Requirement of norD for Brucella suis Virulence in a Murine Model of In Vitro and In Vivo Infection

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A mutant of Brucella suis bearing a Tn5 insertion in norD, the last gene of the operon norEFCBQD, encoding nitric oxide reductase, was unable to survive under anaerobic denitrifying conditions. The norD strain exhibited attenuated multiplication within nitric oxide-producing murine macrophages and rapid elimination in mice, hence demonstrating that norD is essential for Brucella virulence.

The gram-negative intracellular pathogen Brucella sp. is the causative agent of brucellosis, which is transmissible to humans from domestic animal species that are infected with B. abortus, B. suis, or B. melitensis.

Multiplication inside macrophages allows Brucella to be carried throughout the host organism and to colonize specific organs. The pathogenicity of brucellae and chronicity are based on the ability of the pathogen to adapt to the environmental conditions that it encounters in its replicative niche (18) and to avoid the killing mechanisms within macrophages (3,8,10,14).

Intensive studies were performed at the genetic level to investigate the factors that are essential for the adaptation of Brucella to the intracellular conditions (5). Analysis of the intramacrophagic virulome (17) confirmed that the type IV secretion system encoded by the intramacrophagic virulome (17) confirmed that the type IV secretion system encoded by the intramacrophagic virulome (17) confirmed that the type IV secretion system encoded by the virB operon (19) is, to date, the main virulence factor of B. suis. It also revealed that the B. suis replicative niche is characterized by low levels of nutrients and oxygen. The latter parameter was previously observed in the phagosomes of stimulated macrophages (13). Complete genome sequences (6,12,20) have revealed that Brucella possesses all of the genes that are necessary for a complete detoxification pathway resulting in the reduction of nitrate to nitrogen. Genes encoding the four reductases Nar (nitrate reductase), Nir (nitrite reductase), Nor (nitric oxide reductase), and Nos (nitrous oxide reductase) constitute a “denitrification island” in B. suis which shares numerous similarities with that of Sinorhizobium meliloti (21); both organisms belong to the α-subclass of the proteobacteria. The respiratory system could allow Brucella to survive under very low oxygen tension, using nitrogen oxides as terminal electron acceptors. Bacteria may also take advantage of denitrification to cope with nitric oxide (NO) production in the macrophages during the innate response against infection. NO production by infected macrophages is a major defense system in control of Brucella infection in mice (9,16) and possibly, although more controversial, in human infection, as revealed by the use of macrophages transfected with inducible NO synthase (7). Despite the low levels of NO that were released by human macrophages, the nitric oxide reductase of Neisseria meningitidis was found to confer intracellular resistance to NO and allowed its utilization, resulting in the optimal survival of this bacterium in nasopharyngeal mucosa (22). In fact, during an infection of murine macrophages producing high levels of NO, B. abortus displayed increased late survival (23). The authors of that work suggested that expression of nitric and nitrous oxide reductase genes could participate in the bacterial defense against NO. Recent work (1) has shown that nitrite and nitric oxide reductases favored long-term persistence of B. neotomae in mice. Nevertheless, this observation cannot be directly related to NO detoxification since the interferon regulatory factor 1-deficient mice used for in vivo analysis did not produce NO (15). In the present study, we investigated the role of norD, the last gene of the nor operon encoding the nitric oxide reductase, in virulence in a murine model of in vitro and in vivo infection.

A norD mutant of B. suis is affected by high mortality under anaerobic denitrifying conditions. To identify the genes of B. suis that are involved in adaptation to limiting oxygen conditions, 3,840 individual Tn5 transposon mutants of B. suis 1330 (17) were screened under anaerobiosis on Trypticase soy (TS) agar plates supplemented with 20 mM NaNO3. After 14 days of incubation in a jar containing GENbox anaerobic generators (bioMérieux, Marcy l’Etoile, France) (oxygen concentration, <0.1%), six strains were selected for their inability to produce a film (wild-type B. suis did not exhibit true growth under anaerobiosis) (Fig. 1A). Under aerobic conditions, they were characterized by a growth rate which was identical to that of the wild-type strain. Partial sequence data (MilleGen, Toulouse, France) of the genes that are responsible for the growth defect in mutants, obtained as previously described (17), indicated that three of the mutants had transposon insertions in the norEFCD operon of B. suis (TIGR database search using the BLASTN algorithm). One was found in the intergenic region between norB and norQ, and two were found at different positions within norD. This indicated that nitric oxide reductase was a critical factor for the resistance of Brucella to anaerobiosis in the
presence of nitrates. Despite its unknown function, the norD product is needed for nitric oxide reductase activity in Rhodobacter sphaeroides and Paracoccus denitrificans (2, 4).

The norD mutant with the Tn5 insertion site that was the most proximal to the start codon (357 bp) was chosen for further studies. When maintained for 14 days under anaerobiosis in liquid culture in TS medium supplemented with 20 mM NaNO₃, the wild-type strain survived, as the numbers of viable bacteria were identical at the beginning and the end of the experiment (Fig. 1A). In contrast, the survival of the norD mutant was severely impaired, as shown by the more-than-5-log reduction in viable counts. In comparison, a strain deficient in nitrate reductase activity (inactivation of narG) was obtained as previously described (14), following the replacement of the 770-bp NcoI fragment by the kanamycin resistance gene) was less affected, exhibiting a decrease in viability of 1 log. The utilization of nitrogen oxides by the bacteria was assessed by measuring the concentration of NO₂ present in the medium. Nitrates were therefore completely reduced to nitrite in nitrate reductase activity inactivation of

\[ \text{NO}_3^- \rightarrow \text{NO}_2^- \]

In order to check whether the absence of nitrite production by the norD mutant could affect the efficiency of the norD mutant at day 14 of incubation (Fig. 1A).

Lack of norD impairs multiplication of B. suis in NO-producing J774A.1 macrophages. To test whether norD could have an impact on the viability of B. suis inside macrophages producing NO, infections of mouse J774A.1 macrophage-like cells were performed in the absence or presence of Escherichia coli lipopolysaccharide (LPS) and gamma interferon (IFN-γ). This treatment has been described to induce NO production in murine macrophages that are infected with Brucella (9, 23). A total of 2 × 10⁵ cells in 1 ml of Iscove medium supplemented with 5 mM glutamine were infected in 24-well plates at a multiplicity of infection of 20. To stimulate the cells after phagocytosis, E. coli LPS (100 ng ml⁻¹) and mouse recombinant IFN-γ (10 U ml⁻¹) were added to gentamicin-supplemented medium. At 1.5, 7, 24, and 48 h postinfection (p.i.), cells were washed with phosphate-buffered saline and lysed in 0.2% Triton X-100. CFU were determined by plating serial dilutions on TS agar.

The infection of resting macrophages showed that the norD mutant and the wild-type strain displayed similar rates of multiplication (Fig. 2A). On the contrary, activation of J774A.1 cells by LPS and IFN-γ was accompanied by a more-than-thousandfold attenuation of the norD mutant at 48 h p.i. (Fig. 2B) compared to that of the wild-type strain. The lower intracellular growth of the mutant indicated that norD was required for the efficient replication of B. suis within activated murine macrophages. Nitrite analysis showed a production of 15 μM NO at 48 h p.i. in supernatants of LPS- and IFN-γ-activated cells but not in that of nonactivated macrophages (not shown). In order to check whether the attenuation of the norD mutant was specific to NO production, a control with an inducible NO synthase inhibitor was performed using 3 mM l-NAME (Nω-nitro-l-arginine methyl ester) during infections. Upon this

FIG. 1. Survival of B. suis strains and nitrite accumulation under anaerobic conditions. Each strain was previously grown under aerobicism until an optical density of 0.5 was reached. Wild-type (○, narG (■), and norD (●) mutant bacteria were then cultivated in TS medium with 20 mM NaNO₃, in a jar containing GENbox anaerobic generators. At various time points, an aliquot was collected, plated for counts of CFU (A), and centrifuged to measure nitrite concentration in the supernatant (B). Values are representative of at least three independent experiments. Error bars represent the standard error of the means obtained from one experiment performed in triplicate. Statistically significant differences between wild-type and norD data as determined by Student’s t test are indicated.
treatment, intracellular survival of the norD mutant was restored (Fig. 2B), showing that the multiplication defect of this strain was due to NO generation by activated macrophages. The norD::Tn5 strain was complemented in trans with the native norD gene, which was amplified from B. suis DNA and cloned under the control of the lacZ promoter into the replicative plasmid pBBR1MCS4. The complementation of the norD mutant led to the recovery of intracellular growth similar to that of the wild-type strain (Fig. 2B).

Inactivation of norD attenuates virulence of B. suis in the mouse model of infection. The NorD-deficient B. suis strain likely accumulated nitric oxide at a toxic concentration in vitro and displayed an intracellular survival defect in stimulated macrophages releasing NO. Given that NO production is an important component of host defense mechanisms and that oxygen tension is low inside the phagosomes of stimulated macrophages (13), we investigated whether norD was involved in B. suis pathogenicity in a mouse model of infection. The survival of wild-type, norD, and narG strains was measured in 7-week-old female BALB/c mice that were inoculated with 10⁵ CFU of either wild-type B. suis or the norD and narG mutants. At different time points, the spleens of five mice for each B. suis strain were harvested. After homogenization in phosphate-buffered saline, serial dilutions were plated on TS agar to determine bacterial counts. The absence of active NorD severely affected virulence of B. suis, since bacteria harboring the Tn5 transposon in norD did not multiply in the spleens at 7 days postinfection, unlike the virulent strain 1330, and showed a reduction in CFU numbers of approximately 3 logs relative to that of the wild type at 4 weeks postinoculation (Fig. 3). In contrast, the narG strain was not attenuated, exhibiting multiplication and persistence within the spleen until 4 weeks postinoculation similar to that of the parental strain. This result strongly supported the conclusion that, in contrast to narG, norD is necessary for the survival of B. suis in the mouse model, probably because it participates in the nitric oxide reductase activity encoded by norB and norC and in NO detoxification.

Conclusions. This report demonstrated that the inactivation of norD severely impaired virulence of B. suis. According to our knowledge, it is the first direct evidence that a gene of a denitrification pathway plays a crucial role in the pathogenicity of B. suis. This bacterium may have a double advantage from using denitrification: it may provide sufficient energy for the persistence of bacteria under hypoxic conditions or support resistance of bacteria upon NO release by infected macrophages or both. From the results described here, we propose

![FIG. 2. Intracellular growth of B. suis strains in murine J774A.1 cells without (A) and in the presence of (B) E. coli LPS and IFN-γ. Cells were infected with the wild-type strain (○), the norD mutant (□), or the norD mutant complemented in trans (◇). Corresponding closed symbols represent data that were obtained with L-NAME. Error bars represent the standard error of the means for triplicate infections performed in the experiment shown, which is representative of two independent experiments. The significant P values (Student’s t test) for the wild-type strain versus the norD mutant are given.](http://iai.asm.org/)

![FIG. 3. Course of infection of B. suis wild-type strain (●) and the narG (□) and norD (◇) mutants in spleens of BALB/c mice. Recovery of the bacteria was determined at 2, 7, 14, and 28 days postinoculation, with five mice per strain and time point. Error bars represent the standard deviations of the means. Analysis of variance using the Fisher protected least-significant-difference test and the Tukey-Kramer test was performed to determine the level of significance (P) of differences in CFU between the wild-type and the norD mutant.](http://iai.asm.org/)
that norD participates in nitric oxide reductase activity, which in turn could play a critical role in bacterial multiplication in murine models of infection. On the contrary, the nitrate reductase (narG)-deficient strain of B. suis survived rather well under anaerobic conditions and multiplied as well as the wild-type strain within the spleens of infected mice. This is in accordance with the absence of attenuation of the narG and nirK mutants of B. melitensis in vitro in stimulated murine macrophages producing NO and/or in vivo (11). Altogether, our results therefore defined NorD as a virulence factor of Brucella that is most likely involved in the NO detoxification function rather than energy production.

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