similarly to its counterpart in enteric bacteria (15). Expression profiles of *lacZ* transcriptional fusion with the *nasAB* promoter in the Nif$^-$ strain UW1 confirm that *nasAB* transcription is subject to nitrogen regulation

(Table 4). Nitrogen inhibition of the *nasAB* promoter (P_{nasA}) is not complete. This is in agreement with the previous observation that it is quite difficult to cause complete nitrogen repression in *A. vinelandii,* as nitrate and nitrite reductase activities in *A. vinelandii* can not be eliminated even when the concentration of ammonia reaches 1 g/L (R. Dixon, person communication)(24).

The *nasAB* promoter is repressed in the wild-type strain UW136 in the presence of atmospheric N_2. Although the reason for this is unclear, one possibility is that *A. vinelandii* has multiple nitrogen fixation systems and that atmospheric nitrogen represents a more accessible and sustainable nitrogen source than nitrate and nitrite in natural environments. As a result, *nasAB* transcription is repressed unless nitrogen fixation becomes dysfunctional.

Strain UW1 differs from UW136 by a mutation in *nifA,* the gene that encodes NifA, a transcriptional activator of all the other nitrogen fixation (*nif*) operons (20). NifA and its partner protein NifL regulate *nif* operon activation in response to nitrogen, redox, and carbonate levels (27). Sequence analysis failed to identify a region with similarity to the NifA binding signature (5'-TCT-N_{10}-ACA-3') (6) within the *nasAB* promoter region. How *nifA* affects *nasAB* transcription remains unclear.

The 135-nucleotide *nasAB* leader region is involved in both the positive and negative regulation of *nasAB*. The intrinsic terminator structure in the region plays a negative role

in *nasAB* expression in the absence of nitrate/nitrite. Deletion of the terminator hairpin led to constitutive expression of the operon regardless of the nitrogen source present.

There are two other hairpins upstream of the terminator structure. The promoter-proximal hairpin I has a positive role in *nasAB* expression in that deletion of part of it resulted in the loss of *nasAB* expression. However, the mechanism by which this hairpin activates *nasAB* expression is currently unknown. Secondary structure analyses failed to reveal a credible alternative structure formation involving both hairpin I and terminator hairpin III (Want et al, unpublished data). Partial deletion of the center hairpin (II) had no significant effect on Φ(*nasA-lacZ*) expression, suggesting that the formation of an alternative hairpin may be not required for antitermination.

In other work, we used a heterologous expression system to confirm that NasT acts upon the *nasAB* leader region and plays a positive role in *nasAB* expression (Wang et al, submitted for publication). Although the molecular mechanism of NasT-mediated antitermination is unclear, the presence of a putative RNA-binding domain ANTAR homolog at the C-terminal end of NasT suggests that NasT might interact with the *nasAB* leader region directly (27).

Many amino acid biosynthetic operons use translation of a small ORF to influence transcription termination within the leader region (45). The leader region of the *nasAB* operon in *A. vinelandii* contains a small putative eight peptide ORF, analogous to known

amino acid biosynthetic operon. Mutational analysis, however, suggests that this ORF is not involved in the antitermination. The involvement of NasT in *nasAB* antitermination also suggests that the molecular mechanism of *nasAB* antitermination is different from those of amino acid biosynthetic operons.

Antitermination is also involved in nitrate and nitrite induction of the assimilatory nitrate reductase operon *nasFEDCBA* in *Klebsiella oxytoca* (*pneumomiae*) M5al (21). The operon consists of six structural genes, designated *nasFEDCBA*(23). The *nasFED* genes encode an ABC-type nitrate transporter system, *nasA* and *nasC* encode nitrate reductase, and *nasB* encodes a nitrite reductase. Transcription of *nasFEDCBA* is subject to Ntr regulation; transcription is inhibited when ammonia is generated by anaerobic (respiratory) nitrate reduction or supplied as the nitrogen source (7, 22). No *nif* interference of *nasFEDCBA* transcription has been observed.

The 119-nucleotide leader region of the *nasFEDCBA* operon contains an intrinsic transcriptional terminator (8). Antitermination is mediated by a trans-acting protein NasR encoded by a single gene operon located immediately upstream of *nasFEDCBA* (9, 13). NasR has a nitrate- and nitrite-sensing (NIT) domain and a C-terminal ANTAR domain (36, 37). It was demonstrated that NasR interacts with a promoter-proximal hairpin structure in the leader region (8, 9). Thus, *A. vinelandii* is the second diazotroph to utilize antitermination to regulate assimilatory nitrate reductase activity. The difference in

operon organizations, signal cascades, and leader sequences suggests that these two antitermination systems evolved independently.

Based on the available data, a model for *nasAB* regulation is proposed (Figure 6). Transcription of *nasAB* in *A. vinelandii* is attenuated within the operon leader region in the absence of nitrate or nitrite. Transcription attenuation is removed when nitrate or nitrite is available in the environment, possibly via antitermination mediated by the NasS/NasT system.

ACKNOWLEDGMENTS

We thank Bentley A. Fane and Eduardo Santero for discussion, advice, and critical review of the manuscript.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
A. vinelandii strains		
UW136	Rif^r derived from strain UW (ATCC 13705)	(3)
UW1	*nifA* $^-$	(20)
AVW552	UW136 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*)	This study
AVW627	UW136 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*) with +60 ATG→TAG mutation	This study
AVW643	UW136 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*) with +13 to +81 nt deletion	This study
AVW650	UW136 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*) with +14 to +25 nt deletion	This study
AVW663	UW136 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*) with -24/-12 GG/GC → AA/AT mutation	This study
AVW664	UW136 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*) with +53 to +58 nt deletion	This study
AVW672	UW136 *vnf*::Φ(*nasA*$^{-131\ to\ +297}$-*lacZ*)	This study
AVW674	UW136 *vnf*::Φ(*nasA*$^{+89\ to\ +297}$-*lacZ*)	This study
AVW677	UW136 *vnf*::Φ(*nasA*$^{-43\ to\ +297}$-*lacZ*)	This study
AVW678	UW136 *vnf*::Φ (*nasA*$^{-108\ to\ +297}$-*lacZ*)	This study
AVW693	UW136 *vnf*::Φ(*nasA*$^{-168\ to+297}$-*lacZ*)	This study
AVW853	UW136 Δ*nasC*::Gm^r	This study
AVW816	AVW853 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*)	This study
AVW908	UW136 *vnf*::Φ(*nasA*$^{-316\ to\ +297}$-*lacZ*) with +68 to +100 nt deletion	This study
AVW960	UW136 *vnf*::Φ(P_{nasA}-*lacZ*)	This study
AVW961	AVW853 *vnf*::Φ(P_{nasA}-*lacZ*)	This study
AVW962	UW1 *vnf*::Φ(P_{nasA}-*lacZ*)	This study
E. coli strain		
DH5α	*supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 hi-1 relA1*	GIBCO-BRL
Plasmids		
pBluescript II KS(+)	Car^r, cloning vector	Stratagene
pBlue-lacZ	Car^r; pBluescript II KS(+) containing *lacZ* from pSUP102::Tn*5*-B21 at *BamH*I-*Xho*I region	This study
pGEM-T	Car^r; cloning vector	Promega
pIC20H	Car^r; cloning vectors	(26)

pIC-lacZ	Car^r; pICT4 carrying *Xba*I-*lacZ*-*Xho*I from pBlue-lacZ	This study
pICT4	Car^r; pIC20H carrying four tandem copies of *rrnB1* cloned at the *Sph*I site	This study
pJW1	Car^r; pTZ19 carrying *vnf* sequence	(43)
pKT2-lacZ	Kn^r, expression vector	(Pierson, unpublished data)
pPROBE-NT	Promoter-probe vector	(30)
pSUP102::Tn*5*-B21	Cm^r Tet^r, Transposable promoter probe; B21 in pSUP102	(38)
pTn*Mod*-OGm	Gm^r; pMB1, *mob*_ Tn*5 tnp*	(10)
pVnflacZa	Car^r p15A; translational *lacZ* fusion probe vector	This study
pVnflacZb	Car^r p15A; transcriptional *lacZ* fusion probe vector	This study
pWB30	Car^r; pBluescript II KS(+) carrying 7.8-kb *Xho*I-*Xho*I DNA fragment containing the 5' end of *nasA* and its upstream sequence	This study
pWB552	Car^r; pVnflacZa carrying *nasA*$^{-316\ to\ +297}$-*lacZ*	This study
pWB627	Car^r; pWB552 with +60 ATG→TAG mutation	This study
pWB643	Car^r; pWB552 with +13 to +81 nt deletion	This study
pWB650	Car^r; pWB552 with +14 to +25 nt deletion at the *nasA* leader region	This study
pWB663	Car^r; pWB552 with -24/-12 GG/GC → AA/AT mutation	This study
pWB664	Car^r; pWB552 with +53 to +58 nt deletion at the *nasA* leader region	This study
pWB672	Car^r; pVnflacZa carrying *nasA*$^{-131\ to\ +297}$-*lacZ*	This study
pWB674	Car^r; pVnflacZa carrying *nasA*$^{+89\ to\ +297}$-*lacZ*	This study
pWB677	Car^r; pVnflacZa carrying *nasA*$^{-43\ to\ +297}$-*lacZ*	This study
pWB678	Car^r; pVnflacZa carrying *nasA*$^{-108\ to\ +297}$-*lacZ*	This study
pWB680	Car^r; pGEM-T carrying the *ntrC* gene	This study
pWB685	Gm^{r}Car^r; pWB680 with Δ*ntrC*::Gm^r	This study
pWB693	Car^r; pVnflacZa carrying *nasA*$^{-168\ to+297}$-*lacZ*	This study
pWB908	Car^r; pWB552 with +68 to +100 nt deletion	This study
pWB960	Car^r; pVnflacZb carrying *nasA*$^{-316\ to\ +16}$-*lacZ*	This study

pWhite	Car^r; cloning vector	This study
pWvnf	Car^r; pWhite carrying 2.8-kb *vnf* sequence	This study

[a] Abbreviations: Car, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Rif, rifampicin; Tet, tetracycline.

TABLE 2. Primers used in this study

Primer	Designation	Sequence[a] (5'-- 3')	Restriction enzyme[b] and use
1	103NtrCf1	GTTGCCGTCCCTGGTATGG	PCR *ntrC* from the *A. vinelandii* genome
2	106NtrCr1	GTGGACACGGCATAGATTTC	PCR *ntrC* from the *A. vinelandii* genome
3	107NtrCSacIf	AGAGCTCAGCCATGCCCAGGAG	*Sac*I; *ntrC* deletion mutation construction
4	108NtrCSacIr	AGAGCTCGGCTTGGGCAGGTATTC	*Sac*I; *ntrC* deletion mutation construction
5	12XbaI-nasf1	AAATCTAGA AGCAGCGCTTCGAAAC	*Xba*I; PCR *nasA* promoter region
6	1BamH162innasA	TGGATCCATGCCGGCCAGCACCGG AGAG	*Bam*HI; PCR *nasA* promoter region
7	43pnasApolyT	ATCTAGAGGCGGGACGCCTTTTTT G	*Xba*I; PCR Shine-Dalgarno region of *nasA*
8	54 T4f	AGCTGGGGCCGCAATTC	PCR tandem *rrnB1* from pPROBE-NT
9	55T4r	ATTCAGCTTCCGATC	PCR tandem *rrnB1* from pPROBE-NT
10	56lead-1-f1	CCGACAGAACGGGATAGGACAAA GGCGTC	*nasA* leader small ORF start codon mutation

11	57lead-f-r1	CCTATCCCGTTCTGTCAAAGGCGTC	*nasA* leader small ORF start codon mutation
12	602ndloopf	GCAGTGTTTCCCGACAGGAATGGAC AAAGGCGTC	*nasA* leader middle hairpin deletion mutation
13	612ndloopr	CTGTCGGGAAACACTGC	*nasA* leader middle hairpin deletion mutation
14	65Indenf	GGGGATCGGAAGCTGAATG	Confirmation of integration of foreign sequence upstream of *lac*Z in pVnflacZ
15	72pblueconfirmf1	GTCTCATGAGCGGATAC	Confirmation of *vnf* integration into pWhite
16	73pblursalIhindIII rf	AGTCGACAAGCTTCCTCGCTCACTGACTC	*Sal*I, *Hind*III; PCR *bla* and pM1 of pBbluscript II KS (+)
17	74bpluesacIr	AGAGCTC AGGTGGCACTTTTCG	PCR *bla* and pM1 of pBbluscript II KS (+)
18	75pblueconfirmr1	GTATTACCGCCTTTGAGTG	Confirmation of *vnf* integration into pWhite
19	8prior1stloop-PnasA1r	GTGATCTGTCGGTTTTC	*nasA* leader hairpin I deletion mutation
20	9primextenf	AAACCGACAGATCACATGGTAACCGGGGGAC	*nasA* leader hairpin I

21	Deletion-108	AAATCTAGAACCAAGACAGTGCAAG	deletion mutation *Xba*I; Promoter deletion construction
22	Deletion-131	AAATCTAGAAACCCCATA AGAGG	*Xba*I; Promoter deletion construction
23	Deletion-168	AAATCTAGAGCTCTGCTCGCCCTGTTG	*Xba*I; Promoter deletion construction
24	Deletion-43	AAATCTAGAGT CATCCGTGAA AC	*Xba*I; Promoter deletion construction
25	lacZr	CAAGGCGATTAAGTTGGGTAAC	Confirmation of integration of foreign sequence upstream of *lac*Z in pVnflacZ
26	M13f	GTAAAACGACGGCCAGTG	Cloning confirmation
27	M13r	CAGGAAACAGCTATGAC	Cloning confirmation
28	Nasasigma54f	TAACACAGCCCCTATATCGAGAAAACCGAC	σ^{54} binding site mutation
29	Nasasigma54r	TATAGGGGCTGTGTTAGTTTCACGGATGACTG	σ^{54} binding site mutation
30	O31Pnasaf	CAGAACGGGAATGGACAATTTTTGTTTTCCGCTTTC	Terminator hairpin deletion mutation
31	O32pnasar	AATTGTCCATTCCCGTTCTG	Terminator hairpin deletion mutation
32	P92	AAGGATCCGCTTATGTGATCTGTC	*Bam*HI;

		G	Transcriptional fusion construction
33	vnff	AAAGAGCTCATCATCTCGCCCAGTTC	*Sac*I; PCR *vnf* sequence
34	vnfr	AAACGCCTCGAGAAGAGCATG	*Xho*I; PCR *vnf* sequence
35	WB30screenf	CGCCTGGCTCGACGAATG	*nasA* promoter region cloning confirmation
36	WB30screenr	GTCCATTCCCGTTCTGTC	*nasA* promoter region cloning confirmation

[a] The underlined sequences are the restriction sites.

[b] Restriction enzymes that can digest the underlined sequences.

TABLE 3. Expression of Φ(*nasA*-*lacZ*) translational fusion in *A. vinelandii* and its mutants

Promoter region within the *lacZ* fusion	Strain genotype	β-galactosidase activity (Miller units)[a]			
		Nitrogen source			
		NH_4^+	N_2	NO_2^-	NO_3^-
$nasA^{-316 - +297}$	Wild type	69 ± 1	96 ± 4	682 ± 9	478 ± 6
$nasA^{-316 - +297}$	*nif*$^-$	32 ± 32	NT[b]	942 ± 16	1389 ± 26
$nasA^{-316 - +297}$ with σ^{54} binding site GG/GC→AA/AT mutation	Wild type	3 ± 1	5 ± 2	7 ± 1	5 ± 1
$nasA^{-316 - +297}$	Δ*ntrC*::Gm^r	2 ± 0	2 ± 0	23 ± 1	2 ± 0
$nasA^{+89 - +297}$	Wild type	2 ± 1	3 ± 2	3 ± 1	3 ± 0

[a] Cultures were grown in BS medium supplemented with the indicated nitrogen sources. After seven hours, β-galactosidase activity was measured. All data are presented as the mean values of triplicates ± standard deviations from a representative experiment.

[b] NT, not tested.

TABLE 4. Expression of Φ(P_{nasA}-*lacZ*) in *A. vinelandii* and its mutants

Strain genotype	β-galactosidase activity (Miller units)[a]				
	Nitrogen source				
	NH_4^+	N_2	U	NO_2^-	NO_3^-
Wild type	594 ± 15	307 ± 14	324 ± 10	439 ± 10	394 ± 25
ntrC mutant	2 ± 1	8 ± 1	4 ± 1	2 ± 1	3 ± 1
nif$^-$	559 ± 17	NT[b]	3470 ± 34	2087 ±142	2677 ± 32

[a] Cultures were grown in BS medium supplemented with the indicated nitrogen sources. After seven hours, β-galactosidase activity was measured. All data are presented as the mean values of triplicates ± standard deviations from a representative experiment.

[b] NT, not tested.

FIGURE LENDENDS

FIGURE 1. DNA sequences surrounding the *nasAB* operon promoter region. (A) The putative transcription start side is marked with +1; facing arrows denote inverted repeats; the putative σ^{54}-binding sequence is shown in a light grey box, with the conserved GG/GC pairs in bold; the putative NtrC protein binding sites between -90 and -150 are underlined with the consensus NtrC binding sequence shown underneath for comparison; the start codon ATG (+60 - +62) and stop codon TAA (+84 - +86) of the small ORF in the leader region are bold-faced; the putative Shine-Dalgarno sequence (S-D) sequence of the gene *nasA* is boxed. (B) The predicted secondary structure of the *nasAB* leader sequence. The numbers are relative to the putative start base of the transcript.

FIGURE 2. Transcriptional and translational *lacZ* reporter vectors. (A) Plasmid maps and integration into the *vnf* locus of the *A. vinelandii* genome through homologous recombination (not drawn to scale). MCS, multiple cloning site; pMB1, replicon region; $T1_{(4)}$, four tandem copies of the T1 terminator from the *E. coli rrnB1* operon (30); *vnf*, vanadium(V)-containing nitrogenase gene (4). (B) Multiple cloning sites in vectors. SD, Shine-Dalgarno sequence.

FIGURE 3. Time course of $\Phi(nasA^{-316\ -\ +297}$-*lac*Z) expression in *A. vinelandii* UW136. Bacteria were grown on BS medium supplied with the indicated nitrogen source.

FIGURE 4. Expression of the Φ(*nasA-lacZ*) translational fusions with different *nasAB* leader alterations (not drawn to scale). The top line represents the sequence ranges from -316 nt upstream of the *nasAB* transcription start site to the +297 nt (base 162 of the NasA ORF). The bent arrow represents the direction of transcription, +1 represents the first nucleotide of the transcript; facing arrows (designated I, II, and III) represent the three inverted sequences in the *nasA* leader region. The empty rectangles represent the regions deleted; ◊ represents the ATG → TAG mutation. Values on the right show Φ(*nasA-lacZ*) expression in *A. vinelandii*. Cultures were grown in BS medium supplemented with the indicated nitrogen sources and β-galactosidase activity was measured after seven hours. All data are presented as the mean values of three replicates ± standard deviations from a representative experiment.

FIGURE 5. Expression of Φ(*nasA-lacZ*) transcriptional fusion constructs bearing deletions within the promoter region in *A. vinelandii* (not drawn to scale). All deletions were constructed based on the sequence around the *nasA* promoter region in the plasmid pWB908, which has the terminator hairpin in the leader region deleted. (A) The *nasA* promoter region from -316 to +1 is shown, the putative tandem NtrC binding sites are underlined; the 5' ends (-168, -131, -108, -43) of *nasAB* promoter deletion mutations are shown. β-Galactosidase activity of the reporter constructs was measured seven hours after cultures were grown on BS medium supplemented with ammonium (B) or nitrate (C). The activity of pWB908 (with 5'end at -316) was arbitrarily set to 100%, and the

activities of other fusions are represented as a percentage of that value. This experiment has been repeated three times with similar results.

FIGURE 6. Model for *nasAB* regulation. The *nasAB* operon in *A. vinelandii* has an NtrC-dependent promoter, which is subject to global nitrogen regulation. In the wild-type strain, *nasAB* transcription initiation is repressed at basal levels regardless of the presence of ammonia. The terminator structure at the operon leader region regulates transcription negatively. Nitrate/nitrite induces antitermination, which is mediated by NasS/NasT, a nitrate sensor-antiterminator system. NasS regulates the activity of NasT directly through steric hindrance in the absence of nitrate and nitrite.

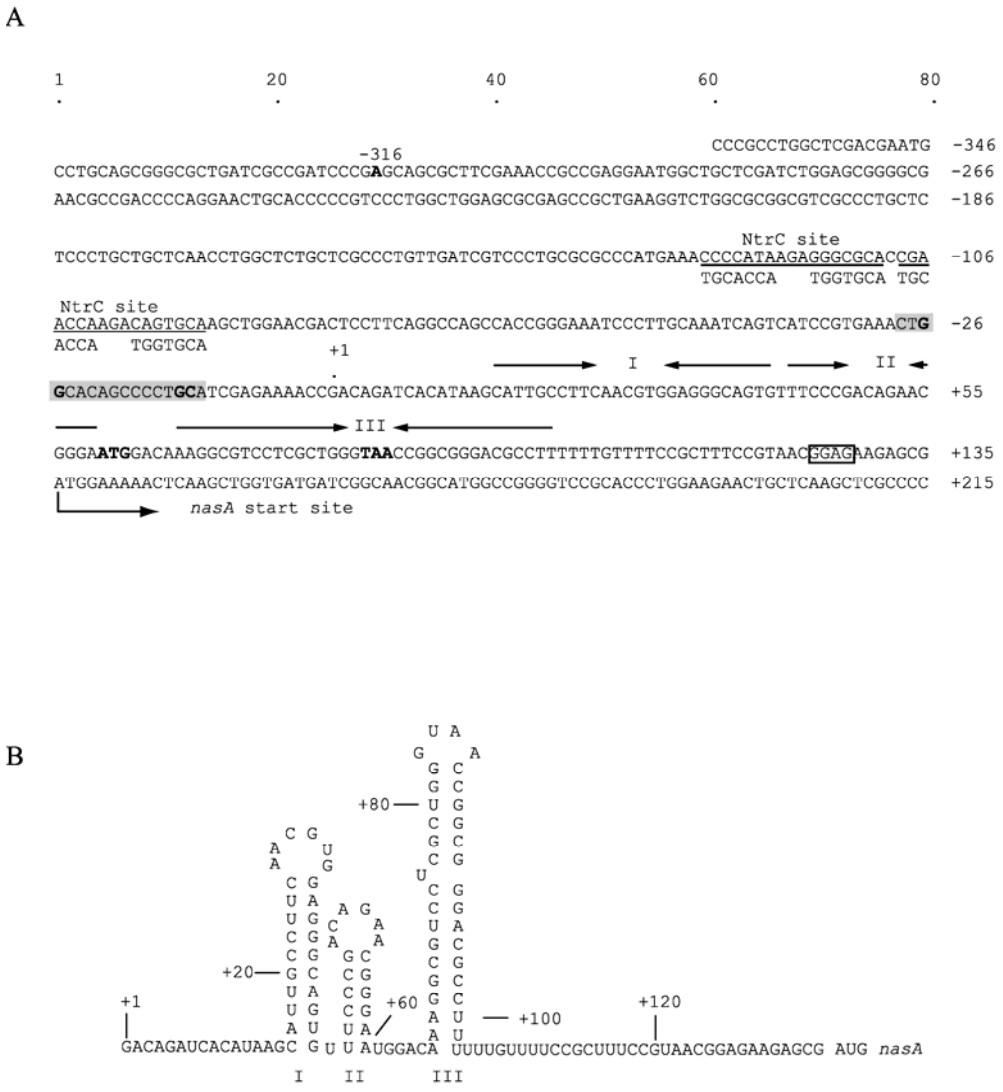
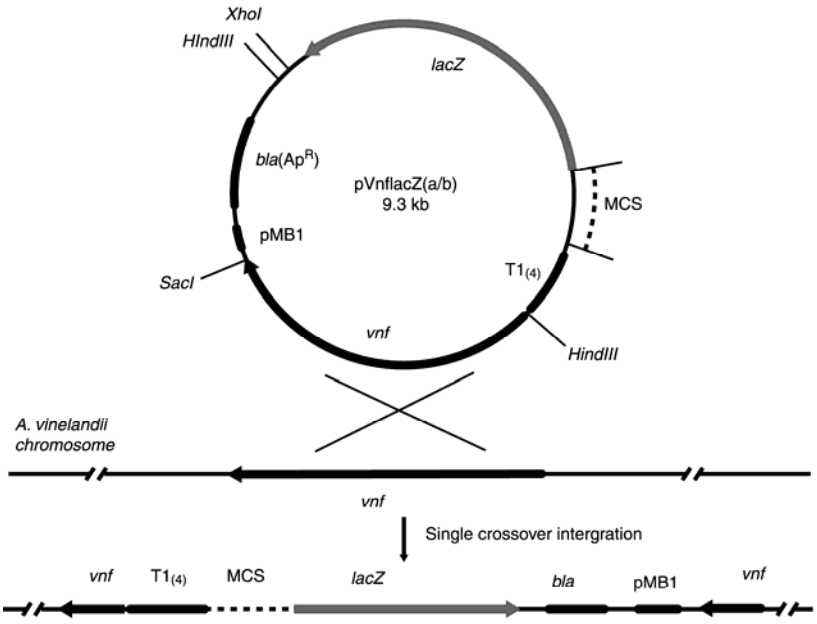

A
1
20
40
60
80
-316
CCCGCCTGGCTCGACGAATG -346
CCTGCAGCGGGCGCTGATCGCCGATCCCGAGCAGCGCTTCGAAACCGCCGAGGAATGGCTGCTCGATCTGGAGCGGGGCG -266
AACGCCGACCCCAGGAACTGCACCCCCGTCCCTGGCTGGAGCGCGAGCCGCTGAAGGTCTGGCGCGGCGTCGCCCTGCTC -186
NtrC site
TCCCTGCTGCTCAACCTGGCTCTGCTCGCCCTGTTGATCGTCCCTGCGCGCCCATGAAACCCCATAAGAGGGCGCACCGA -106
TGCACCA TGGTGCA TGC
NtrC site
ACCAAGACAGTGCAAGCTGGAACGACTCCTTCAGGCCAGCCACCGGGAAATCCCTTGCAAATCAGTCATCCGTGAAACTG -26
ACCA TGGTGCA
+1
I
II
GCACAGCCCCTGCATCGAGAAAACCGACAGATCACATAAGCATTGCCTTCAACGTGGAGGGCAGTGTTTCCCGACAGAAC +55
III
GGGAATGGACAAAGGCGTCCTCGCTGGGTAACCGGCGGGACGCCTTTTTTGTTTTCCGCTTTCCGTAACGGAGAAGAGCG +135
ATGGAAAAACTCAAGCTGGTGATGATCGGCAACGGCATGGCCGGGGTCCGCACCCTGGAAGAACTGCTCAAGCTCGCCCC +215
nasA start site
B
+80
+20
+1
+60
+100
+120
GACAGAUCACAUAAGC GUUAUGGACA UUUUGUUUUCCGCUUUCCGUAACGGAGAAGAGCG AUG nasA
I II III

FIGURE 1

A



B

pVnflacZa: $T1_{(4)}$-*Xba*I-*Bcu*I-*Bam*HI-*lacZ*

pVnflacZb: $T1_{(4)}$-*Xba*I-*Bcu*I-*Bam*HI-*Xba*I-SD-*lacZ*

FIGURE 2

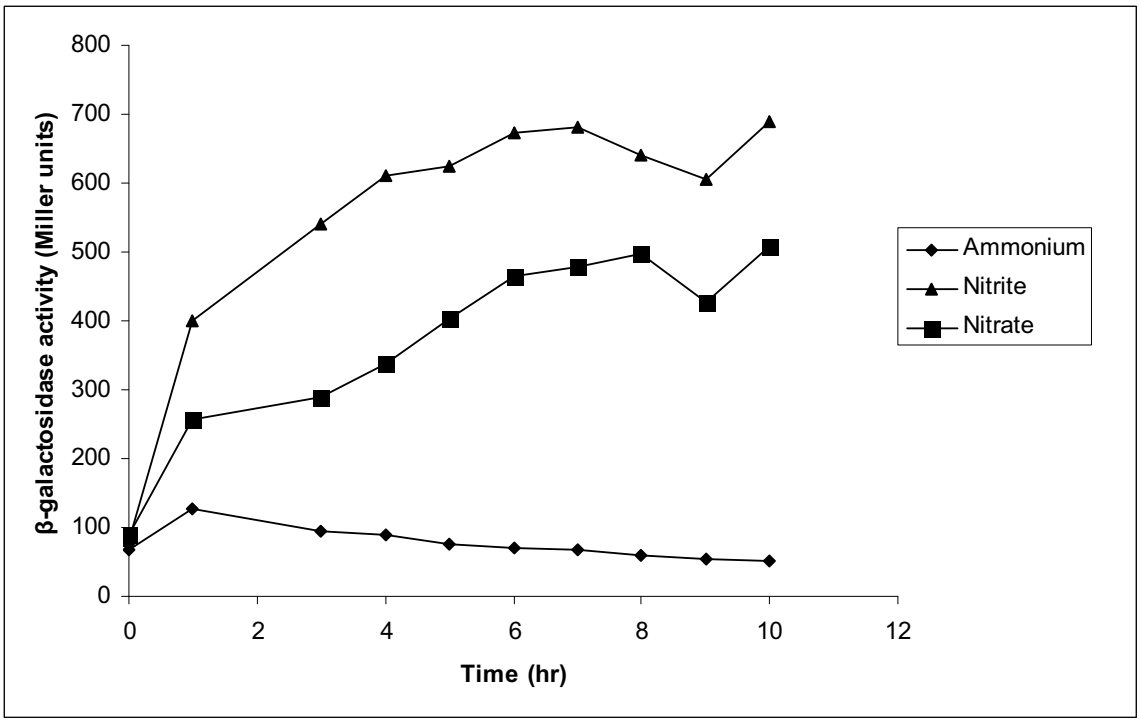
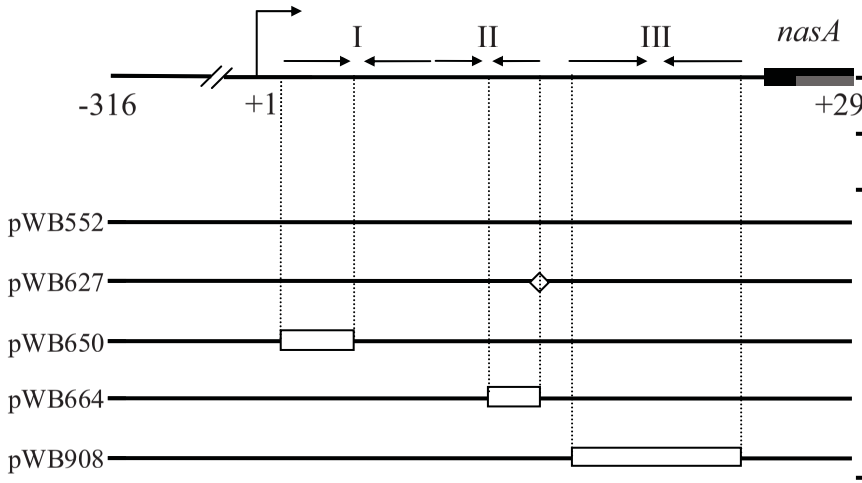

800
700
600
500
400
300
200
100
0
β-galactosidase activity (Miller units)
0
2
4
6
8
10
12
Time (hr)
Ammonium
Nitrite
Nitrate

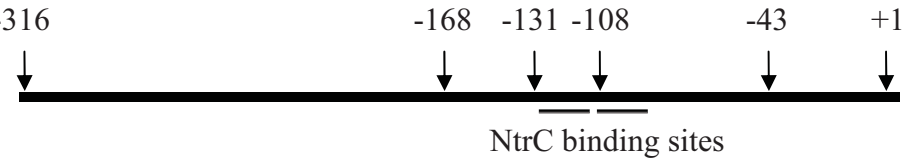
FIGURE 3



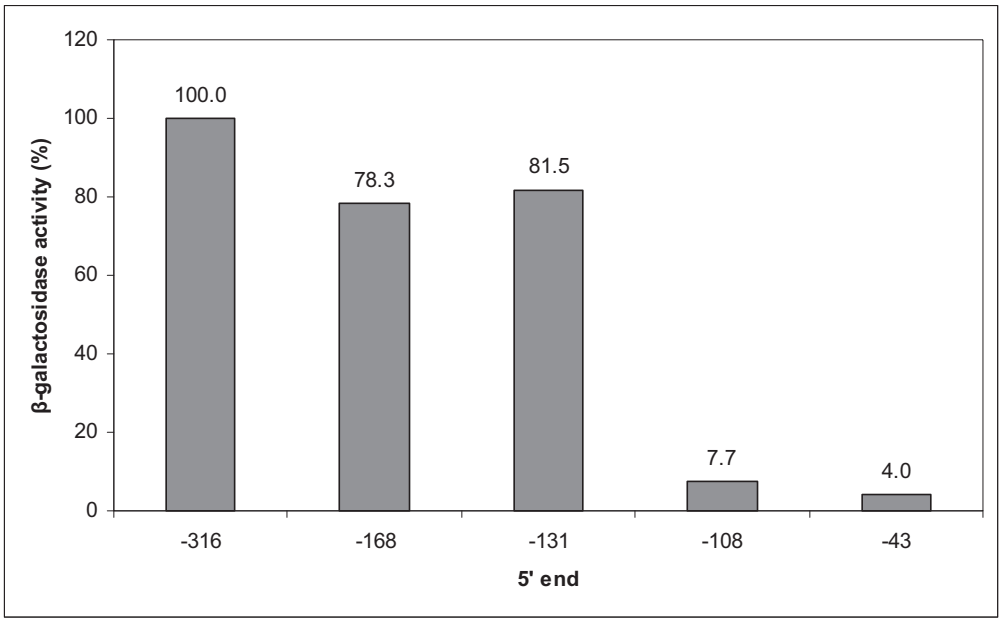
	β-galactosidase activity (Miller Units)			
	NH_4^+	N_2	NO_2^-	NO_3^-
pWB552	69 ± 1	96 ± 4	682 ± 9	478 ± 6
pWB627	26 ± 1	72 ± 2	313 ± 3	479 ± 15
pWB650	6 ± 3	7 ± 3	8 ± 0	8 ± 3
pWB664	36 ± 1	29 ± 1	105 ± 2	304 ± 3
pWB908	1451 ± 6	745 ± 5	1690 ± 34	852 ± 18

FIGURE 4

A


-316
-168
-131
-108
-43
+1
NtrC binding sites

B


β-galactosidase activity (%)
120
100
80
60
40
20
0
100.0
78.3
81.5
7.7
4.0
-316
-168
-131
-108
-43
5' end

C

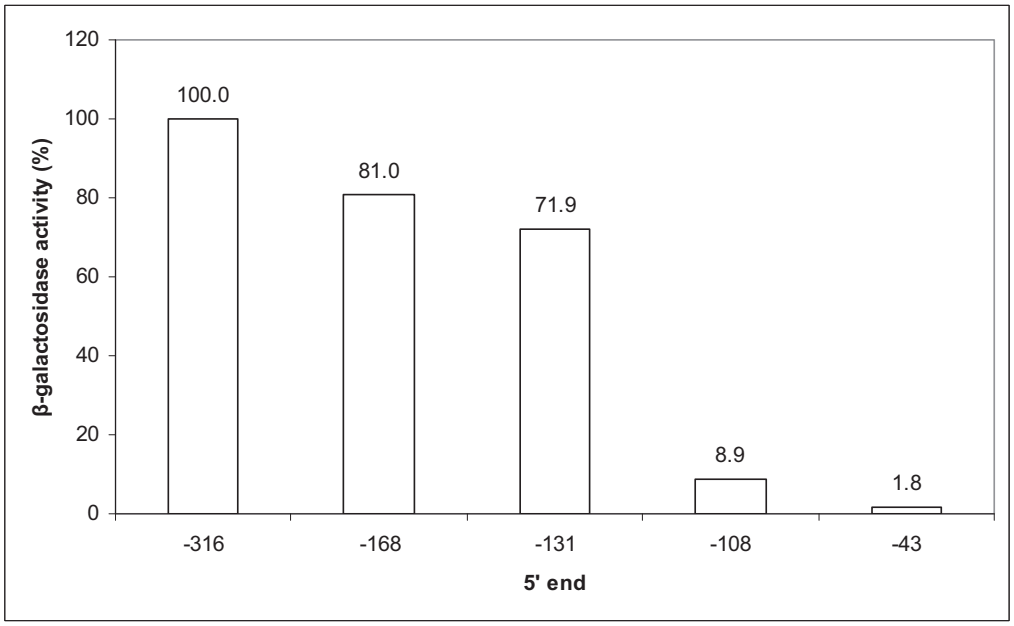

β-galactosidase activity (%)
120
100
80
60
40
20
0
100.0
81.0
71.9
8.9
1.8
-316
-168
-131
-108
-43
5' end

FIGURE 5

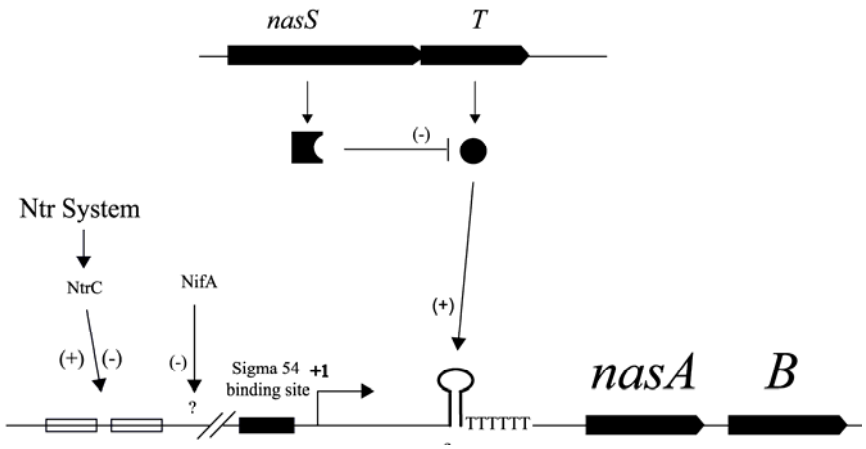

nasS
T
(-)
Ntr System
NtrC
NifA
(+)
(+)
(-)
(-)
Sigma 54
binding site
+1
?
TTTTTT
nasA
B

FIGURE 6

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APPENDIX B. NASS/NAST, A NON-CANONICAL TWO-COMPONENT REGULATORY SYSTEM REGULATES THE ASSIMILATORY NITRATE REDUCTASE OPERON (*NASAB*) IN *AZOTOBACTER VINELANDII* VIA ANTITERMINATION

NasS/NasT, a non-canonical two-component regulatory system regulates the nitrate reductase operon in *Azotobacter vinelandii* via antitermination

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Dedication (in memoriam): This manuscript is dedicated to the memory of Christina Kennedy.

ABSTRACT

Expression of the assimilatory nitrate reductase operon *nasAB* in *Azotobacter vinelandii* is induced by nitrate and nitrite. This operon-specific induction involves antitermination of an intrinsic transcription terminator within the leader region of the *nasA*. Nitrate and nitrite induction also requires the products of the *nasST* operon. NasT is homologous to the antiterminator AmiR in *Pseudomonas aeruginosa.* Mutation of *nasT* abolishes *nasAB* induction. NasS is homologous to the substrate-binding component of ABC-type transporters, but is not required for nitrate transport. Mutation of *nasS* without affecting downstream *nasT* expression leads to constitutive expression of *nasAB*. In this study, a bacterial two-vector system assay demonstrated that NasT has a positive effect on *nasAB* expression and acted within the *nasAB* leader region. A yeast two-hybrid system analysis indicated that NasT formed a dimer or higher level complex, resembling the structure of AmiR. These results suggest *nasT* and *amiR* may represent orthologous genes encoding a family of antitermination regulators. Over-expression of *nasT* and mutational analyses of *nasS* or *nasT* demonstrated that *nasAB* regulation required the maintenance of the endogenous NasS and NasT stoichiometry, which may be achieved through translational coupling. The results presented here support a model that NasS and NasT constitute a nitrate sensor-antiterminator regulatory system, with NasS stringently controlling the activity of NasT by a mechanism requiring stoichiometric interaction between the proteins.

INTRODUCTION

Azotobacter vinelandii strain UW136 is a free-living diazotroph with three genetically distinct nitrogenase systems: the conventional molybdenum-containing nitrogenase system (Nif), a vanadium-containing nitrogenase system (Vnf), and a iron-containing system (Anf) (11, 27). *A. vinelandii* can also use nitrate or nitrite as its sole nitrogen source. Nitrate is sequentially reduced to nitrite and to ammonium before being incorporated into glutamine by glutamine synthetase (35). The assimilatory nitrate reductase (NasB) and nitrite reductase (NasA) are encoded by a two-gene *nasAB* operon (29).

The *nasAB* operon is subject to complex transcriptional regulation. *nasAB* has a NtrC-dependent promoter, which is regulated in response to intracellular nitrogen status. In the wild type strain with a functional Nif system, *nasAB* transcription initiation is repressed to basal levels regardless the presence of ammonia (Wang et al, submitted for publication). The *nasAB* operon is also subject to post-transcriptional attenuation regulation. The 135-nucleotide *nasAB* leader region contains an intrinsic transcriptional terminator that blocks transcription of *nasAB* in the absence of nitrate/nitrite (Wang et al, submitted for publication).

The presence of nitrate or nitrite in the absence of ammonium stimulates antitermination within the *nasAB* leader region. This relief of termination requires the products of the *nasST* operon, which is located 12-kb upstream of the *nasAB* (8). The deduced protein

NasS shares sequence homology with the substrate binding component of ABC-type nitrate transporters in *Klebsiella pneumoniae* and *Synechococcus* sp. Deletion of *nasS*, however, has no effect on nitrate uptake by *A. vinelandii* (8). Insertion mutagenesis of *nasS* without affecting downstream *nasT* expression led to constitutive expression of *nasAB*, suggesting that NasS plays a negative role in *nasAB* expression (8).

NasT is a 192 amino acid polypeptide with a modular structure homologous to AmiR, an mRNA- binding antiterminator in *Pseudomonas aeruginosa* (8, 32). Both proteins have a C-terminal AmiR and NasR Transcription Antitermination Regulator (ANTAR) domain and an N-terminal domain homologous to the receiver domain REC of the response regulator of classic two-component regulatory systems (8, 24, 32). Mutation of *nasT* abolished *nasAB* expression even in the presence of nitrate and nitrite, indicating that it is required for *nasAB* expression (8).

The NasT domain structure and the phenotype of *nasT* mutants suggested that NasS and NasT may mediate nitrate- and nitrite-inducted antitermination within the *nasAB* leader region (18). In this work, a bacterial two-vector system was used to test the regulatory role of NasT. The results confirmed that NasT acts positively within the *nasAB* leader region. In addition, the results of over-expression analyses indicated that the maintenance of correct stoichiometry between NasS and NasT is required for *nasAB* regulation. Furthermore, the results of mutational analyses suggested that translational coupling was used to coordinate NasS and NasT synthesis. The results presented here support the

model that NasS and NasT constitute a sensor-antiterminator system; signal transduction from NasS to NasT is conveyed through protein-protein interactions.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids used in this study are listed in Table 1. *A. vinelandii* and its derivatives were grown on Burk's nitrogen-free salts (BS) medium supplemented with 1% sucrose (34). When needed, BS was supplemented with ammonium acetate, 15 mM; urea, 10 mM; KNO_3, 10 mM; and $NaNO_2$, 2.8 mM. Antibiotics were added where appropriate: Carbenicillin (Car) at 20 μg/ml, Gentamicin (Gm) at 0.05 μg/ml, and Kanamycin (Km) at 2.0 μg/ml. *E. coli* was grown in LB (0.5% NaCl, 1% Tryptone, 0.5% yeast extract) broth or on LB agar (1.5%) at 37^{o}C and antibiotic concentrations were as follows: Chloramphenicol (Cam) at 25 μg/ml, Km at 25 μg/ml. *Saccharomyces cerevisiae* AH109 was grown in YPDA (yeast extract-peptone-dextrose-adenine) or SD (synthetic dropout) media as described by the manufacturer manual for the Matchmaker two-hybrid system (Clontech,).

Bioinformatics. Protein sequence BLAST against the Conserved Domain Database (CDD) was performed at the NCBI website http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. Coil-coil structure prediction was performed at http://www.russell.embl-heidelberg.de/cgi-bin/coils-svr.pl.

DNA manipulation. DNA digestions, ligations, and cloning were conducted following standard protocols (20). For blunt end ligation, DNA fragments were treated with T4 DNA polymerase following the manufacturer instructions (Fermentas, Glen Burnie, MD). All enzymes and the plasmid isolation kit GeneJET plasmid miniprep kit were purchased from Fermentas. DNA purification from agarose gels was performed using the NucleoSpin Extract II (Clontech, Mountain View, CA). *A. vinelandii* transformations were carried out as described previously (30). *E. coli* transformations were performed using the $CaCl_2$ chemical method (20). Yeast transformations were carried out using the LiAc method following the manual for the Matchmaker two-hybrid system (Clontech). Oligonucleotides used in this study are listed in Table 2. Oligonucleotides were purchased from IDT Inc. (Coralville, IA). DNA sequencing was performed at the University of Arizona Genomics and Technology Core (GATC) DNA Sequencing Facility.

Construction of a *A. vinelandii nasST* deletion mutant. The 4.0-kb *Eco*RI fragment carrying *nasST* from the plasmid pMAS20 was subcloned into the *Hin*dIII-*Hin*dIII region of pIC20H via blunt end ligation. The resulting plasmid was cut with *Sal*I and ligated with a *Sac*I-Gm^r-*Sac*I cassette from pTnMod-OGm through blunt end ligation. The resulting plasmid pWB296 was then transformed into the *nif*$^-$ derivative UW1. Transformants were plated on selective BSN media supplemented with gentamicin. Transformants (Gm^r) were patched onto BSN plates supplemented with carbenicillin.

Gm^rCar^s clones were screened and Δ*nasST*::Gm^r allelic replacement was confirmed by colony PCR using the primers nasSf and nasSr.

Construction of P*scrX-nasS* and **P*scrX-nasT* fusions.** The *nasS* gene was amplified using primer pairs PciI-nasS and BglII-nasS from pMAS20. The PCR product was treated with *Pci*I and *Bgl*II and cloned into the *Pci*I-*Bam*HI site downstream of the P*scrX* promoter in the plasmid pDB1332 (12). The resulting plasmid was restricted with *Kpn*I, blunt-ended, and ligated with PCR fragments containing the kanamycin resistance gene from the plasmid pBBR1MCS-2, giving rise to the plasmid pDB1332-S. Using a similar strategy, P*scrX-nasT* fusion was constructed, and the resulting plasmids was designated pDB1332-T.

Construction of plasmids for bacteria two-vector system. To construct a *nasS* expression plasmid, *nasS* was amplified from pMAS20 using primer pairs EcoRI-nasS and BglII-nasS. The PCR product was treated with *Eco*RI and *Bgl*II and cloned into the *Eco*RI-*Bam*HI site of the plasmid pDK6, giving rise to the plasmid pDK6-S. Using a similar strategy, *nasT* was amplified from pMAS20 using primer pairs EcoRI-nasT and BglII-nasT and cloned into the pDK6, giving rise to the plasmid pDK6-T.

The plasmid carrying a P_{lacUV5}-*nasA*-*lacZ* fusion was engineered from the plasmid pBT. The DNA fragment containing chloramphenicol resistance gene, p15A replicon, and *lac*UV5 promoter was amplified from pBT using primer pairs BTr and 76pBTNotIr, and

digested with *Not*I and *Xho*I. The resulting fragment was linked with the *Not*I-*lacZ*-*Xho*I cassette from the plasmid pblue-lacZ, giving rise to plasmid pBTW.

The DNA sequence containing the *nasA* leader and *nasA* 5' end162 nucleotides or its deletion derivatives was amplified from plasmid pWB552, pWB643, pWB650, pWB664, and pWB908 using primer pairs 013NotI77 and 1BamH162innasA, and cloned into the *Not*I-*Bam*HI region of pBTW, giving rise to a series of heterologous fusions.

Construction of plasmids for Yeast two-hybrid system. *nasT* or a truncated *nasT* fragment without C-terminal the ANTAR domain were amplified from pRM19. The PCR products were double digested with *Nde*I and *Eco*RI, and cloned into *Nde*I-*Eco*RI regions of the plasmids pGADT7 and pGBKT7.

Construction of *lac*UV5-*nasST'-lacZ* and *lac*UV5-*nasT'-lacZ* fusions. Two *lac*UV5-*nasST'-lacZ* fusions were constructed. The *nasST'* fragments in both fusions start at the ninth nucleotide of the *nasS* leader and end at the 62nd codon of *nasT* with an *nasS* central in-frame (codon 56 — 388) deletion. One fusion has the original 389th codon CCC (Proline) in *nasS* substituted with a TGA stop codon. The deletion was constructed using the overlap extension method based on two rounds of PCRs (5). Briefly, the first round PCR generated two fragments, which are complementary at the ends with the desired mutation. The second round PCR uses the two fragments from the first round as templates, generating a merged sequence with the desired mutation. The *nasST'*

fragments were digested with *Not*I and *Bam*HI, and cloned into the *Not*I-*Bam*HI region of pBTW. Then, The *Xba*I-*lac*UV5-*nasST'*-*Bam*HI fragment was subcloned into *Xba*I-*Bam*HI region of pVnflacZ, giving rise to plasmids pWB923 and pWB924. Plasmid pWB924 contains the CCC→ TGA nonsense mutation.

The *lac*UV5-*nasT'*-*lacZ* fusions were constructed by the amplification of the *nasT'* fragments from pMAS20 using primer pairs O27NotIinS and 026BamHIinT and subsequent insertion into the *Not*I-*Bam*HI region downstream of *lac*UV5 in pBTW. The *Xba*I-*lac*UV5-*nasT'*-*Bam*HI fragments were subcloned into the *Xba*I-*BamH*I region of pVnflacZa resulting in plasmid pWB903.

β-galactosidase assay. β-galatosidase activities of the fusion plasmids was determined in *E. coli* using the method by Miller (1972) (23). β-galactosidase activity was measured in the yeast two-hybrid systems using the freeze/thaw method following the manual for the Matchmaker two-hybrid system (Clontech).

RESULTS AND DISCUSSION

A homologous *nasST* mutation in *A. vinelandii* could be stably maintained. *A. vinelandii* is unique in that the chromosome number can vary from 2 to 80, depending on growth conditions (19). The multiplicity of *A. vinelandii* genomes results in challenges in creating mutations in essential genes (13, 35). Since essential gene mutations are lethal to the bacterium, *A. vinelandii* will maintain a mixture of chromosomes containing the wild-

type allele and mutated allele marked by an antibiotic resistance gene under the antibiotic selection pressure. Removal of the antibiotic from the medium leads to the rapid elimination of the mutant allele. For non-essential genes, selection for the mutant allele results in full replacement of all wild-type copies.

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In this project, a *nasST* deletion mutant in the Nif$^-$ strain UW1 was constructed. Strain UW1 can not fix atmospheric nitrogen, but can use nitrate or nitrite as its sole nitrogen source (14). The *nasST* deletion mutant has the central *Sal*I-*Sal*I region replaced with a Gm^r cassette, removing 68% of *nasS* from the 3' end and 15% of *nasT* from the 5' end. The mutant cannot grow on medium with nitrate or nitrite as the sole nitrogen source (Figure 1), in agreement with previous observations that *nasST* mutation in wild type *A. vinelandii* represses *nasAB* expression. Colony PCR verified that the mutant contains only the mutant allele (data not shown). These results confirm the previous observation of the inhibition of *nasAB* expression in a *nasST* deletion background is not a result of *nasST* heterozygosity.

To complement the *nasST* mutation, *nasS* or *nasT* were placed under the control of the sucrose-inducible P*scrX* promoter by introduction into the sucrose catabolic regulon (*scr*) region of *A. vinelandii* through homologous recombination. P*scrX* is active in Burk's medium supplemented with 1% sucrose (BS) (12). Expression of *nasT* in the *nasST* deletion mutant restored the strain's ability to grow in BS broth supplemented with nitrate or nitrite as sole nitrogen source while expression of *nasS* had no obvious effect

on mutant growth (Figure 1C and D). These results suggest that NasT is produced in an active form.

NasT acts positively within the *nasAB* leader region. To test whether NasT is involved in regulation within the *nasAB* leader region, a bacterial two-vector system was constructed. The system is composed of a protein expression plasmid and a heterologous fusion plasmid under *lac*UV5 promoter control. The protein expression plasmid was derived from pDK6 that has a *tac* promoter followed by a multiple cloning site (MCS). For protein expression, *nasS* or *nasT* was cloned into the *Eco*RI-*Bam*HI region of the MCS. The plasmid carrying *lac*UV5-*nasA-lacZ* fusion was engineered from the plasmid pBT, which contains a *lac*UV5 promoter upstream of the bacteriophage λ cI gene. To construct the *lac*UV5-*nasA-lacZ* fusion, the λ cI gene was replaced with the *nasA-lacZ* cassette. The cassette has the *nasA* leader sequence extending into *nasA* 5' end 162 nucleotides fused with *lacZ*. The pair of plasmids for both protein expression and *lacZ* fusion were co-transformed into *E. coli* MC1061 for β-galactosidase assays.

The *lac*UV5-*nasA-lacZ* fusion had low level expression of *lacZ* in *E. coli*. Deletion of the terminator hairpin III resulted in a 15-fold increase in β-galactosidase activity (Figure 2). Co-expression of *nasT* with *lac*UV5-*nasA-lacZ* increased *nasA* gene expression more than 30-fold. However, the effect of NasT on *lacZ* expression was partially reduced when hairpin II was disrupted and virtually abolished when part of the promoter-proximal hairpin I was deleted. The results presented here are consistent with the observations of

the *in vivo* mutational analyses of the *nasAB* leader in *A. vinelandii*: deletion of the intrinsic terminator hairpin III led to constitutive expression of Φ(*nasA-lacZ*); the integrity of the promoter-proximal hairpin I is required for antitermination; the regulatory role of the middle hairpin II is not significant, but deletion may affect the stabilities of hairpin I and III. These results suggest that NasT may mediate antitermination within the *nasAB* leader region. However, further work is still needed to further clarify the molecular mechanism of antitermination.

NasT exists as a dimer or oligomer. Protein sequence alignment showed that NasT residues 63 – 192 are homologous to AmiR residues 68 – 196, with 25% identity and 51% similarity (Figure 2C). Residues 113 – 160 of AmiR form a long α-helix secondary structure, and residues 129 – 160 play a major role in protein dimerization via coiled-coil interactions (26). Bioinformatic analysis predicted that residues 111 – 153 region of NasT also formed a α-helix secondary structure with a coiled-coil motif at residues 113 – 140, suggesting the possibility that protein-protein interactions may occur between NasT polypeptides.

Whether NasT subunits interact was tested using a yeast two-hybrid system. The *nasT* gene or a truncated *nasT* was translationally fused to the GAL4 DNA binding and activation domains in plasmids pGBKT7 and pGADT7. The resulting plasmid pairs were transformed into yeast strain AH109, which carries a *lacZ* reporter downstream of the *MEL1* upstream activation sequence. Transcriptional activation of *lacZ* requires that

GAL4 DNA binding and activation domains come into close proximity, a function that is dependent on the interaction of the fused target proteins.

The results indicate that the two fused NasT peptides have a strong interaction (Table 3). Expression of β-galactosidase activity is higher than the positive control interaction between murine p53 and SV40 large T-antigen (9, 17), indicating NasT peptides have strong affinity. The N-terminal REC domain showed weak interaction when separated from the rest of NasT. The difference of affinity strength between the NasT-NasT and REC-REC interaction suggests that the predicted coiled-coil region in NasT plays a role in NasT-NasT interaction.

NasT and AmiR share similar predicted overall structural conformations and both have been shown to play a positive role in antitermination. They may represent a family of regulator proteins, derived from a common ancestor – a fused protein of the domains REC and ANTAR. A bacterial genome database search identified that NasT homologues are widespread among alpha-, beta, and gamma-proteobacteria groups (data not shown), suggesting regulation mediated by this family of regulators may be common in bacteria.

Modular structure of NasS. NasS has a negative effect on *nasAB* expression (8). Genome sequence annotation confirms that *nasS* is 1,248 bases in length. Protein sequence BLAST searches against the NCBI conserved domain database showed that NasS has a conserved central region homologous to TauA (Figure 2A), a periplasmic

component of bacterial ABC-type sulfonate transporters (10). The central domain of NasS is flanked by sequences that have no homology to the databases. NasS is probably a cytoplasmic protein, as no N-terminal membrane signal peptide or transmembrane structure was identified in the predicted NasS amino acid sequence based on bioinformatic analysis.

The lack of DNA or RNA binding domains and the presence of a chemical binding domain in NasS suggest that NasS may act as a nitrate/nitrite sensor instead of an expression regulator. It was predicted that NasS and NasT constitute a sensor – antiterminator system. (18). However, the mechanism of signal transduction between NasS and NasT is unclear. The presence of an N-terminus REC in NasT suggests that phosphorylation may be involved in signal transduction. However, the NasT REC domain lacks the conserved Lysine residue essential for REC phosphorylation (8). In addition, NasS lacks the kinase domain for auto-phosphorylation in response to substrate signal. Combined data suggest that signal transduction between NasS and NasT uses a mechanism other than phosphorylation.

Stoichiometry of NasS and NasT is essential for regulation of *nasAB*. To test whether alterations in the ratio of NasS and NasT affects *nasAB* expression, the translational fusion of *nasA-lacZ* which contains the -316 to +297 region around the *nasA* promoter region was introduced into the wild type strain containing P*scrX – nasS* or P*scrX – nasT* in the *scr* region. The results of β-galactosidase assays show that induction of P*scrX - nasT* led to constitutive expression of Φ(*nasA-lacZ*) fusion independent of nitrogen

source; Induction of P*scrX* -*nasS* led to lower expression of Φ(*nasA*-*lacZ*) under conditions of ammonium, N_2, or nitrite, but not nitrate (Table 4).

The results presented here suggest that maintenance of the correct stoichiometry between NasS and NasT is required for *nasAB* regulation. Over-expression of NasT leads to constitutive *nasAB* expression under all conditions, even those in which NasA/NasB expression is unnecessary.

Translation of *nasS* and *nasT* is coupled. The ORFs of NasS and NasT overlap by 19 bases (Figure 4A). mRNA secondary structure analysis predicted that both the NasT start codon and NasS stop codon are part of an mRNA secondary structure (Figure 4B), resembling the intergenic regions of translationally coupled cistrons (6, 7, 25).

Translational coupling is a type of regulation that ensures the adjacent genes in a polycistronic mRNA expressed in a balanced manner. For two genes involved in translational coupling, translation of the terminal end of the upstream gene is a prerequisite for the translation of the downstream gene, which lacks the ability to recruit ribosomes efficiently. Translational coupling has been demonstrated to be used widely by viruses and bacteria to keep functionally related proteins at consistent ratios (33). For example, AmiC/AmiR and NifL/NifA are two sensor-regulator pairs that use protein-protein interactions to convey signals between them (22, 36) Both signal transduction

systems employ translational coupling to ensure that the sensors and regulators are produced at similar stoichiometric ratios (6, 7, 25).

A *lac*UV5-*nasT'-lacZ* fusion was constructed to test whether *nasT* has its own SD. In this fusion, the NasT ORF start region spanning from 18 nucleotides upstream of the NasT ATG codon to base 186 was fused to *lacZ* (Figure 5). The plasmids carrying the fusions were integrated into the *vnf* locus of the *A. vinelandii* genome using single-crossover recombination (Wang et al, submitted for publication). The *lac*UV5-*nasT'-lacZ* fusion displayed constitutive expression, confirming that *nasT* has its own SD sequence. We suspect this SD is the AGGA sequence eight nucleotides upstream of the ATG start codon (Figure 3B).

To investigate whether translational coupling is involved in *nasST* expression, two *lac*UV5- *nasST'-lacZ* fusions were constructed (Figure 5). The *nasST'* fragments in the fusions range from the *nasS* leader sequence to 186 nucleotides in the 5' end of *nasT* with a deletion of the central region (nts 169-1167) of *nasS*. In addition to deletion of the *nasS* central region, one of the fusions has the original Proline CCC codon (nt 1168-1170) in *nasS* replaced with the translation stop codon TGA. The TGA null mutation in the *nasS* central region should lead to premature termination of translation of *nasS* mRNA and the dissociation of the ribosome before reaching the genuine *nasS* stop codon; In comparsion, a fusion with the *nasS* central deletion but no CCC replacement should result in a shortened polypeptide, but should not affect the translating ribosome from reaching the 3'

end of the ORF. β-galactosidase assays show that premature termination of *nasS* translation reduced *lacZ* fusion expression by three-fold (Figure 4), suggesting that the *in vivo* translation of *nasT* is regulated in response to NasS translation.

CONCLUDING REMARKS

Previous work demonstrated that the products of *nasST* are involved in *nasAB* regulation (8), and potential roles of NasS and NasT in this regulation have been postulated (2, 18, 24, 28). The results presented here provide direct evidence that NasT is produced in an active form and acts within the *nasAB* leader region, possibly as an antiterminator. The modular characteristic of NasS suggests that it functions as a nitrate sensor. The requirement of consistent NasS and NasT stoichiometry for *nasAB* regulation suggests that NasS regulates the activity of NasT using a steric hindrance mechanism.

ACKNOWLEDGEMENTS

We thank Leland S. Pierson III, and Eduardo Santero for discussion, advice, and critical review of the manuscript.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant Characteristic(s)[a]	Source or reference
A. vinelandii		
UW136	Rif^r derived from strain UW (ATCC 13705)	(1)
UW1	*Nif*$^-$	(31)
AVW362	*Nif*$^-$ Δ*nasST*::Gm	This work
AVW599	Δ*scr:: nasS*-Km^r	This work
AVW600	Δ*scr:: nasT*-Km^r	This work
AVW618	Δ*scr:: nasS*-Km^r *vnf*::Φ(*nasA-lacZ*)	This work
AVW620	Δ*scr:: nasT*-Km^r *vnf*::Φ(*nasA-lacZ*)	This work
AVW718	*nif*$^-$Δ*nasST*::Gm^r Δ*scr*:: *nasS*-Km^r	This work
AVW719	*nif*$^-$Δ*nasST*::Gm^r Δ*scr*:: *nasT*-Km^r	This work
AVW903	UW136 derivative, *vnf*::Φ(*lac*UV5-*nasT'-lacZ*)	This work
AVW923	UW136 derivative, *vnf*::Φ(*lac*UV5-*nasST'-lacZ*) with *nasS* (nt 169—1167) deletion	This work
AVW924	UW136 derivative, *vnf*::Φ(*lac*UV5-*nasST'-lacZ*) with *nasS* (nt 169—1167) deletion and nt 1168-1170 CCC→TGA mutation	This work
E. coli		
DH5α	*supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 hi-1 relA1*	GIBCO-BRL
MC1061	F$^-$ Δ(*lacI-lacY*)74 *galE15 galK16 relAl rpsL150 spoTl hsdR2* λ$^-$	(3)
Yeast		
S. cerevisiae AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2:: GAL1$_{UAS}$-GAL1T$_{ATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*	Clontech
Plasmids		
pBBR1MCS-2	Km^r; cloning vector	(16)
pBlue-lacZ	Car^r; pBluescript II KS(+) containing *lacZ* from pSUP102::Tn*5*-B21 at *BamH*I-*Xho*I region	(Wang et al, submitted for publication)
pBT	Cam^r; bait vector of BacterioMatch® II two-hybrid system	Stratagene
pBTW	Cam^r; derivative of pBT, expression vector	This work

pDB1332	Car^r; Expression vector, containing an *A. vinelandii* P*scrX* promoter	(12)
pDB1332-S	Car^rKn^r; pDB1332 carrying *nasS* at the *Pci*I-*Bgal*II region followed by a Kn^r gene	This work
pDB1332-T	Car^rKn^r; pDB1332 carrying *nasT* at the *Pci*I-*Bgal*II region followed by a Kn^r gene	This work
pDK6	Km^r; protein expression vector	(15)
pDK6-S	Km^r; pDK6 carrying *nasS* at *EcoR*I-*Bam*HI region	This work
pDK6-T	Km^r; pDK6 carrying *nasT* at *EcoR*I-*Bam*HI region	This work
pGADT7	Car^r; GAL4 activation domain (AD) fusion two-hybrid vector	Clontech
pGAD7-T	Car^r; Fusion between the GAL4 AD and SV40 large T-antigen,	Clontech
pGAD7-nasT	Car^r; *nasT* cloned into the *Nde*I-*EcoR*I site of pGADT7	This work
pGAD7-nasT$_{REC}$	Car^r; *nasT* N-terminal domain cloned into the *Nde*I-*EcoR*I site of pGADT7	This work
pGBKT7	Km^r; GAL4 binding domain (BD) fusion two-hybrid vector	Clontech
pGBK7-53	Km^r; Fusion between the GAL4 BD and murine p53	Clontech
pGBK-Lam	Km^r; Fusion between the GAL4 BD and human lamin C	Clontech
pGBK7-nasT	Km^r; *nasT* cloned into the *Nde*I-*EcoR*I site of pGBKT7	This work
pGBK7-nasT$_{REC}$	Km^r; *nasT* N-terminal domain cloned into the *Nde*I-*EcoR*I site of pGBKT7	This work
pIC20H	Car^r; cloning vector	(21)
pMAS20	Car^r; pTZ19R carry *nasST* sequence	(8)
pTn*Mod*-OGm	Gm^r; pMB1, *mob*_ Tn5 *tnp*	(4)
pVnflacZa	Car^r; translational *lacZ* fusion probe vector	(Wang et al, submitted for publication)
pWB244	Car^r; pIC20H carrying *nasST* sequece	This work
pWB296	Gm^r; pWB244 Δ*nasST*::Gm^r	This work
pWB552	Car^r, pVnflacZa carrying Φ(*nasA-lacZ*)	(Wang et al, submitted for publication)
pWB555	Cam^r, pBTW carrying *lac*UV5-*nasA-lacZ* fusion	This work

pWB643	Car^r; pVnflacZa carrying Φ(*nasA-lacZ*) with all leader hairpins deleted	(Wang et al, submitted for publication)
pWB650	Car^r; pVnflacZa carrying Φ(*nasA-lacZ*) with the 5' stem of the leader the antiterminator hairpin deleted	(Wang et al, submitted for publication)
pWB664	Car^r; pVnflacZa carrying Φ(*nasA-lacZ*) with the 3' stem of leader middle hairpin deleted	(Wang et al, submitted for publication)
pWB692	Cam^r; pBTW carrying *lac*UV5-*nasA-lacZ* fusion with all leader hairpins deleted	This work
pWB901	Cam^r; pBTW carrying *lac*UV5-*nasA-lacZ* fusion with the 5' stem of the leader the antiterminator hairpin deleted	This work
pWB902	Cam^r; pBTW carrying *lac*UV5-*nasA-lacZ* fusion with the 3' stem of leader middle hairpin deleted	This work
pWB903	Car^r; pVnflacZa carrying *lac*UV5-*T'-lacZ* fusion	This work
pWB908	Car^r; pVnflacZa carrying Φ(*nasA-lacZ*) with the terminator hairpin deleted	(Wang et al, submitted for publication)
pWB910	Cam^r; pBTW carrying *lac*UV5-*nasA-lacZ* fusion with the terminator hairpin deleted	This work
pWB923	Car^r; pVnflacZa carrying *lac*UV5-*nasST'-lacZ* fusion with *nasS* codon 56 — 388 deletion	This work
pWB924	Car^r; pVnflacZa carrying *lac*UV5-*nasST'-lacZ* fusion with *nasS* codon 56 — 388 deletion and 389 CCC→ TGA mutation	This work

[a] Abbreviations: Car, carbenicillin; Cam, Chloramphenicol; Gm, Gentamicin; Km, Kanamycin; Rif, rifampicin.

TABLE 2. Primers used in this study

Name	Sequence (5'-3')[a]	Restriction enzyme[b] and use
1BamH162innasA	TGGATCCATGCCGGCCAGCACCGGAGAG	*Not*I; Clone *nasA-lacZ* fragment
75pblueconfirmr1	GTATTACCGCCTTTGAGTG	Clone *nasA-lacZ* fragment
76pBTNotIr	AGCGGCCGCTCACAATTCCACAC	PCR pBT
BglII-nasS	ATAAGATCTTTAGGAGGATGCGCAG	*Bgl*II; Clone *nasS* into pDB1332, pDK6
BglII-nasT	ATAGATCTCAGCTTCCCAGCATGTCGTGCATG	*Bgl*II; Clone *nasT* into pDB1332, pDK6
BTr	TCCGTTGTGGGGAAAGTTATC	PCR pBT
EcoRI-nasS	ATAGAATTCCATGACAGACCACCACGCA ACTTC	*Eco*RI; Clone *nasS* into pDK6
EcoRI-nasT	TCGAATTCCATGCTGCGCATCCTCCTG	*Eco*RI; Clone *nasT* into pDK6
EcoRITr	AGAATTCTCAGCTTCCC AACATG	*Eco*RI; Clone *nasT* into pGAD7 or pGBK7
EcoRITr2	TTGAATTCTCAGCGCTGCGCCTGGATGCCCTC	*Eco*RI; Clone *nasT* N-terminal domain into pGAD or pBGK
KninBBR1f	GGGCTTACATGGCGATAG	Amply Kn^r cassette from pBBR1MCS2
KninBBR1r	CCGAAGCCCAACCTTTC	Amply Kn^r cassette from pBBR1MCS2
nasSf	CCAGGGACTGTACGGACTGATC	*nasST* deletion confirmation
nasSr	GGCGCATCAG GGTATAG	*nasST* deletion confirmation
NdeITf	ACATATGCTGCGCATCCTCCTGAT C	*Nde*I; Clone *nasT* into pGAD7 or pGBK7
O13NotII+77	AGAGCGGCCGCCGACAGATCACATAAG	*Not*I; Clone *nasA-lacZ* fragment
O20NotI-Sf	AGAGCGGCCGCGGTCCCAACGGCGGGG	*lac*UV5-*nasST'-lacZ* construction
O21nasS	GATCAACCTGGGCTTCATGCCCGCCA	*lac*UV5-*nasST'-*

	CGGCCATGCGCAGC	*lacZ* construction
O22nasS	GATCAACCTGGGCTTCATGTGAGCCA CGGCCATGCGCAGC	*lac*UV5-*nasST'*-*lacZ* construction
O23nasS	CATGAAGCCCAGGTTGATC	*lac*UV5-*nasST'*-*lacZ* construction
O26BamHItr	CCGGATCCATCACGTCGCGGCCGGGA GATTC	*lac*UV5-*nasT'-lacZ* construction
O27NotIinS	CCGCGGCCGCTGACCAAGGACCATCC CCATG	*lac*UV5-*nasT'-lacZ* construction
PciI-nasS	ATACATGTCCGACCACCACGCAACTT C	*Pci*I; Clone *nasS* into pDB1332
PciI-nasT	ATACATGTTGCGCATCCTCCTGATCAA CG	*Pci*I; Clone *nasT* into pDB1332
PDBK	GTCACTTTAAAATTTGTATAA	pGBK clone confirmation
PGAD	AGATGGTGCACGATGCACAG	pGAD clone confirmation
T7	TAATACGACTCACTATAGGGC	pGAD or pGBK clone confirmation

Table 3. NasT dimerization assay in a yeast two-hybrid system[a]

Fusion[b]		**β-Galactosidase activity (Miller units)[c]**
BD	AD	
NasT	NasT	35 ± 3
T_{REC}[f]	T_{REC}	2 ± 0
NasT	Vector only	<1
Vector only	NasT	<1
T_{REC}	Vector only	<1
Vector only	T_{REC}	<1
Control (+)		12 ± 0[d]
Control (-)		1 ± 0[e]

[a] All strains are derivatives of yeast strain AH109 carrying two corresponding plasmids.

[b] BD, fusion to GAL4 DNA-binding domain of plasmid pGADT7; AD, fusion to GAL4 transcriptional activation domain of the plasmid pGBKT7.

[c] β-Galactosidase assays of yeast strains were performed by the protocols supplied by the manufacturer (Clontech). All data are presented as the mean values of three replicates ± standard deviations from a representative experiment.

[de] Positive are plasmids pGBKT7-53 +pGADT7-RecT; negative control are pGBKT7-Lam + pGADT7-RecT.

[f] T_{REC} N-terminal REC domain of NasT.

Table 4. Effect of *nasS* and *nasT* over-expression on Φ(*nasA-lacZ*) expression in *A. vinelandii*

Genotype	β-Galactosidase activity (Miller units) Nitrogen source[a]			
	NH_4^+	N_2	NO_2^-	NO_3^-
Wild type	22 ± 3	26 ± 1	149 ± 3	262 ± 3
Δ*scrX*::*nasS*	13 ± 3	11 ± 3	66 ± 3	288 ± 10
Δ*scrX*::*nasT*	357 ± 8	479 ± 1	420 ± 9	372 ± 10

[a] Cultures were grown in BS medium supplemented with nitrogen sources for seven hours before β-galactosidase activity was measured. All data are presented as the mean values of triplicates ± standard deviations from a representative experiment.

Figure Legends

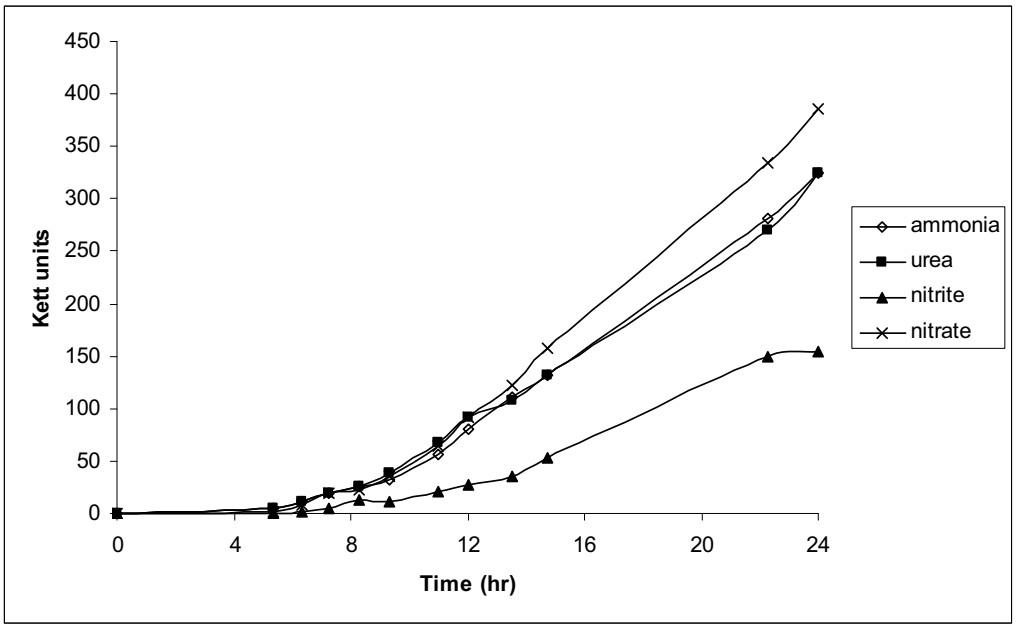
Figure 1. *nasST* mutation and complementation test in *A. vinelandii* UW1. Cultures were grown in BS medium supplemented with nitrogen sources. (A) UW1 (*nif*$^-$). (B) AVW362 (*nif*$^-$Δ*nasST*::Gm^r). (C) AVW718 (*nif*$^-$Δ*nasST*::Gm^r Δ*scr*:: *nasS*-Kn^r). (D) AVW719 (*nif*$^-$Δ*nasST*::Gm^r Δ*scr*:: *nasT*-Kn^r).

Figure 2. Test of NasT regulation at the *nasAB* leader region in *E. coli* 1061. The *nasA-lacZ* cassette is fused the downstream of *lac*UV5 promoter. The *nasS* or *nasT* is induced by *tac* promoter in the plasmid pDK6. The top line represents the sequence ranges from the *nasAB* transcription start site +1 to the +297 nt (the 162nd nt within the *nasA* ORF). The bend arrow represents the transcription direction, facing arrows (designated I, II, and III) represent inverted sequences in the *nasA* leader region. The arrows represent the *lac*UV5 promoter; the empty rectangles represent the deletion regions. The plasmid pairs were co-transformed into *E. coli* M1061 for β-galactosidase activity assays. Two hundred microliter transformant overnight cultures grown in LB broth supplied with kanamycin and chloramphenicol were sequentially pelleted, rinsed with LB broth twice, and diluted in 10 ml of fresh LB broth supplied with kanamycin, chloramphenicol, and 10 μM of IPTG (isopropyl-1-thio-β-D-galactopyranoside), shake for three hours. Two hundred microliter cultures were used for β-galactosidase activity. NT, not tested. All data are presented as the means values for triplicate samples ± standard deviations from a representative experiment.

Figure 3. Modular structures of NasS, NasT, and protein sequence alignment of NasT with AmiR. The numbers above the bar or sequence indicate amino acid residue positions. (A) NasS has a central conserved region homologous to TauA (Shaded area). (B) NasT has an N-Terminal REC domain and C-terminal ANTAR domain (32). (C) Sequence alignment of AmiR and NasT. The coiled-coil regions are underlined with dish-lines. For AmiR, coiled-coil region has been experimentally confirmed (26).

Figure 4. Overlap region of *nasS* and *nasT* open reading frames. The *nasT* start codon ATG and *nasS* stop codon TGA are bold-faced; the presumptive Shine-Dalgarno sequence of *nasT* is underlined. (A) Letters above the DNA sequence represents C-terminus of NasS; letters below the DNA sequence represents N-terminus of NasT. (B) The predicted secondary structure around the *nasS* and *nasT* overlap region.

Figure 5. Translational coupling of *nasS* and *nasT* in *A. vinelandii*. The top line represents the NasS and NasT ORFs with numbers above the bar indicating the nucleotide residues. 186_{nasT} is the 186^{th} nt of *nasT*. The arrows represent the *lac*UV5 promoter; the empty rectangles represent the deletion regions; TGA represents the codon CCC→TGA replacement. Values on the right show β-galactosidase activity. Cultures were grown in BS medium supplemented with the indicated nitrogen sources for seven hours before β-galactosidase activity was measured. All data are presented as the mean values of three replicates ± standard deviations from a representative experiment.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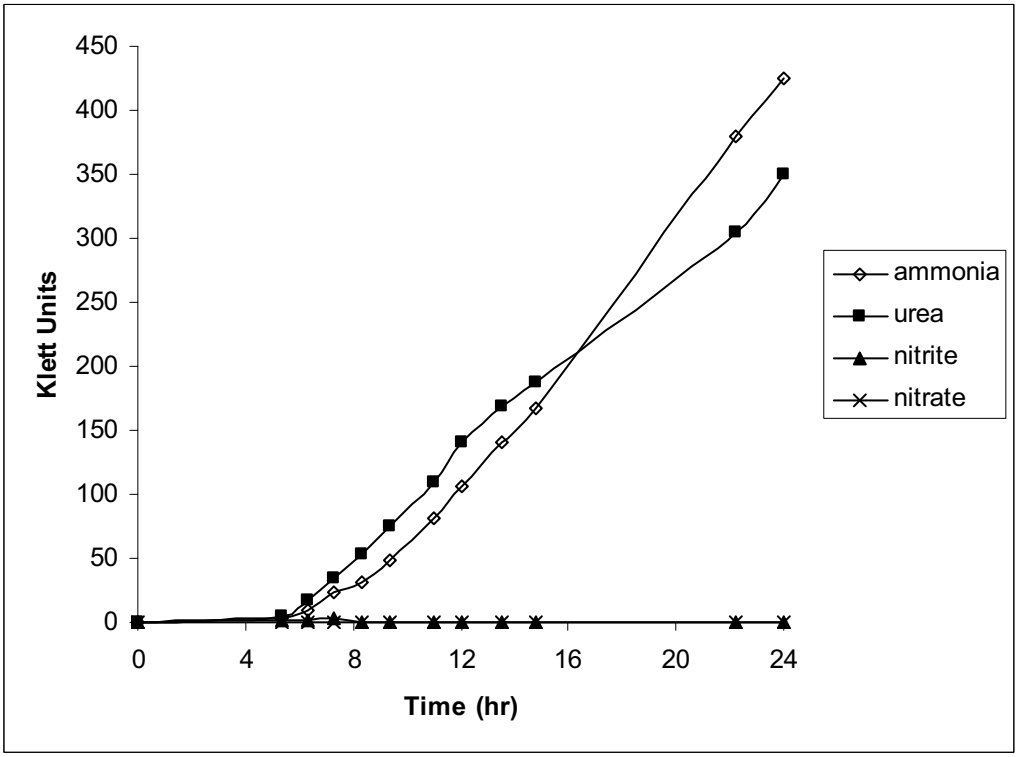
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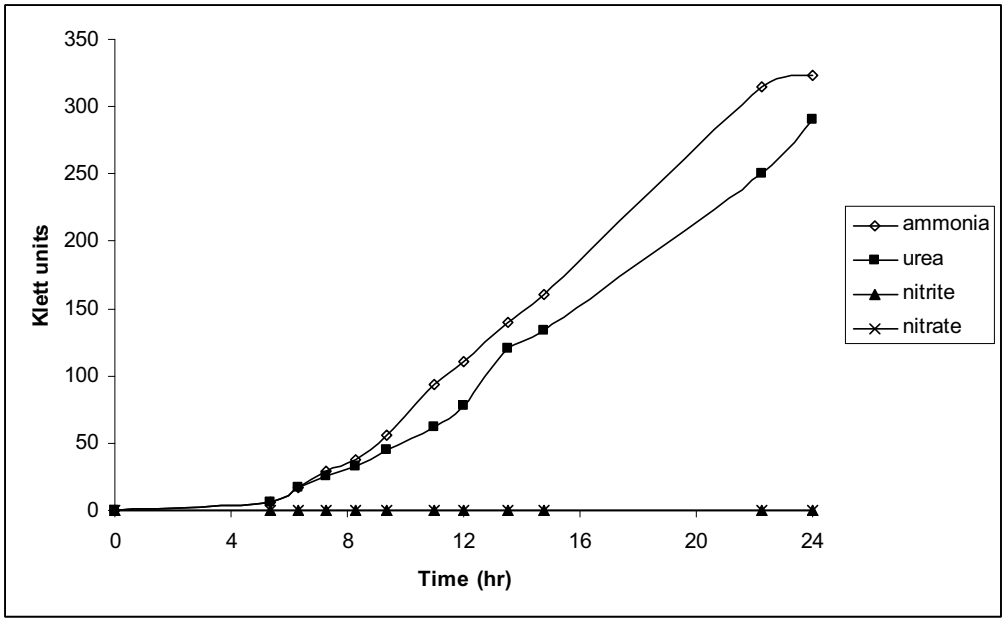
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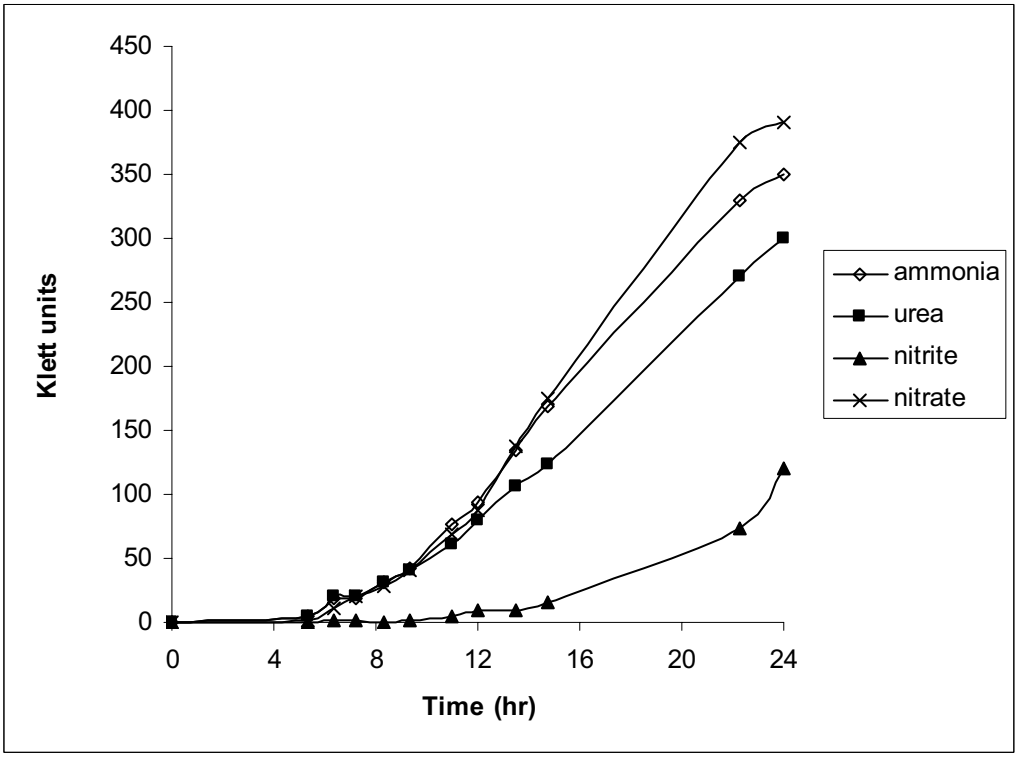
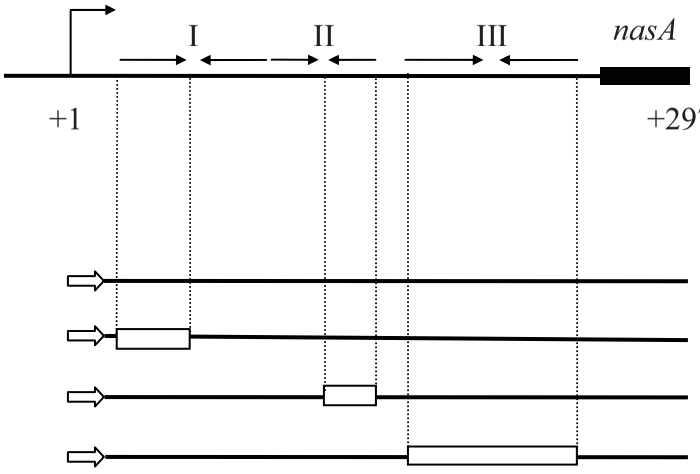
nitrate

C


450
400
350
300
250
200
150
100
50
0
Klett units
0
4
8
12
16
20
24
Time (hr)
ammonia
urea
nitrite
nitrate

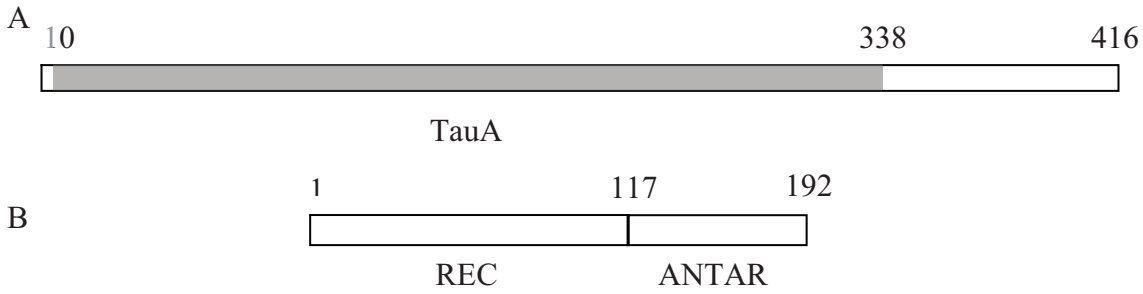
D

Figure 1.



β-Galactosidase activity (Miller Units)			
pDK6 (vector alone)	NasS	NasT	β-galactosidase ratio(+*nasT*/-*nasT*)
235 ± 2	234 ±5	7917 ± 233	33.7
341 ± 18	NT	532 ± 14	1.6
512 ± 4	NT	1788 ± 5	3.5
3524 ± 56	NT	2995 ± 9	0.9

Figure 2.




```
                *         20         *         40         *         60
AmiR : MSANSLLGSLRELQVLVLNPPGEVSDALVLQLIRIGCSVRQCWPPPESFDVPV-------- :  53
NasT : ~~~~~~~~~~~MLRILLINDTPKKVGRLKSALVEAGFEVVD--ESGLTIDLPVRVEAVRP :  47

                *         80         *        100         *        120
AmiR : DVVFTSI-FQNRHHDEIAALLAAGTPRTTLVALVEYESEAVLSQIIELECHGVITQPLDA : 112
NasT : DVILIDTESPGRDVMEQVVLVSRDRPR-PIVMFTDEHDEQVMRQAIQAGVSAYIVEGIQA : 106

                *       140         *       160         *       180
AmiR : HRVLPVL-VSARRISEEMAKLKQKTEQLQERIAGQARINQAKALLMQRHGWDEREAHQYL : 171
NasT : QRLQPILDVAMARFESDQA-LRAQLQAREAQLAERKRVELAKGLLMKMKNCSEEEAYTLM : 165

                *       200
AmiR : SREAMKRREPILKIAQELLGNEPSA~~~ : 196
NasT : RRQAMSRQQKLIQVAEQVIAMHDMLGS* : 192
```

Figure 3

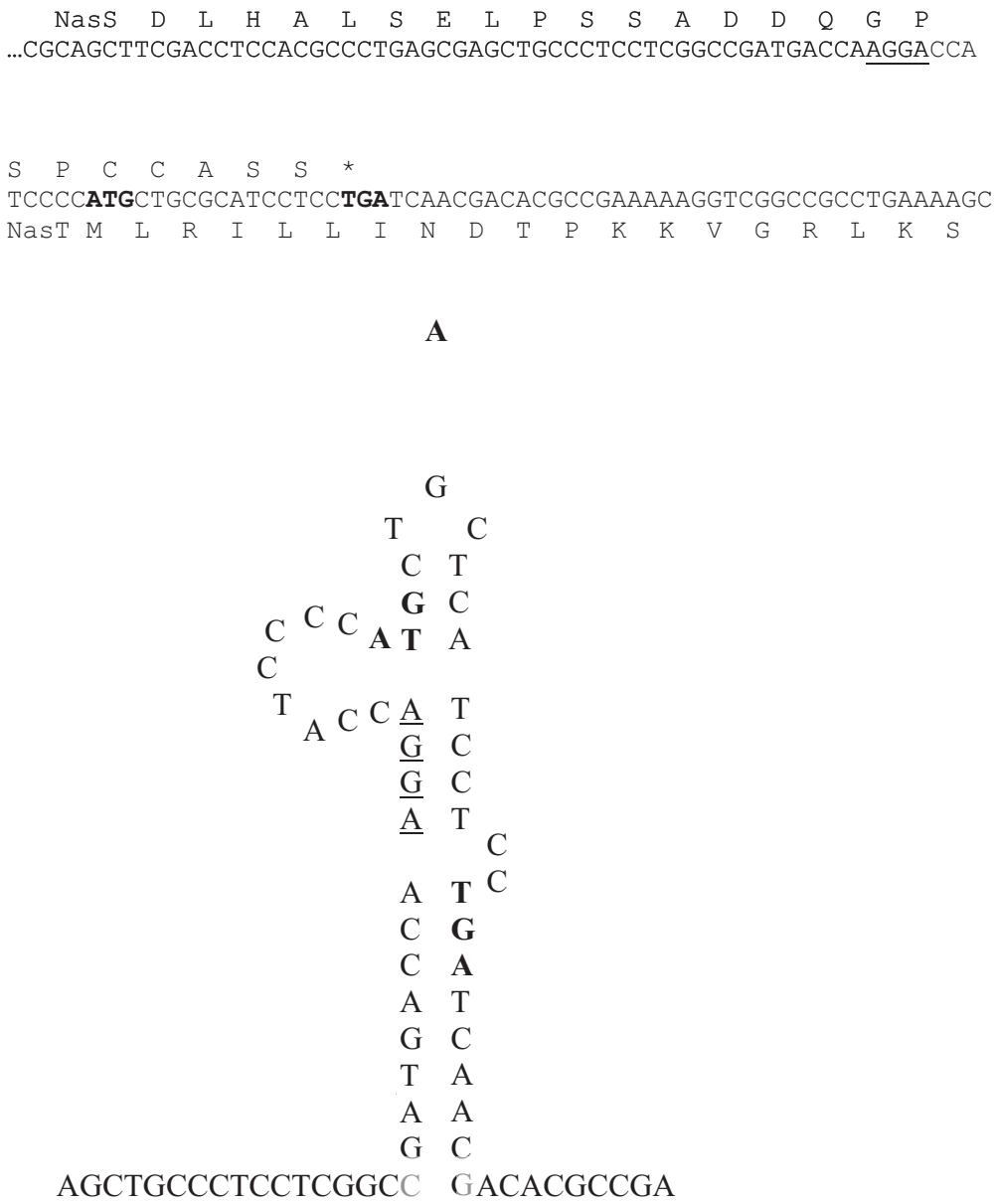
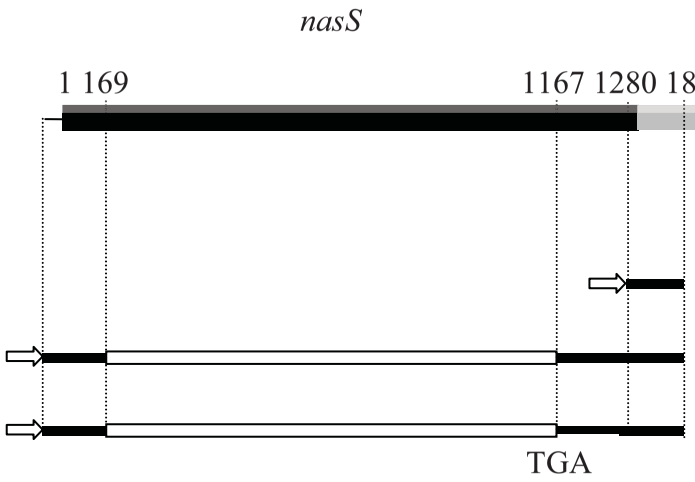

NasS D L H A L S E L P S S A D D Q G P
…CGCAGCTTCGACCTCCACGCCCTGAGCGAGCTGCCCTCCTCGGCCGATGACCAAGGACCA
S P C C A S S *
TCCCCATGCTGCGCATCCTCCTGATCAACGACACGCCGAAAAAGGTCGGCCGCCTGAAAAGC
NasT M L R I L L I N D T P K K V G R L K S
A
AGCTGCCCTCCTCGGCC GACACGCCGA
B

Figure 4



β-galactosidase activity (Miller unites)	
NO_3^-	NH_4^+
461 ± 4	517 ± 11
392 ± 4	354 ± 4
134 ± 1	124 ± 2

Figure 5