**Title and Subtitle**
Cloning, expression and purification of Brucella suis outer membrane proteins

**Authors**
Xuan Z. Ding, Apurba K. Bhattacharjee, Ian T. Paulsen, Mikeljon P. Nikolich, Gary Myers, and David L. Hoover

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Brucella, an aerobic, nonsporeforming, nonmotile Gram-negative cocccobacillus, is a NIH/CDC category B bioterror threat agent that causes incapacitating human illness. Medical defense against the bioterror threat posed by Brucella would be strengthened by development of a human vaccine and improved diagnostic tests. Central to advancement of these goals is discovery of bacterial constituents that are immunogenic or antigenic for humans. Outer membrane proteins (OMPs) are particularly attractive for this purpose. In this study, we cloned, expressed and purified seven predicted OMPs of Brucella suis. The recombinant proteins were fused with 6-his and V5 epitope tags at their C termini to facilitate detection and purification. The B. suis surface genes were PCR synthesized based on their ORF sequences and directly cloned into an entry vector. The recombinant entry constructs were propagated in TOP 10 cells, recombined into a destination vector, PET-DEST42, then transformed into E. coli BL21 cells for IPTG-induced protein expression. The expressed recombinant proteins were confirmed with Western blot analysis using anti-6-his

**Subject Terms**
Brucella suis, outer membrane proteins, gene clone
Cloning, expression, and purification of *Brucella suis* outer membrane proteins

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Abstract

*Brucella*, an aerobic, nonsporeforming, nonmotile Gram-negative coccobacillus, is a NIH/CDC category B bioterror threat agent that causes incapacitating human illness. Medical defense against the bioterror threat posed by *Brucella* would be strengthened by development of a human vaccine and improved diagnostic tests. Central to advancement of these goals is discovery of bacterial constituents that are immunogenic or antigenic for humans. Outer membrane proteins (OMPs) are particularly attractive for this purpose. In this study, we cloned, expressed, and purified seven predicted OMPs of *Brucella suis*. The recombinant proteins were fused with 6-His and V5 epitope tags at their C termini to facilitate detection and purification. The *B. suis* surface genes were PCR synthesized based on their ORF sequences and directly cloned into an entry vector. The recombinant entry constructs were propagated in TOP 10 cells, recombined into a destination vector, pET-DEST42, then transformed into *Escherichia coli* BL21 cells for IPTG-induced protein expression. The expressed recombinant proteins were confirmed with Western blot analysis using anti-6-His antibody conjugated with alkaline phosphatase. These *B. suis* OMPs were captured and purified using a HisGrab plate. The purified recombinant proteins were examined for their binding activity with antiserum. Serum derived from a rabbit immunized intramuscularly with dialyzed cell lysate of *Brucella* rough mutant WRR51. The OMPs were screened using the rabbit antiserum and purified IgG. The results suggested that recombinant *B. suis* OMPs were successfully cloned, expressed and purified. Some of the expressed OMPs showed high binding activity with immunized rabbit antiserum.

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Keywords: *Brucella suis*; Recombinant membrane proteins; Antiserum; IFN-γ; Vaccine; Diagnostic reagents

*Brucella* is a zoonosis that is typically acquired after ingestion of foodstuffs, especially unpasteurized dairy products, or after occupational contact with infected animals. *Brucella* has been considered as an agent for biological warfare since the 1940s [1]. It is highly resistant to drying, easily survives aerosolization in water, and is highly infectious by multiple routes [2]. Human brucellosis is characterized by fever, chills, malaise, and a chronic course that requires six weeks of therapy with at least two antibiotics for cure treatment [3]. There are six recognized species of which *Brucella suis*, *Brucella melitensis*, and *Brucella abortus*, all smooth bacteria [4] that express long-chain O-polysaccharide (OPS) on their outer membrane lipopolysaccharide (LPS), are considered significant bioterror threat agents. All three agents are extremely similar at the DNA level.

To prevent *Brucella* infection, we developed a whole cell vaccine candidate, WR201. The whole cell vaccine has been tested in mice model and demonstrated that it was able to protect mice against intranasal challenge...
with \textit{B. melitensis} 16M [5]. These results indicate that some components of the bacteria function as immunogens, activate immune response, and induce a protective effect. Bacterial outer membranes are basically composed of protein, lipid, and sugar. It has been known that among the bacterial components, outer membrane proteins (OMPs) are particularly attractive used for development of vaccine candidates and diagnostic kits. For this reason, a few \textit{Brucella} OMPs have been cloned, expressed, and identified from different species such as \textit{B. melitensis} 20 kDa [6], 28 kDa [7], and 31 kDa [8] OMPs; \textit{B. abortus} 16.5 kDa [9], 17 kDa [10], 22 kDa [11], 25 kDa [12], and 36 kDa [13] OMPs; and \textit{B. ovis} 25 kDa OMP [14]. The molecular sizes of these OMPs are in the lower range and, they were constructed from a recombinant library and immune reaction as specific diagnostic agents has been examined.

Recently, the complete genomic sequences of \textit{B. melitensis} [15] and \textit{B. suis} [16] were published. The \textit{B. suis} genome contains 3388 predicted open reading frames (ORFs), and versus 3197 ORFs for \textit{B. melitensis}, on both chromosomes. Comparison of these sequences revealed extensive similarity and gene synteny [16]. Indeed, these two species differ by only 74 genes [17]. \textit{B. suis} contains 42 unique genes found in 22 chromosomal regions and \textit{B. melitensis} contains 32 unique genes in 11 locations [15-17]. Similarly, preliminary data from genome sequencing of \textit{B. abortus} indicate that nearly all of this species’ genes are identical to those of the other two. This genome information provides an opportunity to clone any expected gene and express its protein. In this study, we selected seven membrane proteins from \textit{B. suis}, including three large ones of 88, 72, and 68 kDa, cloned them into Gateway system (Invitrogen), and expressed in \textit{Escherichia coli}. Their immune reaction with antiserum derived from immunized rabbit was observed.

### Materials and methods

#### Selection of \textit{Brucella} OMP genes

Seven genes of \textit{B. suis} that encoded different sizes of OMPs were selected and their bioinformation are shown in Table 1. Besides the seven genes, two controls were used for evaluation of gene expression. The negative control was pET-DEST-42 vector alone and the positive control was plasmid pET101/D/lacZ containing the lacZ gene.

#### Preparation of \textit{B. suis} bacteria

One milliliter of \textit{B. suis} bacteria was collected in a 50 ml tube and treated in an equal amount of phenol. After vortex, the bacterial cells were spun down at 1560g for 10 min, at 4 °C. Nine hundred microliters of the upper liquid was collected into a 1.5 ml tube and added 630 μl isopropanol. The sample was centrifuged at 16,000g, for 10 min, at 4 °C. The pellet was washed with 70% alcohol and dissolved in 100 μl of sterile water.

#### PCR synthesis of \textit{B. suis} genes

Primers used for synthesis of the seven \textit{B. suis} genes were designed as 20 mers (Table 2). The forward primers contained an additional CACC sequence at the 5' end followed by the first 20 bases of the ORF including the start codon. The reverse primer was comprised of the last 20 bases of the ORF prior to the stop codon. The PCR was conducted in a final volume of 50 μl that contained 5 μl of 10x ThermalAce reaction buffer, 1 μl of 50 mM dNTP, 0.5 μl of each of 50 μM, 41 μl of PCR water, and 1 μl of ThermalAce. \textit{B. suis} template without primers was used as negative control. The lacZ gene with primers from Invitrogen was used as a positive control. The PCR protocol was according to our previous study.

### Table 1

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<td></td>
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### Table 2

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</tr>
<tr>
<td>7</td>
<td>Cacc atgacgegca gttctaaatt</td>
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</table>
[18] designed as 1 cycle of 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; and 1 cycle of 72 °C for 10 min and then refrigeration at 4 °C.

Preparation of recombinant constructs in entry vector

The PCR products were directly cloned into an entry vector (Invitrogen). Reactions were performed according to the manufacturer's instructions. One microliter of PCR product plus 1 μl water was mixed with 0.5 μl of each salt solution and pENTR/SD/D-TOPO vector. Ligation was conducted at room temperature for 5 min. Three microliters of the ligation reaction was then added to 40 μl of TOP 10 competent cells and incubated at 4 °C for 30 min. After heating at 42 °C for 30 s, the cells were added to 250 μl of culture medium and shaken at 37 °C for 1 h. After the incubation, 20 μl of TOP 10 cells was streaked on culture plate containing trypticase soy agar with 50 μg/ml kanamycin for clone selection. Plates were incubated at 37 °C overnight. Four clones of each gene were picked and cultured in 2 ml of LB broth medium with 50 μg/ml kanamycin for propagation of the plasmid. On the next day, the plasmids were purified using miniprep spin column 250 (Qiagen) and examined by PCR method.

Cloning of genes into destination vectors

One positive entry plasmid for each gene was selected for ligation into Gateway destination vector pET-DEST42. Each ligation reaction (LR) contained 2 μl of buffer, 4 μl of pENTR-gene, 2 μl of pET-DEST42 vector, and 2 μl of LR clonase. The reactions were incubated at 25 °C for 60 min. One microliter of proteinase K was added and the reaction mixture was incubated at 37 °C for another 10 min. One microliter of each LR was transformed into 50 μl of TOP 10 competent E. coli cells and incubated at 4 °C for 30 min. After heating at 42 °C for 30 s, the cells were added to 250 μl of culture medium and shaken at 37 °C for 1 h. Twenty microliters of TOP 10 cells was then streaked on tissue culture plates containing trypticase soy agar with 100 μg/ml ampicillin for clone selection. Plates were incubated at 37 °C overnight. Four clones of each gene were picked and cultured in 2 ml of LB broth medium with 100 μg/ml ampicillin at 37 °C overnight. On the next day, the plasmids were purified and examined by above-mentioned methods.

Expression of recombinant proteins

Two microliters of the purified destination plasmids of each gene was mixed with 50 μl of expression host cells, BL21 Star (DE3), and incubated at 4 °C for 30 min. After heating at 42 °C for 30 s, the cells were added with 250 μl of culture medium and shaken at 37 °C for 1 h. Then, the host cells were transferred into 5 ml of LB solution containing 100 μg/ml ampicillin and shaken at 37 °C overnight. On the next day, 200 μl of culture from each sample was transferred into 4 ml of LB medium containing 1% glucose and 100 μg/ml ampicillin, and shaken at 37 °C for another 2 h. The OD_600 measurements of the cell cultures were within 0.5–0.8 U. Then, the culture was added IPTG (Invitrogen) to 1 μM and grown for 5 h. This optimal time was obtained based on preliminary experiments. After protein induction, cells were centrifuged and resuspended in 400 μl PBS for detection.

Western blot analysis of the expressed recombinant proteins

Seven microliters of protein sample for each gene was mixed with an equal amount of 2x SDS loading buffer. The samples were boiled for 10 min and purified on 13% acrylamide/bis gel. The protein samples were transferred onto nitrocellulose membrane and hybridized overnight with 1:2000 alkaline phosphatase (AP)-conjugated anti-6-His. The membranes were developed with 1 mg/ml of Naphthol AS-MX phosphate (Sigma) and 2 mg/ml of Fast red TR salt (Sigma) in 50 mM Tris buffer.

Purification and detection of recombinant proteins

A HisGrab plate (Pierce) was used to purify the recombinant proteins. One hundred microliters of IPTG-induced recombinant cells was centrifuged and the pellets were resuspended in 100 μl of lysis buffer. The samples were kept at room temperature for 30 min and then frozen and thawed three times. Ten microliters of the cell lysate was mixed with 90 μl of blotting buffer and placed on each well of the plate in triplicate, incubated at room temperature overnight. To detect the recombinant proteins, 100 μl of anti-V5 monoclonal antibody conjugated with AP (1:2000 dilutions) was added to each well and incubated at room temperature for 16 h. After washing three times with 1x PBS, 100 μl of developer buffer was added to each well and incubated at room temperature for 1 h. The protein concentration was determined using OD reader at wavelength 410 nm.

Antiserum screening of recombinant proteins

Rabbit serum was used for screening of the recombinant Brucella proteins. The rabbit was immunized intramuscularly with dialyzed cell lysate of B. melitensis rough mutant WRR51. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and
experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Two doses of vaccine (25 μg of protein per dose) were given 4 weeks apart. Blood was collected from the rabbit 2 weeks after the second dose of vaccine. IgG was prepared from the serum by affinity chromatography on protein G-Sepharose (Pharmacia-LKB Biotech). The IgG concentration in this preparation was 1.8 mg/ml. The immunized rabbit serum was diluted 1:4000 and added to each well of recombinant sample for 16 h of incubation. After washing six times with 1x PBS plus 0.5% Tween 20, the samples were incubated with 1:2000 secondary goat anti-rabbit antibody conjugated with HRP for 16 h. After substrate development, the plate was read with a plate reader.

**Statistical analysis**

All experiments were repeated at least three times. Data were processed with analysis of variance, Newman-Keuls, and Student's t test for comparison of groups. A value of p < 0.05 was used as the significance level for the study.

**Results**

**Construction of B. suis outer membrane genes**

Surface antigens of bacteria have been thought as the best immunogens to stimulate an immune response. Seven OMPs of Brucella with different molecular sizes were selected based on genome sequence of *B. suis* and their bioinformation has been described in Table 1. The OMP genes were PCR synthesized using ThermalAce DNA polymerase (Invitrogen). Genome DNA prepared from *B. suis* was used as template in the PCR. Result shows that single band with correct molecular weight has been amplified for each Brucella gene (Fig. 1).

To provide versatility for additional cloning for future applications, Gateway cloning system (Invitrogen) was selected to express the Brucella genes. The PCR produced Brucella genes were directly cloned into entry vector, pENTR directional TOPO vector (Invitrogen), and propagated in TOP 10 cells. The positive recombinant cells containing Brucella genes were selected on culture plate of LB agar with 50 μg/ml kanamycin. All seven samples resulted in isolated colonies on the selection plates. Four colonies of each gene were picked up and cultured in LB medium overnight for plasmid amplification. Plasmids were purified using QIAprep column (Qiagen) and their molecular sizes of these were examined on 1% agarose gel (Fig. 2). Negative control was pENTR vector alone. The pENTR vector showed a lower molecular weight than the plasmids containing Brucella genes (data not shown). The presence of inserted genes in the plasmids was confirmed by PCR method, using primers designed according to the M13 sequence of the pENTR vector (data not shown). The full-length sequence of the gene inserts was further confirmed using ABI 3730xl capillary sequencing machines analysis by the Institute for Genomic Research. The results show that all the seven plasmids contain an insert that exactly matches OMP genes of *B. suis*.

One positive plasmid for each gene was selected for recombination into Gateway destination vector, pET-DEST42 (Invitrogen). The new constructs were propagated in TOP 10 cells. The positive recombinant cells were selected on culture plate of LB agar with 100 μg/ml ampicillin. Four clones of each gene were picked and cultured in LB medium with 100 μg/ml ampicillin at 37 °C overnight. Plasmids were purified from these samples and molecular sizes of these were examined on 1% agarose gel as described above. Insertion of *B. suis* genes in the destination constructs was confirmed by
PCR method using primers designed according to the attB sequence of the pET-DEST42 vector. Their sequences were attL1 5'-G TAC AAA AAA GCA GGC T-3', and attL2 5'-GTA CAA GAA AGC TGG GT-3'. The PCR result shows that all seven constructs contain their recombinant inserts (Fig. 3).

Protein expression and measurement

Purified plasmids of each *B. suis* OMP were transformed into expression host cells, BL21Star (DE3) for protein expression. The positive control was cells transformed with pET-DEST42-lacZ and negative control was cells transformed with pET-DEST42 vector alone. The host cells were cultured in LB medium containing 1% glucose and 100 μg/ml ampicillin overnight. On the expression day, the recombinant cells were diluted in the ratio 1:20 into fresh LB medium containing 1% glucose and 100 μg/ml ampicillin, and cultured for another 2 h until OD_{600} measurements reached 0.5–0.8 U. Then, the cell culture of each sample was induced with 1 μM IPTG (Invitrogen). From preliminary experiments, the recombinant cells were shown to start expression of protein at 2 h and reached a maximum level at 4–5 h. Thus, optimal time for the IPTG-induced protein expression was 5 h after the chemical induction. After protein induction, cells were collected and resuspended in 1× PBS for detection. The samples were detected on Western blots using 1:2000 diluted monoclonal anti-6-His antibody (Invitrogen) to verify protein expression and their sizing. The protein samples were prepared with equal amount of 2× SDS loading buffer and purified on 13% acrylamide/bis gel as described under Materials and methods. The result shows that the seven OMPs of *B. suis* were over-expressed with correct molecular size in the host cells (Fig. 4).

Purification of *B. suis* OMPs

It was necessary to purify and fix the recombinant proteins in a 96-well plate to detect their immune functions. HisGrab plate (Pierce) was used to purify the *Brucella* proteins in this study. Recombinant cells were treated with cell lysis buffer after the protein expression. HisGrab plate was pre-blotted with blotting buffer and then 10 μl cell sample plus 90 μl buffer were added into each well of the plate. The negative control was *Brucella* cell lysate and the positive was LacZ recombinant protein. Protein samples were incubated at room temperature overnight. After wash, 100 μl of anti-V5 monoclonal antibody conjugated with AP (1:2000) was added to each well and incubated with the sample at room temperature overnight. The bound proteins were detected using phosphatase substrate. The result shows that LacZ protein was recognized by the antibody, but not *Brucella* lysate. All seven *Brucella* recombinant proteins were positively identified by the anti-V5 antibody (Fig. 5).

Screening *Brucella* proteins for immune reactivity against rabbit serum

We have found that intranasal immunization of rhesus macaques with WR201 leads to production of anti-*Brucella* antibody and protects rhesus macaques from infectious brucellosis induced by subsequent aerosol challenge with 16M [5]. To investigate the possibility that the recombinant *Brucella* proteins react with protective antiserum, serum was collected from an immunized rabbit and used as a tool to screen for expressed
Development of a human vaccine and improved diagnostic tests would significantly assist prevention of *Brucella*-induced infection and serve a valuable societal goal. A *Brucella* whole cell vaccine, WR201, has been developed and found that it was attenuated and able to protect wild-type virulent *B. melitensis* induced infection in both mice and monkey models [5]. Based on these data, the discovery of additional immunogenic or antigenic bacterial constituents is a vital component of the vaccine and diagnostics development process. Outer membrane proteins of the bacteria have been reported to play an important role during *Brucella* infection and induction of host immune response. Generally, successful vaccines and diagnostic reagents against their microorganisms primarily induce antibodies against surface structures. These surface proteins of the bacteria have been thought of as useful antigens for development of both diagnostic reagents and vaccine candidates. Some *Brucella* proteins have been cloned and expressed, but they were constructed by traditional methods from an unknown gene library as genome information of the bacteria was not available in those days. Full-length genome sequences of *B. suis* and *B. melitensis* have been published in 2002 [16], and this bioinformation provides an extremely useful opportunity to clone and express the *Brucella* outer membrane proteins. The utility of applying whole-genome sequencing data to development of recombinant proteins has been demonstrated for *Neisseria meningitidis* [19] and *Streptococcus pneumoniae* [20]. In this study, we constructed and expressed seven OMPs of *B. suis* based on open reading frames of the genome information.

The full-length sequence of the seven ORFs was PCR synthesized and cloned into the Gateway expression system. The system provides a benefit for optimal expression of these *Brucella* membrane proteins using alternative destination vectors for different purposes. These expressed recombinant proteins have been designed to be fused with both V5 and 6-His tags at their C termini. Thus, two alternative methods can be used to purify and detect them, either to capture V5 or 6-His tags and then either to detect 6-His or V5 protein tags. The two methods were compared and we found that the recombinant proteins can be captured or detected by using either of the methods (Fig. 7).

Since nickel reagent may be used to capture those proteins fused with 6-His tag, His-Grab plate (Qiagen) was used in the experiment to purify these expressed recombinant proteins. In this study, all expressed proteins were confirmed by Western blot and then purified using the His-Grab plate. High protein purity is required, as the recombinant proteins will be further examined, for their antigenic functions. In another study, we have analyzed four expressed recombinant proteins before and after purification with a Ni-NTA spin column (Pierce) following the manufacturer's instructions. The purification principle of the column is the same as the His-Grab plate, but provides sufficient protein to visualize by gel

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Discussion

Development of a human vaccine and improved diagnostic tests would significantly assist prevention of *Brucella*...
electrophoresis. After elution, the purified proteins were examined using both Coomassie blue stain and Western blot. Only a single band was detected from each of the purified protein samples, while the cell lysates contained multiple bands (data not shown). These results indicate that the expressed recombinant proteins are well purified by using a nickel-based method, suggesting that this method should be adequate for analysis of antigenicity.

It is very possible that certain protein components of the Brucella, but not necessary as opposed to whole cell, may be sufficient to elicit the protective immunity with reduced side effects. These bacterial proteins can be cloned and expressed by using our currently used method and are examined for their antigenic activity. Immunized animals that survived from virulent bacterial challenge may have actively obtained protective immunities. The protective antibodies or/and T lymphocytes derived from these hosts should recognize and react with those antigen proteins that cause the infection and produce the specific protective immunities. Thus, these antibodies and activated T lymphocytes can be used as screening probes to select out those proteins that are immunogenic. Antiserum was collected from the rabbit immunized intramuscularly with dialyzed cell lysate of B. melitensis rough mutant WRR51. The immunized rabbit serum and IgG were diluted 1:4000, and used to screen the recombinant surface antigens. Both the antiserum and derived IgG had strong reaction to the Brucella cell lysate. In fact, we found that using whole cell lysate of WRR51 as antigen we get more than 20 protein bands in Western blot using this antiserum (data not shown). Recombinant OMPs 1 and 2 were also found to have significant detection by the antiserum. The protein samples were prepared by treatment of WRR51 cells and the recombinant proteins with sodium dodecyl sulphate (SDS), therefore, the proteins were in their denatured form. The antibodies generated in rabbit will therefore recognize linear epitopes of the proteins.

Intraperitoneal administration of WR201 to mice leads to not only production of antibody to OMPs, but also induction of spleen cells that produce IFN-γ when cultured with Brucella antigens, and protection against intranasal challenge with 16M [5]. This result indicates that both humoral and cellular immunities may be important in the protection of host against Brucella infection. In a pilot experiment, we observed the effect of the immobilized recombinant surface antigens on IFN-γ production of spleen cells derived from mice immunized orally with 10^11 colony-forming units (CFU) of WR201 for 8 weeks and found that at least two of the recombinant proteins significantly increased IFN-γ (data not shown). These results indicate that surface antigens may play a distinct role in stimulation of host immune system. These recombinant surface antigens will be further examined in vivo model to test their immune stimulation.

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References


