Characterization and Protective Property of Brucella abortus cydC and looP Mutants

Quang Lam Truong, Youngjae Cho, Abhijit Kashinath Barate, Suk Kim, Tae-Wook Hahn

College of Veterinary Medicine, Kangwon National University, Chuncheon, South Korea; College of Veterinary Medicine, Gyeongsang National University, Jinju, South Korea

Brucella abortus readily multiplies in professional or nonprofessional phagocytes in vitro and is highly virulent in mice. Isogenic mutants of B. abortus biovar 1 strain IVKB9007 lacking the ATP/GDP-binding protein motif A (P-loop) (named looP; designated here the IVKB9007 looP::Tn5 mutant) and the ATP-binding/permease protein (cydC; designated here the IVKB9007 cydC::Tn5 mutant) were identified and characterized by transposon mutagenesis using the mini-Tn5Km2 transposon. Both mutants were found to be virtually incapable of intracellular replication in both murine macrophages (RAW264.7) and the HeLa cell line, and their virulence was significantly impaired in BALB/c mice. Respective complementation of the IVKB9007 looP::Tn5 and IVKB9007 cydC::Tn5 mutants restored their ability to survive in vitro and in vivo to a level comparable with that of the wild type. These findings indicate that the cydC and looP genes play important roles in the virulence of B. abortus. In addition, intraperitoneal immunization of mice with a dose of the live IVKB9007 looP::Tn5 and IVKB9007 cydC::Tn5 mutants provided a high degree of protection against challenge with pathogenic B. abortus strain 544. Both mutants should be evaluated further as a live attenuated vaccine against bovine brucellosis for their ability to stimulate a protective immune response.

MATERIALS AND METHODS

Bacterial culture and media. The virulent wild-type B. abortus biovar 1 strain IVKB9007 was isolated from an aborted bovine fetus in South Korea, passaged twice on tryptic soy agar (TSA; Difco, Sparks, MD, USA), and stored frozen at −70°C in 25% glycerol. The B. abortus vaccine strain RB51, the virulent B. abortus challenge strain 544, the B. abortus IVKB9007 strain, and the isogenic IVKB9007 looP::Tn5 and IVKB9007 cydC::Tn5 mutants were routinely grown on TSA or tryptic soy broth (TSB; Difco) containing 5% bovine serum at 37°C in 5% CO2. E. coli DH5α and E. coli S17-1 pir pUT mini-Tn5Km2 were used for transformation and as the donor strain for conjugation, respectively (6). Each E. coli strain was cultivated in Luria-Bertani (LB) broth or agar (Difco, Sparks, MD, USA). If necessary, the medium was supplemented with an appropriate reagent and antibiotics, namely, 0.5% glycerol, 50 μg/ml...
rifampin (RP), 50 μg/ml kanamycin (KM), 30 μg/ml nalidixic acid (NA), and 100 μg/ml ampicillin (AMP). Bacterial strains and plasmids used in this study are described in Table 1.

**Construction of mini-Tn5Km2 mutants.** Wild-type *B. abortus* IVKB9007 was subjected to random transposon mutagenesis using a mini-Tn5Km2 transposon that was delivered on the pUT suicide vector as described previously (6). The transposon mutant was constructed by con- junctal transfer of pUT mini-Tn5Km2 from the *E. coli* S17-1 λpir donor strain into the wild-type *B. abortus* IVKB9007 strain. Transconjugants were isolated on TSA containing 50 μg/ml KM and 30 μg/ml NA to select against both *B. abortus* and *E. coli* donor strains, and mutants were screened for defects in intracellular survival within HeLa cells as described below. All mutants were kept in 25% glycerol in TSB at −70°C until the screening.

**Screening for an intracellular-survival defect of *B. abortus* mutants.** Intracellular-survival assays were performed using the modified method described previously (6). Briefly, HeLa cells were grown and maintained at 37°C in 5% CO₂ in complete medium (CM; Dulbecco’s modified Eagle’s medium [DMEM; Gibco, Carlsbad, CA, USA] containing 10% fetal bovine serum [GenDEPOT, USA]) with an antibiotic or antimycotic (Life Technologies, Gaithersburg, MD, USA). The HeLa cells were dispensed in medium [DMEM; GIBCO, Carlsbad, CA, USA] containing 10% fetal bovine serum [GenDEPOT, USA] for 10 min at room temperature and then incubated at 37°C for 1 h. The plates were centrifuged at 150 × g for 10 min at room temperature. Subsequently, the cells were incubated at 37°C in 5% CO₂ for 1 h, washed twice with sterile phosphate-buffered saline (PBS; pH 7.0), and incubated with CM plus 30 μg/ml gentamicin (GM; Sigma, St. Louis, MO, USA) for 48 h. The infected cells were then washed and lysed with 0.1% Triton X-100 in sterile distilled deionized water (sDDW), and 30 μl of the cell lysate was inoculated onto TSA and incubated at 37°C in 5% CO₂ for 72 h. The mutants showing no growth on TSA were considered intracellular-survival-defective mutants and were selected for further assays.

**Cloning and sequencing of transposon insertion sites.** The DNA sequences flanking the transposon insertion were assembled, and homology searches were carried out using the public BLASTn and BLASTx databases at http://www.ncbi.nlm.nih.gov.

**Complementation of IVKB9007 lOOPl::Tn5 and IVKB9007 cydC::Tn5 mutants.** Intact copies of the cydC and lOOPl genes were amplified by PCR from chromosomal DNA of *B. abortus* IVKB9007 using a high-fidelity *Pfu* X DNA polymerase (SolGent, Daejeon, South Korea) with the primer pairs 5’-AATCTCGAGCTAGCTAATGAGATGGCAGGAGTGAG-3’ (PstI) and 5’-GGCTCTAGAGGCCTGACCTGACG-3’ (XbaI) and 5’-AACCTCGAGCTGACCTGACGAGTGGCAGGAGGAGTTGACTAC-3’ (XbaI) and 5’-AACCTCGAGCTGACCTGACGAGTGGCAGGAGGAGTTGACTAC-3’ (PstI) and 5’-GGCTCTAGAGGCCTGACCTGACGAGTGGCAGGAGGAGTTGACTAC-3’ (XbaI), respectively. The approximately 1.7-kb (cydC) and 1.9-kb (lOOPl) gel-purified products were digested with PstI and XbaI, followed by ligation with similarly cleaved pBBRI-MCS4-Amp’ and pBBRI-looP-Amp’, respectively. Plasmid constructs were verified by PCR, restriction analysis, and DNA sequencing. The plasmids were introduced into the IVKB9007 lOOPl::Tn5 and IVKB9007 cydC::Tn5 mutants via electroporation as described previously (17). The KM- and AMP-resistant transformants were selected, thereby generating the complemented C-IVKB9007 lOOPl::Tn5 and C-IVKB9007 cydC::Tn5 strains. To determine bacterial phenotype, a crystal violet method (18) was performed to con- firm that the transformant mutants and their complementation strains maintained their smooth phenotype.

**Determination of the intracellular growth efficiency of mutants.** The intracellular bacterial growth assays were performed as described previously (19, 20). Briefly, RAW264.7 and HeLa cells were seeded at 10⁵ cells/well into a 24-well tissue culture plate and infected with wild-type IVKB9007 and its isogenic mutants at a multiplicity of infection (MOI) of 20. The plates were centrifuged at 150 × g for 10 min at room temperature and then incubated at 37°C in 5% CO₂ for 1 h. The nonadherent bacteria were removed by washing the plates three times with CM without antibiotics, and then cells were incubated with CM containing 50 μg/ml GM and 30 μg/ml AMP to kill extracellular bacteria. The infected cells were washed twice with CM, the medium was replaced with CM containing 10 μg/ml GM, and the plate was incubated at 37°C for 0, 4, 24, and 48 h. At different times

---

**Table 1: Bacterial strains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics or putative function of the disrupted gene | Reference or source |
|-------------------|---------------------------------------------------------------|---------------------|
| **Bacterial strains** | | |
| *E. coli* DH5α | F^- 80 lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hisD171 (λDE3) relA1 gal phoA supE44 thi-1 gyrA96 | Invitrogen |
| *E. coli* S17-1 λpir | F' araD (lac pro) argE1(recA1) recA56 rif naldA, harboring the suicide vector pUT mini-Tn5Km2, Km' | 6 |
| *B. abortus* RB51 | Rough *B. abortus* vaccine strain RB51 | CVT' |
| *B. abortus* 544 | Smooth virulent *B. abortus* strain 544 (ATCC 23448) | 6 |
| *B. abortus* IVKB9007 | Epidemic strain; smooth virulent *B. abortus* biovar 1 strain isolated from an aborted bovine fetus | This study |
| IVKB9007 lOOPl::Tn5 | Mini-Tn5Km2 insertion in ATP/GDP-binding proteins motif A (P-loop), ABC transporter of IVKB9007 strain, Km' | This study |
| IVKB9007 cydC::Tn5 | Mini-Tn5Km2 insertion in ATP-binding/permease protein, ABC transporter (cydC) of IVKB9007 strain, Km' | This study |
| IVKB9007 C-looP::Tn5 | IVKB9007 lOOPl::Tn5 mutant with plasmid pB4P-looP, Km' Amp' | This study |
| IVKB9007 cydC::Tn5 | IVKB9007 cydC::Tn5 mutant with plasmid pB4cydC, Km' Amp' | This study |

| Plasmids | | |
| pBluescript II KS (+) | ColE1 Amp' | Stratagene |
| pBRII-MCS4 | Broad-host-range cloning vector, Amp' | 40 |
| pB4cydC | PstI/XbaI fragment containing the *B. abortus* IVKB9007 cydC gene cloned into pBBRI-MCS4, Amp' | This study |
| pB4P-looP | PstI/XbaI fragment containing the *B. abortus* IVKB9007 lOOPl gene cloned into pBBRI-MCS4, Amp' | This study |

### Notes

1. The *B. abortus* RB51 strain was provided by Chungang Vaccine Institute (CVI), South Korea.
postinfection (p.i.), the infected cells were washed three times with sterile PBS and lysed with 0.1% Triton X-100 in sDW. Serial dilutions of the lysates were plated on TSA agar to enumerate the CFU. These assays were performed at least three times, and the means ± standard deviations (SD) were calculated.

Measurement of sensitivity of mutants to environmental stresses. The parental IVKB9007 strain and mutants were evaluated for sensitivity to stress conditions and for acid and oxidative-stress tolerance. To measure sensitivity to hydrogen peroxide (H₂O₂), a hydrogen peroxide agar diffusion assay was conducted (9). Briefly, bacterial cultures (optical density at 600 nm [OD₆₀₀] of 0.8) were pelleted by centrifugation, and the pellets were washed twice in PBS and resuspended in PBS at a concentration of approximately 10⁵ CFU/ml. One hundred microliters of each culture were spread on TSA plates in triplicate. Five microliters of 30% H₂O₂ was spotted onto 6-mm-diameter sterile blank paper (BD Bioscience, NJ, USA), and the paper was placed at the center of each plate. After incubation for 3 days at 37°C, the diameter of the zone of bacterial clearance around each disk was measured.

To determine the sensitivity to acid, bacteria were grown to early stationary phase (OD₆₀₀ = 0.8) at 37°C. The cultures were collected and then pelleted by centrifugation at 12,000 × g for 5 min at room temperature, and the pellets were resuspended in TSB adjusted to pH 7.0, 4.5, or 3.5 with concentrated HCl. After 3 h of incubation with agitation at 37°C, CFU were counted by plating of serial dilutions on TSA plates. The assay was repeated at least three times independently, and the means ± SD were calculated.

Assessment of sensitivity to heavy metals and respiratory chain inhibitors was performed as described previously (9). Early-stationary-phase bacteria were serially diluted and plated in triplicate on TSA or TSB supplemented with zinc sulfate (ZnSO₄; Sigma) and sodium azide (NaN₃; Sigma). Bacterial survival was determined after 5 days of incubation by enumerating the number of bacteria grown on plates of TSA and TSA containing ZnSO₄/NaN₃. Experiments were performed at least three times, and the means ± SD were calculated.

Persistence of B. abortus mutants in mice. The virulence in vivo of the wild-type B. abortus IVKB9007 strain and mutants was determined by measuring the persistence of the strains in the spleens of mice after 1, 2, 3, and 7 weeks p.i. All mice were kept under controlled conditions in a microventilation cage system (MVCS; ThreeShine, Daejeon, South Korea). The animal experiments were approved by the Animal Research Committee of Kangwon National University. Female 8-week-old BALB/c mice were inoculated intraperitoneally (i.p.) with 2.0 × 10⁵ CFU/mouse of the wild-type B. abortus IVKB9007 strain, the IVKB9007 looP::Tn5 mutant, or the complemented IVKB9007 C-looP::Tn5 or C-cydD::Tn5 mutant. One hundred microliters of each challenge strain was determined by subtracting the number of CFU on antibiotic-containing agar from the number of CFU on agar without antibiotics. The degrees of protection were expressed as the mean number of CFU ± SD of B. abortus strain 544 for each mouse group obtained after challenge. Log₉₀ units of protection (U) were obtained by subtracting the mean log₁₀ number of CFU for the vaccinated group from the mean log₁₀ number of CFU for the PBS control group.

Statistical analysis. The bacterial CFU, survival, and morphometric analysis data were analyzed using GraphPad Prism (version 5; GraphPad Software, La Jolla, CA, USA). Analyses of variance (ANOVA) and Student t-tests were performed to compare individual mutant groups with groups infected with complementation strains or the wild-type control and for comparing the vaccine group with unvaccinated controls. P-values of less than 0.05 were considered significant.

RESULTS
Isolation of intracellular-survival-defective B. abortus mutants. To identify B. abortus genes required for virulence and intracellular survival, transposon mutagenesis of a wild-type B. abortus IVKB9007 strain was performed as described previously (6). Nine hundred ninety mutants were screened to select mutants defective in intracellular survival within HeLa cells in at least two independent assays. Finally, B. abortus IVKB9007 looP::Tn5 and cydD::Tn5 mutants were selected because, while they retained a smooth phenotype (data not shown), they displayed the most severe defects in intracellular survival in HeLa cells and were used for further analysis.

Determination of the transposon insertion site in B. abortus mutants. To determine the location of the transposon insertion site in the chromosomal DNA of the mutants, the genomic DNA of the mutants was extracted, digested, and cloned into the Bluescript II KS (+) plasmid. The resulting plasmid DNA was isolated and subjected to DNA sequencing using the O’-end primer of the mini-Tn5Km2 transposon (6). The nucleotide and the deduced amino acid sequences flanking the transposon insertions in each mutant were compared with the Brucella sequence database available at the NCBI (BLAST). The genes of the mutants disrupted by transposon insertion were identified by their homology and demonstrated coding sequences which showed that a mini-Tn5Km2 transposon was inserted in the looP and cydD genes, encoding ATP/GDP-binding protein motif A (P-loop) and the ATP-binding/permease protein, respectively. The site of transposon insertion in looP is position 83741, and the gene region spans positions 83622 to 85535 (coding sequence [CD] BruAb2_0083) on B. abortus biovar 1 chromosome II. The transposon insertion site in cydD is at position 726981, nearly overlapping the region of the cydC and cydD genes (the cydC gene region is positions 725304 to 726986 [CDs BruAb2_0713]), and the cydD gene region is positions 726983 to 728719 [CDs BruAb2_0714]) of B. abortus biovar 1 chromosome II.

B. abortus cydC and looP are required for intracellular survival and multiplication. The viability and intracellular growth of the B. abortus IVKB9007 looP::Tn5 and cydD::Tn5 mutants within RAW264.7 and HeLa cells were evaluated at different time points p.i. As shown in Fig. 1, the numbers of intracellular bacteria recovered 0 h p.i. did not differ significantly between the IVKB9007 strain and mutants, indicating that there was no defect in the mutants in internalization and invasion of either cells. However, we found almost a 1-log reduction in the number of intracellular bacteria of the mutant types (B. abortus IVKB9007 looP::Tn5 and cydD::Tn5 mutants in RAW264.7 and HeLa cells) by 24 h p.i., and at 48 h p.i., the mutants were not recovered from either cells.
cydC::Tn5 mutants) at 4 h p.i. compared with the number of bacteria of the B. abortus IVKB9007 strain (P < 0.05). At 24 h p.i., the numbers of CFU of IVKB9007 looP::Tn5 and cydC::Tn5 were dramatically decreased by nearly 1.5 and 2.5 logs, respectively, compared with that of the wild-type IVKB9007 strain (Fig. 1A) (P < 0.001). At 48 h p.i., the numbers of CFU of the B. abortus IVKB9007 looP::Tn5 and cydC::Tn5 mutants continued to decrease, with approximately 3.4- and 4.5-log reductions, respectively, relative to the number for the wild-type strain (P < 0.001). Infection with the complemented C-looP::Tn5 and C-cydC::Tn5 mutants indicated that the introduction of intact copies of the looP and cydC genes in the looP::Tn5 and cydC::Tn5 mutants restored their intracellular-survival capability to near the level of the wild-type B. abortus IVKB9007 strain. This result confirmed that the intracellular growth defects of the looP::Tn5 and cydC::Tn5 mutants were caused by disruption of the looP and cydC genes.

To determine whether sensitivity to respiratory inhibitors and intracellular-stress conditions play a role in the attenuation of the IVKB9007 looP::Tn5 and cydC::Tn5 mutants, the survival ability of all strains under stress conditions was determined. As shown in Fig. 2A, the looP::Tn5 and cydC::Tn5 mutants showed increased sensitivity to respiratory inhibitors (P < 0.001). In particular, the cydC::Tn5 mutant was highly sensitive compared with the parental IVKB9007 strain to the combination of ZnSO4 and NaN3 (P < 0.001). Our results suggest, similarly to the results obtained with Truong et al., that the levels of resistance of the looP::Tn5 and cydC::Tn5 strains in RAW264.7 cells (A) and HeLa cells (B). Both cell types were infected with the IVKB9007 strain and the mutants at an MOI of 20. At the indicated times p.i., the numbers of viable intracellular bacteria were determined by plating serial dilutions of cell lysates. Data points and error bars represent the mean numbers of CFU from triplicates and their standard deviations. The asterisks denote a significant difference from the values for the complementation strains or the parental IVKB9007 strain (**, *P* < 0.001; *, P < 0.05).

**Fig. 1** The cydC and looP genes are required for the virulence of B. abortus in cell infection models. (A and B) Intracellular growth of wild-type B. abortus biovar 1 strain IVKB9007, its looP::Tn5 and cydC::Tn5 mutants, and complemented looP::Tn5 and cydC::Tn5 strains in RAW264.7 cells (A) and HeLa cells (B). Both cell types were infected with the IVKB9007 strain and the mutants at an MOI of 20. At the indicated times p.i., the numbers of viable intracellular bacteria were determined by plating serial dilutions of cell lysates. Data points and error bars represent the mean numbers of CFU from triplicates and their standard deviations. The asterisks denote a significant difference from the values for the complementation strains or the parental IVKB9007 strain (**, *P* < 0.001; *, P < 0.05).
and IVKB9007 cydC::Tn5 mutants are able to induce protection against challenge, mice were immunized with B. abortus IVKB9007 looP::Tn5 and cydC::Tn5 mutants and the commercial RB51 vaccine strain. At 5 weeks p.i., these mice were challenged with the virulent B. abortus 544 strain, and the level of protection against challenge provided by the mutants was assessed (Table 2). At 2 weeks postchallenge, BALB/c mice immunized with the looP::Tn5 and cydC::Tn5 mutants and RB51 strain had significantly fewer splenic bacteria than nonimmunized mice (P < 0.001). Mice vaccinated with the RB51 strain and looP::Tn5 mutant exhibited a significant degree of protection (1.51 U and 2.19 U, respectively) against challenge (P < 0.001). Interestingly, the level of protection following challenge in mice immunized with the cydC::Tn5 mutant (2.31 U) was higher than that in mice immunized with the looP::Tn5 mutant and RB51 strain, even though it was cleared more quickly from the spleen. Splenomegaly postchallenge was significantly reduced following vaccination with either the looP::Tn5 or the cydC::Tn5 mutant compared with that of RB51-vaccinated (P < 0.001) and nonvaccinated (P < 0.001) mice, resulting in significantly lower levels of 544 infection. Taken together, these results suggest that both mutants, especially the cydC::Tn5 mutant, are very safe and show protective ability against virulent B. abortus challenge.

DISCUSSION

A virulence factor is defined as any factor that contributes to the ability of the pathogen to colonize, persist, propagate, and cause disease in hosts. Therefore, to better understand the interplay between Brucella and its host that leads to the development and persistence of disease, it is crucial to identify genes encoding putative virulence determinants. The primary objective of this study was to identify B. abortus mutants that are well attenuated, capable of inducing protection against virulent challenge, and able to be used for a live attenuated vaccine. We produced transposon mutations in the B. abortus IVKB9007 field isolate obtained from a cow and compared the resulting mutants with the parental strain for virulence in vitro and in vivo. In addition, we assessed their ability to provide protection against challenge with a virulent strain. We identified among the mutants the looP::Tn5 and cydC::Tn5 mutants, which have a transposon insertion in the looP and cydC genes, respectively, as meeting the desired criteria.

The P-loop motif is present in ATP/GDP-binding protein Walker A, a member of the ATP-binding cassette (ABC) superfamily of transporters, which are known to be involved in the uptake or secretion of diverse molecules across the bacterial membrane and to be important for survival in the host (21, 22). The looP gene has been identified previously in E. coli, Saccharomyces cerevisiae, and Porphyromonas gingivalis, and the motif is commonly found in many nucleotide-binding proteins, including ATPases, ATP synthases, kinases, elongation factors, and myosin (15, 16, 23). In fact, a mutation within the P-loop ATP-binding motif abolished most of the activity of the ribosome-activated diameter of the clear zone around a disk containing 30% H₂O₂. (C) Sensitivity to acidic stress was determined after all strains were exposed for 3 h to TSB adjusted to pH 7.0, pH 4.4, or pH 3.5. The CFU were enumerated on TSA plates. Data points and error bars represent the means of results from three independent trials and their standard deviations. The asterisks indicate a significant difference in mean values from those for the IVKB9007 strain (**, P < 0.001; *, P < 0.05).
ATPase and reduced its intrinsic ATPase activity, leading to increased oxidative stress and increased sensitivity to respiratory inhibitors (15, 16, 23–26). However, the role of ATP/GDP-binding protein motif A (P-loop) in the virulence of brucelae is still unknown.

The *B. abortus* cydDC genes are located upstream of cydAB, a gene cluster encoding a high-oxygen-affinity cytochrome bd terminal oxidase (9). This experimental finding is consistent with observations for the *E. coli* cydDC genes, which encode a heterodimeric, membrane-bound ABC type of transporter that is required for the cydAB-encoded cytochrome bd terminal-oxidase assembly (13, 27). This transporter functions in energy conservation under microaerobiosis and protects the cell from oxidative stress and respiratory inhibitors (28–30). The cytochrome bd terminal oxidase is used by *Brucella* species to survive inside host cells, where it contributes to intracellular replication (9, 31). It has been previously demonstrated by Kohler et al. that mutation of *Brucella suis* cydD results in defective intracellular growth in cultured macrophages, but its characterization and attenuation in vivo have yet to be reported (8). Moreover, the cytochrome bd oxidase-deficient cydB mutants of *B. abortus* and *B. suis* display very different phenotypes in vitro and in vivo (9, 31); while the *B. abortus* cydB mutant was attenuated, the *B. suis* cydB mutant was hypervirulent. These results suggested a differential use of terminal oxidases in these two species. Therefore, the roles of the cydDC genes in the virulence of *B. suis* and *B. abortus* may differ and should be further evaluated.

In this study, we found that the looP::Tn5 and cydC::Tn5 mutants have a reduced ability compared with that of the parental IVKB9007 strain to survive and proliferate within RAW264.7 and HeLa cells. The mutants also showed heightened sensitivity to respiratory inhibitors, acidic pH, and highly reactive oxygen species. These results suggest that disruption of the looP and cydC genes impaired the ability of the IVKB9007 strain to cope with different environmental stresses and to reach its intracellular replicative niche within the host cell. Similarly to a previously published *B. abortus* cydB mutant (9), our *B. abortus* mutant lacking cydC displayed a sensitivity to environmental stressors that was significantly greater than that of the parental IVKB9007 strain. In addition, we found that the virulences of the looP::Tn5 and cydC::Tn5 mutants were significantly attenuated in mice. The decrease in intracellular numbers of CFU of mutants in vivo is consistent with the intracellular growth defects observed in cell culture. To address the exact role of the looP and cydC genes in *B. abortus* virulence, complementation strains of the looP::Tn5 and cydC::Tn5 mutants were constructed. The complemented strains restored the intracellular survival and replication, persistence, and splenomegaly in mice to the level observed in the parental IVKB9007 strain. Therefore, the data indicated that the looP and cydC genes are involved in the virulence of *B. abortus*.

Of note, the hyperattenuation observed in the cydC::Tn5 mutant was possibly because of a deficiency in cytochrome *bd* terminal oxidase. Consistently with this, the cydC::Tn5 mutant may be attenuated because of insufficient oxygen exposure and heightened sensitivity to environmental stresses, which is essential for the proliferation and metabolism of *Brucella* inside the host. Remarkably, while the *B. abortus* cydB mutant remained viable until 8 weeks p.i. in mice (9), the IVKB9007 cydC::Tn5 mutant was rapidly cleared after 3 weeks p.i.; even the inoculum dose was nearly 10-fold higher than that of the *B. abortus* cydB mutant.
These observations suggest that cydD, a component of the ABC cassette-type transporter, is required for cytochrome bd oxidase activity and that mutation of cydC has a more deleterious effect on *B. abortus* virulence than that of the absence of cydB-encoded cytochrome bd oxidase.

In fact, however, the exact mechanisms responsible for the rapid clearance of the IVKB9007 cydC::Tn5 mutant are still not known. Since we have not measured either cydAB expression or accumulation of the defective cytochrome terminal oxidases in the mutant, we cannot explain the precise mechanism by which cydC contributes to the cytochrome bd oxidase expression. Notably, a recent study on cydX of *B. abortus* demonstrated that the promoter controlling the expression of cydAB and cydX is located between the cydDC and cydAB genes (32). Therefore, it is less likely that mutation of cydC will affect the regulation of cytochrome bd expression. In *E. coli* and other bacterial species where cydC genes, an ATP-binding cassette type, have clearly been identified, these genes are adjacent but separate from cydAB genes (29, 33–35). Mutation of either cydD or cydC displayed complex phenotypes in addition to being required for cytochrome bd oxidase assembly or activity. These include the loss of periplasmic c-type cytochrome and transport of glutathione from the cytoplasm to the periplasm (14, 29, 33). Therefore, it is reasonable to propose that a more severe colonization defect of the IVKB9007 cydC::Tn5 mutant possibly resulted from one of the other phenotypes associated with the lack of glutathione uptake or cytochrome c oxidase assembly.

The differences in survival exhibited by the IVKB9007 looP::Tn5 and cydC::Tn5 mutants might prove to be potential advantages for vaccine strains. It is possible that the rapid clearance of the IVKB9007 cydC::Tn5 mutant may enhance its safety, but this may provide only modest protection, because it may not produce in vivo some key antigens necessary for the induction of a protective immunity. Therefore, the protective efficacy of the IVKB9007 cydC::Tn5 mutant should be investigated. It is of interest that the IVKB9007 cydC::Tn5 mutant showed better protection than the RB51 vaccine strain, as did the IVKB9007 looP::Tn5 mutant. The reason why both mutants showed better protection than RB51 seems to be related to lipopolysaccharide (LPS) biosynthesis. Both mutants retained the smooth phenotype of the parental IVKB9007 strain, while the RB51 strain has a rough phenotype because it does not have the O side chain of LPS (O-LPS) (36). *B. abortus* LPS O antigen is known to be an essential virulence factor, and this antigen plays an important role in eliciting protective immunity against challenge infection with virulent *B. abortus* (36, 37). Therefore, it might be explained that the mice vaccinated with either mutant showed higher protection than the mice vaccinated with RB51 because the mice vaccinated with the mutants may have had protective immunity to smooth LPS. The high level of protection provided by both the IVKB9007 looP::Tn5 and cydC::Tn5 mutants suggests that the mutants retained their immunogenic properties enough to confer protection against wild-type *B. abortus* infection. Further study should be needed for characterizing the cydDC operon and particularly the cydC mutant as a live vaccine.

In present study, a higher level of protection conferred by the IVKB9007 cydC::Tn5 mutant was surprising, although a precise explanation for this observation is not available at this time. We hypothesize that the IVKB9007 cydC::Tn5 mutant retained all the known virulence factors that may contribute to protective immunity, which is similar to the phenotypes observed in *Brucella cydBA* mutants (38, 39). A possible explanation for a slightly lower level of protection observed in mice immunized with the IVKB9007 looP::Tn5 mutant is that disruption of the looP gene may produce unidentified effects on the induction of protective immunity. Future research will be needed to elucidate the exact mechanisms of induction of immunity by the mutants.

In summary, we describe two *B. abortus* transposon mutants, looP and cydC mutants, which exhibit attenuated phenotypes both in cell infection models and in mice and afford better protection than the RB51 vaccine strain. This indicates that both mutants, especially the cydC mutant, might provide useful information for the construction of a new live vaccine candidate against bovine brucellosis.
growth and survival properties of Escherichia coli cydDC and cydAB strains are due to deficiencies in cytochrome bd and are corrected by exogenous catalase and reducing agents. J. Bacteriol. 178:6348–6351.
13. Poole RK, Hatch L, Cleeter MW, Gibson F, Cox GB, Wu G. 1993. Cytochrome bd biosynthesis in Escherichia coli: the sequences of the cydC and cydD genes suggest that they encode the components of an ABC membrane transporter. Mol. Microbiol. 10:421–430. http://dx.doi.org/10.1111/j.1365-2958.1993.tb06273.x.
14. Poole RK, Gibson F, Wu G. 1994. The cydD gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome c and of cytochrome bd in Escherichia coli. FEMS Microbiol. Lett. 117:217–223. http://dx.doi.org/10.1016/S0378-1097(01)00995-9.
15. Fischer H, Glocshuber R. 1994. A point mutation within the ATP-binding site inactivates both catalytic functions of the ATP-dependent protease La (Lon) from Escherichia coli. FEBS Lett. 356:101–103. http://dx.doi.org/10.1016/0014-5793(94)01244-X.
16. Yang H, Hamada K, Terashima H, Iizuta M, Yamaguchi-Sihta E, Konidoh O, Satoh H, Miyazaki M, Arisawa M, Miyamoto C, Kitada K. 1996. A point mutation within each of two ATP-binding motifs inactivates the functions of elongation factor 3. Biochim. Biophys. Acta 1310:303–306. http://dx.doi.org/10.1016/S0167-4889(95)00179-4.
17. McQuiston JR, Schurig GG, Sriranganathan N, Boyle SM. 1995. Transformation of Brucella species with suicide and broad host-range plasmids. Methods Mol. Biol. 47:143–148.
18. White PG, Wilson JB. 1981. Differentiation of smooth and nonsmooth colonies of brucellae. J. Bacteriol. 61:239–240.
19. Watarai M, Kim S, Erdenebastar J, Makino S, Horiechi M, Shirahata T, Sakaguchi S, Katamine S. 2003. Cellular prion protein promotes Brucella infection into macrophages. J. Exp. Med. 198:5–17. http://dx.doi.org/10.1084/jem.20021980.
20. Watarai M, Makino S, Fujii Y, Okamoto K, Shirahata T. 2002. Modulation of Brucella-induced macropinocytosis by lipid rafts mediates intracellular replication. Cell. Microbiol. 4:341–355. http://dx.doi.org/10.1046/j.1462-5822.2002.00195.x.
21. Young J, Holland JB. 1999. ABC transporters: bacterial exporters revisited five years on. Biochim. Biophys. Acta 1461:177–200. http://dx.doi.org/10.1016/S0005-2736(99)00138-3.
22. Fath MI, Kolter R. 1993. ABC transporters: bacterial exporters. Microbiol. Rev. 57:995–1017.
23. Toyoda T, Okano S, Shibata Y, Abiko Y. 2010. Oxidative stress induces phosphorylation of the ABC transporter, ATP-binding protein, in Porphyromonas gingivalis. J. Oral Sci. 52:561–566. http://dx.doi.org/10.2334/josud.52.561.
24. Deypur AT, Krishnan S, Cockburn BN, Schwartz NB. 1998. Deletion and site-directed mutagenesis of the ATP-binding motif (P-loop) in the bifunctional murine ATP-sulphurylase/adenosine 5’-phosphosulphate kinase enzyme. J. Biol. Chem. 273:9450–9456. http://dx.doi.org/10.1074/jbc.273.16.9450.
25. Bowler MJW, Montgomery MG, Leslie AG, Walker JE. 2006. How azide inhibits ATP hydrolysis by the F-ATPases. Proc. Natl. Acad. Sci. U. S. A. 103:8646–8649. http://dx.doi.org/10.1073/pnas.0602915103.
26. Hong S, Pedersen PL. 2008. ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. Microbiol. Mol. Biol. Rev. 72:590–641. http://dx.doi.org/10.1128/MMBR.00016-08.
27. Goldman BS, Kranz RG. 2001. ABC transporters associated with cytochrome c biogenesis. Res. Microbiol. 152:323–329. http://dx.doi.org/10.1016/S0923-2508(01)01203-7.
28. Cook GM, Poole RK. 2000. Oxidase and periplasmic cytochrome assembly in Escherichia coli K-12: CydDC and CcmAB are not required for haem-membrane association. Microbiology 146:527–536.
29. Pittman MS, Robinson HC, Poole RK. 2005. A bacterial glutathione transporter (Escherichia coli CydDC) exports reductant to the periplasm. J. Biol. Chem. 280:32254–32261. http://dx.doi.org/10.1074/jbc.M503075200.
30. Cruz-Ramos H, Cook GM, Wu G, Cleeter MW, Poole RK. 2004. Membrane topology and mutational analysis of Escherichia coli CydDC, an ABC-type cysteine exporter required for cytochrome assembly. Microbiology 150:3415–3427. http://dx.doi.org/10.1099/mic.0.27191-0.
31. Jimenez de Bagues MP, Loisel-Meyer S, Liautard JP, Jubier-Maurin V. 2007. Different roles of the two high-oxygen-affinity terminal oxidases of Brucella suis: cytochrome c oxidase, but not ubiquinol oxidase, is required for persistence in mice. Infect. Immun. 75:531–535. http://dx.doi.org/10.1128/IAI.00185-06.
32. Sun YH, de Jong MF, den Hartigh AB, Roux CM, Rolan HG, Toolsim RM. 2012. The small protein CydX is required for function of cytochrome bd oxidase in Brucella abortus. Front. Cell. Infect. Microbiol. 2:47. http://dx.doi.org/10.3389/fcimb.2012.00047.
33. Cook GM, Membrillo-Hernandez J, Poole RK. 1997. Transcriptional regulation of the cydDC operon, encoding a heterodimeric ABC transport required for assembly of cytochromes c and bd in Escherichia coli K-12: regulation by oxygen and alternative electron acceptors. J. Bacteriol. 179:6523–6530.
34. Way SS, Sallustio S, Magliozzo RS, Goldberg MB. 1999. Impact of either elevated or decreased levels of cytochrome bd expression on Shigella flexneri virulence. J. Bacteriol. 181:1229–1237.
35. Winstedd I, Yoshida K, Fujita Y, von Wachenfeldt C. 1998. Cytochrome bd biosynthesis in Bacillus subtilis: characterization of the cydBcD operon. J. Bacteriol. 180:6571–6580.
36. Stevens MG, Olsen SC, Pugh GW, Jr, Brees D. 1995. Comparison of immune responses and resistance to brucellosis in mice vaccinated with Brucella abortus 19 or RB51. Infect. Immun. 63:264–270.
37. Jimenez de Bagues MP, Elzer PH, Jones SM, Blasco JM, Enright FM, Schurig GG, Winter AJ. 1994. Vaccination with Brucella abortus rough mutant RB51 protects BALB/c mice against virulent strains of Brucella abortus, Brucella melitensis, and Brucella ovis. Infect. Immun. 62:4990–4996.
38. Kahl-McDonagh MM, Elzer PH, Hagius SD, Walker JF, Perry QL, Seabury CM, den Hartigh AB, Tsolis RM, Adams LG, Davis DS, Ficht TA. 2006. Evaluation of novel Brucella melitensis unmarked deletion mutants for safety and efficacy in the goat model of brucellosis. Vaccine 24:5169–5177. http://dx.doi.org/10.1016/j.vaccine.2006.04.005.
39. Ko J, Gendron-Fitzpatrick A, Ficht TA, Spliter GA. 2002. Virulence criteria for Brucella abortus strains as determined by interferon regulatory factor 1-deficient mice. Infect. Immun. 70:7004–7012. http://dx.doi.org/10.1128/IAI.70.7.7004-7012.2002.
40. Kim S, Kurokawa D, Watanabe K, Makino S, Shirahata T, Watarai M. 2004. Brucella abortus nicotinamidase (PncA) contributes to its intracellular replication and infectivity in mice. FEMS Microbiol. Lett. 234:289–295. http://dx.doi.org/10.1111/j.1574-6968.2004.tb09546.x.