INFECTION AND IMMUNITY, Nov. 1991, p. 3863–3868
0019-9567/91/113863-06$02.00/0

Vol. 59, No. 11

# Deletion of the BCSP31 Gene of *Brucella abortus* by Replacement

SHIRLEY M. HALLING,[1]* PHILIPPE G. DETILLEUX,[1] FRED M. TATUM,[1]
BRADLEY A. JUDGE,[2] AND JOHN E. MAYFIELD[2]

*U.S. Department of Agriculture, Agricultural Research Service, National Animal Disease Center, P.O. Box 70, Ames, Iowa 50010,[1] and Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011[2]*

Received 18 April 1991/Accepted 31 July 1991

**The 31-kDa salt-extractable immunogenic protein, BCSP31, was deleted from several *Brucella abortus* strains by replacement with a marker gene encoding resistance to the antibiotics kanamycin and neomycin. The BCSP31 gene replacement plasmids, constructed with ColE1-derived vectors, were introduced by electroporation into *B. abortus* strain 19 (S19), into a rough variant of *B. abortus* S19, and into *B. abortus* S2308, and antibiotic-resistant transformants were isolated. *B. abortus* S19 is an attenuated strain used as a vaccine for prevention of bovine brucellosis in the United States, and *B. abortus* S2308 is a commonly used challenge strain. The antibiotic-resistant isolates were all obtained by recombination; none were spontaneous mutants. Loss of the gene encoding BCSP31 and presence of the marker gene were confirmed by Southern analysis. Vector sequences were either absent or linked to the genome, indicating that ColE1-derived plasmids are not maintained in *B. abortus*. Survival of *B. abortus* mutant strains in the macrophagelike cell line J774 and in HeLa cells was examined and shown to be indistinguishable from that of the parental strain.**

*Brucella abortus* is a facultative intracellular parasite whose primary host is bovines, but which can infect humans, in whom it causes undulant fever. Infection of pregnant cows can cause abortions and is of major economic importance in the southern United States. The use of the current vaccine strain, *B. abortus* strain 19 (S19), in the eradication of bovine brucellosis is limited because field-infected animals cannot be reliably distinguished from vaccinated animals. Application of molecular genetic techniques should lead both to an increased understanding of the molecular basis of pathogenesis and to improved vaccines for combating the disease. Of particular interest to us is the development of a vaccine with diagnostic properties so that vaccinated and naturally infected animals can be reliably distinguished.

Knowledge of the genetics and molecular biology of the organism is quite limited. Only a small number of genes have been cloned (4, 7, 14). The application of several powerful techniques such as transposon mutagenesis and gene replacement has not been widely employed because of the difficulty of introducing foreign DNA into *Brucella* cells (18). Although transformation of nonlaboratory strains of many gram-negative bacteria has been difficult or impossible, it was recently shown that plasmids can be introduced into gram-negative bacteria by electroporation (8).

Gene replacement and gene deletion have been used as a method of constructing vaccine strains that makes it possible to distinguish between vaccinated and infected animals (13, 15). In this report, we describe replacement of the gene encoding the 31-kDa protein, BCSP31 (14), with an antibiotic resistance marker gene. BCSP31 is highly conserved with each known species and biovar (except *Brucella ovis*) expressing the protein, as determined by Western immunoblot analysis (3). BCSP31, which has no known function, is soluble in aqueous buffers and is probably a periplasmic protein (22). The protein frequently elicits an immune response during infection (16, 17, 20). This report demonstrates that loss of BCSP31 does not significantly affect invasion, growth, or replication of *B. abortus* in a macrophagelike cell line, J774, and a nonphagocytic cell line, HeLa.

* Corresponding author.

## MATERIALS AND METHODS

**Materials.** Restriction endonucleases and ligase were purchased from New England Biolabs, Inc. Avian myeloblastosis virus reverse transcriptase was obtained from Bio-Rad, Inc. Radiolabeled [$\alpha$-$^{32}$P]dCTP (3,000 Ci/mmol) was from ICN Biomedicals, Inc., and Zwittergent 3-14 was from Calbiochem Corp. Electroporation was done with a Gene Pulser and Pulse Controller Unit (Bio-Rad). Sterile electroporation cuvettes with 0.2-cm electrode gaps and pUC18 for electroporation were from the same source. Agarose (SeaKem GTG) was from FMC Corp. Gentamicin and streptomycin were from Pfizer, and kanamycin and ampicillin were from Sigma Chemical Co. Tissue culture plates were from Costar, and tissue culture media were from GIBCO Laboratories. The plasmid pKK223-3 was obtained from Pharmacia, Inc.

**Bacterial strains.** *Salmonella typhimurium* SL5283 ($r^-m^+$), a rough strain, was obtained from Ronald Griffith, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, and smooth *S. typhimurium* SL3261 (*aroA*) (10) was obtained from Praxis Biologics, Rochester, N.Y. *B. abortus* S19 original seed culture (serial no. 7901) was obtained from the National Veterinary Services Laboratory, Ames, Iowa. *B. abortus* S19 rough (accession no. 752) was isolated and kindly supplied by Billy Deyoe, National Animal Disease Center. *B. abortus* S2308 was also supplied by Billy Deyoe.

**Plasmids.** The plasmid pBA31-R7, which encodes the protein BCSP31 on a 1.3-kbp *Hin*dIII fragment, was described previously (14). A 4.2-kbp *Eco*RI *Brucella* DNA fragment was cloned into pKK223-3 to generate pBA31-R7. pKK223-3 is a derivative of pBR322 (2), which is a ColE1 plasmid. The plasmid pBA31-R7/Kan7 was constructed for this experiment by replacing the 1.3-kbp *Hin*dIII DNA fragment encoding BCSP31 with a 1.4-kbp fragment encoding the kanamycin-neomycin resistance gene from Tn*5*. The

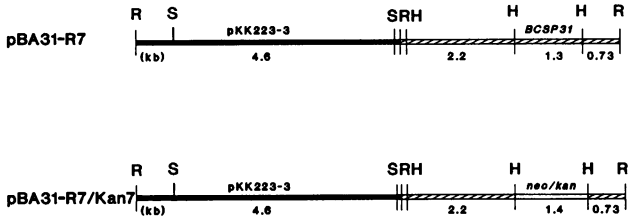


FIG. 1. Diagrammatic restriction map of the plasmids pBA31-R7 and pBA31-R7/Kan7. *B. abortus* S19 sequences are indicated by hatched bars. The antibiotic resistance marker gene is indicated by an open bar. The vector pKK223-3, denoted by a solid bar, is a derivative of pBR322 (2). Abbreviations: R, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

1.4-kbp fragment containing the kanamycin-neomycin resistance gene from Tn*5* was excised from pBRneo (11) by *Hin*dIII and *Bam*HI digestion; the *Bam*HI end was converted to a *Hin*dIII end by ligation of *Bam*HI-*Hin*dIII linker; and the resultant *Hin*dIII fragment was ligated to a partial *Hin*dIII digest of pBA31-R7. Recombinants were isolated, and the plasmids were physically mapped. A clone with the desired configuration was designated pBA31-R7/Kan7. Diagrams of both pBA31-R7 and pBA31-R7/Kan7 are shown in Fig. 1. Plasmid pUC18 was described by Yanisch-Perron et al. (23). The *Eco*RI deletion cassette of pBA31-R7/Kan7 was cloned into the *Eco*RI restriction endonuclease site of pUC9, generating pUCR7/Kan7.

**Media and culturing.** *S. typhimurium* SL5283 and *S. typhimurium* SL3261 were cultured in LB medium (12) to obtain cells for electroporation. After electroporation, *S. typhimurium* was cultured in SOC (9) for 1 h before dilution and plating on LB agar supplemented with 50 μg of ampicillin per ml.

The *B. abortus* S19 strains and *B. abortus* S2308 were recovered from freeze-dried cultures by plating on potato infusion agar (1). After growth was noted, a loopful was streaked on tryptose agar (1) supplemented with heat-inactivated calf serum (5%). Single colonies were spread individually on tryptose agar plates and incubated at 37°C, and after 3 days cultures were harvested in a small amount of sterile saline and stored at −70°C for inoculum. Inoculum was also grown on tryptose agar. For in vitro survival studies, the bacteria were diluted to $1 \times 10^7$ CFU in Dulbecco minimal essential medium (DMEM) supplemented with 5% fetal calf serum (FCS). When the cells were to be used for electroporation, they were harvested in water, washed first with water and then 10% glycerol, and resuspended in 10% glycerol.

Following electroporation, *B. abortus* S19 was cultured in filter-sterilized tryptic soy broth at 37°C for 1 h before plating. *B. abortus* S2308 was cultured longer, for 6 h. Transformants were isolated by plating on tryptose agar containing 25 μg of kanamycin per ml. Kanamycin-neomycin-resistant antibiotic (Kan$^r$) *Brucella* isolates were streaked for single colonies.

**Electroporation.** pUC18 (10 pg or 1 μg) was combined with $2 \times 10^8$ to $5 \times 10^8$ *S. typhimurium* cells immediately before electroporation under the conditions given in Table 1. Largely supercoiled plasmid (2 or 10 μg) was mixed with $5 \times 10^8$ to $7 \times 10^8$ *B. abortus* cells just before electroporation. pBA31-R7/Kan7 was used to transform *B. abortus* S19 rough and smooth strains, and pUCR7/Kan7 was used to transform *B. abortus* S2308. Capacitance was varied as indicated in Table 1. After electroporation, cells were diluted immediately into medium and cultured as described above.

TABLE 1. Transformation of *B. abortus* and *S. typhimurium* strains by electroporation[a]

| Strain | Amt of plasmid DNA (μg) | No. of transformants (CFU) | Capacitance (μF), time constant (ms) |
|---|---|---|---|
| *B. abortus* S19 (rough) | 2 | 5 | 200, 3.7 |
| | 2 | 1 | 400, 7.3 |
| | 10 | 0[b] | 600, 9.7 |
| | 10 | 4 | 400, 7.0 |
| *B. abortus* S19 (smooth) | 10 | 2 | 400, 7.3 |
| | 10 | 1 | 800, 10.3 |
| *S. typhimurium* SL3261 (smooth) | 1 | 25 | 400, 3.7 |
| *S. typhimurium* SL5283 (rough) | $10^{-5}$ | 2,500 | 200, 3.7 |
| *B. abortus* S2308 (smooth) | 10 | 133 | 400, 7.1 |

[a] The initial electric field strength was 25 kV/cm. Cells were prepared for electroporation as described in Materials and Methods. *S. typhimurium* was transformed with pUC18, *B. abortus* S19 was transformed with pBA31-R7/Kan7, and *B. abortus* S2308 was transformed wtih pUCR7/Kan7.
[b] Arcing was observed.

**Isolation of genomic DNA.** Methanol-killed cells (0.1 to 0.2 g [wet weight]) were collected by centrifugation (15 min at 5,000 × *g*) and washed twice in 5 ml of TE (10 mM Tris, 1 mM EDTA [pH 7.5]) before being resuspended in 1 ml of TE. Citric acid (0.1 ml of a 1 M solution) and Zwittergent 3-14 (0.1 ml of a 10% aqueous solution) were added, and the cells were incubated at 50°C for 1 h before being collected by centrifugation. The cells were then washed twice in TE before resuspension in 2 ml of lysozyme buffer (50 mM Tris, 25 mM EDTA [pH 8.0]). Lysozyme (0.2 ml of a 100-mg/ml solution in lysozyme buffer) was added, and the cells were incubated at 37°C for 30 min; proteinase K (25 μl of a 10-mg/ml solution in water or 0.5% sodium dodecyl sulfate [SDS]) was added, and incubation was continued for 15 min; EDTA (200 μl of 0.25 M EDTA [pH 8.0]), RNase A (10 μl of a 10-mg/ml solution), and sarcosine (0.25 ml of a 10% aqueous solution) were added before incubation at 65°C for 30 to 60 min. The preparation was then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated. The recovered DNA was dissolved in 4 ml of TE. Then 4.0 g of cesium chloride and 0.1 ml of 5-mg/ml ethidium bromide were added, and the preparation was centrifuged overnight at 50,000 rpm in a Beckman VTi65 rotor. The DNA was recovered from the gradient, and the ethidium was extracted with isoamyl alcohol (12).

**Treatment of DNA with restriction endonucleases and electrophoresis.** DNA was digested with restriction endonucleases under the conditions described by the manufacturer. pBA31-R7 and pBA31-R7/Kan7 *Hin*dIII fragments used as probes for Southern analysis were obtained from agarose gels following digestion with *Hin*dIII. *Brucella* genomic DNA (2 μg) was treated for 4 h with 10 to 20 U of restriction endonuclease and electrophoresed in the presence of TBE (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA) through 0.75% agarose for 16 h at 25 V in the presence of 0.1 μg of ethidium bromide per ml.

**Probes and Southern blot analysis.** The 1.3-kbp *Hin*dIII fragment containing the BCSP31 gene and the downstream 2.2-kbp *Hin*dIII fragment were isolated from *Hin*dIII-treated pBA31-R7, and the 1.4-kbp *Hin*dIII fragment encoding kanamycin-neomycin resistance (*neo*) was isolated from pBA31-R7/Kan7. pBR322 was isolated from a transformed

strain of *Escherichia coli* HB101 by alkaline SDS lysis and cesium chloride gradient centrifugation (12).

The purified *Hin*dIII DNA fragments isolated for probes were digested with *Hpa*II and labeled by incubation in the presence of reverse transcriptase and [$\alpha$-$^{32}$P]dCTP-radiolabeled nucleotide. After 1 to 3 h at 37°C, the probes were ethanol precipitated. They were then boiled and added to hybridization mixture. Blotting was carried out essentially by the technique of Southern (19), except that Nytran (Schleicher & Schuell, Inc.) was used and, in some cases, gels were not neutralized before blotting (5). Hybridization and washing of the hybridized filters were performed as described by Maniatis et al. (12). The prehybridization solution contained 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's reagent, 0.5% SDS, 0.5 mg of denatured fragmented calf thymus DNA per ml, and 50% formamide. Hybridization solution contained 6× SSC, 5× Denhardt's reagent, 0.5% SDS, 0.1 mg of denatured fragmented calf thymus DNA per ml, 50% formamide, and 1 μg of radiolabeled boiled probe. Hybridization was carried out overnight at 42°C.

**Characterization of *B. abortus* outer membrane.** Acriflavine was used to determine whether the *B. abortus* S19 Kan$^r$ isolates were rough or smooth (1). Rough *Brucella* strains aggregate when mixed with acriflavine.

**Tissue culture cells.** Mouse monocyte-macrophage (J774A.1; ATCC IB61) and human epitheloid carcinoma cells (HeLa; ATCC CCL2) were purchased from the American Type Culture Collection, Rockville, Md. J774 cells were grown in DMEM supplemented with 5% FCS, and HeLa cells were grown in Eagle MEM (EMEM) supplemented with 5% FCS. For monolayer inoculation, 24-well tissue culture plates were seeded with 1 ml of 150,000 to 200,000 cells per ml. After 24 h of incubation, subconfluent monolayers were washed with phosphate-buffered saline (PBS) at 37°C and further incubated in 1 ml of culture medium without antibiotics.

**Monolayer inoculation.** After overnight incubation, medium was aspirated from plates and 1 ml of bacterial suspension was added. The culture plates were centrifuged for 5 min at 800 × *g* at room temperature and placed in an incubator under an atmosphere of 5% $CO_2$ at 37°C. After 2 or 4 h, plates were washed three times with PBS and further incubated with EMEM supplemented with 5% FCS (HeLa cells) or DMEM supplemented with 5% FCS (J774). The tissue culture medium was also supplemented with gentamicin (50 μg/ml) and streptomycin (50 μg/ml) at bactericidal levels to kill extracellular brucellae. Medium was replaced 1 h later and at 24 h. The inoculation period was defined as the period between the exposure of the monolayer to *B. abortus* and the introduction of gentamicin and streptomycin. Experiments were done in duplicate and repeated at least three times for each experimental treatment.

**Enumeration of brucellae.** The number of intracellular viable *B. abortus* was determined at 2, 3, 5, 7, 24, 36, and 48 h postinoculation in J774 cells and at 3, 5, 24, 36, and 48 h in HeLa cells. Except for the 2-h samples, gentamicin and streptomycin were introduced in all J774 samples 2 h postinoculation and in all HeLa cells 4 h postinoculation. The 2-h samples were treated with gentamicin (50 μg/ml) and streptomycin (50 μg/ml) for 1 h before dilution to determine the CFU. After exposure to antibiotics, inoculated monolayers were washed in PBS and incubated for 20 min in 2 ml of a 0.1% solution of deoxycholate in distilled water. This procedure disrupted the host cells without affecting the viability of brucellae. Samples of the lysate were serially diluted in saline for determination of *B. abortus* CFU on tryptose agar plates containing 5% heat-inactivated bovine serum. *Brucella* colonies were identified by colony morphology and growth characteristics.

## RESULTS

**Transformation by electroporation.** Transformation of *Salmonella* species by electroporation has been reported by Taketo (21) and served as a positive control in this work. Rough and smooth strains of *S. typhimurium* were readily transformed to ampicillin resistance by electroporation in the presence of pUC18 (Table 1), and this transformation of *S. typhimurium* to ampicillin resistance was plasmid dependent (data not shown).

Both rough and smooth strains of *B. abortus* S19 were transformed by electroporation in the presence of pBA31-R7/Kan7. The electroporation of *B. abortus* S19 resulted in the isolation of 13 Kan$^r$ mutants (Table 1). No apparent differences in efficiency were noted between the *B. abortus* S19 strains. In a similar experiment, *B. abortus* S2308 was also transformed. Although electroporation of *B. abortus* S2308 was more efficient than that of *B. abortus* S19, the difference may not be significant. Both plasmid sizes and experimental details differed. Because resistance to kanamycin-neomycin could have been caused by spontaneous mutation, by replication of the introduced plasmid, or by recombination, we characterized 10 randomly chosen Kan$^r$ *B. abortus* S19 transformants by Southern analysis to determine whether gene replacement had occurred; those results are reported here. A Kan$^r$ *B. abortus* S2308 strain was similarly analyzed, and these results are reported below. As a control, a ColE1-derived plasmid, pBRneo (11), which encodes the neomycin resistance of Tn*5*, was electroporated into *B. abortus*. No Kan$^r$ mutants were isolated.

**Southern analysis to detect *neo*.** The frequency of spontaneous Kan$^r$ mutations of *B. abortus* S19 was determined to be less than $1 \times 10^{-8}$. Because the efficiency of generating *B. abortus* Kan$^r$ mutations by electroporation was low, we did not know whether the antibiotic-resistant isolates were spontaneous mutants or transformants. To determine the basis of antibiotic resistance, a Southern blot of *Hin*dIII-treated cellular DNA from each of the Kan$^r$ *B. abortus* mutants was hybridized with a radiolabeled *Hin*dIII *neo* probe (Fig. 2A). A 1.4-kbp *Hin*dIII fragment was detected for each of the Kan$^r$ mutants. The probe did not hybridize to DNA from either *B. abortus* S19 (lane 11) or pBA31-R7 (lane 12), which do not contain those sequences. The probe did hybridize to a 1.4-kbp *Hin*dIII fragment of pBA31-R7/Kan7 (lane 13). Thus, resistance to the antibiotic was directly correlated with the introduction of *neo* by electroporation.

**Southern analysis to detect the BCSP31 gene.** A *Hin*dIII Southern blot of genomic DNA of the Kan$^r$ isolates was hybridized with a BCSP31 probe. DNA from four of the Kan$^r$ isolates (Fig. 2B, lanes 1, 2, 6, and 7) did not hybridize to the probe, making them candidates for gene replacement transformants. The probe did hybridize to a 1.3-kbp *Hin*dIII fragment of the other isolates (lanes 3 to 5 and 8 to 10) and to a 1.3-kbp *Hin*dIII fragment of *B. abortus* S19 DNA (lane 11). Western analysis confirmed that BCSP31 was not expressed in gene replacement strains (data not shown).

**Southern analyses of Kan$^r$ mutants for vector sequences.** A *Sal*I Southern blot of cellular DNA from the same 10 Kan$^r$ *B. abortus* mutants was probed with $^{32}$P-radiolabeled pBR322. (The vector, pKK223-3, used in construction of the deletion plasmid is a derivative of pBR322.) The probe hybridized to

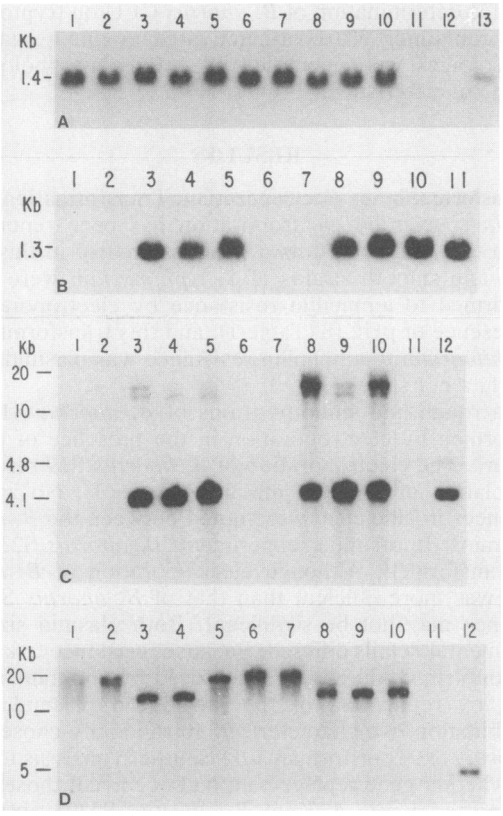


FIG. 2. Southern blots of cellular DNA from *B. abortus* kanamycin-resistant isolates probed with radiolabeled DNA. Lanes 1 to 10 correspond to same individual isolates in each panel. Fragment sizes are indicated in kilobase pairs along the left margin. (A) *Hin*dIII digest hybridized with a $^{32}$P-radiolabeled neomycin resistance gene probe. Lane 11 is total DNA from *B. abortus* S19. Lane 12 is pBA31-R7. Lane 13 is pBA31-R7/Kan7. (B) *Hin*dIII digest hybridized with $^{32}$P-labeled BCSP31 gene. Lane 11 is pBA31-R7. (C) *Sal*I digest hybridized with $^{32}$P-radiolabeled pBR322. Lane 11 is total DNA from *B. abortus* S19. Lane 12 is pBA31-R7/Kan7. (D) *Sal*I digest hybridized with $^{32}$P-radiolabeled *neo* probe. Lane 11 is *B. abortus* S19. Lane 12 is pBA31-R7/Kan7.

a 4.1-kbp *Sal*I DNA fragment in the same six isolates which have the 1.3-kbp *Hin*dIII fragment containing the BCSP31 gene (Fig. 2C, lanes 3 to 5 and 8 to 10). The high-molecular-weight fragments that are visible in lanes 3 to 5 and 8 to 10 are probably due to a junction *Sal*I restriction endonuclease fragment which has homology to the few hundred base pairs of pBR322 sequences which are linked to the *B. abortus* BCSP31 gene of pBA31-R7 (Fig. 1). DNA from the other four isolates (Fig. 2C, lanes 1, 2, 6, and 7) did not hybridize to the pBR322 probe. The lack of hybridization of Kan$^r$ mutants 1, 2, 6, and 7 to both the BCSP31 probe and the pBR322 probe is consistent with the conclusion that these mutants have undergone gene replacement. *B. abortus* S19 DNA did not hybridize to the pBR322 probe (Fig. 2C, lane 11), whereas both the 4.1-kbp *Sal*I fragment and the 4.8-kbp *Sal*I fragment of pBA31-R7 did (Fig. 2C, lane 12). The 4.8-kbp *Sal*I fragment of pBA31-R7 contains only a few hundred base pairs of pBR322 (Fig. 1) and produced a weak signal.

Kan$^r$ *B. abortus* isolates which hybridized to vector sequences potentially contained freely replicating pBA31-R7. To test this possibility, we hybridized a *Sal*I Southern blot of cellular DNA of the 10 Kan$^r$ isolates with a radiolabeled *neo* probe. Three hybridization patterns were noted for the Kan$^r$ isolates. The *Sal*I restriction nuclease fragments were either approximately 14 kbp (Fig. 2D, lanes 3, 4, and 8 to 10), approximately 20 kbp (lane 5), or larger than 20 kbp (lanes 1, 2, 6, and 7). As none of the Kan$^r$ transformants exhibited the linkage pattern of freely replicating pBA31-R7/Kan7, all of the Kan$^r$ isolates appear to be recombinants. DNA from *B. abortus* S19 did not hybridize to the Kan$^r$ probe (lane 11).

**Analyses of the *B. abortus* S2308 antibiotic-resistant mutant.** Cellular DNA was isolated from a *B. abortus* S2308 Kan$^r$ isolate. A *Hin*dIII Southern blot of the DNA from the isolate was hybridized with radioactively labeled probes for the neomycin gene and for DNA sequences encoding BCSP31, as for *B. abortus* S19. The isolate hybridized to the neomycin gene probe, but not the BCSP31 probe (data not shown), indicating that gene replacement had occurred. Western analysis confirmed that BCSP31 was not expressed (data not shown).

**Determination of whether the Kan$^r$ mutants are rough or smooth.** The Kan$^r$ *B. abortus* mutants from smooth cultures were tested to determine whether they remained smooth. Two of two smooth *B. abortus* S19 mutants and three of three rough *B. abortus* S19 mutants reacted like the parental clone. The *B. abortus* S2308 mutant used in the tissue culture studies was also determined to be smooth.

**Kinetics of *B. abortus* intracellular growth.** The number of viable brucellae in J774 and HeLa cells was determined at various times after inoculation (Fig. 3A and B). The number of viable brucellae in the cell lysate increased rapidly in J774 cells, after an initial decrease, reflecting intracellular replication. In HeLa cells, there was a rapid rise in the number of brucellae followed by a slow increase. Although a larger number of brucellae entered J774 cells than HeLa cells, this was followed by a sharper decline in the number of viable intracellular bacteria. This may be explained both by the phagocytic nature of J774 cells and by the more efficient intracellular killing mechanisms of these cells. Survival and growth of mutant strains in both J774 and HeLa cells was indistinguishable from that of parental strains. As previously reported for Vero cells (6), smaller numbers of *B. abortus* S19 than *B. abortus* S2308 entered both J774 and HeLa cells. This difference in infectivity between the strains remained unchanged with the mutant strains. Survival and growth of the mutants in both J774 cells and HeLa cells were similar to those of the parental strains.

## DISCUSSION

Several new strains of *B. abortus* which have the coding sequences for BCSP31 entirely deleted and replaced by the Tn*5* kanamycin-neomycin resistance gene were constructed. Loss of BCSP31 had no effect on the survival and intracellular growth of *B. abortus* in two mammalian cell lines. In addition, we have demonstrated that electroporation is a practical method for introducing foreign DNA into *Brucella* species, that pBR322-derived vectors behave as suicide vectors in *Brucella* species, and that homologous DNA recombination is an efficient mechanism for gene replacement in this organism.

**Nature of the Kan$^r$ transformants.** *B. abortus* was transformed to Kan$^r$ by electroporation of pBA31-R7/Kan7. Ten of the *B. abortus* Kan$^r$ transformants were characterized by Southern analysis to determine whether they were spontaneous mutants or recombinants. In each case, a 1.4-kbp *Hin*dIII fragment which hybridized to the *neo* probe was

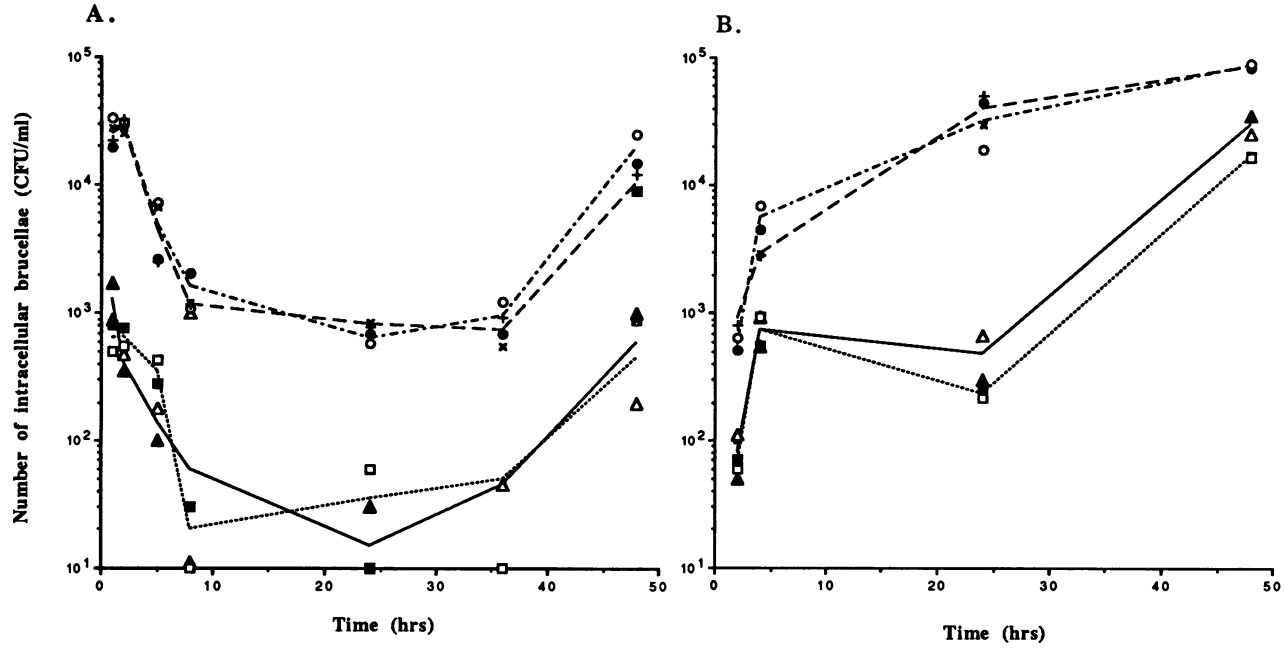


FIG. 3. Survival of *B. abortus* S19, S19 BCS31 deletion, S2308, and S2308 BCS31 deletion in J774 cells (A) and HeLa cells (B). Cell culture lines were infected with $10^7$ CFU of *B. abortus* S19 (▲, △), S19 BCS31 deletion (■, □), S2308 (●, ○), and S2308 BCS31 deletion (+, ×). CFU of *B. abortus* isolated from gentamicin-streptomycin-treated cultures at various times are shown. Results are expressed as the average number of viable *B. abortus* organisms per milliliter.

introduced into the strains. All the isolates were the result of genetic recombination. A ColE1-derived plasmid which had no homology to the cellular DNA did not transform *B. abortus*.

Single- and double-recombination events were anticipated. A single crossover would give rise to a mutant which hybridizes to BCSP31, *neo*, and pBR322 probes. Six of the isolates exhibited this single-crossover genotype (mutants 3, 4, 5, 8, 9, and 10). The desired event, a double crossover due to crossovers in each of the flanking arms, results in gene replacement. DNA from such mutants would not hybridize to a BCSP31 probe or a pBR322 probe, but would hybridize to a *neo* probe. Four of the isolates had the gene replacement genotype. DNA from these isolates is shown in Fig. 2, lanes 1, 2, 6, and 7.

Recombination would be expected to occur more frequently in the longer flanking region. Three genomic hybridization patterns were seen among the isolates probed with *neo* (Fig. 2D). All four of the double recombinants exhibited a single band greater than 20 kbp. A somewhat smaller band was seen in lane 5, and the remaining five samples revealed a single band of about 14 kbp. This pattern is consistent with more frequent recombination within the flanking 2.2-kbp arm than the 0.73-kbp arm.

**ColE1 based plasmid is not maintained in *Brucella* species.** For efficient gene replacement, the deletion plasmid should not replicate. ColE1-derived vectors were used to construct the deletion plasmids. Because ColE1 vectors have a narrow host range, we did not expect these plasmids to replicate in *Brucella* species. The gene replacement plasmid, pBA31-R7/Kan7, introduced into *B. abortus* by electroporation had a ColE1 origin of replication. DNA linkage studies established that the deletion plasmid was not maintained as freely replicating plasmid in *B. abortus* (Fig. 2D). The kanamycin-neomycin resistance gene of freely replicating pBA31-R7/Kan7 is on a 4.8-kbp *Sal*I fragment, but when a *Sal*I Southern blot of DNA from the Kan$^r$ isolates was hybridized to a *neo* probe, the *Sal*I restriction endonuclease fragments that hybridized were much larger than 4.8 kbp. No evidence of free plasmid was observed in any experiment.

To select transformants in these experiments, we needed a selectable marker gene which could be expressed in *Brucella* species. We used the antibiotic resistance gene from Tn*5* because it had been shown previously by Smith and Heffron that kanamycin-neomycin-resistant mutants could be isolated (18).

In transposon mutagenesis experiments by Smith and Heffron, Tn*5* mutants of *B. abortus* S19 were constructed by introduction of Tn*5* either by phage or by mating. Even though *B. abortus* S19 is smooth, a relatively high percentage of the Kan$^r$ isolates were rough (18). The high percentage of rough mutants might have resulted from the presence of a few rough *B. abortus* S19 variants being more efficiently infected by phage or more efficiently transformed by plasmids encoding Tn*5* conjugation. Electroporation of smooth *B. abortus* S19 in the presence of pBA31-R7/Kan7 did not result in the selection of Kan$^r$ rough mutants.

**Survival of *B. abortus* in mammalian cells.** Deletion of the BCSP31 gene has no detectable effect on the invasion, survival, or intracellular growth of *B. abortus* S19 or S2308 within either monocyte-macrophages (J774) or nonphagocytic cells (HeLa). This result suggests the inactivation of this gene may also have little effect on the survival in the host animal. Such a property is a prerequisite for the development of a modified live brucellosis vaccine with a distinctive immunological signature. Experiments in a mouse model and in cattle are now in progress to further explore the usefulness of these deletion mutants.

## ACKNOWLEDGMENTS

We thank Robert Angus and Billy Deyoe for providing *B. abortus* strains, Allen Jensen for help in screening *B. abortus* mutant strains, and Aileen Duit for cell culture work.

This work was supported in part by the competitive Research Grants Office, U.S. Department of Agriculture, under agreement no. 85-CRCR-1-1842.

## REFERENCES

1. **Alton, G. G., L. M. Jones, and D. E. Pietz.** 1975. Laboratory techniques in brucellosis, 2nd ed. Monograph Series no. 55. World Health Organization, Geneva.
2. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
3. **Bricker, B. J., L. B. Tabatabai, B. L. Deyoe, and J. E. Mayfield.** 1988. Conservation of antigenicity in a 31-kDa *Brucella* protein. Vet. Microbiol. **18**:313–325.
4. **Bricker, B. J., L. B. Tabatabai, B. A. Judge, B. L. Deyoe, and J. E. Mayfield.** 1990. Cloning, expression, and occurrence of the *Brucella* Cu-Zn superoxide dismutase. Infect. Immun. **58**:2935–2939.
5. **Chomczynski, P., and P. K. Qasba.** 1984. Alkaline transfer of DNA to plastic membrane. Biochem. Biophys. Res. Commun. **122**:340–344.
6. **Detilleux, P. G., B. L. Deyoe, and N. F. Cheville.** 1990. Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. Infect. Immun. **58**:2320–2328.
7. **Ficht, T. A., S. W. Bearden, B. A. Sowa, and L. G. Adams.** 1988. A 36-kilodalton *Brucella abortus* cell envelope protein is encoded by repeated sequences closely linked in the genomic DNA. Infect. Immun. **56**:2036–2046.
8. **Fiedler, S., and R. Wirth.** 1988. Transformation of bacteria with plasmid DNA by electroporation. Anal. Biochem. **170**:38–44.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
10. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291**:238–239.
11. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Marchioli, C. C., R. J. Yancey, Jr., R. C. Wardley, D. R. Thomsen, and L. E. Post.** 1987. A vaccine strain of pseudorabies virus with deletions in the thymidine kinase and glycoprotein X genes. Am. J. Vet. Res. **48**:1577–1583.
14. **Mayfield, J. E., B. J. Bricker, H. Godfrey, R. M. Crosby, D. J. Knight, S. M. Halling, D. Balinsky, and L. B. Tabatabai.** 1988. The cloning, expression, and nucleotide sequence of a gene coding for an immunogenic *Brucella abortus* protein. Gene **63**:1–9.
15. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
16. **Pugh, G. W., Jr., L. B. Tabatabai, B. J. Bricker, J. E. Mayfield, M. Phillips, T. J. McDonald, and E. S. Zehr.** 1989. Identification of a virulence factor for *Brucella abortus* infection in BALB/c mice. Am. J. Vet. Res. **50**:323–328.
17. **Pugh, G. W., Jr., L. B. Tabatabai, B. J. Bricker, J. E. Mayfield, M. Phillips, E. S. Zehr, and C. A. Belzer.** 1990. Immunogenicity of *Brucella*-extracted and recombinant protein vaccines in CD-1 and BALB/c mice. Am. J. Vet. Res. **51**:1413–1420.
18. **Smith, L. D., and F. Heffron.** 1987. Transposon Tn*5* mutagenesis of *Brucella abortus*. Infect. Immun. **55**:2774–2776.
19. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
20. **Tabatabai, L. B., and B. L. Deyoe.** 1984. Specific enzyme-linked immunosorbent assay for detection of bovine antibody to *Brucella abortus*. J. Clin. Microbiol. **20**:209–213.
21. **Taketo, A.** 1988. DNA transfection of *Escherichia coli* by electroporation. Biochim. Biophys. Acta **949**:318–324.
22. **Xie, W.** 1988. Cellular location and mode of export of three *Brucella abortus* proteins expressed in *Escherichia coli*. M. S. thesis. Iowa State University, Ames.
23. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.