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The NtrY/X two-component system of *Brucella* spp. acts as a redox sensor and regulates the expression of nitrogen respiration enzymes

Mariela del Carmen Carrica,[1] Ignacio Fernandez,[1] Marcelo Adrián Martí,[2] Gastón Paris[1] and Fernando Alberto Goldbaum[1]*

[1]*Fundación Instituto Leloir (IIBBA-CONICET), Av. Patricias Argentinas 435, Buenos Aires, Argentina.*
[2]*Departamento de Química Biológica y INQUIMAE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, C1428EHA, Buenos Aires, Argentina.*

Summary

Brucella **spp. are facultative intracellular bacteria pathogenic for many mammalian species including humans, causing a disease called brucellosis. Learning how** ***Brucella*** **adapts to its intracellular niche is crucial for understanding its pathogenesis mechanism, allowing for the development of new and more effective vaccines and treatments against brucellosis.** ***Brucella*** **pathogenesis resides mostly in its ability to adapt to the harsh environmental conditions encountered during host infection such as the oxygen depletion. The mechanism by which** ***Brucella*** **senses the oxygen tension and triggers its environmental adaptation is unknown. In this work we show that the** ***Brucella abortus*** **NtrY/NtrX two-component system is involved in oxygen sensing through a haem group contained in a Per-ARNT-SIM (PAS) domain of the NtrY histidine kinase. The NtrY haem iron can be reduced to the ferrous form and is rapidly oxidized to the ferric form in presence of oxygen. Importantly, we show that the oxidation state of the haem iron modulates the autokinase activity, being the anoxygenic reduced ferrous form the signalling state of NtrY. Also, we show that** ***ntrY*** **gene expression increases under low oxygen tension and that NtrY transfers its signal to its cognate response regulator NtrX, regulating in this way the expression of nitrogen respiration enzymes. Based on these findings, we postulate that NtrY acts as a redox sensor in** ***Brucella*** **spp.**

Accepted 9 May, 2012. *For correspondence. E-mail fgoldbaum@leloir.org.ar; Tel. (+54) 115 238 7500; Fax (+54) 115 238 7501.

Introduction

Brucella spp. are facultative intracellular Gram-negative bacteria that belong to the α-2-proteobacteria group. They are pathogenic for many mammalian species including humans, causing a disease called brucellosis. This is a chronic infection that produces abortion and sterility in domestic mammals and undulant fever in humans (Corbel, 1997). In comparison with other pathogenic bacteria, *Brucella* spp. lack classical virulence factors and their pathogenesis reside mostly in their ability to survive and multiply within professional and non-professional phagocytes (Detilleux *et al*., 1990; Gorvel and Moreno, 2002). Immediately after phagocytosis *Brucella* cells interact with compartments related to early endosome and subvert the normal vesicular trafficking to finally establish in a specialized compartment with membranes derived from endoplasmic reticulum (Celli *et al*., 2003). During this process *Brucella* have to adapt to harsh conditions encountered inside the host cell, such as nutritional deprivation, exposition to acidic pH, reactive oxygen and nitrogen species and low oxygen tension (Kohler *et al*., 2002; Roop *et al*., 2009). *Brucella* is also exposed to hypoxia in many places during the infection course inside the animal body, such as in the Peyer's patches of the gastrointestinal tract during oral infection and at the phagosomes during intracellular multiplication. In chronic infections, *Brucella* can reside within granulomatous lesions, being exposed to microaerobic and anaerobic areas in the liver, spleen and brain (Ariza *et al*., 2001; Colmenero Jde *et al*., 2002; Sohn *et al*., 2003).

Adaptation to the low oxygen tension involves relevant metabolic changes in gene expression such as increased expression of high-affinity cytochrome oxidases (CydCDAB and CcoNOQP), which allow the efficient respiration at low oxygen concentration, (Loisel-Meyer *et al*., 2005; Jimenez de Bagues *et al*., 2007) and enzymes involved in denitrification pathway (NarGHIJK, NirKV, NorBCDEFQ and NosDFLRXYZ) which allow the bacteria to use nitrate as an alternative terminal electron acceptor

instead of oxygen (DelVecchio *et al*., 2002; Loisel-Meyer *et al*., 2006). Recently, a proteomic analysis of *Brucella suis* grown under oxygen deficiency show also an increase in the expression levels of several enzymes pertaining to various pathways such as denitrification, fatty acid oxidation and citrate fermentation that suggest a metabolic adaptation to low-oxygen condition (Al Dahouk *et al*., 2009). The nature of environmental signals and mechanisms that *Brucella* uses to adapt and respond to hypoxia conditions are largely unknown.

In bacteria, changes of gene expression triggered by environmental signals are modulated mainly by two-component systems, which consist of a sensor histidine kinase (HK) protein that detect and converts the environmental signal into a phosphorylation chain and finally activates a response regulator protein which binds promoter regions and, in turns, modulates gene expression. The PAS domains are usually involved in signal transduction and are very common in sensory HK. Previous studies showed that they can sense different stimuli such as light, oxygen, redox potential and small molecules (Taylor and Zhulin, 1999). The most studied bacterial oxygen sensor is the FixL histidine kinase of *Sinorhizobium meliloti* that can directly sense the oxygen level through a haem cofactor located in its N-terminal PAS domain (Monson *et al*., 1995). Other oxygen sensors that have a haem cofactor situated in an N-terminal PAS domain are the phosphodiesterases ecDOS (direct oxygen sensor) of *Escherichia coli* and AxPDEA-1 of *Acetobacter xylinum* (Delgado-Nixon *et al*., 2000; Chang *et al*., 2001). There are also other proteins that can sense changes in oxygen availability by modification of the redox status of their bound cofactor. Examples of these indirect oxygen sensors are Aer which has a flavin-nucleotide cofactor, and ArcB which has a thiol-based switch (Bibikov *et al*., 1997; Malpica *et al*., 2006).

The *Brucella abortus* S2308 genome has 10 open reading frames (ORFs) that are predicted to encode PAS domains. We focused our analysis in potential haem-binding proteins without an experimentally tested function in *Brucella*. Among them, we detected NtrY as the only gene with altered expression levels in low oxygen tension. NtrY is a putative sensor histidine kinase that belongs to the two-component system NtrY/X. This system was first characterized in the symbiotic nitrogen-fixing bacteria *Azorhizobium caulinodans* and *Azospirillum brasilense* (Pawlowski *et al*., 1991; Ishida *et al*., 2002). In *A. caulinodans* the mutation of *ntrY/X* causes a pleitotropic defect in nitrogen metabolism and alters symbiotic nitrogen fixation, while in *A. brasilense*, *ntrY/X* is involved in nitrate-dependent growth but, in this later case, a direct relationship with symbiosis has not been demonstrated (Ishida *et al*., 2002). It has also been demonstrated that NtrY/X system is involved in photosynthetic gene expression and nitrogen metabolism in the purple non-sulphur photosynthetic bacterium *Rhodobacter capsulatus* (Drepper *et al*., 2006; Gregor *et al*., 2007). Moreover, NtrY/X has been linked to the virulence of the pathogenic bacteria *B. suis* and *Bordetella bronchiseptica*. The insertion of a transposon in the *ntrY* gene affects the intracellular survival of *B. suis* in an *in vitro* human macrophage infection model (Foulongne *et al*., 2000). A mutation in the *ntrY* homologous (named *plrS*) of the respiratory pathogen *B. bronchiseptica* affects bacterial colonization and persistence in the lower respiratory tract of infected rats (Kaut *et al*., 2011). However, the signal sensed by NtrY and its mechanism of action are largely unknown.

In this work, we show that the *Brucella* NtrY histidine kinase has a PAS domain that binds haem as a cofactor. The haem iron can be reduced to Fe^{2+} and is rapidly oxidized to Fe^{3+} in presence of oxygen. Importantly, we demonstrate that the oxidation state of the haem iron modulates histidine kinase autophosphorylation activity, being the anoxygenic reduced ferrous form the signalling state of NtrY. Also, we show that *ntrY* gene expression increases under low oxygen tension and that NtrY transfers the phosphate moiety to its cognate response regulator NtrX, regulating in this way the expression of nitrogen respiration enzymes.

Results

Brucella *NtrY is a PAS domain containing histidine kinase induced under low oxygen tension*

Adaptation of bacteria to harsh environmental conditions through sensor proteins is essential for *Brucella* pathogenesis. Because of the fact that PAS (Per-ARNT-SIM) domains are frequently involved in environmental signal (Taylor and Zhulin, 1999) sensing we conducted a bioinformatics analysis of *B. abortus* genome to detect PAS domain containing proteins.

We found that the *Brucella* genome encodes for 10 proteins with predicted PAS domains, eight are associated with histidine kinase domains and two are associated with phosphodiesterase/diguanylate cyclase domains (Table 1). Four of these genes have experimentally assigned functions different from oxygen sensing: PleC, PdhS and DivJ, which are involved in asymmetric cell division (Hallez *et al*., 2007), and the light-sensor LOV-HK (Swartz *et al*., 2007). Three proteins from the remaining six are predicted to bind haem as cofactor (BAB1_1139, BAB1_2101 and BAB1_0220) as analysed by SUPERFAMILY program (Gough *et al*., 2001). We were particularly interested in these three proteins because PAS domains from oxygen/redox sensors bind haem as a cofactor, thus we decided to test whether the transcriptional levels of them are affected by oxygen tension. For this aim we performed RT-qPCR on *B. abortus* S2308 grown in rich medium under three different oxygen conditions: at normal

Table 1. Predicted PAS domain containing protein in *B. abortus* S2308 genome.

ORF number[a]	Gene name	Assigned function	Effector domain	Predicted cofactor[b]
BAB1_0346	–	None	Histidine kinase, response regulator receiver domain	–
BAB1_0601	DivJ	Cell division	Histidine kinase	–
BAB1_0640	PleC	Cell division	Histidine kinase	Haem
BAB1_1139	NtrY	Homologous to the histidine kinase NtrY involved in nitrogen metabolism and fixation	Histidine kinase	Haem
BAB1_1621	PdhS	Asymmetric cell division	Histidine kinase	Haem
BAB1_1669	–	None	Histidine kinase	Flavin
BAB1_2101	PhoR	Homologous to the histidine kinase PhoR involved in adaptation to low environmental phosphate concentrations	Histidine kinase	Haem
BAB2_0652	LOV-HK	Light-activated histidine kinase involved in virulence	Histidine kinase	Flavin
BAB1_0220	–	None	Diguanylate cyclase/phosphodiesterase	Haem
BAB1_0507	–	None	Diguanylate cyclase/phosphodiesterase	Flavin

a. PAS domain was predicted with pFAM program and are included which have *E*-value lower to 1×10^{-3}.
b. Cofactor predicted with SUPERFAMILY program.

oxygen condition (aerobiosis), under microaerobiosis or incubated in anaerobiosis. We found that BAB1_1139 transcriptional levels are significantly increased at low oxygen tension (Fig. 1). In contrast, the levels of BAB1_0220 and BAB1_2101 showed no significant changed under the conditions assayed (data not shown). BAB1_1139 gene encodes for a histidine kinase homologue to NtrY that is involved in nitrogen metabolism and/or fixation in *A. caulinodans*, *A. brasilense* and *R. capsulatus* (Pawlowski *et al.*, 1991; Ishida *et al.*, 2002; Gregor *et al.*, 2007). A genome-wide study found that a transposon insertion in the *ntrY* gene affects *B. suis* intracellular survival in an *in vitro* human macrophage infection model (Foulongne *et al.*, 2000) and in the respiratory pathogen, *B. bronchiseptica*, a mutation in a gene homologous to *ntrY* (named *plrS*) affects the bacterial colonization and persistence in the lower respiratory tract of infected rats (Kaut *et al.*, 2011). Thus, we decided to further investigate the function of *Brucella* NtrY.

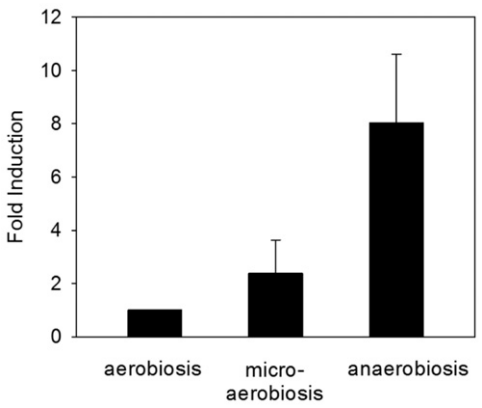

Fig. 1. Induction of NtrY expression under low oxygen tension. *Brucella abortus* S2308 was grown in rich TSB medium at 37°C in different oxygen conditions as detailed in *Experimental procedures*. The expression of *ntrY* was measured by RT-qPCR using RNA extracted from bacteria cultured in the log-phase. Data are the mean ± standard deviation of three independent experiments and are reported as fold induction relative to the expression in aerobic cultures.

NtrY is a haem protein

The NtrY/NtrX two-component system is widely distributed among the α-proteobacteria, including pathogens such as *Bordetella* spp., *Bartonella* spp., *Agrobacterium* and *Neisseria meningitidis* and symbionts such as *Rhizobium*, *Sinorhizobium*, *Bradirhizobium* and *Azorhizobium*. However, the signal sensed by the histidine kinase and the sensing mechanism remain unknown. In the same way that other proteins belonging to the NtrY family, *B. abortus* NtrY contains predicted transmembrane regions at the N-terminus followed by three protein domains: a HAMP (histidine kinases, adenyl cyclases, methyl-accepting chemotaxis protein and phosphatases) domain, a PAS domain and a histidine kinase domain (Fig. 2A). Frequently, PAS domain proteins detect their signal by means of an associated cofactor such as haem. In order to assay the cofactor binding of NtrY we cloned and expressed in *E. coli* the PAS domain (PAS-NtrY) and the PAS domain followed by the histidine kinase domain (PAS-HK-NtrY, Fig. 2A). Both proteins show the typical UV-visible spectrum (for simplification, only the PAS-HK-NtrY spectra is shown) of haem proteins, indicating that they bind haem as cofactor (Fig. 2B). The spectra of purified proteins show that the maximum of the Soret band is at 408 nm while α and β bands are at 533 nm and 558 nm respectively. These values are very similar to those found for *Mycobacterium tuberculosis*, DosS (Kumar *et al.*, 2007) and BarleyHb (Duff *et al.*, 1997) and indicative of a

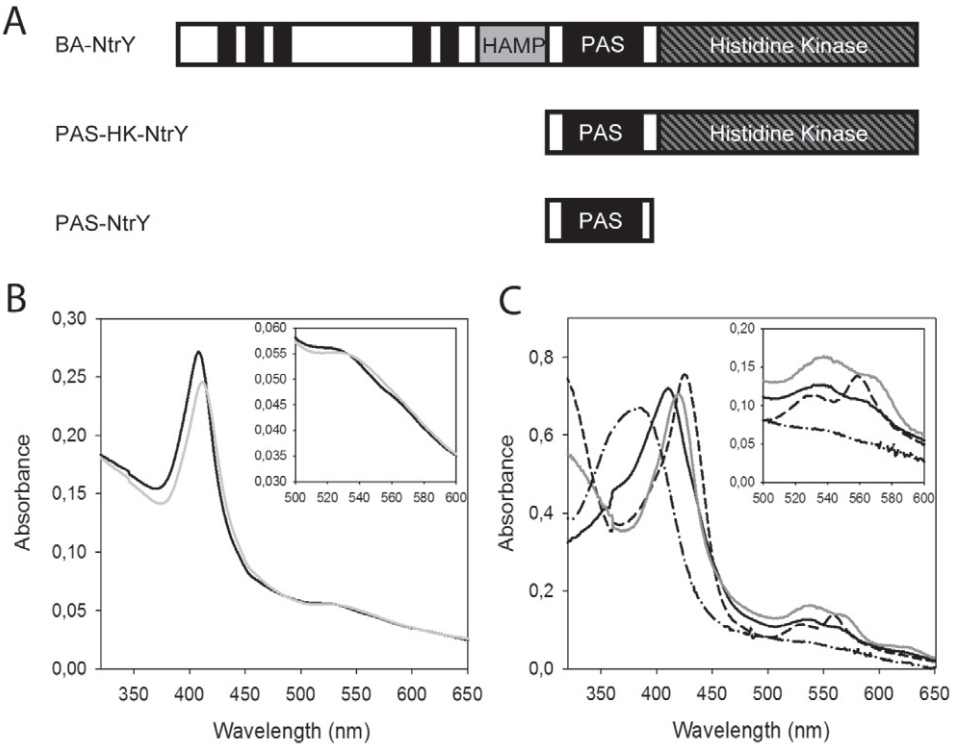

Fig. 2. NtrY is a haem protein.
A. Schematic representation of the NtrY full-length protein and the derived constructs that were used in this work, PAS-NtrY and PAS-HK-NtrY. B and C. (B) UV-visible spectra of PAS-HK-NtrY in the presence of KCN (C) or exposed to DTH with or without NO and CO as ligands. Solid black line Fe^{3+}, light solid grey KCN, short dash Fe^{2+}, solid grey CO, dash dot NO. Identical spectrum were obtained for PAS-NtrY protein. Inserts in (B) and (C) show a zoom between 500 and 600 nm to highlight the α and β bands of each spectrum.

ferric haem (Table 2). To confirm the redox state of the haem we added an excess of cyanide (CN^-) to freshly purified NtrY. The resulting spectrum shows the conversion of α and β bands into a single peak at about 536 nm, which is characteristic of CN bound ferric haem (Kumar *et al.*, 2007), and the shift of the Soret band to 411 nm (Fig. 2B). In order to obtain the spectra of fully reduced protein (Fe^{II}-NtrY), we added sodium dithionite (DTH) to a sample of purified PAS-HK-NtrY contained in a sealed quartz cuvette under Argon atmosphere, and recorded the spectra until no significant changes were observed. The spectrum of reduced PAS-HK-NtrY shows both the typical sharpening and red-shift of the Soret band to 423 nm, and change in relative intensity of the α and β bands (Fig. 2B).

Haem proteins, and especially haem-based sensors (Gilles-Gonzalez and Gonzalez, 2005), commonly bind small gaseous molecules, mainly O_2, nitric oxide (NO) and carbon monoxide (CO), which could be their actual physiological signal. Therefore we analysed each of these possible ligands for their ability to bind to ferrous NtrY. First, to determine if NtrY can bind O_2 we exposed the reduced Fe^{2+} protein to atmospheric oxygen. Importantly, the exposure to atmospheric oxygen rapidly reverted the UV-visible spectra to that of the native ferric protein. This shows that

Table 2. Comparison of optical absorption maxima between NtrY and other haem proteins (nm).

	Fe^{3+}			Deoxy Fe^{2+}			Fe^{2+}–NO			Fe^{2+}–CO			
Protein	γ	β	α	γ	β	α	γ	β	α	γ	β	α	Reference
NtrY	408	533	558	423	527	555	380	–	–	417	534	565	This work
FixL	395	509	645	437	556			–		427	548	560	Gilles-Gonzalez *et al.* (1994)
ecDOS	417	530	564	428	532	563	419	537	563	423	540	570	Sasakura *et al.* (2002)
DosS	409	536	575	430	557		418	542	570	420	535	563	Kumar *et al.* (2007)
BarleyHb	411	534	565	425	539	563	418	545	575	417	537	567	Duff *et al.* (1997)
sGS	393	–	–	431	555		398	537	572	423	541	567	Stone and Marletta (1994)

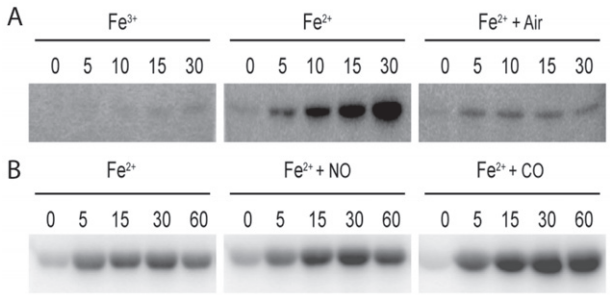

Fig. 3. Kinetics of the autophosphorylation of PAS-HK-NtrY.
A. Autophosphorylation assay of aerobically purified PAS-HK-NtrY (Fe^{3+}) protein and exposed to sodium dithionite in anoxic conditions to generate the Fe^{2+} form and Fe^{2+} form re-exposed to atmospheric oxygen (Fe^{2+}+Air).
B. Ferrous PAS-HK-NtrY binding to nitric oxide and carbon monoxide. Ferrous PAS-HK-NtrY was obtained by exposure to DTH (Fe^{2+}) and incubated with a slightly excess of the NO donor *N*-nitrosomelatonin (Fe^{2+}+NO) or bubbled with CO (Fe^{2+}+CO) A representative of three independent experiments is shown.

oxidation of ferrous NtrY is very fast and that no stable oxygen complex is established. On the other hand, ferrous NtrY exposed to CO shows a shift in the Soret peak from 423 nm to 417 nm which indicates the formation of a typical hexa-coordinated Fe^{2+}–CO complex, as also seen in DosS and BarleyHb (Table 2). Finally, the binding of NO resulted in a broad and less intense Soret band at about 385 nm as well as ill-defined α and β bands, which are indicative of a penta-coordinated haem that resembles the NO-sensing H-NOX domain of the soluble guanylate cyclase (sGC) (Table 2) (Fig. 2C) (Stone and Marletta, 1994). In summary the UV-Vis spectral analysis of NtrY strongly suggests that it is a haem protein which is easily oxidized to a ferric stable state in the presence of oxygen and that is able to form both 6c-Fe2–CO and 5c-Fe2–NO complexes.

NtrY is a redox sensor

Haem-based sensor proteins are key regulators of adaptive responses to redox cellular state or to the presence of ligands such as O_2 and NO. In these proteins, the haem-containing sensory domains can modulate the catalytic activity of the protein and then trigger a specific cellular response. We decided to test if the NtrY histidine kinase activity is affected by the haem iron redox status or the binding of NO and CO ligands. For this aim, autophosphorylation assays were performed with PAS-HK-NtrY aerobically purified or reduced with DTH in anoxic conditions. Results show that deoxy ferrous NtrY possess much higher autokinase activity than ferric NtrY (Fig. 3A). Moreover, we show that after re-exposing an aliquot of deoxy ferrous NtrY to air, the autokinase activity diminishes to the level of the ferric protein suggesting that deoxy ferrous NtrY is the active state of this sensory protein. We also assessed whether CO or NO influence NtrY autokinase activity. For this, we exposed deoxy ferrous NtrY to a NO donor or to gaseous CO and conducted phosphorylation assays. Both, the nitrosylated and carbonylated forms of NtrY showed autokinase activities comparable to levels of active ferrous NtrY (Fig. 3B). In summary, activity assays together with the absorption spectra allow us to propose NtrY as a redox sensor with the ferrous form as the signalling state since the rapidly oxidation of NtrY to the ferric form dramatically decrease the autophosphorylation activity. Ligands such as NO or CO may eventually stabilize the ferrous NO–NtrY or CO–NtrY complexes in the active state.

NtrY transduces the signal through the cognate response regulator NtrX

Using RT-PCR we demonstrated that the *ntrY* gene is located in an operon whose organization is similar to that of other bacteria (*A. caulinodans*, *A. brasilense* and *R. capsulatus*) (Fig. 4A) (Pawlowski *et al.*, 1991; Ishida *et al.*, 2002; Drepper *et al.*, 2006). The genes belonging to the two-component system NtrB/C (*nifR3-ntrB-ntrC*) are located upstream to *ntrY*, whereas its response regulator, *ntrX*, and a putative low-affinity potassium transporter *trkA* are found downstream. In *R. capsulatus* there is a promoter upstream of *ntrY* that acts in concert with another promoter upstream of *nifR3-ntrB-ntrC* (Gregor *et al.*, 2007). The fusion of the DNA regions upstream of *nifR3* and *ntrY* genes (see arrows in Fig. 4A) to LacZ gene confirmed the presence of two independent promoters in the operon (Fig. S1). This result shows that the structure of *Brucella* NtrY/X operon is similar to other alphaproteobacteria (Fig. 4A) (Drepper *et al.*, 2006).

To test the interaction between NtrY and NtrX we used a bacterial two-hybrid (BTH) system with a LacZ reporter strain. For this purpose, we constructed both, a fusion of PAS-HK-NtrY with the repressor protein from bacteriophage λ (λcI) and NtrX fused to the α-subunit of RNA polymerase (RNAP). The constructions were co-transformed in an *E. coli* reporter strain that increased the β-galactosidase activity according to the interaction strength between the two-hybrid proteins. When the *E. coli* reporter strain was co-transformed with the NtrY and NtrX constructs the β-galactosidase activity increase up to the level of a positive control (Fig. 4B), consistent with a strong interaction between the sensor histidine kinase and its cognate response regulator.

Finally, to demonstrate that NtrY specifically transduces the signal through NtrX, we performed time-course phosphotransference assays. For this aim, recombinant full-length NtrX was purified and incubated with phosphorylated PAS-HK-NtrY at equimolar ratio, and aliquots were taken at different times for SDS-PAGE analysis. NtrY specifically transfers its phosphate to NtrX with a fast kinetics

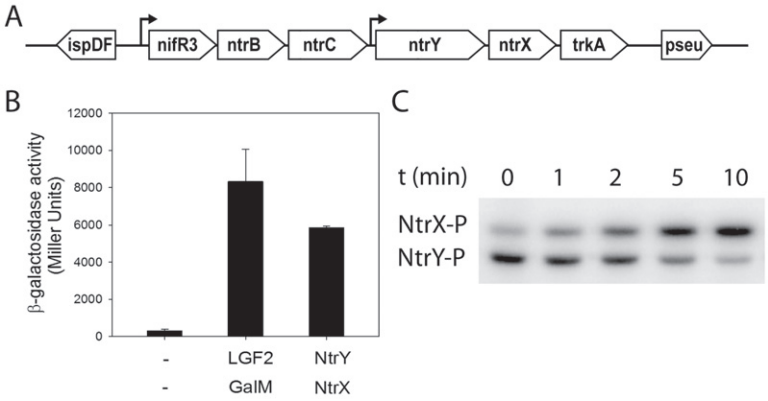

Fig. 4. *Brucella abortus* NtrY genomic context.
A. The genetic organization of the operon containing *nifR3*, *ntrB*, *ntrC*, *ntrY*, *ntrX* and *trkA* was demonstrated by RT-PCR. The arrows in front of *nifR3* and *ntrY* represent promoters whose function was demonstrated by fusion to β-galactosidase gene (see *Results*).
B. Bacterial two-hybrid analysis. PAS-HK-NtrY and full-length NtrX were fused to λcI or RNAP in the pBT and pTRG vector, respectively, and co-transformed in *E. coli* FW102 OL2-62. Fusion to LGF2 and GalM proteins that results in a strong interaction was used as positive control and empty vectors were used as negative control. A representative of three independent experiments is shown. The data shown are mean ± standard deviation of triplicate samples.
C. Phosphotransfer activity. Autoradiogram showing phosphotransfer of ^{32}Pi from PAS-HK-NtrY to recombinant NtrX. PAS-HK-NtrY was reduced with sodium dithionite and incubated with [γ-^{32}P]-ATP for 45 min, followed by incubation with NtrX. The numbers above the gel indicate the time in minutes after the addition of NtrX.

(Fig. 4C). This result clearly indicates that *Brucella* NtrX is the cognate response regulator of NtrY. The incubation of NtrX alone with [γ-^{32}P]-ATP or the previously phosphorylated NtrY with an unrelated response regulator from *B. abortus*, DivK (Hallez *et al*., 2007) show not label in these response regulators further confirming the specificity of interaction between NtrY and NtrX (data not shown).

The NtrY/X two-component system regulates the expression of denitrification pathway genes

To evaluate the role of *B. abortus ntrY/X* genes we constructed a mutant strain deficient in *ntrY/X* by deletion of *ntrY* and insertion of a resistance kanamycin cassette. This mutant strain produces colonies slightly smaller than the wild-type strain on solid media and, in liquid media, replicates to a slower rate than the wild-type strain under aerobiosis or microaerobiosis (Fig. S2). A previous publication showed that a transposon insertion at the *B. suis ntrY* gene affected intracellular survival in an *in vitro* human macrophage infection model (Foulongne *et al*., 2000). Because of this precedent, we analysed the intracellular replication of the *B. abortus ntrY/X* mutant strain, developed for this study, in the murine macrophage cell lines j774 and RAW. The results show that the replication of the mutant strain is lower than the wild-type strain, demonstrating a specific requirement for *ntrY/X* for intracellular replication (Fig. S3).

Brucella possesses all the genes necessary for a complete denitrification pathway, which allows it to use nitrate as final electron acceptor for respiration under low oxygen tension (DelVecchio *et al*., 2002). As NtrY is implicated in redox/oxygen sensing and it transfers the signal to its response regulator NtrX (which has a predicted DNA-binding domain), we hypothesized that this two-component system could regulate the transcriptional levels of specific genes involved in low oxygen tension adaptation. For this reason we decided to test whether the transcriptional levels of the denitrification enzymes are affected in the absence of the *ntrY/X* operon. Using RT-qPCR we analysed in *B. abortus* and the *ntrY* mutant strain the expression profiles of all the operons that encode denitrification enzymes: nitrate reductase (NarGHIJK operon), nitrite reductase (NirKV operon), nitric oxide reductase (NorBCDEFQ operon) and nitrous oxide reductase (NosDFLRXYZ) under aerobiosis or microaerobiosis.

The expression of all the denitrification enzymes is increased in low oxygen tension (Fig. 5). The same results were previously demonstrated for nitrate and nitrous oxide reductases in *B. suis* (Al Dahouk *et al*., 2009) and for nitrate and nitric oxide reductase in *Brucella neotomae* (Baek *et al*., 2004). Interestingly, this increase is significantly affected in the *ntrY* mutant strain, suggesting that the NtrY/X two-component system is involved in the induction of nitrate respiration enzymes by sensing the redox state (Fig. 5).

Discussion

We conducted bioinformatics, biochemical and genetics studies looking for oxygen/redox sensors in *Brucella*. This multidisciplinary analysis allowed us to detect NtrY as a

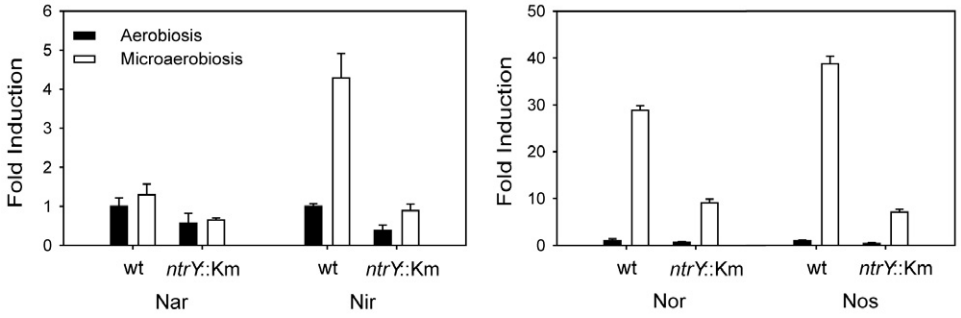

Fig. 5. Expression of enzymes involved in the denitrification pathway. The expression of the operons: nitrate reductase (NarGHIJK), nitrite reductase (NirKV), nitric oxide reductase (NorBCDEFQ) and nitrous oxide reductase (NosDFLRXYZ) were assayed by qRT-PCR in *Brucella abortus* S2308 and in the isogenic *ntrY* mutant strain (*ntrY*::Km) at aerobic (black bars) and microaerobic (white bars) conditions. Bacteria were cultured in rich TSB medium at log-phase in aerated Erlenmeyer or in a microaerobiosis jar and were harvested to extract RNA. Real-time PCR was used to analyse the expression of denitrification enzymes using specific primers. The experiment was repeated three times with similar results. The data shown are mean ± standard deviation of duplicate samples from one representative experiment and is reported as fold induction relative to expression of *B. abortus* S2308 in aerated cultures. Primers for IF-1 gene were used as a housekeeping control.

novel sensor system in α-proteobacteria. Previous to this work, the signal sensed by NtrY was unknown. The present work is the first demonstration that NtrY binds haem as cofactor through its PAS domain and that the oxidation status of the haem-iron modulates the kinase activity. Moreover, we showed that NtrY expression is increased under low oxygen tension.

NtrY belongs to the COG5000 (cluster orthologous group) that at the moment has 192 proteins in 160 proteobacteria species. The mechanism of action described in this work for *B. abortus* NtrY could be relevant for other members of this group, although this remains to be experimentally determined.

The spectra of NtrY-PAS domain are similar to those of other PAS-haem domains such as *E. coli* DOS (Delgado-Nixon *et al*., 2000) and BjFixL (Gong *et al*., 1998). However, the NtrY-PAS domain primary sequence does not clearly align with the sequences of the other sensors. Moreover, the alignment of NtrY sequences from different microorganisms does not show a conserved histidine residue within its PAS domain, as could be expected if the coordination of the haem iron is similar to EcDOS or FixL. Altogether, this suggests that the haem group binds to the PAS domain of NtrY in a different manner to that described for other haem-based redox sensors. We are currently performing structural studies in order to address this question.

We show by UV-visible spectroscopy that deoxy-reduced NtrY rapidly reverts to the oxidized state upon exposure to air, without a stable intermediate oxy-ferrous form. Based on this evidence, we propose that NtrY functions as a redox sensor more than a direct oxygen sensor. A similar redox-mediated mechanism was previously described in *M. tuberculosis* DosS which has a haem GAF domain (Kumar *et al*., 2007). Bacteria growing under microaerobic or anaerobic conditions produce many reducing agents that could be the signal sensed by NtrY such as the reduced quinone pool and NADH. It remains to be determined which is the physiological molecule that reduces NtrY-haem iron.

We have also demonstrated that deoxy ferrous-NtrY binds NO and CO. In the same way than the ferric and ferrous NtrY spectra, the ferrous-CO spectrum is consistent with a hexa-coordinated haem iron. On the other hand, the NO binding produced a spectrum consistent with a penta-coordinated iron haem. This penta-coordinated form is often observed in NO sensors, such as H-NOX, DNR and E75 (Igarashi *et al*., 2004; Reinking *et al*., 2005; Castiglione *et al*., 2009; Derbyshire and Marletta, 2009). The transition from hexa- to penta-coordinated state upon NO binding suggests that this compound triggers the dissociation of the undefined proximal ligand from the iron centre. The binding of NO and CO does not change the NtrY autokinase activity, thus it appears that ferrous-NtrY does not need ligand binding for its function. Similar to that proposed for *M. tuberculosis* DosS, NO and CO ligands could lock these sensor proteins in an active state modulating the persistence of the signal (Kumar *et al*., 2007). The NtrY–NO binding could be physiologically relevant due to the simultaneous presence of NO and low oxygen tension at different stages of *Brucella* infection (intracellular replication inside macrophages, granulomatous lesions). CO, together with NO and hypoxia, has been implicated in the induction of a long bacterial persistence of *M. tuberculosis* inside the host, thus CO is also a relevant host signalling molecule (Kumar *et al*., 2007). It is tempting to think that the same signalling mechanisms are being used by *Brucella*.

As we previously mentioned, NtrY has several putative transmembrane domains suggesting that it is a membrane

protein. Between the third and fourth transmembrane segments of NtrY there is a long amino acid region (100 residues approximately) (Fig. 1A), which is predicted to be localized in the periplasm, and forms a loop that could serve as a second signal-sensing site for another stimuli or a periplasmic redox signal. NtrY also possesses a HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase) domain between the transmembrane helices and the PAS domain. HAMP domains are proposed to link signal transmission between extracellular sensory input domains and cytoplasmic output domains (Appleman *et al*., 2003). This protein architecture could allow NtrY to integrate two or more environmental signals, raising a global response to the conditions found by *Brucella* during its infection.

The phenotypic changes between the NtrY/X mutant strain and the wild type are not very drastic. This could be due to the presence of alternative systems substituting, at least partially, the mutated function. For example, in *Neisseria gonorrhoeae* the FNR mutation (homologous to the anaerobic respiration regulator FNR of *E. coli*) produces total depletion of bacterial growth under anaerobic conditions (Lissenden *et al*., 2000) and a *Shigella flexneri* mutant in FNR is severally impaired in virulence (Marteyn *et al*., 2010). Oppositely, the FNR mutant strain of *B. suis* does not present any defects in virulence (Loisel-Meyer *et al*., 2005). These antecedents suggest a redundancy in *Brucella* oxygen/redox sensor proteins that could mask the bacterial mutant phenotypes. We recently found a genetic interaction between NtrY/X and the redox-responding global two-component regulatory system PrrB/A. A *B. abortus* double mutant strain in *prrB* and *ntrY/X* is severally affected in virulence in cells and mice, meanwhile the simple mutant in *prrB* is not, and a *ntrY/X* mutant is only slightly affected in virulence (M. d. C. Carrica *et al*., unpubl. results). A physical interaction between RegA (PrrA homologous) and NtrX was previously observed in *R. capsulatus*, where it was demonstrated that both response regulators bind closely in the *puf* promoter region (Gregor *et al*., 2007). Thus, it is likely that the functions regulated by the NtrY/X two-component system are also regulated by other regulatory systems that are affected by the absence of PrrB.

Previous evidence links NtrY with bacterial growth under different oxygen tension. *NtrY* and *ntrX* mutant strains in *A. caulinodans* showed an impairment to grow using nitrate as sole nitrogen source, but this defect was only observed under aerobic conditions (Pawlowski *et al*., 1991). In *R. capsulatus* it was demonstrated that NtrX is involved in the regulation of the photosynthetic genes *puf* and *puc*, which are upregulated under low oxygen tension (Gregor *et al*., 2007). In this work we demonstrated that all the enzymes belonging to the denitrification pathway are increased in *B. abortus* under microaerobic conditions.

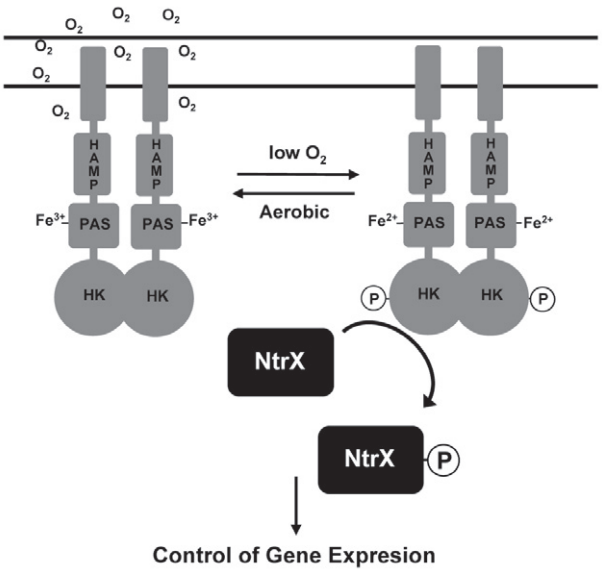

Fig. 6. Model for NtrY/X two-component system action. When bacteria are growing aerobically NtrY is in its inactive, oxidized state. However, under low oxygen tension the haem iron of NtrY is reduced and the histidine kinase activity is induced. Thus NtrY autophosphorylates and transfers the signal to the cognate response regulator NtrX, which, in turn, binds to specific promoter regions and thereby control gene expression.

This induction is partially triggered by the NtrY/X two-component system.

In *Brucella melitensis*, the presence of two Fnr/CRP-type transcriptional regulators involved in the transcriptional control of the denitrification genes, NarR and NnrA, was described (Haine *et al*., 2006). NarR and NnrA do not completely switch off the expression of the denitrification genes, thus it is possible that another regulator, such as NtrX described in this work, may contribute to the regulation of these genes. Alternatively, the NtrY/X system may control the denitrification pathway indirectly through the modulation of the expression of NarR and/or NnrA.

In addition to its role in the denitrification pathway, the nitric oxide reductase (Nor) may also play an important role in the detoxification of the NO produced by macrophages during the innate response against *Brucella* infection (Loisel-Meyer *et al*., 2006). This could contribute to the decreased virulence observed in the NtrY *B. abortus* mutant strain.

In summary, a model could be envisioned based on the results present in this work (Fig. 6). When *Brucella* grows in aerobic conditions, NtrY is in its inactive oxidized form. When oxygen tension decreases, NtrY is reduced by an unknown cellular reductant (e.g. reduced quinone pool or NADH that are produced during anaerobic respiration) and the autophosphorylation is activated. Then, the phosphorylated reduced NtrY transfers the signal to its cognate response regulator NtrX, which in turn interacts with DNA

promoters to activate genes involved in low oxygen tension adaptation and detoxification, such as the denitrification enzymes.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli strains were grown in either solid or liquid Luria–Bertani medium at 37°C and 200 r.p.m. Appropriate antibiotics were added to the following final concentrations: 100 μg ml^{-1} ampicillin, 25 μg ml^{-1} kanamycin, 12.5 μg ml^{-1} tetracycline, 25 μg ml^{-1} chloramphenicol.

All *Brucella* strains used in this study were derived from *B. abortus* 2308 and are listed in Table S1. *Brucella* were grown at 37°C in trypticase soy broth (TSB) or tryptose agar (TA) (DIFCO), supplemented with the 25 μg ml^{-1} kanamycin or 25 μg ml^{-1} ampicillin) when appropriate. All experiments with viable *Brucella* strains were performed in a biosafety level 3 containment laboratory.

DNA manipulations

DNA manipulations were performed according to standard techniques. Plasmids pET24D-PAS-NtrY (encoding NtrY amino acids 394–513), pET24D-PAS-HK-NtrY (encoding NtrY amino acids 402–774) and pET24D-NtrX (encoding full-length NtrX, amino acids 1–453) were generated by polymerase chain reaction (PCR) from *B. abortus* S2308 chromosomal DNA using suitable primers listed in Table 2. Cloning of the products were made between the NcoI and XhoI restriction sites of the pET24D vector (Novagen) for PAS-NtrY and PAS-HK-NtrY and between the BamHI and XhoI sites for NtrX. All the products are under control of the T7 promoter of the vector and are in frame with the six-histidine tag present in the C-terminal end of the proteins. For two-hybrid assays the fragments corresponding to coding regions of PAS-HK-NtrY and full-length NtrX were amplified by PCR using the suitable primers (Table S2) and *B. abortus* 2308 chromosomal DNA as template. The amplified fragments were digested with the NotI and XhoI restriction enzymes and ligated in the same sites from the pBT and pTRG plasmid vectors (BacterioMatch II Two-Hybrid System Kit) (Stratagene) in frame with λ cI and RNAP respectively. The plasmids generated were called pBT-NtrY and pTRG-NtrX. All cloned inserts were DNA sequenced to confirm the absence of mutations.

Expression and purification of recombinant proteins

pET24D plasmids encoding the sequences of PAS-NtrY, PAS-HK-NtrY and NtrX were transformed in *E. coli* BL21(DE3). For PAS-NtrY and PAS-HK-NtrY purification, the cells were grown in LB at 37°C until the A_{600} was 0.5 and the protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 16 h at 28°C. Cells were harvested by centrifugation at 4000 *g* for 20 min, resuspended in lysis buffer [20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF)], and disrupted by sonication with a probe tip sonicator. The total cell lysates were centrifuged at 14 000 *g* for 30 min to remove insoluble protein, cell debris and unbroken cells. The protein in the cell lysate was reconstituted by incubation with haemin (Sigma) dissolved in 0.1 M NaOH at a 2:1 haem/protein ratio at 4°C during 1–2 h. Binding and elution from nickel nitrilotriacetic acid-agarose (Ni-NTA) resin were carried out under native conditions according to the manufacturer's instructions (Qiagen). After binding, the resin was washed with 20–30 ml of washing buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 20 mM imidazole) and then proteins were eluted with 5 ml of elution buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 250 mM imidazole). The eluted proteins were dialysed against 20 mM Tris-HCl pH 8 and 150 mM NaCl. NtrX was purified under denaturing conditions. Cells were grown in LB at 37°C to an A_{600} of 0.5 and induced with 1 mM IPTG for 4 h at 37°C. Then, cells were harvested and resuspended in denaturing buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 8 M Urea), and disrupted by sonication. The total cell lysate was centrifuged at 14 000 *g* for 30 min. Binding and elution from Ni-NTA resin were carried out under denaturing conditions with washing buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 50 mM imidazole, 8 M urea) and elution buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 250 mM imidazole, 8 M urea). Purified NtrX was refolded by dialysis against 20 mM Tris-HCl pH 8 and 300 mM NaCl. The purification procedures of proteins were evaluated in 12–15% SDS-PAGE stained with Coomassie blue.

UV-visible spectral analyses

UV-visible spectra (between 200 and 100 nm) were recorded with a HP8453 diode-array spectrometer in a septum sealed 1 cm path quartz cuvette in 20 mM Tris-HCl pH 8 and 150 mM NaCl at 20°C. To obtain the spectra of totally reduced NtrY, 1 ml solution of protein (*c.* 5×10^{-6} M) was gently flushed with Ar for 10–20 min, and afterwards aliquots (1–5 μl) of freshly prepared anaerobic solution of sodium dithionite (DTH) (*c.* 1 mM) were injected using a Hamilton syringe until total conversion of the spectra was achieved. CO-bound NtrY was obtained by bubbling CO gas to a reduced solution of NtrY. The NO-bound NtrY was obtained by adding to a ferrous NtrY slightly excess of the efficient NO donor *N*-nitrosomelatonin and a short light flash, as described in previous works (De Biase *et al.*, 2005; Suarez *et al.*, 2010). Ferric CN derivative was obtained by the addition of KCN (1 mM) to ferric NtrY.

Phosphorylation assay

The autophosphorylation reaction of the different forms (Fe^{3+}, Fe^{2+}, Fe^{2+} exposed to air, Fe^{2+}–NO and Fe^{2+}–CO) of PAS-HK-NtrY were assayed in a reaction mixture containing 10 μM of protein 0.5 μCi [γ-^{32}P]-ATP (PerkinElmer Life Sciences), 20 mM Tris-HCl, pH 8, 50 mM NaCl, 100 μM ATP and 5 mM $MgCl_2$. The reaction was incubated at room temperature and was stopped at different times by addition of an equal volume of Laemmli's sample buffer. For phosphotransfer analysis an equimolar amount of the purified response regulator NtrX was added to the phosphorylation reaction mixture containing deoxy ferrous-PAS-HK-NtrY previously autophosphorylated for 60 min and aliquots were draw at different times. Samples were separated in 12% SDS-PAGE and, after electrophore-

sis, the gels were dried and exposed to a Storage Phosphor Screen (GE Healthcare). The screen was scanned using a Storm Image and Detection system (Molecular Dynamics).

Bacterial two-hybrid assay

pBT and pTRG plasmids (100 ng each) containing the different inserts were co-transformed by electroporation into FW102 O_L2-62 *E. coli* reporter strain (Deaconescu *et al.*, 2006). Cells were plated on LB agar containing 25 μg ml^{-1} kanamycin, 12.5 μg ml^{-1} tetracycline and 25 μg ml^{-1} chloramphenicol. Two colonies from each plate were selected, transferred to liquid medium with the same antibiotics plus 1 mM IPTG, and incubated at 37°C with agitation. After 24 h, β-galactosidase activity was assayed from 200 μl of these cultures using a standard Miller protocol.

Construction of ntrY *mutant strain*

For the construction of the *ntrY* (BAB1_1139) mutant strain a fragment of 643 pb of the *ntrY* was replaced with a kanamycin resistance cassette. Two PCR fragments were generated from *ntrY*-flanking regions. Oligonucleotides ntrY1 and ntrY2 were used to amplify a 654 bp fragment and oligonucleotides ntrY3 and ntrY4 were used to amplify a 636 bp fragment. Both fragments (containing complementary regions) were ligated by overlapping PCR using oligonucleotides ntrY1 and ntrY4. The resulting fragment containing the flanking *ntrY* regions was cloned into the vector pGEM-T easy (Promega) which is suicide in *Brucella*. Then the kanamycin resistance cassette was introduced in a BamHI site generated in the overlapping PCR product. This plasmid was introduced in *B. abortus* 2308 by electroporation. Homologous recombination events were selected by resistance to kanamycin and sensitivity to ampicillin in TSA plates. The excision of the plasmid and the generation of the mutant strain (named *ntrY*::km) by allelic exchange were confirmed by colony PCR.

Isolation of total RNA from B. abortus *bacterial cell culture*

Brucella abortus strain 2308 and *ntrY* mutant were grown in rich TSB medium at 37°C. Bacteria were grown in aerobic conditions using Erlenmeyer flasks at 200 r.p.m. The microaerobic and anaerobic were generated using an anaerobic jar with the GENbox microaer and anaer generators (Biomerieux). The presence of oxygen was monitored using Anaer Indicator Biomerieux. About 7×10^8 bacteria in log-phase culture were harvested. The supernatant was removed, and the pellet was resuspended in 100 μl of a solution containing 84 μl of TE buffer, 15 μl of 10% SDS and 1 μl of 10 μg μl^{-1} proteinase K. The sample was then incubated at 37°C for 1 h and 600 μl of Qiagen RLT lysis buffer was added. Total RNA was isolated following the Qiagen RNeasy Mini Bacterial protocol. DNA was subsequently removed by digestion with DNase RNase-free according to the manufacturer instructions (Promega). RNA was quantified using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific).

Real-time quantitative RT-PCR assay

Reverse-transcription was performed with a transcriptor first-strand superscript III cDNA kit (Invitrogen) using random decamer primers (Invitrogen) and RNasin ribonuclease inhibitor (Promega). Complementary DNA (cDNA) samples were used as templates in real-time PCRs. Primers were designed with Primer3 program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table 2. PCR products ranged from 90 to 110 bp. Real-time PCRs were performed with SYBR Green in 96-well plates in an Mx3005P Stratagene instrument and analysed with MXPro and LinReg programs. Relative quantification using a standard curve method was performed for each set of primers. Results for each target mRNA were normalized to *B. abortus* initiation factor-1 (IF-1) mRNA.

Determination of operon organization and promoter activity

To determinate the operon organization we performed RT-PCR of intergenic regions using cDNA of *B. abortus* S2308 and primers listed in Table S2.

The promoter regions of the operons *nifR3-ntrB-ntrC* and *ntrY-ntrX-trkA* were fused to the *lacZ* reporter gene. A 4.5 kb fragment containing the *lacZ* promoter-probe cassette was released from plasmid pAB2001 after digestion with XhoI and KpnI restriction enzymes and ligated to the same restrictions sites in pBBR1MSC1 (Kovach *et al.*, 1994), (the resulting vector was called pBBR-LacZ). A fragment of 582 pb upstream of *nifR3* and a fragment of 485 pb upstream of *ntrY* were amplified by PCR using the primers prom-*nifR*ff and prom-*nifR*rev for *nifR3* and, prom-*ntrY*ff and prom-*ntrY*rev primers for *ntrY* (Table S2). The resulting PCR product was digested with the BamHI and XhoI enzymes and cloned in the same sites of the pBBR-LacZ vector in fusion with *lacZ* gene, generating the plasmid pBBR-prom-*nifR*-LacZ and pBBR-prom-*ntrY*-LacZ. This constructs were DNA sequenced with the same primers. For promoter activity determination pBBR-prom-*nifR*-LacZ, pBBR-prom-*ntrY*-LacZ and pBBR-LacZ as negative control were electroporated in *B. abortus* S2308. Bacteria were grown to log-phase and β-galactosidase activity was determined and expressed in Miller units $(A_{420}/\text{volume} \times \text{OD}_{600}) \times 100$.

Macrophage cell infection

J774 and RAW 264.7 murine macrophages were seeded at a density of 2×10^5 cells per well in 24-well culture plates. Stationary-phase cultures of *B. abortus* S2308 and *ntrY*::km were added to the cells at a multiplicity of infection (moi) of 100. Culture plaques containing infected cells were centrifuged at 1000 r.p.m. for 10 min and incubated at 37°C for 60 min to allow bacterial uptake and invasion. The extracellular bacteria were removed by washing twice with PBS and incubated with 50 μg ml^{-1} gentamicin for 1 h. The cells were then incubated with 25 μg ml^{-1} gentamicin for the rest of the experiment. At the indicated times post infection, the cells were lysed with 1 ml of 0.1% Triton X-100 and the number of bacterial cells were determined by plating serial dilutions on TSA agar plates with the appropriate antibiotic.

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