Biochemical Society Transactions www.biochemsoctrans.org

Bacterial nitrate assimilation: gene distribution and regulation

Víctor M. Luque-Almagro*, Andrew J. Gates†, Conrado Moreno-Vivián*, Stuart J. Ferguson‡, David J. Richardson† and M. Dolores Roldán*[1]

*Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, Edificio Severo Ochoa, 1ª planta, Campus de Rabanales, Córdoba, 14071, Spain, †Centre for Metalloprotein Spectroscopy and Biology, School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K. and ‡Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

Abstract

In the context of the global nitrogen cycle, the importance of inorganic nitrate for the nutrition and growth of marine and freshwater autotrophic phytoplankton has long been recognized. In contrast, the utilization of nitrate by heterotrophic bacteria has historically received less attention because the primary role of these organisms has classically been considered to be the decomposition and mineralization of dissolved and particulate organic nitrogen. In the pre-genome sequence era, it was known that some, but not all, heterotrophic bacteria were capable of growth on nitrate as a sole nitrogen source. However, examination of currently available prokaryotic genome sequences suggests that assimilatory nitrate reductase (Nas) systems are widespread phylogenetically in bacterial and archaeal heterotrophs. Until now, regulation of nitrate assimilation has been mainly studied in cyanobacteria. In contrast, in heterotrophic bacterial strains, the study of nitrate assimilation regulation has been limited to *Rhodobacter capsulatus*, *Klebsiella oxytoca*, *Azotobacter vinelandii* and *Bacillus subtilis*. In Gram-negative bacteria, the *nas* genes are subjected to dual control: ammonia repression by the general nitrogen regulatory (Ntr) system and specific nitrate or nitrite induction. The Ntr system is widely distributed in bacteria, whereas the nitrate/nitrite-specific control is variable depending on the organism.

Bacterial distribution of nitrate assimilatory gene clusters

The nitrate-assimilation process begins with the transport of nitrate into the cell. Nitrate is further reduced to nitrite in a two-electron reaction by a cytoplasmic molybdenum-containing nitrate reductase followed by a six-electron nitrite reduction to produce ammonia by a sirohaem-nitrite reductase [1,2]. Genetic characterization of assimilatory nitrate-reducing systems has been focused mainly on cyanobacteria, but also on heterotrophic bacteria such as *Rhodobacter capsulatus* [3,4], *Klebsiella oxytoca* [5], *Azotobacter vinelandii* [6] and the Gram-positive *Bacillus subtilis* [7]. Studies of nitrate assimilation in heterotrophic bacterial species are scarce; however, examination of available genomes suggests that assimilatory nitrate reductases (Nas) are phylogenetically widespread in bacterial and archaeal heterotrophs [2]. In addition, a nitrate assimilation system has been recently characterized in *Paracoccus denitrificans* [8]. In this organism, a genetic and biochemical analysis has revealed novel insights into bacterial nitrate assimilation. This study has identified a key role for a Rieske-type [2Fe–2S] protein, NasG, encoded by the *nasG* gene (also called *nirD*, *nasD* or *nasE* in other organisms), which is conserved in several bacterial *nas* gene clusters (Figure 1). NasG is essential for coupling of NADH oxidation to both nitrate and nitrite reduction. A three-component Rieske Fe–S protein–nitrate–nitrite reductase system has been proposed, where the Rieske Fe–S protein mediates electron transfer from a single NADH-oxidizing site within the nitrite reductase to the sites of nitrate and nitrite reduction present in the nitrite reductase (NasB) and nitrate reductase (NasC) components respectively, since NasC lacks a nicotinamide–nucleotide-binding domain [8]. In the Gram-positive *B. subtilis*, there is an accessory protein to the nitrate reductase, NasB, which contains FAD. Recently, the nitrate assimilation *nas* gene cluster of the Gram-positive actinomycete *Amycolatopsis mediterranei* strain U32 has been described [9]. Nitrate assimilation genes have been also described and characterized in the haloarchaeon *Haloferax mediteraneii* [10].

The *nas* gene cluster of *P. denitrificans* also contains two genes, *nasA* and *nasH*, that code for a nitrate transporter of the MFS (major facilitator superfamily) and for a nitrite transporter that belongs to the formate–nitrite transporter superfamily respectively (Figure 1). ABC (ATP-binding cassette)-type nitrate/nitrite transporters are also encoded in bacterial *nas* clusters (*nasFED* in *Klebsiella pneumoniae* and *R. capsulatus*), mainly in cyanobacteria (*nrtABCD* in *Synechococcus elongatus*). Another gene present in the *nas* region of some bacteria is *cysG* (Figure 1). CysG is an uroporphyrin-III C-methyltransferase involved in the synthesis of sirohaem, the nitrite reductase cofactor. In

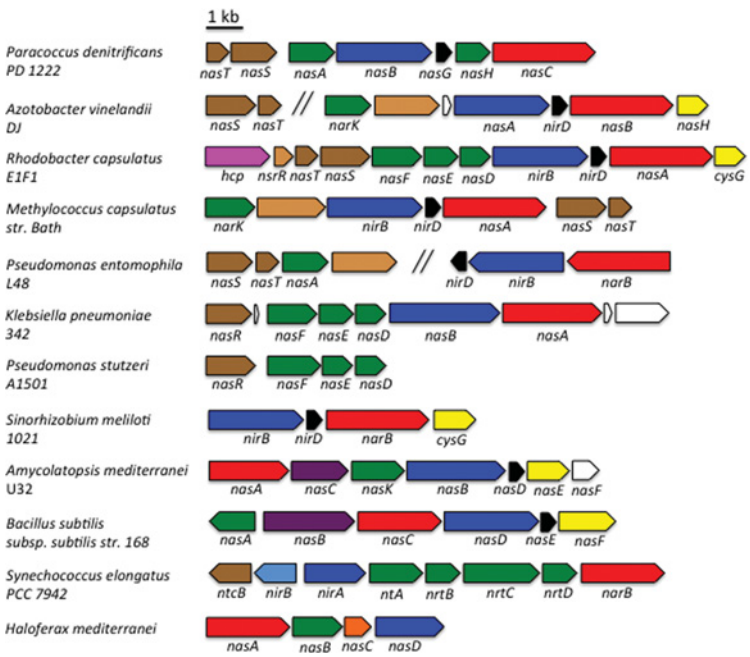
Key words: nitrate assimilation, nitrate reductase, nitrate transport, nitrite reductase, two-component regulatory system.

Abbreviations used: ABC, ATP-binding cassette.

[1]To whom correspondence should be addressed (email bb2rorum@uco.es).

Figure 1 | Bacterial *nas* gene arrangements including regulatory genes for the regulation of nitrate assimilation by the pathway-specific control

Open reading frames that encode the following putative products are coloured as follows: red, nitrate reductase; blue, nitrite reductase; black, Rieske-type ferredoxin; green, NO_3^-/NO_2^- transporters; yellow, uroporphyrin-III C-methyltransferase; brown, nitrate/nitrite regulatory genes; light brown, putative regulatory genes; pink, *hcp* gene encoding a hybrid cluster protein; orange, gene encoding a molybdopterin guanine dinucleotide biosynthesis; purple, FAD-containing proteins; white, other genes.



addition, regulatory genes that respond to the presence of nitrate/nitrite are present in the *nas* gene clusters, such as *ntcB*, *nasR* and *nasS–nasT* genes (Figure 1), and their roles in nitrate assimilation are discussed in the next section. Another regulatory gene is the *R. capsulatus nsrR* gene, which responds to nitrite, acting as a repressor. Curiously, the *hcp* gene encoding a hybrid protein involved in hydroxylamine detoxification is clustered together with the *nas* genes in *R. capsulatus* [3,4] (Figure 1).

The assimilatory reduction of nitrate to ammonium is an energetically expensive process since it requires eight electrons and complex prosthetic groups for the nitrate and nitrite reductase enzymes, in addition to the active nitrate transport. In order to avoid this energetic cost under unnecessary environmental conditions, bacteria have evolved a strict control of the expression of the *nas* system. Thus expression of the *nas* genes is subjected to dual control based on specific nitrate or nitrite induction, and ammonium repression by a general nitrogen-regulatory system.

Nitrate assimilation is regulated by the pathway-specific control

Except in Gram-positive bacteria, nitrate assimilation is regulated by a pathway-specific control through regulatory proteins encoded by genes usually located within the *nas* region (Figure 1). These regulatory proteins belong to three different types: the NtcB regulator of cyanobacteria, and the two-component regulatory system NasST or the NasR regulatory protein of heterotrophic bacteria. NtcB, a LysR family protein, is a transcription activator that enhances transcription in response to nitrite [11]. The molecular mechanism of NasST and NasR is based on nitrate/nitrite sensing and transcription anti-termination. These two functions are performed by either the single-component NasR or the two-component NasST system, where NasS is a nitrate/nitrite sensor and NasT is predicted to be a transcription anti-terminator. NasR and NasT-type proteins are characterized by an RNA-binding domain (ANTAR) involved in transcriptional anti-termination [12]. Sequence analysis suggests that NasR and NasS have two different domains to sense nitrate/nitrite. NasR contains a nitrate- and nitrite-sensing (NIT) domain, also detected in various receptor components of signal transduction pathways in different bacterial lineages [13]. NasS exhibits high sequence similarity to NrtA, the periplasmic component of an ABC-type uptake system for nitrate and nitrite in cyanobacteria [14] (Figure 1), suggesting that NasS could bind nitrate or nitrite by a similar molecular mechanism as NrtA. In addition, the periplasmic leader sequence conserved within cyanobacterial transport systems is absent from the NasS

amino acid sequence. Consequently, NasS is located in the cytoplasm, making its involvement in nitrate uptake unlikely. Instead, NasS may bind nitrate and/or nitrite as part of a cytoplasmic sensing system for transcriptional regulation.

The regulation of nitrate assimilation by NasR has been extensively studied in *K. oxytoca*. In this bacterium, a hairpin structure has been identified in mRNA upstream of the *K. oxytoca nas* operon that causes early termination of transcription [15]. NasR, presumably as a binary complex with nitrate or nitrite, binds to the mRNA transcript, preventing hairpin formation and allowing complete expression of the *nas* genes. The NasST regulatory system has been studied in *A. vinelandii*. In this nitrogen-fixing bacterium, a mutational analysis revealed that NasT is required for the expression of the nitrite–nitrate reductase genes (*nasAB*), whereas NasS plays a negative regulatory role in the synthesis of the nitrate and nitrite reductase [6]. A positive transcriptional regulator was also the function proposed for NasT in *Pseudomonas putida* JLR11A, where a *nasT* mutant was impaired in the use of nitrate and nitrite [16].

Both *nasR* and *nasST* genes are clustered together with other genes involved in nitrate assimilation, but showing different gene arrangement within the *nas* clusters. In some cases, these regulatory genes cluster together genes that code for nitrate/nitrite transporters. In other organisms, the *nasST* genes are located in different loci from the *nas* gene cluster, suggesting that the NasST system could act at distance or even might be involved in the regulation of other metabolic pathways. The analysis of the genome sequences currently available reveals that the NasST system is more widely distributed than NasR. Despite *nasST* having been characterized in *A. vinelandii*, a gammaproteobacterium, genes coding for this two-component regulatory system have been found mainly in Alphaproteobacteria (Rhizobiales and Rhodobacterales) and Betaproteobacteria (Burkholderiales). In contrast, *nasR* is mainly distributed among Gammaproteobacteria (Enterobacteriaceae and Alteromonadales).

In addition to the NtcB, NasR and NasST regulatory systems, some *nas* clusters contain additional putative regulatory genes (Figure 1). For instance, a gene coding for a kinase/phosphatase is present in the *nas* cluster of *A. vinelandii*, *Methylococcus capsulatus* and others, although there is no experimental evidence implicating this gene in regulation of nitrate assimilation. Another example is the *nsrR* gene, which has been identified in the *Rhodobacter capsulatus nas* gene region (Figure 1). The *nsrR* gene product is homologous with a novel nitrite-sensitive transcription repressor of the Rrf2 family that controls expression of the copper nitrite reductase NirK in *Nitrosomonas europea*. Therefore the NsrR protein could repress expression of nitrate assimilation genes in the absence of nitrate or nitrite [4]. Another putative regulatory protein for nitrate assimilation is Hfq, an RNA-binding protein involved in post-transcriptional regulation of gene expression in bacteria. It has been recently described that Hfq is required for optimal nitrate assimilation in the cyanobacterium *Anabaena* sp. strain PCC 7120 and *hfq* orthologues have been annotated in several cyanobacterial genomes [17]. In heterotrophic bacteria, any role of Hfq in the regulation of nitrate assimilation has yet to be esxtablished.

General nitrogen control of the nitrate-assimilation process

In bacteria, carbon, nitrogen and energy status of the cells is sensed and co-ordinated by the PII signal transduction proteins (GlnB and GlnK) [18]. The PII proteins control a wide range of processes related to nitrogen metabolism, such as nitrate assimilation through the global nitrogen control factor NtcA in cyanobacteria [19] or the NtrBC system in heterotrophic bacteria [16,20–25]. Gram-positive bacteria possess a GlnK-like PII protein, which controls the activity of the nitrogen-stress transcription factor TnrA in conjunction with the glutamine synthetase [26].

NtcA mediates ammonia repression of nitrate assimilation in cyanobacteria. The activity of NtcA is indirectly regulated by 2-oxoglutarate through a small PII-binding protein, PipX. The transcriptional activator NtcA is found in all cyanobacterial strains characterized to date [11].

The two-component system NtrBC has been characterized extensively in enteric bacteria [27]. NtrB is a sensor kinase that autophosphorylates on a histidine residue under low nitrogen conditions and also transfers a phosphoryl group to the NtrC response regulator protein on a specific aspartate residue [28,29]. Phosphorylated NtrC acts as a transcriptional activator by oligomerization on the DNA template and has an ATPase activity that is essential for activation of transcription [30,31]. The NtrC members are usually σ^{54}-dependent and they are involved in the transcription of genes related to nitrogen metabolism such as *glnA*, which codes for the glutamine synthetase. However, in *R. capsulatus*, a regulatory two-component NtrBC system has been described in which the NtrC component is not dependent on the σ^{54} factor. The NtrBC system has been described to be the mechanism by which the ammonium represses nitrate assimilation in *K. oxytoca* [20], *A. vinelandii* [21], *Azorhizobium caulinodans* [22], *Azospirillum brasilense* [23], *Rhizobium meliloti* [24], *Pseudomonas aeruginosa* [25] and *Pseudomonas putida* [16]. Downstream of the *ntrBC* genes, some organisms contain the *ntrYX* genes that code for an additional two-regulatory system, NtrYX (Figure 2), which also shows similarity to a sensor/kinase and to regulatory proteins respectively. In *A. caulinodans*, a mutant in the *ntrX* gene was found to be defective in using nitrate as the sole nitrogen source, with reduced *nifA* expression under nitrogen-fixation conditions and a disturbed symbiotic phenotype [22]. In addition, expression of the *ntrYX* operon was derepressed in an *ntrC* mutant grown with nitrate, suggesting an interaction between the *ntrXY/ntrBC* systems [22]. In *A. brasilense*, the NtrYX system is involved in nitrate utilization, and a possible cross-talk between the NtrYX and NtrBC sensor/regulator pairs is also suggested [23,32]. The NtrBC system in *A. brasilense*

Figure 2 | **Bacterial *ntr* gene cluster distribution**

glnA (yellow) codes for the glutamine synthetase, *nifR3* (blue) codes for a TIM-barrel enzyme, *ntrB* (green) and *ntrY* (orange) code for the histidine kinase sensor of the two-regulatory systems NtrBC and NtrYX, and *ntrC* (pink) and *ntrX* (red) code for the response regulatory components. White represents a gene of unknown function.

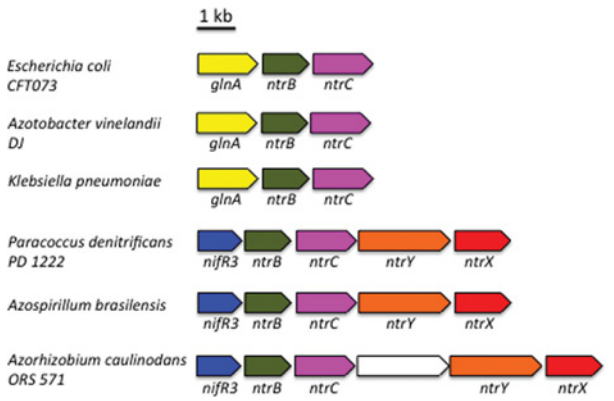
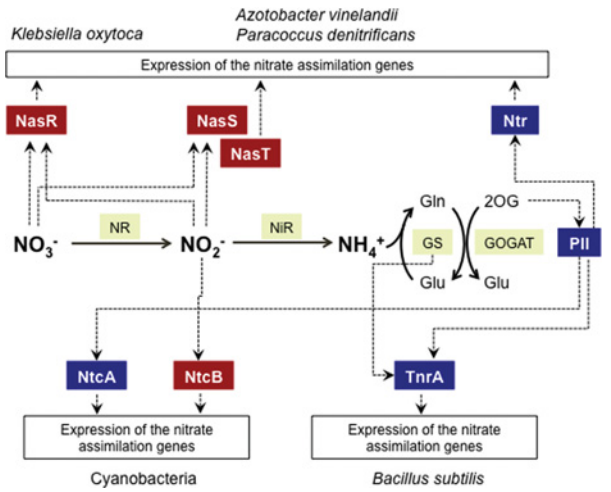


Figure 3 | **Regulatory proteins and signalling network for nitrate and nitrite assimilation in bacteria**

Red boxes, regulatory proteins of the pathway-specific control; blue boxes, regulatory proteins of the general nitrogen control. Abbreviations: GOGAT, glutamate synthase; GS, glutamine synthetase; 2OG, 2-oxoglutarate; NR, nitrate reductase; NiR, nitrite reductase; Gln, glutamine; Glu, glutamate.



is involved in the regulation of nitrate assimilation, the switch-off of nitrogenase by ammonium and ammonium transport [23].

The regulatory protein TnrA of Gram-positive bacteria shows sequence similarity to GlnR, the repressor of the *B. subtilis* glutamine synthetase operon. A *tnrA* mutant of *B. subtilis* was impaired in the use of nitrate as nitrogen source [33]. TnrA activity has been described to be regulated by its interaction with AmtB (ammonia transporter)–GlnK and glutamine synthetase [34].

Intregrating the two regulatory levels that control nitrate assimilation in bacteria

To summarize, nitrate assimilation is subjected to regulation that may differ depending on the organism. In general, nitrate assimilation is controlled at the transcriptional level by nitrate and nitrite induction and by ammonium repression. As an exception, in Gram-positive bacteria such as *B. subtilis*, nitrate assimilation is only regulated by the global nitrogen regulator TnrA, which activates *nas* genes under nitrogen-starvation conditions [35] (Figure 3).

In cyanobacteria, the transcription factor NtcA represses nitrate assimilation genes when ammonium is present, whereas it activates transcription of these genes at a high carbon/nitrogen ratio or nitrogen depletion that is reflected by high 2-oxoglutarate levels through the sensor PII [36–38] (Figure 3). Nitrate assimilation in α-cyanobacteria in only regulated by NtcA; however, β-cyanobacteria require both NtcA and NtcB for expression of the *nas* operon [38]. In these organisms, the regulatory proteins NtcA and NtcB act not only as transcription activators, but also as the sensors of 2-oxoglutarate and nitrite respectively.

In heterotrophs, the only organism in which dual regulation of nitrate/nitrite assimilation has been studied in detail is *K. oxytoca* [20]. In this bacterium, expression of *nas* genes is activated under low nitrogen conditions through the global nitrogen regulator Ntr and by nitrate/nitrite induction through NasR. The *nas* region contains two promoters; one is located upstream of the *nasR* gene and the other upstream of the structural gene operon (*nasF*). It has been demonstrated that these two promoters are targets for the NtrC protein. Thus, under nitrogen-rich conditions, the *nasFEDCBA* operon is repressed both directly (by decreasing Ntr activation of the *nasF* promoter) and indirectly (by decreasing synthesis of the NasR regulatory protein). Because the NasR protein can affect a significant level of transcription anti-termination in the *nasF* operon leader region even in the absence of nitrate or nitrite, it has been proposed that Ntr control of NasR synthesis might provide an additional means of further reducing the basal level of *nasF* operon expression. In addition, an increase in NasR levels under nitrogen-limiting conditions through the phosphorylated form of NtrC serves to sensitize the response of the organism to even relatively low levels of nitrate.

In common with *K. oxytoca*, synthesis of assimilatory nitrate and nitrite reductases in *A. vinelandii* requires the absence of ammonium and the presence of nitrate. In both organisms, equivalent specific regulatory genes (*nasR* and *nasST*) are located upstream of the structural *nas* genes, with an intergenic non-coding region between the regulatory and structural genes. Therefore the dual regulation of nitrate assimilation in *A. vinelandii* is expected to be similar to that described for *K. oxytoca*. Thus expression of the *nasAB* operon under nitrogen-limiting conditions requires the general nitrogen control genes *ntrA* and *ntrC* [39].

However, in contrast with *nasR*, the *nasST* genes involved in the specific regulation by nitrate/nitrite are not regulated by either the Ntr system or the nitrogen source [6]. Accordingly, the integration of nitrate (via NasST) and ammonium (via Ntr) signals in *A. vinelandii*, and of course in other organisms possessing these systems, is still unknown.

Funding

M.D.R. and C.M.-V. thank the Ministerio de Ciencia y Tecnología for supporting the work [grant number BIO2008-04542-C02-01] and the Junta de Andalucía [grant number CVI1728]. V.M.L.-A. was the recipient of a postdoctoral fellowship from the Ministerio de Ciencia y Tecnología, Spain. This work was supported by the Biotechnology and Biological Sciences Research Council [grant numbers BBE0219991 and BBD5230191]. D.J.R. is a Royal Society and Wolfson Foundation for Merit Award Fellow.

References

1 Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R. and Castillo, F. (1999) Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J. Bacteriol. **181**, 6573–6584
2 Richardson, D.J., Berks, B.C., Rusell, D.A., Spiro, S. and Taylor, C.J. (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. **58**, 165–178
3 Cabello, P., Pino, C., Olmo-Mira, M.F., Castillo, F., Roldán, M.D. and Moreno-Vivián, C. (2004) Hydroxylamine assimilation by *Rhodobacter capsulatus* E1F1: requirement of the *hcp* gene (hybrid cluster protein) located in the nitrate assimilation *nas* gene region for hydroxylamine reduction. J. Biol. Chem. **44**, 45485–45494
4 Pino, C., Olmo-Mira, M.F., Cabello, P., Martínez-Luque, M., Castillo, F., Roldán, M.D. and Moreno-Vivián, C. (2006) The assimilatory nitrate reduction system of the phototrophic bacterium *Rhodobacter capsulatus* E1F1. Biochem. Soc. Trans. **34**, 127–129
5 Lin, J.T. and Steward, V. (1998) Nitrate assimilation by bacteria. Adv. Microbiol. Physiol. **39**, 1–30
6 Gutierrez, J.C., Ramos, F., Ludwig, O. and Tortolero, M. (1995) *nasST*, two genes involved in the induction of the assimilatory nitrite-nitrate reductase operon (*nasAB*) of *Azotobacter vinelandii*. Mol. Microbiol. **18**, 579–591
7 Ogawa, K.I., Akagawa, E., Yamane, K., Sun, Z.W., laCelle, M., Zuber, P. and Nakano, M.M. (1995) The *nasB* operon and the *nasA* gene are required for nitrate/nitrite assimilation in *Bacillus subtilis*. J. Bacteriol. **177**, 1409–1413
8 Gates, A.J., Luque-Almagro, V.M., Goddard, A.D., Ferguson, S.J., Roldán, M.D. and Richardson, D.J. (2011) A composite biochemical system for bacterial nitrate and nitrite assimilation as exemplified by *Paracoccus denitrificans*. Biochem. J. **435**, 743–753
9 Shao, Z., Gao, J., Ding, X., Wang, J., Chiao, J. and Zhao, G. (2011) Identification and functional analysis of a nitrate assimilation operon *nasACKBDEF* from *Amycolatopsis mediterranei* U32. Arch. Microbiol. **193**, 463–77
10 Lledó, B., Marhuenda-Egea, F.C., Martínez-Espinosa, R.M. and Bonete, M.J. (2005) Identification and transcriptional analysis of nitrate assimilation genes in the halophilic archeon *Haloferax mediterraneii*. Gene **361**, 80–88
11 Ohashi, Y., Shi, W., Takatani, N., Aichi, M., Maeda, S-i., Watanabe, S., Yoshikawa, H. and Omata, T. (2011) Regulation of nitrate assimilation in cyanobacteria. J. Exp. Bot. **62**, 1411–1424
12 Shu, C.J. and Zhulin, I.B. (2002) ANTAR: an RNA-binding domain in transcription antitermination regulatory proteins. Trends Biochem. Sci. **27**, 3–5
13 Shu, C.J., Ulrich, L.E. and Zhulin, I.B. (2003) The NIT domain: a predicted nitrate-responsive module in bacterial sensory receptors. Trends Biochem. Sci. **28**, 121–124
14 Koropatkin, N.M., Pakrasi, H.B. and Smith, T.J. (2006) Atomic structure of a nitrate-binding protein crucial for photosynthetic productivity. Proc. Natl. Acad. Sci. U.S.A. **103**, 9820–9825
15 Chai, W. and Stewart, V. (1999) RNA sequence requirements for NasR-mediated, nitrate-responsive transcription antitermination of the *Klebsiella oxytoca* M5al *nasF* operon leader. J. Mol. Biol. **292**, 203–216
16 Caballero, A., Esteve-Núñez, A., Zylstra, G.J. and Ramos, J.L. (2005) Assimilation of nitrogen from nitrite and trinitrotoluene in *Pseudomonas putida* JLR11. J. Bacteriol. **187**, 396–399
17 Puerta-Fernández, E. and Vioque, A. (2011) Hfq is required for optimal nitrate assimilation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **193**, 3546–55
18 Forchhammer, K. (2008) P(II) signal transducers: novel functional and structural insights. Trends Microbiol. **16**, 65–72
19 Llácer, J.L., Espinosa, J., Castells, M.A., Contreras, A., Forchhammer, K. and Rubio, V. (2010) Structural basis for the regulation of NtcA-dependent transcription by proteins PipX and PII. Proc. Natl. Acad. Sci. U.S.A. **107**, 15397–15402
20 Wu, S.Q., Chai, W., Lin, J.T. and Stewart, V. (1999) General nitrogen regulation of nitrate assimilation regulatory gene *nasR* expression in *Klebsiella oxytoca* M5a1. J. Bacteriol. **181**, 7274–7284
21 Toukdarian, A. and Kennedy, C. (1986) Regulation of nitrogen metabolism in *Azotobacter vinelandii*: isolation of *ntr* and *glnA* genes and construction of *ntr* mutants. EMBO J. **5**, 399–407
22 Pawlowski, K., Klosse, U. and de Bruijn, F.J. (1991) Characterization of a novel *Azorhizobium caulinodans* ORS571 two-component regulatory system, NtrY/NtrX, involved in nitrogen fixation and metabolism. Mol. Genet. Genomics **231**, 124–138
23 Ishida, M.L., Assumpção, M.C., Machado, H.B., Benelli, E.M., Souza, E.M. and Pedrosa, F.O. (2002) Identification and characterization of the two-component NtrY/NtrX regulatory system in *Azospirillum brasilense*. Braz. J. Med. Biol. Res. **35**, 651–661
24 Szeto, W.W., Nixon, B.T., Ronson, C.W. and Ausubel, F.M. (1987) Identification and characterization of the *Rhizobium meliloti ntrC* gene: *R. meliloti* has separate regulatory pathways for activation of nitrogen fixation genes in free-living and symbiotic cells. J. Bacteriol. **169**, 1423–1432
25 Li, W. and Lu, C.D. (2007) Regulation of carbon and nitrogen utilization by CbrAB and NtrBC two-component systems in *Pseudomonas aeruginosa*. J. Bacteriol. **189**, 5413–5420
26 Kayumov, A., Heinrich, A., Fedorova, K., Ilinsakaya, O. and Forchhammer, K. (2011) Interaction of the general transcription factor TnrA with the PII-like protein GlnK and glutamine synthetase in *Bacillus subtilis*. FEBS J. **278**, 1779–1789
27 Swanson, R.V., Alex, L.A. and Simon, M.I. (1994) Histidine and aspartate phosphorylation: two-component systems and the limits of homology. Trends Biochem. Sci. **19**, 485–490
28 Weiss, V. and Magasanik, B. (1988) Phosphorylation of nitrogen regulator I (NRI) of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **85**, 8919–8923
29 Sanders, D.A., Gillece-Castro, B.L., Burlingame, A.L. and Koshland, D.E. (1992) Phosphorylation site of NtrC, a protein phosphatase whose covalent intermediate activates transcription. J. Bacteriol. **174**, 5117–5122
30 Weiss, V., Claverie-Martin, F. and Magasanik, B. (1992) Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. Proc. Natl. Acad. Sci. U.S.A. **89**, 5088–5092
31 Chen, P. and Reitzer, L.J. (1995) Active contribution of two domains to cooperative DNA binding of the enhancer-binding protein nitrogen regulator I (NtrC) of *Escherichia coli*: stimulation by phosphorylation and the binding of ATP. J. Bacteriol. **177**, 2490–2496
32 Assumpção, M.C., Souza, E.M., Yates, M.G., Pedrosa, F.O. and Benelli, E.M. (2007) Purification and characterization of *Azospirillum brasilense* N-truncated NtrX protein. Protein Expression Purif. **53**, 302–308
33 Wray, Jr, L.V., Ferson, A.E., Rohrer, K. and Fisher, S.H. (1996) TnrA, a transcription factor required for global nitrogen regulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **93**, 8841–8845
34 Leigh, J.A. and Dodsworth, J.A. (2007) Nitrogen regulation in bacteria and archaea. Annu. Rev. Microbiol. **61**, 349–377

35 Nakano, M.M., Hoffmann, T., Zhu, T. and Jahn, D. (1998) Nitrogen and oxygen regulation of *Bacillus subtilis nasDEF* encoding NADH-dependent nitrite reductase by TnrA and ResDE. J. Bacteriol. **180**, 5344–5350
36 Paz-Yepes, J., Flores, E. and Herrero, A. (2003) Transcriptional effects of the signal transduction protein P_{II} (*glnB* gene product) on NtcA-dependent genes in *Synechococcus* sp. PCC 7942. FEBS Lett. **543**, 42–46
37 Forchhammer, K. (2004) Global carbon/nitrogen control by PII signal transduction in cyanobacteria: from signals to targets. FEMS Microbiol. Rev. **28**, 319–333
38 Flores, E., Frias, J.E., Rubio, L.M. and Herrero, A. (2005) Photosynthetic nitrate assimilation in cyanobacteria. Photosynth. Res. **83**, 117–133
39 Ramos, F., Blanco, G., Gutiérrez, J.C., Luque, F. and Tortolero, M. (1993) Identification of an operon involved in the assimilatory nitrate-reducing system of *Azotobacter vinelandii*. Mol. Microbiol. **8**, 1145–1153

Received 3 August 2011
doi:10.1042/BST20110688