

UNIVERSIDADE FEDERAL DO PARANÁ

PALOMA BONATO

**REGULAÇÃO DOS GENES RELACIONADOS AO METABOLISMO
DE NITRATO EM *Herbaspirillum seropedicae***



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Tese apresentada ao Programa
de Pós-graduação em Ciências –
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Doutor em Ciências (Bioquímica).

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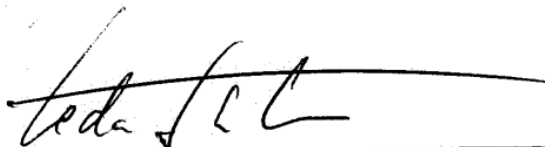
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TERMO DE APROVAÇÃO

PALOMA BONATO

Regulação dos genes relacionados ao metabolismo de nitrato em
Herbaspirillum seropedicae

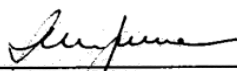
Tese aprovada como requisito parcial para obtenção do grau de Doutor no curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, pela seguinte banca examinadora:



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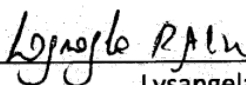
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Dedico esta tese de doutorado a todos aqueles
que direta ou indiretamente contribuíram
para o meu crescimento pessoal e científico.

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Chico Xavier

“Work hard in silence, let your success be your noise.”

Frank Ocean

“The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I've found it!), but 'That's funny...'”

Isaac Asimov

RESUMO

H. seropedicae, membro das β -proteobactérias, é um diazotrofo capaz de colonizar tecidos internos de gramíneas de interesse comercial, como trigo, milho e cana-de-açúcar. O processo de fixação de nitrogênio desta bactéria é bem estudado. Entretanto, embora o nitrato seja a principal fonte de nitrogênio inorgânico encontrado nos solos, o metabolismo deste composto em *H. seropedicae* ainda não é bem conhecido. Análises *in silico* sugerem que *H. seropedicae* apresenta genes codificando para duas enzimas que metabolizam nitrato: nitrato redutase assimilatória (NAS) (genes *HSERO_RS14545nasA*) e nitrato redutase respiratória (NAR) (genes *narGHI*). Além disso, *H. seropedicae* apresenta genes codificando os membros dos sistemas de dois componentes, NtrB/NtrC e NtrY/NtrX e mutantes *ntrC* e *ntrY* não crescem em nitrato como fonte única de nitrogênio, sugerindo que estes sistemas são importantes para regular genes do metabolismo de nitrato. Face ao exposto, o objetivo geral deste trabalho foi avaliar a função das nitrato redutases NAS e NAR de *H. seropedicae*, e entender a função dos sistemas NtrB/NtrC e NtrY/NtrX na regulação de genes acessórios envolvidos no metabolismo do nitrato. A caracterização fisiológica de um mutante na subunidade catalítica de NAS (mutante *nasA*) mostra que esta enzima é a principal forma de *H. seropedicae* assimilar nitrato. Por outro lado, a caracterização de um mutante na subunidade catalítica de NAR (mutante *narG*) sugere que esta enzima não é requerida para assimilar ou respirar nitrato. Entretanto, NAR está envolvida na produção de altas quantidades de nitrito, como também na produção de óxido nítrico. Finalmente, NAR parece ser importante para interação planta-bactéria possivelmente por produzir óxido nítrico. Os sistemas NtrY/NtrX e NtrB/NtrC são requeridos para ativar a transcrição do operon que dirige a transcrição dos genes que codificam NAS, razão pela qual os mutantes *ntrY* e *ntrC* não crescem em nitrato como fonte única de nitrogênio. Caracterizações *in vitro* da proteína NtrX mostraram que esta proteína é dimérica e que se liga diretamente a região promotora de *narXL*, que codifica um par de genes regulatórios relacionados ao metabolismo de nitrato. Análises de “footprinting” com DNase I permitiram a delimitação de uma provável região de ligação de NtrX na região promotora de *narXL*. Análises do transcriptoma global de um mutante *ntrC* por RNA-seq sugerem que o sistema NtrB/NtrC é o regulador chave de *H. seropedicae* em resposta a baixas concentrações de nitrogênio fixado, ativando genes cujos produtos estão envolvidos na recuperação de amônio, e reprimindo genes cujos produtos estão envolvidos no metabolismo energético, além de ativar genes do metabolismo de nitrato, incluindo os genes codificam para transportadores de nitrato (genes *nasFED*), e para NAS (genes *HSERO_RS14545nasA*).

Palavras-chave: *Herbaspirillum seropedicae*. Metabolismo de nitrato. Nitrato redutase assimilatória. Nitrato redutase respiratória.

ABSTRACT

H. seropedicae, a member of the β -proteobacteria, is a diazotroph that colonizes the internal tissues of crops of economic importance, such as wheat, maize and sugar cane. The nitrogen-fixing process of this bacterium is well known. However, although nitrate is the main inorganic nitrogen source found in soils, the metabolism of this compound in *H. seropedicae* is not well known. *In silico* analyses suggest that *H. seropedicae* presents genes coding for two enzymes that metabolize nitrate: the assimilatory nitrate reductase (NAS) (*HSERO_RS14545nasA* genes) and the respiratory nitrate reductase (NAR) (*narGHI* genes). Additionally, *H. seropedicae* presents genes coding for the members of the two component system, NtrB/NtrC and NtrY/NtrX, and *ntrC* and *ntrY* mutants cannot grow on nitrate as the sole nitrogen source, suggesting that these systems are important to regulate genes of nitrate metabolism. Given that, the aim of this study was to evaluate the function of the nitrate reductases NAS and NAR of *H. seropedicae*, and to understand the function of the NtrB/NtrC and NtrY/NtrX systems in the regulation of accessory genes related to nitrate metabolism. Physiological characterization of a knock-out mutant for the catalytic subunit of NAS (*nasA* mutant) shows that it is the main enzyme required to assimilate nitrate. On the other hand, the characterization of a mutant for the catalytic subunit of NAR (*narG* mutant) suggests that this enzyme is not required to assimilate or respire nitrate. However, NAR is involved in the production of high levels of nitrite, and also in the production of nitric oxide. Finally, NAR seems to be important for plant-bacteria interaction probably for producing nitric oxide. The NtrY/NtrX and NtrB/NtrC systems are required to activate the operon that drives the transcription of genes coding for NAS and probably this is the reason the *ntrY* and *ntrC* mutants cannot grow using nitrate as the only nitrogen source. *In vitro* characterization of NtrX protein showed that this protein is dimeric and binds directly to *narXL* promoter, which drives the transcription of genes related to nitrate metabolism. DNase I footprinting narrowed a putative NtrX binding site in the promoter region of *narXL*. RNA-seq analyses with *ntrC* mutant suggest that the NtrB/NtrC system is the master regulator of *H. seropedicae* in response to low levels of fixed nitrogen by activating genes whose products are involved with ammonium scavenging, and repressing genes whose products are involved with energy metabolism. Besides, this system is important to activate genes of nitrate metabolism, including the genes coding for nitrate transporters (*nasFED* genes), and for NAS (*HSERO_RS14545nasA* genes).

Key words: *Herbaspirillum seropedicae*. Nitrate metabolism. Assimilatory nitrate reductase. Respiratory nitrate reductase.

SUMÁRIO

1 REVISÃO BIBLIOGRÁFICA.....	14
1.1 <i>Herbaspirillum seropedicae</i>	14
1.2 CICLO DO NITROGÊNIO.....	14
1.3 METABOLISMO DE NITRATO.....	16
1.3.1 Genes envolvidos na assimilação de nitrato e seus produtos em procariotos.....	17
1.3.2 Genes envolvidos na desnitrificação/dissimilação de nitrato e seus produtos em procariotos.....	19
1.3.3 Nitrato redutase periplasmática (NAP).....	21
1.4 SISTEMAS DE DOIS COMPONENTES.....	22
1.4.1 Sistema NtrB/NtrC.....	23
1.4.2 Sistema NtrY/NtrX.....	24
1.5 METABOLISMO DE NITRATO EM <i>H. seropedicae</i>	25
2 JUSTIFICATIVA, OBJETIVOS E ESTRATÉGIA DE AÇÃO.....	30
2.1 JUSTIFICATIVA.....	30
2.2 OBJETIVOS.....	31
2.2.1 Objetivo Geral.....	31
2.2.2. Objetivos Específicos.....	31
2.3 ESTRATÉGIA DE AÇÃO.....	31
2.3.1 Manuscrito 1.....	31
2.3.2 Manuscrito 2.....	32
2.3.3 Manuscrito 3.....	33
3 MANUSCRITO 1.....	34
ABSTRACT.....	35
INTRODUCTION.....	36
RESULTS.....	38
<i>In silico</i> analyses of genes related to nitrate metabolism in <i>H. seropedicae</i>	38
Nitrogen metabolism-related genes responding to nitrate.....	39
NAR produces nitrite in <i>H. seropedicae</i>	42
NAR produces nitric oxide in <i>H. seropedicae</i>	43
NAR increases carbon consumption in <i>H. seropedicae</i>	44
Involvement of NAR with the plant-bacteria interaction.....	44
DISCUSSION.....	45
EXPERIMENTAL PROCEDURES.....	48
Bacterial strains, plasmids, and growth conditions.....	48
β -galactosidase assays.....	48
Transcription profile by RNA-seq.....	48
Quantitative reverse transcription PCR (RT-qPCR).....	49
Construction of <i>H. seropedicae narG</i> mutant.....	50
NO ₂ ⁻ and NO determination.....	50
Quantification of malate consumption.....	51
Plant-bacteria interaction assay.....	51
ACKNOWLEDGEMENTS.....	52
REFERENCES.....	53
FIGURES.....	57

TABLES.....	60
SUPPLEMENTARY MATERIAL.....	67
References.....	77

4 MANUSCRITO 2.....	78
ABSTRACT.....	79
INTRODUCTION.....	80
MATERIAL AND METHODS.....	82
Bacterial strains and growth conditions.....	82
Plasmids and oligonucleotides.....	82
Mutagenesis of the <i>ntrY</i> gene of <i>H. seropedicae</i>	82
Enzymatic activities.....	82
Protein Expression and Purification.....	83
Electrophoretic mobility shift assay (EMSA).....	83
Gel filtration assay.....	83
DNase I footprinting assay.....	83
Bioinformatics and Phylogenetic analyses.....	84
RESULTS.....	85
DISCUSSION.....	89
REFERENCES.....	92
FIGURES.....	95
TABLES.....	102

5 MANUSCRITO 3.....	106
ABSTRACT.....	106
INTRODUCTION.....	108
MATERIAL AND METHODS.....	110
Whole transcriptional analysis by RNA-seq.....	110
Semi-quantitative PCR.....	110
Bioinformatics analysis.....	111
Construction of <i>H. seropedicae nasA</i> mutant.....	111
RESULTS.....	112
RNA-seq experimental strategy.....	112
Differential expression of <i>H. seropedicae</i> genes under Ammonium-limiting and Ammonium-shock treatments.....	113
Differential expression of <i>H. seropedicae</i> genes under Nitrate-shock and Ammonium-limiting treatments.....	115
Differential expression of <i>H. seropedicae</i> genes under Nitrate-shock and Ammonium-shock treatments.....	116
The regulon of NtrC in response to Ammonium-limiting treatment.....	117
The regulon of NtrC in response to Ammonium-shock treatment.....	118
The regulon of NtrC in response to Nitrate-shock treatment.....	118
Consensus sequence of NtrC of <i>H. seropedicae</i>	119
The importance of NtrB/NtrC system to nitrate metabolism of <i>H. seropedicae</i>	119
DISCUSSION.....	121
REFERENCES.....	125
FIGURES.....	128

SUPPLEMENTARY MATERIAL.....	132
6 CONSIDERAÇÕES FINAIS E PERSPECTIVAS.....	144
6.1 CONSIDERAÇÕES FINAIS.....	144
6.1.1 Função de NAS e NAR de <i>H. seropedicae</i>	144
6.1.2 Regulação de NAS e NAR de <i>H. seropedicae</i>	145
6.1.3 Sistema NtrB/NtrC e NtrY/NtrX de <i>H. seropedicae</i>	146
6.2 ETAPAS FUTURAS.....	147
7 REFERÊNCIAS BIBLIOGRÁFICAS.....	148

LISTA DE ILUSTRAÇÕES

1 REVISÃO BIBLIOGRÁFICA

FIGURA 1 – CICLO DO NITROGÊNIO.....	15
FIGURA 2 – ESQUEMA DO PROCESSO DE TRANSPORTE E REDUÇÃO DE NITRATO A AMÔNIO CATALISADO PELA NITRATO REDUTASE E NITRITO REDUTASE ASSIMILATÓRIAS EM <i>K. pneumoniae</i>	18
FIGURA 3 – MODELO DE REGULAÇÃO DO OPERON <i>nasFEDCBA</i> DE <i>K. pneumoniae</i> EM RESPOSTA A NITRATO E AMÔNIO.....	19
FIGURA 4 – ESQUEMA DO PROCESSO DE TRANSPORTE E REDUÇÃO DE NITRATO A NITRITO CATALISADA PELA NITRATO REDUTASE RESPIRATÓRIA DE <i>E. coli</i>	20
FIGURA 5 – FIGURA 5 – ESQUEMA DOS OPERONS DE <i>H. seropedicae</i> SmR1 ENVOLVIDOS COM O METABOLISMO DE NITRATO E SUA LOCALIZAÇÃO NO GENOMA.....	27

3 MANUSCRITO 1

FIGURE 1 – <i>H. seropedicae</i> GENES INVOLVED IN NITRATE METABOLISM.....	57
FIGURE 2 – CELLULAR GROWTH OF <i>H. seropedicae</i> WILD TYPE STRAIN (SmR1) AND THE <i>narG</i> MUTANT.....	58
FIGURE 3 – CELLULAR GROWTH AND NITRITE PRODUCTION OF <i>H. seropedicae</i> WILD TYPE STRAIN (SmR1) AND THE <i>narG</i> MUTANT GROWN IN NITRATE UNDER MICRO-AERATED CONDITION.....	59
FIGURE S1 - RPKM VALUES OF INDIVIDUAL GENES OF <i>narK₁UGHJlmoaA</i>	74
FIGURE S2 – DNA AMPLIFICATION OF THE INTERGENIC REGION OF <i>narU</i> AND <i>narG</i> EXPRESSED mRNA.....	75
FIGURE S3 – NITRIC OXIDE PRODUCTION BY <i>H. seropedicae</i> STRAINS.....	76

4 MANUSCRITO 2

FIGURE 1 – THE NtrYX AND NtrBC SYSTEMS OF <i>H. seropedicae</i>	95
FIGURE 2 – THE FUNCTION OF NtrYX SYSTEM OVERLAPS WITH THAT OF THE NtrBC SYSTEM REGARDING THE NITRATE METABOLISM.....	96
FIGURE 3 – FIGURE 3 – ESTIMATION OF THE MOLECULAR WEIGHT OF NtrX AND VARIANT PROTEINS.....	97
FIGURE 4 – NtrX PROTEIN BINDS OF <i>H. seropedicae</i> TO <i>narKnirB</i> AND <i>narX</i> PROMOTER REGIONS.....	98
FIGURE 5 – PREDICTION OF THE C-TERMINAL STRUCTURE OF NtrX OF <i>H. seropedicae</i>	99

FIGURE 6 – IDENTIFICATION OF DNA-BINDING SITES OF NtrX.....	101
---	-----

5 MANUSCRITO 3

FIGURE 1 – EFFECT OF NITROGEN LIMITATION AND AMMONIUM OR NITRATE SHOCK ON <i>H. seropedicae</i> SmR1 WILD TYPE STRAIN.....	128
FIGURE 2 – METABOLIC ADAPTATIONS OF <i>H. seropedicae</i> TO NITROGEN-LIMITING TREATMENT AND TO NITRATE-SHOCK.....	129
FIGURE 3 – PUTATIVE NtrC-BINDING SITE OF <i>H. seropedicae</i>	130
FIGURE 4 – HEATMAP WITH GENES RELATED TO NITRATE METABOLISM OF <i>H. seropedicae</i>	131
FIGURE S1 – CULTURES OF <i>H. seropedicae</i> WILD TYPE AND <i>ntrC</i> MUTANT USED IN RNA-SEQ.....	132
FIGURE S2 – PRINCIPAL COMPONENT ANALYSIS (PCA).....	133
FIGURE S3 – HEATMAP WITH GENES INDUCED IN AMMONIUM-LIMITING TREATMENT IN <i>H. seropedicae</i>	134

LISTA DE TABELAS

1 REVISÃO BIBLIOGRÁFICA

TABELA 1 – GENES DE <i>H. seropedicae</i> RELACIONADOS COM O METABOLISMO DE NITRATO.....	28
--	----

3 MANUSCRITO 1

TABLE 1 – PROMOTER ACTIVITY OF <i>narK₁-lacZ</i> FUSION IN <i>H. seropedicae</i> WILD TYPE STRAIN AND IN A <i>fnr</i> TRIPLE MUTANT.....	60
TABLE 2 – DIFFERENTIAL EXPRESSION OF GENES RELATED TO NITRATE METABOLISM IN <i>H. seropedicae</i> CELLS GROWN IN PRESENCE OF NITRATE UNDER OXYGEN-LIMITING CONDITIONS AS REVEALED BY THE RNA-seq ANALYSES.....	61
TABLE 3 – PROMOTER ACTIVITY OF GENES RELATED WITH NITRATE METABOLISM IN <i>H. seropedicae</i> WILD TYPE STRAIN.....	63
TABLE 4 – DIFFERENTIAL EXPRESSION OF GENES RELATED TO NITROGEN METABOLISM IN RESPONSE TO NITRATE.....	64
TABLE 5 – NITRIC OXIDE PRODUCTION BY <i>H. seropedicae</i> WILD TYPE AND <i>fnr</i> OR <i>narG</i> MUTANT STRAINS USING A NITRIC OXIDE-ELECTRODE.....	65
TABLE 6 – QUANTIFICATION OF MALATE LEVELS IN THE SUPERNATANT OF <i>H. seropedicae</i> WILD TYPE STRAIN AND <i>narG</i> MUTANT STRAIN	65
TABLE 7 – DRY WEIGHT OF SHOOTS AND ROOTS OF WHEAT PLANTS INOCULATED WITH <i>H. seropedicae</i> WILD TYPE AND <i>narG</i> STRAIN.....	66
TABLE S1 – STRAINS USED IN THIS WORK.....	67
TABLE S2 – PLASMIDS USED IN THIS WORK.....	68
TABLE S3 – OLIGONUCLEOTIDES USED IN THIS WORK.....	70
TABLE S4 – DIFFERENTIAL EXPRESSION OF <i>H. seropedicae</i> ENERGY PRODUCTION RELATED GENES IN RESPONSE TO NITRATE.....	72

4 MANUSCRITO 2

TABLE 1 – PLASMIDS USED IN THIS WORK.....	102
TABLE 2 – OLIGONUCLEOTIDES USED IN THIS WORK.....	104
TABLE 3 – PROMOTER ACTIVITY OF <i>narX-lacZ</i> OR <i>narK-lacZ</i> FUSIONS IN <i>H. seropedicae</i> WILD TYPE STRAIN AND IN A <i>ntrY</i> MUTANT.....	105

5 MANUSCRITO 3

TABLE S1 – STRAINS USED IN THIS WORK.....135

TABLE S2 – PLASMIDS USED IN THIS WORK.....136

TABLE S3 – OLIGONUCLEOTIDES USED IN THIS WORK.....137

TABLE S4 – GENE SET ANALYSIS WITH GO TERMS AND KEGG TERMS.....138

1 REVISÃO BIBLIOGRÁFICA

1.1 *Herbaspirillum seropedicae*

Herbaspirillum seropedicae é uma bactéria diazotrófica, membro da classe β -proteobacteria, que coloniza a superfície da raiz e tecidos internos de gramíneas de interesse comercial, como trigo, milho, arroz e cana-de-açúcar, sem causar danos à planta hospedeira (Baldani, Baldani et al. 1986, Olivares, Baldani et al. 1996, Roncato-Maccari, Ramos et al. 2003). Entretanto, apresenta uma baixa taxa de sobrevivência em solo livre de plantas (Olivares, Baldani et al. 1996).

H. seropedicae tem se mostrado um excelente modelo de estudo para interação endofítico-gramínea, por várias razões, dentre elas: (i) é facilmente cultivável em condições de laboratório, (ii) apresenta seu genoma completo sequenciado (Pedrosa, Monteiro et al. 2011), e (iii) existem várias ferramentas para modificação genética desta bactéria, as quais permitem, por exemplo, a construção de estirpes mutantes ou a avaliação da expressão de genes através de fusões com genes repórter.

Por ser um microrganismo diazotrófico, o metabolismo de nitrogênio de *H. seropedicae*, incluindo genes e seus produtos envolvidos na fixação de nitrogênio são bem estudados (Chubatsu, Monteiro et al. 2012). Entretanto, apesar de nitrato ser a principal fonte de nitrogênio inorgânico encontrado no solo, o metabolismo deste composto em *H. seropedicae* não é tão bem conhecido (Dechorgnat, Nguyen et al. 2011).

1.2 CICLO DO NITROGÊNIO

O nitrogênio é um dos elementos mais importantes para organismos vivos, uma vez que é requerido para a síntese de proteínas, ácidos nucleicos e cofatores, como NAD(P)H e FADH₂ (Canfield, Glazer et al. 2010). O ciclo do nitrogênio, envolve várias reações redox, onde o nitrogênio varia entre vários estados de oxidação (de +5 a -3). Os principais processos deste ciclo são catalisados por bactérias (Figura 1).

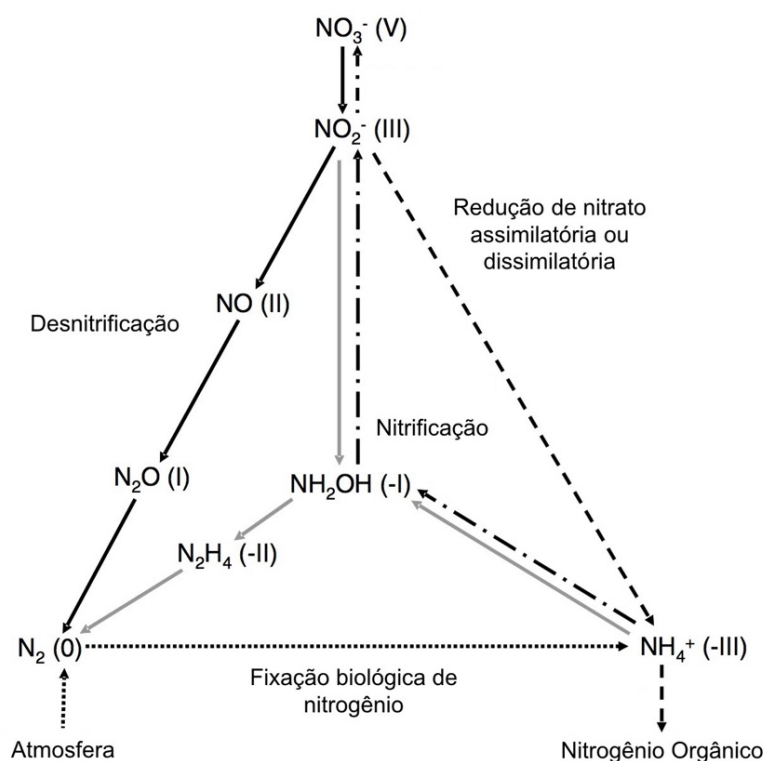


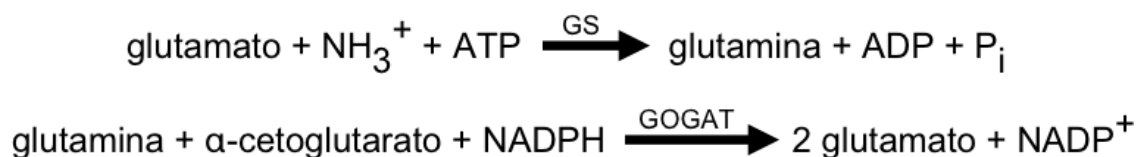
FIGURA 1 – CICLO DO NITROGÊNIO

Esquema mostrando as principais formas de nitrogênio potencialmente utilizáveis a nível biológico e seus respectivos estados de oxidação. As rotas metabólicas envolvendo cada forma estão indicadas. Os números em algarismos romanos indicam a variação do estado de oxidação de nitrogênio (Modificado de Gonzalez, Correia et al. 2006). Copyright © 2005 Elsevier Inc. All rights reserved.

O processo de fixação biológica de nitrogênio envolve a redução de nitrogênio atmosférico (N_2) a amônio por organismos conhecidos como diazotrofos. Por outro lado, na nitrificação, o amônio é convertido a nitrato. No processo conhecido como desnitrificação, nitrato e nitrito são reduzidos a óxido nítrico, óxido nitroso e a nitrogênio gasoso. Além disso, nitrato pode ainda ser sucessivamente reduzido a nitrito e a amônio nos processos conhecidos como redução de nitrato assimilatória e redução de nitrato dissimilatória (Figura 1) (Richardson and Watmough 1999).

O amônio gerado através dos processos de fixação de nitrogênio e redução de nitrato assimilatória pode então ser incorporado a biomoléculas através da via glutamina sintetase/glutamato sintase (GS/GOGAT) (Equação 1). As moléculas de glutamina e glutamato gerados são as principais doadoras de nitrogênio nas vias biossintéticas (Merrick and Edwards 1995).

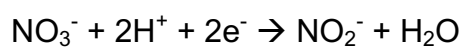
Equação 1:



1.3 METABOLISMO DE NITRATO

O nitrato pode apresentar diversas funções em bactérias, entre elas, fonte de nitrogênio ou aceitor final na cadeia de elétrons. Procariotos apresentam três tipos diferentes de nitrato redutases, as quais catalisam a seguinte reação (Equação 2):

Equação 2:



Todas as nitrato redutases apresentam o cofator molibdo-bis(molibdopterina guanina dinucleotídeo) (cofator Mo-bisMGD) em seu sítio ativo. Entretanto, elas são diferentes entre si tanto em relação à função e localização celular, como também em estrutura, organização de genes e regulação (Moreno-Vivián, Cabello et al. 1999). A principal função da nitrato redutase assimilatória (NAS) é utilizar nitrato como fonte de nitrogênio. A nitrato redutase respiratória (NAR) está associada à membrana citoplasmática e utiliza o nitrato como aceitor alternativo de elétrons, permitindo a síntese de ATP na ausência de oxigênio. Finalmente, a nitrato redutase periplasmática (NAP) está localizada no espaço periplasmático e normalmente está associada à dissipação do excesso de equivalentes redutores (Moreno-Vivián, Cabello et al. 1999, Richardson and Watmough 1999, Arruebarrena Di Palma, Pereyra et al. 2013).

Conforme mencionado anteriormente, o nitrito gerado pelas enzimas NAS, NAR e NAP apresenta duas vias principais na célula. A primeira via, conhecida por redução de nitrato assimilatória e dissimilatória (Figura 2), envolve a redução de nitrito a amônio por uma nitrito reductase. Por outro lado, a segunda via, conhecida

por desnitrificação, envolve a redução sucessiva de nitrito a óxido nítrico, óxido nitroso e a nitrogênio atmosférico (Moreno-Vivián, Cabello et al. 1999, Richardson and Watmough 1999, Arruebarrena Di Palma, Pereyra et al. 2013).

1.3.1 Genes envolvidos na assimilação de nitrato e seus produtos em procariotos

Análises de genomas mostram que genes que codificam para as NAS são amplamente distribuídos (Richardson, Berks et al. 2001). A enzima NAS contém vários centros Fe-S em sua estrutura, mas não apresenta grupos heme, como NAR (Moreno-Vivián, Cabello et al. 1999, Richardson and Watmough 1999, Arruebarrena Di Palma, Pereyra et al. 2013).

Em organismos heterótrofos, o principal modelo de estudo de NAS é o da enterobactéria *Klebsiella pneumoniae* (Luque-Almagro, Gates et al. 2011). Este organismo é capaz de assimilar nitrato ou nitrito como fontes únicas de nitrogênio e os genes estruturais requeridos para assimilação destas duas fontes de nitrogênio estão contidos no operon *nasFEDCBA*. NAS de *K. pneumoniae* é dependente de NADH e é constituído por duas proteínas: NasA (codificada pelo gene *nasA*) e NasC (codificada pelo gene *nasC*) (Figura 2). A proteína NasA é a subunidade maior e catalítica que contém o cofator Mo-bisMGD e vários centros Fe-S. Por outro lado, NasC transfere elétrons de NAD(P)H para NasA. Além disso, *K. pneumoniae* também apresenta a nitrito redutase NasB (codificada pelo gene *nasB*), a qual catalisa a conversão de nitrito a amônio (Lin, Goldman et al. 1993, Lin, Goldman et al. 1994). Os genes *nasFED* codificam para um sistema de transporte de nitrato/nitrito do tipo ABC (ATP-binding casete), o qual é dependente da hidrólise de ATP (Wu and Stewart 1998). À montante do operon *nasFEDCBA*, está localizado o gene *nasR*, o qual é expresso independentemente do operon *nasFEDCBA* e codifica para o regulador transcricional, NasR (Goldman, Lin et al. 1994). NasR apresenta um domínio sensor de nitrato ou nitrito (NIT) e um domínio de ligação à mRNA (ANTAR) e regula a transcrição de genes alvo através de um mecanismo de anti-terminação (Boudes, Lazar et al. 2012).

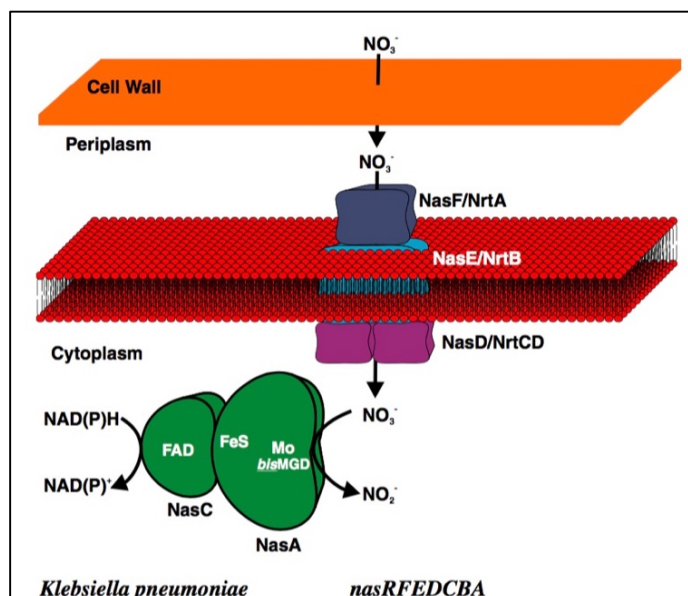


FIGURA 2 – ESQUEMA DO PROCESSO DE TRANSPORTE E REDUÇÃO DE NITRATO A AMÔNIO CATALISADO PELA NITRATO REDUTASE E NITRITO REDUTASE ASSIMILATÓRIAS EM *K. pneumoniae*

Na parte superior da figura está representado um esquema contendo as subunidades de NAS e sistema transportador de nitrato. Na parte inferior está representada a organização gênica dos genes relacionados a NAS de *K. pneumoniae* (Modificado de Gonzalez, Correia et al. 2006). Copyright © 2005 Elsevier Inc. All rights reserved.

A sucessiva redução de nitrato a nitrito e nitrito a amônio é uma reação que demanda alto custo energético, uma vez que em conjunto as nitrato e nitrito redutases utilizam oito elétrons para converter nitrato (N +V) a amônio (N -III). Devido a esta alta demanda energética, em *K. pneumoniae* o processo de assimilação de nitrato está regulado em dois níveis: repressão em altos níveis de amônio e indução por nitrato/nitrito. A proteína NtrC ativa a expressão do operon *nasFEDCBA* em resposta a baixos níveis de amônio. Entretanto, na região líder do transcrito do gene *nasF*, há um grampo terminador de transcrição, bloqueando a transcrição dos genes *nasFEDCBA* (Figura 3). Na presença de nitrato, NasR interage com a região líder do transcrito de *nasF*, interrompendo o grampo terminador e os genes *nasFEDCBA* são transcritos. Em resumo, a expressão do operon *nasFEDCBA* é ativado em baixos níveis de amônio, através da ativação da transcrição pela proteína NtrC e na presença de nitrato, auxiliado pela proteína NasR que remove o grampo terminador (Goldman, Lin et al. 1994, Lin and Stewart 1996).

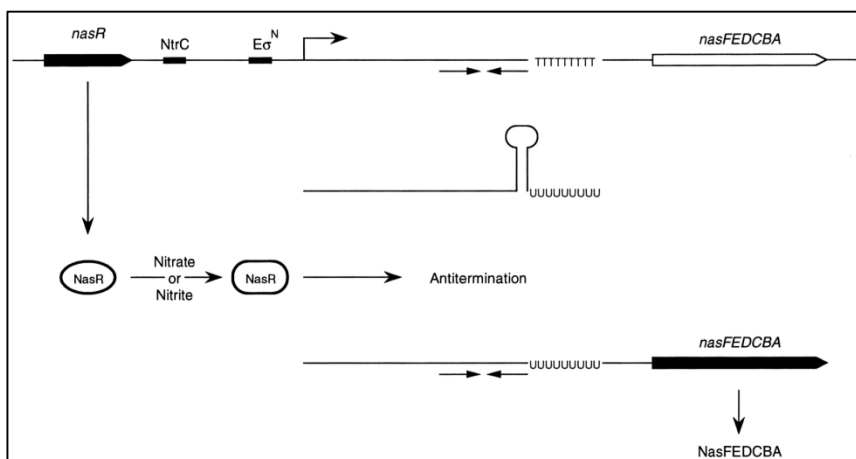


FIGURA 3 – MODELO DE REGULAÇÃO DO OPERON *nasFEDCBA* DE *K. pneumoniae* EM RESPOSTA A NITRATO E AMÔNIO

Os retângulos em preto representam os sítios de ligação reconhecidos por NtrC ou pela RNA polimerase associada ao fator sigma N, conforme indicado (Lin and Stewart 1996). Copyright © 1996 Academic Press. All rights reserved.

1.3.2 Genes envolvidos na desnitrificação/dissimilação de nitrato e seus produtos em procariotos

A enzima NAR é amplamente distribuída entre enterobactérias e foi bem caracterizado principalmente em *E. coli* (Philippot and Hojberg 1999). Neste organismo, NAR é codificado pelo operon *narKGHJ* e é composto por três subunidades. A subunidade catalítica NarG (codificada por *narG*), contém um centro Fe-S e o cofator Mo-bisMGD, enquanto que a subunidade NarH (codificada pelo gene *narH*), contém quatro centros Fe-S. Finalmente, a subunidade NarI (codificada pelo gene *narI*) é uma proteína transmembrana e ancora as proteínas NarGH no lado citoplasmático da membrana celular. NarI contém dois grupos hemes e o sítio Q, onde quinol se liga e é re-oxidado (Figura 4) (Blasco, Guigliarelli et al. 2001, Bertero, Rothery et al. 2003). Foi proposto que o produto do gene *narJ* codifica para uma chaperona requerida para inserir o cofator Mo-bisMGD na subunidade catalítica NarG (Blasco, Dos Santos et al. 1998). Finalmente, o gene *narK* codifica para um transportador de nitrato para o interior da célula (Moir and Wood 2001).

NAR utiliza o "pool" de quinol como doador fisiológico de elétrons para a redução de nitrato a nitrito, gerando um gradiente de prótons através da membrana (força próton motiva), que dirige a formação de ATP através da ATPase F_1F_0 .

ATP através da ATPase F_1F_0 (Philippot and Hojberg 1999, Bertero, Rothery et al. 2003).

A expressão dos genes codificando para NAR é normalmente induzida por nitrato e condições limitantes de oxigênio, mas insensível aos níveis de amônio. Em *E. coli*, o operon *narKGHJ* é regulado pelo sistema de dois componentes NarX/NarL codificado pelos genes *narXL*. A proteína NarX é transmembrana, enquanto que NarL é uma proteína ligadora de DNA. Em resposta a nitrato, a proteína NarX se autofosforila e transfere esse grupo fosforil para NarL, que por sua vez liga-se em seu sítio específico em *narKGHJ*, ativando a transcrição deste operon na presença de nitrato (Schroder, Darie et al. 1993, Darwin, Tyson et al. 1997). A proteína Fnr, que regula a transcrição de genes em resposta a baixos níveis de oxigênio, também é requerida para ativar a expressão do operon *narKGHJ* em *E. coli* (Self, Grunden et al. 1999).

Assim com em *E. coli*, o patógeno oportunista *Pseudomonas aeruginosa* também apresenta os genes *nar* e é capaz de respirar nitrato. A expressão do operon *narK₁K₂GHJ* é induzida por nitrato, via sistema NarXL e em anaerobiose via proteína Anr, uma proteína homóloga à Fnr de *E. coli* (Schreiber, Krieger et al. 2007).

Os genes *nirBD* de *E. coli* codificam para uma nitrito redutase dependente de NADH, NirBD, que converte nitrito a amônio. A expressão dos genes *nirBD* é induzida nas mesmas condições que NAR: por nitrato, via sistema NarX/NarL e em anaerobiose via Fnr. Foi proposto que NirBD de *E. coli* tem como função principal detoxificar o nitrito produzido por NAR (Jayaraman, Peakman et al. 1987, Page, Griffiths et al. 1990, Harborne, Griffiths et al. 1992).

1.3.3 Nitrato redutase periplasmática (NAP)

A nitrato redutase periplasmática, ou NAP, é um heterodímero que está localizado no espaço periplasmático de bactérias gram-negativas. A subunidade catalítica NapA contém o cofator Mo-bisMGD, enquanto que NapB contém dois grupos heme envolvidos no transporte de elétrons para a subunidade catalítica. A organização gênica dos genes *nap*, e a regulação da expressão deles é bem variável. Além disso, NAPs de diferentes organismos apresentam diferentes funções

fisiológicas, dentre elas desnitrificação aeróbia, adaptação de crescimento a condições de baixa aeração ou manutenção do equilíbrio redox. O nitrito gerado por NAP pode ser utilizado como fonte de nitrogênio, apesar de não parecer ser esta a sua principal função. Finalmente, não está associado diretamente à produção de uma força próton-motiva, como ocorre a NAR (Moreno-Vivián, Cabello et al. 1999, Richardson and Watmough 1999, Morozkina and Zvyagilskaya 2007).

1.4 SISTEMAS DE DOIS COMPONENTES

Bactérias encontradas no ambiente, como *H. seropedicae*, estão constantemente sujeitas a variações ambientais e, com isso, devem sensorar o meio e modular uma reação em resposta a sinais específicos, evitando gasto energético com a síntese de proteínas que não são requeridas em um dado momento. O principal sistema encontrado em procariotos para sensorar o ambiente é o sistema de dois componentes. Como o nome sugere, este sistema é em geral constituído de dois componentes, a proteína Histidina quinase (HK) e a proteína Reguladora de resposta (RR). A proteína HK geralmente é transmembrana e em resposta a um sinal específico ela se autofosforila e, em seguida, transfere esse grupo fosforil para o seu par, a proteína RR. Esta, por sua vez, quando fosforilada apresenta afinidade ao DNA e regula a transcrição de genes alvo. Desta forma, o sistema de dois componentes é um mecanismo que acopla um estímulo extracelular (sinal ambiental) à expressão de genes específicos (Stock and Da Re 2000).

H. seropedicae apresenta três sistemas da família do sistema de dois componentes que provavelmente estão relacionados com o metabolismo de nitrato: NarX/NarL, NtrB/NtrC e NtrY/NtrX. O sistema NarX/NarL já foi brevemente descrito na seção anterior (seção 1.3.2), enquanto que os sistemas NtrB/NtrC e NtrY/NtrX serão abordados nas próximas seções.

1.4.1 Sistema NtrB/NtrC

Como mencionado anteriormente, as proteínas NtrB e NtrC são membros do sistema de dois componentes, sendo que NtrB é a proteína histidina quinase e NtrC, a reguladora de resposta. O sistema NtrB/NtrC é amplamente encontrado em bactérias e modula a expressão de genes alvo em resposta a baixos níveis intracelulares de nitrogênio (Merrick and Edwards 1995). Ao contrário de outras histidinas quinases, a proteína NtrB não é transmembrana, mas apresenta um domínio sensor PAS na sua região N-terminal, e o domínio requerido para atividade de histidina quinase na região C-terminal. Já a proteína NtrC apresenta três regiões principais: o domínio que recebe a fosforilação de NtrB, na região N-terminal; o domínio AAA que interage com o fator sigma N na região central; e o motivo de ligação ao DNA na região C-terminal. O mecanismo de modulação da atividade do sistema NtrB/NtrC em resposta aos níveis de nitrogênio é complexo e envolve o sistema regulatório de nitrogênio, ou sistema Ntr.

O sistema Ntr foi bem caracterizado em *E. coli* e contém sete proteínas principais: as proteínas membro do sistema de dois componentes, NtrB e NtrC; as proteínas PII, GlnB e GlnK; e as enzimas glutamina sintetase (GS), GlnD e GlnE. A enzima GS é uma das principais formas de assimilação de nitrogênio e condensa glutamato e amônio, gerando glutamina, com o custo da hidrólise de 1 ATP (Equação 1). A enzima GlnE atua como uma adenililtransferase, transferindo um grupo AMP de ATP para uma subunidade de GS, inativando-a. A proteína GlnB também está sujeita a modificação covalente catalisada por GlnD, que tem atividade de Uridililtransferase/Uridilil-removedora. Em altos níveis de nitrogênio, glutamina atua como indicador de suficiência de nitrogênio e modula principalmente duas enzimas: (i) aumenta a adenilação de GS, inativando a enzima, e (ii) diminui a atividade de uridililação de GlnD e, por sua vez, diminuindo o grau de uridililação de GlnB. Quando GlnB está livre, sem modificação covalente, ela interage com NtrB, impedindo esta de fosforilar NtrC. Desta forma, NtrC não ativa a transcrição de promotores alvo. Por outro lado, em baixos níveis de nitrogênio, α -cetoglutarato atua como indicador de insuficiência de nitrogênio por inibir a interação de GlnB com NtrB. Desta forma, NtrB fica livre para fosforilar NtrC, estimulando a atividade ligadora de DNA de NtrC, a qual ativa a transcrição de vários genes cujos produtos

são requeridos para o metabolismo em baixo nitrogênio (Merrick and Edwards 1995, Reitzer 2003, Leigh and Dodsworth 2007).

O sistema Ntr de *H. seropedicae* é muito semelhante ao de *E. coli* (Chubatsu, Monteiro et al. 2012). NtrC regula genes relacionados com a fixação de nitrogênio, através da ativação de expressão da proteína NifA (codificada pelo gene *nifA*) e também é requerida para ativar a transcrição de *glnA* em resposta a baixos níveis de nitrogênio (Pedrosa, Teixeira et al. 1997, Persuhn, Souza et al. 2000, Souza, Pedrosa et al. 2000, Schwab, Souza et al. 2007). Além disso, um mutante *ntrC* não é capaz de crescer em nitrato, alanina, prolina ou serina como fontes únicas de nitrogênio, reforçando que a proteína NtrC de *H. seropedicae* é requerida para a mobilização de fontes alternativas de nitrogênio em condições limitantes de íons amônio (Persuhn, Souza et al. 2000, Gusso, de Souza et al. 2008).

Com o sequenciamento do genoma de *H. seropedicae*, foi identificado outro par da família de dois componentes, homólogo ao sistema NtrB/NtrC. Este novo sistema está principalmente presente em α e β -proteobactérias e foi denominado NtrY/NtrX (Kloepper and Schroth 1978, Alves 2006, Pedrosa, Monteiro et al. 2011, Attack, Srikhanta et al. 2013).

1.4.2 Sistema NtrY/NtrX

Com o sequenciamento do genoma de *H. seropedicae*, foi identificado outro par da família de dois componentes, homólogo ao sistema NtrB/NtrC. Este novo sistema está principalmente presente em α e β -proteobactérias e foi denominado NtrY/NtrX (Pedrosa, Monteiro et al. 2011, Attack, Srikhanta et al. 2013).

A proteína histidina quinase NtrY apresenta domínios transmembrana e está provavelmente associada à membrana e contém um domínio C-terminal HATPaseC que fosforila NtrX. O regulador de resposta NtrX contém dois domínios principais: o domínio REC que é fosforilado por NtrY e um domínio ligador de DNA hélice-volta-hélice. Em geral, NtrX também apresenta um domínio central AAA+, o qual sugere que esta proteína ativa a transcrição de genes alvo de forma dependente do fator sigma N (análises SMART; <http://smart.embl-heidelberg.de/>). Entretanto a proteína NtrX de *H. seropedicae* e de bactérias relacionadas não apresenta o domínio

central, AAA+, sugerindo que regula a transcrição de genes alvo de forma independente do fator sigma N (Osaki 2009).

A função do sistema NtrY/NtrX ainda não é completamente entendida. Em *Azorhizobium caulinodans* e *Rhizobium tropici* este sistema parece estar associado à fixação de nitrogênio e formação de nódulos em leguminosas (Pawlowski, Klosse et al. 1991, Nogales, Campos et al. 2002). Além disso, o sistema NtrY/NtrX de *Azospirillum brasilense* e *A. caulinodans* é requerido para o metabolismo de nitrato (Pawlowski, Klosse et al. 1991, Ishida, Assumpção et al. 2002). Na bactéria fotossintética *Rhodobacter capsulatus*, além de estar envolvido com metabolismo de nitrogênio, o sistema NtrY/NtrX também regula a formação de complexos fotossintéticos em resposta ao estado redox da célula (Drepper, Wiethaus et al. 2006, Gregor, Zeller et al. 2007).

O sistema NtrY/NtrX é um importante fator de virulência em várias bactérias patogênicas, como *Brucella suis*, *Brucella abortus*, *Neisseria gonorrhoeae* e *Bordetella bronchiseptica* (Foulongne, Bourg et al. 2000, Kaut, Duncan et al. 2011, Atack, Srikhanta et al. 2013, Carrica, Fernandez et al. 2013). Em *B. abortus* foi proposto que o sistema NtrY/NtrX é requerido para a adaptação da bactéria em condições limitantes de oxigênio. Em baixas tensões de oxigênio, o domínio PAS de NtrY é reduzido, induzindo a atividade de autoquinase da proteína, a qual transfere o grupo fosforil para NtrX, que regula a transcrição de genes alvo (Carrica, Fernandez et al. 2012).

Poucos estudos foram realizados em relação à interação de NtrX a promotores alvo. Ensaio de retardamento de gel mostraram que NtrX de *R. capsulatus* liga-se ao promotor do operon *puf*, cujos produtos estão envolvidos na formação de pigmentos fotossintéticos (Gregor, Zeller et al. 2007). Já em *Ehrlichia chaffeensis*, NtrX liga-se diretamente às regiões promotoras de *glnA* e *putA*, genes que codificam para proteínas relacionadas ao metabolismo de nitrogênio (Cheng, Lin et al. 2014).

1.5 METABOLISMO DE NITRATO EM *H. seropedicae*

Análises *in silico* sugerem que *H. seropedicae* apresenta 18 prováveis genes relacionados com o metabolismo de nitrato, os quais estão localizados em quatro

regiões diferentes do genoma (Figura 5 e Tabela 1), incluindo genes que codificam para duas nitrato redutases: NAR e NAS, e uma nitrito redutase. O cluster *narK₁UGHJlmoaAnarXL* contém os genes estruturais e regulatórios relacionados à NAR. O gene *moaA* codifica para uma enzima que sintetiza o cofator Mo-bisMGD de NAR. Os genes *narXL* codificam para os membros do sistema de dois componentes NarX/NarL. Em uma segunda região do genoma é encontrado o operon *narKnirBDHSERO_RS14545nasA*, contendo genes codificando para NAS e nitrito redutase. Os genes *nasFED* e *nasR*, localizados numa terceira e quarta região do genoma, codificam para um sistema de transporte de nitrato e para uma proteína regulatória, respectivamente (Pedrosa, Monteiro et al. 2011). Entretanto, *H. seropedicae* não apresenta genes codificando para a nitrato redutase periplasmática (NAP). Além disso, *H. seropedicae* também não apresenta genes codificando enzimas que convertem óxido nítrico a óxido nitroso (óxido nítrico redutase) e óxido nitroso a nitrogênio (óxido nitroso redutase).

Dados fisiológicos mostram que um mutante na subunidade catalítica de NAS de *H. seropedicae* (mutante *nasA*) perdeu a capacidade de crescer em nitrato como fonte única de nitrogênio, sugerindo que NAS é a principal via de assimilação de nitrato neste microrganismo (Bonato 2012). Entretanto, apesar de *H. seropedicae* apresentar genes relacionados com a respiração de nitrato (genes *narGHI*), este microrganismo não é capaz de crescer em nitrato em anaerobiose utilizando nitrato como aceitador terminal de elétrons na cadeia de fosforilação oxidativa (Baldani, Baldani et al. 1986). Um mutante na subunidade catalítica de NAR de *H. seropedicae* (mutante *narG*) apresenta perfil de crescimento em nitrato similar ao selvagem em aerobiose, sugerindo que NAR não é essencial para assimilação de nitrato. Com isso, a função de NAR em *H. seropedicae* ainda não é bem entendida (Bonato 2012).

Os mutantes *ntrC* e *ntrY* de *H. seropedicae* também não crescem aerobicamente em nitrato como fonte única de nitrogênio, sugerindo que os sistemas NtrB/NtrC e NtrY/NtrX são requeridos para a regulação de genes envolvidos com o metabolismo de nitrato neste microrganismo (Persuhn, Souza et al. 2000, Alves 2006). Ensaio de β -galactosidase com *H. seropedicae* selvagem e mutantes *ntrC* e *ntrY* sugerem que os sistemas NtrB/NtrC e NtrY/NtrX são requeridos para ativar os promotores dos operons *nasFED* e *narKnirBDHSERO_RS14545nasA* (Alves 2006, Miguel 2011, Bonato 2012). Por outro lado, a expressão dos promotores de *narXL* e

narK, *UGHJlmoaA* é induzida por nitrato e em baixos níveis de oxigênio via proteína Fnr (Bonato 2012, Batista, Sfeir et al. 2013).

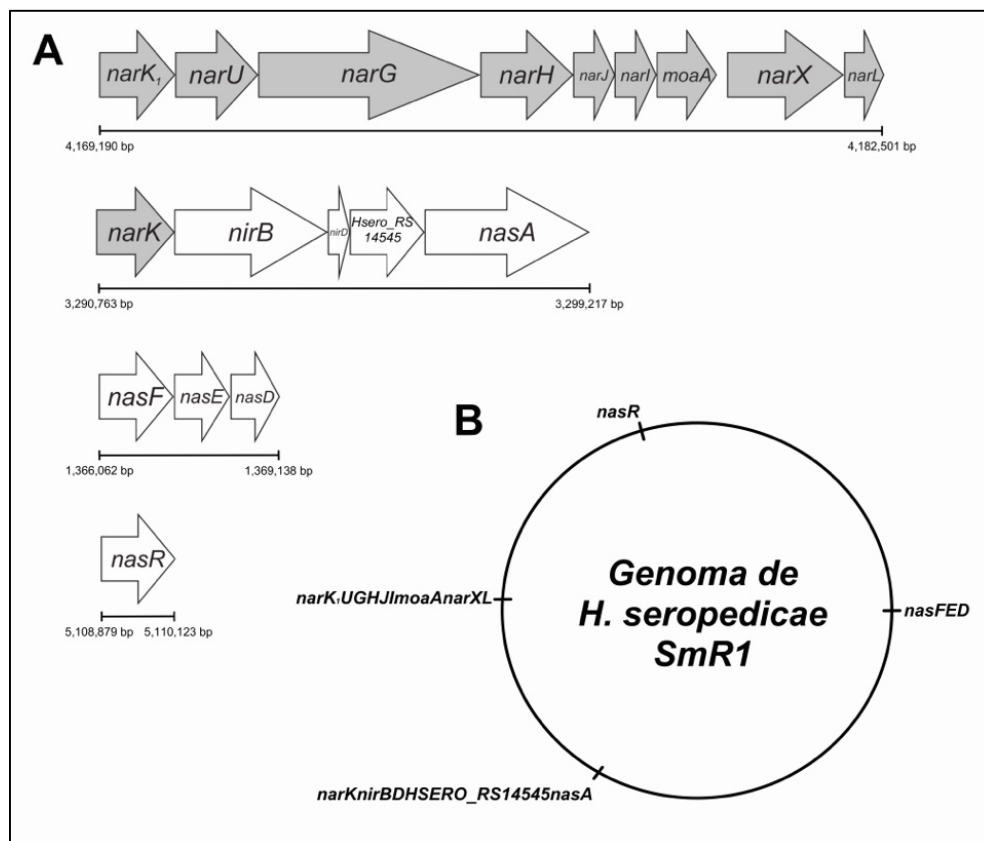


FIGURA 5 – ESQUEMA DOS OPERONS DE *H. seropedicae* SmR1 ENVOLVIDOS COM O METABOLISMO DE NITRATO E SUA LOCALIZAÇÃO NO GENOMA

(A) Em cinza estão representados genes relacionados com NAR, enquanto que em branco estão representados genes relacionados com NAS e Nitrito redutase NirBD. Os números representam o contexto gênico no genoma de *H. seropedicae* (Número de acesso no NCBI: NC_014323). (B) Esquema do genoma de *H. seropedicae* evidenciando a localização dos operons mostrados em "A".

TABELA 1 – GENES DE *H. seropedicae* SmR1 RELACIONADOS COM O METABOLISMO DE NITRATO

Nome do gene	Antigo ID (NCBI)	Novo ID (NCBI)	Anotação
Genes estruturais relacionados com a Nitrato redutase respiratória			
<i>narK₁</i>	<i>Hsero_3638</i>	<i>HSERO_RS18155</i>	Transportador de nitrato/nitrito
<i>narU</i>	<i>Hsero_3639</i>	<i>HSERO_RS18160</i>	Transportador de nitrato/nitrito
<i>narG</i>	<i>Hsero_3640</i>	<i>HSERO_RS18165</i>	Nitrato redutase respiratória (subunidade alfa)
<i>narH</i>	<i>Hsero_3641</i>	<i>HSERO_RS18170</i>	Nitrato redutase respiratória (subunidade beta)
<i>narJ</i>	<i>Hsero_3642</i>	<i>HSERO_RS18175</i>	Chaperona requerida para maturação e atividade de Nitrato redutase respiratória
<i>narI</i>	<i>Hsero_3643</i>	<i>HSERO_RS18180</i>	Nitrato redutase respiratória (subunidade gama)
<i>moaA</i>	<i>Hsero_3644</i>	<i>HSERO_RS18185</i>	Enzima envolvida na biossíntese do cofator molibdênio
Genes estruturais relacionados com a Nitrato redutase assimilatória			
<i>narK</i>	<i>Hsero_2896</i>	<i>HSERO_RS14530</i>	Transportador de nitrato/nitrito
<i>nirB</i>	<i>Hsero_2897</i>	<i>HSERO_RS14535</i>	Nitrito redutase (subunidade maior)
<i>nirD</i>	<i>Hsero_2898</i>	<i>HSERO_RS14540</i>	Nitrito redutase (subunidade menor)
<i>HSERO_RS14545</i>	<i>Hsero_2899</i>	<i>HSERO_RS14545</i>	Nitrato redutase assimilatória (subunidade maior)
<i>nasA</i>	<i>Hsero_2900</i>	<i>HSERO_RS14550</i>	Nitrato redutase assimilatória (subunidade menor)
<i>nasF</i>	<i>Hsero_1207</i>	<i>HSERO_RS06055</i>	Transportador de nitrato do tipo ABC (componente periplasmático)
<i>nasE</i>	<i>Hsero_1208</i>	<i>HSERO_RS06060</i>	Transportador de nitrato do tipo ABC (permease)
<i>nasD</i>	<i>Hsero_1209</i>	<i>HSERO_RS06065</i>	Transportador de nitrato do tipo ABC (ATPase)
Genes que codificam para proteínas envolvidas na regulação do metabolismo de nitrato			
<i>narX</i>	<i>Hsero_3645</i>	<i>HSERO_RS18190</i>	Sistema de dois componentes (histidina quinase)

<i>narL</i>	<i>Hsero_3646</i>	<i>HSERO_RS18195</i>	Sistema de dois componentes (regulador de resposta)
<i>nasR</i>	<i>Hsero_4469</i>	<i>HSERO_RS22335</i>	Regulador transcricional
<i>ntrY</i>	<i>Hsero_0070</i>	<i>HSERO_RS00345</i>	Sistema de dois componentes (histidina quinase)
<i>ntrX</i>	<i>Hsero_0069</i>	<i>HSERO_RS00340</i>	Sistema de dois componentes (regulador de resposta)
<i>ntrB</i>	<i>Hsero_3126</i>	<i>HSERO_RS15685</i>	Sistema de dois componentes (histidina quinase)
<i>ntrC</i>	<i>Hsero_3125</i>	<i>HSERO_RS15680</i>	Sistema de dois componentes (regulador de resposta)

2 JUSTIFICATIVA, OBJETIVOS E ESTRATÉGIA DE AÇÃO

2.1 JUSTIFICATIVA

H. seropedicae é uma bactéria fixadora de nitrogênio encontrada em associação com gramíneas de interesse comercial e, por isso, tem um potencial uso como biofertilizante. Deste modo, o metabolismo de nitrogênio desta bactéria, em especial a fixação biológica de nitrogênio, tem sido alvo de vários estudos nos últimos anos. Entretanto, embora o nitrato seja a principal fonte de nitrogênio inorgânico encontrada no solo (Dechorgnat, Nguyen et al. 2011), o metabolismo deste composto em *H. seropedicae* ainda não é tão bem conhecido. Foram encontrados no genoma de *H. seropedicae* genes que codificam para duas prováveis nitrato redutases. A nitrato redutase assimilatória (NAS) é essencial para a assimilação de nitrato (Bonato 2012), enquanto que a real função da nitrato redutase respiratória (NAR) não é conhecida uma vez que *H. seropedicae* parece não ser capaz de respirar nitrato, pois é incapaz de crescer anaerobicamente na presença de nitrato (Bonato 2012).

Embora a regulação de genes que codificam proteínas estruturais das nitrato redutases (*HSERO_RS14545nasA* e *narGHI*), transportadores de nitrato (*nasFED*, *narK* e *narK_{1U}*) e de prováveis proteínas regulatórias de NAR e NAS ter sido estudada, ainda há vários pontos a serem elucidados. Os mutantes *ntrC* e *ntrY* de *H. seropedicae* não crescem em nitrato como fonte única de nitrogênio, demonstrando que os sistemas NtrB/NtrC e NtrY/NtrX são importantes para a regulação de genes relacionados com o metabolismo do nitrato. O sistema NtrB/NtrC é bem conhecido por ser um regulador central em resposta a baixos níveis de nitrogênio, entretanto, a sua função na regulação de genes cujos produtos estão envolvidos com o metabolismo de nitrato é menos estudada. A função do sistema NtrY/NtrX ainda não é bem entendida, uma vez que em alguns organismos está associado ao metabolismo de nitrogênio, enquanto que em outros está relacionado ao sensoramento do estado redox da célula.

2.2 OBJETIVOS

2.2.1 Objetivo Geral

Determinar a função dos produtos dos genes que codificam para as nitrato redutase assimilatória e respiratória e determinar o envolvimento dos sistemas de dois componentes NtrB/NtrC e NtrY/NtrX na regulação de expressão dos genes do metabolismo de nitrato de *H. seropedicae*.

2.2.2. Objetivos Específicos

- ✓ Validar o transcriptoma global de *H. seropedicae* crescido em nitrato e comparado com amônio, realizado através de RNA-seq por Bonato (2012) (manuscrito 1).
- ✓ Caracterizar estirpe mutante *narG* (manuscrito 1).
- ✓ Identificar o sítio de ligação ao DNA da proteína NtrX (manuscrito 2).
- ✓ Avaliar o transcriptoma global das estirpes *H. seropedicae* SmR1 selvagem e do mutante *ntrC* em três condições de nitrogênio diferentes: baixo amônio, choque de amônio (NH₄Cl 10 mM) e choque de nitrato (KNO₃ 10 mM) por 30 minutos (manuscrito 3).

2.3 ESTRATÉGIA DE AÇÃO

Esta tese está estruturada no formato de artigo científico, e consiste em três manuscritos.

2.3.1 Manuscrito 1

Recentemente foi realizada uma análise transcriptômica global por RNA-seq de *H. seropedicae* SmR1 crescido na presença de nitrato de potássio 10 mM e coletado em DO₆₀₀ 0,8 (Bonato 2012). Estes resultados foram comparados com aqueles obtidos da mesma estirpe cultivada na presença de cloreto de amônio 20 mM como fonte única de nitrogênio (Tadra-Sfeir, Faoro et al. 2015). O software CLC

Genomics Workbench 4.8 foi utilizado para realizar análise comparativa e estatística, conforme descrito em Bonato (2012).

No presente trabalho de doutorado, os dados de RNA-seq mencionados anteriormente foram novamente analisados utilizando o pacote DESeq (pacote de R do projeto Bioconductor), que é um dos métodos atualmente mais utilizados para a análise de dados de RNA-seq. Além disso, os dados de expressão de genes relacionados ao metabolismo de nitrato foram validados através de ensaios de β -galactosidase e PCR em tempo real.

Como os genes codificando para NAR (*narGHI*) estavam entre os mais induzidos na presença de nitrato, o mutante *narG* de *H. seropedicae* construído por Bonato (2012) também foi utilizado para complementar os dados obtidos através de RNA-seq. No presente trabalho, a mutação no gene *narG* foi confirmada através de ensaios de complementação gênica. Além disso a capacidade de produzir nitrito, consumir malato e de colonizar trigo comum (*Triticum aestivum*) do mutante *narG* foi comparada à estirpe selvagem. Finalmente, o Dr Marcelo Bueno Batista em colaboração com o laboratório da Prof^a Dr^a Cecília M. Creus (Unidad Integrada Balcarce, INTA-UNMdP, Balcarce, Buenos Aires, Argentina) dosou óxido nítrico na estirpe selvagem e no mutante *narG*.

2.3.2 Manuscrito 2

Alves (2006) construiu e caracterizou fenotipicamente um mutante *ntrY* de *H. seropedicae*, denominado A01. Como principais resultados foi mostrado que o sistema NtrY/NtrX é requerido para o crescimento de *H. seropedicae* em nitrato, mas não para a atividade da nitrogenase ou da glutamina sintetase (Alves 2006). Além disso, Osaki (2009) expressou, purificou e caracterizou bioquimicamente a proteína NtrX. Como principais resultados, mostrou que a proteína reguladora de transcrição NtrX liga-se às regiões promotoras dos operons *narXL* e *narKnirBDHSERO_RS14545nasA* (Osaki 2009).

Baseado nos resultados mencionados acima, neste presente trabalho de doutorado foi proposta a caracterização *in vitro* da proteína NtrX de *H. seropedicae* e a identificação do sítio de ligação de NtrX ao DNA. Foram realizados ensaios de β -galactosidase para confirmar que NtrX regula as regiões promotoras de *narX* e

narKnirB. O estado de oligomerização de NtrX foi caracterizado por cromatografia de exclusão de massa molecular. Em estágio sanduíche realizado no laboratório do Prof Dr Martin Buck (Imperial College London – Londres/Reino Unido), a atividade de ligação de NtrX ao DNA foi determinada por ensaios de retardamento de migração em eletroforese em gel (“band-shift”) utilizando a proteína completa ou apenas região C-terminal. Além disso a região de ligação de NtrX no promotor de *narX* foi identificada através de ensaios de “footprinting” por DNase I.

2.3.3 Manuscrito 3

O sistema NtrB/NtrC de *H. seropedicae* e outras bactérias é o regulador principal em resposta a baixos níveis de amônio fixado intracelulares. Como mencionado anteriormente, um mutante *ntrC* de *H. seropedicae*, denominado estirpe DCP286A, não é capaz de crescer em nitrato como fonte única de nitrogênio (Persuhn, Souza et al. 2000). Com base nestes dados, no presente trabalho nós propusemos a identificação de genes regulados pelo sistema NtrB/NtrC tanto em resposta a baixos níveis de amônio fixado e de nitrato. Para isso, foram construídas bibliotecas de RNA-seq das estirpes selvagem SmR1 e mutante *ntrC* de *H. seropedicae* cultivados em baixos níveis de amônio (cloreto de amônio 1 mM), em nitrato (10 mM), e em altos níveis de amônio (cloreto de amônio 10 mM). As análises estatísticas foram realizadas utilizando DESeq 2.0 e edgeR (pacotes de R do projeto Bioconductor). O pacote mgsa foi utilizado para identificar grupos de genes diferencialmente expressos em cada tratamento e o pacote Pheatmap foi utilizado para gerar os “heatmaps”.

O mutante *nasA* de *H. seropedicae* construído por Bonato (2012) não cresce em nitrato como fonte única de nitrogênio. Este dado também foi incluído no manuscrito para corroborar que a nitrato redutase assimilatória é a principal forma de assimilação de nitrato neste microrganismo.

3 MANUSCRITO 1

RNA-seq analyses reveal insights into the function of respiratory nitrate reductase of the diazotroph *Herbaspirillum seropedicae*

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Key words: *Herbaspirillum seropedicae*, respiratory nitrate reductase, nitrate metabolism

Running title: Insights into nitrate reductase of *H. seropedicae*

Originality-Significance Statement: This work shows that despite being highly expressed in the presence of nitrate genes coding for a respiratory nitrate reductase of *H. seropedicae* do not support nitrate respiration. Our data support the involvement of this enzyme in redox power dissipation and confirm that it is the main source of NO production, an important signal molecule in plants. We show for the first time that the NAR activity in *H. seropedicae* is important for plant-bacterial interaction.

ABSTRACT

Herbaspirillum seropedicae is a nitrogen-fixing β -proteobacterium that associates with roots of gramineous plants. *In silico* analyses revealed that the *H. seropedicae* genome has genes encoding a putative respiratory (NAR) and an assimilatory nitrate reductase (NAS). To date, little is known about nitrate metabolism in *H. seropedicae*, and, as this bacterium cannot respire nitrate, the function of NAR remains unknown. Therefore, this study aimed to investigate the function of NAR in *H. seropedicae* and how it reduces nitrate under oxygen-limiting conditions. RNA-seq transcriptional profile in a condition where NAR genes were expressed allowed us to pinpoint genes important for nitrate metabolism in *H. seropedicae*, including nitrate transporters and regulatory proteins. Additionally, both RNA-seq data and physiological characterization of a mutant in the catalytic subunit of NAR (*narG* mutant) showed that NAR is not required for nitrate assimilation but is required for: (i) production of high levels of nitrite, (ii) production of NO, and (iii) dissipation of redox power, which in turn may increase carbon consumption. In addition, wheat plants showed a significant increase in shoot dry weight when inoculated with *H. seropedicae* wild type, but not with the *narG* mutant, suggesting that NAR is important to *H. seropedicae*-wheat interaction.

INTRODUCTION

Herbaspirillum seropedicae is a nitrogen-fixing β -proteobacterium that associates with roots of gramineous plants of economic importance including wheat, maize and sugarcane (Baldani *et al.*, 1986; Olivares *et al.*, 1996). Nitrogen metabolism in this bacterium, in particular regulation of nitrogen fixation, has been the object of several studies (Chubatsu *et al.*, 2012). However, the metabolism of nitrate, which is the main inorganic nitrogen source in the soil (Dechorgnat *et al.*, 2011), has not been well studied.

Nitrate is usually found in small concentrations in soil at micromolar level. However, the increasing usage of nitrogen fertilizers is leading to an accumulation of environmental nitrate up to milimolar levels (revised by Dechorgnat *et al.*, 2011). Nitrate is a key compound in the nitrogen cycle as it can have different cellular functions depending on how it is metabolized. Bacteria can use nitrate as nitrogen source, as terminal electron acceptor, or to dissipate reducing equivalents.

The nitrate reductases catalyze the reduction of nitrate to nitrite. In prokaryotes there are three types of nitrate reductases. Nitrate assimilation involves the reduction of nitrate to nitrite by a cytoplasmatic assimilatory nitrate reductase (NAS), followed by reduction of nitrite to ammonium. Genes encoding NAS are widespread in bacteria, however biochemical and physiological studies were conducted in only few bacterial genera (Moreno-Vivián and Flores, 2007). Furthermore, both membrane-bound respiratory nitrate reductase (NAR) and periplasmic nitrate reductase (NAP) are responsible for a process called denitrification, which involves nitrate reduction to nitrite by one of the nitrate reductases, followed by the successive reduction of nitrite, nitric oxide, and nitrous oxide to atmospheric nitrogen by other enzymes. This process is usually coupled to the generation of a proton motive force that can be used to produce ATP. However, in some bacteria, NAP dissipates reducing equivalents to help maintaining the redox homeostasis rather than being coupled to the generation of proton motive force (Berks *et al.*, 1995).

H. seropedicae bears putative genes encoding both assimilatory (*nasA* and *Hsero_RS14545* genes) and respiratory nitrate reductases (*narGHI*) (Fig. 1)

(Pedrosa *et al.*, 2011). *H. seropedicae* uses NAS to assimilate nitrate as this bacterium is able to grow aerobically in the presence of nitrate as the sole nitrogen source (Baldani *et al.*, 1986). However, the presence of genes encoding NAR in the *H. seropedicae* genome is more intriguing since this microorganism is not able to grow on nitrate anaerobically, nor is able to denitrify (Baldani *et al.*, 1986), raising the question about the role of NAR in the *H. seropedicae* metabolism.

Considering that little is known about nitrate metabolism in *H. seropedicae*, and that the presence of *narGHI* genes in this bacterium is intriguing, the aim of this study was to investigate the function of NAR in *H. seropedicae* and how it metabolizes nitrate under oxygen-limiting conditions. We found that *narGHI* genes of *H. seropedicae* are expressed in the presence of nitrate under oxygen-limiting conditions. A whole transcriptional profile of *H. seropedicae* grown on nitrate was performed and compared to that of cells grown on ammonium, both under oxygen-limiting conditions, which allow the expression of the *narGHI* genes. Our analysis was mainly focused on genes related to nitrogen metabolism, however, some other interesting metabolic pathways were revealed to be nitrate-induced and were also explored. These results allow us to gain insights into the function of NAR in *H. seropedicae*.

RESULTS

In silico analyses of genes related to nitrate metabolism in *H. seropedicae*

The *H. seropedicae* SmR1 genome contains genes related to nitrate metabolism in four clusters (Pedrosa *et al.*, 2011). The first one contains two probable operons, *narK₁UGHJlmoaA* and *narXL* (Fig. 1). The *narGHI* genes encode a putative respiratory nitrate reductase complex (NAR) whereas the *narK₁U* genes encode nitrate/nitrite antiporters of the major facilitator superfamily (MFS). The *narJ* gene encodes a putative chaperone and the *moaA* product is probably involved in synthesis of the molybdenum cofactor, both functions required for nitrate reductase maturation and activity in *Escherichia coli* (Liu and DeMoss, 1997; Blasco *et al.*, 1998). Expression of *narGHI* genes in *E. coli* and *P. aeruginosa* is regulated by NarX and NarL, a two-component system, and is induced by nitrate and oxygen-limiting conditions (Schroder *et al.*, 1993; Schreiber *et al.*, 2007). In *H. seropedicae* *narXL* are located downstream from the *moaA* gene.

In a second region of *H. seropedicae* genome, another *narK* homolog, coding for a nitrate transporter of the MFS family is found upstream the *nirBD* genes, which code for a nitrite reductase. Downstream from *nirBD*, genes encoding putative assimilatory nitrate reductase (NAS) (*nasA* and *Hsero_RS14545*) are found.

The *nasFED* genes that encode an ABC-type nitrate transport system are found in a third region of the *H. seropedicae* SmR1 chromosome. In the gram-negative bacterium *Klebsiella oxytoca*, *nasFED* expression is controlled by the NasR protein in response to nitrate. The *H. seropedicae* homolog *nasR* gene is found in a fourth region of the genome (Pedrosa *et al.*, 2011).

In silico revealed there are no genes coding for a NAP homolog system. Therefore, *H. seropedicae* only contains putative structural and regulatory genes related to NAR and NAS. However, as also reported by Baldani and colleagues (1986), no anaerobic growth of *H. seropedicae* in the presence of nitrate was observed.

Nitrogen metabolism-related genes responding to nitrate

In *E. coli* and *P. aeruginosa*, the expression of *narK* and *narG* are induced by nitrate in anaerobiosis (Kolesnikow *et al.*, 1992; Tseng *et al.*, 1994; Schreiber *et al.*, 2007). Although *H. seropedicae* cannot grow anaerobically using nitrate, a previous report using RNA-seq transcriptome approach showed expression of *narK₁UGHJlmoaA* is dependent on Fnr (Batista *et al.*, 2013) implying an oxygen control of this operon.

Expression of the *narK₁* promoter was assayed using a *lacZ* fusion in both *H. seropedicae* wild type and in a triple *fnr* mutant in the presence of nitrate and under high or oxygen-limiting conditions (Table 1). β -galactosidase assays revealed that the *narK₁* promoter is expressed in the wild type strain in response to nitrate and under oxygen-limiting conditions. Cells grown in the presence of ammonium under oxygen-limiting condition showed basal level of *narK₁* expression as revealed by the β -galactosidase activity. On the other hand, in agreement with the *fnr* RNA-seq transcriptome data (Batista *et al.*, 2013), only basal levels of expression were detected in the *fnr* mutant strain. These results indicate that one or more Fnr proteins of *H. seropedicae* are the main activators of the *narK₁* operon in response to oxygen-limiting levels, but only in the presence of nitrate.

The results above suggest that NAR may have a function in response to nitrate and under oxygen-limiting conditions. To gain insights into the role of NAR in *H. seropedicae* metabolism and also in how this bacterium can metabolize nitrate in an oxygen-limiting condition, we conducted a whole transcriptional profile of this bacterium grown on nitrate at late exponential phase (OD₆₀₀ 0.8) and shaking at 120 rpm, conditions with oxygen concentrations low enough to activate the *narK₁* promoter (data not shown). This RNA-seq profile of *H. seropedicae* grown on nitrate was compared to cells grown under the same conditions, in the presence of ammonium as the only nitrogen source (Tadra-Sfeir *et al.*, 2015).

The RNA-seq profile revealed that changing the nitrogen source from ammonium to nitrate caused modifications in the pattern of gene expression in *H. seropedicae*. Out of 3880 expressed genes, 1456 were differentially expressed, of which 774 were nitrate-induced and 682 nitrate-repressed.

The *narK₁UGHJlmoaA* operon was among the most highly expressed genes in response to nitrate, with fold-change values ranging from 697 to 19,086 (Table 2). The high expression levels of some of these genes was confirmed with both RT-qPCR and β -galactosidase assays. RT-qPCR assays showed that *narU* and *narG* genes are 912 and 1,621-fold induced by nitrate, respectively (Table 2). β -galactosidase assays with *lacZ* reporter gene fusion containing *narK₁* or *narG* promoters also confirmed that these genes are induced by nitrate (9-fold and 21-fold increase, respectively) (Table 3).

Analyses of the reads mapping immediately upstream of the *narK₁* gene allowed us to predict the transcription start site of the *narK₁UGHJlmoaA* operon, which is located 26 bp upstream from the putative translation start codon. Further analyses of the RPKM values in the nitrate library showed a difference of about 2-fold between the RPKM values of *narK₁U* genes compared with *narGH* (Supplementary Material Fig. S1). This finding suggested the presence of a promoter between *narU* and *narG*, which was confirmed by using a reporter gene fusion containing their intergenic region (*pnarG-lacZ*) (Table 3). In order to verify if *narG* was expressed independently of *narK₁*, DNA amplification was carried out using cDNA synthesized from mRNA of *H. seropedicae* grown on nitrate. Primers annealing within *narU* and *narG* were used and a single amplicon was obtained, showing that *narG* is co-transcribed with *narU* gene (Supplementary material, Fig. S2). These results suggested that the *narGHJlmoaA* genes are transcribed from two distinct promoters, one located upstream from *narK₁* and the other upstream from *narG*, both responding to nitrate.

The regulatory genes *narXL* are located downstream from the *moaA* gene. The RNA-seq profile also showed that *narXL* were induced by nitrate with a fold change of 10.7 and 23.7 respectively (Table 2). The *narX* induction by nitrate was also confirmed using RT-qPCR (Table 2) and by a *pnarX-lacZ* fusion (Table 3). Since the expression of the *narXL* genes of *H. seropedicae* is induced by nitrate it is possible that the products of these genes regulate the transcription of the *narGHI* genes in this bacterium similar to *E. coli* and *P. aeruginosa* (Schroder *et al.*, 1993; Schreiber *et al.*, 2007).

RNA-seq profile indicated that expression of the *narKnirBDHsero_RS14545nasA* cluster was induced in the nitrate libraries, with fold change values ranging from 8.5 to 29.4, which were confirmed by RT-qPCR assays for *narK*, *nasA* and *nirD* (Table 2). In addition, β -galactosidase assays of *H. seropedicae* containing a *pnarK-lacZ* fusion showed an increase of around 50-fold in the presence of nitrate (Table 3). Both the genetic organization and the co-regulation of these genes suggest that they are co-transcribed, forming an operon (*narKnirBDHsero_RS14545nasA*). The structure of this operon is similar to that of *Ralstonia eutropha* H16, *R. solanacearum* and *R. metallidurans* genomes (Pedrosa *et al.*, 2011).

Although the *nasFED* operon, which codes for a nitrate transporter, showed an induction by nitrate with fold change ranging from 3 to 7.5, statistical analysis indicated a *p-value* higher than 0.05 (Table 2). However, gene reporter assays with a *pnasF-lacZ* fusion confirmed a significant up-regulation of this operon promoter by nitrate (Table 3).

On the other hand, the putative regulator *nasR* gene was not induced by nitrate as compared to ammonium in the RNA-seq assay (Table 2), and the RT-qPCR assay showed a slight induction on nitrate (1.9-fold).

Taken together, the above results indicate that genes predicted to be involved in nitrate metabolism in *H. seropedicae* are indeed up-regulated in response to nitrate. In addition, RNA-seq assay also revealed increase in expression by nitrate of other genes related to nitrogen metabolism. Genes that encode glutamate synthase, *gltD* and *gltB*, were induced 6.0- and 4.9-fold, respectively by nitrate (Table 4). On the other hand, the expression of the glutamine synthetase (*glnA*) was not regulated.

Another gene involved in nitrogen metabolism that was found to be highly nitrate-induced was the *Hsero_RS12535* gene (former *Hsero_2508*) (Table 4). This gene product shares around 40% identity with HmpA of *E. coli*, a nitric oxide dioxygenase that converts nitric oxide to nitrate in aerobiosis and to nitrous oxide in anaerobiosis (Poole and Hughes, 2000). The high expression of this gene in *H. seropedicae* is intriguing since *in silico* analyses did not find other genes related to

nitric oxide metabolism in *H. seropedicae*. The relevance of *Hsero_RS12535* gene in nitrate metabolism of *H. seropedicae* will be further discussed.

NAR produces nitrite in *H. seropedicae*

The RNA-seq transcriptional profile of *H. seropedicae* conducted in this study showed that genes encoding the putative NAR of *H. seropedicae* are highly transcribed in response to nitrate (Table 2), although its role in the nitrate metabolism in *H. seropedicae* is unclear. Thus, to investigate a possible function of *narGHI* genes in *H. seropedicae*, a knockout strain containing an *in-frame* deletion of the *narG* gene was obtained.

Growth profile of *H. seropedicae* wild type strain (SmR1) and the *narG* mutant in the presence of ammonium or nitrate as nitrogen source were very similar in aerobiosis (Fig. 2). On the other hand, in microaerobiosis both strains grew similarly up to 6 hours, but then the *narG* mutant continued to grow whereas the wild type growth halted (Fig. 3A). We then measured the nitrite concentration in the growth media for each strain under these conditions. While the mutant strain produced only small amounts of nitrite (up to 0.05 mM) throughout the whole growth, the wild type strain produced large amounts of nitrite (up to 6 mM) (Fig. 3B). Nitrite accumulation by the wild type strain correlates with the observed impaired growth suggesting that the nitrite concentration was sufficient to inhibit bacterial growth. Meanwhile, under the same conditions, the *narG* mutant continued to grow due to the lower amounts of nitrite produced. Complementation of the *narG* mutant with the *narG* gene restored its capacity to produce nitrite by 65% after 24 hours of growth (data not shown). As discussed before, the *narG* gene is transcribed from two promoter regions: (i) one upstream from *narK₁* gene and other (ii) upstream from *narG* gene. However, for the complementation assays, the *narG* gene contained only the second promoter region. This explains the partial complementation of the nitrite production (65%).

Taken together, these results indicate that NAR of *H. seropedicae* is responsible for the high production of nitrite by the wild type strain. These experiments were performed in a “closed” system, in which the nitrite concentration

increased to toxic levels for *H. seropedicae*. However, considering a natural environment it is most likely that nitrite diffuses out and probably would not reach a toxic level to *H. seropedicae*.

NAR produces nitric oxide in *H. seropedicae*

Hsero_RS12535 was highly induced in response to nitrate (1507.6-fold) (Table 4). This gene encodes a putative nitric oxide dioxygenase, which in *E. coli* can convert nitric oxide to nitrate or to nitrous oxide under aerobiosis or anaerobiosis, respectively (Poole and Hughes, 2000). *In silico* analyses revealed that *H. seropedicae* does not contain enzymes that directly produce nitric oxide nor is it able to reduce nitrous oxide to nitrogen. However, recent studies have reported that NAR of *E. coli* and *Salmonella* reduce nitrite to nitric oxide at low levels of nitrate (Vine and Cole, 2011). In a similar way, NAR of *H. seropedicae* could also reduce nitrite to nitric oxide. This nitric oxide may then be removed from the cell by the *Hsero_RS12535* product. To test this hypothesis, nitric oxide production by the wild type strain and the *narG* mutant was evaluated using a NO-electrode. Production of NO by the wild type strain was significantly higher as compared to the *narG* mutant (about 3-fold) (Table 5). Production of NO by the *fnr* mutant strain was very similar to *narG*, consistent with the fact that one Fnr protein seems to be one of the key regulators of the *narK₁UGHJlmoaA* operon (see Table 1). Additionally, although the production of NO by *narG* and *fnr* mutants was low, it is still significant, which suggests that *H. seropedicae* may possess other mechanisms than NAR to produce NO. These NO measurements were confirmed using a fluorimetric assay with DAF-2DAA, a NO-specific probe (Supplementary Material Fig. S3). Taken together these results suggest that, in addition to producing nitrite, *H. seropedicae* NAR is also able to produce nitric oxide, probably from nitrite. Finally, the *Hsero_RS12535* gene product could have a role in the detoxification of excess of nitric oxide produced by the bacterium through NAR.

NAR increases carbon consumption in *H. seropedicae*

Further investigations in the RNA-seq profile of *H. seropedicae* grown in nitrate showed an interesting up-regulation of genes involved with energy production and conversion. Several genes involved in the citrate cycle (TCA) and oxidative phosphorylation were up-regulated in the nitrate libraries (Supplementary Material Table S4). These data suggest that the carbon consumption was increased in cells grown on nitrate. To test this hypothesis, we measured the consumption of malate, the carbon source used in all growth conditions, by the *H. seropedicae* wild type strain grown on nitrate or on ammonium as nitrogen source (Table 6). The wild type strain consumed two times more malate when using nitrate as compared to ammonium. On the other hand, the *narG* mutant strain showed malate consumption similar to the wild type grown on ammonium (Table 6). These results suggest that NAR was responsible for the increase in malate catabolism on nitrate, probably by acting as a sink of the reducing power.

Involvement of NAR with the plant-bacteria interaction

Involvement of NO with legumes-Rhizobia symbiosis has been described (Boscari *et al.*, 2013; Hichri *et al.*, 2015), as well as, the influence of NO on lateral root formation in the association *Azospirillum*-tomato (Creus *et al.*, 2005; Molina-Favero *et al.*, 2008). Considering that *H. seropedicae* is able to associate with gramineae, and that NAR is involved in NO production, we evaluated whether NAR would have a role in *Herbaspirillum*-plant interaction. Wheat plants were inoculated with *H. seropedicae* wild type or *narG* mutant strains using a hydroponic system. After 12 days, a significant difference in dry weight of shoots and roots was observed (Table 7). Wheat plants inoculated with the *narG* mutant strain showed a decrease in shoots and roots dry weight as compared with plants inoculated with the wild type strain. These results suggest that the absence of a functional NAR system affects plant development.

DISCUSSION

H. seropedicae strain SmR1 genome analyses revealed its genetic potential in using nitrate as an alternative electron acceptor, since genes encoding the respiratory nitrate reductase (*narGHI*) were identified (Fig. 1). Despite of that, *H. seropedicae* is impaired to sustain growth anaerobically using nitrate as the terminal electron acceptor (Baldani *et al.*, 1986). Gene reporter expression analyses showed that the *narK*₁ promoter, which drives the expression of *narGHI* genes, is expressed under nitrate and oxygen-limiting condition. To further investigate the metabolic context of NAR, and better understand its role, we conducted a whole transcriptional RNA-seq in a condition known to induce *narK*₁ (Table 1). Indeed, the NAR-encoding operon, *narK*₁*UGHJImoaA*, was the most induced operon in response to nitrate (Table 2). Physiological characterization of *narG* mutant strain showed that NAR is a functional enzyme responsible for high production of nitrite under oxygen-limiting conditions. However, as this bacterium is unable to grow using nitrate as the terminal electron acceptor in the absence of oxygen, we hypothesize that nitrate reduction to nitrite by NAR is either not coupled to the generation of a proton motive force, or the proton motive force generated is not sufficient to sustain bacterium growth under anaerobiosis.

Similar to *H. seropedicae*, the *Mycobacterium tuberculosis* genome also presents *nar* genes and it cannot grow on nitrate under anaerobiosis. The *narGHI* genes of this bacterium are expressed aerobically and NAR was shown to be essential to nitrate assimilation, function primary attributed to NAS (Sohaskey and Wayne, 2003; Malm *et al.*, 2009). On the other hand, in *H. seropedicae* the *narGHI* genes are expressed under low-oxygen conditions in presence of nitrate. Additionally, a *narG* mutant showed similar growth on nitrate as compared to the wild type strain under aerobiosis. Both results suggest that NAR from *H. seropedicae*, as opposed to *M. tuberculosis*, has not a nitrate assimilatory function.

NAR of the gram-positive and obligate aerobe *Streptomyces coelicolor* was suggested to have a function in dissipating redox power, rather than nitrate respiration (Fischer *et al.*, 2010). Since NAR of *H. seropedicae* is neither related to nitrate assimilation nor to nitrate respiration, this enzymatic complex may be

important for dissipation of excess reducing power, since no NAP encoding genes were found in *H. seropedicae* genome. *H. seropedicae* is usually grown with malate as carbon source. Wild type strain cultured in nitrate showed an increase in malate consumption (Table 6), whereas the *narG* mutant shows a similar malate consumption either on nitrate or ammonium and similar to the wild type strain on ammonium. This result suggests that NAR could be acting as a sink of reducing power leading to an increase of malate consumption. Dissipation of reducing equivalents can be important to *H. seropedicae* considering its natural environment, roots of gramineous plants such as wheat and maize. The radicular system of these plants exudate organic acids, including malate and succinate (Carvalhais *et al.*, 2010; de Andrade *et al.*, 2011) which can be used as carbon source by bacteria. Since in soil we have the presence of nitrate, which induces the expression of *narGHI* genes, NAR could help to sustain the bacterium growth by the oxidation of the excess of reducing power generated by the oxidation of organic acids.

In this study we also determined that NAR of *H. seropedicae* is involved in producing nitric oxide, an important signaling molecule during plant-bacteria interaction. Previous studies have already suggested the production of NO by NAR of other bacteria. Both in *E. coli* and in *Salmonella typhimurium* NAR was shown to produce NO from nitrite in low concentrations of nitrate (Gilberthorpe and Poole, 2008; Vine and Cole, 2011; Rowley *et al.*, 2012). This production of NO can be relevant to *H. seropedicae*, since it is found in association with plants. It is well documented that NO is an important signaling molecule in plants, being also related to plant-growth promotion (Cohen *et al.*, 2010). Additionally, NO produced by the plant growth promoting bacterium *Azospirillum brasilense* was shown to promote growth of tomato roots (Creus *et al.*, 2005; Molina-Favero *et al.*, 2008; Cohen *et al.*, 2010).

Interestingly, wheat plants inoculated with *H. seropedicae narG* mutant showed a decrease in dry weight of shoots and roots when compared with plants inoculated with the wild type strain. These results suggest that NAR can have a role in maintaining a benefic association between wheat and *Herbaspirillum*. The impaired NO production by the *narG* mutant may be a factor, reinforcing the role of bacterial NO production with plant association as also described with *Azospirillum*

and rhizobia (Creus *et al.*, 2005; Hichri *et al.*, 2015). However, further investigation is required to determine the role of NAR in plants colonized with *H. seropedicae*.

In addition to the NAR enzyme complex, *H. seropedicae* also contains genes (*Hsero_RS14545nasA*) encoding to a second nitrate reductase, NAS. Our transcriptional analysis revealed that these genes were also induced by nitrate. Probably NAS is the main enzyme involved in nitrate assimilation, as the *narG* mutant could grow well on nitrate as the only nitrogen source. In other bacteria, such as *Klebsiella pneumoniae*, the *nasA* gene is also related to nitrate assimilation (Lin and Stewart, 1998).

H. seropedicae presents genes encoding only one nitrite reductase, the *nirBD* genes. In *E. coli*, these genes are expressed under anaerobiosis and the nitrite reductase is important to nitrite detoxification (Cole, 1996; Wang and Gunsalus, 2000). However, in *H. seropedicae*, under oxygen-limiting condition, we see high accumulation of nitrite (around 80% of the initial nitrate is converted to nitrite), which suggests that *nirBD* genes are not expressed in this condition. These genes are clustered with *Hsero_RS14545nasA* genes that encode to NAS, and probably co-expressed with them. Together these results suggested that in *H. seropedicae*, *nirBD* products have a nitrite assimilatory function rather than nitrite detoxification. This same function was attributed to the product of *nirBD* genes of *M. tuberculosis* (Malm *et al.*, 2009) and *S. coelicolor* (Fischer *et al.*, 2012).

To our knowledge, this is the first study focusing on the nitrate metabolism of *H. seropedicae*. Based on the RNA-seq assay we were able to identify some genes whose products are important in the nitrate metabolism of *H. seropedicae*. Characterization of the NAR system in *H. seropedicae* indicated its role in redox power dissipation, nitrite and NO formation but not in nitrate respiration. Also, NAR seems to be required for the establishment of a beneficial association with gramineous plants, an effect which will be further pursued.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and growth conditions

H. seropedicae and *E. coli* strains, and plasmids used in this study are listed in Tables S1 and S2 (Supplementary Material), respectively. *E. coli* strains were grown at 37°C in LB medium (Sambrook *et al.*, 1989). *H. seropedicae* strains were grown at 30°C and 120 rpm in NFbHP-malate medium (Klassen *et al.*, 1997) supplemented with 10 mmol/L KNO₃ or 20 mmol/L NH₄Cl as nitrogen source.

β-galactosidase assays

Regulatory regions of the *narK*₁, *narG*, *narX*, *narK*, and *nasF* genes were amplified using oligonucleotides described in Table S3 (Supplementary Material) and cloned into pPW452 or pMP220 vectors (Supplementary Material, Table S2). Constructed plasmids were transferred to *H. seropedicae* wild type cells or *fnr* triple mutant by conjugation. Cells were cultivated on ammonium or on nitrate as the only source of nitrogen, and harvested at OD₆₀₀ of 0.8. For assays with *narK*₁ promoter in two different levels of oxygen, cells were cultured on ammonium or nitrate for six hours with 5% of initial oxygen level and shaken in a sealed flask or under aerated condition (final OD₆₀₀ around 0.3). β-galactosidase activity was measured as previously described (Miller, 1992), and expressed as specific activity (nmol of o-nitrophenol produced per min per mg protein). Protein concentration was determined using the Bradford method (Bradford, 1976).

Transcription profile by RNA-seq

H. seropedicae wild type strain was grown on nitrate and harvested at an OD₆₀₀ of 0.8. Total RNA was isolated using RiboPure™-Bacteria Kit (Ambion), followed by rRNA depletion using MICROBExpress™ Bacterial mRNA Enrichment Kit. Sequencing libraries were prepared using SOLiD™ Whole Transcriptome Analysis Kit and SOLiD™ Transcriptome Multiplexing Kit (Life Technologies), and

sequenced using a SOLiD 4 platform according to the manufacturer's instructions. Two DNA libraries were constructed from two independent cultures and reads were mapped against the *H. seropedicae* SmR1 genome (NC_014323) using CLC Genomics Workbench 4.8 (tolerance of 2 mismatches, 0.9 and 0.8 minimum length fraction and minimum similarity fraction, respectively). More than 10 million reads of both DNA libraries were uniquely mapped in coding regions of the *H. seropedicae* genome. These DNA libraries were called NT-1 and NT-2 and were compared to libraries constructed from cells grown on ammonium as the only nitrogen source (20 mmol/L) (AM-1 and AM-2 DNA libraries) (Tadra-Sfeir *et al.*, 2015). The DESeq software (R package) was used to calculate fold-changes and to perform statistical analysis (Anders and Huber, 2010), and CLC Genomics Workbench was used to calculate RPKM (Reads Per Kilobase of exon per Million of mapped reads). Only genes with at least 3 times coverage in at least one of the libraries (NT-1 and NT-2 or AM-1 and AM-2) were considered expressed for this analysis. Genes with at least two-fold change and p-value FDR corrected lower than 0.05 were considered as differentially expressed. The sequence data of NT libraries and AM libraries are available in ArrayExpress database under accession numbers E-MTAB-2842 and E-MTAB-3435, respectively.

Quantitative reverse transcription PCR (RT-qPCR)

In order to confirm differential expression in the RNA-seq profile, *H. seropedicae* wild type strain was grown on nitrate or on ammonium and harvested at OD₆₀₀ of 0.8. Total RNA was isolated using RiboPure™-Bacteria Kit (Ambion). Two micrograms of RNA was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligonucleotides are listed on Table S3 (Supplementary Material), and the *rpoC* gene was used as the reference gene.

Construction of *H. seropedicae narG* mutant

H. seropedicae narG mutant strain was constructed by homologous recombination, deleting 3651 bp (out of 3729 bp) of *narG* internal region. Fragments located upstream (UP) and downstream (DOWN) of *narG* were amplified, and ligated using an *Xba*I restriction site. The UP-DOWN fragment was digested with *Bam*HI/*Sa*II and ligated into the pSUP202 suicide vector (*Bam*HI/*Sa*II digested) to yield plasmid pSUP-*narG*. A *Bam*HI fragment containing the *sacB*Km fragment from pMH1701 was then ligated into this plasmid to generate the suicide plasmid pSUP-*narG-sacB*Km. This plasmid was then transferred to *H. seropedicae* SmR1 wild type strain by bi-parental conjugation using *E. coli* S17.1 strain as donor strain. Kanamycin resistant transconjugants resulting from the first recombination event were selected and then plated on 5% sucrose for selection of the final mutant strain. Sucrose resistant and kanamycin sensitive strains, resulting from the second recombination event were screened by PCR for confirmation of *narG* deletion.

For complementation of *narG* mutant phenotype, a fragment of 4.8 Kb containing the *narG* gene and including its promoter region was amplified using Phusion® High-Fidelity DNA polymerase (NEB) and cloned into pLAFR3.18 vector, generating pLAFR-*narG* plasmid.

NO₂⁻ and NO determination

NO₂⁻ levels of cell-free medium of cultured cells were evaluated using spectrophotometric assay previously described (Xu *et al.*, 2000) and expressed as mmol/L.

For determination of NO, cells were cultivated on nitrate and harvested at OD₆₀₀ of 0.8. Production of NO was evaluated using an NO-specific amiNO-2000 microelectrode coupled to the in NO-T-II system (Innovative Instruments, Inc), as previously described (Arruebarrena Di Palma *et al.*, 2013), and monitored in real-time during 20 minutes and was expressed as nmol per min per mg of protein. A second method was used to determine NO production, using the NO-specific fluorescent probe DAF-FM Diacetate (Molecular Probes®) as previously described (Molina-

Favero *et al.*, 2008). NO scavenger Carboxy-PTIO (Sigma) was used as negative control to confirm NO specific measurement. Fluorescence intensity was measured with a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Scientific) every 2 min for 60 minutes. NO production was expressed as relative fluorescence units (RFU) per mg of protein.

Quantification of malate

Malate concentration in the cell-free medium of cultured cells was measured by ultra fast liquid chromatography (UFLC Prominence Shimadzu), using UV detector at 217 nm on a XR-ODS ShimPack column (30 x 3 mm, 2.2 µm particles) at 0.2 mL/min of acetic acid and tributylamine/methanol mobile phase. The NFbHP medium containing malate concentration from 3.7 to 37 mM was used as standard.

Plant-bacteria interaction assay

Triticum aestivum (CD-104 cv.) seeds were surface-sterilized as previously described (Camilios-Neto *et al.*, 2014) and germinated in water agar plates at 30°C for 24 h under darkness. Germinated seedlings were transferred to sterile glass tubes containing 25 mL Hoagland's nutrient solution (Hoagland, 1950) without carbon or nitrogen source (two seedlings per tube). Seedlings were inoculated with *H. seropedicae* wild type or *narG* mutant strains at a final concentration of 5×10^5 CFU/mL. Seedlings were then cultivated at 30°C under 14 h light period for 12 days.

Bacterial cells for inoculation were prepared by growing *H. seropedicae* wild type or *narG* mutant strains in NFbHP-20mM NH₄Cl at 30°C and 120 rpm to an OD₆₀₀ around 1.0. Then, 1 mL of the cultures were centrifuged (15 seconds, 12100 x g), resuspended in 1 mL of Hoagland's nutrient solution, and diluted 10 times in Hoagland's solution. Seedlings were inoculated with 0.25 mL of these bacterial suspensions.

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FIGURES

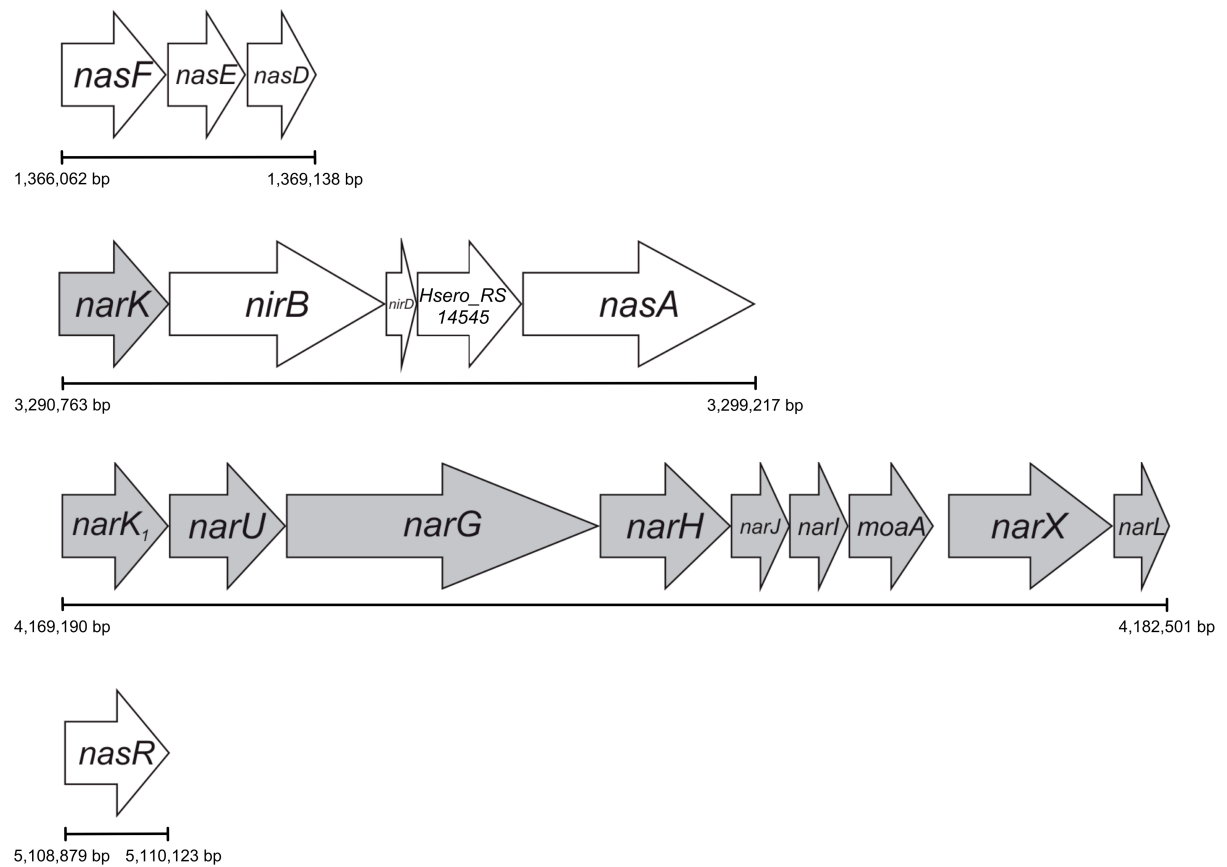


FIGURE 1 – *H. seropedicae* GENES INVOLVED IN NITRATE METABOLISM

Genes related to the respiratory or assimilatory nitrate reductases are shown in grey or white, respectively. Numbers represent the gene local context in *H. seropedicae* genome (NCBI accession number NC_014323).

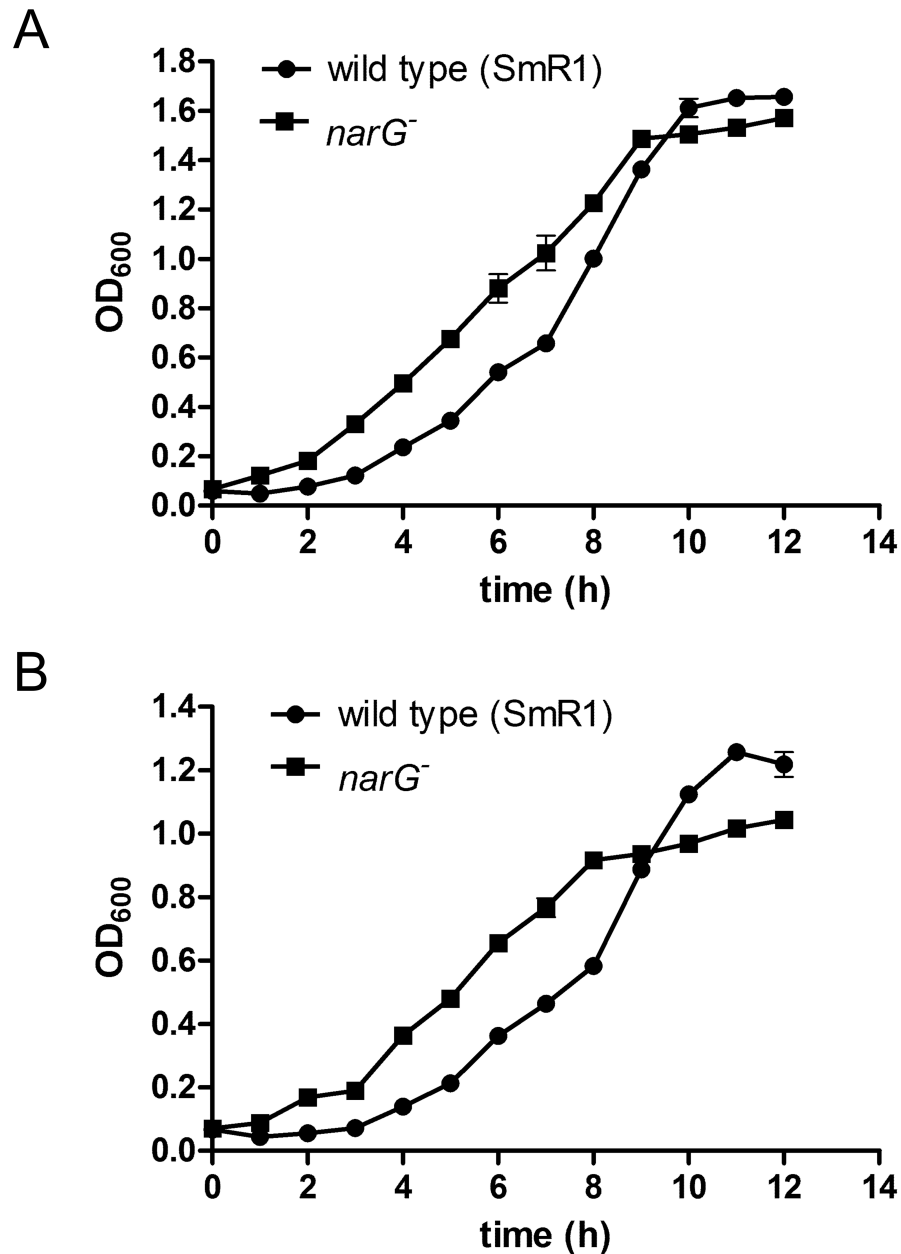


FIGURE 2 – CELLULAR GROWTH OF THE *H. seropedicae* WILD TYPE STRAIN (SmR1) AND THE *narG* MUTANT

Cells were grown with ammonium (panel A) or nitrate (panel B) as nitrogen source, under aerobic conditions. At the indicated time, absorbance at 600 nm was determined. Data represent the mean and standard deviation of two biological replicates.

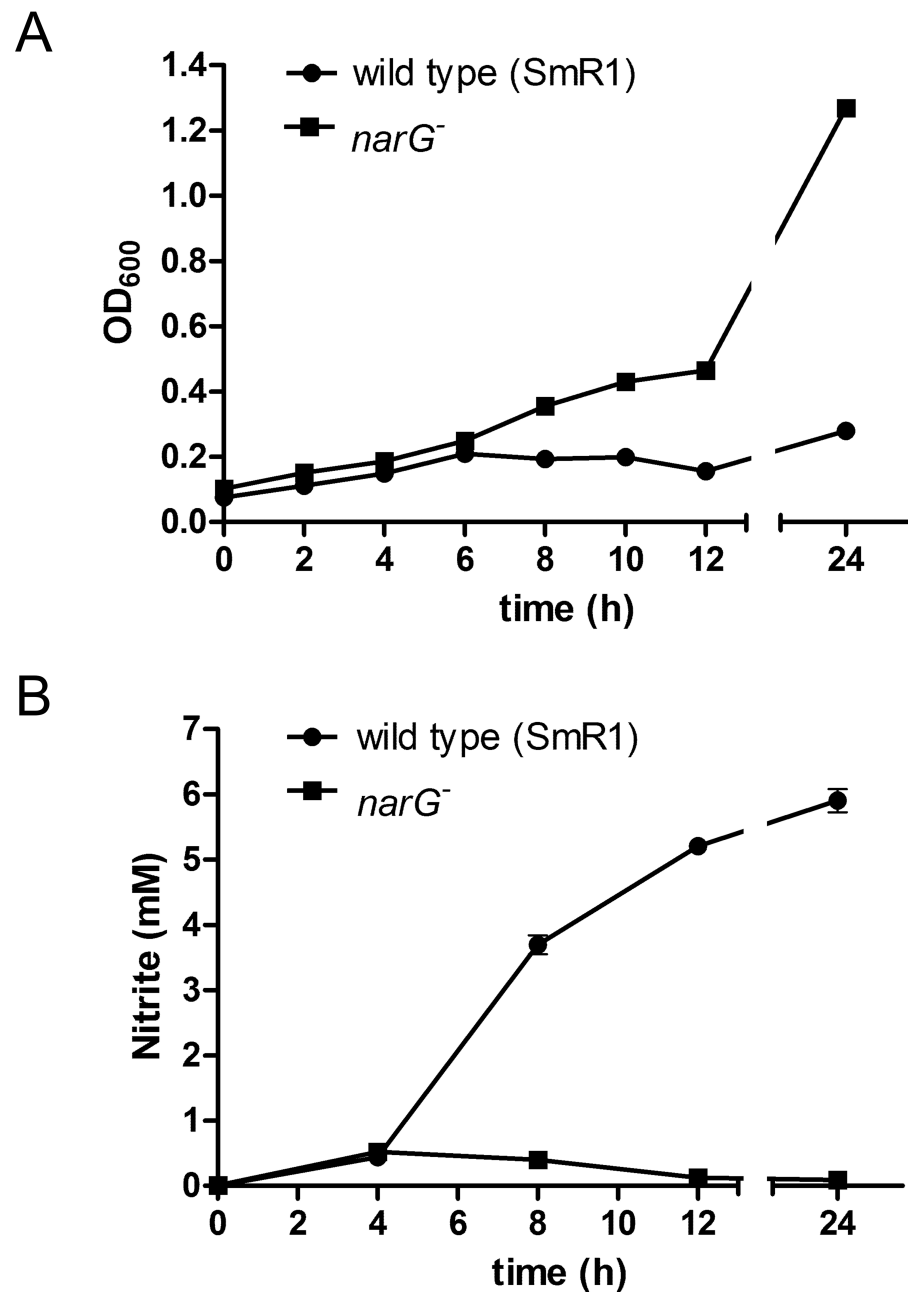


FIGURE 3 – CELLULAR GROWTH AND NITRITE PRODUCTION OF *H. seropedicae* WILD TYPE STRAIN SmR1 AND THE *narG* MUTANT GROWN IN NITRATE UNDER MICRO-AERATED CONDITION

At the indicated time, samples were removed to determine absorbance at 600 nm for cellular growth (Panel A) and nitrite concentration (Panel B) as described in Material and Methods. Data represent the mean and standard deviation of two biological replicates.

TABLES

TABLE 1 – PROMOTER ACTIVITY OF *narK₁-lacZ* FUSION IN *H. seropedicae* WILD TYPE STRAIN AND IN A *fnr* TRIPLE MUTANT

Condition	Wild type (SmR1)	<i>fnr</i>
Nitrate	24.7 ± 6.0	7.5 ± 4.8
Nitrate ↓O ₂	719.7 ± 43.5	38.2 ± 8.1
Ammonium ↓O ₂	44.7 ± 9.3	12.7 ± 4.8

β-galactosidase activity was determined in *H. seropedicae* wild type strain (SmR1) and a triple *fnr* mutant strain (MB231) carrying the pMP-*narK₁* plasmid as described in Material and Methods. Cells were cultivated with NH₄Cl or KNO₃, aerobically or under low O₂ (↓O₂), for six hours. (↓O₂) corresponds to 5% initial oxygen in the gas phase volume in culture grown in sealed flasks. β-galactosidase are indicated as specific activity (nmol of o-nitrophenol produced per minute per mg of protein). Data are indicated as mean ± standard deviation of biological duplicates.

TABLE 2 – DIFFERENTIAL EXPRESSION OF GENES RELATED TO NITRATE METABOLISM IN *H. seropedicae* CELLS GROWN IN THE PRESENCE OF NITRATE UNDER OXYGEN-LIMITING CONDITIONS AS REVEALED BY THE RNA-SEQ ANALYSES

Gene name	Fold Change ¹	<i>p</i> -value (FDR corrected) ¹	Ratio RT-qPCR ²	Annotation ³
Structural genes related to the respiratory nitrate reductase				
<i>narK₁</i>	4754.0	< 0.01	ND**	nitrite/nitrate transporter
<i>narU</i>	19086.4	< 0.01	912±332	nitrite/nitrate transporter
<i>narG</i>	5174.2	< 0.01	1621±652	respiratory nitrate reductase (alpha subunit)
<i>narH</i>	2054.1	< 0.01	ND	respiratory nitrate reductase (beta subunit)
<i>narJ</i>	1284.5	< 0.01	ND	respiratory nitrate reductase (chaperone)
<i>narI</i>	1574.0	< 0.01	ND	respiratory nitrate reductase (gamma subunit)
<i>moaA</i>	697.4	< 0.01	ND	molybdenum cofactor biosynthesis enzyme A
Structural genes related to the assimilatory nitrate reductase				
<i>narK</i>	19.7	0.01	2.8±0.6	nitrate/nitrite transporter
<i>nirB</i>	27.7	0.02	ND	nitrite reductase (large subunit)
<i>nirD</i>	29.4	0.03	24.0±10.6	nitrite reductase (small subunit)
<i>Hsero_RS14545</i>	9.9	0.06	ND	assimilatory nitrate reductase (small subunit)
<i>nasA</i>	8.5	0.04	9.7±2.8	assimilatory nitrate reductase (large subunit)
<i>nasF</i>	7.5	0.29*	ND	ABC-type nitrate transporter, periplasmic component

<i>nasE</i>	4.5	0.29 [*]	ND	ABC-type nitrate transporter, permease component
<i>nasD</i>	3.0	0.16 [*]	ND	ABC-type nitrate transporter, ATPase component protein
Regulatory genes				
<i>narX</i>	10.7	< 0.01	3.7±0.0	nitrate/nitrite sensor histidine kinase transcription regulator
<i>narL</i>	23.7	< 0.01	ND	nitrate/nitrite response regulator
<i>nasR</i>	1.6	0.18 [*]	1.9±0.6	nitrate response regulator

¹The fold change and *p*-value FDR corrected were calculated by using the software DESeq (R package).

² Ratio RT-qPCR was calculated using *rpoC* as endogenous control. Data are indicated as mean ± standard deviations of two biological replicates.

³Function based on *H. seropedicae* genome annotation.

^{*} *p*-values were above the cut-off (0.05).

^{**} ND: not determined.

TABLE 3 – PROMOTER ACTIVITY OF GENES RELATED WITH NITRATE METABOLISM IN *H. seropedicae* WILD TYPE STRAIN

	Ammonium	Nitrate
<i>pnarK₁-lacZ</i>	66.7±11.7	636.5 ± 105.9
<i>pnarG-lacZ</i>	248.0 ± 24.5	5181.2 ± 760.2
<i>pnarX-lacZ</i>	1409.1 ± 161.1	2990.5 ± 430.3
<i>pnarK-lacZ</i>	113.5 ± 26.1	5813.4 ± 1006.1
<i>pnasF-lacZ</i>	26.6 ± 3.8	487.1 ± 53.6
pPW452	4.9 ± 2.8	6.5 ± 3.5
pMP220	131.5 ± 7.4	183.8± 40.6

H. seropedicae wild type strain (SmR1) harboring plasmids carrying the indicated promoter regions fused to *lacZ* were assayed for β -galactosidase activity. Cells were cultivated with NH_4Cl or KNO_3 and harvested at $\text{OD}_{600} = 0.8$ as described for the RNA-seq approach. Expression levels of the promoter regions are indicated as nmol of o-nitrophenol produced per min per mg of protein. Data are indicated as mean $\pm$ standard deviation of biological duplicates and correspond to a representative of three experiments performed independently. Control plasmids (pPW452 and pMP220) indicate the β -galactosidase background activity.

TABLE 4 – DIFFERENTIAL EXPRESSION OF GENES RELATED TO NITROGEN METABOLISM IN RESPONSE TO NITRATE

Gene name	Fold Change ¹	<i>p</i> -value (FDR corrected) ¹	Annotation ²
<i>gltD</i> (<i>Hsero_RS19590</i>)	6.0	0.00	glutamate synthase (small subunit)
<i>gltB</i> (<i>Hsero_RS19595</i>)	4.9	0.00	glutamate synthase (large subunit)
<i>glnA</i> (<i>Hsero_RS15690</i>)	-1.4	0.60	glutamine synthetase
<i>Hsero_RS12535</i>	1507.6	0.00	nitric oxide dioxygenase

¹The fold change and *p*-value FDR corrected were calculated by using the software DESeq (R package).

²Function based on *H. seropedicae* genome annotation. Positive values represent activation of genes in response to nitrate, whereas the negative number indicates repression.

TABLE 5 – NITRIC OXIDE PRODUCTION BY *H. seropedicae* WILD TYPE AND *fnr* OR *narG* MUTANT STRAINS USING A NITRIC OXIDE-ELECTRODE

Strain	Nitric oxide production
Wild type (SmR1)	8.93 ± 0.07
<i>narG</i>	3.56 ± 0.66
<i>fnr</i> (MB231)	3.23 ± 0.25

H. seropedicae wild type (SmR1), *narG* and the triple *fnr* mutant (MB231) were cultivated on nitrate and harvested at OD₆₀₀ = 0.8, as described for the RNA-seq approach. Nitric oxide production was measured using a NO-electrode and the results are presented in nmol per min per mg of protein. Data indicate the mean and standard deviations of experiments performed in biological duplicate.

TABLE 6 – QUANTIFICATION OF MALATE LEVELS IN THE SUPERNATANT OF *H. seropedicae* WILD TYPE STRAIN AND *narG* MUTANT STRAIN

N source	Wild type (SmR1)	<i>narG</i>
Nitrate	8.7 ± 1.0	15.4 ± 1.5
Ammonium	17.1 ± 1.4	19.9 ± 0.78

H. seropedicae wild type strain and *narG* mutant were grown on nitrate or ammonium, as indicated. Cells were harvested at OD₆₀₀ = 0.8, as described for the RNA-seq approach. Data are indicated as the final concentration of malate present in the supernatant of cells and represent the mean and standard deviation of experiments performed in biological duplicate. The initial concentration of malate was 37 mM.

TABLE 7 – DRY WEIGHT OF SHOOTS AND ROOTS OF WHEAT PLANTS INOCULATED WITH *H. seropedicae* WILD TYPE AND *narG* STRAIN

<i>H. seropedice</i> strain	Shoot (mg)	Root (mg)
Wild type (SmR1)	20.5 ± 0.2	55.7 ± 0.8
<i>narG</i> mutant	16.9 ± 0.3 ^a	43.4 ± 0.9 ^b

Seedlings were inoculated with 5×10^5 CFU/mL and kept for 12 days using a hydroponic system. Data represent the mean ± standard deviation of dry weight of shoots and roots of 30 wheat plants inoculated with the indicated *H. seropedicae* strains. Means are significantly smaller for plants inoculated with the *narG* mutant strain: ^a $p=1.0 \times 10^{-4}$; ^b $p=7.0 \times 10^{-5}$.

SUPPLEMENTARY MATERIAL

TABLE S1 – STRAINS USED IN THIS WORK

Strain	Genotype	Reference
<i>Escherichia coli</i> DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ^- <i>rpsL</i> <i>nupG</i>	Invitrogen
<i>Escherichia coli</i> S17.1	Tra ⁺ , (Sm ^R)	(Simon et al., 1983)
<i>Herbaspirillum</i> <i>seropedicae</i> SmR1	wt, Nif ⁺ , (Sm ^R)	(Souza et al., 2000)
<i>Herbaspirillum</i> <i>seropedicae</i> MB231	<i>fnr</i> ₁ ⁻ , <i>fnr</i> ₂ ⁻ e <i>fnr</i> ₃ ⁻ (Sm ^R)	(Batista et al., 2013)
<i>Herbaspirillum</i> <i>seropedicae</i> (<i>narG</i>)	<i>narG</i> ⁻ (Sm ^R)	This work

TABLE S2 – PLASMIDS USED IN THIS WORK

Plasmids	Characteristics	Reference
pMP220	Contains the promoter-less <i>lacZ</i> gene (Tc ^R)	(Spaink et al., 1987)
pPW452	Contains the promoter-less <i>lacZ</i> gene (Tc ^R)	(Woodley et al., 1996)
pPW- <i>narX</i>	<i>Pst</i> I/ <i>Bgl</i> II fragment of 721 pb containing the promoter region of <i>narX</i> of <i>H. seropedicae</i> inserted into pPW452 (<i>Pst</i> I/ <i>Bgl</i> II)	This work
pMP- <i>narK</i> ₁	<i>Bgl</i> II/ <i>Pst</i> I fragment of 711 pb containing the promoter region of <i>narK</i> ₁ of <i>H. seropedicae</i> inserted into pMP220 (<i>Bgl</i> II e <i>Pst</i> I) (Tc ^R)	This work
pMP- <i>nasF</i>	<i>Bam</i> HI/ <i>Eco</i> RI fragment of 916 pb containing the promoter region of <i>nasF</i> of <i>H. seropedicae</i> inserted into pMP220 (<i>Bgl</i> II/ <i>Eco</i> RI) (Tc ^R)	This work
pMP- <i>narG</i>	<i>Bgl</i> II/ <i>Eco</i> RI fragment of 502 pb containing the promoter region of <i>narG</i> of <i>H. seropedicae</i> inserted into pMP220 (<i>Bgl</i> II/ <i>Eco</i> RI) (Tc ^R)	This work
pPW- <i>narKnirB</i>	<i>Xba</i> I/ <i>Pst</i> I fragment of 432 pb containing the promoter region of <i>narK</i> of <i>H. seropedicae</i> inserted into pMP220 (<i>Xba</i> I/ <i>Pst</i> I) (Tc ^R)	This work
pMH1701	Contains <i>sacB</i> -Km fragment (Km ^R)	(Hynes et al., 1989)
pSUP202	(Amp ^R , Cm ^R , Tc ^R)	(Simon et al., 1983)
pSUP- <i>narG</i>	<i>Bam</i> HI/ <i>Sal</i> I fragment of 1.1 Kb containing fragments located upstream (UP) and downstream (DOWN) of <i>narG</i> ligated (UP-DOWN fragment). This UPF-DOWN fragment was inserted into pSUP202 vector (<i>Bam</i> HI/ <i>Sal</i> I) (Amp ^R , Cm ^R)	This work

pSUP- <i>narG-sacB</i>	pSUP- <i>narG</i> plasmid containing <i>sacB</i> Km fragment (Amp ^R , Cm ^R , Km ^R)	This work
pLAFR3.18	pLAFR vector containing the polycloning site of pTZ18R (Tc ^R , Cm ^R)	(Souza et al., 1999)
pLAFR- <i>narG</i>	<i>Xba</i> I/ <i>Hind</i> III fragment of 4.8 Kb containing <i>narG</i> gene and its promoter region inserted into pLAFR3.18	This work

TABLE S3 – OLIGONUCLEOTIDES USED IN THIS WORK

Oligonucleotides	Sequence	Reference
Oligonucleotides used for RT-qPCR		
RT _{rpoC}	Forward 5' AAGCAAGTCCAGCAAAACGAA 3'	(Tadra-Sfeir et al., 2011)
	Reverse 5' CCACGAGCGGATCTTGTCA 3'	
RT _{narU}	Forward 5' TTTCATCTGGGTCCCCTTCA 3	This work
	Reverse 5' GTGTGCGGAGGCGATGTC 3'	
RT _{narK}	Forward 5' CGTGTGGATGCTCTTCATTACCTA 3'	This work
	Reverse 5' CGTAGTAGATGGCAGCGATGTT 3'	
RT _{narG}	Forward 5' AAGTCGGGCGCTGTCAAC 3'	This work
	Reverse 5' GGAACGCCAGACGAACAT 3'	
RT _{narX}	Forward 5' GTCCAACATCCGCAAGCAT 3'	This work
	Reverse 5' TCGTCGGACACGGTCATG 3'	
RT _{nasA}	Forward 5' ACAATTGCTGACCGAGGACTACTA 3'	This work
	Reverse 5' CGTGAATTGGTGTCTGATGTTG 3'	
RT _{nasR}	Forward 5' AAGCAGGGCTTGAACGAAGA 3'	This work
	Reverse 5' GGCAATACGCCGGTTCTG 3'	
RT _{nirD}	Forward 5' TCGGCAACCTGGGTGAAC 3'	This work
	Reverse 5' GTTGGCGGGCTCTTCCA 3'	
Oligonucleotides used for construction of <i>narG</i> mutant		
narG UP	Forward 5' GCTCTTCCTGGATCCCTTC 3'	This work

narG DOWN	Reverse	This work
	5' ATGAATTTCTAGAGGTCCAGA 3'	
	Forward	
	5' CGTCCTGCTCTAGATGAACA 3'	
	Reverse	
	5' GTGCTGTCGACTTCCTTCTG 3'	

TABLE S4 – DIFFERENTIAL EXPRESSION OF *H. seropedicae* ENERGY PRODUCTION RELATED GENES IN RESPONSE TO NITRATE

Gene	Fold Change ¹	<i>p</i> -value (FDR corrected) ¹
Pyruvate deshydrogenase		
<i>aceE</i> (Hsero_RS07305)	3.6	< 0.01
<i>aceF</i> (Hsero_RS07310)	12.1	< 0.01
<i>lpdA</i> (Hsero_RS07315)	6.5	< 0.01
Citric acid (TCA) cycle		
<i>gltA</i> (Hsero_RS14890)	6.8	< 0.01
<i>acnA</i> (Hsero_RS14940)	9.9	< 0.01
<i>acnA</i> (Hsero_RS11425)	4.6	< 0.01
<i>icd</i> (Hsero_RS12570)	7.2	< 0.01
<i>lpdA</i> (Hsero_RS14875)	8.9	< 0.01
<i>sucB</i> (Hsero_RS14880)	7.7	< 0.01
<i>sucA</i> (Hsero_RS14885)	7.3	< 0.01
<i>sucC</i> (Hsero_RS03280)	3.5	0.01
<i>sucD</i> (Hsero_RS03285)	3.3	< 0.01
<i>sdhB</i> (Hsero_RS14900)	9.5	< 0.01
<i>sdhA</i> (Hsero_RS14905)	6.2	< 0.01
<i>sdhC</i> (Hsero_RS14915)	9.0	< 0.01
<i>fumC</i> (Hsero_RS07610)	2.3	0.01
NADH dehydrogenase		
<i>nuoA</i> (Hsero_RS08820)	2.6	< 0.01
<i>nuoH</i> (Hsero_RS08855)	3.5	< 0.01

<i>nuoI</i> (Hsero_RS08860)	2.6	< 0.01
<i>nuoJ</i> (Hsero_RS08865)	2.8	0.03
<i>nuoK</i> (Hsero_RS08870)	8.3	< 0.01
<i>nuoL</i> (Hsero_RS08875)	5.1	< 0.01
<i>nuoM</i> (Hsero_RS08880)	4.6	< 0.01
<i>nuoN</i> (Hsero_RS08885)	2.7	0.01
Cytochrome reductase		
<i>petC</i> (Hsero_RS20235)	8.5	< 0.01
<i>petB</i> (Hsero_RS20240)	2.6	0.04
<i>petA</i> (Hsero_RS20245)	5.3	< 0.01
Cytochrome oxidase		
<i>cyoD</i> (Hsero_RS12305)	8.5	< 0.01
<i>cyoC</i> (Hsero_RS12310)	7.1	< 0.01
<i>cyoB</i> (Hsero_RS12315)	6.2	< 0.01
<i>cyoA</i> (Hsero_RS12320)	4.2	< 0.01
<i>cydA</i> (Hsero_RS15340)	5.0	< 0.01
<i>cydB</i> (Hsero_RS15345)	5.4	< 0.01
<i>fixG</i> (Hsero_RS16035)	4.1	0.02
<i>fixP</i> (Hsero_RS16040)	4.3	< 0.01
<i>fixO</i> (Hsero_RS16050)	4.6	< 0.01
Transhydrogenase		
<i>pntAa</i> (Hsero_RS03480)	3.2	< 0.01
<i>pntAb</i> (Hsero_RS03490)	5.3	< 0.01
<i>pntB</i> (Hsero_RS03495)	4.2	< 0.01

¹The fold change and *p*-value FDR corrected were calculated by using the software DESeq (R package)

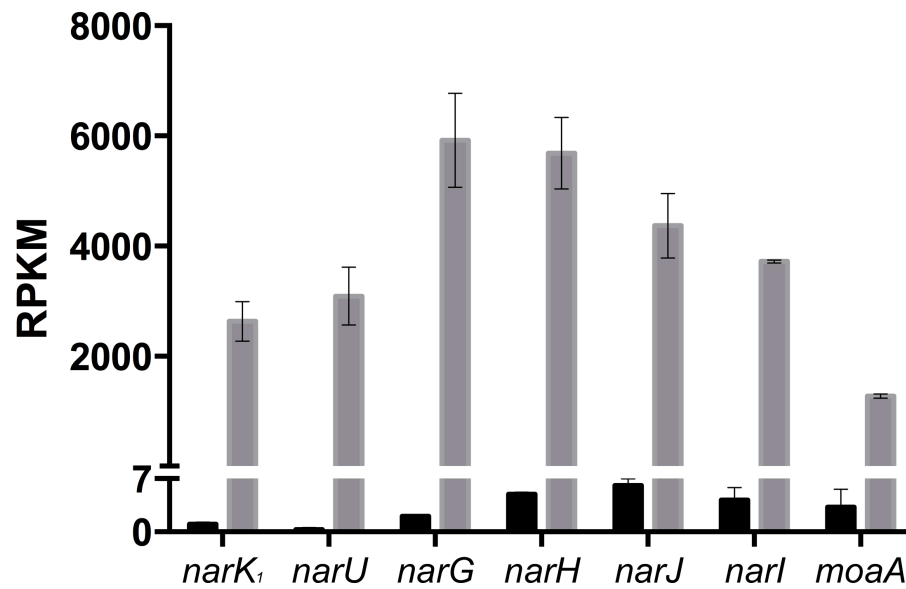


FIGURE S1 - RPKM VALUES OF INDIVIDUAL GENES OF *narK₁UGHJImoaA*
Data correspond to mean and standard deviation from the nitrate libraries (NT-1 and NT-2) and ammonium libraries (AM-1 and AM-2) and are indicated by grey or black columns, respectively. RPKM was calculated using CLC Genomics Workbench 4.8.

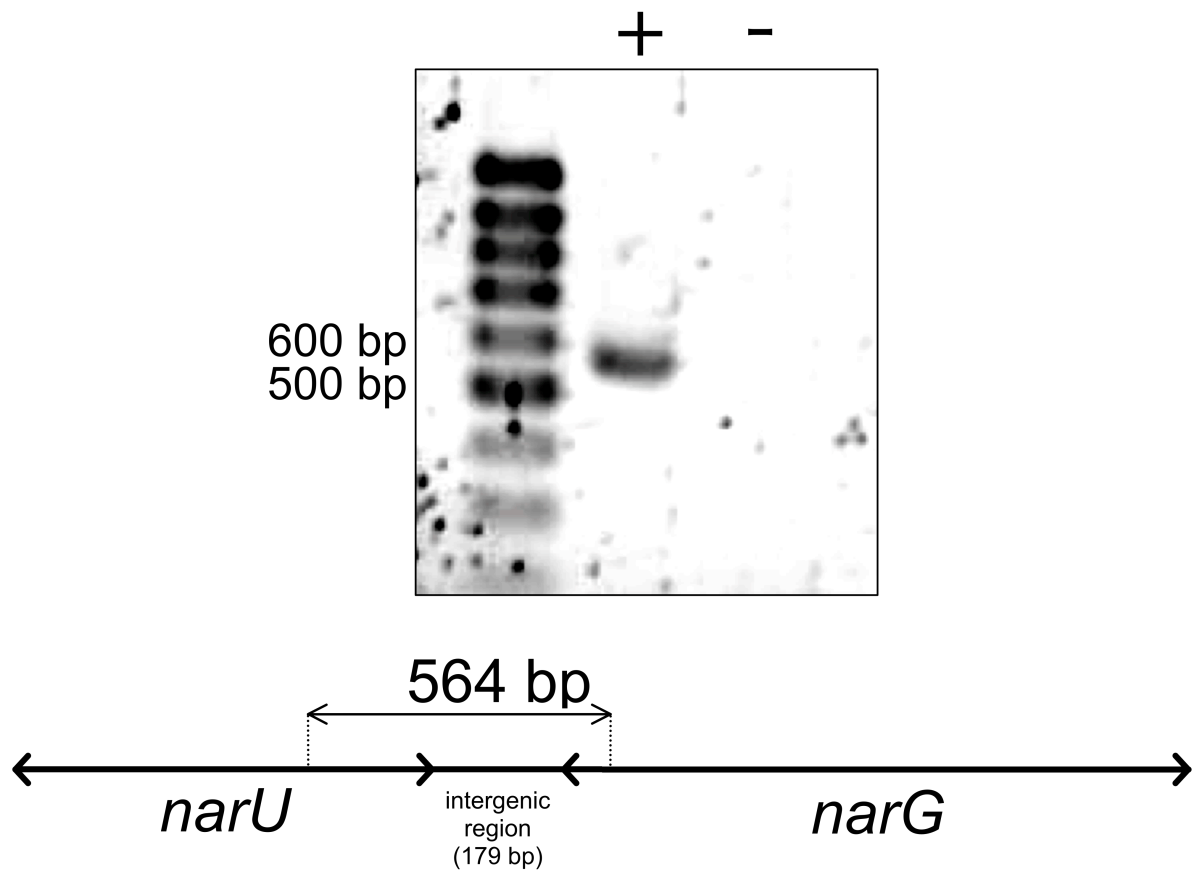


FIGURE S2 – DNA AMPLIFICATION OF THE INTERGENIC REGION OF *narU* AND *narG* EXPRESSED mRNA

RNA was isolated from *H. seropedicae* wild type strain grown on nitrate and used to synthesize cDNA. Primers annealing to 3'end of *narU* and 5'end of *narG*, as indicated at the lower panel were used in a PCR reaction with the produced cDNA (+) or unprocessed RNA (-) as template. Samples were analyzed on a 1% agarose with a 100 bp DNA ladder as molecular marker (upper panel). Expected size of the PCR product is also indicated.

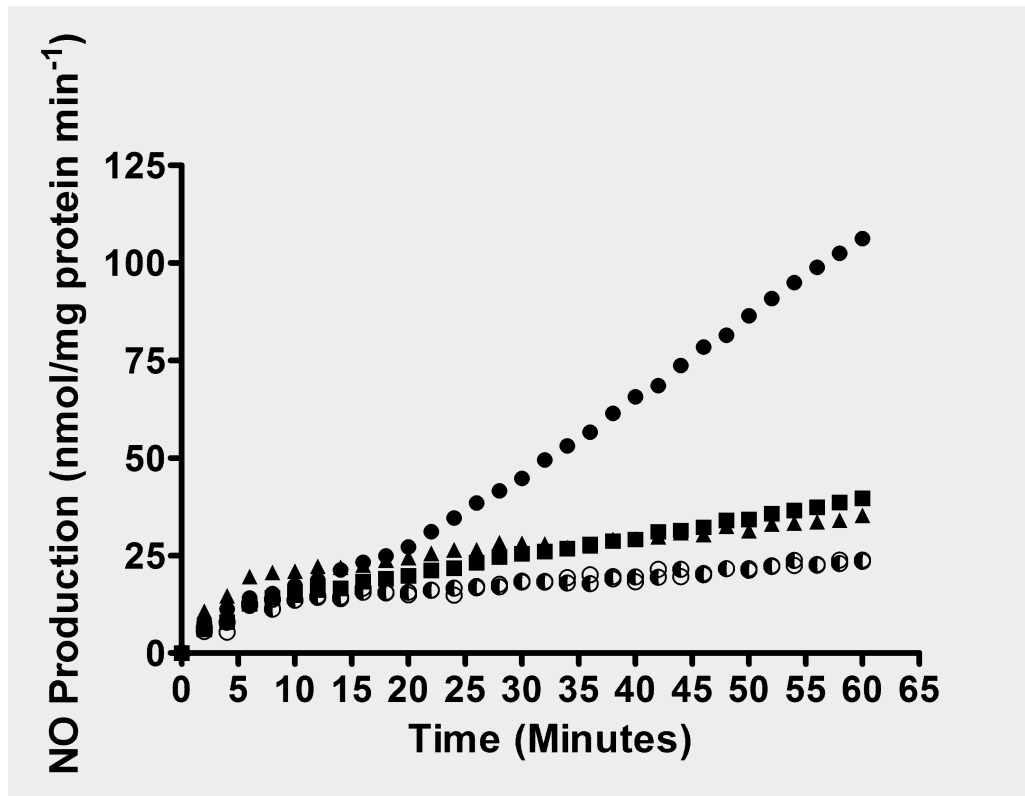


FIGURE S3 – NITRIC OXIDE PRODUCTION BY *H. seropedicae* STRAINS

Cells were cultivated on nitrate and collected at $OD_{600} = 0.8$, as described for the RNA-seq. Wild type strain (SmR1) (closed circles), *narG* mutant (closed squares) and the *fnr* triple mutant (MB231) (closed triangles) were assayed as described in Material and Methods. As negative controls were used the wild type strain without the addition of the NO-specific fluorescent probe DAF-FM (half closed circles) and the wild type strain with DAF but in the presence of the NO scavenger CPTIO (open circles). Graph is a representative of a biological duplicate.

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4 MANUSCRITO 2

The NtrY/NtrX two component system of *Herbaspirillum seropedicae* SmR1 strain

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Key words: *Herbaspirillum seropedicae*, NtrY, NtrX, nitrate metabolism

ABSTRACT

Herbaspirillum seropedicae is a β -Proteobacterium diazotroph found endophytically associated with graminea such as rice, sorghum and sugar cane. This bacterium has two Ntr systems, NtrB/NtrC and NtrY/NtrX, with a genomic organization which differs from that of the α -Proteobacteria. The NtrB/NtrC system is well studied and controls pathways involved in mobilizing nitrogen sources in absence or low concentration of ammonium ions. However, the function/role of the NtrY/NtrX system in *H. seropedicae* has not been addressed. NtrY is a histidine kinase sensor protein likely to be found associated with the membrane and NtrX is the response regulator partner. In this work we report the *in vivo* and *in vitro* characterization of the NtrY/NtrX system of *H. seropedicae*. An *ntrY* mutant was constructed and analyzed. It showed wild type ability in fixing nitrogen, but impairment in using nitrate as the sole nitrogen source. Gene fusion assays indicated that NtrY/NtrX is required for expression of genes coding for the assimilatory nitrate reductase as well as the two component system NarX/NarL (*narK* and *narX* promoters, respectively). The NtrX protein was expressed and purified showing DNA binding activity with the *narK* and *narX* promoters. A putative binding site at the *narX* promoter for the NtrX protein was determined by footprinting. *In silico* analyses revealed the presence of this binding site in other promoter regions of *H. seropedicae* that are related to nitrate metabolism, 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 bacteria survival. The two-component regulators systems constitute the main sensors of environmental changes. This system is composed by the sensor histidine kinase (HK) and the response regulator (RR) (Stock and Da Re 2000). One of most studied two-component system consists of the HK NtrB and RR NtrC proteins. Under low intracellular levels of nitrogen, NtrB autophosphorylates and then transfers this phosphoryl group to NtrC, which in turn modulates the expression of genes related with nitrogen metabolism (Dixon and Kahn 2004). In recent years authors have drawn their attention to a less-known two-component system and second member of the Ntr system, the NtrY/NtrX. The NtrY and NtrX proteins share amino acid sequence homology with NtrB and NtrC and might have originated from duplication and differentiation of the *ntrBC* genes (Capra et al. 2012). NtrY, the HK, is believed to be a transmembrane protein whereas NtrX, the RR, contains a helix-turn-helix motif, which interacts with DNA.

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 (Pawlowski et al. 1991). In *Azospirillum brasilense*, also a nitrogen-fixing α -Proteobacterium, NtrY/NtrX is also required to regulate nitrate metabolism (Ishida et al. 2002). This system has also been described to be involved in nitrogen fixation and nodule formation in *Rhizobium tropici* (Nogales et al. 2002). In the photosynthetic bacterium *Rhodobacter capsulatus*, besides being important to the nitrogen metabolism, the NtrYX system also regulates the formation of photosynthetic complexes (Gregor et al. 2007). Recently, Carrica and collaborators (2012) showed that NtrY of the α -proteobacterium *Brucella abortus* could sense the redox state of the cell by phosphorylating NtrX in response to oxygen limiting conditions. In the β -proteobacterium, *Neisseria gonorrhoeae* NtrY/NtrX are important to activate the transcription of genes involved in the synthesis of respiratory enzymes (Atack et al. 2013).

The endophytic nitrogen-fixing β -proteobacterium *Herbaspirillum seropedicae* presents genes encoding for the two Ntr systems: NtrB/NtrC and NtrY/NtrX (Pedrosa et al. 2011; Capra et al. 2012). The NtrB/NtrC system of this bacterium is well studied

and it is important to signal absence or low ammonium concentration and directing metabolism to use of alternative sources of nitrogen, including nitrogen fixation (Chubatsu et al. 2012). However, 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 consensus NtrX binding site was firstly identified in promoters involved in nitrate metabolism in *H. seropedicae*.

MATERIAL AND METHODS

Bacterial strains and growth conditions

H. seropedicae SmR1 wild type and mutant strains were grown at 30°C and 120 rpm in NFbHP-malate medium supplemented with 20 mmol/L NH₄Cl 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 1, and 2, respectively.

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

H. seropedicae ntrY mutant strain (named A01) was obtained by insertion of a kanamycin resistance cassette into a BglII 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 and ethylene formation was quantified using gas chromatography as previously described (Pedrosa and Yates 1984). Nitrogenase activity was expressed as nmol/L ethylene min⁻¹ mg⁻¹ protein. Protein concentration was determined by Bradford method (Bradford 1976).

The glutamine synthetase (GS) activity was determined as previously described and adapted to microplate (Bender and Magasanik 1977). GS activity was expressed as μmol/L γ-Glutamyl hydroxamate min⁻¹ mg⁻¹ protein.

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-terminal region. Protein overexpression was carried out at 16°C overnight to increase protein solubility. Proteins were purified by affinity chromatograph using HiTrap Chelating HP columns. When high purity was required, a second chromatographic step was performed using HiTrap Heparin HP column. Proteins were stored in HEPES containing buffer (50 mmol/L HEPES pH 8.0, 250 mmol/L NaCl, 50% glycerol).

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 concentrations) with 10 nM of DNA radio labeled (ATP [γ ³²P]) in 10 μ L reactions containing TAP buffer (Popham et al. 1989), and Tween-20 0.01% at 30°C for 10 minutes. The reactions were loaded in native gel and run at +4°C.

Gel filtration assay

The molecular weight 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 on the AKTA system (GE Healthcare), at flow rate of 0.5 mL/min 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 μ L reactions as described in EMSA section. The reactions were digested with DNase I, ethanol precipitated, resuspended in

loading buffer and loaded on 6% DNA sequencing 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/>) (Marchler-Bauer et al. 2015). The PredictProtein was used to predict the transmembrane segments of NtrY (Yachdav et al. 2014).

The three-dimensional structure of the C-terminal domain of NtrX from *H. seropedicae* was predicted using the I-TASSER server (Yang et al. 2015). This NtrX structure was aligned with the structure of NtrC4 bound to double DNA from *Aquifex aeolicus* (Vidangos et al. 2014) using the PyMol software (pymol.org).

RESULTS

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. Since then, different organisms have been described 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 to *ntrBC* suggesting duplication and differentiation of *ntrBC* genes (Capra et al. 2012). However, in *H. seropedicae*, as well as in several β -Proteobacteria, the *ntrYX* genes are not located in proximity to *ntrBC*, therefore sharing different gene organization with α -Proteobacteria (Figure 1A). In *H. seropedicae* strain SmR1, *ntrBC* (locus tag Hsero_RS15685 and Hsero_RS15680) form an operon with *glnA* which codes for glutamine synthetase (Schwab et al. 2007; Pedrosa et al. 2011) and *ntrYX* (locus tag Hsero_RS00345 and Hsero_RS00340) are located downstream of genes coding for a RNA methyltransferase and a proline-rich protein (Pedrosa et al. 2011).

H. seropedicae NtrB and NtrY are histidine kinases which 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 which may be involved in signal transduction (Figure 1B). The *H. seropedicae* NtrY protein also shows transmembrane motifs and a HAMP domain suggesting it is a membrane-bound protein, different from the cytoplasmatic NtrB. Overall, NtrB and NtrY show a 30% identity mainly at the HisKA and HATPase domains as indicated by Blast comparison.

Both the NtrC and NtrX response regulators proteins show a REC domain and a HTH motif (Figure 1B). The REC domain, located at the N-terminal region, receives the phosphoryl group from the histidine kinase (Galperin 2010), whereas the HTH motif, located at the C-terminal region, is mainly associated with DNA-binding (Brennan and Matthews 1989). The *H. seropedicae* NtrC protein also shows an AAA+ domain, found at the central part of the protein, and most likely involved in interaction with the σ^N -RNA polymerase holoenzyme (Studholme and Dixon 2003). On the other hand, *H. seropedicae* NtrX does not show the AAA+ domain, which indicate it is a σ^{70} dependent transcriptional activator. Analysis also indicated that although α -Proteobacteria NtrX shows a typical REC-AAA⁺-HTH domains organization, most β -

Proteobacteria NtrX lacks the AAA+ domain (SMART analysis; <http://smart.embl-heidelberg.de/>).

The *H. seropedicae* NtrB/NtrC system was shown to be required for nitrogen fixation as well as ammonia assimilation in *H. seropedicae* (Persuhn et al. 2000) since NtrC is the main activator required for *nifA* and *glnAntrBC* expression. Previous results also indicated that NtrB/NtrC is involved in nitrate metabolism since an *ntrC* mutant cannot grow on nitrate as the only nitrogen source (Persuhn et al. 2000).

In order to evaluate the importance of the NtrY/NtrX system on the nitrogen metabolism in *H. seropedicae*, an insertional *ntrY* mutant was constructed. The *ntrY* mutant grows similar to the wild type using ammonium as the sole nitrogen source (data not shown), but failed to grow on nitrate as the only nitrogen source (Figure 2A) similarly to the *ntrC* mutant (Persuhn et al. 2000). 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 ((Persuhn et al. 2000) and Figure 2B and 2C) since NtrC is the main transcriptional activator for *nifA*, *glnAntrBC* and *nlnAglnKamtB* operons in *H. seropedicae* as in other bacteria (Persuhn et al. 2000; Wassem et al. 2002; Schwab et al. 2007; Noindorf et al. 2011). On the other hand, *ntrY* mutant showed nitrogenase and glutamine synthetase activities similar to the wild type strain (Figure 2B, C) indicating that the *ntrY* product is not involved with nitrogen fixation or ammonia assimilation.

Use of nitrate as the only 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_RS14545nasA* operon codes for a nitrate/nitrite antiporter (*narK*), the assimilatory nitrate reductase and the nitrite reductase, the genes *Hsero_RS14545nasA* and *nirBD*, respectively. The *narXL* operon codes for members of a two component system family known in other bacteria to regulate nitrate metabolism. Transcriptional fusions *narK::lacZ* and *narX::lacZ* were assayed in the wild type and *ntrY* mutant strains in the presence of ammonium or ammonium+nitrate (Table 3). In the wild type strain expression of *narK::lacZ* is basal when cultivated in

ammonium, whereas high β -galactosidase activity is observed when nitrate is present. However, lower β -galactosidase activity is observed in *ntrY* mutant cultivated 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 low β -galactosidase activity was observed in the *ntrY* mutant.

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, whereas ^{His}NtrX-CTD indicates a His-tagged fused C-terminal domain (HTH) of NtrX.

Considering that some response regulators may form dimers (Robinson et al. 2000), gel-filtration chromatography was used to determine the oligomerization state of NtrX in solution (Figure 3). The calculated molecular weights 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 profile compatible to dimers, therefore suggesting that NtrX assembles itself as dimers through the C-terminal domain. Given that, the subsequent *in vitro* assays were conducted considering ^{His}NtrX and ^{His}NtrX-CTD as 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 migration rate of the DNA fragments (Figure 4). The DNA-protein complex was observed even in the presence of calf thymus DNA and disruption of the complex was observed when the reaction was carried out in the presence of non labeled homologous 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 migration rate (data not shown).

To investigate which amino acids residues are important for NtrX-DNA interaction, a three-dimensional molecular structure of NtrX is of value. However, as the protein structure of NtrX is not known, a model of the DNA-binding C-terminal

domain of NtrX from *H. seropedicae* using the I-TASSER server (Yang et al. 2015) was built. Afterwards, using the PyMol software we were able to align both NtrX protein from *H. seropedicae* and NtrC4 protein from *Aquifex aeolicus*, whose structure is the closest one to NtrX and was determined with the protein bound to DNA (Vidangos et al. 2014). This alignment allowed visualizing the NtrX amino acids most probably in close contact with the DNA at the complex. This procedure allowed us to choose six candidate amino acids potentially important for protein binding to the consensus site: Met₂₁₆, Thr₂₂₈, His₂₂₉, Tyr₂₃₁, Arg₂₃₂ and Gln₂₃₆ (Figure 5A). Six variants of NtrX were constructed replacing each of those amino acids to alanine and tested for DNA-binding activity as determined by 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. The only exception was the ^{His}NtrXArg₂₃₂Ala protein, which was not able to bind to DNA in the tested condition (data not shown). This may suggest the importance of Arg₂₃₂ for DNA binding.

In order to identify the potential site for NtrX binding, a DNaseI footprinting assay was carried out. Since a Cy3-labelled DNA (*narX* promoter region) was used in this experiment, a higher concentration of protein was required. Comparing with the full length NtrX, the N-truncated protein (^{His}NtrX-CTD) is more soluble allowing achieving higher concentration of the purified protein. Moreover, the ^{His}NtrX-CTD protein showed DNA binding activity similar to the full length NtrX (data not shown). Therefore, the footprinting assays were performed using the ^{His}NtrX-CTD protein.

Reactions were carried out using increasing concentration of the ^{His}NtrX-CTD protein and the *narX* promoter region labeled with Cy3 on forward (Figure 6A) and reverse (Figure 6B) strands. Protected and hyperreactive DNA bands from DNase I cutting were observed on the same region on both strands. These results allowed to narrow the DNA binding site of NtrX to approximately 34 base pairs at the *narX* promoter (Figure 6C). This potential DNA binding site found within the *narX* promoter was compared with other promoters of *H. seropedicae* that are related to nitrate metabolism. Interestingly, similar sequences were found in two other promoters: *narKnirB* and *nasFED*, which codes for a putative nitrate ABC type transporter (Figure 6D).

DISCUSSION

Two component systems are the main mechanism by which bacteria can sense and transduce signals to adapt to a new metabolic condition. Nitrogen is an essential element for growth and ammonium is the main source for most organisms. Therefore, in the absence or low concentration of ammonium, bacteria begin to synthesize proteins allowing usage of alternative forms of nitrogen. For most bacteria, NtrB/NtrC is the main two component system controlling these pathways.

In 1991 Pawlowski and colleagues described two genes located downstream of *ntrBC* in *A. caulinodans* that were involved with nitrogen fixation and nitrate metabolism. These genes, *ntrYX*, code for another two-component system, and due to location and similarity it has been suggested that *ntrYX* were the result of gene duplication and differentiation (Capra et al. 2012). This gene organization has been observed in several α -Proteobacteria. However, in most β -Proteobacteria, *ntrYX* are located far from *ntrBC* and show a distinct gene organization. *In silico* analysis also show that β -Proteobacteria NtrX, the response regulator, lacks the AAA⁺ domain related to interaction with σ^N RNA polymerase as compared to those from α -Proteobacteria.

Although the NtrY/NtrX two component system is found in several organisms, its functional characterization is restricted to few of them. Besides *A. caulinodans* (Pawlowski et al. 1991), involvement of NtrY/NtrX with nitrate metabolism has also been described for *Azospirillum brasilense* (Ishida et al. 2002) and *Rhodobacter capsulatus* (Drepper et al. 2006), 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 N₂ by nitrogen fixation (Persuhn et al. 2000; Wasseem et al. 2002; Schwab et al. 2007; Noindorf et al. 2011). Although several studies have been conducted in this organism on the regulation of nitrogen fixation, *H. seropedicae* nitrate metabolism is mainly unknown. It was proposed by Pershun and colleagues (2000) that the NtrB/NtrC system regulates nitrate metabolism since the *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.

However, NtrY/NtrX is not involved with nitrogen fixation or ammonium assimilation by glutamine synthetase.

In order to determine the involvement of the NtrY/NtrX system on nitrate assimilation, *narK::lacZ* and *narX::lacZ* fusions were assayed in the wild type and *ntrY* mutant. The *narK* promoter is directly involved with nitrate assimilation since it controls the expression of a nitrate transporter, the assimilatory nitrate reductase and the nitrite reductase (Bonato submitted). The *narX* promoter controls the expression of the two component system NarX/NarL (Bonato submitted). Expression of both promoters was lower in the *ntrY* mutant indicating the involvement of NtrY/NtrX system in the regulation of both promoters. The direct binding of NtrX as shown by the band-shift experiments support this fact. Furthermore, a consensus DNA binding site for NtrX was found in the *narX*, *narK*, and *nasF* promoters (Figure 6D).

These results indicated a direct role for the NtrY/NtrX system on nitrate metabolism in *H. seropedicae*. However, gene expression regulation does not rely only on NtrY/NtrX since an *ntrC* mutant is also impaired in growing using nitrate as the only nitrogen source. Dependence on NtrC for nitrate assimilation cannot be explained by NtrB/NtrC controlling expression of *ntrYX*. RNA-seq transcriptome analyses showed that *ntrYX* expression is constitutive, and not regulated by nitrate (Bonato et al., submitted). Furthermore, a *lacZ* fusion with the promoter of the operon containing *ntrYX* showed similar activity for the wild type and the *ntrC* mutant (data not shown).

Nitrate assimilation has a high energy cost involving not only a high number of electrons for reduction but also assembling and usage of different proteins (Luque-Almagro et al. 2011), as a result pathway regulation does not rely only on the nitrate availability of but also requires absence or low concentration of ammonium. In *H. seropedicae* both NtrB/NtrC and NtrY/NtrX systems seem to be required for nitrate assimilation.

Another aspect is the report showing that in *Brucella spp*, under oxygen-limiting conditions, NtrY phosphorylates NtrX leading to expression of genes involved in adaptation to low oxygen levels (Carrica et al. 2012). It was shown that *Brucella* NtrY is a haem protein which responds to cellular redox state (Carrica et al. 2013). Also, in the diazotroph *R. capsulatus*, NtrX coordinately participates with RegA in controlling gene expression in response to nitrogen and redox level (Gregor et al. 2007). Furthermore, nitrate metabolism control in *H. seropedicae* also involves oxygen levels. Recently, a transcriptome analysis of *H. seropedicae fnr* mutant revealed that

expression of *narXL*, as well as genes related to respiratory nitrate reductase (NAR) were down regulated (Batista et al. 2013) indicating that O₂ levels or cellular redox state also play a role on nitrate metabolism in *H. seropedicae*. The NAR system in this bacterium is not involved in nitrate respiration (Baldani et al. 1986), but rather involved in NO production and cellular redox regulation (Bonato et al, submitted).

In this paper, we report the role of a second Ntr system, NtrY/NtrX, present in *H. seropedicae*. Our results indicate distinct roles for NtrB/NtrC and NtrY/NtrX in controlling nitrogen metabolism in this bacterium: while NtrB/NtrC is involved in the general response to nitrogen-limiting conditions, the NtrY/NtrX seems to have evolved to specifically regulate the nitrate metabolism. The NtrX protein binds specifically to the *narK**nirB* and *narX* promoters region indicating a direct role on gene expression. Moreover, a putative binding site for NtrX was determined.

Nitrate metabolism in *H. seropedicae* has an intrinsic regulation involving signaling for low ammonium concentration, nitrate availability and O₂ and/or redox level. How those parameters communicate with each other leading to usage of nitrate will be further investigated.

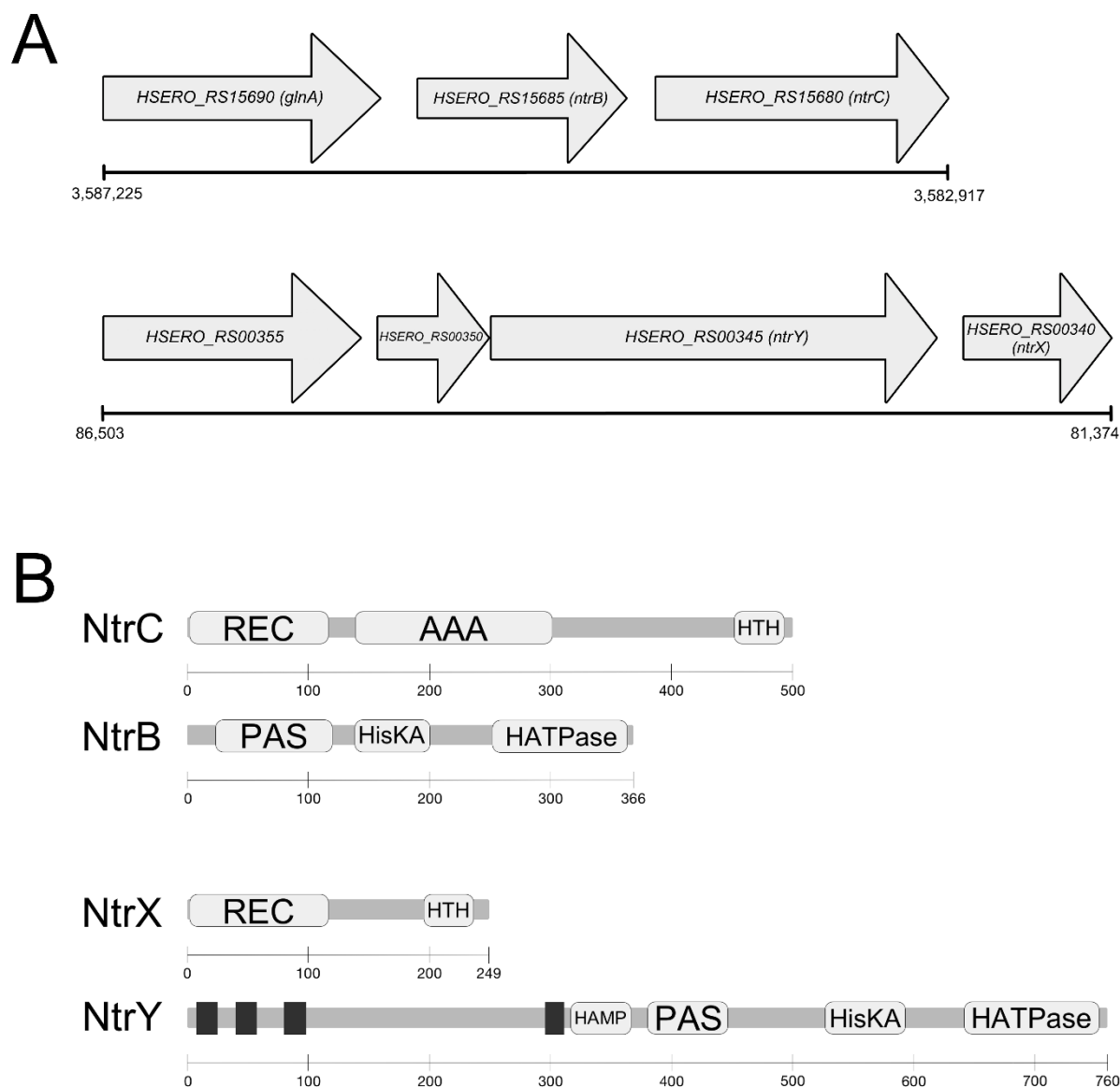
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FIGURES

FIGURE 1 – THE NtrYX AND NtrBC SYSTEMS OF *H. seropedicae*

Panel A: Genomic context of *ntrBC* genes (at the top) and *ntrYX* (at the bottom). Numbers represent the gene location in *H. seropedicae* genome (NCBI accession number NC_014323).

Panel B: Schematic structure with main domains, motifs or transmembrane segments of NtrC, NtrB, NtrX and NtrY proteins.

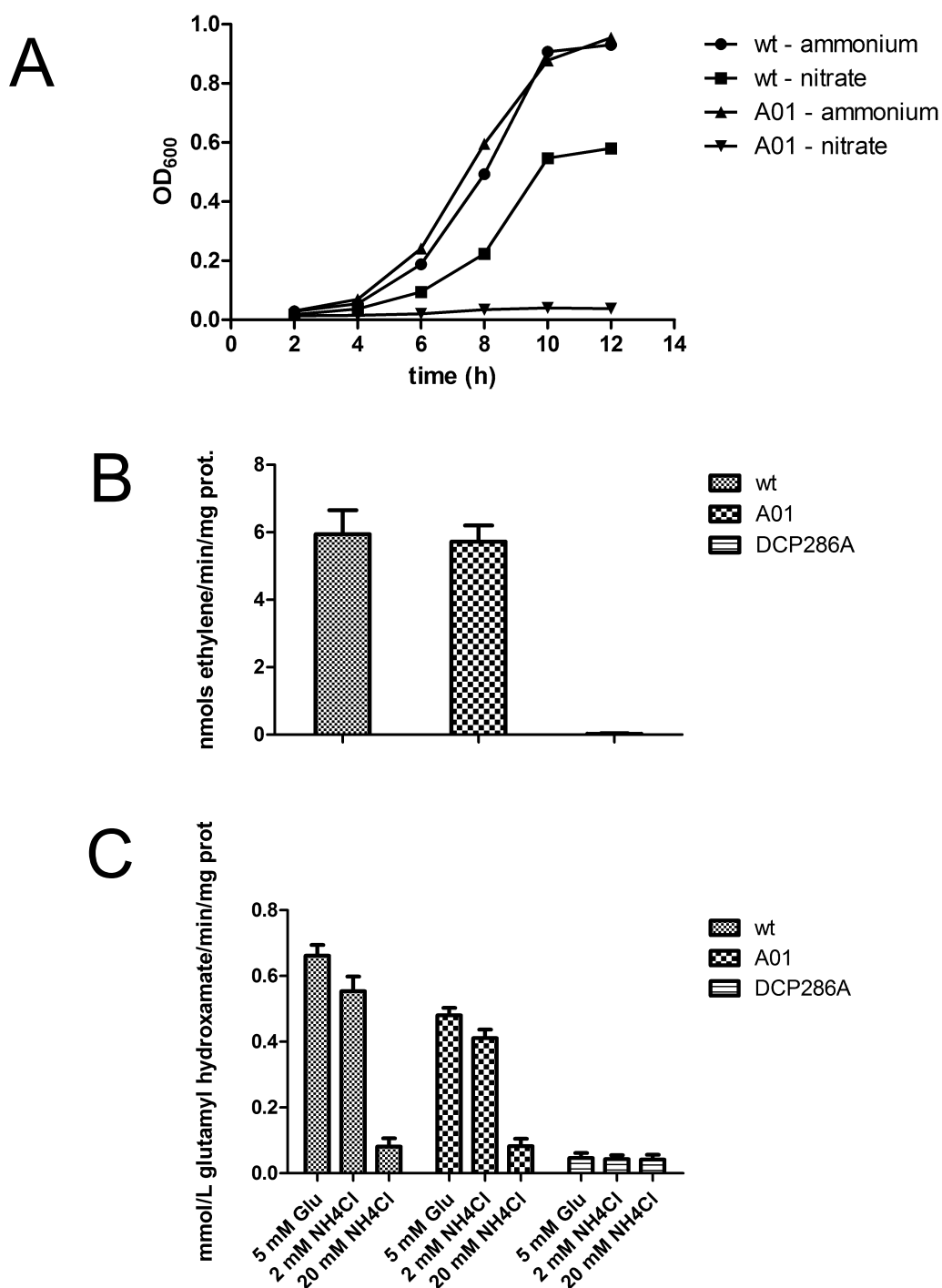


FIGURE 2 – THE FUNCTION OF THE NtrYX SYSTEM OVERLAPS WITH THAT OF THE NtrBC SYSTEM REGARDING THE NITRATE METABOLISM

(A) Growth of *H. seropedicae* SmR1 wild type, and *ntrY* mutant strain on ammonium or nitrate as the only nitrogen source. Data represent a representative experiment. (B) Nitrogenase or (C) GS activity of *H. seropedicae* SmR1 wild type, A01 (*ntrY* mutant), and DCP286A (*ntrC* mutant) strains. GS activity was measured in low (NH₄Cl 2 mmol/L or glutamate 5 mmol/L) or high levels of nitrogen (NH₄Cl 20 mmol/L). In B and C data represent mean $\pm$ standard deviation of at least three experiments.

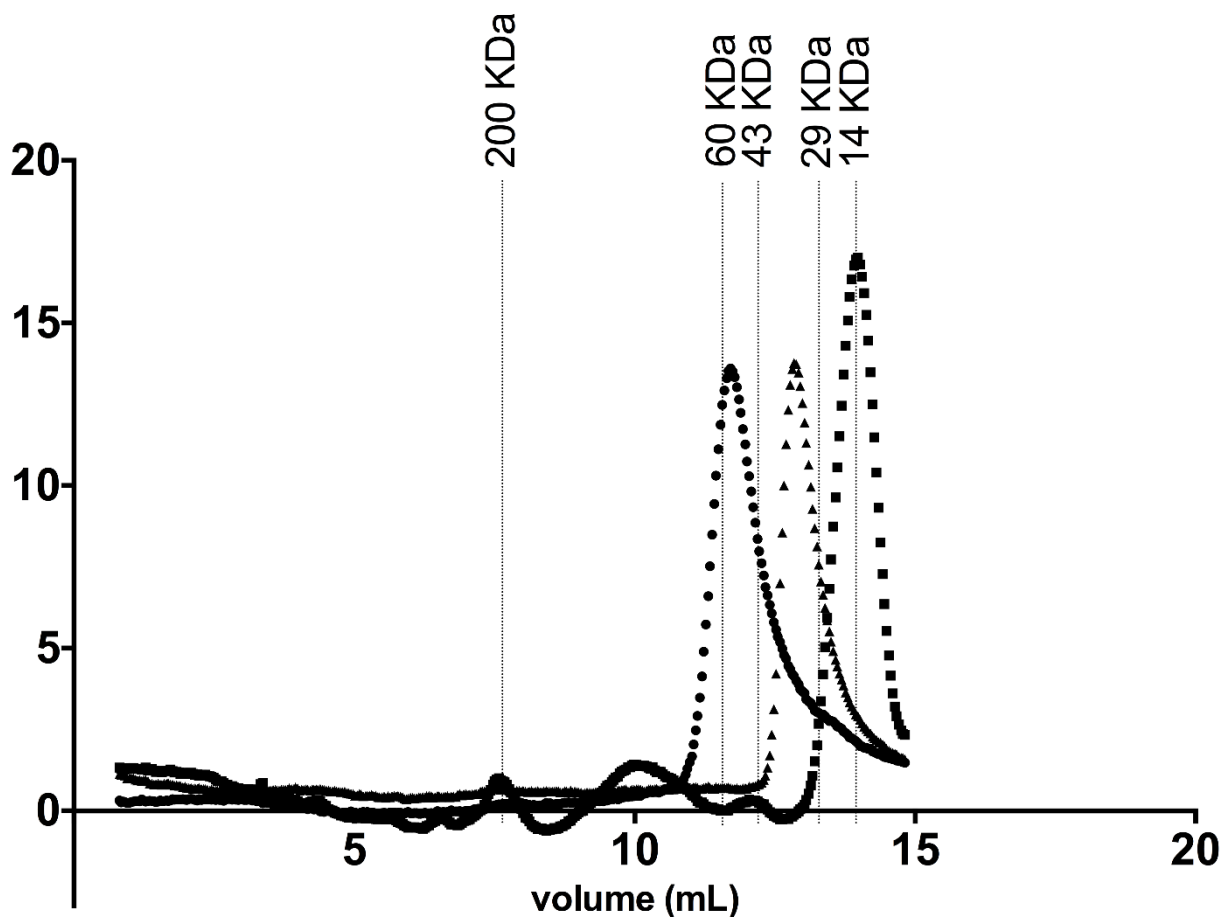


FIGURE 3 – ESTIMATION OF THE MOLECULAR WEIGHT OF NtrX AND VARIANT PROTEINS

The molecular weight of wt ^{His}NtrX (circles), ^{His}NtrX NTD (squares) and ^{His}NtrX CTD (triangles) proteins was estimated by gel filtration using sepharose 12 column. The molecular weights of standard proteins are marked by the vertical lines in KDa.

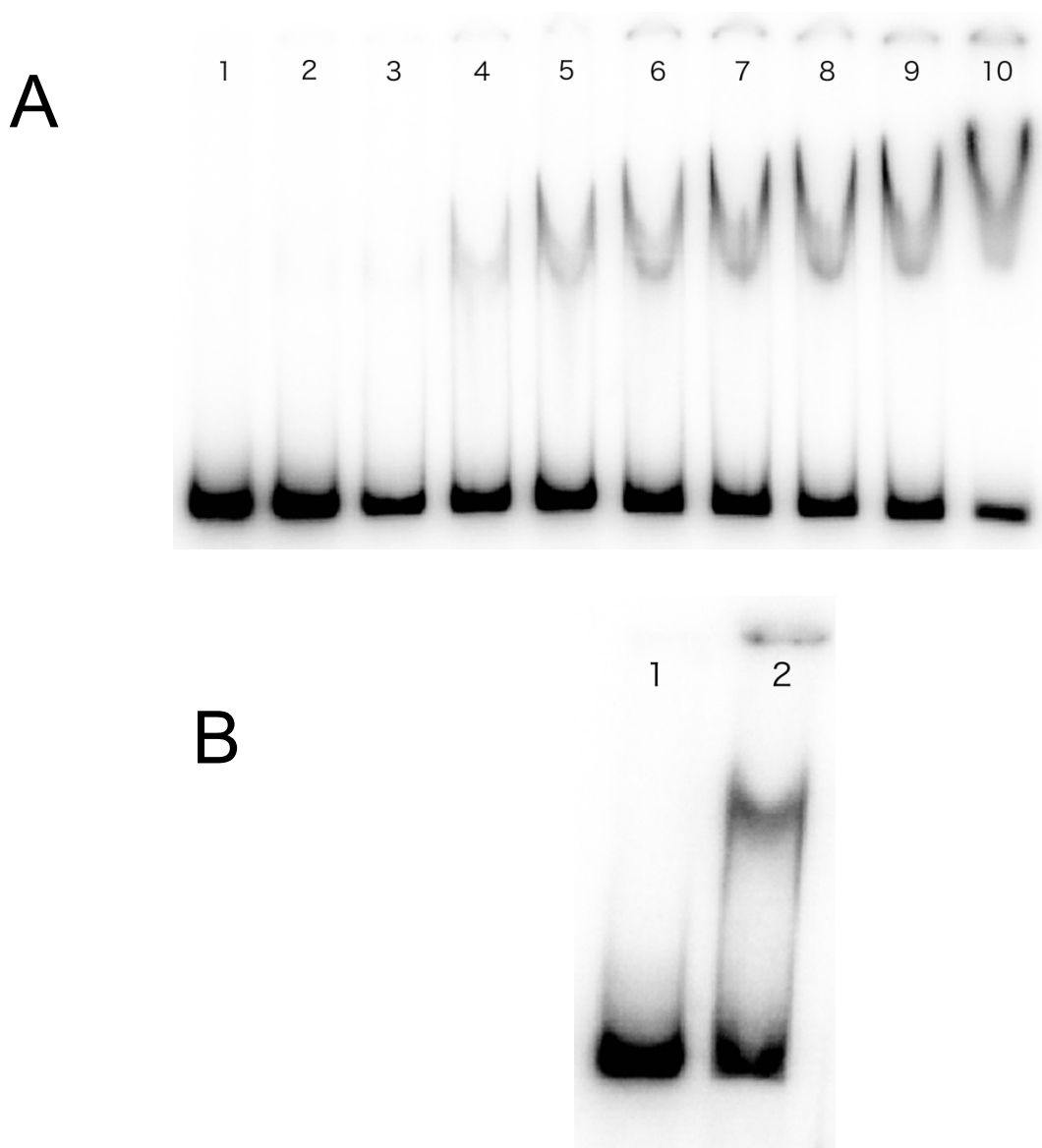


FIGURE 4 – THE NtrX PROTEIN OF *H. seropedicae* BINDS TO *narKnirB* AND *narX* PROMOTER REGIONS

(A) NtrX binds to *narK* promoter. Increasing concentrations of NtrX were incubated with DNA containing *narK* promoter and the reactions were loaded in native gels. Lane 1: only DNA, and lanes 2-10: 0.1, 0.2, 0.5, 0.7, 1, 1.3, 1.5, 2 and 3 μM wt ^{His}NtrX (considered as dimer). (B) NtrX binds to *narX* promoter. DNA containing *narX* promoter was assayed in absence (lane 1) or the presence of 1 μM wt ^{His}NtrX protein (lane 2).

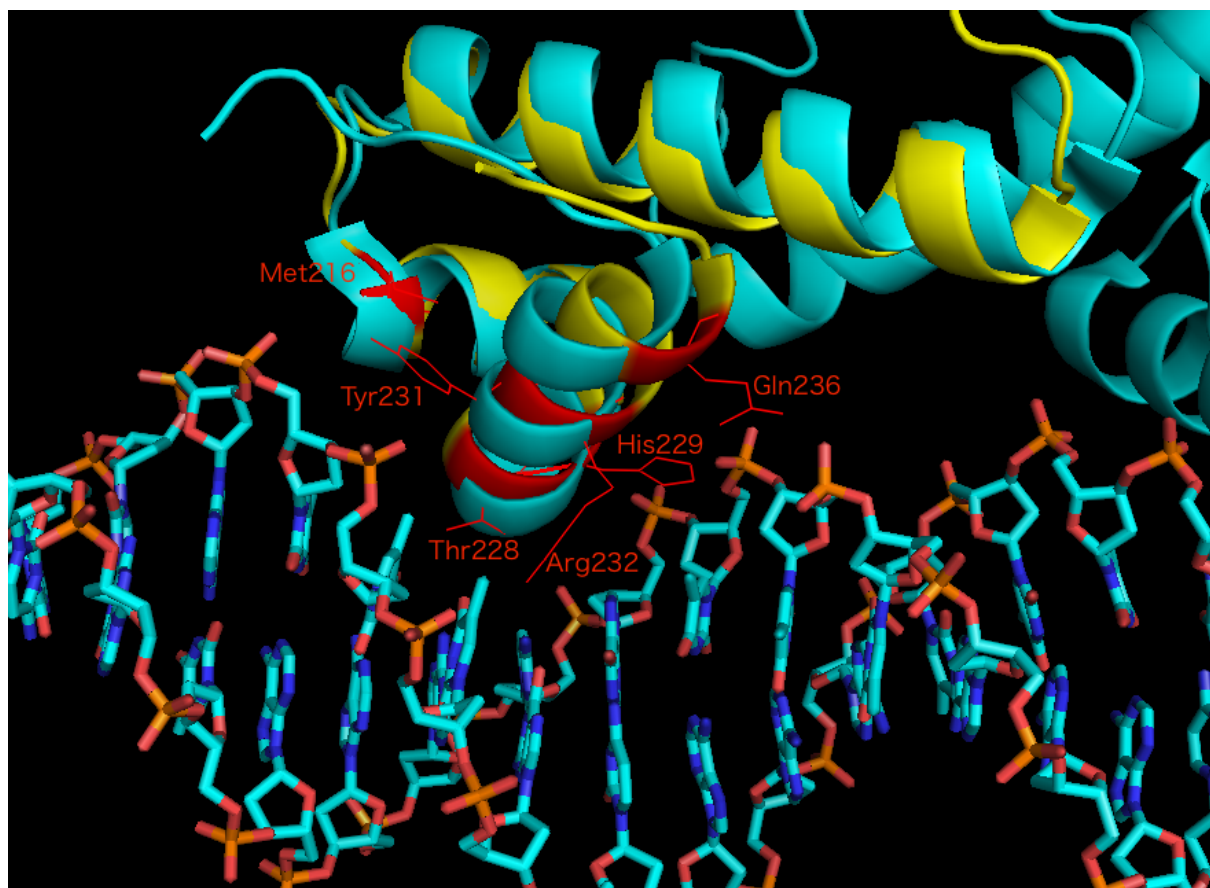
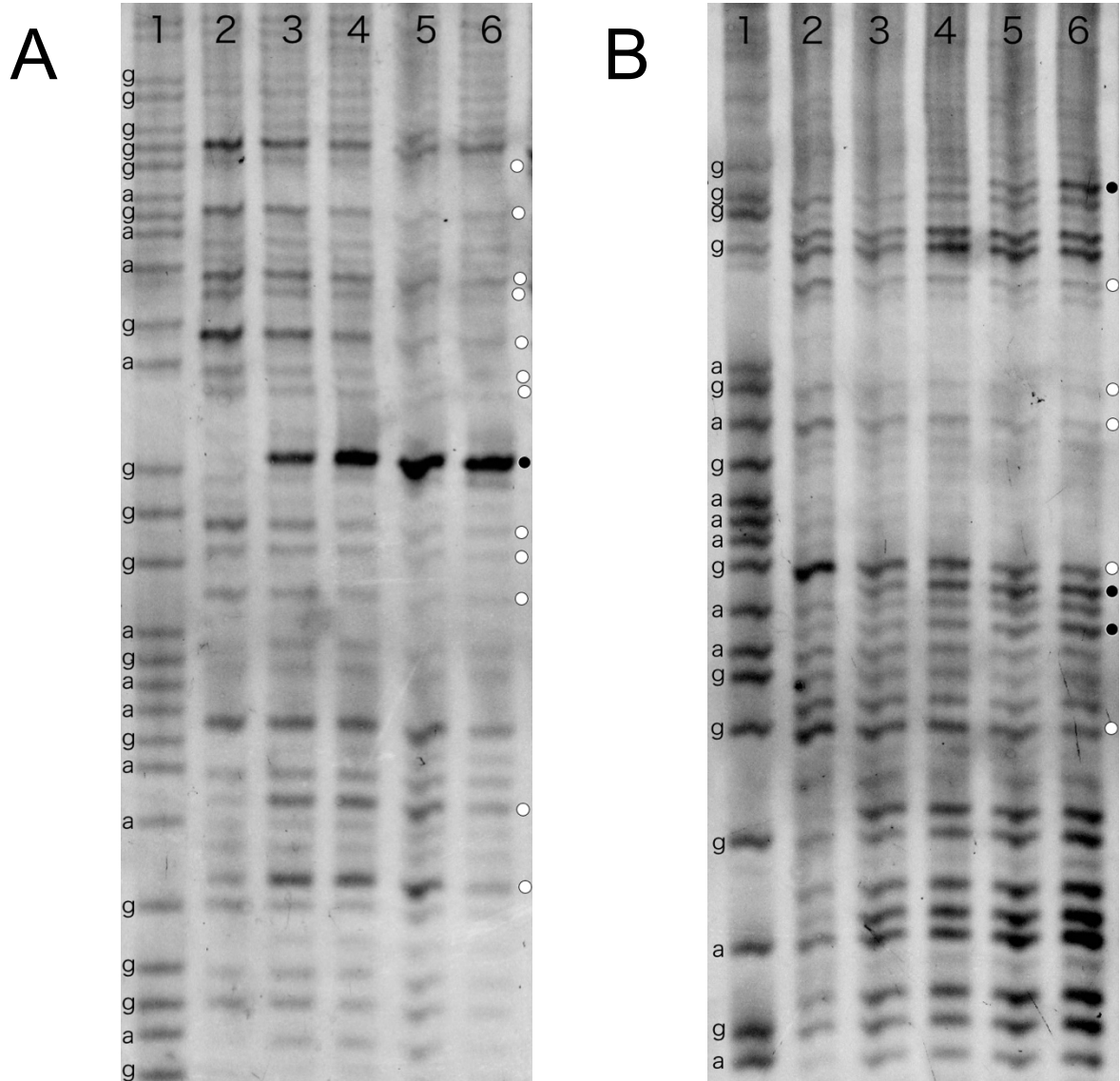


FIGURE 5 – PREDICTION OF THE C-TERMINAL STRUCTURE OF NtrX OF *H. seropedicae*

The NtrX structure (in yellow) was aligned with the structure of NtrC bound to DNA of *Aquifex aeolicus* (in blue) using the PyMol software. Each amino acid shown in red was changed to alanine yielding mutant proteins.



C

gccaacagaagatcgtgcgtttcatgtcacagacg

D

```
>narX  gccacagaagatcgtgcgtttcatgtcacagacg
>narK  gacaatggcgtccgcttgaaccgagggcacggcgc
>nasF  accaacggcggtcgcgccgtccaaggacaaagggcg
          **   *   *   **               *   *   **   *
          ca   g   g   cg               c   g   ca   g
```

FIGURE 6 – IDENTIFICATION OF DNA-BINDING SITES OF 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 hyperreactive bands (black circles). Crescent concentrations of ^{His}NtrX CTD protein from zero, 20, 50, 100 to 200 μ M (lanes 2 to 6, respectively) were incubated with DNA containing the *narX* promoter, followed by digestion with DNase I. (C) The putative DNA-binding site of NtrX in *narX* promoter was reduced to 34 bp. The protected bands (white circles) and hyperreactive bands (black circles) pointed in the figures A and B are shown above and below the bases, respectively. (D) Comparison between the DNA-binding site of NtrX in *narX* promoter and similar sequences present in *narK* and *nasF* promoter regions.

TABLES

TABLE 1 – PLASMIDS USED IN THIS WORK

Plasmids	Characteristics	Reference
p05061H07	Contains fragment of <i>ntrY</i> gene inserted into pUC19 (Amp ^R)	(Pedrosa et al. 2011) (GENOPAR)
pH07	Contains kanamycin resistance cassette inserted into BglII site in the central region of <i>ntrY</i> gene from p05061H07 (Amp ^R , Km ^R)	This work
pLAFR3.18	pLAFR vector containing the policloning site of pTZ18R (Tc ^R , Cm ^R)	(Souza et al. 2000)
pAA27D07	Cosmid containing around 26 Kb of <i>H. seropedicae</i> genomic region, including the <i>ntrYX</i> genes, inserted into pLAFR3.18 plasmid	(Pedrosa et al. 2011) (GENOPAR)
pPW452	Contains the promoter-less <i>lacZ</i> gene (Tc ^R)	(Woodley et al. 1996)
pPW-<i>narX</i>	Contains the promoter region of <i>narXL</i> of <i>H. seropedicae</i> inserted into pPW452 plasmid (Tc ^R)	This work
pPW-<i>narKnirB</i>	Contains the promoter region of <i>narKnirB</i> of <i>H. seropedicae</i> inserted into pPW452 plasmid (Tc ^R)	This work
pET28-<i>ntrX</i>	NdeI-BamHI fragment containing the <i>ntrX</i> gene of <i>H. seropedicae</i> inserted into pET28a. Overexpresses the wt ^{His} NtrX protein (Km ^R)	This work
pET28-<i>ntrX</i> 141-249	Contains the <i>ntrX</i> gene with NtrX ₁₃₉₋₁₄₀ codons changed to generate NdeI cleavage site (catatg). NdeI-BamHI fragment was inserted into pET28a. Overexpresses the ^{His} NtrX CTD protein (Km ^R)	This work
pET28-<i>ntrX</i> Gly141TGA	Contains the <i>ntrX</i> gene with the NtrX ₁₄₁ codon changed from “ggc” (coding for glycine) to “tga” (stop codon) by mutagenic PCR using the pET28- <i>ntrX</i>	This work

	plasmid and oligonucleotides described in Table 2. Overexpresses the ^{His} NtrX NTD protein (Km ^R)	
pCR2.1-<i>narX</i>	Contains the promoter region of <i>narXL</i> of <i>H. seropedicae</i> inserted into pCR2.1-TOPO plasmid (Amp ^R , Km ^R)	This work
pCR2.1-<i>narK</i>	Contains the promoter region of <i>narKnirB</i> of <i>H. seropedicae</i> inserted into pCR2.1-TOPO plasmid (Amp ^R , Km ^R)	This work

TABLE 2 – OLIGONUCLEOTIDES USED IN THIS WORK

Oligonucleotides	Sequence	Reference
<i>ntrX</i> (pET28)	Forward 5' AAGCAGCATATGGCTAAC 3'	This work
	Reverse 5' ACCGTGAGGATCCCGTTTG 3'	
<i>ntrX</i> 141-249	Forward 5' GTGGCCGCCGTGCATATGGGCGCCGCCGAC 3'	This work
<i>ntrX</i> Gly141TGA	Forward 5' CGCCGTGGTCAACTGAGCCGCCGACGGT 3'	This work
	Reverse 5' ACCGTCGGCGGCTCAGTTGACCACGGCG 3'	This work
narX-binding	Forward 5' TCGAACTGAAGCCGGAACGC 3'	This work
	Reverse 5' ATTTCCAGCGCCAGTCCCAG 3'	
narK-binding	Forward 5' AATGGGCATCATCGCCTTCCA 3'	This work
	Reverse 5' TGCCTCGGTTCAAGCGGACG 3'	
narX-footprint Cy3for	Forward 5' [Cy3]TCGAACTGAAGCCGGAACGC 3'	This work
	Reverse 5' CATCGTTGCCAGCATGTATGGAA 3'	
narX-footprint Cy3rev	Forward 5' TCGAACTGAAGCCGGAACGC 3'	This work
	Reverse 5' [Cy3]GCAGAAAGCCTGGGAAACTGC 3'	

TABLE 3 – PROMOTER ACTIVITY OF *narX-lacZ* OR *narK-lacZ* FUSIONS IN *H. seropedicae* WILD TYPE STRAIN AND IN A *ntrY* MUTANT

	Nitrogen source	SmR1	<i>ntrY</i>
empty vector	NO ₃ ⁻	9.8 ± 1.2	15.8 ± 7.9
<i>narX-lacZ</i>	NO ₃ ⁻	136.8 ± 43.2	27.5 ± 2.2
<i>narK-lacZ</i>	NO ₃ ⁻	514.9 ± 31.2	231.6 ± 7.4
empty vector	NH ₄ ⁺	14.3 ± 1.2	33.2 ± 1.4
<i>narX-lacZ</i>	NH ₄ ⁺	168.8 ± 7.8	76.6 ± 20.4
<i>narK-lacZ</i>	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. Cells were cultivated with NH₄Cl or KNO₃, aerobically for six hours. β-galactosidase are indicated as specific activity (nmol of o-nitrophenol produced per minute per mg of protein). Data are indicated as mean ± standard deviation of biological triplicates.

5 MANUSCRITO 3

Response of *Herbaspirillum seropedicae* SmR1 to ammonium limitation and to nitrate, and involvement of the NtrB/NtrC system

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WASSEM, Roseli; SOUZA, Emanuel Maltempo; PEDROSA, Fábio Oliveira;
CHUBATSU, Leda Satie

ABSTRACT

Herbaspirillum seropedicae SmR1 is a diazotrophic and endophytic β -proteobacterium found in association with crops of economic importance. The two component system NtrB and NtrC proteins of this bacterium regulate the expression of genes in response to nitrogen levels. An *ntrC* mutant of *H. seropedicae* cannot grow on nitrate, suggesting that the NtrB/NtrC system is important for nitrate assimilation in aerobiosis. In order to identify the main metabolic routes activated in response to different nitrogen sources, (ammonium-limiting, ammonium-shock or nitrate-shock conditions) and determine the NtrC regulon in response to low nitrogen and to nitrate, RNA-seq profiles of *H. seropedicae* wild type or *ntrC* mutant grown on ammonium-limiting treatment and submitted to nitrate-shock or ammonium-shock for 30 minutes, were conducted. We have found that in response to ammonium-limiting treatment, *H. seropedicae* modulates its metabolism in order to scavenge ammonium by activating alternative metabolic pathways to use different sources of nitrogen and by increasing proteolysis. Additionally, it modulates its carbon metabolism, storing carbon as glycogen, and slowing down its metabolism. The NtrB/NtrC system was found to regulate most of these pathways, indicating that it is the master regulator of *H. seropedicae* in response to fluctuations in the levels of assimilated nitrogen. The main operon induced in response to nitrate-shock was *narKnirBDHSERO_RS14545nasA*, which contains genes encoding for the assimilatory nitrate reductase (NAS). This enzyme is essential to nitrate assimilation in *H. seropedicae*, since a mutant for the catalytic domain of NAS (*nasA* mutant) failed to grow on nitrate, similarly to the *ntrC* mutant. The RNA-seq profiles showed

that NtrC is required to activate this operon, reinforcing the importance of NtrB/NtrC system in nitrate assimilation by *H. seropedicae*.

INTRODUCTION

Herbaspirillum seropedicae SmR1 is an endophytic β -proteobacterium found in association with roots and aerial parts of crops of economic importance, such as wheat and sugar cane (Baldani, Baldani et al. 1986). As *H. seropedicae* can fix atmospheric nitrogen into ammonium, the regulation of nitrogen-fixation genes in this bacterium is well understood and is under control of the Ntr system (Chubatsu, Monteiro et al. 2012). The main components of this system in *H. seropedicae* are the two component system NtrB/NtrC, the PII family, GlnB and GlnK, and the PII modifier GlnD protein, among others. Under high levels of intracellular ammonium, GlnB interacts with NtrB, preventing it to phosphorylate NtrC. If NtrC is not phosphorylated, it cannot activate the transcription of genes. At low levels of nitrogen, GlnD uridylylates GlnB. In this condition, NtrB is free to phosphorylate NtrC, which in turn, activates the transcription of genes required to adapt the metabolism to low levels of nitrogen (Chubatsu, Monteiro et al. 2012).

However, considering that nitrate is the main inorganic source of nitrogen found in soil, attention has been drawn to the nitrate metabolism of *H. seropedicae* (Bonato, unpublished). *H. seropedicae* has regulatory and structural genes related to two nitrate reductases: the respiratory nitrate reductase (NAR) and the assimilatory nitrate reductase (NAS) (Pedrosa, Monteiro et al. 2011). The NAR system of *H. seropedicae* seems to be involved with high production of nitrite under low levels of oxygen, and also in the production of nitric oxide. Additionally, the expression of NAR-encoding genes is highly induced in response to nitrate and to oxygen-limiting levels, via Fnr proteins. On the other hand, NAS has not been characterized yet, but its expression is induced in the presence of nitrate (Bonato, unpublished).

An *ntrC* mutant of *H. seropedicae* failed to grow on nitrate as the only nitrogen source, suggesting that the NtrB/NtrC system induces genes required for nitrate assimilation (Persuhn, Souza et al. 2000). Interestingly, *ntrC* mutants of other bacteria, such as *Azospirillum brasilense* (Machado, Yates et al. 1995), *Azotobacter vinelandii* (Toukdarian and Kennedy 1986), *Bradyrhizobium japonicum* (Martin, Chapman et al. 1988), *Rhizobium meliloti* (Szeto, Nixon et al. 1987), *Agrobacterium tumefaciens* (Rossbach, Schell et al. 1987), and *Pseudomonas aeruginosa* PAO1 (Li and Lu 2007) also cannot grow on nitrate. The regulon of NtrC in low nitrogen was defined by using microarray in *Escherichia coli* (Zimmer, Soupene et al. 2000),

Sinorhizobium meliloti (Davalos, Fourment et al. 2004), *Pseudomonas putida* (Hervás, Canosa et al. 2008) and *Bradyrhizobium japonicum* (Franck, Qiu et al. 2015). However, the regulon of NtrB/NtrC in response to nitrate was never reported.

The first aim of this study was to pinpoint the main routes of *H. seropedicae* activated in response to low levels of nitrogen and after addition of ammonium, or nitrate. We also aimed to define the regulon of the NtrB/NtrC system of *H. seropedicae* in response to low nitrogen and to nitrate. To achieve that, we determined a whole transcriptional profile of *H. seropedicae* SmR1 wild type and *ntrC* mutant grown in low levels of nitrogen (ammonium-limiting treatment), and after 30 minutes of addition of ammonium (ammonium-shock) or nitrate (nitrate-shock). Our results indicate that the NtrB/NtrC system is the main regulator of *H. seropedicae* in low levels of nitrogen, activating routes that scavenge ammonium, and repressing routes involved with the energy metabolism. *H. seropedicae* quickly adapted to ammonium, but the adaptation to nitrate was slower, supporting that ammonium is the preferred nitrogen source. NAS is the main enzyme required to assimilate nitrate in *H. seropedicae*, since a knockout mutant in the subunit containing the catalytic site of NAS failed to grow on nitrate, as did the *ntrC* mutant. Finally, NtrC protein activates the operon encoding NAS (*narKnirBDHSERO_RS14545nasA*), highlighting the importance of NtrB/NtrC also in the nitrate assimilation by *H. seropedicae*.

MATERIAL AND METHODS

Whole transcriptional analysis by RNA-seq

All strains and plasmids used in this study are listed in Tables S1 and S2, respectively. *H. seropedicae* SmR1 wild type (wt) or *ntrC* mutant strains were cultivated in NFbHP-malate (Klassen, Pedrosa et al. 1997) supplemented with 2 mM NH_4Cl medium at 30 °C and 120 rpm. Cells were collected at $\text{OD}_{600 \text{ nm}}$ around 0.9 and re-inoculated in NFbHP-malate supplemented with 1 mM NH_4Cl , and cultivated aerobically at 30 °C and 250 rpm (50 mL of medium in erlenmeyers of 250 mL). When cells reached OD_{600} 0.4 (after four hours of growth), they were divided into three parts: (i) one stored in RNA-later solution (ammonium-limiting treatment) and the other two were incubated for further 30 minutes after addition of (ii) 10 mM NH_4Cl (ammonium-shock treatment) or (iii) 10 mM KNO_3 (nitrate-shock treatment). After 30 minutes, cells were collected and stored in RNA-later. The experiments were conducted in biological replicates.

The RNA extraction from cells stored in RNA-later was performed using the Trizol method. The purified RNA was precipitated with 10 M LiCl, and contaminant DNA was removed using TURBO™ DNase Treatment (Ambion). Ribosomal RNA (rRNA) was removed using the Ribo-Zero rRNA Removal (Gram-negative bacteria) kit (Epibio). The cDNA libraries were constructed using Ion Total RNA-Seq Kit v2 kit (Applied Biosystems) and sequenced in an Ion Proton™ Sequencer.

Semi-quantitative PCR

In order to perform semi-quantitative PCR, 1.3 ug total RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The PCR amplification (10 μL) was performed with 1 μL of cDNA 1:10 as template, and GO Taq Flexi DNA polymerase kit (Promega) according to manufacturer's instructions. Oligonucleotides are listed in Table S3. The PCR reactions were submitted to (i) denaturation at 95 °C for 2 minutes, and (ii) 25 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 15 seconds. The PCR products were analyzed in 3% agarose gels.

Bioinformatics analysis

Reads obtained from the sequencing were trimmed using CLC Genomics Workbench (v. 7.5.2) to remove reads smaller than 20 nucleotides and with Phred quality lower than 20. Trimmed reads were mapped against the *H. seropedicae* SmR1 genome (NC_014323.1) using CLC Genomics Workbench (v. 7.5.2), with 0.9 and 0.9 minimum length fraction and minimum similarity fraction, respectively. Differentially expressed genes were analyzed using DESeq 2.0 and edgeR (Robinson, McCarthy et al. 2010, Love, Huber et al. 2014). It was considered as differentially expressed the genes with log2FoldChange over 1.0 or below -1.0 and *p*-value FDR-corrected < 0.05 in both DESeq 2.0 and edgeR. The heatmaps were constructed using pheatmap (Kolde, R).

The Gene set analysis was performed using mgsa (Bauer, Gagneur et al. 2010), which is based in the hypergeometric test, with inPopulation genes > 10 genes and estimate > 0.5. The Gene Ontology (GO) terms of *H. seropedicae* were recovered using Blast2GO software (Conesa, Götz et al. 2005), whereas the KEGG terms were recovered using the function “kegg.gsets” from gage (Luo, Friedman et al. 2009). DESeq 2.0, edgeR, mgsa, and gage are R packages from Bioconductor project. Pheatmap is R package from CRAN (Comprehensive R Archive Network).

Promoter sequences of genes activated by NtrC protein were scanned for the NtrC binding-site predicted for *Salmonella thyphimurium* (TGACCCNNNNTGGTGCA) (Ferro-Luzzi Ames and Nikaido 1985). Only promoter sequences with sigma N consensus were considered in the analysis, since NtrC activates the transcription of target genes with RNA polymerase associated with this sigma factor (YTGGCACGNNNNTTGCW) (Barrios, Valderrama et al. 1999). The sequence logo for the NtrC consensus of *H. seropedicae* was obtained by using the Meme Suite 4.11.0 (Bailey and Elkan 1994).

Construction of *H. seropedicae* *nasA* mutant

The *nasA* mutant was obtained by homologous recombination, deleting 2715 bp (out of 2769 bp) of the *nasA* coding region. DNA fragments located upstream and downstream the *nasA* gene were amplified by PCR, and ligated into the pSUP202

suicide vector, generating the pSUP-*nasA* plasmid. A DNA fragment containing the gene encoding for saccharase (*sacB*Km) was ligated into pSUP-*nasA*, generating the pSUP-*nasA-sacB*Km plasmid. Then, this plasmid was transferred into *H. seropedicae* wild type by conjugation. Kanamycin resistant colonies (resulting from the first recombination event) were plated on 5% sucrose plates for selection of colonies resulting from the second recombination event. The final mutant strain was screened by PCR for confirmation of the deletion.

RESULTS

RNA-seq experimental strategy

The *ntrC* mutant of *H. seropedicae* does not grow on nitrate as the only nitrogen source (Persuhn, Souza et al. 2000), suggesting that the NtrB/NtrC system is a key regulator of the expression of genes required to nitrate assimilation. Additionally, this system is an important regulator in response to low levels of nitrogen in Enterobacteria and in *H. seropedicae* (Merrick and Edwards 1995, Chubatsu, Monteiro et al. 2012). Given that, we aimed to identify the genes regulated by the NtrB/NtrC system in *H. seropedicae*, in response to low nitrogen levels and to nitrate. We analyzed the growth profile of the wild type strain growth on media containing 1 mM NH₄Cl, a nitrogen-limiting concentration (Figure 1). Cell growth halted at OD₆₀₀ 0.4, indicating that in this OD, the nitrogen levels were too low to sustain growth; the number of cells was sufficient for RNA extraction (around 1x10⁸ cells/mL) (Figure 1A). The establishment of these treatments was necessary since the *ntrC* mutant does not grow on nitrate (Persuhn, Souza et al. 2000), and similar growth conditions should be applied for both wild type and *ntrC* mutant.

To evaluate the time necessary to achieve the transcriptional response to ammonium- or nitrate-shock, similar experiments as describe above were repeated and when cells reached OD 0.4, they were divided into three parts. The first part was stored in RNA-later. This treatment was denominated ammonium-limiting treatment. The other two parts were transferred into new ernlenmeyers: in one of them it was added 10 mM NH₄Cl (ammonium-shock treatment) and in the other, 10 mM KNO₃ (nitrate-shock treatment). After 30 and 60 minutes of nitrate-shock or after 30 minutes of ammonium-shock, cells were collected prior to RNA extraction and semi-

quantitative PCR. Results revealed that 30 minutes after nitrate-shock was sufficient to activate genes related to nitrate assimilation (*nasA*, *nirD* and *nasF* genes) (Figure 1C). From the experiments described above, the RNA-seq experimental design used in this study was defined: *H. seropedicae* wild type or *ntrC* mutant were grown on 1 mM NH₄Cl until cells reached OD₆₀₀ 0.4 (ammonium-limiting treatment). At this point, cells were submitted to 30 minutes of ammonium- or nitrate-shock (Figure S1A and S1B).

The first analyses with the RNA-seq profile aimed to identify genes required to the metabolism of *H. seropedicae* in ammonium-limiting, ammonium-shock, or nitrate-shock treatment. To achieve that, the transcriptional profiles of *H. seropedicae* wild type grown in the three different nitrogen treatments were compared with each other (ammonium-limiting x ammonium-shock; ammonium-limiting x nitrate-shock; ammonium-shock x nitrate-shock). Then, to identify the regulon of NtrC in the three different nitrogen treatments, the transcriptional profile of wild type was compared to the *ntrC* mutant, in each nitrogen treatment. Gene set analysis with KEGG and GO terms were conducted in all comparisons in order to identify groups of genes that were over-represented in each treatment.

Differential expression of *H. seropedicae* genes under Ammonium-limiting and Ammonium-shock treatments

The RNA-seq profile of *H. seropedicae* wild type strain grown in ammonium-limiting treatment was compared with the profile of that strain submitted to an ammonium-shock (wt -N x +NH₄). Downregulated genes were considered as induced in the ammonium-limiting treatment, whereas the upregulated genes were considered as induced in the ammonium-shock treatment. It was found that 335 genes were induced in the ammonium-limiting treatment and 405 genes induced in the ammonium-shock treatment.

Gene set analysis with KEGG terms revealed that the “nitrogen metabolism” term was over-represented under ammonium-limiting treatment (Table S4). Genes encoding proteins of the Ntr system were induced in this condition: *glnAntrBC* and *nImAglnKamtB* genes. Previous work by Schwab et al (2007), Persuhn et al (2000) and Noindorf et al (2011) have shown that the *H. seropedicae* *glnAntrBC* and *nImAglnKamtB* operons are induced under nitrogen limiting treatments and their

expression is dependent on NtrC. The *gltBD* genes coding for glutamate synthase (GOGAT) are not regulated by nitrogen levels. The *nImAglnKamtB* operon codes for NImA, a periplasmic protein with unknown function, for GlnK, a PII-like protein and an ammonium permease AmtB. GlnK was shown to be required for an active NifA for nitrogen fixation (Noindorf et al 2011). The *glnB* gene codes for the PII protein and it was previously shown to be constitutively expressed (Benelli et al 1997), a result confirmed by the present transcriptome analysis. Interestingly, the expression of the *rpoN* gene, encoding the alternative sigma N, was induced under low nitrogen. This sigma factor drives the RNA polymerase to the promoter regions of genes whose products are required in low nitrogen metabolism.

Another gene induced by ammonium-limitation was *nifA*. This gene codes for the transcriptional activator NifA necessary for expression of genes involved with nitrogen fixation. Wassem et al (2002) showed that *nifA* expression was dependent on NtrC under low ammonium levels.

The expression of genes encoding transporter systems for nitrogen compounds was also induced under ammonium-limiting treatment, including those transporters for urea (*urtABCDE*), nitrate (*nasFED* and *nark*), and amino acids (*livKMG*). Genes encoding enzymes that catabolyze alternative sources of nitrogen were also induced, including nitrite (*nirB*) and urea (*HSERO_RS22340* and *HSERO_RS07105*).

The KEGG term “nicotinate and nicotinamide metabolism” was also over-represented under ammonium-limiting treatment. Interestingly all the genes encoding for enzymes that convert nicotinate to malate, a process that generates ammonium ions, were induced in this condition. This result suggests that nicotinate can also be metabolized in order to generate ammonium under ammonium-limiting treatment. The use of nicotinate as nitrogen source was previously reported in *Bacillus niancini* (Hirschberg and Ensign 1972, Kaiser, Feng et al. 1996). Interestingly other genes encoding enzymes that metabolize other nitrogenous compounds, such as arginine and urea were also induced (Figure 2, Figure S3).

The third and last KEGG terms over-represented under ammonium-limiting treatment was the “starch and sucrose metabolism”. Among these genes are those encoding enzymes that synthesize trehalose (*ostAB*), and encoding the glycogen synthase (*glgAB*). *H. seropedicae* can also store carbon in the form of polyhydroxyalcanoate (PHA). However, from all genes related to PHA found in *H.*

seropedicae genome, only the *phaZ* gene (*HSERO_RS03195*), encoding for PHA depolymerase, was induced under ammonium-limiting treatment. These results suggest that under ammonium limitation, *H. seropedicae* stores carbon as glycogen and as trehalose, but not as PHA.

One gene encoding a peptidase (*HSERO_RS21705*) and two genes encoding putative proteases (*HSERO_RS03170* and *HSERO_RS00825*) were also induced in the ammonium-limiting treatment. The protein encoded by *HSERO_RS03170* presents 45% similarity with a protease of *Pseudomonas putida* also found to be induced in low levels of ammonium (Hervás, Canosa et al. 2008).

Many KEGG and GO terms were over-represented in the ammonium-shock treatment. From GO analyses, the terms “generation of precursor metabolites and energy” (GO:0006091), “translation factor activity, nucleic acid binding” (GO:0008135), “unfolded protein binding” (GO:0051082), and “membrane organization” (GO:0061024) were induced. From KEGG terms analysis, the over-represented pathways were: “citrate cycle (TCA cycle)”, “oxidative phosphorylation”, “2-Oxocarboxylic acid metabolism”, “ribosome”, “RNA degradation” and “Protein export”. Most of these categories are related to the energy metabolism, suggesting that 30 minutes after ammonium addition was sufficient to over-activate the pathways involved in the energy metabolism of *H. seropedicae*.

Differential expression of *H. seropedicae* genes under Nitrate-shock and Ammonium-limiting treatments

The transcriptional profile of *H. seropedicae* wild type grown on ammonium-limiting treatment was compared to Nitrate-shock treatment (wt -N x +NO₃). Genes found to be downregulated in the presence of nitrate were considered as induced in the ammonium-limiting treatment, whereas genes found to be upregulated were considered as induced in the nitrate-shock treatment. Only 66 genes were induced in the ammonium-limiting treatment and 58 were induced in the nitrate-shock treatment. This shows that both treatments are very similar, as observed in the principal component analysis (PCA) (Figure S2).

Interestingly, the “nitrogen metabolism” KEGG term was over-represented in the nitrate-shock treatment. The genes of this category that were upregulated are related to the nitrate metabolism, and are contained in two operons,

narKnirBDHSERO_RS14545nasA, and *nasFED*. Both operons were the most induced in response to nitrate. The *nasFED* and *narK* encode to nitrate transporters, whereas *HSERO_RS14545nasA* and *nirBD* encode for the assimilatory nitrate reductase and nitrite reductase, respectively. These results are in agreement with previously observed (Figure 1C), and suggest that these genes are required for nitrate assimilation in *H. seropedicae*.

Two categories were over-represented in the ammonium-limiting treatment: the GO term “structural molecule activity” (GO:0005198), and the KEGG term “Ribosome”. Other genes related with DNA, RNA and protein metabolism were also over-represented in this treatment. Together these results suggest that after 30 minutes of nitrate-shock, the cellular machinery slowed down the protein synthesis to direct its metabolism towards the assimilation of nitrate.

Differential expression of *H. seropedicae* genes under Nitrate-shock and Ammonium-shock treatments

A last comparison was conducted between the RNA-profile obtained from *H. seropedicae* wild type grown on different nitrogen sources. The level of RNA expression of cells submitted to ammonium-shock was compared to that of nitrate-shock (wt +NH₄ x +NO₃). Upregulated genes were considered as those induced following a nitrate-shock, whereas the downregulated genes were considered as those induced following an ammonium-shock treatment. 425 genes were induced following the nitrate-shock, and 544 genes were induced after the ammonium-shock.

Most of the induced genes in the nitrate-shock treatment compared to the ammonium-shock were also found to be induced in the ammonium-limiting treatment compared to ammonium shock treatment, including genes encoding transporter systems for urea, nitrate, amino acids, and ammonia, assimilatory nitrate reductase and nitrite reductase, urea carboxylase, proteins of the Ntr system, proteases/peptidase, and proteins involved in glycogen synthesis. Given that, we focused in the analysis of genes induced by the nitrate-shock treatment, but not induced in the ammonium-limiting treatment (compared to the ammonium-shock treatment). Gene set analysis with this new group of genes revealed that the “flagellar assembly” KEGG term was over-represented following the nitrate-shock.

All categories over-represented in the ammonium-shock treatment were related to the energy metabolism, such as: “translation factor activity, nucleic acid binding” (GO:0008135), “citrate cycle (TCA cycle)”, “oxidative phosphorylation”, “biosynthesis of amino acids” and “ribosome”. Probably this is related to the different growth rates observed between cells grown on ammonium and on nitrate. As expected, cell growth recovered faster in ammonium when compared to nitrate, as observed in the Figure 1B.

The regulon of NtrC in response to Ammonium-limiting treatment

Data from RNA-seq analysis of the *H. seropedicae* SmR1 wild type in ammonium-limiting treatment was compared to the *ntrC* mutant grown under the same nitrogen treatment (wt -N x *ntrC* -N). The genes that were downregulated or upregulated were considered as activated or repressed by NtrC protein, respectively. The results showed that 261 genes were activated and 273 genes were repressed in the *ntrC* mutant regarding the wild type strain.

As expected, NtrC activated genes of the “nitrogen metabolism” KEGG term (Table S4). Interestingly, NtrC seems to activate direct or indirectly most of the pathways induced in response to low nitrogen treatment (in comparison with ammonium-shock treatment) (Figure 2, Figure S3), including transporters of nitrogenous compounds such as nitrate, amino acids, urea, and ammonium, and enzymes that metabolize nitrate, arginine, and nicotinate. Additionally, genes encoding protease/peptidases (*HSERO_RS03170*, *HSERO_RS00825*, *HSERO_RS21705*), the NifA protein and nitrogenase were also activated by NtrC. Together these results suggest that NtrB/NtrC system is the main activator of pathways that scavenge alternative nitrogenous compounds in response to ammonium limitation in *H. seropedicae*, replenishing the cell for these ions.

Regarding the carbon metabolism, the NtrB/NtrC system also activates genes encoding enzymes that synthesize trehalose (*ostAB* genes). However, genes encoding to the glycogen pathway (*glgAB* genes), which were induced in low-nitrogen treatment were not regulated by NtrB/NtrC system.

Four KEGG terms were over-represented: “citrate cycle (TCA cycle)”, “oxidative phosphorylation”, “ribosome” and “RNA degradation”. The only GO term

over-represented was “generation of precursor metabolites and energy” (GO:0006091). All these categories are involved with energy metabolism.

The regulon of NtrC in response to Ammonium-shock treatment

The RNA-seq profile of *H. seropedicae* wild type submitted to ammonium-shock treatment was compared to the profile of the *ntrC* mutant under the same nitrogen treatment (wt +NH₄ x *ntrC* +NH₄). The downregulated genes were considered as activated by NtrB/NtrC system, whereas the upregulated genes were considered as repressed by this system. Only 71 genes were activated by NtrC, and gene set analysis did not reveal GO terms or KEGG terms activated by NtrC. On the other hand, 116 genes were repressed by NtrC protein. Additionally, the three KEGG terms that were repressed by NtrC were related to energy metabolism (“oxidative phosphorylation”, “amino sugar and nucleotide sugar metabolism”, and “methane metabolism”).

The regulon of NtrC in response to Nitrate-shock treatment

A final comparison between the RNA-seq profiles was conducted between wild type and *ntrC* mutant, both submitted to nitrate-shock (wt +NO₃ x *ntrC* +NO₃). As in the previous sections, the downregulated genes were considered as activated by NtrC protein, while the upregulated genes were repressed by this protein. The results showed 405 genes activated, and 412 genes repressed by NtrB/NtrC system.

Gene set analysis reveals that the categories from KEGG terms “nicotinate and nicotinamide metabolism”, “nitrogen metabolism”, and the category from GO “nitrogen cycle metabolic process” (GO:0071941) were activated by NtrC protein. These categories comprise many of the genes previously discussed. The NtrB/NtrC system activated genes encoding transporter systems for amino acids, urea, ammonium and nitrate. Genes encoding the nitrogenase, one protease (*HSERO_RS03170*), and enzymes that metabolize nicotinate and nitrate were also activated by NtrC in response to nitrate. All these genes were also activated by NtrC in response to low nitrogen. In conclusion, the most important genes activated by NtrC in response to low nitrogen, were the same that are activated by NtrC in response to nitrate. The PCA confirms that the RNA-profile of wild type grown in low

nitrogen and in nitrate were more similar to each other as compared to the ammonium-shock treatment; as yet the RNA-profile of *ntrC* grown in both nitrogen treatments were also very similar (Figure S2).

As observed in the previous sections, all the KEGG terms and GO terms repressed by NtrC are related to energy metabolism. Five KEGG terms were repressed: “oxidative phosphorylation”, “lipopolysaccharide biosynthesis”, “ribosome”, “citrate cycle (TCA cycle)”, and “RNA degradation”. The two GO term repressed were “generation of precursor metabolites and energy” (GO:0006091) and “translation factor activity, nucleic acid binding” (GO:0008135).

Consensus sequence of NtrC of *H. seropedicae*

The promoter regions of operons activated by NtrB/NtrC system in response to nitrate-shock and ammonium-limiting treatments were scanned for the DNA-consensus sequence for the binding of NtrC proposed for *S. typhimurium*. Good matches were found for eleven promoter regions. Eight of these promoters are related to the metabolism of nitrogen: *narKnirBDHSERO_RS14545nasA*, *nasFED*, *urtABCDE*, *nImAglInKamtB*, *HSERO_RS22330nasR*, *HSERO_RS22365-22360-22355-22350-22345gatA*, *nifA*, and *glnAntrBC*. The other three operons are *HSERO_RS03190-03185*, encoding for glutathione S-transferase and a protein containing a cupin domain, *HSERO_RS06665-06670-06675-06680-06685* and *iorAHSERO_RS06615*. The sequences of these 11 promoters were aligned and a consensus sequence of NtrC of *H. seropedicae* was proposed (Figure 3).

The importance of NtrB/NtrC system to nitrate metabolism of *H. seropedicae*

As the *ntrC* mutant DCP286A does not grow on nitrate as the sole nitrogen source, one of the aims of this study was to investigate the role of NtrB/NtrC system in the nitrate metabolism of *H. seropedicae*. As previously discussed, the comparison between the RNA-seq profile of wild type grown in ammonium-limiting with wild type submitted to nitrate-shock showed that these treatments were similar. However, two operons were highly activated in response to nitrate, both of them encoding genes related to nitrate metabolism: *narKnirBDHSERO_RS14545nasA* and *nasFED*. The

promoter region of both promoters presents NtrC consensus sequences, suggesting that NtrC activates directly both promoter regions. Interestingly only genes related to NAS were highly induced in response to nitrate in the tested treatments of this paper, whereas genes related to the respiratory nitrate reductase (NAR) remained constitutive expressed (Figure 4).

The *HSERO_RS14545* and *nasA* genes encodes to a putative assimilatory nitrate reductase. In order to verify if this enzymatic complex is required for nitrate assimilation, we constructed a knockout strain with a deletion of the *nasA* gene. Interestingly, this mutant grows well on ammonium, but fails to grow on nitrate as the only nitrogen source (data not shown), supporting that NAS is the main enzyme required to assimilate nitrate in *H. seropedicae*.

DISCUSSION

One of the aims of this study was to identify the main pathways of *H. seropedicae* required to its metabolism under nitrogen-limiting treatment and after ammonium-shock or nitrate-shock. In Enterobacteria, the NtrB/NtrC system is the master regulator in response to low nitrogen (Merrick and Edwards 1995), and *ntrC* mutants of *H. seropedicae* and other bacteria are impaired in growing on nitrate (Toukdarian and Kennedy 1986, Rossbach, Schell et al. 1987, Szeto, Nixon et al. 1987, Martin, Chapman et al. 1988, Machado, Yates et al. 1995, Persuhn, Souza et al. 2000, Li and Lu 2007). Given that, in this study we also intended to define the regulon of the transcriptional regulator protein NtrC of *H. seropedicae* in response to the nitrogen source and level.

The main pathways activated under ammonium-limiting treatment when compared to ammonium-shock are summarized in Figure 2. Most of the activated pathways are involved with nitrogen metabolism, from transporter systems to enzymatic complexes that metabolize nitrogenous compounds to generate ammonium ions. Genes encoding the NtrBC two component system (*ntrBC* genes) and gene encoding the alternative sigma N (*rpoN* gene) were also induced. Interestingly, three genes encoding proteases/peptidase were activated in *H. seropedicae* in response to ammonium-limiting treatment, as previously reported in *Pseudomonas putida* transcriptome grown in low levels of nitrogen (Hervás, Canosa et al. 2008). Additionally, proteolysis is important to the survival of mycobacteria in nitrogen starvation conditions as this process recycles amino acids (Elharar, Roth et al. 2014), supporting proteolysis as an ammonium scavenger. Another process of *H. seropedicae* regulated in response to low nitrogen was the energy metabolism: genes encoding enzymes of the TCA cycle, oxidative phosphorylation, and protein synthesis were repressed in *H. seropedicae*. *Pseudomonas pseudoalcaligenes* and *Mycobacterium tuberculosis* also blocked protein synthesis in response to low nitrogen levels (Luque-Almagro, Escribano et al. 2015, Williams, Jenkins et al. 2015). These results suggest that repression of protein synthesis is an important process used by bacteria to deal with nitrogen-starving condition. Consistent with these data, the *glgAB* genes, coding to glycogen synthase, were induced, suggesting that *H. seropedicae* stores carbon as glycogen under these treatment. Synthesis of glycogen under ammonium limitation was also reported to *Pseudomonas putida* (Hervás,

Canosa et al. 2008) and is probably the mechanism by which the cell represses the catabolism of carbon and reduces the energy metabolism. Together, these data support the view that in response to low nitrogen, *H. seropedicae* activates the catabolism of alternative sources of nitrogen, and proteolysis to increase the level of intracellular level of ammonium. Additionally, *H. seropedicae* also regulates its carbon metabolism by storing carbon, as glycogen, and slowing down the energy metabolism probably to avoid the waste of energy when fixed nitrogen is limiting. The *ostAB* genes, encoding to enzymes that synthesize trehalose were also induced. The accumulation of trehalose upon nitrogen limitation was reported in *Corynebacterium glutamicum* and *Ectothiorhodospira halochloris*, and is important to osmoregulation (Galinski and Herzog 1990, Wolf, Krämer et al. 2003), and could have the same function in *H. seropedicae*, preparing the cells for a period of starvation.

The NtrC protein activates the expression of most of the important pathways involved with nitrogen metabolism, that were induced in the ammonium-limiting treatment of many of the genes required to *H. seropedicae* metabolism in low nitrogen, which are represented in Figure 2 probably by binding directly to their promoter regions and in association with RNA polymerase-sigma N. Among genes activated directly by NtrC are genes related to metabolism of nitrate and urea. Additionally, NtrC also activates indirectly most of the genes represented in Figure 2 (indicated by arrows), as no NtrC-binding sites were found in their promoter regions, including the three genes involved with proteolysis. Similarly to *H. seropedicae*, microarray transcriptome analyses with wild type and *ntrC* mutants of *E.coli*, *S. meliloti*, *P. putida*, and *B. japonicum*, also showed that the main NtrB/NtrC system response to ammonium limitation was to induce metabolic pathways in order to scavenge ammonium (Davalos, Fourment et al. 2004, Hervás, Canosa et al. 2008, Franck, Qiu et al. 2015). Regarding the carbon metabolism, NtrC protein of *H. seropedicae* did not regulate the genes involved in glycogen synthesis, as in *P. putida* (Hervás, Canosa et al. 2008). However, in the three nitrogen treatments tested in this study, NtrC repressed genes related to the energy metabolism. Similarly, the NtrC of *P. putida* also repressed carbon metabolism in response to low nitrogen (Hervás, Canosa et al. 2008).

Even in the absence of urea and nitrate, genes encoding to transporter systems and enzymes that metabolize these compounds were highly induced in the ammonium-limiting treatment in *H. seropedicae*. Genes involved in transport and

metabolism of urea were also upregulated in response to low nitrogen in other bacteria present in soil or found in association with plant roots, such as *P. putida*, *P. pseudoalcaligenes*, and *B. japonicum* (Hervás, Canosa et al. 2008, Franck, Qiu et al. 2015, Luque-Almagro, Escribano et al. 2015). Additionally, genes involved in the metabolism of nitrate were also induced in *P. pseudoalcaligenes* (Luque-Almagro, Escribano et al. 2015). Similarly, the obligate pathogen *M. tuberculosis* upregulates genes involved with the nitrate metabolism in ammonium limitation, even in the absence of nitrate. It was proposed that this pathogen lost many pathways involved with assimilation of nitrogen, but kept genes required to assimilate nitrate as this compound is commonly produced in its vertebrate host (Williams, Jenkins et al. 2015).

Most of the pathways activated by the ammonium-shock, when compared to nitrate-shock, were also related to the energy metabolism. This result indicates that 30 minutes of addition of ammonium was sufficient to *H. seropedicae* to adjust its metabolism to the assimilation of this compound. On the other hand, the adaptation of *H. seropedicae* after nitrate-shock was slower, suggesting that this bacterium prefers ammonium as the nitrogen source upon nitrate.

The nitrate-shock and ammonium-limiting treatments were very similar. However, two main operons were activated by the nitrate-shock, *narKnirBDHSERO_RS14545nasA* and *nasFED*, and both are related to nitrate metabolism. Interestingly, a knock-out mutant in the NAS catalytic subunit (*nasA* mutant) does not grow on nitrate as the nitrogen source. *H. seropedicae* also presents a second nitrate reductase, NAR, however its role in nitrate assimilation was discarded as a mutant in the catalytic site of NAR (*narG* mutant) grows well on nitrate as the only nitrogen source (Bonato, unpublished). Our data also show that genes related to NAR were constitutive in all tested treatments in this study, but genes related to NAS were highly induced in the presence of nitrate (Figure 4). Together, these data support that NAS is the main pathway used by *H. seropedicae* to assimilate nitrate. Additionally, the results suggest that the *ntrC* mutant cannot grow on nitrate because the NtrB/NtrC system is required to activate the operon encoding NAS (*narKnirBDHSERO_RS14545nasA*). This activation is direct since the promoter of *narK* presents an NtrC binding-site. The NtrB/NtrC system of *Klebsiella pneumoniae* also induces the expression of NAS in response to nitrogen limitation.

However, in this bacterium the *nasA* and *nasFED* genes are organized in a single operon (*nasFEDCBA*) (Goldman, Lin et al. 1994).

In conclusion, in response to ammonium-limiting treatment, *H. seropedicae* scavenges nitrogen and represses the carbon catabolism by modulating four important processes: (i) activates operons involved in the catabolism of alternative sources of nitrogen; (ii) increases the amino acid turnover through proteolysis; (iii) represses the energy metabolism and protein synthesis; and (iv) activates the carbon storage as glycogen. Additionally, the NtrB/NtrC system of *H. seropedicae* regulates the first three processes mentioned above, suggesting that this system is the master regulator of *H. seropedicae* in response to ammonium-limiting treatment. Interestingly, *H. seropedicae* quickly adapted to ammonium-shock, but the adaptation to nitrate-shock was slower, supporting that ammonium is the preferred nitrogen source. Additionally, NAS is the main pathway required to assimilate nitrate in *H. seropedicae* and its expression is activated directly by NtrC, highlighting the importance of the NtrB/NtrC system to nitrate metabolism.

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FIGURES

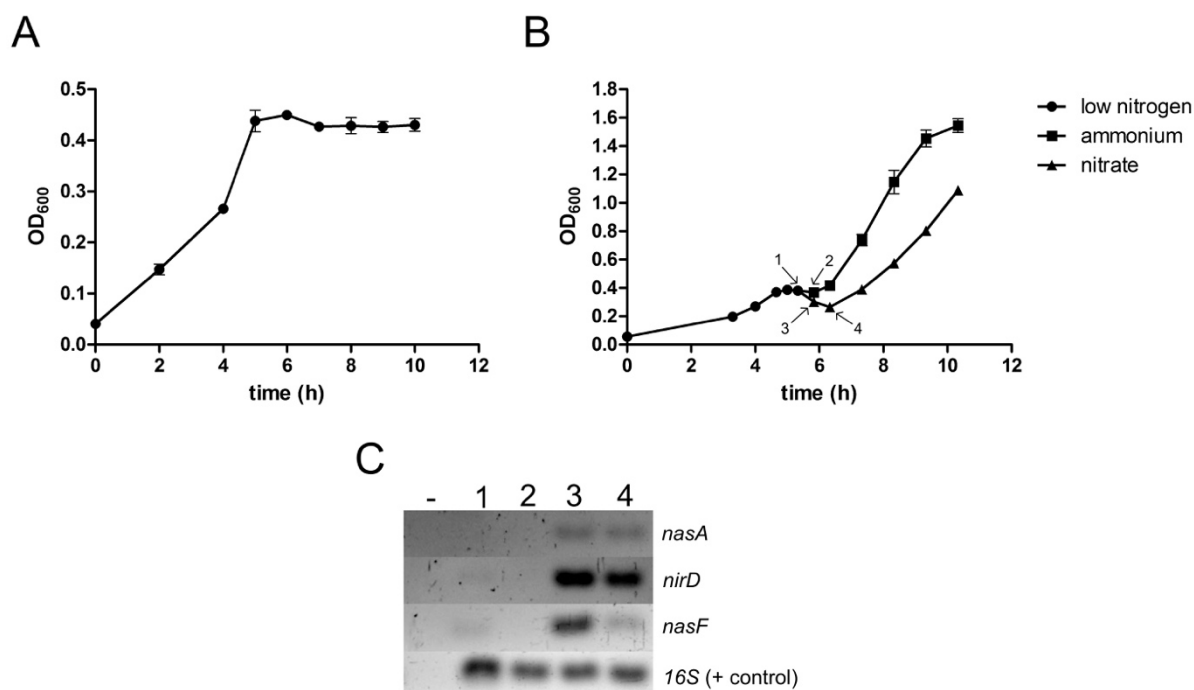


FIGURE 1 – EFFECT OF NITROGEN LIMITATION AND AMMONIUM OR NITRATE SHOCK ON *H. seropedicae* SmR1 WILD TYPE STRAIN

(Panel A) Growth curve of *H. seropedicae* grown in NFbHP-malate supplemented with 1 mM NH_4Cl . (Panel B) Cells were grown at the same treatment described in panel A; at OD₆₀₀ 0.4, culture was divided in three parts. The first one was stored in RNA-later (sample 1), and the second and third fractions were transferred each to two different flasks: in one of them it was added 10 mM NH_4Cl and in the other 10 mM KNO_3 . Cells cultured on NH_4Cl were collected after 30 minutes of growth (sample 2) whereas cells cultured on KNO_3 were collected after 30 (sample 3) and 60 minutes (sample 4) of growth. Cells were stored in RNA-later and RNA was extracted. (Panel C) Semi-quantitative PCR with cDNA produced from RNA extracted as shown in Panel B and oligonucleotides annealing in genes encoding to subunits of the assimilatory nitrate reductase (*nasA*), nitrite reductase (*nirD*) and to a nitrate transporter (*nasF*). The 16S rRNA was used as positive control (+ control), while “-” corresponds to the negative control (without addition of cDNA).

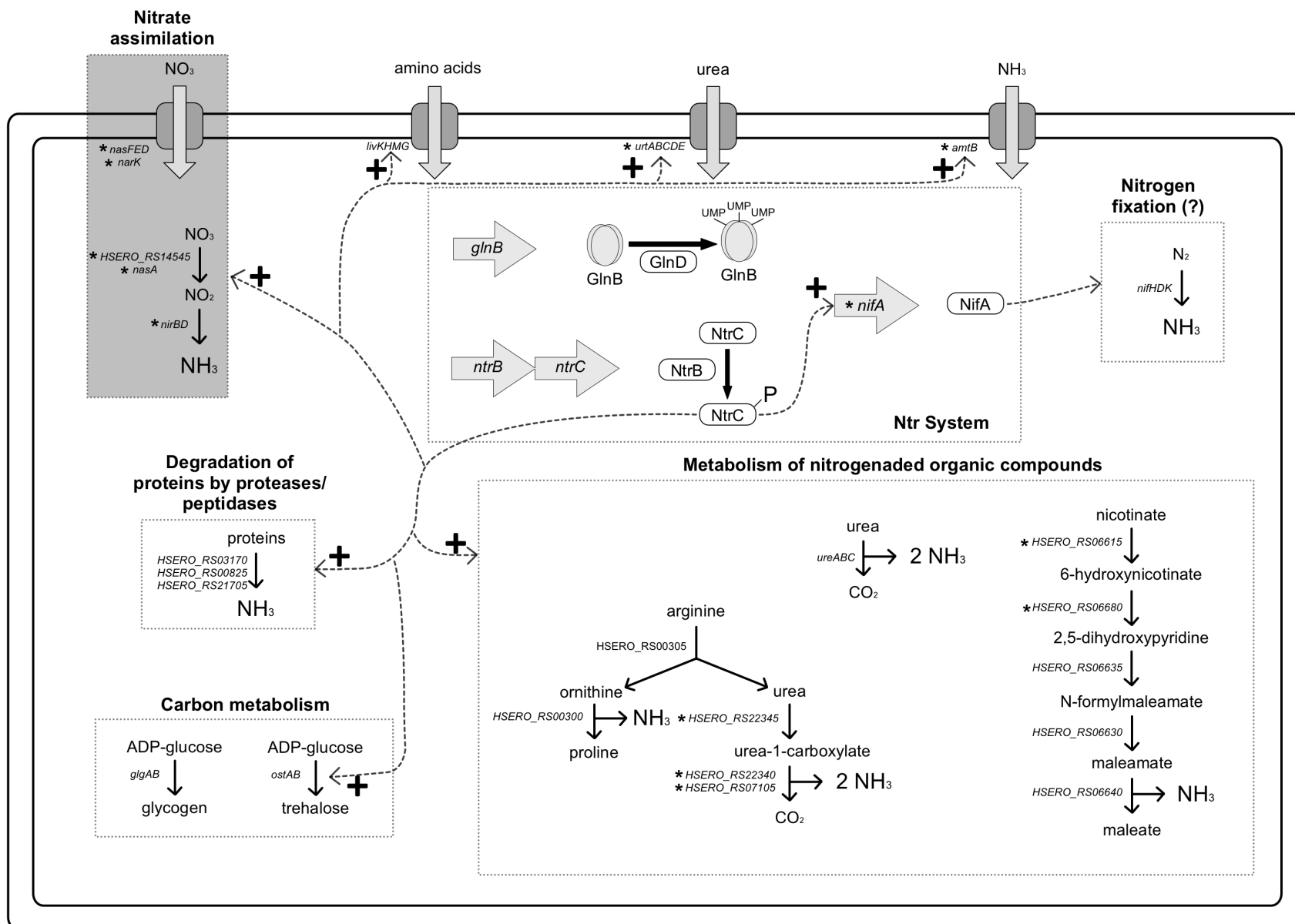


FIGURE 2 – METABOLIC ADAPTATIONS OF *H. seropedicae* TO NITROGEN-LIMITING TREATMENT AND TO NITRATE-SHOCK

All the pathways represented are induced in ammonium-limiting treatment. In grey, the pathway required for nitrate assimilation. Pathways that were activated by NtrC protein are represented with “+”. Genes within operons that presents consensus sequence for NtrC protein are represented with “*”. The expression of *glnB* and *glnD* were constitutive.

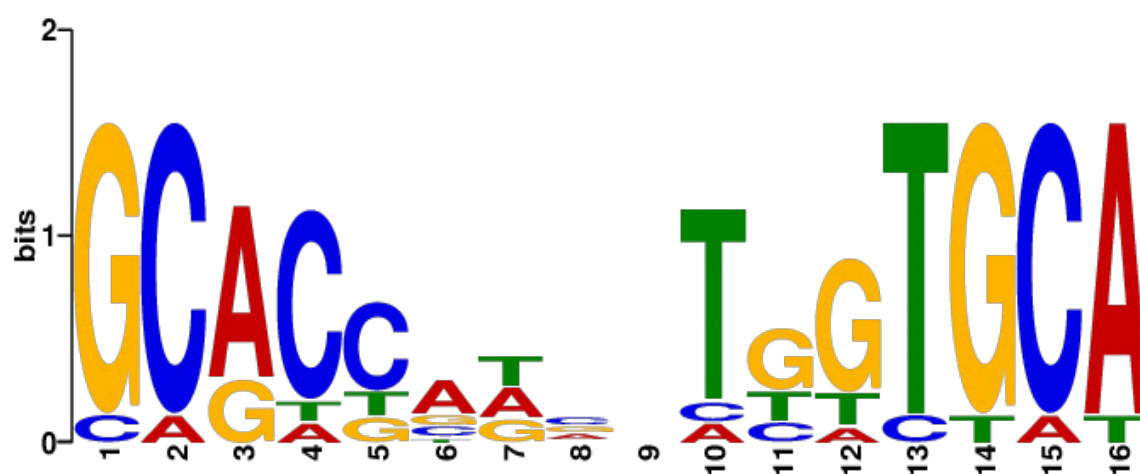


FIGURE 3 – PUTATIVE NtrC-BINDING SITE OF *H. seropedicae*

Several promoter regions of genes responding to NtrC were analyzed using the MEME Software

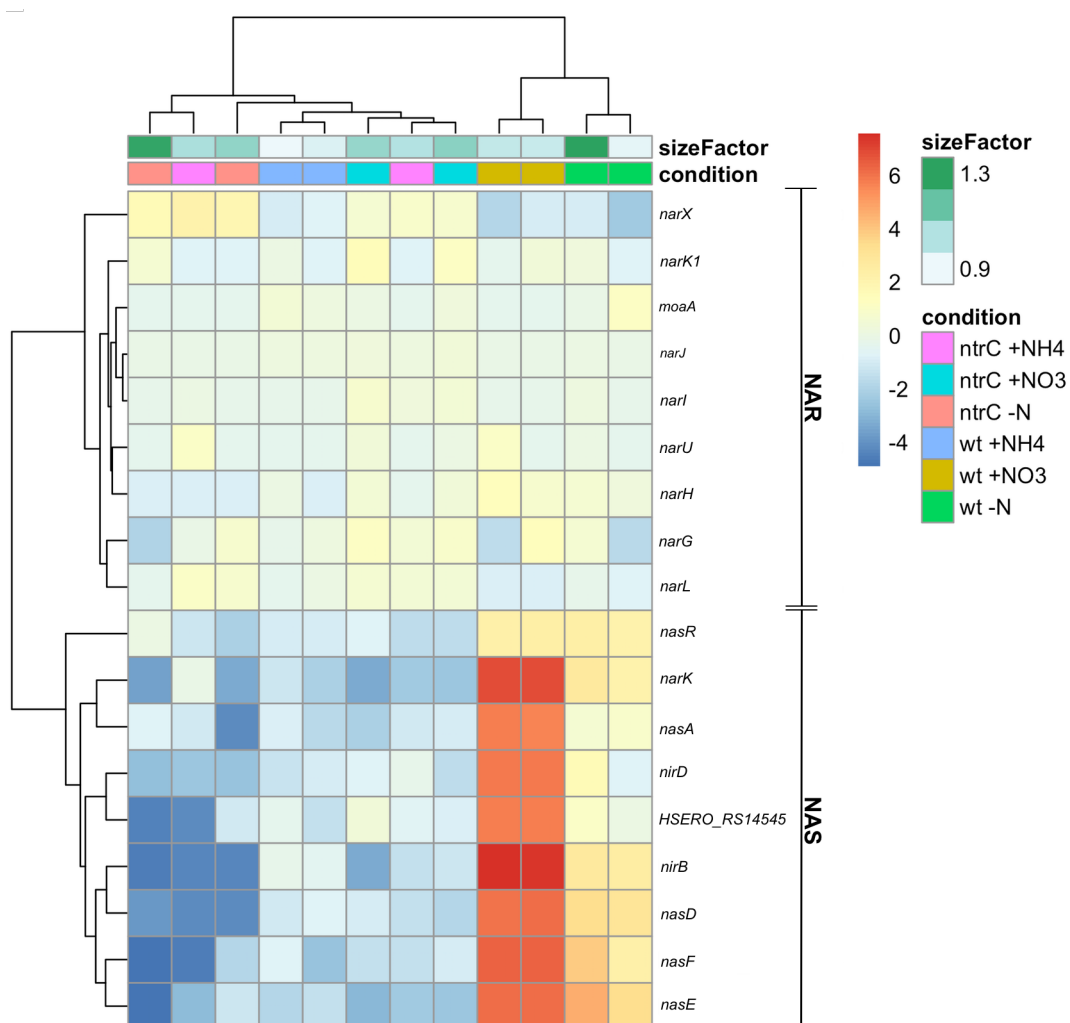


FIGURE 4 – HEATMAP WITH GENES RELATED TO NITRATE METABOLISM OF *H. seropedicae*

All the cDNA libraries built in this study were used to construct the heatmap. NAS corresponds to genes putatively involved with the assimilation of nitrate (*narKnirBDHSERO_RS14545nasA*, *nasFED*, and *nasR*), and NAR corresponds to genes related to the respiratory nitrate reductase (*narK₁UGHJlmoaA* and *narXL*).

SUPPLEMENTARY MATERIAL

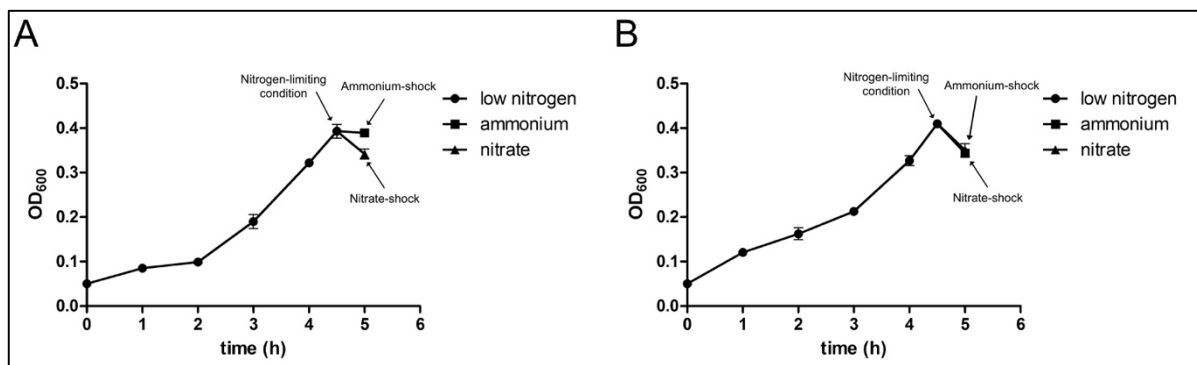


FIGURE S1 – CULTURES OF *H. seropedicae* WILD TYPE AND *ntrC* MUTANT USED IN RNA-SEQ

H. seropedicae wild type (Panel A) or *ntrC* mutant (Panel B) were grown in NFbHP-malate supplemented with 1 mM NH₄Cl until reaching OD₆₀₀ 0.4 (ammonium-limiting treatment). At this point, 10 mmol/L NH₄Cl or 10 mmol/L NaNO₃ was added. Cells were incubated for 30 minutes before harvesting. Arrows indicate samples used for RNA extraction.

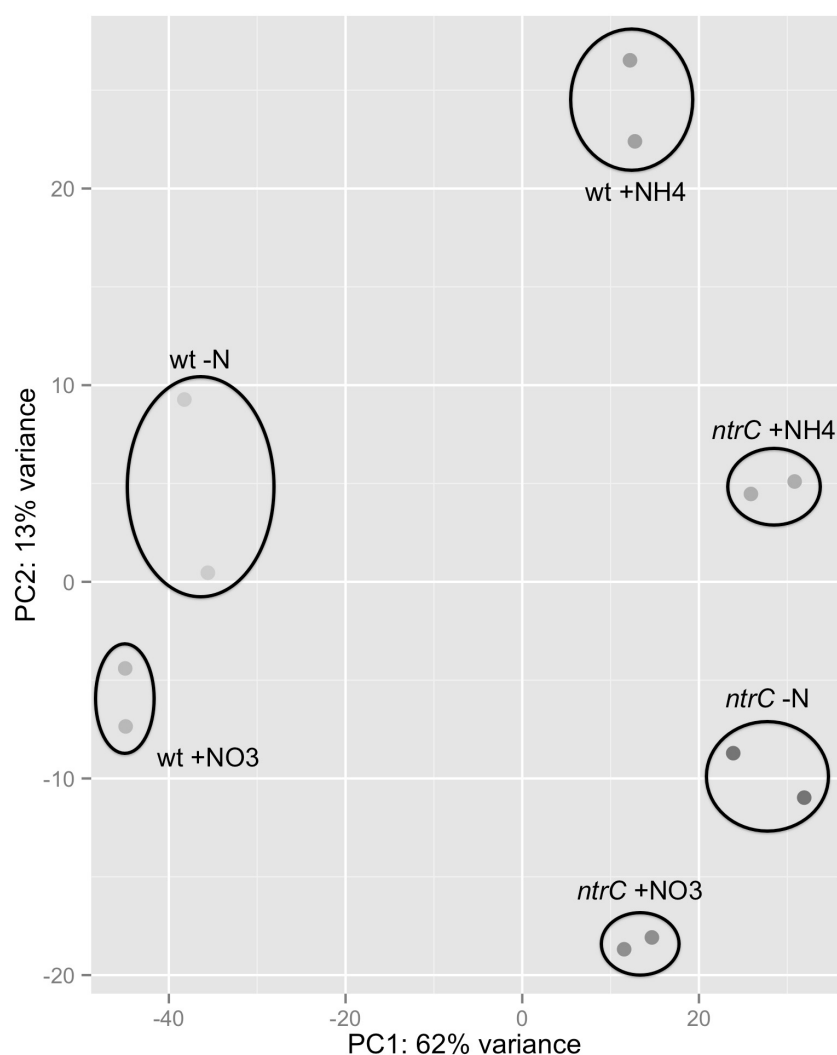


FIGURE S2 – PRINCIPAL COMPONENT ANALYSIS (PCA)

Principal component analysis of all libraries used in this study. The terms “-N”, “+NH₄”, and “+NO₃” refer to ammonium-limiting, ammonium-shock and nitrate-shock treatments, respectively.

All the cDNA libraries built in this study were used to construct the heatmap. All the genes shown in Figure 2, are represented, except for genes related to nitrate metabolism (represented in Figure 4).

TABLE S1 – STRAINS USED IN THIS WORK

Strain	Genotype	Reference
<i>Escherichia coli</i> DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80/ <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 endA1</i> <i>araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL nupG</i>	Invitrogen
<i>Escherichia coli</i> S17.1	Tra ⁺ , (Sm ^R)	Simon, Priefer et al. (1983)
<i>Herbaspirillum</i> <i>seropedicae</i> SmR1	wt, Nif ⁺ , (Sm ^R)	Souza, Pedrosa et al. (2000)
<i>Herbaspirillum</i> <i>seropedicae</i> DCP286A	<i>ntrC</i> :: Tn5-B20	(Persuhn, Souza et al. 2000)
<i>Herbaspirillum</i> <i>seropedicae</i> <i>nasA</i>	<i>nasA</i> ⁻ (Sm ^R)	This work

TABLE S2 – PLASMIDS USED IN THIS WORK

Plasmid	Relevant properties	Reference
pMH1701	Contains <i>sacB</i> -Km fragment (Km ^R)	Hynes, Quandt et al. (1989)
pSUP202	(Amp ^R , Cm ^R , Tc ^R)	Simon, Priefer et al. (1983)
pSUP- <i>nasA</i>	<i>Bam</i> HI/ <i>Sal</i> I fragment of 1.2 Kb containing fragments located upstream (UP) and downstream (DOWN) of <i>nasA</i> ligated (UP-DOWN fragment). This UP-DOWN fragment was inserted into pSUP202 vector (<i>Bam</i> HI/ <i>Sal</i> I) (Amp ^R , Cm ^R)	This work
pSUP- <i>nasA-sacB</i>	pSUP- <i>nasA</i> plasmid containing <i>sacB</i> Km fragment (Amp ^R , Cm ^R , Km ^R)	This work

TABLE S3 – OLIGONUCLEOTIDES USED IN THIS WORK

Oligonucleotides	Sequence	Reference
Oligonucleotides used for semi-quantitative PCR		
RT16S	Forward 5' TGTCAGGGAAGAAACGGTTTTG 3'	Tadra-Sfeir, Souza et al. (2011)
	Reverse 5' AGTTAGCCGGTGCTTATTCTTCA 3'	
RTnasA	Forward 5' ACAATTGCTGACCGAGGACTACTA 3'	Bonato, unpublished
	Reverse 5' CGTGAATTGGTGTCTGATGTTG 3'	
RTnasF	Forward 5' CCGACAAGCGCGAATACAC 3'	Bonato, unpublished
	Reverse 5' CCAGCCAGTAATACAGCCACATC 3'	
RTnirD	Forward 5' TCGGCAACCTGGGTGAAC 3'	Bonato, unpublished
	Reverse 5' GTTGGCGGGCTCTTCCA 3'	
Oligonucleotides used for construction of <i>nasA</i> mutant		
<i>nasA</i> UP	Forward 5' TCAAGGATCCCAGCAGCAT 3'	This work
	Reverse 5' AGCGGAGTCTAGAAAAGATTG 3'	
<i>nasA</i> DOWN	Forward 5' GAGGTCAGACTCTAGAACGC 3'	This work
	Reverse 5' CAACATTGTCTGACCGAATCC 3'	

TABLE S4 – GENE SET ANALYSIS WITH GO TERMS AND KEGG TERMS

1x2 (wt -N x +NH4)					
GROUPS INDUCED IN THE AMMONIUM-LIMITING TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
hse00500	21	6	0.9848	Starch and sucrose metabolism	
hse00760	24	6	0.9598	Nicotinate and nicotinamide metabolism	
hse00910	27	7	0.9852	Nitrogen metabolism	
GROUPS INDUCED IN THE AMMONIUM-SHOCK TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
GO:0006091	97	32	1	generation of precursor metabolites and energy	The chemical reactions and pathways resulting in the formation of precursor metabolites, substances from which energy is derived, and any process involved in the liberation of energy from these substances.
GO:0008135	20	13	0.8048	translation factor activity, nucleic acid binding	Functions during translation by interacting selectively and non-covalently with nucleic acids during polypeptide synthesis at the ribosome.
GO:0051082	14	7	0.7832	unfolded protein binding	Interacting selectively and non-covalently with an unfolded protein.

GO:0061024	12	5	0.8736	membrane organization	A process which results in the assembly, arrangement of constituent parts, or disassembly of a membrane. A membrane is a double layer of lipid molecules that encloses all cells, and, in eukaryotes, many organelles; may be a single or double lipid bilayer; also includes associated proteins.
hse00020	24	15	1	Citrate cycle (TCA cycle)	
hse00190	51	25	1	Oxidative phosphorylation	
hse01210	33	14	0.7698	2-Oxocarboxylic acid metabolism	
hse03010	62	52	1	Ribosome	
hse03018	14	6	0.9016	RNA degradation	
hse03060	16	8	0.9956	Protein export	

1x3 (wt -N x +NO3)					
GROUPS INDUCED IN THE AMMONIUM-LIMITING TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
GO:0005198	63	12	0.7172	structural molecule activity	The action of a molecule that contributes to the structural integrity of a complex or assembly within or outside a cell.
hse03010	62	11	1	Ribosome	
GROUPS INDUCED IN THE NITRATE-SHOCK TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
hse00910	27	8	1	Nitrogen metabolism	

2x3 (wt +NH4 x +NO3)					
GROUPS INDUCED IN THE AMMONIUM-SHOCK TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
GO:0008135	20	16	1	translation factor activity, nucleic acid binding	Functions during translation by interacting selectively and non-covalently with nucleic acids during polypeptide synthesis at the ribosome
hse00020	24	13	0.9728	Citrate cycle (TCA cycle)	
hse00190	51	28	1	Oxidative phosphorylation	
hse00450	11	4	0.5424	Selenocompound metabolism	
hse00540	21	8	0.7422	Lipopolysaccharide biosynthesis	
hse00740	10	4	0.5226	Riboflavin metabolism	
hse01230	129	49	0.7526	Biosynthesis of amino acids	
hse03010	62	51	1	Ribosome	
hse03060	16	10	0.9996	Protein export	
GROUPS INDUCED IN THE NITRATE-SHOCK TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
GO:0071941	25	13	1	nitrogen cycle metabolic process	A nitrogen compound metabolic process that contributes to the nitrogen cycle. The nitrogen cycle is a series of metabolic pathways by which nitrogen is converted between various forms and redox states; it encompasses pathways in which nitrogen is acted upon directly, such as nitrification, denitrification, nitrogen fixation, and mineralization

hse00910	27	11	0.9952	Nitrogen metabolism
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1x4 (wt -N x ntrC -N)					
GROUPS ACTIVATED BY NtrC IN RESPONSE TO AMMONIUM-LIMITING TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
hse00910	27	6	0.5726	Nitrogen metabolism	
GROUPS REPRESSED BY NtrC IN RESPONSE TO AMMONIUM-LIMITING TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
GO:0006091	97	27	1	generation of precursor metabolites and energy	The chemical reactions and pathways resulting in the formation of precursor metabolites, substances from which energy is derived, and any process involved in the liberation of energy from these substances.
hse00020	24	9	0.9442	Citrate cycle (TCA cycle)	
hse00190	51	26	1	Oxidative phosphorylation	
hse02030	66	12	0.5806	Bacterial chemotaxis	
hse03010	62	19	1	Ribosome	
hse03018	14	6	0.994	RNA degradation	

2x5 (wt +NH4 x ntrC +NH4)					
GROUPS ACTIVATED BY NtrC IN RESPONSE TO AMMONIUM-SHOCK TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
-	-	-	-	-	
GROUPS REPRESSED BY NtrC IN RESPONSE TO AMMONIUM-SHOCK TREATMENT					

	inPopulation	inStudySet	estimate	term	definition
hse00190	51	7	1	Oxidative phosphorylation	
hse00520	38	4	0.7686	Amino sugar and nucleotide sugar metabolism	
hse00680	25	4	0.9766	Methane metabolism	

3x6 (wt +NO3 x ntrC +NO3)					
GROUPS ACTIVATED BY NtrC IN RESPONSE TO NITRATE-SHOCK TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
GO:0071941	25	13	1	nitrogen cycle metabolic process	A nitrogen compound metabolic process that contributes to the nitrogen cycle. The nitrogen cycle is a series of metabolic pathways by which nitrogen is converted between various forms and redox states; it encompasses pathways in which nitrogen is acted upon directly, such as nitrification, denitrification, nitrogen fixation, and mineralization.
hse00760	24	7	0.9332	Nicotinate and nicotinamide metabolism	
hse00910	27	13	1	Nitrogen metabolism	
GROUPS REPRESSED BY NtrC IN RESPONSE TO NITRATE-SHOCK TREATMENT					
	inPopulation	inStudySet	estimate	term	definition
GO:0006091	97	33	1	generation of precursor metabolites and energy	The chemical reactions and pathways resulting in the formation of precursor metabolites, substances from which energy is derived, and any process involved in the liberation

					of energy from these substances.
					Functions during translation by interacting selectively and non-covalently with nucleic acids during polypeptide synthesis at the ribosome.
GO:0008135	20	8	0.6826	translation factor activity, nucleic acid binding	
hse00190	51	30	1	Oxidative phosphorylation	
hse00540	21	7	0.8714	Lipopolysaccharide biosynthesis	
hse03010	62	25	1	Ribosome	
hse00020	24	8	0.9614	Citrate cycle (TCA cycle)	
hse03018	14	5	0.8414	RNA degradation	

6 CONSIDERAÇÕES FINAIS E ETAPAS FUTURAS

6.1 CONSIDERAÇÕES FINAIS

6.1.1 Função de NAS e NAR de *H. seropedicae*

Análises *in silico* do genoma de *H. seropedicae* mostram que este microrganismo apresenta genes estruturais codificando para duas nitrato redutases: nitrato redutase assimilatória (NAS) (genes *nasAH*SERO_RS14545) e nitrato redutase respiratória (NAR) (genes *narGHI*).

Um mutante nocaute na subunidade catalítica de NAS (mutante *nasA*) não é capaz de crescer em nitrato como fonte única de nitrogênio, enquanto que um mutante para NAR (mutante *narG*) apresenta crescimento em nitrato comparável ao selvagem. Estes resultados sugerem que NAS é a principal forma de assimilação de nitrato em *H. seropedicae*. Além disso, *H. seropedicae* apresenta genes codificando para uma única nitrito redutase (genes *nirBD*), os quais estão contidos no mesmo operon que os genes codificando para NAS (*narKnirBDH*SERO_RS14545*nasA*). Dados de expressão dos dois ensaios de RNA-seq realizados na presença de nitrato (em DO₆₀₀ 0,8 ou realizado em DO₆₀₀ 0,4 e após choque de nitrato por 30 minutos (“nitrate-shock treatment”)) sugerem que os genes *narKnirBDH*SERO_RS14545*nasA* são co-transcritos e formam um único operon. Juntos, estes dados mostram que *H. seropedicae* assimila nitrato através da conversão de nitrato a nitrito, através de NAS, e nitrito a amônio através da nitrito redutase codificada pelos genes *nirBD*.

Um mutante *narG* de *H. seropedicae* cresce bem em nitrato como única fonte de nitrogênio, sugerindo que NAR não é requerido para assimilar nitrato. Além disso, *H. seropedicae* não é capaz de crescer em anaerobiose utilizando nitrato como aceptor de elétrons, o que sugere que NAR também não seja requerido para desnitrificar. Entretanto, o mutante *narG* perdeu a capacidade de produzir nitrito em condições limitantes de oxigênio. A caracterização fisiológica do mutante *narG* mostrou que NAR produz óxido nítrico como produto secundário, como ocorre em *E. coli* e *Salmonella* (Gilberthorpe and Poole 2008, Vine and Cole 2011, Rowley, Hensen et al. 2012). Além disso, NAR está relacionada com o aumento do consumo da fonte de carbono, o que possivelmente está relacionado com o aumento na

produção e consumo de NADH, doador indireto de elétrons para redução de nitrato a nitrito. Finalmente, plântulas de trigo inoculadas com mutante *narG* apresentaram peso seco inferior ao de plântulas inoculadas com *H. seropedicae* selvagem. Uma vez que óxido nítrico já foi mostrado ser importante na interação planta-bactéria no modelo *A. brasilense*-tomate (Creus, Graziano et al. 2005, Molina-Favero, Creus et al. 2008), é possível que o óxido nítrico produzido por NAR de *H. seropedicae* também esteja envolvido na interação *H. seropedicae*-trigo.

6.1.2 Regulação de NAS e NAR de *H. seropedicae*

Análise transcriptômica (RNA-seq) realizada na presença de nitrato e em baixa DO₆₀₀ (“nitrate-shock treatment”), condição bem aerada, mostrou que genes relacionados à NAR (*narK₁UGHJI*) estavam pouco expressos, enquanto que genes codificando para NAS estavam bem induzidos. Por outro lado, na análise de RNA-seq realizada na presença de nitrato, mas em DO₆₀₀ mais alta (0,8), condição com limitação de oxigênio, mostrou que os genes relacionados a NAR estavam entre os mais expressos, mas genes relacionados a NAS estavam induzidos. Estes dados sugerem que NAS é importante para assimilar nitrato, enquanto que NAR somente é expressa em condição de oxigênio limitante.

A expressão dos genes *narK₁UGHJI* é induzida em resposta a baixos níveis de oxigênio, via Fnr, e em resposta a nitrato. A expressão de *narXL* também é induzida em resposta a baixos níveis de oxigênio via Fnr e em resposta a nitrato. Ensaios de β-galactosidase com fusão *narX-lacZ* em mutante *ntrY* sugerem o sistema NtrY/NtrX é requerido para ativação desta região promotora em resposta a nitrato (ALVES, 2006). Além disso, ensaios de interação proteína-DNA e “footprinting” com DNase I mostram que NtrX liga-se diretamente ao promotor de *narX* (Bonato 2009).

H. seropedicae apresenta vários genes relacionados com NAS: os genes *nasFED*, codificando para transportadores de nitrato do tipo ABC, gene *nasR*, codificando para a proteína regulatória NasR e os genes do operon *narKnirBDHSERO_RS14545nasA*, que codifica para um transportador de nitrato, codificando para NAS e para nitrito redutase. A expressão de todos esses genes foi induzida em resposta a baixos níveis de amônio e na presença de nitrato. Além disso, o sistema de dois componentes NtrB/NtrC é requerido para induzir a

expressão destes genes, provavelmente em resposta a baixos níveis de nitrogênio. O sistema NtrY/NtrX também é requerido para ativar a expressão do operon *narKnirBDHSERO_RS14545nasA* e *nasFED* (Alves 2006), provavelmente em resposta a nitrato.

6.1.3 Sistema NtrB/NtrC e NtrY/NtrX de *H. seropedicae*

O sistema NtrB/NtrC de *H. seropedicae*, assim como de outras bactérias é o regulador central em resposta a limitação de amônio, ativando genes cujos produtos estão relacionados a vias com o intuito de gerar amônio, como exemplo: proteases/peptidases, transportadores de compostos nitrogenados (aminoácidos, ureia, etanolamina e amônio), ou enzimas que metabolizam estes compostos. O sistema NtrB/NtrC também está envolvido na repressão do metabolismo energético e na síntese de proteínas provavelmente para evitar gasto energético em condição onde amônio é limitante. O sistema NtrB/NtrC também é importante para ativar genes relacionados a NAS, em resposta a baixo nitrogênio, como mencionado na seção anterior. O mutante *ntrC* não cresce em nitrato como fonte única de nitrogênio por ser requerido para expressão do operon *narKnirBDHSERO_RS14545nasA*, uma vez que NAS é essencial para *H. seropedicae* assimilar nitrato.

O sistema NtrY/NtrX de *H. seropedicae* é importante para o metabolismo de nitrato, uma vez que o mutante *ntrY* não cresce em nitrato como fonte única de nitrogênio. Entretanto, este sistema não é requerido para controlar genes envolvidos com a fixação de nitrogênio ou na assimilação de nitrogênio via glutamina sintetase (Alves 2006). Além disso, o sistema NtrY/NtrX é importante para ativar a expressão dos operons *narKnirBDHSERO_RS14545nasA* e *narXL* (Alves 2006). Caracterização *in vitro* da proteína NtrX sugere que esta proteína é mais estável na forma dimérica. Além disso, NtrX liga-se diretamente ao promotor de *narXL* e ensaios de footprinting com DNase I, mostram que NtrX provavelmente se liga numa região localizada entre -166 e -133 pb, considerando o início de tradução do gene *narX*.

Da mesma forma que observado para o mutante *ntrC*, o mutante *ntrY* não cresce em nitrato por ser importante para expressão do operon *narKnirBDHSERO_RS14545nasA* (Alves 2006). Assim, os resultados sugerem que

a expressão dos genes envolvidos com a nitrato redutase assimilatória depende dos sistemas NtrB/NtrC e NtrY/NtrX.

6.2 ETAPAS FUTURAS

- Realizar transcriptoma do mutante *ntrY* em condições limitantes de nitrogênio e após 30 minutos de choque com amônio, ou nitrato (“ammonium-limiting treatment”, “nitrate-shock treatment”, “ammonium-shock treatment”), com o intuito de identificar genes regulados direta ou indiretamente por NtrY/NtrX em resposta a baixo ou alto amônio ou nitrato.
- Realizar ChIP-seq com proteína NtrX com o intuito de identificar promotores alvos reconhecidos diretamente por NtrX, além de propor um sítio consenso reconhecido por esta proteína.
- Construir mutante para o gene *nasR* para avaliar se a proteína reguladora NasR de *H. seropedicae* é importante para assimilação de nitrato como ocorre em *K. pneumoniae*.

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