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# The two-component systems PrrBA and NtrYX co-ordinately regulate the adaptation of *Brucella abortus* to an oxygen-limited environment

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## Summary

***Brucella*** **is the causative agent of the zoonotic disease brucellosis, which is endemic in many parts of the world. The success of** ***Brucella*** **as pathogen relies in its ability to adapt to the harsh environmental conditions found in mammalian hosts. One of its main adaptations is the induction of the expression of different genes involved in respiration at low oxygen tension. In this report we describe a regulatory network involved in this adaptation. We show that** ***Brucella abortus*** **PrrBA is a functional two-component signal transduction system that responds to the redox status and acts as a global regulator controlling the expression of the regulatory proteins NtrY, FnrN and NnrA, which are involved in the adaptation to survive at low oxygen tension. We also show that the two-component systems PrrBA and NtrYX co-ordinately regulate the expression of denitrification and high-affinity cytochrome oxidase genes. Strikingly, a double mutant strain in the** ***prrB*** **and** ***ntrY*** **genes is severely impaired in growth and virulence, while the** ***ntrY*** **and** ***prrB*** **single mutant strains are similar to wild-type** ***B. abortus*****. The proposed regulatory network may contribute to understand the mechanisms used by** ***Brucella*** **for a successful adaptation to its replicative niche inside mammalian cells.**

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## 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 an illness called brucellosis. This bacterium causes a chronic infection that produces abortion and sterility in domestic mammals, leading to severe economic losses and a chronic debilitating febrile disease in humans (Corbel, 1997).

In comparison with other pathogenic bacteria, *Brucella* lacks classical virulence factors such as exotoxins, invasive proteases, toxic lipopolysaccharide, capsules, virulence plasmids and lysogenic phages. The virulence of *Brucella* resides mostly in its ability to survive and multiply within professional and non-professional phagocytes (Detilleux *et al*., 1990; Gorvel and Moreno, 2002). This intracellular residence within the macrophage is a hallmark in the life cycle of *Brucella* (Celli *et al*., 2003). The adaptation of *Brucella* to the host environment includes the correct regulation of genes involved in virulence and metabolism. The conditions encountered by *Brucella* inside the host cell are nutritional deprivation, exposure to acidic pH, reactive oxygen and nitrogen species and low oxygen tension. It was postulated that in the intracellular niche of *Brucella* nitrogen oxide species are present, which could be used for respiration instead of oxygen (Kohler *et al*., 2002; Roop *et al*., 2009).

Several bacteria have the capability to induce metabolic changes upon sensing of oxygen-limiting conditions. This adaptation is required to survive in harsh environments (Bueno *et al*., 2012). For example, in *Mycobacterium tuberculosis*, hypoxia induces dormancy, which is important for the survival of bacteria during long periods of time inside the host (Wayne and Sohaskey, 2001). The adaptation of *Brucella* to the conditions encountered inside the replicative niche is also probably triggered by environmental signals including low oxygen tension. Like many bacteria, *Brucella* uses two-component systems to sense and adapt to environmental changing conditions (Gao and Stock, 2009). Recently, we have described *Brucella abortus* NtrYX as a redox sensor regulatory two-component system that is involved in the adaptation to

oxygen-limiting conditions (Carrica *et al*., 2012). The NtrY sensor histidine kinase is a haem protein that is activated under low oxygen tension and regulates the expression of denitrification genes allowing respiration of nitrate instead of oxygen (DelVecchio *et al*., 2002). In *Brucella*, this pathway is upregulated under low oxygen tension and is involved in the bacterial virulence (Baek *et al*., 2004; Haine *et al*., 2006; Loisel-Meyer *et al*., 2006; Al Dahouk *et al*., 2009).

In addition to the NtrYX system, the transcriptional regulators NnrA, NarR and FnrN belonging to the Crp/Fnr family are involved in the adaptation to microaerobiosis in *Brucella* (Baek *et al*., 2004; Loisel-Meyer *et al*., 2005; Haine *et al*., 2006). In *Brucella melitensis*, NnrA and NarR are involved in the regulation of the denitrification pathway in response to oxygen limitation and increase of nitrogen oxides concentration (Baek *et al*., 2004; Haine *et al*., 2006). These proteins are proposed as nitric oxide and nitrate sensors respectively (Korner *et al*., 2003). FnrN is suggested as a direct oxygen sensor and is involved in the induction, under microaerobiosis, of the high-affinity cytochrome *cbb3* oxidase (ccoNOPQ operon) in *B. suis* (Kiley and Beinert, 1998; Loisel-Meyer *et al*., 2005). *Brucella* mutants in high-affinity cytochrome oxidases are less virulent indicating that this metabolic adaptation is crucial for a successful bacterial infection (Endley *et al*., 2001; Kim *et al*., 2003; Jimenez de Bagues *et al*., 2007).

PrrBA (also called RegBA) is a global regulator two-component system, present in almost all alphaproteobacteria, which regulates many metabolic pathways including the denitrification and high-affinity cytochrome oxidase genes (Elsen *et al*., 2004; Wu and Bauer, 2008). PrrB from *Rhodobacter capsulatus* is a membrane-bound histidine kinase that senses the redox state by two mechanisms: a periplasmatic ubiquinone binding region and a redox-active cysteine residue present in the cytosolic region (Swem *et al*., 2003; 2006). Recently, Wu *et al*. demonstrated that a sulphenic acid modification at this cysteine plays a key regulatory role *in vivo* as a redox switch (Wu *et al*., 2013). In the human pathogen *M. tuberculosis*, PrrBA is expressed during intracellular replication in human macrophages and is essential for bacterial viability (Ewann *et al*., 2004; Haydel *et al*., 2012). The genomes of *Brucella* encode for a homologous PrrBA system. A proteomic analysis showed that the PrrA protein is differentially expressed during *Brucella* intracellular replication (Lamontagne *et al*., 2009).

In this report we describe a regulatory network involved in the adaptation of *Brucella* to oxygen-limiting conditions. We show that the *B. abortus* PrrBA two-component system is a redox sensor involved in the regulation of NtrYX, FnrN and NnrA. We also show that the two-component systems PrrBA and NtrYX co-ordinately regulate the expression of denitrification and high-affinity cytochrome oxidase genes. Strikingly, a double mutant strain in the *prrB* and *ntrY* genes is severely impaired in growth and virulence. These results highlight the importance of redox sensor regulatory systems in the adaptation of *Brucella* to the conditions encountered during host infection.

## Results

### *The* B. abortus *PrrBA two-component system is activated by a reducing environment*

The *Brucella* spp. genomes code for proteins homologous to the sensor histidine kinase PrrB (BA-PrrB) and its cognate response regulator PrrA (BA-PrrA) (BAB1_0132 and BAB1_0136 ORFs in *B. abortus* S2308 genome respectively). BA-PrrB and BA-PrrA proteins are similar to previously characterized PrrBA proteins from α-proteobacteria (*R. capsulatus*, *Rhodobacter sphaeroides*, *Agrobacterium tumefaciens*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*) (Fig. S1). The characteristic PrrB sequence motifs are conserved in BA-PrrB, including a conserved H-Box (which comprises the phosphoacceptor histidine residue), a quinone binding site in the transmembrane regions and a redox-active cysteine residue within the redox box located in the cytoplasmic region (Elsen *et al*., 2004) (Fig. S1A). BA-PrrA also presents two characteristic sequence motifs: a phosphate-accepting aspartate residue and a highly conserved HTH–DNA binding motif (Elsen *et al*., 2004) (Fig. S1B).

The *R. capsulatus* PrrB histidine kinase increases its autokinase activity under reducing conditions. Two mechanisms have been proposed to sense changes in redox state: (i) direct binding of quinone to the conserved periplasmic site and (ii) by means of a conserved reactive cysteine located in the cytoplasmic redox box (Swem *et al*., 2003; 2006). Mutation of this residue showed both an unregulated histidine kinase activity and lack of control of gene expression under different oxygen conditions (Swem *et al*., 2003).

In order to study the regulatory role of this cysteine residue in BA-PrrB, we constructed a recombinant protein that contains the redox box and lacks the transmembrane regions. We analysed its autokinase activity in presence and absence of a reducing agent (DTT). The autophosphorylation activity is increased under reducing conditions (Fig. 1A). This increase in the autophosphorylation activity is specific for BA-PrrB since a non-related histidine kinase from *B. abortus* does not show changes in autophosphorylation upon addition of DTT (Fig. 1A).

In addition, we performed time-course phosphotransfer assays between phosphorylated BA-PrrB and its predicted response regulator BA-PrrA. BA-PrrB was activated with 10 mM DTT and incubated with radioactive ATP for 60 min. At this point a stoichiometric amount of BA-PrrA was added. The phosphoryl group was

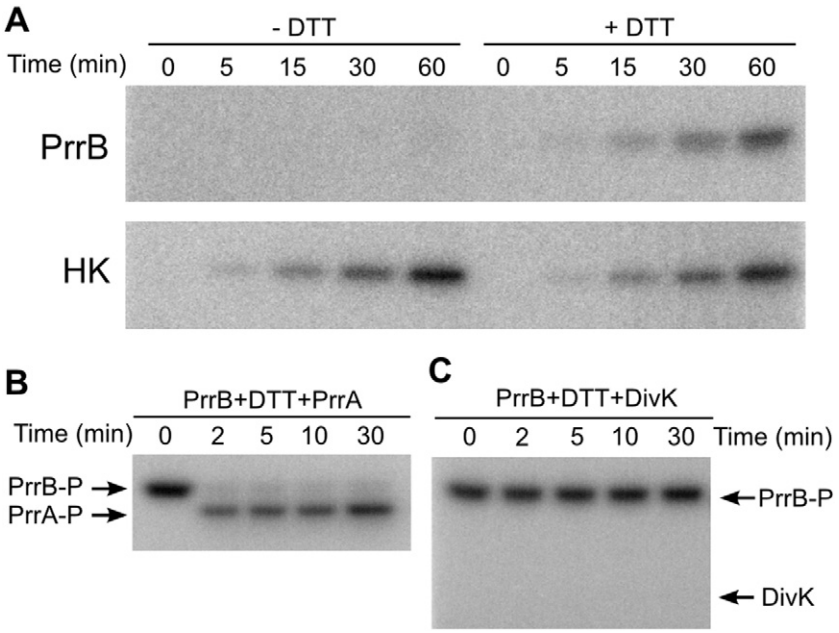


**Fig. 1.** PrrBA is a two-component system activated by redox state.
A. Autophosphorylation assays of purified BA-PrrB and control histidine kinase (HK). Each protein (2.5 μM) was incubated with or without 10 mM DTT and [γ-$^{32}$P]-ATP at the indicated times (see *Experimental procedures* section for details).
B. Autoradiogram showing the phosphotransfer activity from BA-PrrB to BA-PrrA. Purified BA-PrrB was reduced with 10 mM DTT and incubated with [γ-$^{32}$P]-ATP for 1 h, followed by incubation with an equimolar amount of purified BA-PrrA.
C. Autoradiogram showing the lack of phosphotransfer activity from BA-PrrB to DivK, under the same experimental conditions. The numbers above the gel indicate the time in minutes after the addition of BA-PrrA or DivK.

completely transferred from the histidine kinase to the response regulator in less than 2 min (Fig. 1B) demonstrating that BA-PrrA is the response regulator partner of BA-PrrB. This phosphotransfer reaction is specific since phosphorylated BA-PrrB was unable to transfer the phosphoryl group to the unrelated response regulator from *B. abortus*, DivK (Fig. 1C). The fast phosphotransfer kinetics between BA-PrrB and BA-PrrA and the absence of phosphotransfer between BA-PrrB and DivK indicate that PrrBA form a two-component system. The conserved homology and common activation mechanisms between PrrB from *R. capsulatus* and *B. abortus* suggest that BA-PrrB is functional *in vivo* and it could act as a redox sensing histidine kinase.

### B. abortus *PrrB controls the induction of other regulatory genes in microaerobiosis*

The PrrBA two-component system has been described in *R. sphaeroides*, *R. capsulatus*, *B. japonicum* and *A. tumefaciens* as a global regulator for metabolic adaptation to low oxygen tension conditions (Bauer *et al*., 1998; Baek *et al*., 2008; Eraso *et al*., 2008). Oxygen and/or redox sensor systems present in bacteria are interconnected to induce metabolic adaptation in response to low oxygen tension (Torres *et al*., 2011; Bueno *et al*., 2012). In *Brucella*, we have previously shown that NtrYX is a two-component redox sensor system (Carrica *et al*., 2012). Thus, we wondered if there is a cross regulation between PrrBA and NtrYX. With this aim, we constructed two *B. abortus* mutant strains, one deficient in the *prrB* gene (by deletion of *prrB* and insertion of a resistance kanamycin cassette) and another in the *ntrY* gene (previously described in Carrica *et al*., 2012). We cultured *B. abortus* wild-type and mutant strains in aerobiosis and microaerobiosis, and the expression of the *ntrY* and *prrB* genes was analysed by qRT-PCR. Under microaerobiosis, PrrBA strongly regulates the expression of *ntrY* (Fig. 2A) while NtrYX does not affect the expression of *prrB* (Fig. 2B). The NarR, NnrA and FnrN transcriptional regulators are also involved in the metabolic adaptation to microaerobiosis (Baek *et al*., 2004; Loisel-Meyer *et al*., 2005; Haine *et al*., 2006). However, in *B. abortus*, NarR is absent due to a genomic deletion that also includes the *narK* gene (Chain *et al*., 2005). Thus, we evaluated the expression of *nnrA* and *fnrN* in *B. abortus* wild-type and the mutant strains *prrB* and *ntrY* under aerobic or microaerobic conditions. Loisel-Meyer *et al*. showed that the expression of *fnrN* is induced under microaerobic conditions (Loisel-Meyer *et al*., 2005). We demonstrated that this induction is abolished in the *prrB* mutant strain but is not affected in the *ntrY* mutant (Fig. 2C). In addition, we observed a strong induction of *nnrA* gene expression in microaerobiosis and that this induction is affected in both mutant strains (Fig. 2D). Together, these results suggest that the PrrBA system may function as a global regulator of the NtrY, FnrN and NnrA regulatory systems, whereas NtrYX system only affects the expression of *nnrA*.

### *PrrBA and NtrYX act in concert to regulate high-affinity cytochrome* cbb3 *oxidase and denitrification genes*

It has been previously demonstrated that the PrrBA two-component system from *Rhodobacter* spp. regulates the

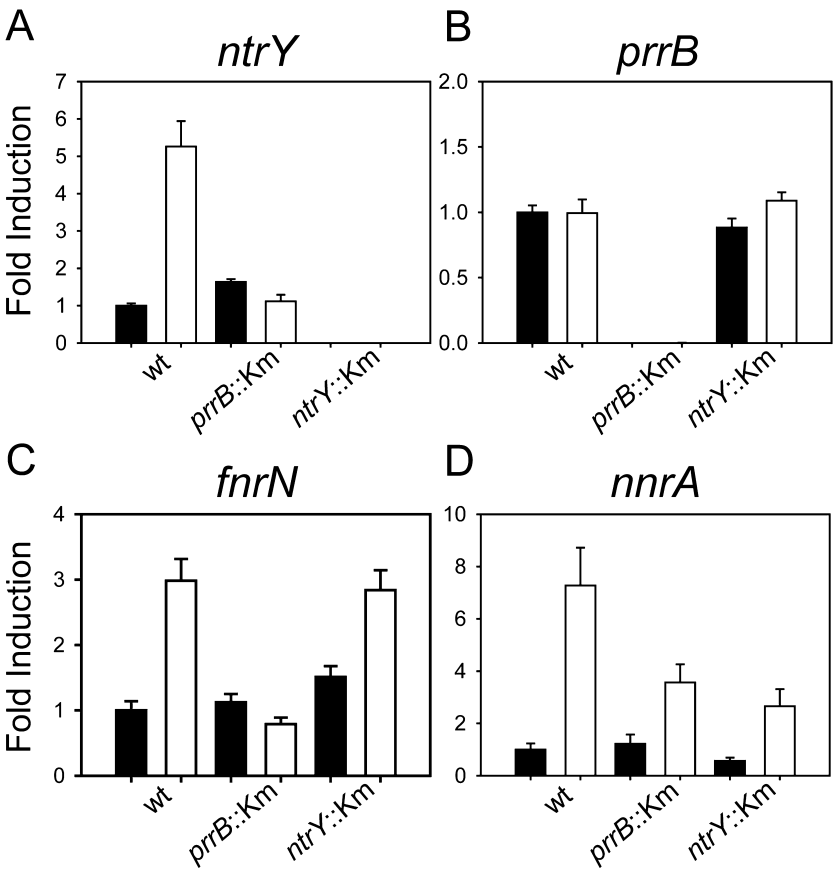


**Fig. 2.** Expression of *Brucella* regulatory genes. The expression of *ntrY* (A), *prrB* (B), *fnrN* (C) and *nnrA* (D) genes were analysed by qRT-PCR in *B. abortus* S2308 and in the isogenic *prrB* (*prrB*::Km) and *ntrY* (*ntrY*::Km) mutant strains under aerobic (black bars) and microaerobic (white bars) conditions using specific primers. The experiments were repeated at least three times with similar results. The data shown are mean ± standard deviation of duplicate samples from one representative experiment and are reported as fold induction relative to expression of these genes in *B. abortus* S2308 in aerated cultures. Expression of the IF-1 gene was used as a housekeeping control.

expression of high-affinity cytochrome oxidases and denitrification genes (Swem *et al.*, 2001; Laratta *et al.*, 2002). Recently, we have reported that in *B. abortus* NtrYX controls the induction of denitrification genes (Carrica *et al.*, 2012). In addition, the results showed in Fig. 2 suggest that PrrBA and NtrYX have overlapping functions in the regulation of the above mentioned respiratory electron transfer systems in *Brucella*. To confirm this observation we constructed a double mutant strain in *ntrY* and *prrB* (by an in-frame deletion of the *prrB* gene in the *ntrY* mutant strain) and the expression of cytochrome oxidases and denitrification genes was evaluated by qRT-PCR. The *B. abortus* wild-type, *ntrY*, *prrB* and *ntrY-prrB* mutant strains were grown under aerobic or microaerobic conditions. In the single mutants *ntrY* and *prrB*, we observed a clear decrease in the expression of the cytochrome *c* type-*cbb3* oxidase (*ccoNOQP operon*) and denitrification genes (*narGHIJK*, *nirKV*, *norBCDEF* and *nosDFLRXYZ* operons) (Fig. 3) indicating that PrrBA and NtrYX positively regulate the expression of these genes. Strikingly, the double mutant *ntrY-prrB* showed an almost complete lack of expression of all these genes under microaerobic conditions (Fig. 3). In contrast, the cytochrome *bd* oxidase (*cydB*) is regulated by PrrBA but is not significantly affected by NtrYX (Fig. 3). These results suggest that at least one of the two-component systems, either PrrBA or NtrYX, is needed for the induction of the denitrification and cytochrome *cbb3* genes under microaerobic conditions. Overall, data are consistent with a model in which PrrBA acts upstream of NtrYX, with NtrYX having some activity in the absence of PrrB.

### *PrrA is a direct regulator of denitrification, cytochrome oxidases and regulatory genes*

PrrA is a response regulator comprising a C-terminal domain with DNA binding capacity (Elsen *et al.*, 2004). We performed electrophoretic mobility shift assays (EMSA) to determine if BA-PrrBA only regulates the expression of denitrification and cytochrome oxidase genes through NtrYX, FnrN and NnrA or if BA-PrrA also directly binds promoter regions of the target genes. Increasing concentrations of phosphorylated BA-PrrA were incubated with DNA probes corresponding to the promoter regions of nitrite (pNir) and nitric oxide reductases (pNor), cytochrome *c* type-*cbb3* oxidase (pccoN), *fnrN* (pfnrN), *nifN3* (pnifR3) and *ntrY* (pYX) genes (Fig. 4A). Figure 4B shows that BA-PrrA binds to all these promoter regions (Fig. 4B). The binding of BA-PrrA to the promoter DNA probes is specific since an unrelated DNA

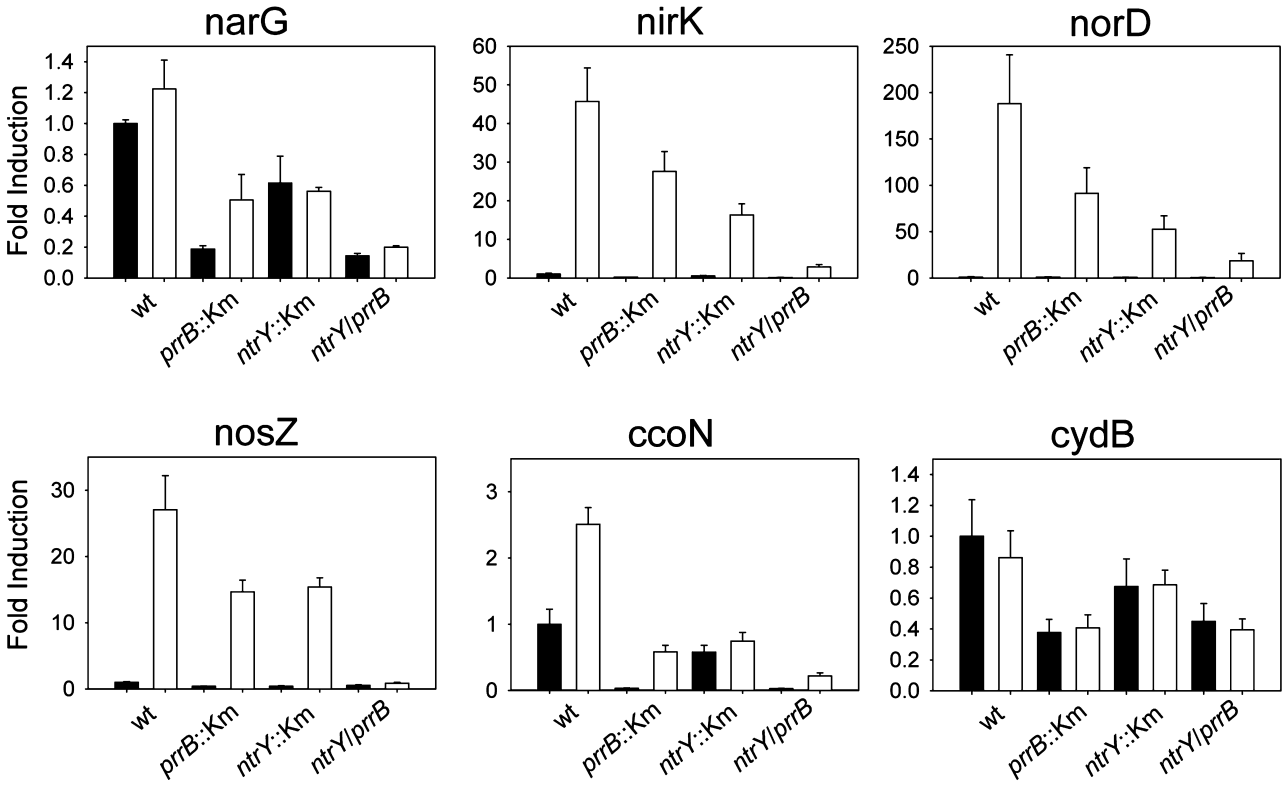


**Fig. 3.** Expression of high-affinity cytochrome oxidases and denitrification genes. The expression of one gene of each operon was measured: *ccoN* from cytochrome *c*-type *cbb3* oxidase, *cydB* from cytochrome *bd* ubiquinol oxidase, *narG* from nitrate reductase, *nirK* from nitrite reductase, *norD* from nitric oxide reductase and *nosZ* from nitrous oxide reductase. The expression of each gene was assayed by qRT-PCR in *B. abortus* S2308 wild-type, *prrB* and *ntrY* single mutants and, the *ntrY-prrB* double mutant under aerobic (black bars) and microaerobic (white bars) conditions using specific primers. The experiment was repeated at least 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. Expression of the IF-1 gene was used as a housekeeping control.

control probe incubated with the same amounts of protein did not show any gel shift (Fig. 4C). The binding specificity of BA-PrrA to the pNir promoter was further confirmed by a competition assay: when the pNir–BA-PrrA complex was incubated with an excess of unlabelled control DNA, no changes were observed in the mobility shift; however, when the unlabelled pNir probe was added, a clear increment of free labelled probe was observed (Fig. 4C).

Thus, we were able to confirm by EMSA that most of the genes previously described by qRT-PCR analysis of the *prrB* mutant strain are being directly regulated by BA-PrrA. Besides, as the *nnrA* gene forms an operon with *nirKV* (Fig. S2), we were able to demonstrate that BA-PrrA also directly regulates *nnrA* expression by binding to the promoter upstream to *nirK*.

Noteworthy, in the case of the *nifR3-ntrBC-ntrYX-trkA* operon, BA-PrrA binds to two promoter regions: one upstream to *nifR3* and the other upstream to the *ntrY* genes (Fig. 4A and B) indicating that the PrrBA system regulates not only NtrYX but also the NtrBC two-component signal transduction system, shown to play a central regulatory role in nitrogen assimilation (Reitzer and Schneider, 2001; Ninfa and Jiang, 2005).

### *NtrYX and PrrBA systems act co-ordinately in the virulence of* B. abortus

Adaptation to oxygen-limiting conditions is relevant for *Brucella* pathogenesis, thus sensing of redox state, as an indirect manner to measure the oxygen concentration, could also be important for bacterial infection. We have shown that NtrYX and PrrBA are redox sensors that control relevant genes for intracellular replication. Thus, we decided to evaluate the replication and virulence of the *prrB*, *ntrY* and the *ntrY-prrB* double mutant strains.

Under both aerobic and microaerobic conditions, the *prrB* and *ntrY* single mutants showed no significant difference in growth relative to the *B. abortus* wild-type strain (Fig. 5A). However, the double mutant exhibited growth impairment under both conditions: in aerobiosis we observed a protracted growth lag of the double mutant compared with the other strains, without significant differ-

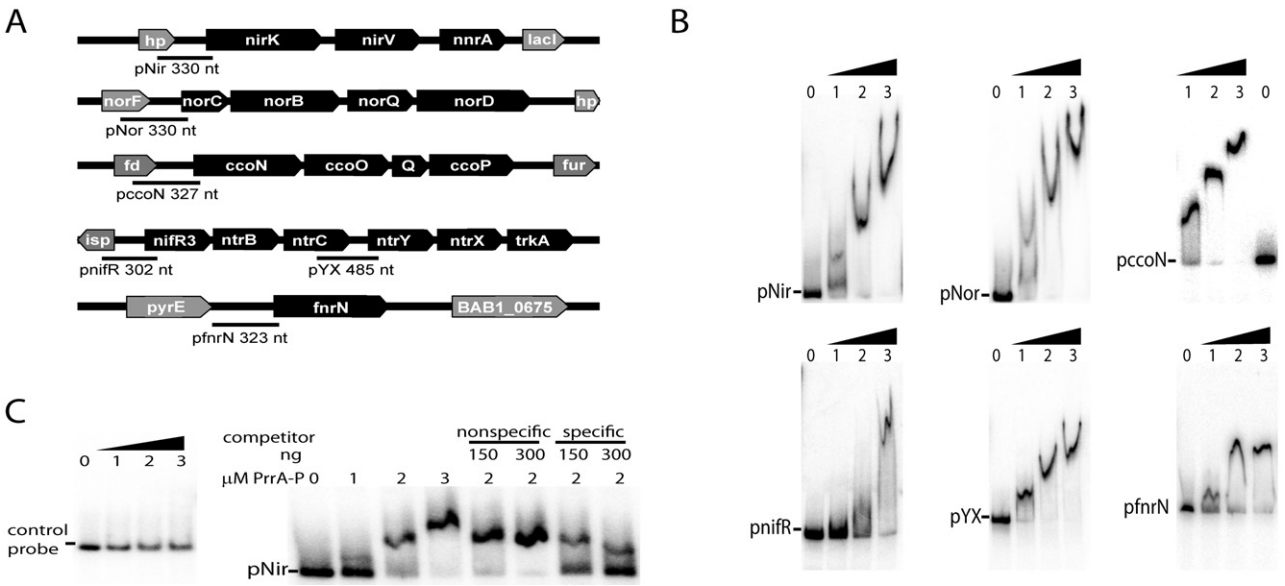


**Fig. 4.** Binding of BA-PrrA to specific promoter regions.
A. Schematic representation of *B. abortus* genomic regions corresponding to the zone used in EMSA assays. Black boxes correspond to genes that are predicted (norCBQD and ccoNOQP) or demonstrated (nirKV-nnrA and nifR3-ntrBC-ntrYX-trkA) as belonging to the same operon. Solid lines below the genomic regions represent probes used in EMSA assays (probe length is indicated).
B. Phosphorylated BA-PrrA (BA-PrrA-P) binding to nirKV-nnrA (pNir), norCBQD (pNor), cconNOQP (pccoN), nifR-ntrBC-ntrYX-trkA (pnifR and pYX) and fnrN (pfnrN) promoter regions evaluated by EMSA. The numbers above the gels indicate the concentration in µM of BA-PrrA-P used in the assay.
C. EMSA assay with a control probe (left) and competition assay (right). pNir probe, BA-PrrA-P and 150 or 300 ng of unlabelled competitors were used for the competition assays. The non-specific competitor corresponds to the probe previously used as control and specific competitor corresponds to pNir unlabelled probe.

ences in the growth rate; under microaerobiosis all strains showed a similar lag phase but the double mutant showed a slight decrease in the growth rate (Fig. 5A).

In order to evaluate the virulence of all strains, we performed infection assays in macrophages and mice. The *prrB* mutant is not attenuated in mammalian cells or mice infections while the *ntrY* mutant only exhibits modest attenuation in cells and is not affected in mice. Strikingly, the *ntrY-prrB* double mutant exhibits severe attenuation in virulence in both cells and mice infections (Fig. 5B and C). This synergistic effect suggests that PrrBA and NtrYX cooperatively participate in regulatory pathways that control genes important for intracellular replication and mice infection.

## Discussion

The results presented in this work allow us to propose a complex regulatory network for *Brucella* adaptation to the oxygen-limiting conditions that are present in the replicative niche. We demonstrated that *B. abortus* PrrBA is a functional two-component signal transduction system that is sensitive to the redox status and acts as a global regulator controlling the microaerobic expression of NtrY, FnrN and NnrA. Besides, we showed that NtrYX is also involved in NnrA regulation. PrrBA and NtrYX act cooperatively in the control of the expression of dentrification pathway and high-affinity cytochrome oxidase genes. We demonstrated that this cooperative effect of PrrBA and NtrYX also affects the growth and virulence of *Brucella*.

PrrBA has been characterized as global regulator of gene expression (Elsen *et al*., 2004). A combined transcriptome and proteome analysis revealed that approximately 25% of the genome of *R. sphaeroides* is regulated directly or indirectly by PrrA (Eraso *et al*., 2008). Thus, other transcriptional regulators might act in co-ordination with PrrBA in order to fine tune the expression of specific genes. For example, in *R. capsulatus* the expression of cytochrome *cbb3* and ubiquinol oxidases is controlled by multiple regulatory proteins: RegBA-FnrL-HvrA and RegBA-FnrL-HvrA-CrtJ-AerR respectively (Swem and Bauer, 2002). In this work we showed that PrrBA from *Brucella* regulates the expression of denitrification pathway and high-affinity cytochrome oxidase genes. Denitrification genes are also regulated by NtrYX and NnrA and cytochrome *cbb3* oxidase is regulated by NtrYX and FnrN (Baek *et al*., 2004; Loisel-Meyer *et al*., 2005; Haine *et al*., 2006).

Frequently, bacteria integrate several regulatory systems in order to sense different environmental signals, constituting sophisticated regulatory networks (Bueno *et al*., 2012). For example, in *B. japonicum* the denitrifica-

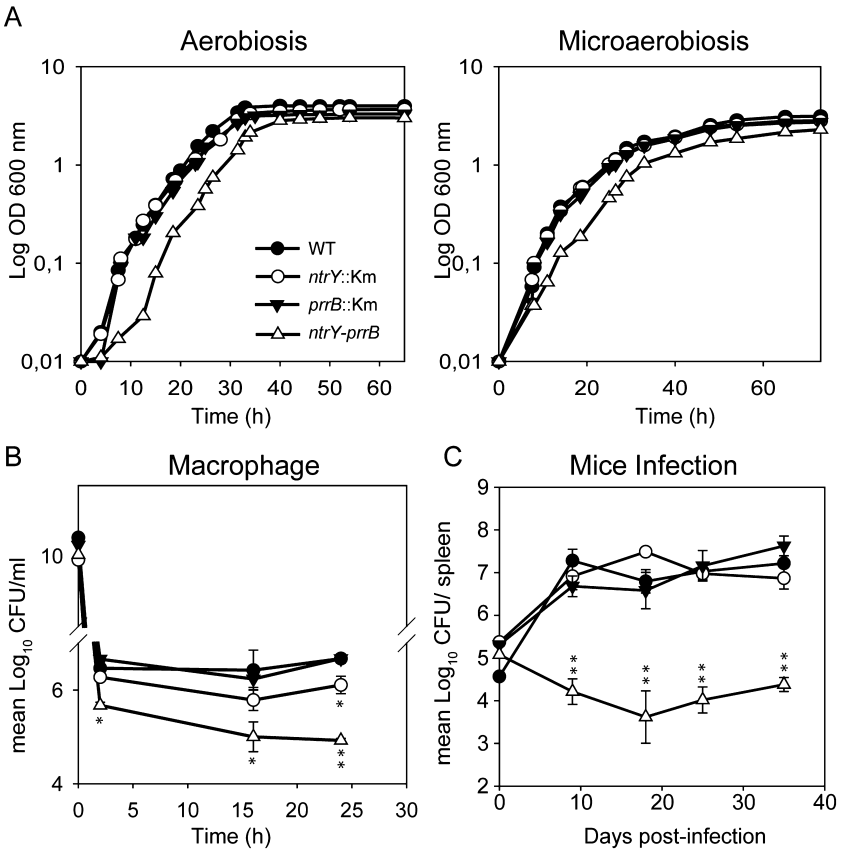


**Fig. 5.** Growth and virulence assays of *B. abortus* S2308 (close circles), *ntrY::Km* mutant (open circles), *prrB::Km* mutant (close triangles) and double mutant *ntrY-prrB* (open triangles) strains.
A. Growth curve in aerobiosis or microaerobiosis. A saturated culture of each strain was diluted 1:200 in TSB and cultivated in aerobic or microaerobic conditions. The $OD_{600}$ was measured at the indicated time points until 68 h and data are presented in a semilog plot. Growth curves of *B. abortus* 2308 and *ntrY-prrB* double mutant strains show statistically significant differences (*P*-value < 0.01) using two-tailed paired *t*-test. Data shown are representative from four independent performed experiments.
B. Invasion and intracellular replications of the different strains in the J774 murine macrophage cell line. Macrophages cells were infected with a moi = 100, cells were lysed at 2, 16 and 24 h post infection and the amount of viable bacteria was determined by plating in TSB agar media. The reported values are the means of duplicates samples ± standard deviation. The experiment was repeated at least three times.
C. Infection of BALB/c mice. Groups of five mice were intraperitoneally infected with $2 \times 10^5$ cfu of each strain. After 9, 18, 25 and 35 days post infection animals were euthanized and their spleens removed to perform the quantification of viable bacteria. Each data point is the mean ± standard deviation of cfu of five mice spleens. The differences between groups were analysed by a *t*-test.
Significant differences between the mutants and parent strains are indicated with **P*-values < 0.05 or ***P*-values < 0.01.

tion genes are controlled by a regulatory cascade comprising FixLJ-$FixK_2$-NnrR (Torres *et al.*, 2011). In *A. tumefaciens*, these genes are co-ordinately regulated by ActRS (homologous to PrrBA) and the cascade FnrN-NnrR (Baek *et al.*, 2008). The model presented in Fig. 6, showing the role of different regulatory proteins in the adaptation of *Brucella* to low-oxygen tension, constitutes a new example of this type of complex regulatory networks. As observed in the model, the PrrBA system is upstream of an intricate network controlling the expression of the two-component system NtrYX and the transcriptional regulators FnrN and NnrA. The latter is also regulated by NtrYX.

The results presented in this work indicate that PrrBA and NtrYX regulate the expression of the cytochrome *cbb3* oxidase. In a previous work it was shown that, under microaerobiosis, FnrN mediates the induction of the cytochrome *cbb3* oxidase in *B. suis* (Loisel-Meyer *et al.*, 2005). Thus, we propose at least three mechanisms for this regulation: (i) PrrBA directly regulates the expression of cytochrome *cbb3* by interaction of BA-PrrA with its promoter region, (ii) a regulatory cascade involving PrrBA and FnrN and (iii) a regulatory cascade involving PrrBA and NtrYX (Fig. 6).

We also propose three mechanisms for the regulation of the denitrification pathway by PrrBA: (i) direct binding of BA-PrrA to the operons involved in denitrification, (ii) a regulatory cascade involving PrrBA and NnrA and (iii) the same cascade but with NtrYX acting as intermediate (Fig. 6) (Haine *et al.*, 2006; Baek *et al.*, 2004, Carrica *et al.*, 2012).

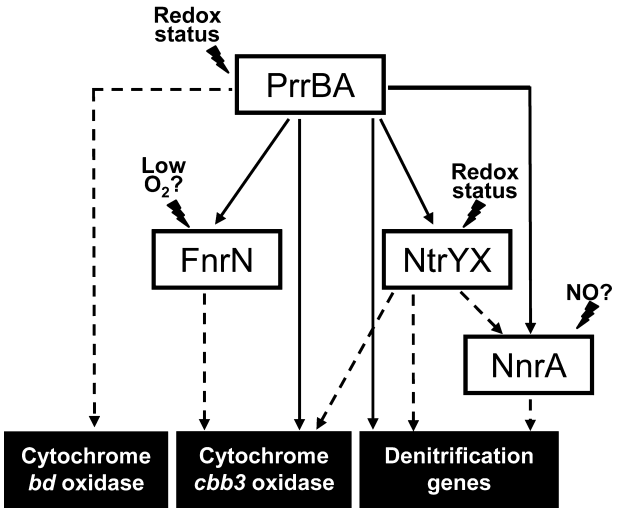


**Fig. 6.** Regulatory network proposed for adaptation of *Brucella* spp. to its replicative niche. The dashed lines indicate interactions demonstrated by gene expression experiments. The solid lines indicate direct interactions between phosphorylated BA-PrrA and DNA regulatory regions demonstrated by EMSA experiments. All observed interactions correspond to positive regulation. The environmental signals proposed to activate each regulatory sensor protein are indicated.

We also hypothesize that NtrYX may directly interact with the operons involved in denitrification, although this need experimental demonstration. An additional mechanism is involved in the regulation of the denitrification pathway in other bacteria. The homologues from *Brucella* FnrN in *Escherichia coli*, *Bacillus subtilis* and *Paracoccus denitrificans* are involved in the regulation of denitrification genes (Tseng *et al*., 1996; Reents *et al*., 2006; Bouchal *et al*., 2010). Interestingly, in *Brucella* the promoter region of *nir* has three putative binding sites for CRP/FNR type regulators. Future experiments are needed to demonstrate these additional mechanisms for regulation of the denitrification pathways.

While the expression of cytochrome *cbb3* oxidase and denitrification pathway genes is decreased under microaerobiosis in the *ntrY* and *prrB* single mutant strains, strikingly they are almost completely turned-off in the double mutant. This result suggests that the two-component systems PrrBA and NtrYX have compensatory functions in the control of the expression of these genes under low-oxygen tension.

Independent studies have confirmed that genes encoding for high-affinity cytochrome oxidases and denitrification pathway enzymes are required for the wild-type virulence (Endley *et al*., 2001; Kohler *et al*., 2002; Kim *et al*., 2003; Haine *et al*., 2006; Loisel-Meyer *et al*., 2006; Jimenez de Bagues *et al*., 2007). We have demonstrated that the *B. abortus ntrY-prrB* double mutant strain is impaired for *in vitro* growth and macrophage and mice virulence while the single mutant strains are not significantly affected in these phenotypes. This suggests a synergistic effect of PrrBA and NtrYX due to the compensatory function in the induction of denitrification and high-affinity cytochrome oxydase genes under microaerobiosis (Roop and Caswell, 2012).

We showed that PrrBA and NtrYX regulate the expression of the transcriptional regulator *nnrA* under oxygen-limiting conditions. Additionally, *nnrA* expression is strongly decreased in the *ntrY-prrB* double mutant strain under the same conditions (Fig. S3). Haine *et al*. demonstrated in *B. melitensis* that the mutation of *nnrA* severely affects bacterial virulence, concluding that, in addition to denitrification genes, this transcriptional regulator controls other genes that are important for infectivity (Haine *et al*., 2006). The strong virulence attenuation of the double mutant might be due to changes in the expression of those genes regulated by NnrA.

The status of nitrogen sources in host cells has been suggested to be critical for the intracellular survival of *Brucella* (Foulongne *et al*., 2000). The NtrBC system plays a central regulatory role in nitrogen assimilation (Atkinson and Ninfa, 1998). Therefore, the activation of NtrBC by PrrBA and NtrYX could expand the regulatory network used by *Brucella* for the adaptation to the intracellular life and might explain the defect of the double mutant to grow in aerobiosis.

In addition, we have observed that the *ntrY-prrB* double mutant strain is sensitive to oxidative stress (unpubl. data). *Brucella* is exposed to reactive oxygen species generated by the oxidative burst of macrophages and by their own aerobic metabolism (Roop *et al*., 2009). Thus, PrrBA and NtrYX might be involved in the regulation of genes needed to overcome oxidative stress. Further experiments are needed to confirm this hypothesis.

In summary, we propose that the metabolic adaptation of *Brucella* to the conditions found in the replicative niche depends on regulatory systems able to sense and integrate different environmental signals: oxygen tension by FnrN (Kiley and Beinert, 1998; Loisel-Meyer *et al*., 2005), nitric oxide by NnrA (Korner *et al*., 2003; Haine *et al*., 2006), nitrogen cellular status by NtrBC (Anjum *et al*., 2002) and redox status by PrrBA (this work) and NtrYX (Carrica *et al*., 2012). Although some of the proposed functions need to be demonstrated in *Brucella*, we believe that the regulatory network proposed in this work contributes to shed light into the mechanisms of bacterial pathogenesis.

## Experimental procedures

### *Bacterial strains and growth conditions*

*Escherichia coli* cells 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.

All *Brucella* strains used in this study were derived from *B. abortus* 2308 and are listed in Table S1. *Brucella* cells were grown at 37°C in trypticase soy broth (TSB) or tryptose agar (TA) (DIFCO), supplemented with 25 μg ml$^{-1}$ kanamycin 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-PrrB (encoding PrrB residues 184–430) and pET24D-PrrA (encoding full-length PrrA, residues 1–190) were constructed by PCR amplification of *Brucella abortus* S2308 chromosomal DNA using suitable primers listed in Table S2 and cloned into the NcoI and XhoI restriction sites from the pET24D vector (Novagen). All genes are under control of the T7 promoter and are in frame with a six-histidine tag at the C-terminus. All cloned inserts were DNA sequenced to confirm the absence of mutations.

### *Expression and purification of recombinant proteins*

The pET24D-PrrB and pET24D-PrrA plasmids were transformed in *E. coli* BL21(DE3) and bacteria were grown in autoinducing medium (Studier, 2005) at 37°C until the $OD_{600}$ was 0.5. Then, the temperature was lowered to 28°C and the incubation was continued with agitation for 16 h. Cells were harvested by centrifugation at 4000 *g* for 20 min, resuspended in lysis buffer [20 mM Tris-HCl pH 8, 200 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF)], and disrupted by sonication with a probe tip sonicator. The total-cell lysates were ultracentrifuged at 35 000 *g* for 60 min to remove insoluble protein, cell debris and unbroken cells. The supernatant was applied to a HisTrap HP column (GE Healthcare) pre-equilibrated with 20 mM Tris-HCl pH 8, 200 mM NaCl, 20 mM imidazole and 1 mM PMSF using an FPLC apparatus (Gilson model 320) connected to a UV/Vis detector (Gilson model 152). Proteins were then eluted using a lineal imidazole gradient and selected fractions were dialysed against 20 mM Tris-HCl pH 8 and 150 mM NaCl. Finally, the dialysed fractions were further purified by gel filtration on a Superdex 75 column (GE Healthcare). Elution was performed with 20 mM Tris-HCl, 200 mM NaCl and 1 mM PMSF. Protein purity was evaluated by SDS-PAGE (12%) with Coomassie Brilliant Blue R staining.

To purify PrrB under reducing conditions, 20 mM dithiothreitol (DTT) was added to the lysis buffer and 1 mM DTT in the subsequent purification buffers.

### *Phosphorylation assays*

The autophosphorylation reaction of PrrB was assayed in a reaction mixture containing 18 μM PrrB (as monomer), 0.5 μCi of [γ-$^{32}$P]-ATP (PerkinElmer Life Sciences), 20 mM Tris-HCl pH 8, 50 mM NaCl, 100 μM ATP and 5 mM $MgCl_2$, in presence or absence of 10 mM DTT. The reaction was incubated at room temperature and stopped at different times by addition of an equal volume of Laemmli sample buffer. For phosphotransfer experiments, an equimolar amount of the purified response regulator PrrA was added to the phosphorylation reaction mixture containing PrrB previously autophosphorylated for 60 min in the presence of DTT, and aliquots were taken at different times. Stopped samples were separated on 12% SDS-PAGE gels and, after electrophoresis, 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).

### *Construction of prrB and ntrY-prrB mutant strains*

For the construction of the *prrB* (BAB1_0132) mutant strain, a fragment of 1212 bp of the *prrB* gene was replaced by a kanamycin resistance cassette. Two PCR fragments generated from regions flanking *prrB* using oligonucleotides prrB1 and prrB2 (708 bp fragment) and prrB3 and prrB4 (723 bp fragment) containing overlapping regions were ligated by overlapping PCR using oligonucleotides prrB1 and prrB4. The resulting PCR product was cloned into the pGEM-T Easy vector (Promega) generating pGEM-ΔprrB, which is able to replicate in *Brucella*. Then, a kanamycin resistance cassette was introduced in a BamHI site generated during the overlapping PCR to obtain the pGEM-ΔprrB::Km vector. This plasmid was introduced in *B. abortus* 2308 by electroporation and homologous recombination events were selected by resistance to kanamycin and sensitivity to ampicillin in TSA agar plates.

For the construction of the *ntrY-prrB* double mutant strain the *B. subtilis sacB* gene encoding levansucrase, which induces lethality upon exposure to 5% (w/v) sucrose in the growth medium, was cloned into the PstI site of pGEM-ΔprrB to generate pGEM-ΔprrB-sacB. This plasmid was introduced in a *B. abortus ntrY* defective strain by electrophoration (*ntrY*::Km, previously published, Carrica *et al*., 2012) and the single recombinant strain was selected by ampicillin resistance. Then, bacteria were grown in TSB in the absence of antibiotic to promote a second recombination event consisting in the excision of the plasmid and generation of the mutant strain by allelic exchange during 16 h. The double recombinant strains were selected in TSA plates supplemented with 10% (w/v) sucrose. Deletion of prrB was confirmed by colony PCR with oligonucleotides prrB1 and prrB4.

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

*Brucella abortus* 2308 and *ntrY*, *prrB* and *ntrY-prrB* strains were grown in rich TSB medium at 37°C. Under aerobic conditions bacteria were grown in erlenmeyers in a rotary shaker at 200 r.p.m. The microaerobic condition was generated by incubation in an anaerobic jar containing GENbox microaer generator (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 (Promega) according to the manufacturer instructions. 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 SuperScritpt 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 the Primer3 program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table S2. 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 the MXPro and LinReg programs. Relative quantification using a standard curve method was performed for each set of primers. The results for each target mRNA were normalized to the amount of the *B. abortus* initiation factor-1 (IF-1) mRNA.

### *Electrophoretic mobility shift assays (EMSA)*

Probes were internally labelled by PCR through inclusion of 40 μCi of [α-$^{32}$P]-dCTP in the reaction mixture and subsequently purified on native polyacrylamide gels. Probes pnir, pnor, pccoN, pnifR, pYX, pfnrN and a control probe were generated by PCR using *Taq*, primers listed in Table S2, and genomic DNA of *B. abortus* S2308 as template. The EMSAs were performed in a volume of 20 μl containing DNA-binding buffer (20 mM Tris-HCl pH 8.0, 5 μg ml$^{-1}$ BSA, 1 mM DTT, 75 mM KCl, 5 mM $MgCl_2$, 1 μg DNA salmon sperm, 4% glycerol), 5000 cpm of the $^{32}$P-labelled probe and PrrA recombinant protein. After incubation at room temperature for 30 min, the protein–DNA complexes were separated from the free probes by electrophoresis in 8% non-denaturing polyacrylamide gels at a constant voltage of 220 V. The results of the EMSAs were visualized by exposure of the gels to X-ray films (Amersham Hyperfilm). Phosphorylated PrrA (PrrA-P) was generated as described above and the phosphotransfer mixture was directly added to the binding reaction.

### *Macrophage cell infection*

Cells of the murine macrophage cell line J774 were grown in 24-well culture plates at $2 \times 10^5$ cells per well in RPMI media during 24 h. Stationary-phase cultures of *B. abortus* S2308 and *ntrY*::km, *prrB*::km and *ntrY-prrB* 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 1 h to allow bacterial uptake and invasion. The extracellular bacteria were removed by washing twice with PBS and incubated with 50 μg ml$^{-1}$ gentamicin in RPMI for 1 h. Cells were then incubated with 25 μg ml$^{-1}$ gentamicin in RPMI 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.

### *Mice infection*

Sixty-day-old female BALB/c mice were inoculated intraperitoneally with $2 \times 10^5$ cfu of the indicated strains. At 9, 18, 25 or 35 days p.i. mice were sacrificed; spleens were removed and homogenized in 2 ml of PBS. Tissue homogenates were serially diluted and plated on TSA plates with the appropriate antibiotics to determine colony-forming units per spleen (cfu spleen$^{-1}$). Values are expressed as mean ± standard errors of the mean (N = 5). All research involving animals has been conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experimental protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) from the Leloir Institute.

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## Supporting information

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