Vector Development for the Expression of Foreign Proteins in the Vaccine Strain \textit{Brucella abortus} S19

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Brucellosis remains a major zoonosis in several countries (3). In cattle, brucellosis is the consequence of infection with the facultative intracellular pathogen \textit{Brucella abortus}, which causes abortion and infertility in cattle and a clinical manifestation known as undulant fever in humans (15). To control the infection, especially in countries with large cattle populations, vaccination with attenuated strain \textit{B. abortus} S19 is a widely accepted approach (16). The outstanding characteristics of this strain are its low pathogenicity and the high level of protection conferred. S19 has an as-yet-uncharacterized alteration but is effective at preventing abortions caused by infections with field strains of \textit{B. abortus} (16). However, the antigenic similarity between S19 and virulent field strains, mainly in the immunodominant lipopolysaccharide antigen, hampers discrimination between infected and vaccinated animals. This is due to the occurrence and persistence of serum antibodies following strain S19 vaccination, which interferes with the detection of infected animals (2, 23). Alternative ways to work out these problems by using a specific monoclonal antibody or by using a deletion mutant as a vaccine strain have been described (17, 20). Other untested alternatives are the expression of a foreign protein in \textit{B. abortus} S19. This would result in a tagged vaccine with a distinctive immunological signature, allowing easy differentiation between vaccinated and infected animals.

\textit{B. abortus} is a well-known Th1 response inducer (5, 21) and, in addition, has been used as a carrier to induce a T-cell-independent immune response against molecules conjugated with the bacterium (7, 24). Thus, the strong humoral and cellular responses it generates in the host make \textit{B. abortus} S19 an attractive alternative as a live carrier of heterologous antigens. For tagging of the available S19 vaccine and its possible use as a live vaccine carrier, it is necessary to express foreign proteins in \textit{Brucella} without affecting its immunological properties. In this report, we describe the development of an expression vector for \textit{Brucella} using the promoter and secretion signals from \textit{bcsp31}, a gene encoding an immunodominant \textit{Brucella} protein (14). The application of this strategy in the generation of a tagged \textit{B. abortus} S19 vaccine is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Attenuated vaccine strain \textit{B. abortus} S19 was obtained from the Comision Nacional de Energia Atomica, Division Agropecuaria, Buenos Aires, Argentina. For mating experiments, \textit{B. abortus} S19 was grown at 37°C on a rotary shaker (200 rpm) for 24 to 48 h in tryptic soy broth containing 5 µg of nalidixic acid per ml. For all experiments, \textit{B. abortus} S19 or the recombinant strain carrying plasmid pBEV was grown at 37°C for 48 h in tryptic soy agar (TSA) or in TSA containing 50 µg of carbenicillin per ml in the case of the recombinant strain. \textit{Escherichia coli} DH5α(F′) was used for the construction of plasmid pBEV and in all cloning experiments. \textit{E. coli} DH5α(Nα) was used as the donor strain in biparental mating procedures.

Construction of an expression vector for \textit{Brucella}. A 250-bp DNA fragment encoding the putative promoter region, the start codon, and the first 31 codons, corresponding to the signal peptide, of the \textit{bcsp31} gene of \textit{B. abortus} S19 described by Mayfield et al. (14), was amplified by PCR using the upper primer 5′-gACTggATCCgCggCCgCCTgCAA-3′ and the lower primer 5′-ACTggTACCCgggCTgCgAA-3′. These primers contain BamHI and KpnI sites (underlined), respectively, to facilitate the cloning procedures. As template DNA, a pUC9-derived vector containing the entire \textit{bcsp31} gene previously constructed in our laboratory was used. The 250-bp fragment was inserted into the BamHI- KpnI sites of the pUC19 plasmid polylinker. The resulting DNA construct was introduced into the competent strain \textit{E. coli} DH5α(F′) as described by Inoue et al. (9), and the construction was analyzed by restriction analysis and DNA sequencing. The recombinant plasmid containing the promoter region, the start codon, and the signal peptide encoding the \textit{Brucella} \textit{bcsp31} gene, together with a linker sequence to facilitate the construction of a recombinant DNA expressing a fusion protein under the control of the \textit{Brucella} promoter, was designated pUC-PROM.

Because pUC-PROM is a ColE1-based plasmid, it is incapable of autonomous replication in \textit{Brucella} spp. (8). A 250-bp \textit{BamHI-EcoRI} fragment having the \textit{Brucella} promoter and the region encoding the secretory signal and carrying the linker sequence was excised from pUC-PROM and inserted into the \textit{BamHI- EcoRI} sites of the broad-host-range plasmid, pBBR4MCS, described by Kovach et al. (10, 11). The resulting pBBR4MCS-based construct was designated pBEV. As a reporter gene for the construction, we used the sequence encoding an antigen of \textit{Trypanosoma cruzi} consisting of 14 tandemly repeated units, each 12 amino acids long (19) (Fig. 1). An 850-bp EcoRI fragment encoding these repeats was inserted in frame into the EcoRI sites of pBEV. The resulting recombinant plasmid, pBEV-REP, was introduced into competent strain DH5α(F′), and the expression of the new recombinant fusion protein was analyzed by DNA sequencing and Western blotting. \textit{E. coli} DH5α carrying pBEV or pBEV-REP was used as the donor for conjugative transfer of this plasmid to \textit{B. abortus} S19.

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Experimental infection of mice. Nine-week-old female BALB/c mice were injected intraperitoneally with approximately $2 \times 10^7$ CFU of brucellae in 0.2 ml of NaCl (150 mM). Groups of eight mice were injected either with B. abortus S19 or with the recombinant strain B. abortus S19(pBEV-REP).

Two mice in each group were examined at each sampling period. At 10, 18, 23, and 30 days postinfection, mice were bled from the retroorbital sinus or by heart puncture. Sera were collected and stored at $-20^\circ$C until used. Spleens were removed, weighed, and cut into thirds. The tissue used for bacterial counting was weighed and homogenized in 1 ml of NaCl (150 mM). Tissue homogenates were serially diluted and plated in duplicate on TSA or on TSA containing 50-g/ml carbenicillin in the case of the recombinant strain. Colonies were counted after 4 days of incubation at 37°C.

Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (12). Periplasmic extracts were obtained by the Zwittergent-lysozyme extraction method developed by Stabel et al. (22). Periplasmic fraction, protoplastic fraction, and whole-cell lysates were solubilized in Laemmli buffer at 100°C, electrophoresed by SDS-10% PAGE, and transferred to nitrocellulose filters. The filters were reacted with sera from infected mice. The development of an expression vector for $B. abortus$ requires that the gene to be expressed be under the control of a constitutive promoter, due to the impossibility of using an inducer in the infected animal. We chose the $bcsp31$ promoter because the BCSP31 protein is expressed during the complete life cycle of the bacteria. Moreover, antibodies to the BCSP31 protein are detectable by using sera of vaccinated and infected animals (1, 8). The fact that this promoter is also functional in $E. coli$ facilitates the construction of the expression vector (14). A 250-bp fragment containing the putative promoter, regulatory sequences, and the region of $bcsp31$, encoding the first 31 amino acids, which includes the signal peptide, was cloned into broad-host-range plasmid pBBR4MCS, a plasmid that is replicative in $Brucella$ (10, 11). Downstream of the codon for Pro-31, a linker sequence containing KpnI, SacI, and EcoRI sites was added to allow in-frame fusions. We named this vector pBEV (see Materials and Methods). As a reporter protein, we used a molecular tag made up of a repetitive antigen from the protozoan parasite $T. cruzi$ (19). A scheme of the resulting construct is shown in Fig. 1. The corresponding reporter gene, consisting of the 5’ and 3’ nonrepeat coding regions with a core of 14 tandemly arranged 36-base-long repeats, was inserted into the correct reading frame in the EcoRI site of the linker. This plasmid was named pBEV-REP.

$B. abortus$ S19 was transformed by biparental conjugation with either plasmid pBEV (control) or pBEV-REP. The expression of the reporter protein was analyzed in whole-cell extracts and periplasmic and protoplastic fractions by Western

RESULTS

Construction of an expression vector for $B. abortus$ and expression of a reporter protein. The development of an expression vector for $Brucella$ requires that the gene to be expressed be under the control of a constitutive promoter, due to the impossibility of using an inducer in the infected animal. We chose the $bcsp31$ promoter because the BCSP31 protein is expressed during the complete life cycle of the bacteria. Moreover, antibodies to the BCSP31 protein are detectable by using sera of vaccinated and infected animals (1, 8). The fact that this promoter is also functional in $E. coli$ facilitates the construction of the expression vector (14). A 250-bp fragment containing the putative promoter, regulatory sequences, and the region of $bcsp31$, encoding the first 31 amino acids, which includes the signal peptide, was cloned into broad-host-range plasmid pBBR4MCS, a plasmid that is replicative in $Brucella$ (10, 11). Downstream of the codon for Pro-31, a linker sequence containing KpnI, SacI, and EcoRI sites was added to allow in-frame fusions. We named this vector pBEV (see Materials and Methods). As a reporter protein, we used a molecular tag made up of a repetitive antigen from the protozoan parasite $T. cruzi$ (19). A scheme of the resulting construct is shown in Fig. 1. The corresponding reporter gene, consisting of the 5’ and 3’ nonrepeat coding regions with a core of 14 tandemly arranged 36-base-long repeats, was inserted into the correct reading frame in the EcoRI site of the linker. This plasmid was named pBEV-REP.

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FIG. 1. Diagrammatic representation of plasmid pBEV-REP. The thin line represents pBBR4MCS sequences. The unshaded box represents the cloned $B. abortus$ $S19$ fragment containing the promoter (Prom), regulatory sequences, and signal peptide (SP) of the $bcsp31$ gene. The nucleotide and peptide sequences of the first 31 amino acids and the linker sequences are indicated. The shaded box represents the repetitive $T. cruzi$ reporter protein. The consensus sequence of the repeat is indicated.
blotting using specific rabbit antiserum raised against the reporter antigen.

As seen in Fig. 2, two strongly reactive bands with apparent molecular masses of 55 and 45 kDa were visible in whole-cell extracts of bacteria transformed with pBEV-REP (Fig. 2, lane 3). Bands likely to have resulted from degradation of the repetitive units were also observed (18, 19). The recombinant product was translocated to the periplasmic space (Fig. 2, lane 1) and was almost undetectable in the protoplastic fraction (Fig. 2, lane 2). The recombinant protein was also expressed in the donor E. coli strain carrying pBEV-REP (Fig. 2, lane 5). This was not unexpected, since the BCSP31 promoter had previously been reported to be active in E. coli (14). The serum failed to react with whole-cell extracts from B. abortus S19 that had been transformed with pBEV, which lacks the DNA insert encoding the reporter protein (Fig. 2, lane 4).

Transformed and control brucellae grew at similar rates in culture media, and no alteration of the morphological characteristics of the transformed bacteria was observed (results not shown).

The reporter protein expressed in B. abortus S19 is immunogenic in the course of an experimental infection. The next question was whether the reporter protein expressed by the transformed B. abortus is able to generate an immune response in the course of an experimental infection in the mouse model. B. abortus S19 and B. abortus S19(pBEV-REP) were used to infect BALB/c mice (see Materials and Methods), and at different days postinfection, sera were collected and analyzed for the presence of specific antibodies against a recombinant GST-REPEATS fusion protein by Western blotting (18) (Fig. 3). Antibodies against the reporter protein in sera from animals infected with B. abortus S19(pBEV-REP) were detectable after 18 days of infection (Fig. 3B). Antibodies against the reporter protein were not detectable in sera from animals infected with B. abortus S19 (Fig. 3A).

The antibody response against the Brucella LPS and T. cruzi repeats was quantified by a kinetics-based ELISA as described in Materials and Methods. The anti-LPS titers elicited by B. abortus S-19 and B. abortus S-19(pBEV-REP) reached similar kinetic equivalent values after 30 days of infection. Anti-T. cruzi repeat antibodies were detected as soon as 10 days postinfection and showed a constant increase until the end of the sampling time (30 days postinfection) (Fig. 4). As expected, no anti-T. cruzi repeat antibodies were detected in mice infected with B. abortus S-19. Thus, the expression of the recombinant repetitive protein in the periplasmic space does not alter the serological response against the immunodominant Brucella antigen.

B. abortus S19 and the recombinant bacteria generate similar infections in mice. Basic parameters of Brucella infection were analyzed to find out if there was any gross modification of the infection process due to expression of the foreign protein. Mice were infected with either parental B. abortus S19 or B. abortus S19(pBEV-REP); animals were sacrificed at different times after infection, and the spleen weights and the numbers of CFU recovered from the spleens were analyzed (Table 1). Mice infected with S19 (six animals) and S19(pBEV-REP) (eight animals) controlled the infection and survived. Significant splenomegaly, a characteristic consequence of Brucella infection, was observed in both groups of animals starting at about days 18 to 20 of infection (Table 1). The numbers of CFU recovered from the spleen were similar for S19(pBEV) and S19(pBEV-REP). The numbers of CFU clearly decreased

<table>
<thead>
<tr>
<th>Day postinfection</th>
<th>S. abortus S-19</th>
<th>S. abortus S-19(pBEV-REP)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Spleen wt (g)</td>
<td>No. of CFU/g of spleen</td>
</tr>
<tr>
<td>0</td>
<td>0.10</td>
<td>ND^b</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18</td>
<td>0.60</td>
<td>1.6 × 10^7</td>
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<tr>
<td>23</td>
<td>0.82</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>0.93</td>
<td>2.7 × 10^3</td>
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<td></td>
<td>0.54</td>
<td>0.42</td>
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^a Mice were inoculated intraperitoneally with 10^7 CFU of B. abortus S19 or 10^7 CFU of B. abortus S19(pBEV-REP). At the indicated days postinfection, two mice per group were killed and the spleens were removed and weighed. The spleens were pooled, homogenized, and plated on tryptic soy broth agar with or without carbenicillin.

^b ND, not determined.
to similar levels at day 30 after infection, indicating that \textit{B. abortus} growth was controlled in both cases.

It was possible to isolate plasmid pBEV-REP from spleen-derived colonies recovered 23 days postinfection. This result shows that the plasmid was stably maintained by intracellular \textit{Brucella} without any selective pressure. Similar data was reported by Elzer et al. (6) for broad-host-range plasmid pBBR4MCS.

**DISCUSSION**

In designing an expression vector for \textit{B. abortus}, we took into consideration some aspects for the selection of the gene to be used. First, the gene promoter selected should constitutively express the recombinant product at levels compatible with the generation of a specific immune response. Second, the sequences selected should encode a signal peptide in order to secrete the recombinant product into the periplasm of the bacteria. This should prevent toxicity and the generation of inclusion bodies frequently found when a recombinant protein is expressed constitutively or at high levels by a strong promoter. We chose the regulatory sequences and the secretory signal of the gene encoding BCS3P1, a periplasmic \textit{B. abortus} protein which is highly antigenic during natural infections and after vaccination (1, 14). In this work, we showed that a recombinant protein can be stably expressed in \textit{Brucella} with the designed vector. Most of the recombinant protein was detected after vaccination (1, 14). In this work, we showed that a recombinant protein can be stably expressed in \textit{Brucella} with the designed vector. Most of the recombinant protein was detected after vaccination (1, 14).

The recombinant bacteria generate a strong antibody response in mice against the heterologous protein. This result indicates that the expression vector is stably maintained without selective pressure and that the promoter sequence selected actively expresses the reporter protein during the infection process.

The expression of the repetitive antigen in \textit{Brucella} does not alter its growth pattern, and in a preliminary study, it failed to generate a toxic or lethal effect in the BALB/c mouse model.

Previous studies have shown that pBBR4MCS replicates in all \textit{Brucella} species (6). Therefore, the strategy we describe here can be extended to the other live \textit{Brucella} vaccines, including \textit{B. melitensis} Rev1, \textit{B. suis} S2 and \textit{B. abortus} RB51.

Although in this work we have expressed a reporter protein to demonstrate the feasibility of the approach, it might be possible to express in this vector epitopes protective against other cattle pathogens. The advantage of using a live, attenuated bacterial carrier like \textit{B. abortus} S19 is the strong immune response it generates after immunization. In the model that we tested in this work, an autonomous replicating vector with an antibiotic resistance marker was used. In developing a live vaccine carrier, however, it might be convenient to generate a vector that integrates itself into the bacterial genome to prevent it from being lost after successive divisions in the absence of any antibiotic selective pressure.

Another possible application of the approach described is tagging of the \textit{Brucella} vaccine. A major problem in many countries in which vaccination against \textit{Brucella} is mandatory is the difficulty in differentiating between vaccinated and infected animals (13). The antibody response is, in both cases, directed to similar antigens, particularly if a complex antigenic bacterial extract is used as the reagent for antibody detection. The use of a vaccine having a distinctive immunological signature as an antigenic tag might allow quick identification of immunized animals through a simple ELISA using either synthetic peptides or a recombinant protein. Different synthetic repeats could be included on \textit{Brucella} in different vaccination campaigns or to label products from different companies.

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