Characterization and protective property of *Brucella abortus* cydC and looP mutants

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Running title: cydC and P-loop are involved in virulence of *Brucella abortus*

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Key words: *Brucella abortus*, transposon mutagenesis, stress adaptation, attenuation, protection
Abstract

Brucella abortus readily multiplies in professional or nonprofessional phagocytes in vitro and is highly virulent in mice. Isogenic mutants of B. abortus (BA7) lacking the ATP/GDP-binding proteins motif A (P-loop) (named as looP and designated hereafter as looP::Tn5 BA7 mutant) and the ATP-binding/permease protein (cydC; designated hereafter as cydC::Tn5 BA7 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 looP::Tn5 BA7 and cydC::Tn5 BA7 mutants restored their ability to survive in vitro and in vivo to a level comparable with that of 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 live looP::Tn5 BA7 and cydC::Tn5 BA7 mutants provided a high degree of protection against challenge with pathogenic B. abortus 544 strain. Both mutants should be evaluated further as a live attenuated vaccine against bovine brucellosis for their ability to stimulate a protective immune response.
Introduction

*B. abortus*, the causative agent of bovine brucellosis, causes abortion and reduced fertility in cattle and undulant fever in humans (1). Unlike other pathogenic bacteria, *Brucella* do not have classical virulence factors. A key aspect of the virulence of *B. abortus* is the ability to invade, survive and proliferate within host phagocytic cells, successfully bypassing the bactericidal activities of phagocytes and thereby establishing long-lasting chronic infections (2, 3). The *B. abortus* vaccine strains S19 and RB51 have been used worldwide for the prevention of brucellosis in cattle, but have several drawbacks, including interference with diagnosis, residual virulence, and some pathogenicity for humans as well as cattle (4, 5). Therefore, understanding the virulence of *B. abortus* on a genetic level and the host response against *B. abortus* will provide important information for the development of a new vaccine for controlling brucellosis.

Transposon mutagenesis is an extensively used approach for the identification of genes involved in the virulence of bacterial pathogens (6-8). To understand the sustained intracellular residence of brucellae in host cells, a transposon-based approach has been employed to identify genes in brucellae that are critically required for lipopolysaccharide biosynthesis, metabolic processes, stress responses, nutrient deprivation, and invasion of and survival in host cells. Mutations in these genes led to the attenuation of *Brucella*, recognized by reduced intracellular survival and replication, or rapid clearance of the mutants from both macrophages and mice, compared with a parental strain (6-11). However, these attenuated mutants were considered either not safe or not sufficiently studied for use as possible vaccines in animals. Therefore, identification of additional vaccine targets is required.

In the present study, in an effort to identify additional genes that control the intracellular fate of *B. abortus*, transposon-generated mutants were selected based on decreased survival in
HeLa cells. We describe here the identification of transposon ATP-binding/permease protein (cydC) and ATP/GDP-binding-proteins motif A (P-loop) B. abortus mutants. The cydC gene has been well described in *Escherichia coli*, and was originally identified by its requirement for assembly of the cytochrome *bd*-type terminal oxidase (12-14). Mutants defective in cydC have a deficiency of cytochrome *bd* oxidase, an inability to exit from stationary phase, and are hypersensitive to zinc and oxidative stress. However, the *looP* gene has not been fully characterized (15, 16). The roles of the *looP* and *cydC* genes in the virulence of *B. abortus* are not well established. In the present study, both *looP* and *cydC* mutants of *B. abortus* were characterized and evaluated for intracellular replication in cell models, their virulence in mice, and their ability to induce protection against a wild-type *B. abortus* challenge in mice.

**Materials and Methods**

**Bacterial culture and media.** The virulent wild-type *B. abortus* biovar 1 (BA7) 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, virulent *B. abortus* challenge strain 544, BA7 strain and the isogenic *looP*:Tn5 BA7 and *cydC*:Tn5 BA7 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 λpir 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 broth or agar (LB; Difco, Sparks, MD, USA). If necessary, the medium was supplemented with appropriate reagents and antibiotics as indicated: 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. *B. abortus* wild-type BA7 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 conjugal transfer of pUT mini-Tn5Km2 from *E. coli* S17-1 λpir donor strain into the *B. abortus* wild-type BA 7 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 screening.

Screening for 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] and antibiotic–antimycotic (Life Technologies, Gaithersburg, MD, USA). The HeLa cells were dispensed into 96-well tissue culture plates 24 h prior to bacterial infection. The mutants were deposited onto the cells grown in 96-well microplates, and 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 adjacent to the transposon insertion sites were determined as described previously (6). Briefly, chromosomal DNA was isolated from transposon mutants and digested with one of the several restriction enzymes (EcoRI or KpnI; New England BioLabs, Hitchin, UK) that cut at either end of the transposon, leaving the KM resistance gene intact. The digested fragments were ligated into similarly cleaved pBluescript II KS (+) (Stratagene, La Jolla, CA, USA) and transformed into competent E. coli DH5α (Invitrogen, Carlsbad, CA, USA). The transformants were selected on LB agar plates containing 50 µg/ml KM and 100 µg/ml AMP. The plasmid DNA from each transformant, containing both transposon and flanking DNA, was purified using a HiYield Plasmid mini kit (RBC Bioscience, Taipei, Taiwan), and automated sequence analysis was performed (Bionic, South Korea) using the mini-Tn5Km2 transposon O’-end primer (5’-CCTCTAGAGTCGACCTGCAG-3’). The DNA sequences flanking the transposon insertion were assembled, and homology searches were carried out using the public databases BLASTn and BLASTx at http://www.ncbi.nlm.nih.gov.

Complementation of looP::Tn5 BA7 and cydC::Tn5 BA7 mutants. Intact copies of the cydC and looP genes were amplified by PCR from chromosomal DNA of B. abortus BA7 using a high-fidelity Pfu-X DNA polymerase (SolGent, Daejeon, South Korea) with the primers 5’-AAC TGC AGT CAC CTC ATG GTT TTG AGG-3’ (PstI) and 5’-GCT CTA GAG CAT GAA GGG TTT GCA TTC AC-3’ (XbaI), and 5’-AAC TGC AGA TGC GCG CCA GGG CAT C-3’ (PstI) and 5’-GCT CTA GAG CTC AAT TGG TTT CGG TGA AC-3’ (XbaI), respectively. The approximately 1.7 kb (cydC) and 1.9 kb (looP) gel-purified products were digested with PstI and XbaI, followed by ligation with similarly cleaved pBBR1MSC-4 to yield the complementation constructs pB4cydC and pB4P-loop, respectively. Plasmid constructs were verified by PCR,
restriction analysis, and DNA sequencing. The plasmids were introduced into looP::Tn5 BA7 and cydC::Tn5 BA7 mutants via electroporation as described previously (17). The KM- and AMP-resistant transformants were selected, thereby generating complemented C-looP::Tn5 BA7 and C-cydC::Tn5 BA7 strains. To determine bacterial phenotype, a crystal violet method (18) was performed to confirm that the transposon 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 10^5 cells/well into a 24-well tissue culture plate and infected with wild-type BA7 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% CO_2 for 1 h. The nonadherent bacteria were removed by washing three times with CM without antibiotics, and then cells were incubated with CM containing 50 μg/ml GM to kill extracellular bacteria. The infected cells were then washed twice with CM, the media replaced with CM containing 10 μg/ml GM, and incubated at 37 °C for 0, 4, 24 and 48 h. At different times postinfection (p.i.), the infected cells were washed three times with sterile PBS and lysed with 0.1% Triton X-100 in sDDW. Serial dilutions of the lysates were plated on TSA agar to enumerate the colony-forming units (CFU). These assays were performed at least three times and the means and standard deviations (mean ± SD) were calculated.

Measurement of sensitivity of mutants to environmental stresses. Parental BA7 strain and mutants were evaluated for sensitivity to stress conditions, acid, and oxidative stress tolerance. To measure sensitivity to hydrogen peroxide (H_2O_2), a hydrogen peroxide agar diffusion assay was conducted (9). Briefly, bacterial cultures (OD_{600} 0.8) were pelleted by
centrifugation, and the pellets were washed twice in PBS and resuspended in PBS at a concentration of approximately $10^8$ CFU/ml. One hundred microliters of each culture was spread on TSA plates in triplicate. Five microliters of 30% H$_2$O$_2$ 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 (OD$_{600}$ 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, and 3.5, respectively, with concentrated HCl. After 3 h of incubation with agitation at 37 °C, CFUs were determined by plating of serial dilutions on TSA plates. The assay was repeated at least three times independently and the mean ± 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 TSA supplemented with zinc sulfate (ZnSO$_4$, Sigma) and sodium azide (NaN$_3$, Sigma). Bacterial survival was determined after 5 days of incubation by enumerating the number of bacteria grown on plates of TSA and TSA containing ZnSO$_4$/NaN$_3$. Experiments were performed at least three times and the mean ± SD were calculated.

**Persistence of *B. abortus* mutants in mice.** The virulence *in vivo* of wild-type BA7 strain and the mutants was determined by measuring the persistence of the strains in the spleens of mice after 1, 2, 3, and 7 weeks p.i. (WPI). All mice were kept under controlled conditions in Micro Ventilation 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 $2.0 \times 10^5$ CFU/mouse of wild-type BA7, \textit{looP}::Tn5 BA7 and \textit{cydC}::Tn5 BA7 mutants or complemented C-\textit{looP}::Tn5 BA7 and C-\textit{cydC}::Tn5 BA7 mutants, or $2.5 \times 10^8$ CFU/mouse of rough vaccine strain RB51, in 0.2 ml of PBS. As a control, one group (n = 5) of mice was inoculated with sterile saline. At 1, 2, 3, and 7 WPI, mice from each group (n = 5) were euthanized by cervical dislocation, and their spleens were removed aseptically and weighed. The spleens were homogenized in 1 ml sterile PBS using a Tissue Lyser system (QIAGEN, Valencia, CA, USA), and spleen homogenates were serially diluted in PBS and plated in duplicate on TSA agar. Plates were then incubated for 3–5 days at 37 °C with 5% CO$_2$ to assess the spleen colonization.

**Protective efficacy of the mutants against challenge with 544 strain.** At 5 weeks postvaccination, groups of five mice were challenged i.p. with $1.5 \times 10^5$ CFU/mouse of virulent \textit{B. abortus} 544 strain. At 2 weeks postchallenge, the mice were euthanized by cervical dislocation, and the spleens were aseptically removed, weighed, and homogenized in 1 ml PBS. Serial dilutions of homogenized spleens were plated in duplicate onto TSA plates or TSA plates supplemented with 50 µg/ml KM or 50 µg/ml RP. After incubation for 3–5 days at 37 °C in 5% CO$_2$, bacterial colonies were enumerated, and the CFU per spleen was calculated. To differentiate between the mutants or RB51 vaccine strain and the challenge strain, different antibiotics were added to TSA according to the antibiotic resistance phenotype of the mutants (KM$^R$) or RB51 vaccine strain (RP$^R$), and the number of CFU of challenge strain was determined by subtracting the CFU on antibiotic-containing agar from the CFU on agar without antibiotics. The degrees of protection were expressed as the mean CFU ± SD of \textit{B. abortus} strain 544 for each mouse group obtained after challenge. Log$_{10}$ units of protection (U) were obtained by
subtracting the mean log_{10} CFU for the vaccinated group from the mean log_{10} 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 (ANOVAs) and Student t tests were performed to compare individual mutant groups with complementation strains or wild-type control, and for comparing the vaccine group with unvaccinated controls. P values 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 wild-type B. abortus BA7 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, looP::Tn5 BA7 and cydC::Tn5 BA7 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 pBluescript 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 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 cydC genes encoding ATP/GDP-binding proteins motif A (P-loop) and ATP-binding/permease protein, respectively. The site of transposon insertion in looP is 83,741, gene region 83,622–85,535 (CDS BruAb2_0083) on B. abortus biovar 1 chromosome II. Transposon insertion site in cydC is at 726,981, near-overlapping region of the cydC and cydD genes [cydC gene region 725,304–726,986 (CDS BruAb2_0713) and cydD gene region 726,983–728,719 (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 looP::Tn5 BA7 and cydC::Tn5 BA7 mutants within RAW264.7 and HeLa cells were evaluated at different time points p.i. As shown in Fig. 1, the number of intracellular bacteria recovered 0 h p.i. did not differ significantly between BA7 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 mutant types (looP::Tn5 BA7 and cydC::Tn5 BA7) at 4 h p.i. compared with that of the BA7 strain ($P < 0.05$). At 24 h p.i., the CFU of looP::Tn5 BA7 and cydC::Tn5 BA7 were dramatically decreased by nearly 1.5 and 2.5 logs, respectively, compared with those of the BA7 strain (Fig. 1A, $P < 0.001$). At 48 h p.i., the CFU of looP::Tn5 BA7 and cydC::Tn5 BA7 mutants continued to decrease, with approximately 3.4 and 4.5 log reduction, respectively, relative to the BA7 strain ($P < 0.001$). Infection with complemented C-looP::Tn5 BA7 and C-cydC::Tn5 BA7 mutants indicated that the introduction of an intact copy of looP and cydC genes in looP::Tn5 BA7 and cydC::Tn5 BA7 mutants restored their intracellular survival capability to near wild-type BA7 level. This result confirmed that the intracellular growth defects of looP::Tn5 BA7 and cydC::Tn5 BA7 mutants were caused by disruption of the looP and cydC genes.
To determine whether sensitivity to respiratory inhibitors and intracellular stress conditions plays a role in the attenuation of *looP::Tn5 BA7* and *cydC::Tn5 BA7* mutants, the survival ability of all strains under stress conditions was determined. As shown in Fig. 2A, *looP::Tn5 BA7* and *cydC::Tn5 BA7* mutants showed increased sensitivity to respiratory inhibitors (*P* < 0.001). In particular, the *cydC::Tn5 BA7* mutant was highly sensitive compared with the parental *BA7* strain to the combination of ZnSO₄ and NaN₃ (*P* < 0.001). Our results suggest, similarly to the results obtained with *E. coli cydDC* and *B. abortus cydB* (9, 12), that the *cydC* gene is required for a functional cytochrome *bd* oxidase of *B. abortus*.

Oxidative killing was determined by measuring the diameter of the clearance zone around a disk containing 30% hydrogen peroxide. The zone of inhibition for *looP::Tn5 BA7* and *cydC::Tn5 BA7* mutants was significantly larger than the zone of inhibition for *BA7*, indicating that the mutants are more susceptible to oxidative stress (*P* < 0.001, Fig. 2B). Fig. 2C shows that the resistance of *BA7* and the mutants to pH 7.0 was similar. However, there are remarkable differences between *BA7* and the mutants in sensitivity to low pH. The CFU of *looP::Tn5 BA7* and *cydC::Tn5 BA7* mutants were significantly reduced by 2.5 to 5 log at pH 4.4 and 3.5, respectively (*P* < 0.001). Complemented strains (C-*looP::Tn5 BA7* and C-*cydC::Tn5 BA7*) of *looP::Tn5 BA7* and *cydC::Tn5 BA7* mutants with intact *cydC* and *looP* genes showed restored survival abilities, to nearly wild-type level, in low pH (Fig. 2). Taken together, these findings indicated that the *looP* and *cydC* genes are involved in *Brucella* stress adaptation, and the intracellular growth defects of the *looP::Tn5 BA7* and *cydC::Tn5 BA7* mutants may be caused by increased sensitivity to a harsh intracellular environment.

The *looP::Tn5 BA7* and *cydC::Tn5 BA7* mutants are attenuated in BALB/c mice. To assess the persistence and level of splenic colonization of tested strains in mice, the same dose
(10^5 CFU) of BA7, looP::Tn5 BA7, cydC::Tn5 BA7, and complemented C-looP::Tn5 BA7 and C-cydC::Tn5 BA7 mutants were inoculated i.p. into BALB/c mice. At 1, 2, 3, and 7 WPI, five mice from each group were sacrificed, spleens were removed, and CFU were counted. As shown in Fig. 3A, a significant difference in CFUs between BA7 and the mutants was observed at all times tested. While BA7 colonizes and replicates rapidly in the spleen, without dramatic decreases in numbers, the colonization of looP::Tn5 BA7 and cydC::Tn5 BA7 in the spleen was significantly reduced at 1 and 2 WPI (P < 0.001). The CFUs in the spleen dropped sharply thereafter; in particular, there were no detectable CFU in the spleens of mice inoculated with cydC::Tn5 BA7 mutant at 3 and 7 WPI, whereas nearly 2.6 log CFU of the looP::Tn5 BA7 mutant were still detected at 7 WPI (P < 0.001).

Spleen weights were compared between the groups. The weight of the spleens in mice that received looP::Tn5 BA7 and cydC::Tn5 BA7 mutants was significantly lower than that in BA7-infected mice during the experimental period (P < 0.001), as was evident by the lack of splenomegaly (Fig. 3B). Additionally, the decreased virulence of looP::Tn5 BA7 and cydC::Tn5 BA7 mutants could be fully restored to wild-type levels by complementation, as shown by C-looP::Tn5 BA7 and C-cydC::Tn5 BA7 (Fig. 3). From these results, we can conclude that mutations in looP and cydC genes of B. abortus BA7 conferred the attenuated phenotype, and resulted in a major reduction in virulence.

**Immunization with the B. abortus mutants conferred protection in mice.** To determine whether looP::Tn5 BA7 and cydC::Tn5 BA7 mutants are able to induce protection against challenge, mice were immunized with looP::Tn5 BA7 and cydC::Tn5 BA7 mutants and the commercial RB51 vaccine strain. At 5 WPI, 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 BA7 and cydC::Tn5 BA7 mutants and the RB51 strain had significantly fewer splenic bacteria than nonimmunized mice ($P < 0.001$). Mice vaccinated with RB51 strain and looP::Tn5 BA7 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 BA7 mutant (2.31 U) was higher than that in mice immunized with the looP::Tn5 BA7 mutant and RB51 strain, even though it was cleared more quickly from the spleen. Significantly reduced splenomegaly post-challenge was observed following vaccination with either looP::Tn5 BA7 or cydC::Tn5 BA7 mutants compared with that in RB51-vaccinated ($P < 0.001$) and nonvaccinated mice ($P < 0.001$), resulting in significantly lower levels of infection. Taken together, these results suggest that both mutants, especially cydC::Tn5 BA7, 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 and capable of inducing protection against virulent challenge, and could be used for a live attenuated vaccine. We produced transposon mutants in the $B. abortus$ BA7 field isolate obtained from a cow, and compared these 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. Of the
mutants, we identified $\text{looP}::\text{Tn5 BA7}$ and $\text{cydC}::\text{Tn5 BA7}$, which have a transposon insertion in the $\text{looP}$ and $\text{cydC}$ genes, respectively, as meeting the desired criteria.

The P-loop motif is present in ATP/GDP-binding proteins 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 $\text{looP}$ gene has been identified previously in $\text{E. coli}$, $\text{Saccharomyces cerevisiae}$, and $\text{Porphyromonas gingivalis}$, 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 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 proteins motif A (P-loop) in the virulence of brucellae is still unknown.

The $\text{B. abortus cydDC}$ genes are located upstream of $\text{cydAB}$, a gene cluster encoding a high-oxygen affinity cytochrome $\text{bd}$ terminal oxidase (9). This experimental finding is consistent with observations for the $\text{E. coli cydDC}$ genes, which encodes a heterodimeric, membrane-bound ABC type of transporter that is required for the $\text{cydAB}$-encoded cytochrome $\text{bd}$ 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 $\text{bd}$ terminal oxidase is used by $\text{Brucella}$ 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 $\text{B. suis cydD}$ results in defective intracellular growth in cultured macrophages, but its characterization and attenuation in vivo has yet to be reported (8). Moreover, the cytochrome $\text{bd}$
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 role of the cydDC genes in virulence of B. suis and B. abortus may differ, and should be further evaluated.

In this study, we found that looP::Tn5 BA7 and cydC::Tn5 BA7 mutants have reduced ability compared with the parental BA7 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 looP and cydC genes impaired the ability of the BA7 strain to cope with different environmental stresses and to reach its intracellular replicative niche within the host cell. Similarly to a B. abortus cydB mutant (9), the B. abortus mutant lacking cydC displayed significantly increased sensitivity to environmental stressors compared with the parental BA7 strain. In addition, we also found that the virulence of looP::Tn5 BA7 and cydC::Tn5 BA7 mutants were significantly attenuated in mice. The decrease in intracellular CFU of mutants in vivo is consistent with the intracellular growth defects observed in cell culture. To address the exact role of looP and cydC genes in B. abortus virulence, complementation strains of looP::Tn5 BA7 and cydC::Tn5 BA7 mutants were constructed. The complemented mutants had restored intracellular survival and replication, persistence and splenomegaly in mice to the level observed with the parental BA7 strain. Therefore, the data indicated that looP and cydC genes are involved in the virulence of B. abortus.

Of note, the hyperattenuation observed in the cydC::Tn5 BA7 mutant was possibly because of a deficiency in cytochrome bd terminal oxidase. Consistent with this, the cydC::Tn5 BA7
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 cyb* mutant remained viable until 8 WPI in mice (9), the *cydC*::Tn5 BA7 mutant was rapidly cleared after 3 WPI, even the inoculum dose was nearly 10-fold higher than that of *B. abortus cydB* mutant. These observations suggest that the *cydC*, a component of 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 *cydC*::Tn5 BA7 mutant are still not known. Since we have not measured either *cydAB* expression or accumulation of the defective cytochrome terminal oxidases in 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* has 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 the cytochrome *bd* expression.

In *E. coli* and other bacterial species where *cydDC* genes, an ATP-binding cassette-type, have been clearly identified, these genes are adjacent but separate from *cydAB* genes (33-36). 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, 33, 34). Therefore, it is reasonable to propose that a more severe colonization defect of *cydC*::Tn5 BA7 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 looP::Tn5 BA7 and cydC::Tn5 BA7 mutants might have potential advantages to be vaccine strains. It is possible that the rapid clearance of the cydC::Tn5 BA7 mutant may enhance its safety, but this could 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 cydC::Tn5 BA7 mutant should be investigated. Interestingly, cydC::Tn5 BA7 mutant showed better protection than RB51 vaccine strain but it showed as looP::Tn5 BA7 did. The reason why both mutants showed better protection than the RB51 seems to be related to lipopolysaccharide (LPS) biosynthesis. Both mutants retained smooth phenotype like the parental BA7 strain, while RB51 strain has a rough phenotype because it does not have O side-chain of LPS (O-LPS) (37). *B. abortus* LPS O-antigen is known to be essential virulence factor, and this antigen plays an important role in eliciting protective immunity against challenge infection with virulent *B. abortus* (37, 38). Therefore, it could be explained that the mice vaccinated with either mutant showed higher protection level compared with the mice vaccinated with the RB51 because the mice vaccinated with the mutants may have protective immunity to smooth LPS. The high level of protection provided by both looP::Tn5 BA7 and cydC::Tn5 BA7 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, particularly cydC mutant for a live vaccine.

In present study, a higher level of protection conferred by cydC::Tn5 BA7 mutant was surprising, although a precise explanations for this observation is not available at this time. We hypothesize that cydC::Tn5 BA7 mutant retained all the known virulence factors that may contribute to protective immunity similar to the phenotypes observed in *Brucella cydBA* mutants.
(39, 40). A possible explanation for a slightly lower level of protection observed in mice immunized with the looP::Tn5 BA7 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, 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, could provide useful information for the construction of a new live vaccine candidate against bovine brucellosis.

Acknowledgments. This research was supported by the Basic Science Research Program through the National Research Foundation of South Korea (NRF) funded by the Ministry of Education, Science and Technology (grant no. 2012R1A1A4A01015303).

References


Figure legends

Figure 1. The cydC and looP genes are required for virulence of B. abortus in the cell infection models. The intracellular growth of B. abortus wild-type BA7, looP::Tn5 BA7, cydC::Tn5 BA7 mutants and complementation of looP::Tn5 BA7 (C-looP::Tn5 BA7) and cydC::Tn5 BA7 (C-cydC::Tn5 BA7) mutants in RAW264.7 cells (A) and HeLa cells (B). Both cell types were infected with BA7 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 CFU in triplicate and their standard deviations. The asterisks denote a significant difference compared with the values of the complementation strains or the parental BA7 strain (**, \( P < 0.001 \); *, \( P < 0.05 \)).

Figure 2. Role of looP and cydC genes in sensitivity to three different types of stress. (A) Sensitivity of mutants to the respiratory chain inhibitors zinc sulfate and sodium azide. Cells grown to stationary phase were serially diluted and plated on TSA or TSA supplemented with 0.15 mM ZnSO4 and 0.15 mM NaN3 for enumeration of surviving bacteria. (B) Hydrogen peroxide sensitivity assay. The parental BA7 strain and mutants were examined for sensitivity to \( \text{H}_2\text{O}_2 \). Resistance to \( \text{H}_2\text{O}_2 \) was determined by measuring the diameter of the clear zone around a disk containing 30% \( \text{H}_2\text{O}_2 \). (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 numbers of CFU were enumerated on TSA plates. Data points and error bars represent the means of the three independent trials and their standard deviations. The asterisks indicate a significant difference in mean values from those of BA7 strain (**, \( P < 0.001 \); *, \( P < 0.05 \)).

Figure 3. Persistence of looP::Tn5 BA7 and cydC::Tn5 BA7 mutants in BALB/c mice. The mouse groups were infected i.p. with \( 10^5 \) CFU of BA7, looP::Tn5 BA7, cydC::Tn5 BA7, C-
looP::Tn5 BA7 or C-cydC::Tn5 BA7 mutants. At 1, 2, 3, and 7 WPI, five mice from each group were euthanized, and their spleens were removed and weighed. (A) The CFU was determined by serial dilution of spleen homogenates and plating onto TSA. (B) Spleen weights were measured at indicated times. Error bars represent the standard deviations and the asterisks denote values that are significantly different between C-looP::Tn5 BA7, C-cydC::Tn5 BA7 or BA7 strains and mutants at each time point as determined by ANOVAs (*, P < 0.001).
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant characteristics/Putative function of the disrupted gene</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>E. coli</em> DH5α</td>
<td>F-φ80 lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (r−, m γ−) gal’ phoA supE44 λ thi1 gyrA96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> S17-1 λpir</td>
<td>F araD Δ(lac pro) argE(Am) recA56 rif naiA, harboring the suicide vector pUT mini-Tn5Km2, KM R</td>
<td>(6)</td>
</tr>
<tr>
<td><em>B. abortus</em> RB51</td>
<td>Rough, <em>B. abortus</em> vaccine strain RB51 (RB51)</td>
<td>CVI a</td>
</tr>
<tr>
<td><em>B. abortus</em> 544</td>
<td>Smooth, virulent <em>B. abortus</em> strain 544 (ATCC 23448)</td>
<td>(6)</td>
</tr>
<tr>
<td><em>B. abortus</em> IVKB9007</td>
<td>Epidemic strain, smooth, virulent <em>B. abortus</em> biovar 1 isolated from aborted bovine fetus (BA7)</td>
<td>This study</td>
</tr>
<tr>
<td><em>looP</em>::Tn5 BA7</td>
<td>Mini-Tn5Km2 insertion in ATP/GDP-binding proteins motif A (P-loop), ABC transporter of BA7 strain, KM R</td>
<td>This study</td>
</tr>
<tr>
<td><em>cydC</em>::Tn5 BA7</td>
<td>Mini-Tn5Km2 insertion in ATP-binding/permease protein, ABC transporter (cydC) of BA7 strain, KM R</td>
<td>This study</td>
</tr>
<tr>
<td><em>C- looP</em>::Tn5 BA7</td>
<td><em>looP</em>::Tn5 BA7 mutant with plasmid pB4P-loop, KM R, AMP R</td>
<td>This study</td>
</tr>
<tr>
<td><em>C-cydC</em>::Tn5 BA7</td>
<td><em>cydC</em>::Tn5 BA7 mutant with plasmid pB4cydC, KM R, AMP R</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pBluescript II KS (+)</td>
<td>CoEl1, bla, AMP R</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBBRI-MCS4</td>
<td>Broad-host-range cloning vector, AMP R</td>
<td>(41)</td>
</tr>
<tr>
<td>pB4cydC</td>
<td><em>PstI/XbaI</em> fragment containing the <em>B. abortus</em> BA7 <em>cydC</em> gene cloned in pBBRI-MCS4, AMP R</td>
<td>This study</td>
</tr>
<tr>
<td>pB4P-loop</td>
<td><em>PstI/XbaI</em> fragment containing the <em>B. abortus</em> BA7 <em>looP</em> gene cloned in pBBRI-MCS4, AMP R</td>
<td>This study</td>
</tr>
</tbody>
</table>

a The *B. abortus* RB51 strain was provided by Chungang Vaccine Institute (CVI), South Korea.
Table 2. Protection provided to BALB/c mice against challenge with virulent *B. abortus* 544 strain by vaccination with *B. abortus* vaccine strain RB51, *looP*:Tn5 BA7, or *cydC*:Tn5 BA7 mutants

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Log$_{10}$ CFU of <em>B. abortus</em> 544 in spleen (mean ± SD)</th>
<th>Log$_{10}$ units of protection</th>
<th>Spleen weight (mg, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>5.76 ± 0.38</td>
<td></td>
<td>356 ± 24</td>
</tr>
<tr>
<td><em>B. abortus</em> RB51</td>
<td>4.25 ± 0.32</td>
<td>1.51$^b$</td>
<td>171 ± 18$^b$</td>
</tr>
<tr>
<td><em>looP</em>:Tn5 BA7</td>
<td>3.57 ± 0.27</td>
<td>2.19$^{b,c}$</td>
<td>123 ± 8$^{b,d}$</td>
</tr>
<tr>
<td><em>cydC</em>:Tn5 BA7</td>
<td>3.45 ± 0.21</td>
<td>2.31$^{b,c}$</td>
<td>114 ± 6$^{b,d}$</td>
</tr>
</tbody>
</table>

$^a$ Mice inoculated with PBS, 10$^5$ CFU/dose of *looP*:Tn5 BA7 and *cydC*:Tn5 BA7 mutants or 10$^8$ CFU/dose of *B. abortus* RB51 vaccine strain. At 5 weeks postinoculation, all groups were challenged i.p. with 10$^5$ CFU/dose of *B. abortus* 544, and splenic CFUs and spleen weight were determined at 2 weeks post-challenge.

$^b$ Significant difference between vaccinated groups and PBS control group ($P < 0.001$).

$^c,d$ Significant difference between the mice groups immunized with mutants and the *B. abortus* RB51 vaccine strain ($^c P < 0.05$; $^d P < 0.001$).