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Subcloning and Expression of the *Brucella abortus L7/L12* Ribosomal Gene and T-Lymphocyte Recognition of the Recombinant Protein

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The *Brucella abortus L7/L12* ribosomal gene was amplified by PCR and subcloned into the prokaryotic expression vector pMAL-c2. *Escherichia coli* DH5α was transformed with the pMAL-L7/L12 construct, and gene expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside). The resulting fusion protein was purified by affinity chromatography and confirmed by Western blot (immunoblot) analysis using an anti-maltose-binding protein antibody. Additionally, purified recombinant L7/L12 protein induced T-lymphocyte proliferation of *B. abortus*-primed bovine peripheral blood mononuclear cells. Phenotypic analysis of the proliferating cell population demonstrated an increase in the percentage of $CD4^+$ T lymphocytes when peripheral blood mononuclear cells were cultured with recombinant L7/L12 compared with cells cultured in medium alone. Subcloning and expression of a *B. abortus* gene encoding a previously demonstrated immunodominant protein for bovine lymphocytes are important steps in selecting *Brucella* proteins that have potential as a component of a genetically engineered candidate vaccine.

Brucella abortus is a gram-negative intracellular bacterium infecting animals and humans (19). *B. abortus* infection causes spontaneous abortions in cattle and persistent undulant fever, endocarditis, arthritis, and meningitis in humans (27). Little is known regarding the cellular and molecular mechanisms used by *B. abortus* to penetrate and resist destruction within the macrophage, the principal host cell of residence for the bacterium. Gamma interferon is a cytokine produced by activated T lymphocytes and NK cells that plays an important role in antimicrobial activity through macrophage activation (1). This cytokine is capable of upregulating major pathways described for microbial killing by macrophages (4, 18, 24). Because *B. abortus* resides and replicates within the cytoplasm of mononuclear phagocytes and that cytokines such as gamma interferon upregulate macrophage killing activity, a T-lymphocyte-mediated response is crucial for host protection.

Few *B. abortus* genes have been cloned and the proteins that they encode have been characterized (6, 12, 15, 23, 29). Furthermore, little is understood regarding the importance of these bacterial proteins in the immune response. In addition, the potential for these proteins to elicit a protective cellular response against bovine brucellosis has not been investigated. Therefore, a greater understanding of the relationship of bacterial proteins with host protection and disease is required. Given that lymphocytes proliferate to bacterial antigens and that cytokines activate macrophage killing of intracellular bacteria, the identification of individual *B. abortus* proteins that trigger T lymphocytes is crucial to define how the immune system functions in this disease. At present, there is no information regarding recombinant *B. abortus* proteins that are able to activate a specific T-cell subset critical for providing host protection against this bacterium. This paper describes the subcloning of the *L7/L12* ribosomal gene from *B. abortus*, its expression in *Escherichia coli*, and the bovine T-lymphocyte recognition of the recombinant protein produced.

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Six cattle were kept at the Department of Animal Health and Biomedical Sciences, University of Wisconsin—Madison. Three animals received three injections of the attenuated live *B. abortus* vaccine and were the source of antigen-primed lymphocytes 1 year after the final vaccination. The other three animals were not vaccinated and served as negative controls.

The *B. abortus L7/L12* ribosomal gene was amplified by PCR and subcloned into the expression vector pMAL-c2 (New England Biolabs, Beverly, Mass.). Primers, containing one artificial restriction site at each end, were constructed according to the *L7/L12* nucleotide sequence (Genbank accession no. L19101 [21]). The primer sequences were (sense) 5′-CGCG GATCTAGAAAAATGGCTGATCTCGCAAAG-3′ (*Xba*I) and (antisense) 5′-GCGGGGCTGCAGCCAAACTTACTT GAGTTCAAC-3′ (*Pst*I). PCR was performed with a 100-μl volume containing 100 ng of DNA template (pBluescript II SK^- carrying the *L10* and *L7/L12* genes [21]), 1 μM of each primer, 2.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 1× PCR buffer, and 2.5 U of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). PCR amplification was conducted with a DNA thermal cycler (Perkin-Elmer Cetus), using the following conditions: denaturation at 94°C for 90 s, annealing at 50°C for 90 s, and extension at 72°C for 150 s (25 cycles). The PCR-amplified product was purified by Geneclean (Bio 101, Inc., La Jolla, Calif.) and digested with *Xba*I and *Pst*I restriction endonucleases (GIBCO BRL, Gaithersburg, Md.). After digestion, the PCR product was purified again by the same procedure and ligated to the predigested pMAL-c2 vector, using DNA ligase (GIBCO BRL). The ligation reaction was used to transform *E. coli* DH5α, and single recombinant clones were selected. Plasmid DNA was extracted by Magic Miniprep (Promega Corp., Madison, Wis.) and digested by *Xba*I and *Pst*I to verify the presence of the insert. Positive *E. coli* clones possessing the pMAL-L7/L12 construct were selected, and expression of the fusion protein was induced by 0.6 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After IPTG induction, bacterial cells were harvested by centrifugation at 4,000 × *g* for 20 min, and the supernatant

was discarded. The cell pellet was frozen in a dry ice-methanol bath at −70°C and allowed to thaw to 37°C. The pellet was resuspended in 100 ml of phosphate buffer saline (PBS; pH 8.4) containing 25 mg of lysozyme. The resuspended pellet was frozen and thawed three more times. The resulting suspension was homogenized to achieve uniformity and then incubated at 37°C for 30 min, with homogenization every 10 min. The suspension was centrifuged at 9,000 × *g* for 30 min, and the supernatant containing crude extract was loaded onto a polyacrylamide gel to confirm the presence of the expressed fusion protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* lysates containing the induced MBP-L7/L12 fusion protein were diluted 1:5 with PBS. Then, the suspension was loaded into an amylose resin column (New England Biolabs) and washed 10 times with PBS, and the fusion protein was eluted with PBS plus 10 mM maltose. The purified fusion protein was cleaved with factor Xa protease, which recognizes a specific amino acid sequence between the maltose-binding protein (MBP) and the L7/L12 protein. After cleavage, recombinant L7/L12 (rL7/L12) was purified by rebinding of MBP to the amylose resin. Pure rL7/L12 was concentrated in PBS, using a centricon-10 microconcentrator (AMICON, Beverly, Mass.), and the protein concentration was determined by a Bio-Rad (Hercules, Calif.) protein assay.

For SDS-PAGE lysates from induced and uninduced *E. coli* carrying the pMAL-L7/L12 construct, purified and cleaved MBP-L7/L12 fusion protein, pure MBP, and purified rL7/L12 were solubilized in sample buffer containing 2% SDS and 5% 2-mercaptoethanol and processed according to the method of Laemmli (10). Before electrophoresis an equal volume of 2× concentrated sample buffer was added, and the mixture was boiled for 3 min and loaded onto the gel. Protein samples and molecular weight markers were analyzed on SDS–15% PAGE gels and visualized by Coomassie blue staining.

For Western blot (immunoblot) analysis, the gels were electroblotted onto nitrocellulose at 75 V for 2 h. Transfers were carried out in 25 mM Tris–192 mM glycine–20% methanol (3, 26). The blotted nitrocellulose was blocked with skim milk for 2 h. Then, rabbit anti-MBP serum was used at a 1:5,000 dilution during incubation for 2 h (at room temperature). After reactions with the primary antibody, the blots were washed three times with TBST (0.5 M NaCl–0.02 M Tris [pH 7.5], 0.05% Tween 20) and incubated for 1 h with a goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Promega) at a 1:10,000 dilution in TBST. Then, the blots were washed three times with TBST, and the reactions were developed by using nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) purchased from Promega.

To express the *B. abortus L7/L12* gene in *E. coli*, PCR amplification of the target gene was done with further subcloning of *L7/L12* into pMAL-c2. This vector expresses foreign sequences as a fusion protein with the 42.7-kDa MBP (8). Expression of a 55-kDa fusion protein was observed by SDS-PAGE containing lysates from IPTG-induced *E. coli* that contained the pMAL-L7/L12 construct (Fig. 1A). To confirm that the expressed fusion protein was MBP-L7/L12, Western blot analysis was performed, using rabbit anti-MBP antibody. Figure 1B shows the anti-MBP recognition of the MBP-L7/L12 fusion protein. After expression, the fusion protein was purified in an amylose affinity column, and the interval necessary for cleavage of L7/L12 from MBP was determined by kinetic experiments at 2, 4, 8, 14 h with factor Xa treatment. Incubation of factor Xa protease with the fusion protein for 14 h resulted in total cleavage of MBP from L7/L12 (Fig. 2). Cleavage of the fusion protein shown in Fig. 2 confirmed the expected molecular masses of MBP and L7/L12, 42.7 and 12 kDa, respectively. rL7/L12 was purified by rebinding MBP to the amylose resin. Figure 3A shows the isolated rL7/L12 on an SDS-PAGE after purification. Western blot analysis demonstrated that the anti-MBP antibody did not recognize the separated rL7/L12 (Fig. 3B).

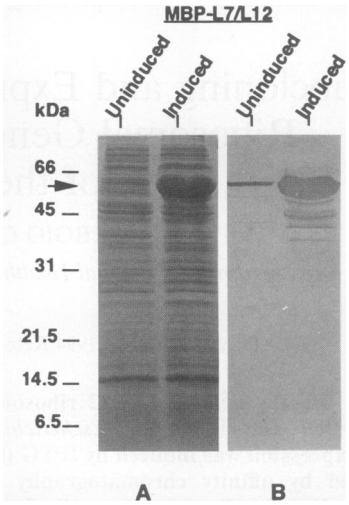

FIG. 1. SDS-PAGE profile and corresponding Western blot analysis of the recombinant MBP-L7/L12 fusion protein. (A) Coomassie blue-stained SDS–15% PAGE of lysates from uninduced and induced *E. coli* expressing the pMAL-L7/L12 construct. (B) Immunoblot of the recombinant MBP-L7/L12 fusion protein probed with rabbit anti-MBP antibody.

Peripheral blood mononuclear (PBM) cells were isolated from cattle blood samples by density gradient centrifugation with Lymphoprep (Nycomed AS Diagnostics, Olso, Norway). The cells were washed three times with PBS containing 100 U of penicillin per ml and 100 μg of streptomycin per ml. The PBM cells were then suspended in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) supplemented with 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 50 μM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated fetal bovine serum (Sigma). Isolated PBM cells were cultured for 4 days at a density of 5×10^5 cells per well in 96-well plates

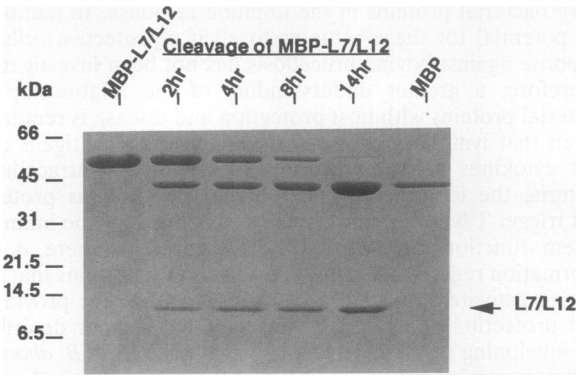

FIG. 2. SDS-PAGE analysis of MBP-L7/L12 fusion protein cleavage by treatment with factor Xa protease at 2, 4, 8, and 14 h. Pure MBP and noncleaved MBP-L7/L12 were used as controls. Molecular mass markers are shown on the left, and pure L7/L12, released from MBP, is indicated by the arrow on the right.

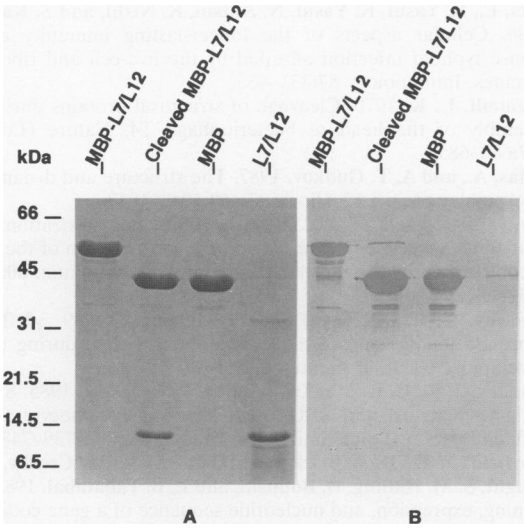

FIG. 3. (A) Coomassie blue-stained SDS–15% PAGE profile of MBP-L7/L12 fusion protein, cleaved MBP-L7/L12, pure MBP, and purified L7/L12 ribosomal protein. (B) Electrotransferred samples from SDS-PAGE were analyzed by immunoblotting with rabbit anti-MBP antibody.

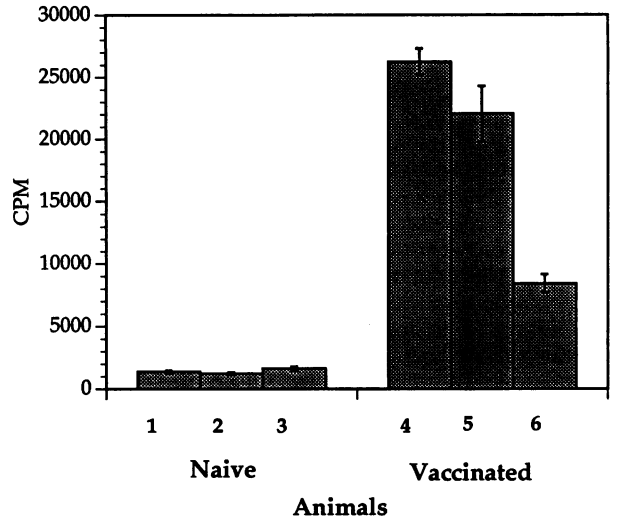

FIG. 4. Proliferative response of PBM cells from *B. abortus*-vaccinated cattle and naive animals. PBM cells were stimulated with 50 μg of rL7/L12 or concanavalin A per ml. Concanavalin A-stimulated cells from both groups of animals had responses of >100,000 cpm (data not shown). Results are expressed as mean cpm. Error bars indicate standard errors of the means.

with the addition of 50 μg of rL7/L12 (optimal concentration was determined after testing 1 to 100 μg/ml) or concanavalin A (2.5 μg/ml) as a T-cell-activating control in a final volume of 200 μl per well. After this culture period, lymphocytes were pulsed for the final 18 h with 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.) in each well. The cells were harvested onto glass fiber filters, and the radioactivity was determined in a liquid scintillation counter. Assays were performed in triplicate.

Primed bovine PBM cells from three different animals proliferated to the rL7/L12 protein, whereas cells from the naive group did not (Fig. 4). Concanavalin A was used as a T-cell-activating control in this experiment, and the counts from both the naive and vaccinated group were above 100,000 cpm (data not shown). The lymphocyte population engaged in this response was analyzed by flow cytometry. Phenotypic surface analysis was conducted on proliferating cells 7 days following stimulation with the rL7/L12. To analyze the membrane phenotypic markers, PBM cells (10^6) in 0.1 ml of PBS containing 1% bovine serum albumin (Sigma) and 0.05 ml of an anti-bovine surface marker monoclonal antibody were incubated on ice for 30 min. The cells were washed twice with PBS prior to suspension in 50 μl (0.5 μg) of anti-mouse immunoglobulin-dichlorotriazinylaminofluorescein (Ig-DTAF; H & L Jackson Laboratories, Avondale, Pa.). The cells were incubated in the dark for 30 min on ice, washed twice, suspended in PBS, and analyzed by flow cytometry (Becton Dickinson, Mountain View, Calif.). The monoclonal antibodies used were MM1A (anti-CD3 [5]), IL-A12 (anti-CD4 [2]), SBU-T8 (anti-CD8 [14]), 86D (anti-γδ T-cell receptor [13]), and 33 (anti-bovine IgM [20]). The cells that proliferated in response to the recombinant protein (their approximate percentages ± standard deviations are given in parentheses) were $CD4^+$ (20.7% ± 0.8%), $CD8^+$ (13.2% ± 1.6%), IgM (10.7% ± 2.1%), and γδ-T-cell receptor (4.5% ± 1.0%). We detected a twofold increase in the percentage of $CD4^+$ T lymphocytes when PBM cells were stimulated in vitro with the rL7/L12 (20.7% ± 0.8%) compared with cells cultured in medium alone (10.8% ± 1.5%). However, no significant change in the percentage of the other cell types was detected (data not shown).

In this study, we have subcloned and expressed a *B. abortus* DNA fragment which encodes the L7/L12 ribosomal protein previously shown to be immunodominant in cattle (3). The *L7/L12* gene encodes a 50S ribosomal protein and is essential in bacterial ribosomes for the proper function of factors involved in protein synthesis, such as elongation factor G and elongation factor Tu (11). L7/L12 consists of an N-terminal domain that anchors the L10 ribosomal protein and a C-terminal domain which is important in the binding of elongation factors G and Tu. The L7/L12 ribosomal protein is essential for the maximal rate of protein synthesis and the low frequency of errors (22).

The in vivo prokaryotic protein expression system used here produced large amounts of the recombinant fusion polypeptide and proved to be a suitable system for expressing foreign sequences and purifying the resultant fusion protein. The pMAL-c2 vector has been used for gene expression of other immunodominant proteins, such as the GroES heat shock protein from *Mycobacterium leprae* (16). Having manufactured the recombinant protein, we demonstrated that L7/L12 is able to specifically stimulate PBM cells from *B. abortus*-primed animals. Phenotypic analysis of the proliferating cells demonstrated that the percentage of $CD4^+$ T cells was twofold higher when PBM cells were stimulated with rL7/L12 than with medium alone. Other investigators have recently confirmed the immunogenic nature of an analogous protein, when they reported the L7/L12 ribosomal protein from *Mycobacterium bovis* as a strong delayed-type hypersensitivity stimulus for sensitized guinea pigs (25). Furthermore, inflammatory reactions including the delayed-type hypersensitivity response in brucellosis and listeriosis have been shown to be mediated exclusively by $CD4^+$ T cells (17).

Ribosomal preparations from several pathogens, including *B. abortus*, have been shown to be highly protective vaccines (7). However, the components of these preparations have not

yet been characterized. A *Salmonella typhimurium* ribosomal vaccine afforded cell-mediated protection, based on the capacity of primed T cells to respond to *Salmonella* antigens and to activate macrophages (9). The mechanism by which the host responds to and eliminates infection is a central issue in our understanding of the immune response to pathogens. For many intracellular organisms, such as *B. abortus*, T-cell-mediated immunity is critical for host protection. Because T cells play a major role in protection against *Brucella* infection through cytokine secretion, such as that of gamma interferon (17), the identification of specific *Brucella* antigens that induce a T-lymphocyte response is an important element in designing new molecular candidate vaccines. Soluble *Brucella* proteins have been isolated and tested for their ability to stimulate primed murine T lymphocytes by cell proliferation and cytokine production (28). These fractions induced T-lymphocyte proliferation and elicited gamma interferon production by $CD4^+$ T cells, but none of the proteins have been purified and characterized, suggesting that a number of proteins rather than a single moiety possess these antigenic determinants.

This is the first report of a *B. abortus* T-cell-reactive ribosomal protein. However, further characterization of the T-lymphocyte subset involved in this response by phenotypic surface analysis and cytokine profile is required. Identifying proteins from a given pathogen that favor a T-cell response and the desired cytokine profile may provide important approaches for diagnosis and alternative vaccine strategies. Subcloning and expression of the *B. abortus L7/L12* gene provides an opportunity to immunologically characterize a specific ribosomal protein which may clarify why ribosomal vaccines confer protection against intracellular pathogens.

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