



The role of NtrC in the adaptation of *Herbaspirillum seropedicae* SmR1 to nitrogen limitation and to nitrate

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Abstract

The RNA-Seq profiling of *Herbaspirillum seropedicae* SmR1 wild-type and *ntrC* mutant was performed under aerobic and three nitrogen conditions (ammonium limitation, ammonium shock, and nitrate shock) to identify the major metabolic pathways modulated by these nitrogen sources and those dependent on NtrC. Under ammonium limitation, *H. seropedicae* scavenges nitrogen compounds by activating transporter systems and metabolic pathways to utilize different nitrogen sources and by increasing proteolysis, along with genes involved in carbon storage, cell protection, and redox balance, while downregulating those involved in energy metabolism and protein synthesis. Growth on nitrate depends on the *narKnirBDHsero_2899nasA operon* responding to nitrate and NtrC. Ammonium shock resulted in a higher number of genes differently expressed when compared to nitrate. Our results showed that NtrC activates a network of transcriptional regulators to prepare the cell for nitrogen starvation, and also synchronizes nitrogen metabolism with carbon and redox balance pathways.

Keywords *Herbaspirillum seropedicae* · Nitrogen metabolism · NtrC regulon · Nitrate metabolism · Assimilatory Nitrate Reductase

Introduction

Herbaspirillum seropedicae SmR1 is a diazotrophic, endophytic β -proteobacterium that associates with the aerial and underground parts of many plants, including crops of economic importance such as sugarcane and wheat (Baldani et al. 1986). Genes involved in the fixation of atmospheric nitrogen as well as ammonia assimilation are under the control of the Ntr system (Chubatsu et al. 2012), whose major components include the two-component system NtrB/NtrC, the PII family proteins GlnB and GlnK, and

the post-translational modifying PII enzyme GlnD. At high intracellular ammonium levels, GlnB interacts with NtrB and prevents it from phosphorylating NtrC. Non-phosphorylated NtrC is unable to activate transcription. When cellular nitrogen levels are low, GlnD uridylylates the PII proteins. Uridylylated GlnB is unable to interact with NtrB. In this way, NtrB can phosphorylate NtrC, which in turn activates the transcription of genes required for metabolic adaptation to low nitrogen levels (Chubatsu et al. 2012).

Nitrate is the major inorganic nitrogen source in soil, and *H. seropedicae* has regulatory and structural genes for two nitrate reductases: respiratory nitrate reductase (NAR) and assimilatory nitrate reductase (NAS) (Pedrosa et al. 2011). NAR produces mainly nitrite at low oxygen levels, but also nitric oxide (Bonato et al. 2016). Consequently, NAR-encoding genes are modulated by low oxygen levels (via Fnr proteins) and by nitrate (Bonato et al. 2016; Batista et al. 2013). NAS is also induced by nitrate, but its function has not yet been characterized in *H. seropedicae* (Bonato et al. 2016).

The *H. seropedicae ntrC* mutant was unable to use nitrate as the sole nitrogen source, suggesting that NtrC induces genes for nitrate assimilation (Persuhn et al. 2000).

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Likewise, *ntnC* mutants of other Proteobacteria, including *Agrobacterium tumefaciens* (Rossbach et al. 1987), *Azospirillum brasilense* (Machado et al. 1995), *Azotobacter vinelandii* (Toukdarian and Kennedy 1986), *Bradyrhizobium japonicum* (Martin et al. 1988), *Pseudomonas aeruginosa* PAO1 (Li and Lu 2007), *Ensifer meliloti* (formerly *Rhizobium meliloti* and *Sinorhizobium meliloti*) (Szeto et al. 1987) and *Burkholderia cenocepacia* (Liu et al. 2017) are also unable to grow with nitrate as the sole nitrogen source. In previous studies, the NtrC regulon was defined using microarrays in *B. japonicum* (Franck et al. 2015), *Escherichia coli* (Zimmer et al. 2000), *P. putida* (Hervás et al. 2008), *E. meliloti* (Davalos et al. 2004) and *B. cenocepacia* as a response to low nitrogen treatments, but not as a response to nitrate.

In this work, we investigated the role of NtrC in the adaptation of *H. seropedicae* from nitrogen limitation to a 30-min ammonium or nitrate shock. Our results indicate that NtrC was necessary to scavenge nitrogen, but also to redirect carbon metabolism to storage and control redox balance. *H. seropedicae* adapted rapidly to ammonium, but adaptation to nitrate was slower and depended on the NAS operon (*narKnirBDHsero_2899nasA*). NtrC is essential for the activation of this operon and emerges as master regulator not only under ammonium-limiting conditions but also linking nitrogen and carbon metabolism to cellular redox under nitrogen-limiting conditions.

Materials and methods

Global transcriptal profile by RNA-Seq

All strains and plasmids used in this study are listed in Tables S1 and S2. Wild-type *H. seropedicae* SmR1 or the *ntnC* mutant were cultured in NFbHP-malate (Klassen et al. 1997) supplemented with 2 mM NH₄Cl medium at 30 °C and 120 rpm. Cells were harvested at OD_{600nm} ≈ 0.9, re-inoculated in NFbHP-malate supplemented with 1 mM NH₄Cl (1/10 dilution; OD₆₀₀ ≈ 0.09), and cultured aerobically at 30 °C and 250 rpm (50 mL medium in 250 mL Erlenmeyer flask). When the cells reached an OD₆₀₀ of 0.4 (after 4 h of growth), they were divided into three parts: (i) one was kept in RNAlater™ stabilizing solution (Thermo Fisher Scientific, Waltham, MA, USA) (ammonium-limiting condition or NH₄-LC) and the other two were incubated for another 30 min after adding (ii) 10 mM NH₄Cl (ammonium-shock condition or NH₄-SC) or (iii) 10 mM KNO₃ (nitrate-shock condition or NO₃-SC). After incubation, cells were collected and stored in RNAlater™. The experiments were performed in biological duplicates.

Trizol™ (Thermo Fisher Scientific) was used to extract total RNA from cells stored in RNAlater™, and the purified RNA was precipitated with 10 M LiCl. Contaminant

DNA was degraded with TURBO™ DNase (Thermo Fisher Scientific). Ribosomal RNA (rRNA) was removed using the Ribo-Zero rRNA Removal Kit (Gram-negative bacteria) (Illumina, San Diego, CA, USA). cDNA libraries were constructed using the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) and sequenced in an Ion Proton™ Sequencer (Thermo Fisher Scientific).

Qualitative RT-PCR

The oligonucleotides used in this work are listed in Table S3. cDNA was obtained from 1.3 µg total RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) (total volume 20 µL). For qualitative RT-PCR, 1 µL of the cDNA synthesis reaction (1/10 dilution) was used as template and GO Taq Flexi DNA polymerase kit (Promega, Madison, WI, USA) was used for amplification according to the manufacturer's instructions (total volume 10 µL). PCR was performed as follows: (i) denaturation at 95 °C for 2 min, (ii) 25 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 15 s. The PCR products were analyzed by electrophoresis in 3% agarose gels.

Transcriptomic data analysis

Sequencing reads were trimmed using the CLC Genomics Workbench (Qiagen, Hilden, Germany) to remove reads smaller than 20 nucleotides and a quality score lower than 20. The trimmed reads were mapped against the *H. seropedicae* SmR1 genome (NC_014323.1) with a minimum length fraction of 0.9 and a minimum similarity fraction of 0.9 using the CLC Genomics Workbench. Differentially expressed genes were analyzed using DESeq 2.0 and edgeR (Love et al. 2014; Robinson et al. 2010). Genes were considered differentially expressed if the log₂-fold-change was ≥ 1.0 or ≤ -1.0 and the FDR-corrected the *p*-value < 0.05 in both DESeq 2.0 and edgeR. Gene Set Enrichment Analysis (GSEA) was performed using mgsa (Bauer et al. 2010) with differentially expressed genes, considering in Population genes ≥ 10 genes and estimates ≥ 0.5. Pathways from the KEGG database (Kanehisa et al. 2014) were recovered using the "kegg.gsets" function of GAGE (Luo et al. 2009). GSEA with KEGG pathways was performed to identify groups of genes that were regulated in each nitrogen treatment or by NtrC (Table S4). DESeq 2.0, edgeR, mgsa and gage are R packages from the Bioconductor project (Gentleman et al. 2004). The heatmap was constructed using the R package pheatmap (<https://CRAN.R-project.org/package=pheatmap>).

To identify genes whose expression is directly or indirectly regulated by NtrC, the transcriptional profile of the *H. seropedicae* wild-type and the *ntnC* mutant was compared in each nitrogen treatment (NH₄-LC, NO₃-SC, and NH₄-SC). Genes that were downregulated in *ntnC* were considered to be

activated by NtrC, whereas upregulated genes were considered to be repressed by NtrC. Furthermore, it was considered strong evidence for direct regulation by NtrC if a gene (1) was induced under nitrogen limitation (\log_2 -fold-change ≥ 1.0 ; p -value FDR-corrected < 0.05), (2) its expression was dependent on NtrC activation (\log_2 -fold-change ≤ -1.0 ; p -value FDR-corrected < 0.05), and (3) it showed a conserved putative Sigma 54 and NtrC binding sites in its promoter region.

Construction of a *H. seropedicae nasA* mutant

The *nasA* mutant was obtained by homologous recombination, deleting 2715 bp (out of 2769 bp) of the *nasA* coding sequence. DNA fragments located upstream and downstream of *nasA* were amplified by PCR and ligated into the pSUP202 suicide vector (Table S2) (Simon et al. 1983), resulting in the pSUP-*nasA* plasmid. A DNA fragment containing the gene encoding for the levansucrase and kanamycin resistance cassette (*sacB Km*) was ligated into pSUP-*nasA*, generating the pSUP-*nasA-sacB Km* plasmid. Subsequently, this plasmid was transferred into the *H. seropedicae* wild-type strain by conjugation using *E. coli* S17.1 as the donor strain. Kanamycin-resistant colonies (from the first recombination event) were plated on 5% (w/v) sucrose plates to select colonies from the second recombination event. The final *nasA* deletion mutant was screened by PCR.

Construction of the *phaZ2::lacZ* transcription fusion plasmid

The promoter region of *phaZ2* (Hsero_0639) was amplified and cloned into the pMP220 vector (Spaink et al. 1987) (Table S2). This fragment (509 bp) corresponds to the entire intergenic region between *Hsero_0638* and *phaZ2*, including the 3' end the coding sequence of *Hsero_0638* in order to include a putative NtrC binding site. The plasmids were transformed into the *E. coli* ET8000 wild-type and the *ntrC* NT8000 strains (Table S1), and the cells were cultured in high or low ammonium concentration. β -galactosidase activity was determined as described (Miller 1972).

Data availability

The sequencing data have been deposited in the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>) under the accession number E-MTAB-8238.

Results and discussion

RNA-Seq experimental strategy

To determine the sampling strategy for RNA-Seq experiments, the *H. seropedicae* wild-type strain was grown in medium containing 1 mM NH_4Cl , a nitrogen-limiting concentration. The cells stopped growing at an OD_{600} of 0.4 when the NH_4Cl ran out (Fig. 1A). The cell number achieved (approximately 1×10^8 cells $\times \text{mL}^{-1}$) was sufficient for RNA extraction. This strategy of starting the culture with 1 mM NH_4Cl ($\text{NH}_4\text{-LC}$) was necessary because the *ntrC* mutant does not grow on nitrate as the sole nitrogen source (Persuhn et al. 2000). The cells were then subjected to ammonium shock ($\text{NH}_4\text{-SC}$) or nitrate shock ($\text{NO}_3\text{-SC}$) (Fig. 1B). Thirty minutes after the nitrate shock was sufficient to activate genes related to nitrate assimilation (*nasA*, *nirD* and *nasF* genes) (Fig. 1C). On this basis, the RNA-Seq experimental design used in this study was established (Figs. 1D and E). Whole transcriptome shotgun sequencing was performed by repeating these experiments with both the wild type and the *ntrC* mutant under these three nitrogen conditions.

Modulation of gene expression by nitrogen

Principal component analysis (PCA) using data from all transcriptomes showed a clear difference between cells under nitrogen deprivation and 30 min after ammonium or nitrate shock for both the wild-type strain and the *ntrC* mutant (Fig. 2A).

The addition of ammonium or nitrate to a culture grown under nitrogen-limiting conditions led to a shift in metabolism with several genes being up- or downregulated. An overall view of the differential gene expression as a \log_2 -fold-change is shown for the wild-type strain after ammonium and nitrate shock (Fig. 2B). The ammonium shock resulted in a higher number of genes that had a \log_2 -fold-change higher or lower than +1 or -1 compared to the nitrate shock, indicating a more dramatic change than the nitrate shock (Fig. 2B) and suggesting that nitrate was metabolized more slowly than ammonium. By comparing the wild-type strain growing under nitrogen limitation to ammonium shock, we were able to identify the major genes for ammonium assimilation in *H. seropedicae*. Our analysis revealed that 405 genes were downregulated by ammonium shock. On the other hand, 335 genes were upregulated indicating that these are required for bacterial metabolism under ammonium limitation (Fig. 3A). When comparing nitrate shock to the ammonium-limiting condition, we found 56 downregulated genes. Additionally, 66

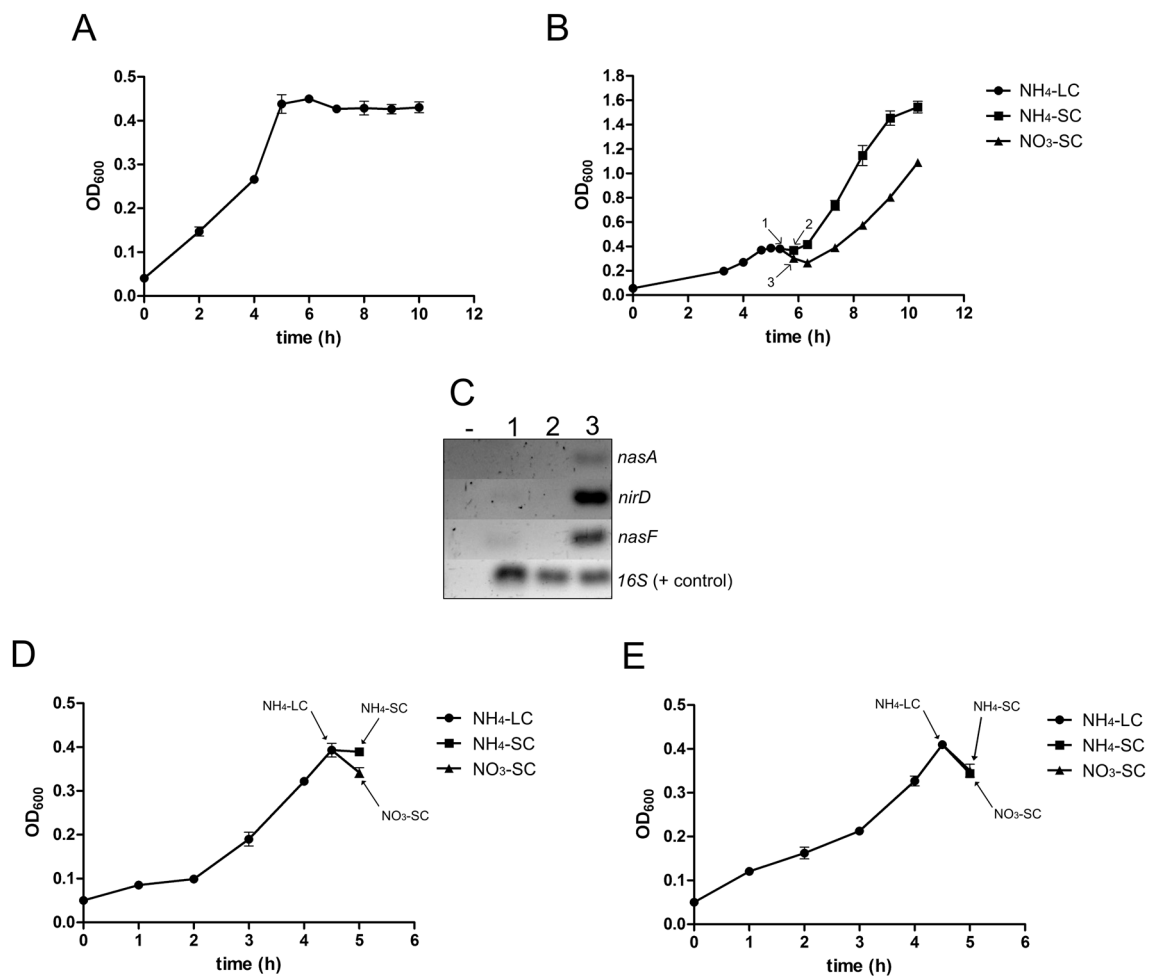


Fig. 1 Effect of nitrogen limitation and ammonium or nitrate shock on wild-type *H. seropedicae* Smr1 and *ntrC* mutant. Growth of *H. seropedicae* Smr1 in NFbHP-malate supplemented with 1 mM NH₄Cl (A). Cells were grown as described in A and then exposed to ammonium or nitrate shock. The numbers from 1 to 3 refer to: (1) ammonium-limiting condition (NH₄-LC), (2) 30 min after ammonium shock (NH₄-SC) and (3) 30 min after nitrate shock (NO₃-SC) (B). Qualitative RT-PCR with cDNA prepared from RNA extracted as in B (arrows 1–3) and specific primers for subunits of assimi-

tory nitrate reductase (*nasA*), nitrite reductase (*nirD*) and a nitrate transporter (*nasF*). 16S rRNA was used as a positive control (+control), while “-” corresponds to the negative controls (without addition of cDNA) (C). The *H. seropedicae* wild-type strain (D) or the *ntrC* mutant (E) were grown in NFbHP-malate supplemented with 1 mM NH₄Cl to an OD₆₀₀ of 0.4 and then submitted to ammonium or nitrate shock for 30 min. The arrows indicate the samples used for RNA extraction

genes were upregulated, suggesting that their expression was induced to adapt cell metabolism to nitrate (Fig. 3B).

Comparison of total gene expression of the wild-type strain and the *ntrC* mutant showed that a larger number of genes were expressed differently under nitrogen limitation than under ammonium shock conditions (seen as log₂-fold-change higher or lower than +1 or -1) (Fig. 2C). In response to nitrogen limitation, 268 genes were activated and 259 genes were repressed directly or indirectly by NtrC (Fig. 3A). On the other hand, NtrC regulated fewer genes in response to ammonium shock, as 115 genes were repressed and 69 genes were activated by NtrC, emphasizing the important role of the *ntrC* product in the metabolic changes required to overcome ammonium limitation.

Modulation of gene expression by ammonium limitation

The Nitrogen Metabolism pathway was induced under NH₄-LC (Table S4), including genes encoding proteins of the Ntr System (*glnAntrBC* and *nlmAglnKamtB*) and the *nifA* gene. The expression of *glnAntrBC*, *nlmAglnKamtB* and *nifA* was dependent on NtrC under ammonium limitation (Table S5), which is consistent with biochemical data from previous reports (Noindorf et al. 2011, 2006; Schwab et al. 2007; Persuhn et al. 2000). Moreover the promoter region of these operons has conserved putative NtrC and Sigma 54 binding sites (Table S6). These operons encode the ammonium assimilation enzyme glutamine synthetase, the two-component system NtrB/NtrC, the ammonium permease

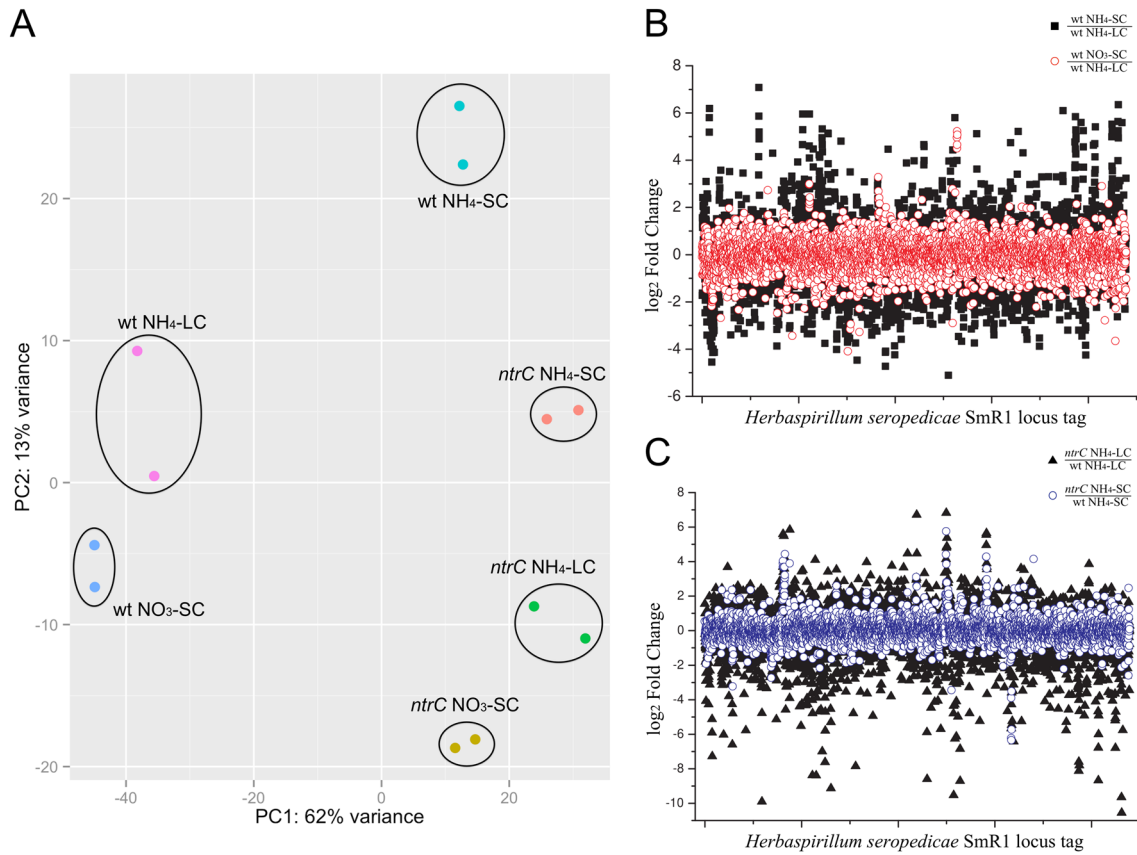


Fig. 2 Principal component analyses (PCA) of all RNA-Seq libraries used in this study (A). Overall view of differential expression (log₂-fold-change) of each gene in *H. seropedicae* SmR1 using the genome locus tag position for mapping compared to the wild-type strain after

ammonium- or nitrate-shock (B) or compared between the *ntrC* mutant and the wild-type strain under low ammonium concentration (NH₄-LC) and after the ammonium shock (NH₄-SC) (C)

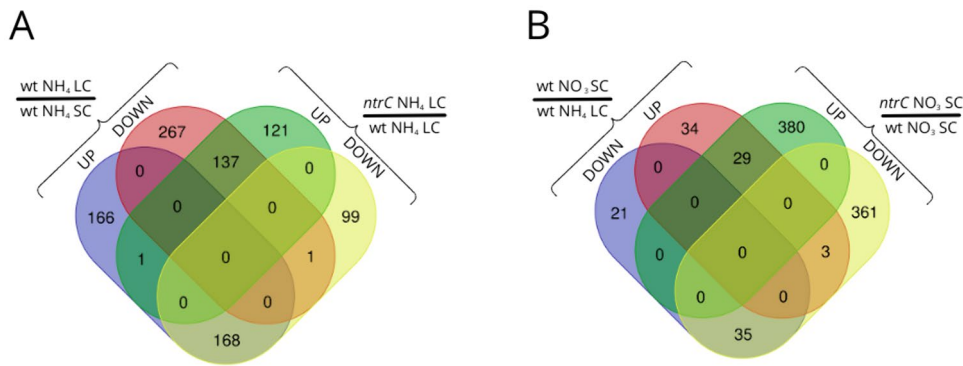


Fig. 3 Venn diagrams comparing differentially expressed genes between *H. seropedicae* wild-type and *ntrC* mutant. A On the left, up- and downregulated genes (referred to as UP and DOWN, respectively) by ammonium limitation when compared to ammonium shock. On the right, up- (or "repressed" via NtrC) and downregulated (or "activated" via NtrC) genes under ammonium limitation, when com-

paring the *ntrC* mutant to the wild-type strain. B On the left, up- and downregulated genes by nitrate shock when compared to ammonium shock. On the right, up- (or "repressed" via NtrC) and downregulated (or "activated" by NtrC) genes under nitrate conditions, when comparing the *ntrC* mutant to the wild-type strain

Table 1 Promoter activity of *phaZ2::lacZ* fusion under high or low ammonium concentration

Plasmid	β-galactosidase activity (Miller units)		
	ET8000		NT8000
	+N	-N	-N
pMP220 (control)	10.0 ± 0.8	1.8 ± 0.5	0.7 ± 0.0
pRP3 (<i>phaZ2::lacZ</i>)	7.8 ± 0.01	138.5 ± 3.5	0.9 ± 0.4

E. coli ET8000 (wild type) and NT8000 (*ntrC*) strains carrying indicated plasmids were grown in M9 minimal medium with 10 mM (+N) or 1 mM (-N) NH₄Cl. β-galactosidase activity was determined and is indicated as Miller units. Data correspond to a representative experiment and are indicated as mean ± standard deviation of biological duplicate

AmtB and the PII protein, GlnK, which is required for the activity of NifA (Noindorf et al. 2011), as well as NifA, which is the transcriptional activator of the *nif* genes required for the nitrogenase complex (Pedrosa et al. 2001). Although the expression of *nifA* exhibited a log₂-fold-change of 5.8, the overall expression of *nif* genes was only slightly induced, most likely due to lower NifA activity under the more aerobic condition performed in our experiments, as NifA has been shown to be inactivated by O₂ (Souza et al. 1999).

Sigma 54 (Sigma N, σ^N) directs RNA polymerase to the -24/-12 promoters in genes regulated by AAA+ domain-containing transcriptional activators such as NtrC and NifA. Sigma 54 is encoded by the *rpoN* gene, which was slightly induced by ammonium limitation when compared to nitrogen-repleted condition in *H. seropedicae*, as also observed in *Paraburkholderia phymatum* (Lardi et al. 2017).

Another operon that has been described as strongly induced under nitrogen-limiting conditions in *E. coli* is *yeaGH*, which encodes a putative Ser protein kinase and a conserved protein, respectively (Switzer et al. 2018a, b). Although the role of these gene products in nitrogen metabolism has not been fully characterized, a role in methionine metabolism in *E. coli* has been reported (Switzer et al. 2018a, b). Under our experimental conditions, *prkA* (*yeaG*) and *yeaH* genes were highly induced in NH₄-LC (Table S5), and while NH₄-SC condition decreased the expression of *prkA* (*yeaG*) and *yeaH* genes, NO₃-SC failed to restore the basal expression level, suggesting that *yeaGH* expression was responsive to ammonium but not nitrate under our conditions.

Genes encoding transporter systems for nitrogen-containing compounds were also induced under nitrogen limitation, including amino acids (*livKMG*), nitrate (*nasFED* and *narK*), and urea (*urtABCDE*) transporter systems (Table S5). The Nicotinate and Nicotinamide metabolism pathway was also activated, including genes encoding enzymes that convert nicotinate to maleate, a pathway that generates ammonium ions (Table S4). Other genes encoding enzymes involved in

the catabolism of nitrogenous compounds were also induced, including arginine, nitrite and urea (Table S5). The expression of most of these genes was dependent on NtrC, including transporters of nitrogenous compounds (amino acids, ammonium, nitrate, urea) and enzymes involved in the catabolism of arginine, nicotinate, urea, and nitrate (Tables S5 and S6). Genes encoding a peptidase (*Hsero_4343*) and two putative proteases (*Hsero_0634* and *yibP* (*Hsero_0164*)) were also induced under NH₄-LC, and the expression of the latter two genes was considered as directly activated by NtrC (Table S6).

Carbon metabolism was also directly affected by the nitrogen-limiting condition (Tables S4 and S5). Among the activated genes of the Starch and Sucrose metabolism pathway are those encoding enzymes involved in trehalose (*otsAB*) and glycogen (*glgAB*) synthesis, suggesting carbon utilization and/or storage in the form of trehalose and glycogen under nitrogen-deficient conditions (Tables S4 and S5). Additionally, in silico analyses suggest that NtrC activates genes encoding enzymes involved in trehalose synthesis (*otsAB*) (Table S6). Accumulation of trehalose under nitrogen limitation has also been reported in *Corynebacterium glutamicum* as well as in *Ectothiorhodospira halochloris* (Galinski and Herzog 1990; Wolf et al. 2003), and synthesis of glycogen under ammonium limitation has also been reported in *P. putida* (Hervás et al. 2008). An intrinsic connection between trehalose and glycogen metabolism in bacteria has been described (Chandra et al. 2011). Although trehalose accumulation may serve as a glucose store, this disaccharide has also been associated with cellular signaling as well as with a bioprotective stress response (Chandra et al. 2011).

H. seropedicae can also store carbon as polyhydroxybutyrate (PHB) (Catalán et al. 2007), a polymer also associated with cell protection (Batista et al. 2018; Sacomboio et al. 2017). Most of the genes involved in PHB metabolism were expressed under our experimental conditions but were not regulated by nitrogen limitation or NtrC. Our experiments were performed under high aeration conditions, supporting the role of Fnr and thus O₂ in regulating PHB synthesis in *H. seropedicae* (Batista et al. 2018) and also suggesting that PHB accumulation increases at low O₂ and is not solely due to nitrogen-limiting conditions causing C/N imbalance. Under our experimental conditions, only two genes involved in PHB metabolism were strongly regulated under nitrogen limitation: *phaP2* (*Hsero_4759*) and *phaZ2* (*Hsero_0639*), which encode a phasin and a putative PHB depolymerase, respectively (Table S5). Although *phaP2* and *phaZ2* are clearly upregulated at low ammonium concentrations, they appear to play a secondary role in PHB metabolism, as the major functions associated with them are carried out by other proteins under normal growth conditions. In *H. seropedicae*, the major phasin covering PHB granules is

PhaP1 (Hsero_1639), and although PhaP2 was also found associated with PHB granules, its levels and expression were very low (Tirapelle et al. 2013). It has also been shown that PhaP2 was able to replace PhaP1 in a $\Delta phaP1$ mutant and function as a replacement protein to cover PHB granules (Tirapelle et al. 2013; Alves et al. 2016). Under our conditions, *phaP1* and *phaP2* exhibited different expression patterns, with *phaP2* being strongly induced under nitrogen limitation, and hence further studies are needed to determine the role of PhaP2 under these conditions. In *H. seropedicae*, PHB mobilization is mainly through the PHB depolymerase encoded by *phaZ1* (Hsero_1622), which appears to be constitutively expressed (unpublished results). However, similar to *phaP2*, the *phaZ2* gene (which is hardly expressed at high ammonium concentration) was highly expressed under $\text{NH}_4\text{-LC}$ and strongly dependent on NtrC (Table S5), a result supported by in silico analyses (Table S6) and gene fusion assays (*phaZ2::lacZ*) carried out in *E. coli* wild type and *ntrC* mutant strains (Table 1). This result also adds to the findings by Sacomboio and colleagues (Sacomboio et al. 2017), who showed that the accumulation of PHB was higher in the *ntrC* mutant. In that report, the increase in PHB synthesis was associated with a higher availability of NADPH in the *ntrC* mutant. Furthermore, *phaZ2* regulation by NtrC, which likely contributes to PHB mobilization, may lead to increased availability of NAD(P)H, which is required for assimilation of other nitrogen compounds under ammonium limitation. These results reinforce that the physiological function of PHB seems to go beyond carbon storage and plays a crucial role in the cellular redox balance (Batista et al. 2018; Sacomboio et al. 2017).

A gene locating on near the *phaZ2* gene with opposite direction is the *gst* gene (Hsero_0638), which encodes a glutathione S-transferase (GST) and whose expression was also highly induced by ammonium limitation (Table S5). Sigma 54 binding sites were identified in the intergenic region between *gst* and *phaZ2* for each gene, revealing an interesting aspect of the orchestration of transcription of both genes (Table S6). Glutathione S-transferases form a superfamily of proteins involved in cellular detoxification by conjugating glutathione to various compounds (Allocati et al. 2009), and although they are usually associated with defense against reactive oxygen species, their physiological role appears to encompass a broader range of compounds (Sheehan et al. 2001; Vuilleumier 1997). Several GST-encoding genes are usually found in an organism, but their substrate specificity is more difficult to determine (Vuilleumier 1997). Furthermore, these genes can be constitutively expressed or induced by different compounds or cellular conditions (Vuilleumier 1997). In *Azospirillum brasilense*, one GST has been shown to be upregulated at low ammonium concentrations and its expression is dependent on NtrC (Kukulj et al. 2020). The genome of *H. seropedicae* contains 11 genes annotated as

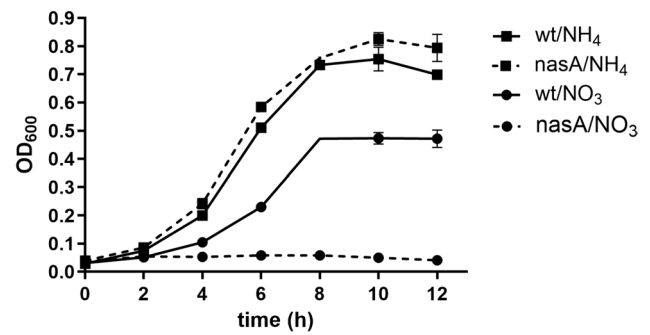
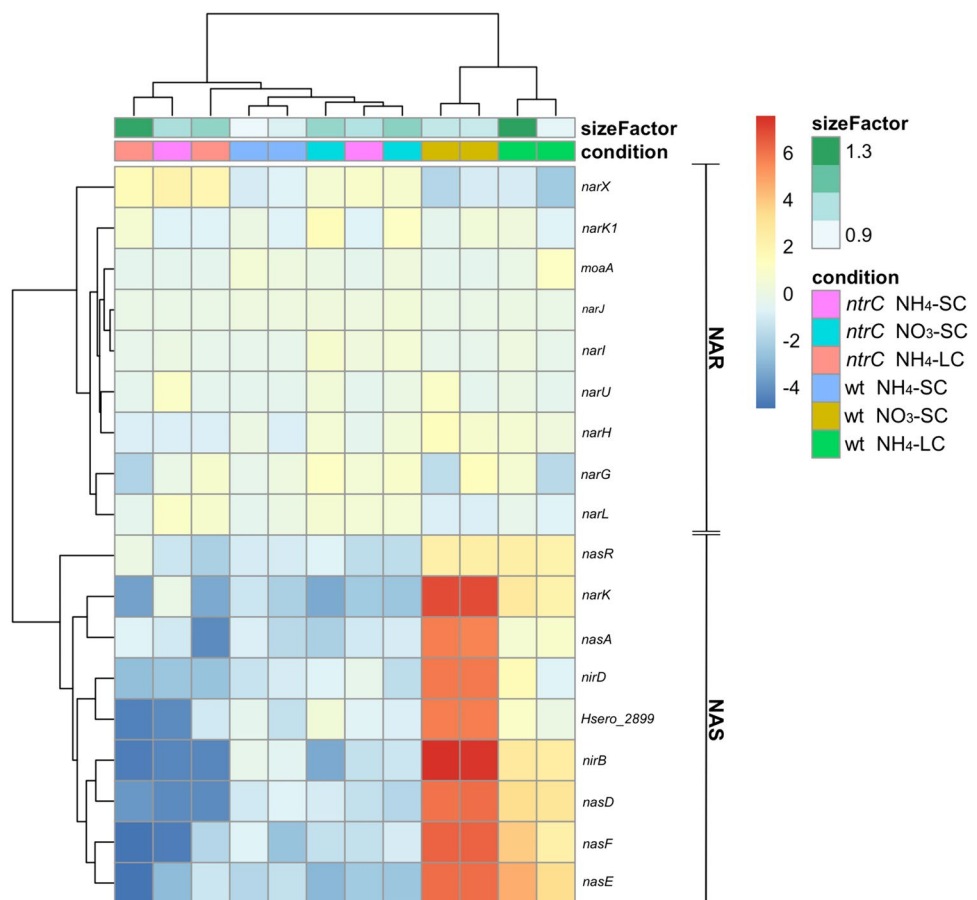


Fig. 4 Cellular growth of *H. seropedicae* SmR1 wild-type strain (wt) and the *nasA* mutant (*nasA*) grown in ammonium (NH_4) or nitrate (NO_3) as the sole nitrogen sources. At the indicated times, samples were taken to measure absorbance at 600 nm for cellular growth. The results represent the profile of experiments conducted in biological duplicates. Data are presented as the mean and standard deviation of two technical replicates

gst with different sequence similarities. A multiple sequence alignment showed that *Hsero_4283* has the highest similarity (60%) to *Hsero_0638* and showed a slight up-regulation at low ammonium concentration (log₂-fold-change of 2.4 compared to 7.1, Table S5). Transcription of *Hsero_0638* was 5.4-fold higher than that of *Hsero_4283* under $\text{NH}_4\text{-LC}$, suggesting a more relevant role under ammonium limitation (data not shown). Although putative NtrC binding sites were found upstream of the promoter regions of *Hsero_0638* and *Hsero_4283*, their expression was not found to be dependent on NtrC (*p*-values were higher than 0.05) (Tables S5 and S6). However, the role of NtrC in the regulation of *gst* genes should be further investigated in *H. seropedicae*. The other *gst* genes in *H. seropedicae* were not regulated by nitrogen limitation under our experimental conditions.

Nitrogen limitation also promotes the synthesis of exopolysaccharide (EPS) in bacteria (Liu et al. 2017; Janczarek 2011). In *H. seropedicae*, there is a cluster of 28 genes potentially involved in EPS biosynthesis and secretion (Balsanelli et al. 2014). Our results indicated that among these genes, *epsLBDAFG* were upregulated under $\text{NH}_4\text{-LC}$ (Table S5). These gene products are involved in EPS biosynthesis, as an *epsB* mutant showed a decrease in EPS production (Balsanelli et al. 2014). The *epsB* mutant was found to be more sensitive to low phenol and SDS concentrations, corroborating the association of EPS production and protection from chemical stress; however, no significant effect on the colonization profile of maize was observed (Balsanelli et al. 2014). It has also been described that exopolysaccharide production is regulated by NtrC (Janczarek 2011; Liu et al. 2017). In *H. seropedicae*, although RNA-seq analysis point to a possible role of NtrC in the regulation of the *epsLBDAFG* operon (Table S5), and a putative NtrC binding site was identified in the DNA region upstream of *epsL*, a Sigma 54 binding

Fig. 5 Heatmap of the expression of genes related to nitrate metabolism in *H. seropedicae*. All data obtained from RNA-seq libraries, conducted in duplicates, were included in the analysis, but here we chose to represent only NAS genes (involved in nitrate assimilation—*narK*, *nirBD*, *Hsero_2899*, *nasA*, *nasFED*, and *nasR*) and NAR genes (related to respiratory nitrate reductase—*narK1UGHJI*, *moaA*, and *narXL*). The data was normalized using DESeq2 and the heatmap was generated using pheatmap. Samples and genes are hierarchically clustered. ‘Size factor’ indicates the relative size of each library, with greener colors representing larger libraries



site was not found in this promoter. Therefore, NtrC could not be considered a direct regulator of *eps* genes.

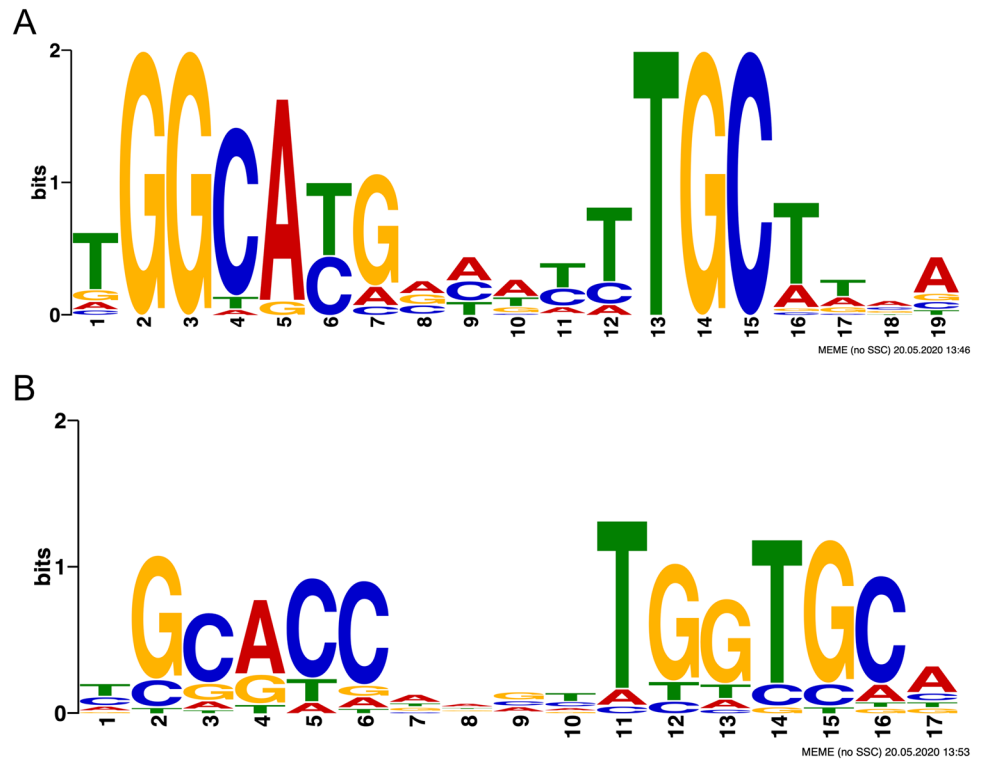
Nitrogen limitation is a stressful condition for bacteria, and the accumulation of guanosine pentaphosphate and tetraphosphate ((p)ppGpp) has been described under these conditions (Ronneau and Hallez 2019). Brown (2019) has shown in *E. coli* that *relA*, which encodes the (p)ppGpp synthetase, is regulated by NtrC and thus there is a coupling between nitrogen status and the stringent response (Brown 2019). *H. seropedicae* has two genes involved in (p)ppGpp synthesis, *relA* and *spoT*. However, under our experimental conditions, neither the expression of *relA* (Hsero_1973) nor *spoT* (Hsero_2128) was upregulated by ammonium limitation or was dependent on NtrC (Tables S5 and S6). It is important to emphasize that our experiments were performed under high O₂ levels and low cell density, conditions under which the stringent response might have less influence. The effects of these factors on (p)ppGpp production should be considered (Ronneau and Hallez 2019).

Under ammonium limitation, *H. seropedicae* downregulated the expression of several pathways related to protein synthesis, such as Ribosome, RNA degradation, and Protein export (Tables S4 and S5). Similarly, several genes encoding the ribosome were also repressed under nitrate shock (Tables

S4 and S5), implying that repression of protein synthesis is an important process that bacteria use to adapt to more severe conditions and to cope with nitrogen starvation, as was also observed in *P. pseudoalcaligenes* and *Mycobacterium tuberculosis*, which also block protein synthesis in response to nitrogen limitation (Ronneau and Hallez 2019; Williams et al. 2015).

Metabolic pathways related to Energy Metabolism, such as Citrate cycle (TCA cycle), Oxidative phosphorylation and 2-Oxocarboxylic acid metabolism, were also repressed under ammonium limitation (Tables S4 and S5). Some of those pathways repressed by ammonium limitation were shown to be repressed by NtrC, including TCA cycle, Oxidative phosphorylation, Ribosome and RNA degradation (Tables S4 and S5). However, this result does not seem to be a consequence of differences in growth between wild-type and *ntrC* mutant, as both strains grew at similar rates under nitrogen limitation (Fig. 1D and E). In addition, these results are consistent with previous transcriptome analyses using *ntrC* mutants of *P. putida* and *B. cenocepacia*, reinforcing the hypothesis that NtrC may repress carbon metabolism under nitrogen limitation (Hervás et al. 2008; Liu et al. 2017).

Fig. 6 Putative Sigma 54 and NtrC consensus sequences of *H. seropedicae*. Proposed consensus sequence for Sigma 54 (A) and NtrC of *H. seropedicae* (B) generated with MEME Suite



Modulation of gene expression by nitrate shock

Overall, the major genes and KEGG pathways that were regulated by NtrC in response to low nitrogen were also regulated by NtrC in response to nitrate, indicating an overlap between the two responses (Table S4).

Although the transcripts induced by $\text{NO}_3\text{-SC}$ and $\text{NH}_4\text{-LC}$ were similar (Fig. 2B), two major operons were highly activated by nitrate: *narKnirBDHsero_2899nasA* (NAS operon) and *nasFED*, both of which are involved in nitrate assimilation and whose expression is dependent on NtrC (Tables S5 and S6). To test the role of NasA in nitrate assimilation, a mutant of *H. seropedicae* carrying a *nasA* deletion was constructed. The *nasA* mutant grew on ammonium as the wild type, but was unable to grow on nitrate as the sole nitrogen source, supporting the hypothesis that NAS is essential for nitrate assimilation in *H. seropedicae* (Fig. 4). The expression of *nasA* is highly increased after nitrate shock and is dependent on NtrC (Tables S5 and S6). The *Klebsiella oxytoca* M5al *nasR* gene encodes a regulatory protein which seems to inhibit transcription termination through

interaction with *nasF* leader RNA (Chai and Stewart 1998). In *H. seropedicae*, *nasR* was also upregulated by nitrate and dependent on NtrC (Tables S5 and S6) implying a regulatory role on nitrate assimilation.

Genes encoding a second nitrate reductase (the respiratory nitrate reductase or NAR) are found in *H. seropedicae*: *narK₁UGHJImoaA* (Bonato et al. 2016). NAR is not required for nitrate assimilation but it seems to be involved in nitrite and NO production as well as redox power dissipation (Bonato et al. 2016). The operon located downstream of the *moaA* gene is *narXL*, which encodes a two-component system, and that was not differently expressed under our experimental conditions. This is in agreement with previous results showing that the *narXL* expression as well as the *narK₁UGHJImoaA* expression is dependent on both nitrate and Fnr (Bonato et al. 2016; Batista et al. 2013). Under the higher aeration conditions used in this work, these genes were expressed at basal levels under all conditions tested (Fig. 5), showing that genes related to NAR are not upregulated by nitrogen limitation and further supporting the role of low oxygen concentrations in the activation of genes

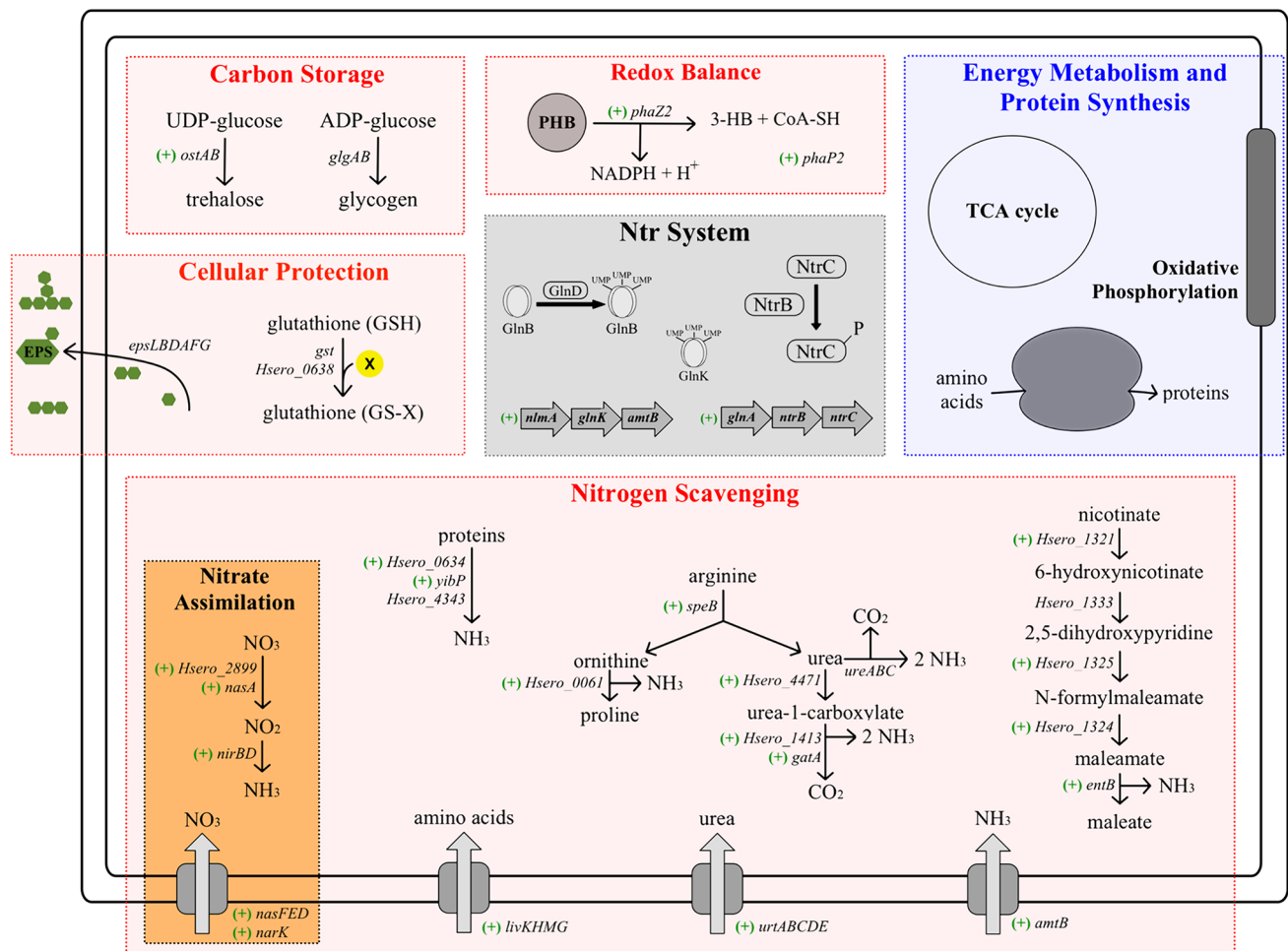


Fig. 7 Metabolic adaptations of *H. seropedicae* to nitrogen limitation and nitrate shock. The main metabolic pathways activated under NH₄-LC (nitrogen scavenging, carbon storage, cellular protection and redox balance) are shown in red, while the metabolic pathways repressed under these conditions (energy metabolism and protein synthesis) are shown in blue. The pathway that was activated under NH₄-LC and NO₃-SC and is required for nitrate assimilation (nitrate assimilation) is shown in orange. The Ntr system is shown in gray. The expression of *glnB* and *glnD* was constitutive. When the GlnB

protein is uridylylated, NtrB is free to phosphorylate NtrC. The phosphorylated form of NtrC (NtrC-P) regulates gene expression. There is strong evidence that NtrC-P directly activates the transcription of genes marked with “(+)”. Genes were considered with strong evidence of direct activation by NtrC whether: (1) their expression was induced under ammonium limitation and (2) dependent on NtrC and have putative (3) NtrC and (4) Sigma 54 binding sites on their promoter region (see also Table S6)

encoding this enzymatic complex, while genes related to NAS were highly induced by nitrate (Fig. 5).

Taken together, these data support the view that NAS is the major pathway used by *H. seropedicae* to assimilate nitrate and suggest that the *ntrC* mutant cannot grow on nitrate because NtrC is required for activation of the operon encoding NAS (*narKnirBDHsero_2899nasA*). This NtrC-dependent activation probably occurs directly because the promoter of *narK* has a highly conserved NtrC binding site (Table S6).

Consensus sequences for Sigma 54 and NtrC

To identify the consensus binding site sequences of Sigma 54 in *H. seropedicae*, we first selected genes that were highly responsive to NH₄-LC compared to NH₄-SC (log₂-fold-change ≥ 3.0) and those that were strongly activated by NtrC under nitrogen limitation (comparison of *ntrC* mutants and wild-type strains under NH₄-LC; log₂-fold-change ≤ -3.0). The intersection of these criteria resulted in 72 genes grouped into 42 operons.

The 500 base pairs upstream of these 42 operon genes were analyzed using the MEME Suite (Bailey and Elkan 1994). For 27 of these genes, the software identified the motif consensus shown in Fig. 6A, which is very similar to

the Sigma 54-dependent motif proposed by Barrios and colleagues (Barrios et al. 1999). Comparing these sequences, we derived the motif: **TGGCAYGNNNNTTGCW** (where Y stands for T or C, and W stands for A or T).

Since NtrC activates transcription of target genes via sigma 54, the 27 promoter regions with sigma 54 binding sites were used as the basis for an initial search using the MEME Suite to identify a putative NtrC binding site. When no results were found, a manual search was performed using the proposed consensus sequence for *Salmonella typhimurium* as a reference (Ferro-Luzzi Ames and Nikaido 1985). We found that 23 sequences contained both putative Sigma 54 and NtrC binding sequences. After aligning these sequences with the MEME Suite, we proposed a consensus sequence for NtrC in *H. seropedicae* (Fig. 6B). Highly similar sequences to this consensus sequence were found in the *nifA* promoter region of *H. seropedicae* and were shown to bind the NtrC protein and the Sigma 54 holoenzyme via a DNA footprint assay (Wassem et al. 2000).

Conclusion remarks

The major genes activated in *H. seropedicae* under nitrogen limitation are summarized in Fig. 7, and the Ntr system was key in activating the expression of most of them. Our results show that *H. seropedicae* copes with nitrogen limitation by assimilating, recycling and utilizing different forms of nitrogen, controlling redox levels and promoting cell defense (with EPS synthesis and GST expression). Cell metabolism is reduced by storing carbon in the form of trehalose and glycogen and slowing down energy metabolism and protein synthesis. On the other hand, nitrate seems to induce a more restricted response involving genes specifically to deal with this nitrogen compound, including nitrate assimilation, which occurs via the NAS pathway and whose expression is dependent on NtrC. This transcriptional regulator directly activates the transcription of several genes involved in trehalose storage, redox balance and metabolic pathways involved in nitrogen scavenging. These data demonstrate that NtrC activates a network of transcriptional regulators to prepare the cell for nitrogen starvation. Furthermore, these results show that NtrC is a major regulator that acts not only globally on nitrogen, but also at nodes of carbon metabolism and redox control.

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Author contributions P.B. designed and performed the experiments, analyzed the data and wrote the manuscript. D.C.-N. designed RNA-Seq experimental strategy and assisted with RNA extraction. M.T.-S. prepared and sequenced the RNA-Seq libraries. F.J.T.M. constructed

plasmids and performed promoter gene fusion experiments. R.W., M.M.S., E.M.S., and F.O.P. supervised the study. L.S.C. supervised the study and wrote the manuscript.

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Declarations

Competing interests We declare that we have no conflict of interest.

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