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RESEARCH LETTER

Translational coupling of *nasST* expression in *Azotobacter vinelandii* prevents overexpression of the *nasT* gene

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Abstract

The *nasST* operon encodes the transcriptional regulators of assimilatory nitrate reductase operons in phylogenetically diverse bacteria. NasT is a RNA-binding antiterminator and helps RNA polymerase read through the regulatory terminator sequences upstream of the structural genes. NasS senses nitrate and nitrite and regulates the activity of NasT through stoichiometric interaction. In this study, we analyzed the *nasST* sequence in *Azotobacter vinelandii* and revealed that the *nasS* and *nasT* genes overlap by 19 nucleotides. Our genetic analyses suggested that translational initiation of NasT was coupled with NasS translation, a regulatory mechanism that prevents overproduction of NasT. The significance of tight control of *nasT* expression was demonstrated in a *nasT*-overexpression strain, where expression of the assimilatory nitrate reductase operon was deregulated.

Introduction

The protein products of the *nasST* operon constitute a nitrate and nitrite sensor/transcriptional antiterminator regulatory system that regulates induction of assimilatory nitrate reductase operons in *Azotobacter vinelandii*, *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *P. putida Rhodobacter capsulatus*, and potentially many other bacterial species (Gutierrez *et al.*, 1995; Morth *et al.*, 2004; Caballero *et al.*, 2005; Pino *et al.*, 2006; Luque-Almagro *et al.*, 2011, 2013; Romeo *et al.*, 2012). A recent publication also showed that the NasS/NasT system is indirectly involved in the regulation of the genes that encode nitrous oxide reductase and periplasmic nitrate reductase in the dissimilatory denitrification pathway in *Bradyrhizobium japonicum* (Sanchez *et al.*, 2014). NasT is synthesized as an active RNA-binding regulator and helps RNA polymerase read through the regulatory terminator sequence that is upstream of associated structural genes (Ramesh *et al.*, 2012; Wang *et al.*, 2012). The genetic function of NasT is under the strict control of NasS. Unlike classic two-component regulatory systems where the sensor protein modulates the activity of the cognate regulator protein through covalent modification, NasS regulates NasT through direct protein–protein interaction (Lin & Stewart, 1998; Luque-Almagro *et al.*, 2013). In the absence of cellular nitrite and nitrate, NasS sequesters NasT, and they form a heterotetramer composed of two units of each protein (Luque-Almagro *et al.*, 2013; Sanchez *et al.*, 2014). Conversely, binding of nitrate or nitrite to NasS triggers protein conformational changes and drives the dissociation of the complex, relieving NasT from the constraint.

Stoichiometric interaction of NasS and NasT implies that there should be regulatory mechanisms that coordinate the expression of NasS and NasT, because oversupplied NasT proteins would drive the transcription of the

FEMS MICROBIOLOGY LETTERS

target operons in the absence of nitrate and nitrite. The *nasST* operon was first identified in *A. vinelandii* as essential for the induction of the assimilatory nitrate reductase operon (Gutierrez *et al.*, 1995). In this study, we continued the analysis of the *nasST* operon in *A. vinelandii* and showed that the *nasS* and *nasT* genes overlap by 19 nucleotides (nt). Gene overlapping has often been linked with translational coupling, a regulatory mechanism that coordinates the translational initiation of the downstream gene with the translation of the upstream gene (Merino *et al.*, 2008; Levin-Karp *et al.*, 2013). We thus explored a potential expressional linkage between the *nasS* and *nasT* genes in this bacterium. In addition, we tested the effect of *nasT* overexpression on the regulation of the assimilatory nitrate reductase operon.

Materials and methods

Strains, plasmids, and growth conditions

All strains and plasmids used in this study are listed in (Table 1). *Azotobacter vinelandii* and derivative strains were grown in Burk's nitrogen-free salts (BS) medium (Newton *et al.*, 1953) supplemented with 1% sucrose or glucose as indicated. Nitrogen sources were supplied into the BS medium at the following concentrations: 15 mM ammonium acetate, 10 mM urea, 10 mM KNO_3, and 2.8 mM $NaNO_2$. Antibiotics were added when appropriate at the following concentrations: carbenicillin (20 μg mL^{-1}), gentamicin (0.05 μg mL^{-1}), and kanamycin (2.0 μg mL^{-1}). *Escherichia coli* was grown in Luria broth (0.5% NaCl, 1% Tryptone, and 0.5% yeast extract) or 1.5% LB agar at 37 °C. When appropriate, antibiotics were added as follows: carbenicillin (200 μg mL^{-1}), chloramphenicol (34 μg mL^{-1}), gentamicin (15 μg mL^{-1}), and kanamycin (25 μg mL^{-1}).

Bioinformatic analysis

The sequences of *A. vinelandii* genomes are available at GenBank under accession numbers CP001157.1 and CP005094.1 (Setubal *et al.*, 2009; Noar & Bruno-Barcena, 2013). DNA secondary structure analysis was performed using MFOLD (Zuker, 2003).

DNA manipulation

DNA isolation, digestion, ligation, and cloning were conducted following standard protocols (Sambrook & Russell, 2001). All enzymes were purchased from Fermentas (Glen Burnie, MD). Plasmid isolations were carried out using the GeneJET plasmid miniprep kit (Fermentas). DNA was purified from agarose gels using NucleoSpin Extract II (Clontech Laboratories, Mountain View, CA). *Azotobacter vinelandii* transformations were performed as previously described (Wang, 2009). The oligonucleotides used in this study (Table 2) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). DNA sequencing was performed at the University of Arizona Genomics and Technology Core DNA Sequencing Facility (Tucson, AZ).

Pfu DNA polymerase (Stratagene, La Jolla, CA) was used for PCR in this study. The PCR was carried out as follows: 92 °C for 2 min, then 30 cycles (92 °C for 1 min, 55 °C for 1 min, and 68 °C for 1 min for extension) for amplification, and 1 extension step at 72 °C for 5 min.

Generation of P*lac*UV5- *nasT′-′lacZ* and P*lac*UV5- *nasS′nasT′-′lacZ* reporter strains

The *nasT′* fragment that covers *nasT* 5′-end 186 nucleotides and immediate upstream 18 nucleotides was amplified from pMAS20 using primer pairs 026BamHItr and O27NotIinS (Table 2) and cloned into the NotI–BamHI region downstream of P*lac*UV5 in the plasmid pBTW. The resulting XbaI-P*lac*UV5-*nasT′*-BamHI fragment was subcloned into the XbaI-BamHI region of plasmid pVnflacZa, leading to plasmid pWB903. The *nasS′nasT′* fragments in the P*lac*UV5-*nasS′nasT′-′lacZ* constructs in plasmids pWB923 and pWB924 were created using the overlap extension PCR method as shown in our previous work (Wang, 2009). The *A. vinelandii* transformants with the integrated *nasT′-′lacZ* fusions in the nonessential *vnf* locus were screened as described previously.

Generation of *A. vinelandii nasST* deletion mutants

The 4.0-kb EcoRI fragment containing *nasST* from plasmid pMAS20 was subcloned into the HindIII–HindIII region of plasmid pIC20H via blunt end ligation. The resulting plasmid was digested with SalI and fused with a *Sac*I-Gm^r-*Sac*I cassette from pTnMod-OGm via blunt end ligation, leading to plasmid pWB296. To construct the *nasST* knockout strain, pWB296 was transformed into *A. vinelandii* UW1. Transformants were plated on ammonium-containing BS (BSN) medium supplemented with gentamicin (Gm). Multiple Gm^r transformants were patched onto BSN plates supplemented with carbenicillin (Car). Gm^rCar^s clones were screened, and the Δ*nasST*:: Gm^r allelic replacement was confirmed by colony PCR using primer pairs nasSf and nasSr (Table 2).

Generation of P*scrX-nasS* and P*scrX-nasT* expression strains

The *nasT* gene was amplified from plasmid pMAS20 using primer pairs PciI-nasT and BglII-nasT (Table 2).

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)	Bishop & Brill (1977)
UW1	*Nif*$^-$	Shah *et al.* (1973)
AVW362	UW1 Δ*nasST*::*Gm*^{r}	This work
AVW552	UW136 *vnf*::Φ(*nasA′-′lacZ*)	Wang (2009)
AVW600	UW136 Δ*scr*:: *nasT-Km*^{r} *vnf*::Φ(*nasA′-′lacZ*)	This work
AVW718	UW1Δ*nasST*::*Gm*^{r} Δ*scr*:: *nasS-Km*^{r}	This work
AVW719	UW1Δ*nasST*::*Gm*^{r} Δ*scr*:: *nasT-Km*^{r}	This work
AVW903	UW136 *vnf*::Φ(P*lac*UV5-*nasT′-′lacZ*)	This work
AVW923	UW136 *vnf*::Φ(P*lac*UV5-*nasS′T′-′lacZ*) with *nasS* codons 57 - 389 replaced with CCC	This work
AVW924	UW136 *vnf*::Φ(P*lac*UV5-*nasS′T′-′lacZ*) with *nasS* codons 57 - 389 replaced with TGA	This work
Plasmids		
pBBR1MCS-2	Km^r; cloning vector	Kovach *et al.* (1995)
pBlue-lacZ	Car^r; pBluescript II KS(+) containing *lac*Z from pSUP102::Tn*5*-B21 at BamHI–XhoI region	Wang (2009)
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	Johnson *et al.* (2006)
pDB1332-S	Car^{r}Km^r; pDB1332 carrying *nasS* at the PciI–BglII region followed by a Km^r gene	This work
pDB1332-T	Car^{r}Km^r; pDB1332 carrying *nasT* at the PciI–BglII region followed by a Km^r gene	This work
pIC20H	Car^r; cloning vector	Marsh *et al.* (1984)
pMAS20	Car^r; pTZ19R carry the *nasST* sequence	Gutierrez *et al.* (1995)
pTn*Mod*-OGm	Gm^r; pMB1, *mob*_ Tn5 *tnp*	Dennis & Zylstra (1998)
pVnflacZa	Car^r; translational *lacZ* fusion probe vector	Wang *et al.* (2012)
pWB244	Car^r; pIC20H carrying the *nasST* sequence	This work
pWB296	Gm^r; pWB244 Δ*nasST*::*Gm*^{r}	This work
pWB552	Car^r, pVnflacZa carrying Φ(*nasA′-′lacZ*)	Wang (2009)
pWB903	Car^r; pVnflacZa carrying P*lac*UV5-*nas′T′-′lacZ* fusion	This work
pWB923	Car^r; pVnflacZa carrying P*lac*UV5-*nasS′T′-′lacZ* fusion with *nasS* codons 57 - 389 replaced with CCC	This work
pWB924	Car^r; same as pWB923 except the introduced CCC was replaced with TGA	This work

[a]Car, Carbenicillin; Cam, Chloramphenicol; Gm, Gentamicin; Km, Kanamycin.

Table 2. Primers used in this study

Primer	Sequence* (5′–3′)	Restriction enzyme† and use
BglII-nasS	ATAAGATCTTTAGGAGGATGCGCAG	BglII; Clone *nasS* into pDB1332
BglII-nasT	ATAGATCTCAGCTTCCCAGCATGTCGTGCATG	BglII; Clone *nasT* into pDB1332
KninBBR1f	GGGCTTACATGGCGATAG	Amplify Km^r cassette from pBBR1MCS2
KninBBR1r	CCGAAGCCCAACCTTTC	Amplify Km^r cassette from pBBR1MCS2
nasSf	CCAGGGACTGTACGGACTGATC	*nasST* deletion confirmation
nasSr	GGCGCATCAGGGTATAG	*nasST* deletion confirmation
O20NotI-Sf	AGAGCGGCCGCGGTCCCAACGGCGGGG	P*lac*UV5-*nasS′T′-lacZ* construction
O21nasS	GATCAACCTGGGCTTCATGCCCGCCACGGCCATGCGCAGC	P*lac*UV5-*nasS′T′-lacZ* construction
O22nasS	GATCAACCTGGGCTTCATGTGAGCCACGGCCATGCGCAGC	P*lac*UV5-*nasS′T′-lacZ* construction
O23nasS	CATGAAGCCCAGGTTGATC	P*lac*UV5-*nasS′T′-lacZ* construction
O26BamHItr	CCGGATCCATCACGTCGCGGCCGGGAGATTC	P*lac*UV5-*nasT′-lacZ* construction
O27NotIinS	CCGCGGCCGCTGACCAAGGACCATCCCCATG	P*lac*UV5-*nasT′-lacZ* construction
PciI-nasS	ATACATGTCCGACCACCACGCAACTTC	PciI; Clone *nasS* into pDB1332
PciI-nasT	ATACATGTTGCGCATCCTCCTGATCAACG	PciI; Clone *nasT* into pDB1332

*Underlined sequences indicate restriction sites.
†Restriction enzymes that can digest the underlined sequences.

The PCR product was digested with PciI and BglII and cloned into the PciI–BglII site downstream of the P*scrX* promoter in plasmid pDB1332 (Johnson *et al.*, 2006). The resulting plasmid containing the P*scrX-nasT* fusion was digested with KpnI, blunt-ended, and ligated with PCR fragments containing the kanamycin (Km) resistance gene from pBBR1MCS-2, leading to plasmid pDB1332-T. pDB1332-T was transformed into *A. vinelandii*, and the P*scrX-nasT* fusion was integrated into the sucrose catabolic regulon (*scr*) via double cross-over. The

A. vinelandii Km^rCar^s transformants containing the P*scrX-nasT* fusion were screened as described for generation of *nasST* deletion mutants. Using the same strategy, we made plasmid pDB1332-S that contains the fusion P*scrX-nasS* and created the *A. vinelandii* Km^rCar^s transformants containing the P*scrX-nasS* fusion in the *scr* region.

β-galactosidase assay

β-galactosidase activity in *A. vinelandii* was assayed as described previously (Walmsley & Kennedy, 1991). Briefly, 2 mL 24 h cultures were centrifuged (3000 ***g***), rinsed once with 1 mL complete Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 10 mM dithiothreitol), and re-centrifuged. Cell pellets were dissolved in 1 mL complete Z buffer, and 0.2 mL was used for β-galactosidase assays as described previously. Results were reported as Miller units (MU) (Sambrook & Russell, 2001).

Results and discussion

nasS and *nasT* sequence analysis

In previous work, we cloned *nasS* and *nasT* from the *A. vinelandii* and expressed them in *E. coli* for functional analysis (Wang *et al.*, 2012). Before cloning, we sequenced the *nasST* operon and compared the result with an earlier analysis (Gutierrez *et al.*, 1995). The sequencing results confirmed the accuracy of the *nasT* sequence from the earlier analysis, but also identified a frameshift error that shortened the *nasS* 3′ end by *c.* 160 nt. The redefined *nasS* gene is 1323-nt in length and has its 3′ end extend into the 5′ end of the *nasT* gene by 19-nt (Fig. 1a and Supporting Information, Data S1a). This observation was confirmed later by genome sequencing and annotation (Noar & Bruno-Barcena, 2013). Sequence overlapping is common for gene pairs that are under the control of translational coupling, as independent translational initiation of the downstream gene is

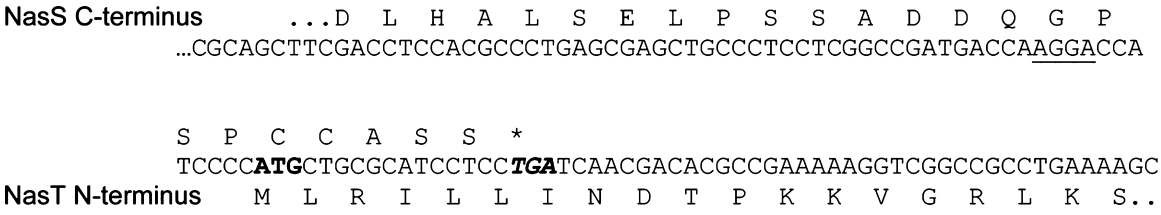

Fig. 1. Characteristics of the overlapping region of *nasS* and *nasT*. The *nasT* start codon [ATG] is bold-faced, and *nasS* stop codon [TGA] bold-faced and italicized; the presumed Shine–Dalgarno sequence of *nasT* is underlined. Letters above the DNA sequence represent the C-terminus of the NasS protein, and letters below the DNA sequence represent the N-terminus of the NasT protein. A star represents the stop codon [TGA].

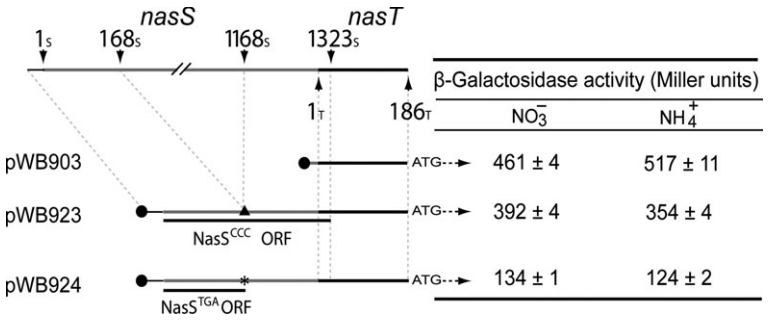

Fig. 2. Expression of the translational *nasT′-′lacZ* fusions in *A. vinelandii*. Left: The top line represents a part of the *nasST* operon (not drawn to scale and see Data S1a for the DNA sequence). From left to right: leader sequence, black line; *nasS*, gray line; *nasT*, black line. The 1_S, 168_S, $1{,}168_S$, and $1{,}323_S$ indicate the nucleotides of the *nasS* gene, and 1_T and 186_T indicate the nucleotides of the *nasT* gene. The *nasS′nasT′* sequences in the *lacZ* fusion constructs are illustrated: *nasT′* in pWB903 includes the 18-nt sequence 5′-TGACCAAGGACCATCCCC-3′ upstream of the *nasT* start codon; the *nasS′* sequences in pWB923 and pWB924 have the *nasS* nt 169 to nt 1167 replaced with either the CCC codon (pWB923) or the TGA codon (pWB924); the relative positions of new ORFs are indicated under the respective DNA sequences. Solid circles represent the P*lac*UV5 promoter; ATG in combination with dash arrow represents the *lacZ* gene. Right: The results of β-galactosidase activity analysis. Bacteria cells were grown in BS medium supplemented with the indicated nitrogen sources, and β-galactosidase activity was measured 7 h after inoculation. All data were means of three replicates ± SDs from a representative experiment.

restricted (Levin-Karp *et al.*, 2013). The presence of this sequence feature in the *nasST* operon could be an indication that *nasST* is under the control of translational coupling.

Efficient *nasT′-′lacZ* expression is dependent on NasS translation

To analyze *nasT* translation, we made two *nasS′nasT′-′lacZ* constructs and analyzed their expression *in vivo*. The carrier of the *lacZ* reporter gene is pVnflanZa, which contains a *c.* 2.8 kb *vnf* sequence that mediates the integration of the plasmid into the nonessential *vnf* locus of the *A. vinelandii* genome via homologous recombination (Wang *et al.*, 2012). The *lacZ* gene in the plasmid begins with the sequence ATGGATCC (BamHI restriction site is underlined) and was fused with *nasT* codon 62 in-frame. In one *nasS′nasT′-′lacZ* construct, the sequence of NasS codons 57-389 was replaced with a proline codon CCC, and the in-frame substitution construct was designated $nasS^{CCC}nasT'$-′*lacZ* (Fig. 2 and Data S1a). For comparative analysis, we replaced this introduced CCC codon with the stop codon TGA, leading to another construct, $nasS^{TGA}nasT'$-′*lacZ*. The introduced TGA marks the 3′ end of $nasS^{TGA}$ that is separated from *nasT* by *c.* 140 nt. To drive transcription of the constructs, we introduced the constitutive P*lac*UV5 promoter upstream of the *nasS′* leader sequence. The plasmids carrying these fusions were then transformed into *A. vinelandii* for expression analysis. The transformant containing $nasS^{TGA}nasT'$-′*lacZ* exhibited cellular β-galactosidase activity of *c.* 130 MU, suggesting that *nasT′-′lacZ* in the transcript underwent

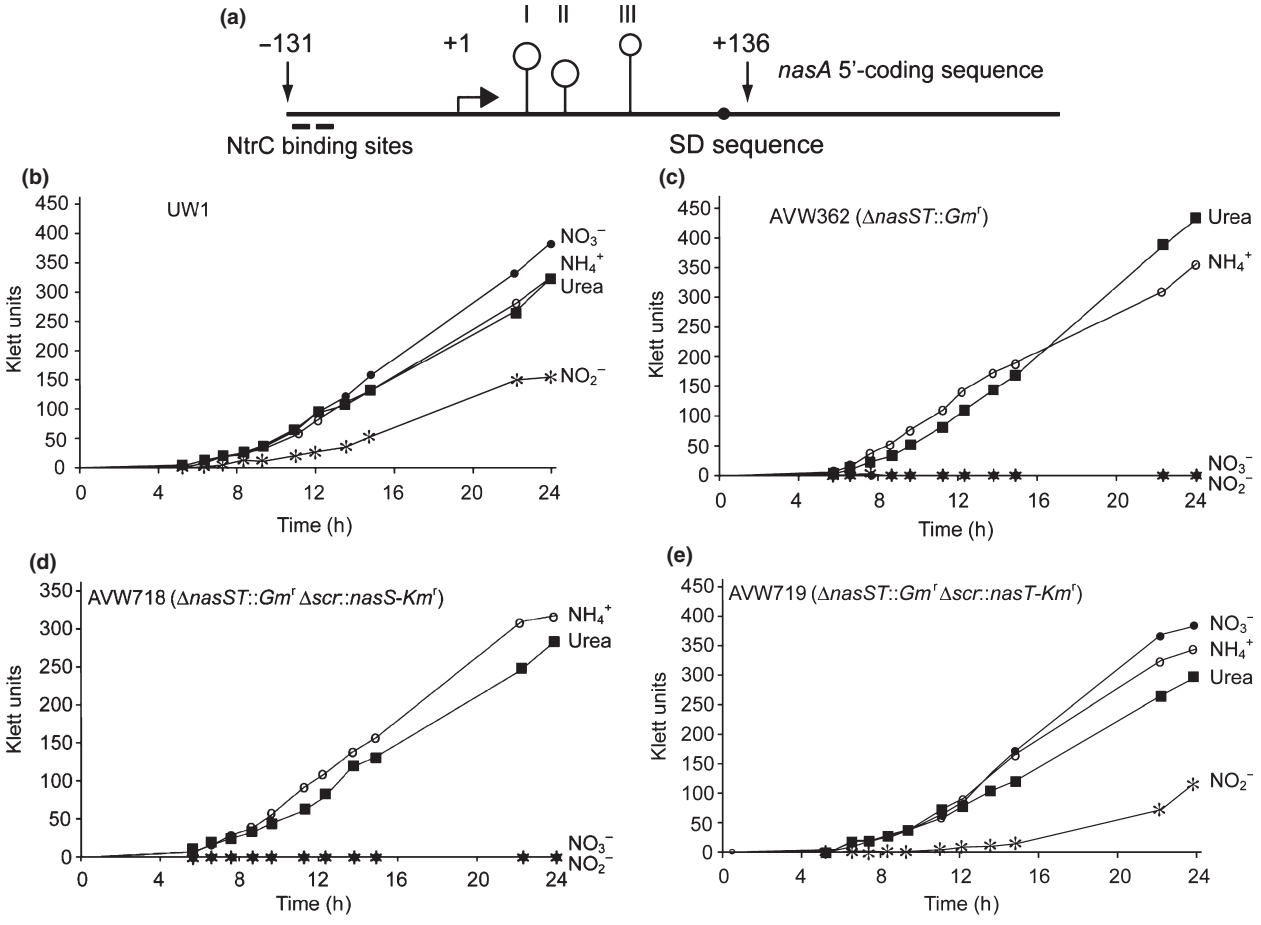

Fig. 3. Induced NasT proteins in the *nasST* mutant supported the utilization of nitrate or nitrite. (a) Schematic representation of the sequences around the 5′ leader region (not drawn to scale) of the *nasACBH* operon (Wang *et al.*, 2012). The bent arrow and + 1 represent the direction of transcription and initiation site; lollipops represent hairpin structures involved in transcriptional termination (Hairpin III) and antitermination (Hairpins I & II) regulation; the Shine–Dalgarno sequence is indicated with a black circle; and NtrC-binding sites are underlined. (b–e) *A. vinelandii* strains were grown in BS medium supplemented with the indicated nitrogen sources: (*) NO_2^-; (●) NO_3^-; (■) urea; and (○) NH_4^+. The genotypes of the bacteria are indicated in the charts: Δ*nasST::Gm*^{r}, *nasST* knockout; Δ*scr::nasS-Km*^{r}, containing a P*scrX-nasS* fusion at the *scr* locus; Δ*scr::nasT-Km*^{r}, containing a P*scrX-nasT* fusion at the *scr* locus.

independent translational initiation; however, the expression was only about one-third of that from *nasS*^{CCC}*nasT*′-′*lacZ*, indicating that translation of the NasS^{CCC} C-terminus prompted the expression of the overlapped *nasT*′-′*lacZ*.

The results of *nasS*^{CCC}*nasT*′-′*lacZ* expression analysis suggested that *nasT* has its own SD sequence that drives translation. In a related analysis, we removed the majority of the *nasS*′ in the *nasS*′*nasT*′-′*lacZ* sequence and fused the P*lac*UV5 promoter with nucleotide 18 immediately upstream of *nasT*′-′*lacZ*. Expression analyses showed that the *A. vinelandii* transformant containing this *nasT*′-′*lacZ* construct exhibited cellular β-galactosidase activities slightly higher than, but similar to, those of the *nasS*^{CCC}*nasT*′-′*lacZ* construct (Fig. 2). This observation confirmed that *nasT* has its own SD sequence, highly possibly the AGGA sequence 8 nt upstream of the *nasT* start codon (Fig. 1).

According to one model of translational coupling, limitations on independent translation of the downstream gene could be due to the sequestration of the SD sequence by secondary structures; disruption of the folding by the ribosome that translates the upstream gene would expose the SD sequence and facilitate the formation of the translational initiation complex and subsequent translation (Govantes *et al.*, 1998). Our sequence analysis did suggest that the translational initiation region of *nasT* could form a potential secondary structure (Data S1a). It will be interesting to learn whether the molecular mechanism underlying *nasST* translational coupling is in accordance with the established model.

nasT overexpression deregulated *nasA*′-′*lacZ* expression

The target sequence of NasT in *A. vinelandii* is located within the 5′-leader sequence of the assimilatory nitrate reductase operon *nasACBH* (Fig. 3a). Although transcription of the *nasACBH* operon is under the control of a constitutively activated promoter, P_{nasA}, transcription prematurely terminates upstream of *nasA* in the absence of a functional NasT (Wang *et al.*, 2012). In one analysis, we inactivated the *nasST* operon by replacing the SalI–SalI region of *nasST* in *A. vinelandii* strain UW1 (*nif*$^-$) with a gentamicin gene, a construct that removed 68% of the *nasS* sequence and 15% of *nasT* (Data S1a and b). This *nasST* knockout prevented the bacterium from using nitrate and nitrite (Fig. 3c). For complementation

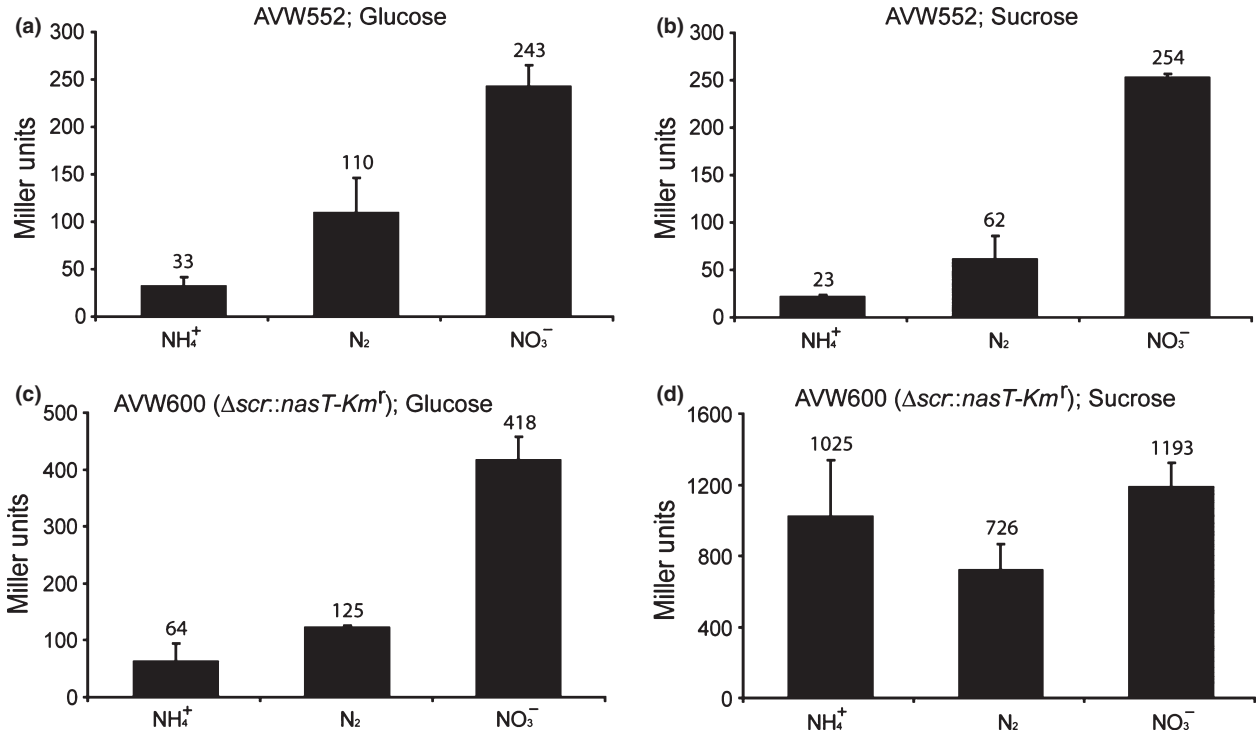

Fig. 4. Effect of *nasT* overexpression on expression of the reporter fusion *nasA*′-′*lacZ*. Bacteria were grown in BS medium supplemented with nitrogen sources as indicated. The carbon source was either glucose or sucrose as indicated. The *nasA*′-′*lacZ* fusion was integrated into the nonessential *vnf* locus of the genomes of AVW552 and AVW600. Genotype: Δ*scr::nasT-Km*^{r}, containing a P*scrX-nasT* fusion at the *scr* locus. All data were means of three replicates ± SDs from a representative experiment.

analysis, we introduced a copy of the *nasT* gene downstream of the sucrose-inducible promoter P*scrX* within the sucrose catabolic regulon (*scr*) region of the *nasST* mutant (Johnson *et al.*, 2006). As Fig. 3e shows induction of P*scrX-nasT* (Δ*scr::nasT-Km*^{r}) by sucrose restored the ability of the *nasST* mutant to utilize nitrate and nitrite for growth, suggesting that the cloned *nasT* gene product was synthesized as a functional regulator.

We then constructed a P*scrX-nasT* fusion in the genome of *A. vinelandii* AVW552 for *nasT* overexpression analysis. AVW552 has the intact *nasST* operon and contains a *nasA′-′lacZ* translational fusion at the *vnf* locus of the genome (Wang *et al.*, 2012). *nasA′-′lacZ* has been used as an expression reporter for studying the transcriptional regulation of the *nasACBH* operon (Wang *et al.*, 2012, 2014). The *nasA′* in the fusion is a 613-nt sequence containing the P_{nasA} promoter extended on to *nasA* codon 54. The activity of P_{nasA} in AVW552 remains relatively constant, regardless of the presence of ammonium, a genetic feature that distinguishes it from NtrC-regulated promoters in other bacteria (Wang *et al.*, 2012). Variations of NasA–LacZ expression under different nitrogen conditions solely reflect the regulatory activity of the NasS/NasT system (Wang *et al.*, 2012, 2014). As shown in Fig. 4a and b, the NasA–LacZ fusion protein was induced specifically by nitrate.

Introduction of *nasT* downstream of the P*scrX* promoter in AVW552 led to strain AVW600. As shown in Fig. 4c, *nasA′-′lacZ* in AVW600 exhibited an expression pattern similar to that of the parental strain AVW552 (Fig. 4a) when the growth media were supplied with glucose as the carbon source, indicating that the P*scrX-nasT* construct had no obvious effect on *nasA′-′lacZ* expression. However, the expression pattern of *nasA′-′lacZ* in AVW600 was dramatically changed when the glucose in the media was replaced with sucrose (Fig. 4d); the bacterium exhibited relatively constant and much higher levels of NasA–LacZ expression under all tested nitrogen conditions, resembling the expression pattern of the *nasA′-′lacZ* deletion mutant that lacks the sequences involved in termination/antitermination in our previous analyses (Wang *et al.*, 2012, 2014). This observation suggests that regulation of *nasA′-′lacZ* expression in AVW600 was disrupted when the P*scrX-nasT* construct was induced by sucrose, highlighting the genetic significance of preventing overexpression of *nasT*.

In summary, this study revealed the translational control of *nasT* expression in *A. vinelandii*, and the significance of this regulation is tied to the genetic function of the NasS/NasT system. The homologs of the *nasST* operon were identified in the genomes of phylogenetically diverse bacteria (Luque-Almagro *et al.*, 2011). Studies on the NasS/NasT systems in multiple bacteria have shown that the modular features and genetic functions of the NasS and NasT proteins from different bacteria are highly conserved (Gutierrez *et al.*, 1995; Romeo *et al.*, 2012; Luque-Almagro *et al.*, 2013; Sanchez *et al.*, 2014). However, the DNA sequences of the *nasST* operons vary considerably among different bacteria. For example, in *P. denitrificans*, the genes are arranged in the orientation of 5′-*nasT*-*nasS*-3′ instead of 5′-*nasS*-*nasT*-3′ as in *A. vinelandii*. Despite the difference of gene organization, the *nasT* and *nasS* genes in *P. denitrificans* showed a 15-nt overlap, suggesting that this *nasTS* operon might also be under the control of translational coupling. It will be of interest to learn whether the mechanisms that coordinated NasS and NasT expression are conserved in different bacteria.

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Dedication (in memoriam): This manuscript is dedicated to the memory of Christina Kennedy (1945–2009).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data. S1. Sequencing features of the *nasST* operon and NasS and NasT proteins.