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NOTES

NnrA Is Required for Full Virulence and Regulates Several *Brucella melitensis* Denitrification Genes

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We identified two regulators of denitrification genes in *Brucella melitensis* 16M: NarR, which regulates the nitrate reductase (*nar*) operon, and NnrA, which is involved in the expression of the last three reductases of the denitrification pathway (*nirK*, *norB*, and *nosZ*). NnrA is required for virulence in mice and for intracellular resistance to nitric oxide.

Brucella species are facultative intracellular pathogens that infect animals and occasionally humans (10). Recently, analysis of the complete *Brucella melitensis* sequence suggested the presence of an anaerobic electron transfer system and the enzymes allowing the reduction of nitrate into dinitrogen gas (nitrate, nitrite, NO [1] and nitrous oxide reductases) (3). These reactions are collectively named denitrification (17) and could allow *Brucella* to grow under low-oxygen conditions by respiration of nitrate (11).

Since the replicative compartment of *Brucella suis* is characterized by low oxygen tension, it was postulated that, during the infectious process, *Brucella* could use denitrification to survive using nitrogen oxides as terminal electron acceptors (6). Another potential role of this system is limiting the production of reactive nitrogen intermediates by the host during infection. Indeed, *Brucella abortus* was shown to counteract the effect of NO after 24 h of infection in activated macrophages (14).

When analyzing the *B. melitensis* genome, we observed that three predicted coding sequences for Crp/Fnr regulators (*narR*, *nnrA*, and *nnrB*) are located near the genes putatively involved in denitrification (Fig. 1). NarR belongs to the NarR subfamily of the Dnr cluster, while NnrA and NnrB belong to the NnrR cluster, according to the Korner classification (7). Three other Crp/Fnr genes are predicted from the genomic sequence (5); two of them belong to the A cluster while the last belongs to the FnrN family (7). The *B. melitensis* genome contains the *narKGHJI*, *nirKV*, *norEFCBQD*, and *nosRZDFYSLX* gene clusters that are coding for a respiratory membrane-bound nitrate reductase (Nar, with the nitrite extrusion protein NarK), a copper nitrite reductase (Nir), a NO reductase (Nor), and a nitrous oxide reductase (Nos), respectively. The *narR* gene is found next to genes encoding the nitrate reductase, *nnrA* is next to genes encoding the nitrite and NO reductases, and *nnrB* is located downstream of genes encoding the nitrous oxide reductase. Furthermore, the regulators encoded by *narR*, *nnrA*, and *nnrB* genes are homologous to regulators involved in denitrification in other bacteria. Indeed, NarR from *B. melitensis* is homologous to the *Paracoccus pantotrophus* NarR, a regulator of the nitrate reductase genes (16). NnrB and NnrA belong to the NnrR family, comprising the NnrR regulator from *Rhodobacter sphaeroides* 2.4.3, which controls the expression of the nitrite and NO reductases (12).

As a starting hypothesis, we postulated that the genomic colocalization between the denitrification genes and *narR*, *nnrA*, and *nnrB* could indicate that these Crp/Fnr regulators are involved in the transcriptional control of the denitrification genes. Since homology analyses support this hypothesis, we constructed mutants for NarR, NnrA, and NnrB regulators and for the catalytic subunits of the four reductases (*narG*, *nirK*, *norB*, and *nosZ*) of the denitrification process by using an integrative disruption strategy (5). Mutant genotypes were confirmed by Southern blotting as previously reported (5). We also constructed plasmidic promoter-*lacZ* fusions using the pBBRMCS1*lacZ* vector (S. Léonard, unpublished data), a derivative of pBBR1MCS (9), in order to follow the activity of *narK*, *nirK*, *norC*, and *nosR* promoters in the *narR*, *nnrA*, and *nnrB* mutants. These promoters, here defined as a 500-bp fragment upstream and including the predicted ATG, were amplified by PCR and inserted in frame with the *lacZ* coding sequence. The primer sequences are available upon request.

An evaluation of nitrite concentration in anaerobic conditions and in the presence of nitrate (15) showed that strains with *nnrA* and *nirK* mutants accumulate more nitrite than the wild-type (wt) strain, while *narR* and *narG* mutants accumulate a lower concentration of nitrite and no detectable nitrite, respectively (data not shown). These mutant phenotypes are complemented by the presence of an *nnrA*, *nirK*, *narR*, or *narG* coding sequence on a pBBRMCS4GW plasmid (data not shown). One interpretation for the nitrite accumulation data is that *narR* could activate the *nar* operon, probably through the promoter located upstream of *narK*, encoding a nitrite extrusion protein, and that *nnrA* could activate *nirK*. We therefore tested the activity of P*narK* in the *narR*

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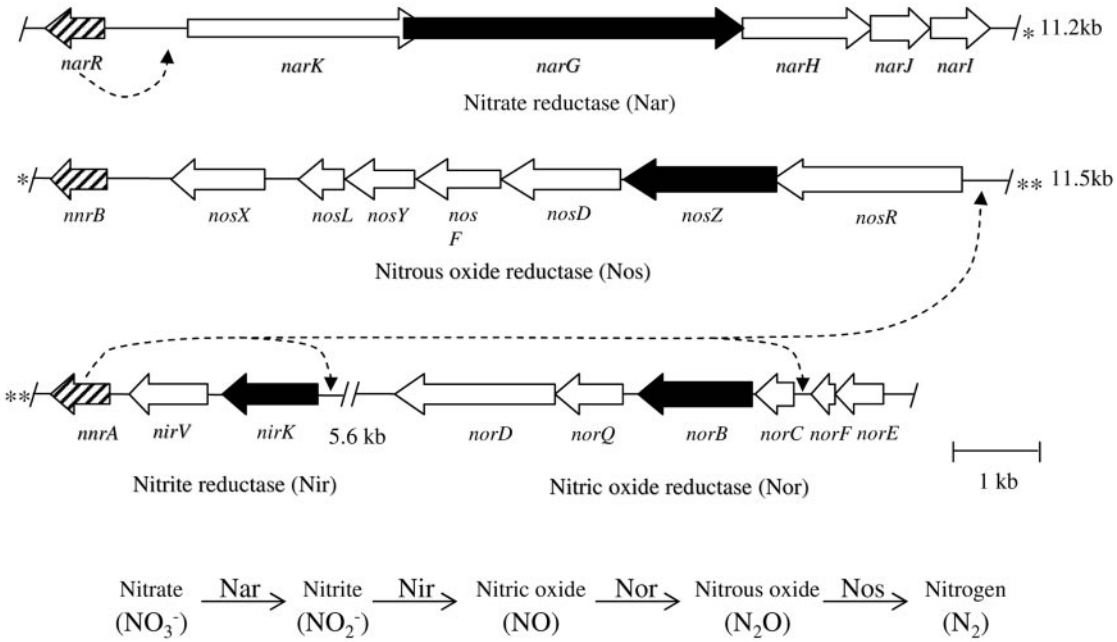

FIG. 1. Organization of the denitrifying reductase gene clusters in the *B. melitensis* 16M genome. The data presented here support a model (depicted as discontinuous lines) in which *nnrA* is a regulator of *nir*, *nor*, and *nos* genes, while *narR* is required for full *nar* expression. Each predicted coding sequence is indicated by an arrow. The arrows in contact and in the same orientation are expected to be coding sequences organized in operons. The genes coding for the catalytic subunit of the reductases are colored in black, and regulator genes are hatched. The three regions are located on the same portion of the small chromosome of *B. melitensis* 16M, as indicated by the short distances separating the *nar*, *nos*, *nir*, and *nor* loci. The overlap of *narK* and *narG* coding sequences is 86 bp long. The *narR* coding sequence (BMEII0947) is found close to genes encoding nitrate reductase homologs (*nar* genes and *narK*, encoding a putative nitrite extrusion protein), *nnrA* (BMEII0986) is next to genes encoding the nitrite and nitric oxide reductase homologs (*nir* and *nor* genes, respectively), and *nnrB* (BMEII0966) is downstream of *nos* genes, encoding the nitrous oxide reductase homolog. At the bottom, a summary of the denitrification pathway is shown. *, region located between *narI* and *nnrB*; **, region located between *nosR* and *nnrA*.

mutant and the activity of P*nirK* in the *nnrA* mutant by using the *lacZ* fusions described in the previous paragraph. Overnight cultures were diluted to an optical density at 600 nm of 0.1 in 2YT medium supplemented with 10 mM $NaNO_3$ and incubated for 3 days in microaerobic conditions (using the CampyGen system, Oxoid) which allow a slow growth of *B. melitensis* 16M. We observed that the transcription from the P*narK* promoter is lower in the *narR* mutant than in the wild-type strain (Table 1). A stronger effect was reported for NarR in the *narK* gene in *Paracoccus* (16), although the target promoter is also not completely switched off in the *narR* mutant of *Paracoccus*. In the *nnrA* mutant, the transcription from the P*nirK* promoter is much lower than that from the wild-type control (Table 1). We also tested the effect of *nnrA* and *nnrB* mutations on *norC* and *nosR* promoters that may control the expression of the *norB* and *nosZ* coding sequences. We observed that in the *nnrA* mutant, the transcription from both promoters is strongly reduced, while *nnrB* mutation does not affect P*norC* or P*nosR* (Table 1).

TABLE 1. β-Galactosidase activities from the reductase promoter-*lacZ* fusions in wt or mutant *B. melitensis* strains

Fusion	Strain	β-Galactosidase activity[a]
P*narK-lacZ*	wt	1,091 ± 159
	narR	457 ± 51
P*nirK-lacZ*	wt	49 ± 12
	nnrA	1 ± 0.6
P*norC-lacZ*	wt	640 ± 25
	nnrA	9 ± 5
	nnrB	698 ± 46
P*nosR-lacZ*	wt	304 ± 81
	nnrA	4 ± 2
	nnrB	395 ± 61

[a] Bacteria were grown microaerobically in rich medium (2YT) supplemented with 10 mM of $NaNO_3$ before β-galactosidase activity was determined. Activity (in Miller units) (8) is the average of three independent clones.

NnrA probably interacts with the *Escherichia coli* transcription machinery to modulate *B. melitensis* promoters *nirK*, *norC*, and *nosR* because when *nnrA* is used in a heterologous transcription interference assay with *E. coli* strain DH10B, transcription from these promoters is significantly reduced relative to a control in which *nnrA* is absent (Fig. 2). For the heterologous transcription interference assay, a plasmid encoding the regulator of interest is cotransformed in a host without close homologs to this regulator, with another plasmid bearing a promoter-reporter fusion. If the regulator is able to interfere with the basal transcription of the reporter, it is likely that the regulator binds to the promoter fused to the reporter. In this study, the regulator is encoded by pMR-nnrA, the empty vector pMR10 is used as a control, the host is *E. coli* strain DH10B, and each promoter tested is fused to the *lacZ* reporter as indicated in Fig. 2. The *narK* promoter, which was not

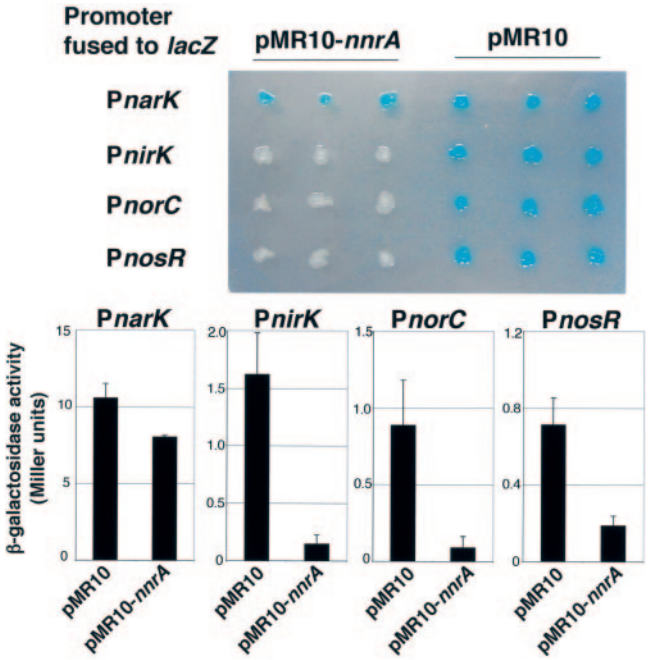

FIG. 2. Heterologous transcriptional interference test using *nnrA* of *B. melitensis* with the *narK*, *nirK*, *norC*, and *nosR* promoters. *E. coli* strains contain a pMR10 plasmid bearing *nnrA* (or a pMR10 without *nnrA*) and a pBBR-MCS1*lacZ* derivative, in which a promoter is fused to the *lacZ* reporter. It is expected that *nnrA* expression from pMR10-nnrA will interfere with a promoter-*lacZ* fusion if NnrA is able to modulate the promoter tested, probably by direct binding. In the upper panel, *E. coli* strains were grown overnight (in aerobic conditions, without the addition of nitrate) on LB medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml), chloramphenicol (20 μg/ml), and kanamycin (50 μg/ml) in order to select for strains harboring a pMR10-derived plasmid and pBBRMCS carrying a fusion to the *lacZ* reporter. In these experiments, *nnrA* is expressed under the control of an *E. coli lac* promoter. The strains having the pMR10-*nnrA* plasmid and the P*nirK-lacZ*, P*norC-lacZ*, and P*nosR-lacZ* fusions are less stained than the controls (pMR10-containing strains and the strain containing pMR10-*nnrA* and the P*narK-lacZ* fusion). Three independent clones are shown. These interferences of *nnrA* with *nirK*, *norC*, and *nosR* promoters that were observed with X-Gal staining are confirmed by a standard β-galactosidase (β-gal) assay (lower panel) made with clones grown overnight in liquid LB medium supplemented with chloramphenicol (20 μg/ml) and kanamycin (50 μg/ml) (8). The indicated values are the averages of measurements of three independent clones, and each error bar corresponds to one standard deviation.

affected by *nnrA* mutation in *B. melitensis*, is much less sensitive to the presence of *nnrA* than the other promoters tested. Moreover, sequence analysis of the *nirK*, *norC*, and *nosR* promoters reveals that each contains a putative NnrA binding site. Indeed, since NnrA is homologous to NnrR of *R. sphaeroides*, it is expected to recognize similar sites on DNA close to the TTGCG(N)$_4$CACAA sequence found upstream of *nnrS* in *R. sphaeroides* (2). Similar sites are found in *B. melitensis* promoters *nirK* (AGGCGTGAA CACAA, at 198 nucleotides [nt] upstream of ATG), *norC* (TTGCCTATTCGCAA, at 129 nt upstream of ATG), and *nosR* (TTGCGTCATAT CAA, at 221 nt upstream of ATG) but not in the *narK* promoter, which also suggests that NnrA modulates transcription from the *nirK*, *norC*, and *nosR* promoters in *B. melitensis*.

TABLE 2. Virulence of the mutants in BALB/c mice after intraperitoneal infection[a]

Strain	Attenuation in mice
narR	0.9 ± 0.1
nnrA	2.4 ± 0.5
nnrB	0.8 ± 0.1
narG	0.5 ± 0.1
nirK	0.6 ± 0.1
norB	0.6 ± 0.1
nosZ	0.7 ± 0.1

[a] Virulence is expressed as the log of attenuation of the mutant compared to the wild-type strain. Each group tested contained four mice at 4 weeks postinfection. Differences larger than 0.25 log units are statistically significant (P was <0.001 using standard ANOVA test). In each experiment, close to 4.5 log CFU was recovered from the spleen of mice infected with the wild-type strain.

In order to test the importance of denitrification control in *Brucella*'s virulence, we tested the residual virulence of the *narR*, *nnrA*, and *nnrB* mutants. The reductase mutants were also tested as a reference. We evaluated the residual virulence of those mutants in a mouse model of infection by using the intraperitoneal route (Table 2). Briefly, 5×10^5 CFU was injected into 7-week-old BALB/c mice and, 4 weeks later, spleens were recovered, homogenized in phosphate-buffered saline with 0.1% Triton X-100, and plated on 2YT medium for CFU counting. The *nnrA* mutant is the only mutant strongly attenuated (about a 100-fold reduction in the number of CFU per spleen) after 4 weeks of infection relative to the wild-type strain. The mutants in genes coding for the reductases NnrB and NarR are slightly attenuated (0.5 to 0.9 log units of attenuation) (Table 2). From these data, we propose that *nnrA* function in mice is not restricted in its capacity to activate *nir*, *nor*, and *nos* genes in the appropriate conditions.

We also compared the phenotypes of *nnrA* and *norB* reductase mutants in activated macrophages. Indeed, it has been proposed that nitric oxide reductase eliminates the NO produced by the host cells and thus prevents the killing of intracellular *Brucella* by reactive nitrogen intermediates like peroxynitrite generated from NO (14). If NnrA is also an activator of *nor* genes ex vivo, the *nnrA* and *norB* mutants are expected to share similar phenotypes during a cellular infection. In order to test this hypothesis, J774A.1 macrophages were cultured in Iscove medium supplemented with 5 mM glutamine (Gibco BRL) and 10% fetal calf serum (Sigma Chimie) at 37°C in 5% CO_2 and were treated or not treated with gamma interferon (IFN-γ) (10 U/ml of mrIFN-γ; Pharmingen, San Diego, CA) before being infected with the *norB* mutant as well as with the *nnrA* mutant (Fig. 3). The *narG* mutant was used as a negative control in this experiment. Unstimulated macrophages did not release any detectable NO (4) (data not shown), and in those conditions, the mutants appeared to be as virulent as was the wild type (Fig. 3). In IFN-γ-activated cells, which release the nitrogen radical upon infection (4), the growth of the *narG* mutant was not affected. On the contrary, we observed a clear difference between the wild type and the *norB* or *nnrA* mutants at 48 h of infection. Indeed, the number of wild-type bacteria strongly increased between 7 and 48 h of infection for the wild-type strain, while the multiplication of the *norB* and *nnrA* mutants was clearly affected (about 10-fold reductions at 48 h relative to unactivated macrophage

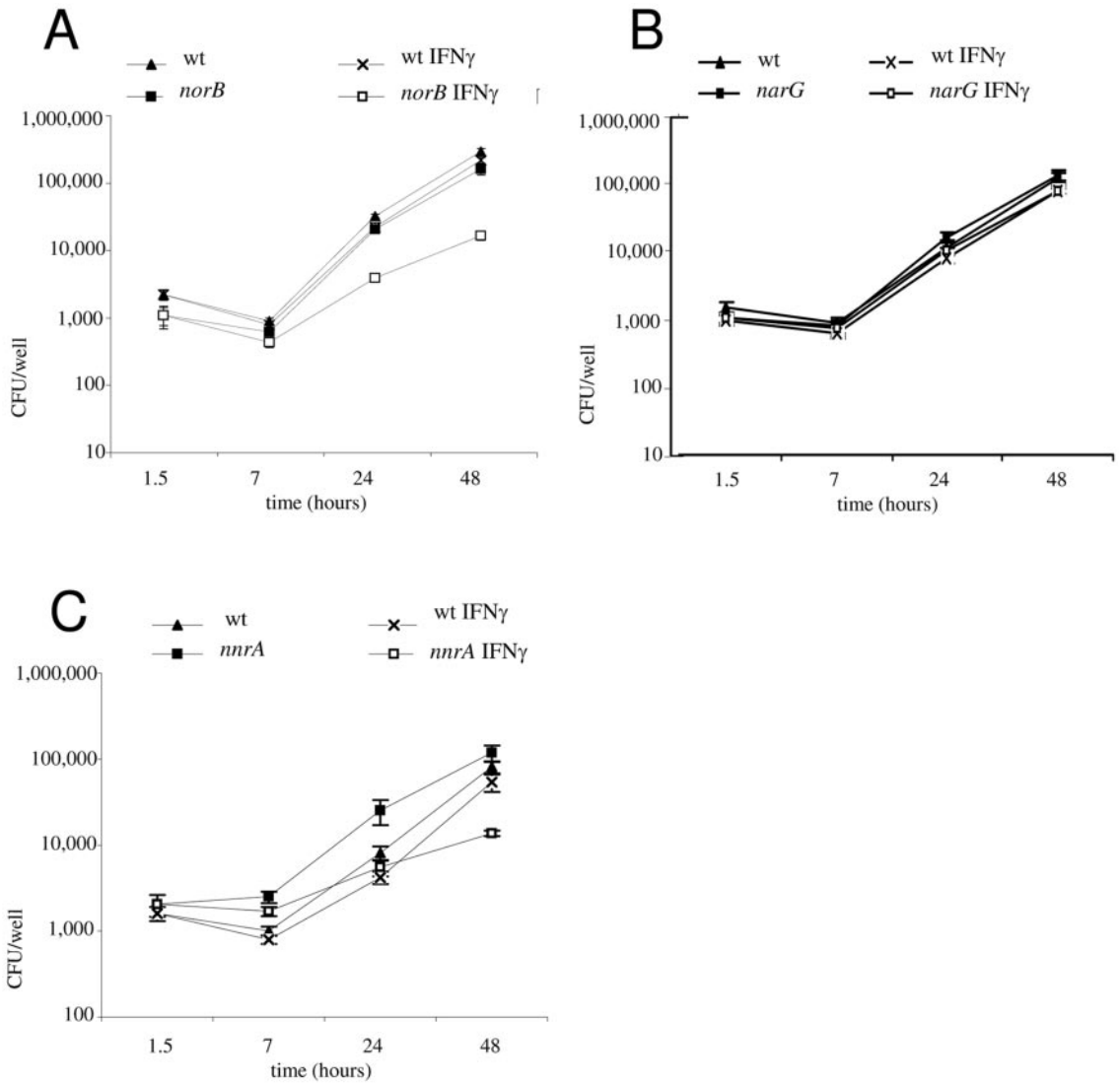

FIG. 3. Growth of wt and *norB* (A), *narG* (B), and *nnrA* (C) mutants of *B. melitensis* in J774A.1 macrophages with or without IFN-γ stimulation. Cells treated or not treated with IFN-γ (10 U/ml) were infected under the conditions indicated in Materials and Methods. At the indicated time p.i., the number of intracellular bacteria was measured and expressed in CFU/well. Measurements performed (six replicates) ± standard deviations are presented. At 48 h p.i., the difference between activated and inactivated samples is statistically significant ($P < 0.001$ in a standard *t* test) for the *norB* and *nnrA* mutants (panels A and C).

condition). Measurements at 48 h postinfection (p.i.) revealed a concentration of nitrite (expected to result from NO oxidation) ranging around 15 ± 5 μM in supernatants of cells infected with the different *Brucella* strains (mutants or wild type), while nitrite was undetectable from the supernatants of cells infected in the absence of IFN-γ (data not shown). In order to check whether NO production was indeed involved in the lower resistance of *norB* and *nnrA* mutants in activated macrophages, we performed the same infections but in the presence of 2 mM L-NAME (Nω-nitro-L-arginine-methyl-ester), an inhibitor of NO synthase. In the presence of L-NAME, the survival of *norB* and *nnrA* mutants at 48 h p.i. was almost restored, confirming that the reduction of CFU of *norB* and *nnrA* mutants in activated macrophages is NO-dependent (Table 3). The *norB* and *nnrA* mutants are therefore sensitive to NO and its derivatives, which was not observed with the wild-type strain and with the *narG* mutant. Sensitivity of *nnrA* and *norB* mutants to NO and its derivatives was confirmed using sin-1, a generator of NO and peroxynitrite (data not shown). These

TABLE 3. L-NAME, an inhibitor of NO synthase, is able to suppress the survival defect of *norB* and *nnrA* mutants in activated macrophages[a]

Mutant strain	Suppression activity with (CFU [10^4/well]):		
	Control	IFN-γ	IFN-γ and L-NAME
norB	12.5 ± 1.0	2.4 ± 0.2	7.8 ± 1.1
nnrA	10.4 ± 0.5	2.4 ± 0.3	7.8 ± 1.9

[a] Murine macrophages are activated using IFN-γ, and L-NAME (2 mM) was added to inhibit the NO synthase activity. Six replicates were performed for each condition. Both treatments, activation with IFN-γ and inhibition with L-NAME, give a statistically significant test (P was <0.001 using standard ANOVA test).

results are in accordance with an *nnrA* role as activator of *norB*.

It is noticeable that the *norB* mutant is attenuated in activated macrophages (Fig. 3) but only slightly attenuated in mice at 4 weeks postinfection (Table 2). Many reasons could explain this apparent discrepancy, including a minor or redundant role of bacterial destruction by NO and its derivatives in this mouse infection model.

In conclusion, our data demonstrate that NnrA-regulated genes, probably different from denitrification genes, are of importance for *Brucella* virulence. Further experiments will be required to identify new NnrA target genes, since more than 80 putative NnrA binding sites were found in a sequence analysis of the *B. melitensis* genome using the DNA pattern search at the RSA Tools website (http://rsat.ulb.ac.be/rsat/) (13), using default parameters and the NnrR predicted binding site [TTGCG$(1)_4$CACAA] with the possibility of one substitution. It will also be interesting to discover the signal(s) perceived by or transmitted to NnrA; NO could be one of the obvious candidates.

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