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The NtrY–NtrX two-component system is involved in controlling nitrate assimilation in *Herbaspirillum seropedicae* strain SmR1

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Herbaspirillum seropedicae is a diazotrophic β-Proteobacterium found endophytically associated with gramineae (Poaceae or graminaceous plants) such as rice, sorghum and sugar cane. In this work we show that nitrate-dependent growth in this organism is regulated by the master nitrogen regulatory two-component system NtrB–NtrC, and by NtrY–NtrX, which functions to specifically regulate nitrate metabolism. NtrY is a histidine kinase sensor protein predicted to be associated with the membrane and NtrX is the response regulator partner. The *ntrYntrX* genes are widely distributed in Proteobacteria. In α-Proteobacteria they are frequently located downstream from *ntrBC*, whereas in β-Proteobacteria these genes are located downstream from genes encoding an RNA methyltransferase and a proline-rich protein with unknown function. The NtrX protein of α-Proteobacteria has an AAA+ domain, absent in those from β-Proteobacteria. An *ntrY* mutant of *H. seropedicae* showed the wild-type nitrogen fixation phenotype, but the nitrate-dependent growth was abolished. Gene fusion assays indicated that NtrY is involved in the expression of genes coding for the assimilatory nitrate reductase as well as the nitrate-responsive two-component system NarX–NarL (*narK* and *narX* promoters, respectively). The purified NtrX protein was capable of binding the *narK* and *narX* promoters, and the binding site at the *narX* promoter for the NtrX protein was determined by DNA footprinting. *In silico* analyses revealed similar sequences in other promoter regions of *H. seropedicae* that are related to nitrate assimilation, supporting the role of the NtrY–NtrX system in regulating nitrate metabolism in *H. seropedicae*.

Introduction

The ability to sense and respond to environmental changes is crucial for bacterial survival. The two-component regulator systems constitute the main sensors of environmental changes. Typically, these systems comprise pairs of sensor histidine kinases (HKs) and response regulators (RRs) [1]. One of most studied two-component systems consists of the HK NtrB and the RR NtrC, the main players in the regulatory cascade controlling nitrogen metabolism in Proteobacteria. Under low intracellular levels of combined

Abbreviations
EMSA, electrophoresis mobility shift assay; HK, histidine kinase; RR, response regulator.

nitrogen, NtrB autophosphorylates and then transfers this phosphoryl group to NtrC, which in turn modulates the expression of genes related with nitrogen metabolism [2]. A second two-component system, the NtrY–NtrX HK–RR system, has also been associated with regulation of nitrogen metabolism in Proteobacteria. The NtrY and NtrX proteins share similarity with NtrB and NtrC and may well have originated from a duplication and differentiation of the *ntrBC* genes [3].

The NtrY–NtrX system was first described and analyzed in the nitrogen-fixing α-Proteobacterium *Azorhizobium caulinodans* where NtrY–NtrX was shown to be important for nitrogen fixation and nitrate metabolism [4]. In *Azospirillum brasilense*, a nitrogen-fixing α-Proteobacterium, NtrY–NtrX is involved in regulating nitrate metabolism [5]. This system is also involved in nitrogen fixation and nodule formation in *Rhizobium tropici* [6]. In the photosynthetic bacterium *Rhodobacter capsulatus*, in addition to nitrogen metabolism, the NtrYX system regulates the formation of photosynthetic complexes [7]. Recently, Carrica and collaborators [8,9] showed that NtrY of the α-Proteobacterium *Brucella abortus* is a heme protein able to sense the redox state of the cell and so phosphorylate NtrX in response to oxygen-limiting conditions. In the β-Proteobacterium *Neisseria gonorrhoeae*, NtrY–NtrX is important for the activation of transcription of genes involved in the synthesis of respiratory enzymes [10].

The endophytic nitrogen-fixing β-Proteobacterium *Herbaspirillum seropedicae* encodes genes for both NtrB–NtrC and NtrY–NtrX systems [11]. The NtrB–NtrC system was shown to be required for nitrogen fixation as well as ammonia assimilation in *H. seropedicae* [12] since NtrC is the main activator required for *nifA* and *glnAntrBC* expression [13,14]. Previous results also indicated that NtrB–NtrC is involved in nitrate metabolism since an *ntrC* mutant cannot grow on nitrate as the sole nitrogen source [12]. On the other hand, the role of the NtrY–NtrX system of *H. seropedicae* has not been addressed. In this work, an *ntrY* mutant was constructed and analyzed, showing its involvement in the regulation of nitrate metabolism but not in nitrogen fixation. The NtrX protein was shown to bind specifically to the *narK* and *narX* promoters, and a potential NtrX binding site was identified in promoters involved in nitrate metabolism in *H. seropedicae*.

Results

Bioinformatics

The two-component system NtrY–NtrX was first described by Pawlowski and colleagues (1991) in *Azorhizobium caulinodans* and shown to be important for nitrogen fixation and nitrate metabolism [4]. Since then, a range of organisms have been shown to have *ntrYX* genes although few have been characterized. In *A. caulinodans*, as well as in other α-Proteobacteria, the *ntrYX* genes are located downstream from *ntrBC* suggesting duplication and differentiation of *ntrBC* genes [3]. However, in *H. seropedicae*, as well as in several β-Proteobacteria, the *ntrYX* genes are not located in proximity to *ntrBC*, illustrating the different gene organizations from α-Proteobacteria (Fig. 1A). In *H. seropedicae* strain SmR1, *ntrBC* (locus tag Hsero_RS15685 and Hsero_RS15680) forms an operon with *glnA*, which encodes glutamine synthetase [11,13], whereas *ntrYntrX* (locus tag Hsero_RS00345 and Hsero_RS00340) is located downstream from genes coding for an RNA methyltransferase and a proline-rich protein [11]. This organization is highly conserved in β-Proteobacteria.

By amino acid similarity with other biochemically characterized HK–RR systems, *H. seropedicae* NtrB and NtrY are histidine kinases that transfer the phosphoryl group to the response regulators NtrC and NtrX, respectively. Both NtrB and NtrY show HisKA and HATPase domains involved in autophosphorylation and also a PAS domain that may be involved in signal transduction (Fig. 1B). The *H. seropedicae* NtrY protein also shows transmembrane motifs and a HAMP domain, suggesting it is a membrane-bound protein, contrasting with the cytoplasmatic NtrB. Overall, NtrB and NtrY share 30% identity, mainly at the HisKA and HATPase domains.

Both NtrC and NtrX response regulator proteins show a REC domain and a HTH motif (Fig. 1B). The REC domain, located at the N-terminal region, receives the phosphoryl group from the histidine kinase [15], whereas the HTH motif, located at the C-terminal region, directs DNA-binding [16]. The *H. seropedicae* NtrC protein also has an AAA+ domain, found at the central part of the protein, involved in interaction with the σ^N-RNA polymerase [17]. On the other hand, *H. seropedicae* NtrX does not have the AAA+ domain, which indicates it is not a σ^N activator, but possibly a σ^{70} transcriptional regulator. Analysis also indicated that although α-Proteobacterial NtrX proteins have a typical REC–AAA$^+$–HTH domain organization, most β-Proteobacterial NtrXs lack the AAA+ domain (SMART analysis; http://smart.embl-heidelberg.de/).

Genetic analysis of *ntrY*

In order to evaluate the importance of the NtrY–NtrX system for nitrogen metabolism in *H. seropedicae*, an

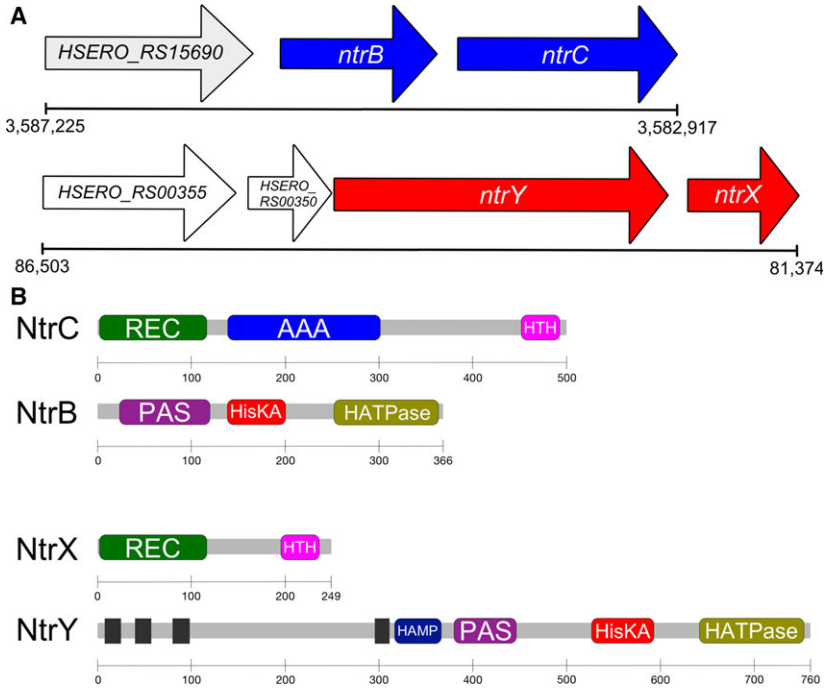

Fig. 1. The NtrYX and NtrBC systems of *H. seropedicae*. (A) Genomic context of *ntrBC* (top) and *ntrYX* (bottom) genes. Numbers represent the gene location in *H. seropedicae* genome (NCBI accession number NC_014323). Hsero_RS00355 and Hsero_RS00350 code for RNA methyltransferase and a proline-rich protein, respectivelly. Gene locus tag is also indicated. (B) Schematic protein domain organization of the *H. seropedicae* NtrC, NtrB, NtrX and NtrY proteins. Numbers indicate position at the primary structure. Black boxes indicate transmembrane segments of NtrX.

insertional *ntrY* mutant was constructed. The *ntrY* mutant (A01 strain) grows at a similar rate to the wild-type (SmR1) using ammonium as the sole nitrogen source, but failed to grow on nitrate as the sole nitrogen source (Fig. 2A), a phenotype similarly to that of an *ntrC* mutant (DCP286A strain) [12]. Complementation of the *ntrY* mutant with a cosmid containing the *ntrYX* genes restored the growth on nitrate (data not shown).

Nitrogenase and glutamine synthetase activities are impaired in the *ntrC* mutant as previously described [12] (and see Fig. 2B,C) since NtrC is the main transcriptional activator for *nifA*, *glnAntrBC* and *nlmAglnKamtB* operons in *H. seropedicae* [12–14,18]. On the other hand, the *ntrY* mutant showed nitrogenase and glutamine synthetase activities similar to the wild-type strain (Fig. 2B,C) indicating that the *ntrY* product is not involved with nitrogen fixation or ammonia assimilation.

Analysis using a semi-quantitative RT-PCR from wild-type and the *ntrY* mutant indicated no polar effect on *ntrX* expression (Fig. 3A). Moreover, the inability to use RT-PCR to amplify an intergenic region between *ntrY* and *ntrX* suggested that these genes are not cotranscribed. This intergenic region was amplified using genomic DNA as a template, supporting the inference that *ntrY* and *ntrX* are not part of an operon. Results also indicated that *ntrX* expression level is higher than *ntrY* (Fig. 3B). An RNA-seq transcriptome analysis carried out with cells grown in the presence of nitrate also indicated that *ntrX* has a higher level of transcription (reads per kilobase per million mapped reads (RPKM) 145 ± 17 for *ntrX* and 63 ± 10 for *ntrY*) [19]. Despite the fact that *ntrY* and *ntrX* seem not to be cotranscribed, the gene synteny and small intergenic region of 134 bp between both genes is in agreement with their products functioning together, as proposed by Williams and Whitworth [20].

Gene expression regulation by NtrY–NtrX

Use of nitrate as sole nitrogen source requires transporters and enzymes allowing nitrate import and reduction of nitrate to nitrite and then to ammonium. Considering that the *ntrY* mutant is impaired in using nitrate as the sole nitrogen source, the effect of *ntrY* mutation on transcriptional activation of promoters of genes involved in nitrate metabolism was addressed using *lacZ* fusions. The *narKnirBDHsero_RS14545-nasA* operon codes for a nitrate–nitrite antiporter (*narK*), the assimilatory nitrate reductase and nitrite reductase. The *narXL* operon codes for members of a two-component system family known in other bacteria to regulate nitrate metabolism [21]. Transcriptional fusions *narK::lacZ* and *narX::lacZ* were assayed in the wild-type and *ntrY* mutant strains in the presence of ammonium or nitrate (Table 1). In the wild-type strain expression of *narK::lacZ* is basal when cultivated in ammonium, whereas higher β-galactosidase activity is observed when nitrate is present. However, a lower

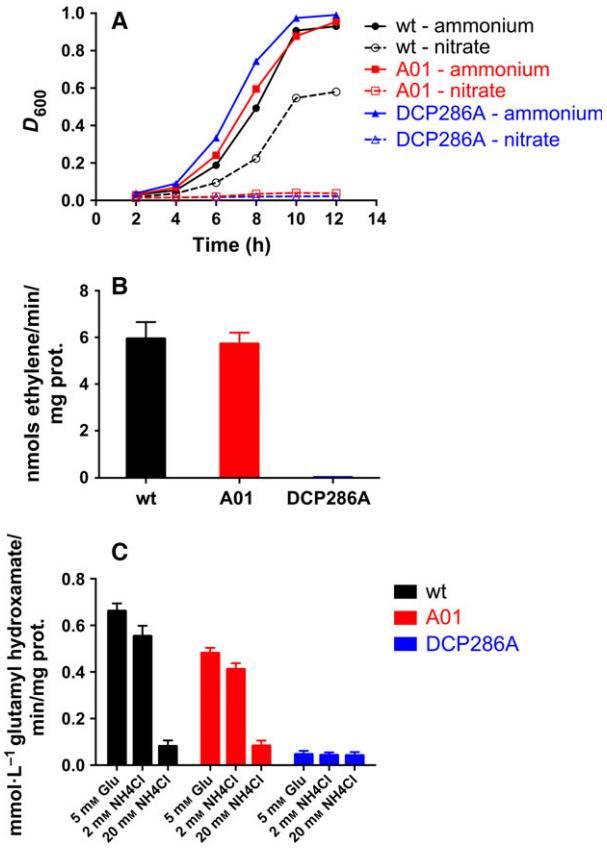

Fig. 2. The function of the NtrYX system overlaps with that of the NtrBC system regarding nitrate metabolism. (A) Growth of *H. seropedicae* SmR1 (wild-type, wt), A01 (*ntrY* mutant) and DCP286A (*ntrC* mutant) strains on ammonium or nitrate as the sole nitrogen source. Data from a representative experiment. (B) Nitrogenase activity of *H. seropedicae* SmR1 wild-type, A01 and DCP286A strains. (C) Glutamine synthetase activity was determined in cells grown at low (NH_4Cl 2 mmol·L^{-1} or glutamate 5 mmol·L^{-1}) or high levels of nitrogen (NH_4Cl 20 mmol·L^{-1}). In (B) and (C) data represent mean ± SD of at least three experiments.

β-galactosidase activity is observed in *ntrY* mutant in the presence of nitrate. These results indicate that the expression of *narKnirBDHsero_RS14545nasA* is induced by nitrate and involves the NtrY–NtrX system. Under the conditions tested the expression of *narX::lacZ* also seems to be dependent on NtrY–NtrX since lower β-galactosidase activity was observed in the *ntrY* mutant.

Promoter DNA-binding functionality of NtrX

In order to determine whether the involvement of NtrY–NtrX in regulating the *narK* and *narX* promoter was based on the direct binding of NtrX to these promoters, the *H. seropedicae* NtrX protein was expressed and purified as a His-tagged fusion protein (hereafter indicated as ^{His}NtrX). Also, N- and C-truncated forms of NtrX were obtained. ^{His}NtrX-NTD indicates a His-tagged fused N-terminal domain (REC) of NtrX, and ^{His}NtrX-CTD indicates a His-tagged fused C-terminal domain (HTH) of NtrX.

Considering that some response regulators may form dimers [22], gel-filtration chromatography was used to determine the oligomerization state of NtrX in solution. The calculated molecular masses of ^{His}NtrX, ^{His}NtrX-NTD and ^{His}NtrX-CTD as monomers were 29, 14 and 17 kDa, respectively; however, these proteins were eluted as 56, 14 and 35 kDa, respectively, suggesting that only the ^{His}NtrX-NTD is a monomer in solution. Both ^{His}NtrX and ^{His}NtrX-CTD showed elution profiles compatible with being dimers, suggesting that NtrX assembles itself as dimers through its C-terminal domain. Subsequent *in vitro* assays were conducted assuming that ^{His}NtrX and ^{His}NtrX-CTD were dimers.

Purified ^{His}NtrX was assayed for DNA binding activity using labeled DNA fragments of *narX* and *narK* promoter regions. Results indicated that ^{His}NtrX was able to bind to these promoters as revealed by a decrease in the gel migration of the DNA fragments (Fig. 4A,B). The DNA–protein complex was observed in the presence of non-specific competitor DNA, calf thymus DNA, and disruption of the complex was observed when the binding reaction was carried out in the presence of non-labeled cognate promoter DNA (data not shown). The truncated forms of NtrX were also tested for DNA binding, and only ^{His}NtrX-CTD, which carries the HTH domain, was able to decrease the DNA mobility (Fig. 4C and data not shown).

To help investigate amino acid residues important for the NtrX–DNA interaction, a model of the DNA-binding C-terminal domain of NtrX from *H. seropedicae* using the I-TASSER server [23] was built. Subsequently in CCP4 MG we aligned the C-terminal domain of NtrX from *H. seropedicae* and NtrC4 from *Aquifex aeolicus*, whose structure was determined bound to DNA [24]. This alignment allowed visualizing the NtrX amino acids predicted to be in close contact with the promoter DNA. We choose six candidate amino acids potentially important for protein binding to the consensus DNA site: Met_{216}, Thr_{228}, His_{229}, Tyr_{231}, Arg_{232} and Gln_{236} (Fig. 5A). Six variants of NtrX were constructed replacing each of those amino acids by alanine and tested for DNA-binding activity in the band-shift assay. A His-tagged fusion protein of each variant was compared with the ^{His}NtrX. In general, these mutations did not cause dramatic changes in the ability of NtrX to bind to the *narX* promoter region.

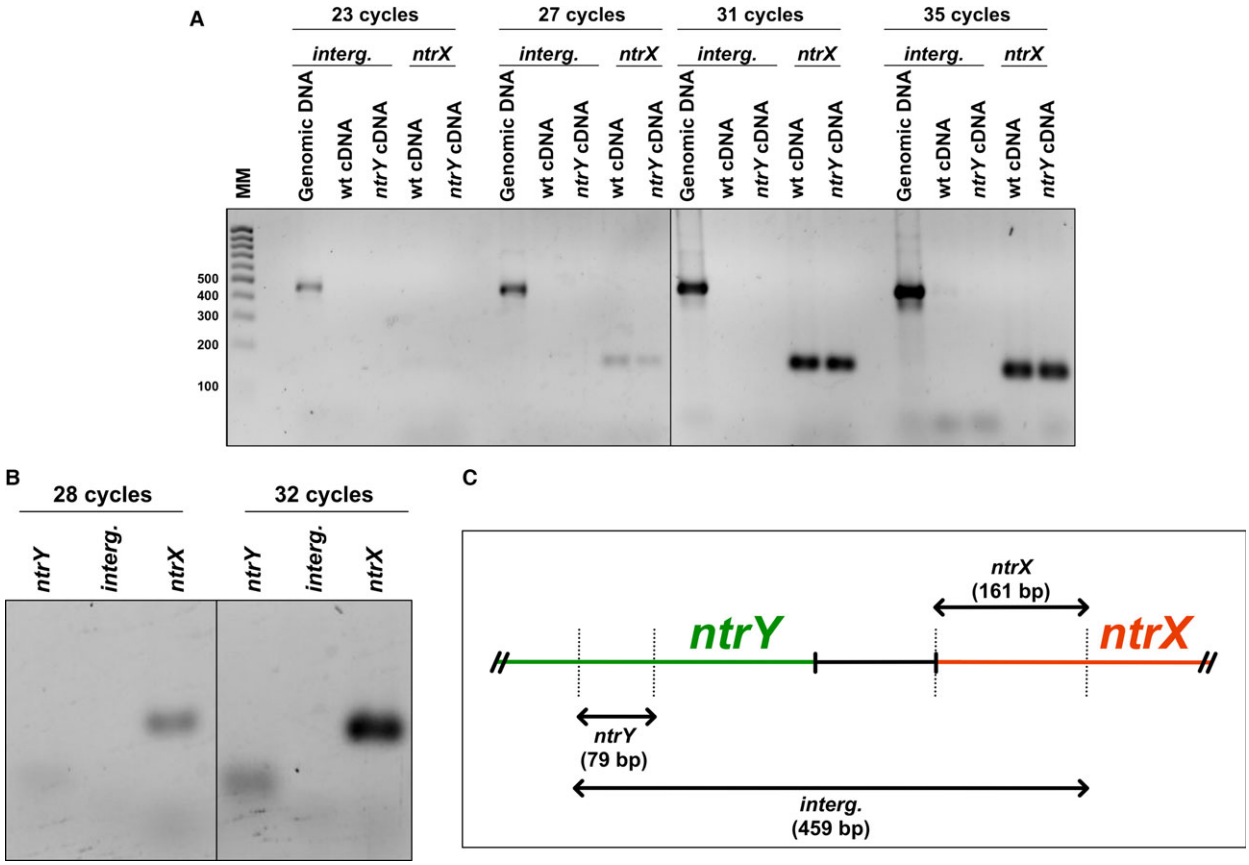

Fig. 3. *ntrY* and *ntrX* are independently transcribed. (A) Semi-quantitative PCR was conducted with cDNA synthesized from *H. seropedicae* wild-type (wt) ('wt cDNA') or *ntrY* mutant ('*ntrY* cDNA') submitted to nitrate-shock, and oligonucleotides amplifying the coding region of *ntrX* gene, or the intergenic region between *ntrY* and *ntrX* genes ('interg.'). Genomic DNA extracted from *H. seropedicae* was used as a positive control to amplify the intergenic region. Molecular marker (MM) is indicated in bp. (B) The cDNA synthesized from *H. seropedicae* wt submitted to nitrate-shock was used as the template to amplify the coding regions of *ntrY*, *ntrX* or the intergenic region between both genes. (C) Schematic representation of genomic location of *H. seropedicae* covering the regions used to amplify *ntrY*, *ntrX* or the intergenic region. The expected sizes of the PCR products are indicated.

Table 1. Promoter activity of *narX::lacZ* or *narK::lacZ* fusions in *H. seropedicae* wild-type strain and in *ntrY* mutant.

plasmid	Nitrogen source	Wild-type (SmR1)	*ntrY* (A01)
Empty vector	NO_3^-	9.8 ± 1.2	15.8 ± 7.9
narX::lacZ	NO_3^-	136.8 ± 43.2	27.5 ± 2.2
narK::lacZ	NO_3^-	514.9 ± 31.2	231.6 ± 7.4
Empty vector	NH_4^+	14.3 ± 1.2	33.2 ± 1.4
narX::lacZ	NH_4^+	168.8 ± 7.8	76.6 ± 20.4
narK::lacZ	NH_4^+	9.0 ± 4.7	29.7 ± 2.0

β-Galactosidase activity was determined in *H. seropedicae* wild-type strain (SmR1) and *ntrY* mutant (A01) and is indicated as specific activity (nmol of *o*-nitrophenol produced per minute per milligram of protein). Data are indicated as mean ± SD of biological triplicates.

The only exception was the ^{His}NtrXArg$_{232}$Ala protein, which was not able to bind to DNA (Fig. 5B). This suggests that Arg$_{232}$ is important for DNA binding.

In order to identify the potential site for NtrX binding, a DNase I footprinting assay was carried out. Since a Cy3-labeled DNA (*narX* promoter region) was used in this experiment, a higher concentration of protein was required to visualize DNA binding. Therefore, the N-truncated protein (^{His}NtrX-CTD) was used in that assay due to its higher solubility as compared with the full length NtrX. Moreover, the ^{His}NtrX-CTD protein showed DNA binding activity similar to the full length NtrX (Fig. 4C).

Reactions were carried out using increasing concentration of the ^{His}NtrX-CTD protein and the *narX* promoter region labeled with Cy3 on top (Fig. 6A) and bottom strands (Fig. 6B). Protected and hyper-reactive DNA bands from DNase I cutting were observed on the same region on both DNA strands. These results allowed locating of the DNA binding site of NtrX to

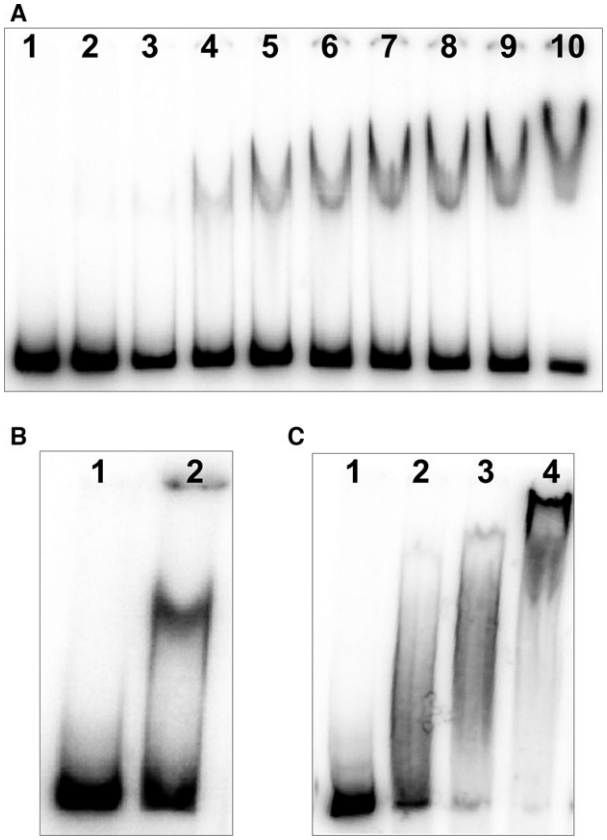

Fig. 4. The NtrX protein of *H. seropedicae* binds to *narK* and *narX* promoter regions. (A) Electrophoresis mobility shift assay (EMSA) was carried out using purified ^{His}NtrX and the ^{32}P-labeled *narK* promoter. Lane 1: no protein added; lanes 2–10: 0.1, 0.2, 0.5, 0.7, 1, 1.3, 1.5, 2 and 3 μM of ^{His}NtrX. (B) EMSA using ^{32}P-labeled *narX* promoter. Lane 1: no protein added; lane 2: 1 μM of ^{His}NtrX protein. (C) EMSA was carried out using purified ^{His}NtrX-CTD and the ^{32}P-labeled *narX* promoter. Lane 1: no protein added; lanes 2–4: 1, 2.5 and 10 μM of ^{His}NtrX-CTD. Protein concentration was determined considering ^{His}NtrX and ^{His}NtrX-CTD as homodimers.

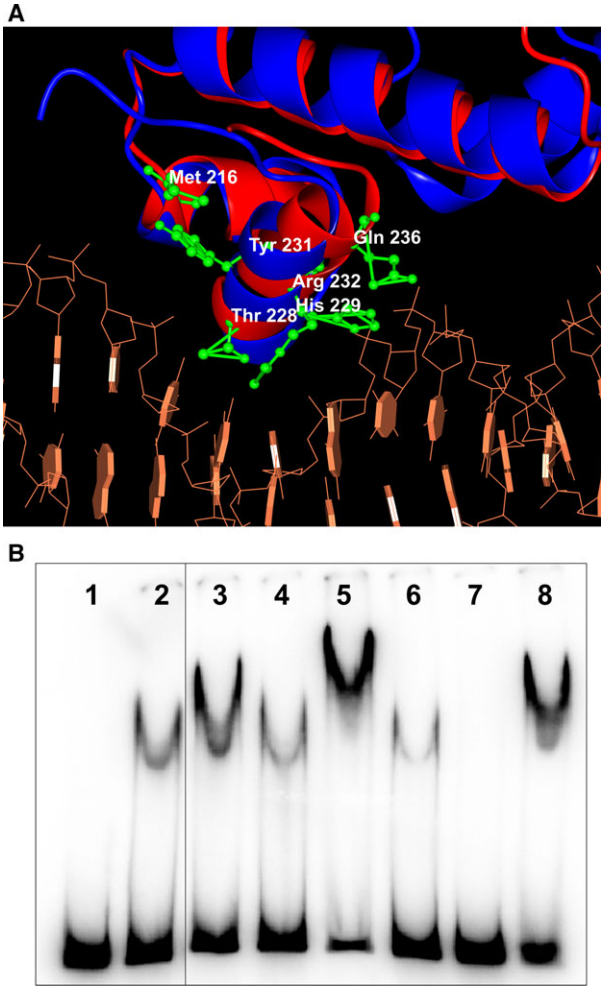

Fig. 5. Arginine 232 is essential for DNA-binding activity of NtrX. (A) Prediction of the C-terminal structure of NtrX of *H. seropedicae* using the I-TASSER server. This NtrX structure (in red) was aligned with the structure of NtrC bound to DNA of *Aquifex aeolicus* (in blue) using the CCP4 MG software. Each amino acid shown in green was changed to alanine yielding variants proteins. (B) EMSA using variant proteins and the ^{32}P-labeled *narX* promoter. Lane 1: no protein added; lane 2: wild-type ^{His}NtrX; lanes 3–8: ^{His}NtrX variants carrying alanine substituents: Met_{216}Ala, Thr_{228}Ala, His_{229}Ala, Tyr_{231}Ala, Arg_{232}Ala and Gln_{236}Ala. Assay was carried out with 1 μM of purified protein. Lanes irrelevant for this analysis were removed as indicated by a line between lanes 2 and 3.

approximately 34 bp interval at the *narX* promoter (Fig. 6C). This sequence is located at the 3′ region of the *moaA* gene and upstream of a putative −10/−35 promoter (Fig. 7A). This DNA binding site found within the *narX* promoter was compared with other promoter regions of *H. seropedicae* that are related to nitrate metabolism. Interestingly, similar sequences were found in two other promoters, *narK* and *nasFED*, the latter coding for a putative ABC-type nitrate transporter (Fig. 7B). Gregor and collaborators [7], working with NtrX from the α-Proteobacterium *R. capsulatus*, noted a protected sequence within the *puf* promoter region using DNA footprinting assay. Comparison analysis indicated a partial overlap between the protected sequence from *R. capsulatus* and that from *H. seropedicae narX*. This overlapping sequence involves 23 nucleotides at the 3′ end of the *narX* protected region and the 5′ end of the *puf* protected region (Fig. 7C).

Discussion

Two-component systems are the main sensory regulatory systems by which bacteria can transduce environmental signals to achieve a new adaptive metabolic condition.

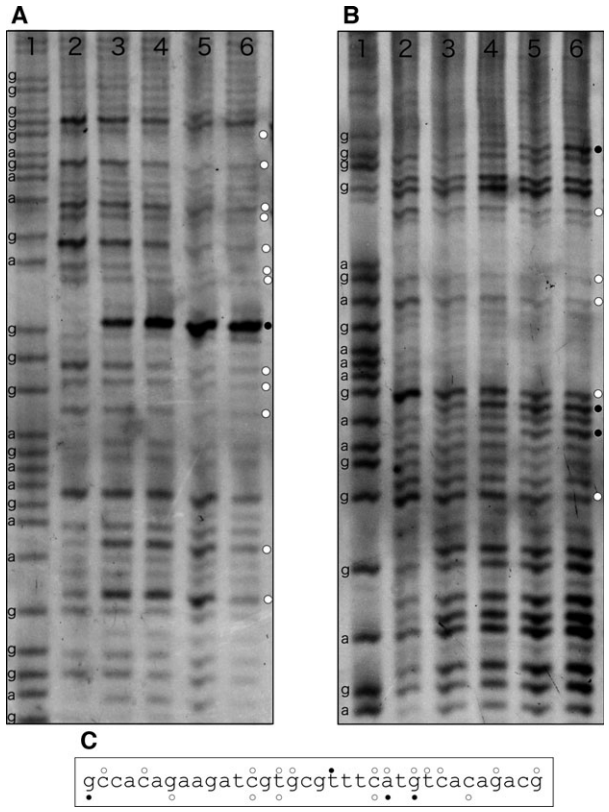

Fig. 6. Identification of DNA-binding site of *H. seropedicae* NtrX. DNase I footprinting assay with ^{His}NtrX CTD protein and *narX* promoter labeled with fluorophores in the forward strand (A) or reverse strand (B). The G + A ladder (lane 1) was used to estimate the position of protected (white circles) or hyper-reactive bands (black circles). Increasing concentrations of ^{His}NtrX CTD protein of 0, 20, 50, 100 and 200 μM (lanes 2–6, respectively) were incubated with the Cy3-labeled *narX* promoter, followed by digestion with DNase I. (C) The DNA-binding site of NtrX in *narX* promoter. The protected bands (white circles) and hyper-reactive bands (black circles) indicated in (A) and (B) are shown above and below the bases, respectively.

Nitrogen is an essential element for growth and ammonium is the main combined nitrogen source for most organisms. Therefore, in the absence or low concentration of ammonium, bacteria have to mobilize alternative sources of nitrogen in order to maintain growth. For most Proteobacteria, NtrB–NtrC is the main two-component system controlling these pathways.

In 1991 Pawlowski and colleagues described two genes, *ntrYX*, coding for another two-component system involved with nitrogen fixation and nitrate metabolism in *A. Caulinodans* [4]. Although the NtrY–NtrX two-component system is found in several organisms, its functional characterization is restricted to a few studies. Besides *A. caulinodans* [4], involvement of NtrY–NtrX in nitrate metabolism has also been described for *Azospirillum brasilense* [5] and *Rhodobacter capsulatus* [25]; however, it is unclear how the intrinsic communication among proteins and/or pathways occurs.

In *H. seropedicae* nitrogen metabolism is controlled mainly by the NtrB–NtrC system, which is responsible for activating transcription of genes involved in using alternative nitrogen sources including dinitrogen gas [12–14,18]. Although several studies have been conducted in this organism on the regulation of nitrogen fixation, *H. seropedicae* nitrate metabolism is mainly unknown. Pershun and colleagues [12] proposed that the NtrB–NtrC system regulates nitrate assimilation, since an *ntrC* mutant was impaired in growing on nitrate as the sole nitrogen source. In our work, a similar phenotype for nitrate growth was also observed with the *ntrY* mutant. On the other hand, NtrY is not involved in the regulation of nitrogen fixation or ammonium assimilation by glutamine synthetase in *H. seropedicae*, contrasting with roles described in *A. caulinodans* [4].

We found that inactivating *ntrY* had no polar effect on *ntrX* expression. In fact, *ntrX* seems to be independently expressed and at a higher level than *ntrY* even though *ntrX* is located only 134 bp downstream of *ntrY*, with no indication of a putative promoter. RNA-seq transcriptome analyses showed that both *ntrY* and *ntrX* genes are constitutive, and apparently not regulated by nitrate or NtrC (P. Bonato, D. Camilios-Neto, M. Z. Tadra-Sfeir, R. Wassem, E. M. Souza, F. O. Pedrosa, L. S. Chubatsu, unpublished data).

Although independently expressed it is plausible that NtrY and NtrX do function together as a distinct two-component system [20]. So far we have been unable to obtain a stable *ntrX* mutant suggesting that NtrX is essential in *H. seropedicae*. Difficulties in obtaining *ntrX* mutants were also described for *A. brasilense* and *R. capsulatus* [5,7].

In order to determine the involvement of the NtrY in nitrate metabolism, *narK::lacZ* and *narX::lacZ* gene fusions were assayed in the wild-type and *ntrY* mutant backgrounds. The *narK* promoter appears to be directly involved with nitrate assimilation since it controls the expression of a nitrate transporter, the assimilatory nitrate and nitrite reductases. The *narX* promoter, on the other hand, controls the expression of the two-component system NarX–NarL. Expression of both promoters was lower in the *ntrY* mutant indicating the involvement of the NtrY–NtrX system in the regulation of both promoters. Our direct binding of NtrX to both promoters, demonstrated by the band-shift experiments, supports this hypothesis. Furthermore, a similar DNA binding site for NtrX found in the *narX* promoter is also evident in the *narK* and

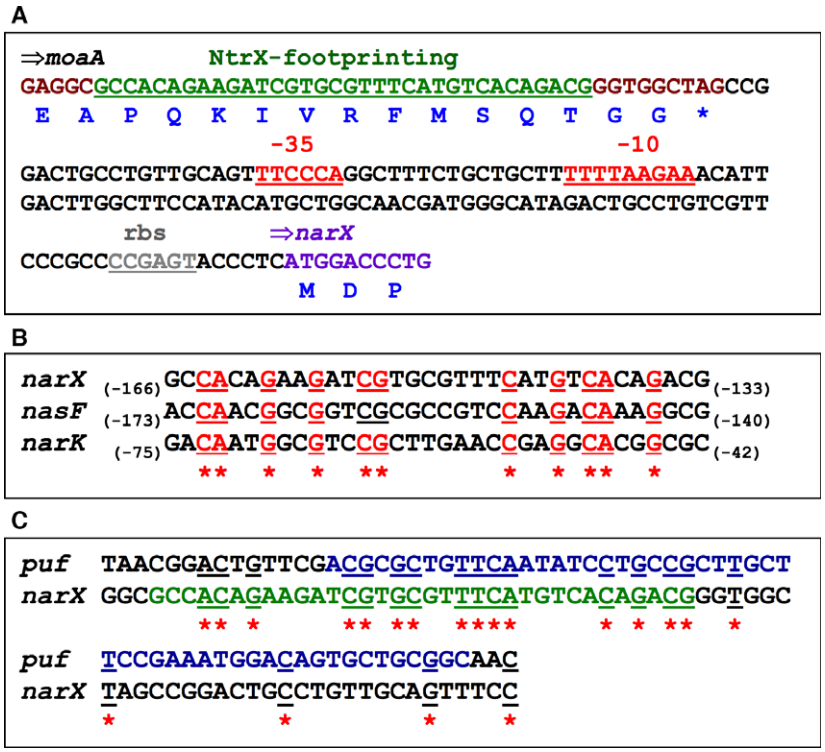

Fig. 7. Analysis of the NtrX binding site. (A) the *narX* promoter region showing the NtrX-footprinting (in green) and putative -10/-35 promoter (in red). Region includes the 3′ end of *moaA* and the 5′ end of *narX*. rbs indicates a ribosomal binding sequence (in gray). Translated amino acid sequence is indicated in blue. (B) Comparison between the DNA-binding site of NtrX in the *narX* promoter and similar sequences in *narK* and *nasF* promoter regions. Identical nucleotides (*) are underlined in red. Numbers indicate location from the start codon. (C) Sequence alignment of *H. seropedicae* NtrX *narX* DNA binding sequence (green) and the *R. capsulatus puf* promoter region protected sequence (blue) as described by Gregor *et al.* [7]. Identical nucleotides (*) are underlined.

nasF promoters supporting the case for a direct involvement of NtrX in nitrate assimilation.

Together, the DNA binding location within the *narK* promoter, the *narK::lacZ* data and expression of *ntrX* in the *ntrY* mutant suggests that NtrX likely acts as a repressor that can be released through the action of NtrY in response to nitrate. Data indicate that *narK* is expressed under ammonium-limiting conditions and in the presence of nitrate. Ammonium-limiting growth conditions are responded to by the NtrB–NtrC system, and nitrate may be sensed by the NtrY–NtrX system. In the *ntrY* mutant, NtrX is probably bound to the promoter, consistent with the observed lower *narK::lacZ* expression. In the wild-type strain, it seems that NtrY relieves the negative effect of NtrX in response to nitrate, so allowing activation of its expression by NtrC. Notably, potential binding sites for NtrC and σ^N-RNA polymerase are observed upstream of the potential NtrX binding site within the *narK* promoter (data not shown). Regulation of *narXL* must also involve NtrY–NtrX since a decrease in activity of the *narX::lacZ* fusion as well as DNA binding by NtrX was observed. Regulation of *narXL* expression, however, shows some differences from the *narK* promoter since it also involves a response to O_2 levels. RNA-seq analyses indicated that expression of *narXL* is increased under low O_2 levels with involvement of Fnr [26], and is also higher if nitrate is present [19]. Although NarX–NarL target genes are currently unknown in *H. seropedicae*, *narXL* is located downstream of genes coding for a respiratory nitrate reductase complex, suggesting a potential role similar to those in enterobacteria [19,21]. An NtrX DNA binding site within the *narX* promoter is located upstream of a putative $-35/-10$ promoter suggesting a promoter activation role.

Nitrate assimilation has a high energy cost involving not only a high number of electrons for reduction of NO_3^- to NH_4^+, but also for the synthesis and activity of the reductases [27]. As a consequence, regulation of the nitrate assimilation pathway does not rely only on the nitrate availability but also requires absence or low concentrations of ammonium ions. In *H. seropedicae* both NtrB–NtrC and NtrY–NtrX systems seem to be required for nitrate assimilation, which may reveal a novel facet of the regulation of nitrate assimilation in *H. seropedicae* that is likely to be operational in a wide range of β-Proteobacteria.

Materials and methods

Bacterial strains and growth conditions

Herbaspirillum seropedicae SmR1 wild-type and mutant strains were grown at 30 °C and 120 r.p.m. in NFbHP–malate medium [28] supplemented with 20 mmol·L^{-1}

NH_4Cl or the indicated nitrogen source. *E. coli* strains were grown at 37 °C in LB medium.

Plasmids and oligonucleotides

All the plasmids and oligonucleotides used in this work are listed in Tables 2 and 3, respectively.

Mutagenesis of the *ntrY* gene of *H. seropedicae*

Herbaspirillum seropedicae ntrY mutant strain (A01) was obtained by insertion of a kanamycin resistance cassette into a *Bgl*II site present in the central region of the *ntrY* gene. For strain complementation, the pAA27D07 cosmid containing about 26 kb of the region including the *ntrYX* genes was inserted into the pLAFR3.18 plasmid and transformed into the A01 mutant strain.

Enzymatic activities

The nitrogenase activity was determined by the acetylene reduction assay. Cultures were grown in nitrogen-free NFbHP–malate semi-solid medium and ethylene formation was quantified using gas chromatography as previously described [29]. Nitrogenase activity was expressed as nanomoles ethylene per minute per milligram protein. Protein concentration was determined by the Bradford method [30] using bovine serum albumin as standard.

Glutamine synthetase activity was determined as previously described and adapted to a microtiter plate reader [31]. It was expressed as micromoles γ-glutamyl hydroxamate per minute per milligram protein.

β-Galactosidase activity was determined in cells carrying *lacZ* fusion as described [32]. Cells grown overnight in NH_4Cl were harvested and inoculated in medium with 20 mM NH_4Cl or with 10 mM KNO_3 plus 1 mM NH_4Cl. After incubation for 6 h, β-galactosidase activity was determined.

Protein expression and purification

Expression plasmids containing the *ntrX* gene from *H. seropedicae* were transformed into *E. coli* BL21(DE3) strain and used to overexpress the NtrX protein (wild-type or variants) fused to a His tag at the N terminus. Protein overexpression was carried out at 16 °C overnight to increase protein solubility. Proteins were purified by affinity chromatography using HiTrap Chelating HP columns and FPLC (ÄKTA, GE Healthcare, Buckinghamshire, UK). When high purity was required, a second chromatographic step was performed using a HiTrap Heparin HP column. Proteins were stored in HEPES-containing buffer (50 mmol·L^{-1} HEPES pH 8.0, 250 mmol·L^{-1} NaCl, 50% glycerol) at −20 °C.

Electrophoretic mobility shift assay

For electrophoretic mobility shift assay (EMSA), fragments of DNA containing *narX* or *narK* promoters were obtained by PCR amplification using oligonucleotides described in Table 2. The NtrX proteins were incubated (at indicated

Table 2. Plasmids used in this work.

Plasmids	Characteristics	Reference
p05061H07	Contains fragment of *ntrY* gene inserted into pUC19 (Amp^R)	[11]
pH07	Contains kanamycin resistance cassette inserted into BglII site in the central region of *ntrY* gene from p05061H07 (Amp^R, Km^R)	This work
pLAFR3.18	pLAFR vector containing the policloning site of pTZ18R (Tc^R, Cm^R)	[37]
pAA27D07	Cosmid containing around 26 kb of *H. seropedicae* genomic region, including the *ntrYX* genes, inserted into pLAFR3.18 plasmid	[11]
pPW452	Contains the promoter-less *lacZ* gene (Tc^R)	[38]
pPW-*narX*	Contains the promoter region of *narXL* of *H. seropedicae* inserted into pPW452 plasmid (Tc^R). Fragment corresponds to position −721 to +2 considering the *narX* start codon	This work
pPW-*narKnirB*	Contains the promoter region of *narK* of *H. seropedicae* inserted into pPW452 plasmid (Tc^R). Fragment corresponds to position −381 to +39 considering the *narK* start codon	This work
pET28-*ntrX*	*Nde*I–*Bam*HI fragment containing the *ntrX* gene of *H. seropedicae* inserted into pET28a. Overexpresses the wt ^{His}NtrX protein (Km^R)	This work
pET28-*ntrX* 141-249	Contains the *ntrX* gene with $NtrX_{139–140}$ codons changed to generate *Nde*I cleavage site (catatg). *Nde*I–*Bam*HI fragment was inserted into pET28a. Overexpresses the ^{His}NtrX CTD protein (Km^R)	This work
pET28-*ntrX* Gly141TGA	Contains the *ntrX* gene with the $NtrX_{141}$ codon changed from 'ggc' (coding for glycine) to 'tga' (stop codon) by mutagenic PCR using the pET28-*ntrX* plasmid and oligonucleotides described in Table 3. Overexpresses the ^{His}NtrX NTD protein (Km^R)	This work
pCR2.1-*narX*	Contains the promoter region of *narXL* of *H. seropedicae* inserted into pCR2.1-TOPO plasmid (Amp^R, Km^R)	This work
pCR2.1-*narK*	Contains the promoter region of *narK* of *H. seropedicae* inserted into pCR2.1-TOPO plasmid (Amp^R, Km^R)	This work

Table 3. Oligonucleotides used in this work.

Oligonucleotides	Sequence	Reference
ntrX (pET28)	Forward 5′ AAGCAGCATATGGCTAAC 3′ Reverse 5′ ACCGTGAGGATCCCGTTTG 3′	This work
ntrX 141-249	Forward 5′ GTGGCCGCCGTGCATATGGGCGCCGCCGAC 3′	This work
ntrX Gly141TGA	Forward 5′ CGCCGTGGTCAACTGAGCCGCCGACGGT 3′ Reverse 5′ ACCGTCGGCGGCTCAGTTGACCACGGCG 3′	This work
narX-binding	Forward 5′ TCGAACTGAAGCCGGAACGC 3′ Reverse 5′ ATTTCCAGCGCCAGTCCCAG 3′	This work
narK-binding	Forward 5′ AATGGGCATCATCGCCTTCCA 3′ Reverse 5′ TGCCTCGGTTCAAGCGGACG 3′	This work
narX-footprint Cy3for	Forward 5′ [Cy3]TCGAACTGAAGCCGGAACGC 3′ Reverse 5′ CATCGTTGCCAGCATGTATGGAA 3′	This work
narX-footprint Cy3rev	Forward 5′ TCGAACTGAAGCCGGAACGC 3′ Reverse 5′ [Cy3]GCAGAAAGCCTGGGAAACTGC 3′	This work
ntrY RT for	5′ GCGCCTTCGAACCCTATGT 3′	This work
ntrY RT rev	5′ TCTTCGACGATTTTCACCAT 3′	This work
ntrX chec.mut for	5′ CAGCACATGGCTAACATCCTGGTC 3′	This work
ntrYX 3 rev	5′ TCGAGCAGCACGAGGTCGG 3′	This work

concentrations) with 10 nM of radiolabeled DNA ([γ^{32}P] ATP) in 10 μL reactions containing TAP buffer [33], and Tween-20 0.01% at 30 °C for 10 min. Reactions were loaded onto native gel and run at +4 °C.

Gel filtration assay

The molecular mass of full length ^{His}NtrX and truncated proteins ^{His}NtrX-NTD and ^{His}NtrX-CTD was determined using a calibrated size exclusion chromatography Superose 12 column (GE Healthcare) coupled to the ÄKTA system (GE Healthcare), at a flow rate of 0.5 mL·min^{-1} and using the HEPES buffer without glycerol.

DNase I footprinting assay

Fragments of DNA containing *narX* promoter labeled with fluorophores in the forward or reverse strands were obtained by PCR amplification using one of the Cy3-labeled oligonucleotides described in Table 2. The NtrX proteins were incubated with 500 nM of fluorescent labeled DNA in 10 μL reactions as described in the EMSA section. The reactions were digested with DNase I, ethanol precipitated, dissolved in denaturing loading buffer and loaded on 6% DNA sequencing gels.

RNA extraction and semi-quantitative PCR

Herbaspirillum seropedicae wild-type or *ntrY* mutant strains were cultivated in NFbHP–malate supplemented with 1 mM NH_4Cl at 30 °C and 250 r.p.m. When cells reached D_{600} 0.4, KNO_3 was added at 10 mM final concentration (nitrate-shock). After 30 min, cells were collected and RNA was extracted using the Trizol method. Contaminant DNA was removed using TURBO DNase treatment (Ambion; Thermo Fisher Scientific, Waltham, MA, USA). RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific). The PCR amplification (10 μL) was performed with 1 μL of 1 : 10 cDNA dilution as template, Taq DNA polymerase, dNTPs 200 μM, oligonucleotides 0.5 μM, and 2.5 mM $MgCl_2$. Oligonucleotides are listed in Table 2. The PCR reactions were submitted to a step of denaturation at 95 °C for 1 min, followed by 23–35 cycles of 95 °C for

30 s, 68 °C for 30 s and 72 °C for 20 s. PCR products were analyzed in 3% agarose gels.

Bioinformatics and phylogenetic analyses

Amino acids sequences of the NtrY, NtrX, NtrB and NtrC proteins were retrieved from NCBI and the main domains and HTH motifs were identified using the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd/) [30]. PredictProtein was used to predict the transmembrane segments of NtrY [34].

The three-dimensional structure of the C-terminal domain of NtrX from *H. seropedicae* was predicted using the I-TASSER server [23]. This NtrX structure was aligned with the structure of NtrC4 bound to double DNA from *Aquifex aeolicus* [24] using the CCP4 MG software [35].

Potential σ^{70} promoters were located using the BPROM program [36].

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Author contributions

PB performed and planned experiments and wrote the paper. LRA performed and planned experiments and analyzed data. JHO performed and planned experiments and analyzed data. LUR planned experiments and analyzed data. FOP planned experiments and wrote the paper. EMS planned experiments, analyzed data and wrote the paper. NZ planned experiments. JS planned experiments. MB planned experiments, analyzed data and wrote the paper. RW planned experiments, analyzed data and wrote the paper. LSC planned experiments, analyzed data and wrote the paper.

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