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A 36-Kilodalton *Brucella abortus* Cell Envelope Protein Is Encoded by Repeated Sequences Closely Linked in the Genomic DNA

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Recombinant bacteriophage expressing *Brucella abortus* antigens have been isolated from a λgt11 expression library by using antibody raised against a sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified cell envelope protein of 36 kilodaltons. Fusion products expressed by these recombinants vary in apparent molecular mass by sodium dodecyl sulfate-polyacrylamide gel electrophoresis but only slightly exceed the size of β-galactosidase. Western blot (immunoblot) analysis of crude lysates derived from λgt11 lysogens indicates that the fusion products react specifically with the original antisera used for recombinant selection and selectively bind antibody directed against the 36-kilodalton cell envelope protein. Analysis of the DNA inserts from 11 independently selected recombinants reveals similar-size *Eco*RI fragments which range in size from 150 to 300 base pairs (bp), all of which cross-hybridize via Southern blot analysis. Three independently selected *Eco*RI inserts ranging in size from 200 to 270 bp have been subcloned into M13mp18 and sequenced; all three contain a common region of about 200 bp. Southern blot analysis of *B. abortus* genomic DNAs digested with *Eco*RI, *Pst*I, or *Dde*I indicates the presence of two fragments which hybridize to these DNA probes while single *Bam*HI and *Hin*dIII fragments hybridize. The absence of these sites from the internal DNA sequence of the cloned probes suggests the presence of more than one copy of these sequences within the *B. abortus* genome. The same DNA probes have been used to select genomic clones of approximately 20 kbp from a λ2001 library. The λ2001 recombinants contain single *Bam*HI fragments and two *Pst*I fragments which hybridize to these probes, suggesting that the repeated sequences are restricted to a limited region of the *Brucella* genome. An oligonucleotide probe constructed on the basis of the amino-terminal sequence of the mature gene product hybridizes to the same *Bam*HI and *Pst*I fragments as the λgt11-derived DNA probe. Although the relative positions of the oligonucleotide sequences and the λgt11 insert within the genes is not known, the two sequences flank a region which corresponds to at least 40% of the size of the predicted gene. Additional experimentation must be performed to determine whether these sequences represent either two complete structural genes encoding major cell envelope proteins or repetitive sequences within a single structural gene.

The gram-negative, facultative intracellular bacterium *Brucella abortus* is currently the second leading cause of livestock loss of cattle in the continental United States (14). Infection with this organism frequently results in abortion or the birth of severely debilitated calves (2, 44). In addition, *B. abortus* can cause human disease, with infections typically referred to as undulant fever (repeated attacks of fever), although a broad range of symptoms has been observed (29, 48, 56, 66). Infection by *B. abortus*, either in man or in cattle, results from the ingestion of contaminated bovine products (44, 48, 66). *B. abortus* is typically isolated from the tissues of the lymphatic system or the mammary gland of the infected host (2, 23). Intracellular uptake and survival within the cells of the reticuloendothelial system inhibit the curative effects of antibiotic therapy (29, 48, 66). The currently approved vaccine strain, S19, has several disadvantages, including human infectivity and virulence, and a low level of persistently infected, vaccinated cattle (10, 15, 45). The search for improved vaccines or diagnostic reagents capable of distinguishing field strain infections from S19-vaccinated or persistently infected animals comprises most current *Brucella* research (9, 12, 28, 46, 50, 51, 64, 65).

The development of an effective nonviable subcellular vaccine presumably requires both humoral and cell-mediated immune responses (4, 5, 12, 24–26, 29, 30, 38, 39, 57, 58). Humoral and cellular immunity elicited by outer membrane proteins (OMPs) has been implicated in naturally occurring and experimental infections, including brucellosis (4, 50, 51), and purified OMPs have been used to stimulate protective immunity against various bacterial infections (17, 32). The major OMPs of *B. abortus* represent potential candidates as broad-range immunogens because of their conservation between strains (13, 49, 61, 62) and their capacity to elicit both humoral and cell-mediated immune responses during natural infection (4, 50, 51; L. G. Adams, unpublished results). However, examination of the protective immunity induced by *B. abortus* cell envelope (outer and cytoplasmic membranes) proteins is complicated by the presence of large amounts of immunogenic lipopolysaccharide (LPS) which contaminates these cell fractions (49, 54, 61, 62). Recent vaccine trials have indicated that a substantial level of protective immunity against abortion and infection is stimulated by rough-cell envelopes lacking extensive LPS (Adams, unpublished). The cloning and expression of the *B. abortus* genes encoding the cell envelope proteins in *Escherichia coli* offer the advantage of obtaining individual antigens or epitopes free of contaminating LPS and in sufficient quantities to permit testing for the cellular or humoral immune response each elicits in cattle (9).

Characterization of the OMPs of *B. abortus* by Verstreate and co-workers (61, 62) identified three major classes as defined by their apparent molecular masses on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Group 1 ranged from 88 to 94 kilodaltons (kDa), group 2 ranged from 35 to 40

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kDa, and group 3 ranged from 25 to 30 kDa. Douglas and co-workers (13) have characterized the group 2 OMPs of *B. abortus* as porins on the basis of their in vitro activity. The active component of this group was not identified, and several bands were shown to share structural homology via partial V8 protease digestion. It was hypothesized that the group 2 proteins varied in apparent molecular mass because of secondary modification. Santos and co-workers (49, 62) examined a number of rough *Brucella* strains lacking extensive LPS and demonstrated, on the basis of their apparent molecular masses and antigenic relatedness, that the group 2 and 3 OMPs are conserved within a species and, to some extent, between species. However, variation in the molecular masses of several OMPs, including the group 2 proteins, was observed when these strains were grown in vitro under different growth conditions. Correlation of this altered mobility with any specific physiological or metabolic requirement such as those which cause the shift in expression of the *ompF* and *ompC* genes in *E. coli* has not been established (34). We report here the cloning of a *B. abortus* gene encoding a major cell envelope protein by using the λgt11 system. By using this system, expression of *B. abortus* antigens is controlled by *lac*-PO (promoter-operator). This procedure assures the expression and detection of all *Brucella* gene products, including those which may not be expressed from the *Brucella* promoters in *E. coli*. We also present evidence that this gene, or portions thereof, is repeated within a short stretch of genomic DNA. The implications of either arrangement with regard to the nature and expression of gene products are discussed.

MATERIALS AND METHODS

Bacterial strains and cultivation. *B. abortus* smooth strains 19 and 2308 and rough strains R2308, RB51, and 45/20 were obtained from Billy Deyoe at the National Animal Disease Center in Ames, Iowa. *E. coli* BNN 97 [=BNN 93(λgt11)] {*hsd R hsdM*$^+$ *supE thr leu thi lacY1 tonA21* [λgt11 = *lac5* nin5 cI857 Sam100]}, Y1088 [*supE supF metB trpR hsdR hsdM*$^+$ *tonA21 strA lacU169 proC*::Tn*5* (pMC9)], and Y1090 [Δ*lacU169 proA* Δ*lon araD139 strA supF* [*trpC22*::Tn*10*] (pMC9)] were obtained from the American Type Culture Collection in Atlanta, Ga., and MC4100 [F$^-$ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] was obtained from Ry Young at Texas A&M University. *E. coli* LE392 [F$^-$ *supF supE hsdR galK trpR metB lacY tonA*] and P2 392 [LE392(P2)] were obtained from Stratagene, Inc. *E. coli* HB101 [*hsdS20* (r_B^- m_B^-) *redA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44*] and JM107 [(r_K^- m_K^+) *endA1 gyrA96 thi hsdR17 supE44 relA1 traD36* Δ(*lac proAB*)/F′ *proAB lacI*^{q} ZΔM15] were obtained from Bethesda Research Laboratories, Inc. *Brucella* species designations for all strains were confirmed by standard biotyping procedures (2). Plasmids pMY222 (*ompF*) and pMY150 (*ompC*) were the kind gift of Masayori Inouye at the State University of New York at Stony Brook.

B. abortus cultivated on potato infusion agar (Difco Laboratories) was used in most experiments. Broth cultures of strains 19 and 2308 were grown by Billy Deyoe and used while viable for the isolation of DNA for construction of genomic libraries. All harvests were tested for purity by colony morphology, Gram stain, and biovar analysis (2). Unless stated otherwise, *B. abortus* was routinely killed in 0.85% (wt/vol) saline containing 0.1% (wt/vol) phenol with incubation at 60°C for 1 to 16 h prior to manipulation. *E. coli* was grown either in liquid media or solid culture by using NZCYM (36).

Extraction of *B. abortus* genomic DNA. Packed cells (5 ml) grown in liquid broth were suspended in 10 ml of buffer (50 mM Tris hydrochloride [pH 8.0], 50 mM EDTA, 0.1 M NaCl). Lysozyme was added to a concentration of 100 μg/ml, and the mixture was incubated for 1 h at 37°C. The cells were lysed by the addition of an equal volume of 8 M guanidine hydrochloride (Sigma Chemical Co.) in TE buffer (10 mM Tris hydrochloride [pH 7.5], 1.0 mM EDTA) and homogenized with 10 strokes of a Dounce homogenizer with a type A pestle. The DNA was overlaid onto a step gradient composed of 2 ml each of 4.4 M and 5.7 M CsCl and banded by centrifugation in a Beckman SW41 rotor at 100,000 × *g* for 22 h. The DNA band was drawn off by side puncture of the tube with an 18-gauge needle, diluted fivefold with sterile distilled water, incubated at 37°C with RNase A (10 μg/ml), and extracted once with an equal volume of water-saturated phenol and then three times with an equal volume of ether. The DNA was then precipitated by the addition of 2 volumes of 95% (vol/vol) ethanol. The purified DNA was pelleted by centrifugation at 12,000 × *g*, dried in vacuo, and dissolved in TE buffer at a final concentration of 1.0 mg/ml as determined by optical density readings at 260 nm.

Growth of bacteriophage λgt11 and isolation of DNA. *E. coli* BNN 97 (λgt11 lysogen) was grown and bacteriophage were purified as described elsewhere (36, 37, 68). λgt11 DNA was extracted from the purified phage as described by Maniatis (36, 37). The concentration of the DNA was determined by optical density at 260 nm, and the DNA was diluted to a final concentration of 1.0 mg/ml for storage at 4°C.

Construction of λgt11 and λ2001 libraries containing *B. abortus* genomic DNA. λgt11 library construction was performed as described by Young and Davis (67, 68). *B. abortus* genomic DNA, isolated as described above, was digested with DNase I at 15°C in the presence of 1.0 mM $MnCl_2$ to an average size of 500 to 3,000 base pairs (bp) as determined by electrophoresis on a 0.8% (wt/vol) agarose gel in Tris borate buffer (TBE) containing 0.89 M Tris borate, 0.089 M boric acid, and 0.002 M EDTA (3, 36). The reactions were stopped by the addition of EDTA to a final concentration of 10 mM and extracted successively with phenol, phenol-chloroform (1:1), and chloroform. The DNA was ethanol precipitated and pelleted by centrifugation at 12,000 × *g*. The DNA was size separated by electrophoresis on a 0.8% (wt/vol) agarose gel; the region of the gel corresponding to fragments between 500 and 3,000 bp was excised, and the DNA was electroeluted (52). Electroeluted DNA was purified over Elutip-d columns (Schleicher & Schuell, Inc.) and precipitated with ethanol. Over 500,000 recombinant bacteriophage were generated as judged by their inability to produce blue plaques in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and had insert sizes ranging from 500 to 3,000 bp as determined by random selection of recombinant bacteriophage (data not shown). *B. abortus* S19 and S2308 libraries were amplified by plating on a lawn of *E. coli* Y1088 r^-m^+ (67, 68). In the construction of the genomic libraries of λ2001, partial digestion of genomic DNAs with *Sau*3AI was performed as described elsewhere (27, 36, 37) and the gel-purified fragments were cloned into λ2001 arms digested with *Bam*HI by the manufacturer (Stratagene, Inc.). In vitro packaging and library amplification were performed as described elsewhere (18, 19, 37).

Immunological screening of recombinant libraries. Screening was performed essentially as described by Young and Davis (67, 68) with the following modifications. The rabbit antisera was diluted 1:100 with antibody buffer (TBST [10

mM Tris hydrochloride {pH 8.0}, 150 mM NaCl, 0.05% {vol/vol} Tween 20] plus 1% [wt/vol] gelatin plus 0.01% [wt/vol] thimerosal) and preadsorbed by overnight incubation with nitrocellulose circles (15 cm) dipped in sonicated cell extract from λgt11-infected *E. coli* Y1089 (68). The preadsorbed antibody was diluted fivefold with antibody buffer to a working concentration of 1:500. Goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories) was used as a secondary antibody, and color development was tested as described by Blake et al. (8). Gelatin (Bio-Rad Laboratories) was used exclusively as the blocking agent.

Selection of recombinant bacteriophage by hybridization. The genomic libraries constructed in λ2001 were screened by using hybridization probes specific for *B. abortus* DNA and labeled as described below. Bacteriophage were plated onto a lawn of *E. coli* P2 392, and recombinant selection via plaque hybridization was performed as described elsewhere (36, 37). Recombinant phage were considered pure when 100% of the plaques reacted positively with the hybridization probe.

Preparation of *B. abortus* cell envelopes. *B. abortus* were grown as described above and suspended at a concentration of 10^9 to 10^{10} cells per ml in Tris-buffered saline (TBS). Following irradiation with ^{60}Co, cell envelopes were prepared essentially as described by Sowa et al. (54, 55) with modifications described elsewhere (B. A. Sowa, unpublished results). A rough mutant lacking extensive LPS was used to avoid contamination of protein samples (49, 61).

Preparation of rabbit antisera directed against *B. abortus* antigens. *B. abortus* cell envelopes were resuspended in Laemmli sample buffer and boiled prior to electrophoresis on a 6 to 12% polyacrylamide gradient gel (33, 55). The bands were visualized by staining with Coomassie brilliant blue and excised from the gel with a scalpel. The protein was electroeluted by using an Isco electroelution apparatus (21). Antisera used to screen the expression libraries were obtained by subcutaneous inoculation of rabbits as described by Vaitukaitis (60) with the following modifications. The initial inoculation was 5 μg of SDS-polyacrylamide gel electrophoresis (PAGE)-purified envelope protein in Freund complete adjuvant, boosted 4 weeks later with 5 μg in Freund incomplete adjuvant; the rabbits were bled 12 weeks later.

Fusion product synthesis and identification by Western blot (immunoblot) analysis. *E. coli* MC4100 was lysogenized with the recombinant bacteriophage, and synthesis of *lacZ* fusion products was thermally induced in broth cultures (68). Lysogens of the recombinant bacteriophage were produced by using *E. coli* MC4100 rather than *E. coli* Y1089 because of improved yields of fusion product. Two hours after induction, the cells were pelleted and lysed by freeze-thawing three times in dry ice-ethanol. The viscosity was reduced by brief sonication, and the lysate was clarified by centrifugation at 12,000 × *g* for 20 min. The proteins were precipitated by the addition of 2 volumes of a saturated solution of ammonium sulfate, and the samples were stored as a slurry at 4°C. Samples containing 10 to 25 μg of protein were centrifuged, and the pellets were suspended in Laemmli sample buffer and boiled for 5 min just prior to electrophoresis. Electrophoresis was performed on an 8 to 14% (wt/vol) polyacrylamide gradient gel essentially as described by Laemmli, and the gel was either stained with Coomassie brilliant blue (33) or the proteins were transferred to nitrocellulose for Western blot analysis as described by Towbin et al. (59). Detection of fusion products on Western blots was performed by using primary rabbit antisera and alkaline phosphatase conjugated goat anti-rabbit IgG second antibody as described above (8).

Antibody selection by recombinant fusion products. Antisera (1:100 dilution) were preadsorbed as described above, as well as by incubation for 16 to 24 h at room temperature with nitrocellulose (Schleicher & Schuell, BA 85) circles previously overlaid on NZCYM plates containing 50,000 to 100,000 nonrecombinant λgt11. The nitrocellulose circles were overlaid following a 3- to 4-h growth period at 42°C, at which time plaque development was just visible on a lawn of *E. coli* Y1090, and incubated an additional 16 h at 38°C. The preadsorbed antisera were then incubated with similarly prepared nitrocellulose circles which had been overlaid on *E. coli* Y1090 lawns infected with recombinant λgt11 clones. After a 16- to 24-h incubation, the filters were extensively washed with TBST followed by several washes of TBS to remove the detergent. The specifically bound antibody was eluted by incubation in buffer containing 0.1 M glycine (pH 2.6) and 0.15 M NaCl for 15 min at room temperature. The eluted antibody solution was neutralized by the dropwise addition of 1.0 M Tris hydrochloride (pH 8.0) and diluted with 2 volumes of antibody buffer. Development of Western blot strips of *B. abortus* cell envelope proteins was performed with a 1:10 dilution of the selected antibodies, and the strips were developed with alkaline phosphatase-conjugated goat anti-rabbit IgG.

Miniprep isolation of λgt11 recombinant DNA. Bacteriophage isolation, DNA extraction, and restriction enzyme analysis of recombinant DNA were performed as described elsewhere (36). End labeling of restriction fragments was performed at 37°C for 20 min by using DNA polymerase I (Klenow fragment) (Boehringer Mannheim Biochemicals) and [α-^{32}P]dATP (3,000 Ci/mmol, 10 μCi/μl; New England Nuclear Corp.).

Subcloning of *Eco*RI fragments into M13mp18. DNA isolated from recombinant λgt11 clones containing *B. abortus* DNA inserts was digested with *Eco*RI endonuclease, and the enzyme was heat inactivated at 70°C for 10 min. Samples of these digests were mixed with *Eco*RI-digested replicative form of M13mp18 (Bethesda Research Laboratories) at a molar ratio of insert to M13 of up to 4:1 and ligated as described above. Ligation mixes were transformed into competent *E. coli* HB101, mixed with *E. coli* JM107 in soft agar with X-Gal (50 μg/ml), and spread on YT agar plates (36, 68). Colorless plaques containing inserts were selected, and single-stranded and double-stranded DNAs were isolated, as described elsewhere, to determine the size and orientation of the DNA insert (20, 41). DNA sequences were determined for both strands by the dideoxy method with 7-deazaGTP (Boehringer Mannheim) in place of dGTP to eliminate compression problems due to G+C-rich regions. Hybridization probes specific for the *B. abortus* inserts were prepared by using the single-stranded DNAs as templates and were labeled as described by Hu et al. (20). This procedure typically yielded probes with specific activities ranging from 0.5×10^9 to 1.0×10^9 cpm/μg of added DNA. These probes were used for selection of genomic clones from the λ2001 libraries and for Southern blot analysis of restriction digests of genomic clones and genomic DNAs.

Preparation of *B. abortus* genomic DNAs for Southern blot analysis. Genomic DNAs were extracted as described previously and digested with restriction enzymes (5 to 10 U/μg of DNA) in 100-μl reaction volumes overnight at the appropriate temperature in buffer supplied by the manufacturer. Transfer of DNA fragments to nitrocellulose, hybridization,

and washing were performed under high-stringency conditions as described by Southern (53). Alternatively, transfer to nylon membranes was performed in 0.4 M NaOH for 4 to 16 h following brief treatment of the gel with 0.25 N HCl. The membrane was then rinsed once in 2× SSPE (2× SSPE is 0.3 M NaCl, 20 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA with the pH adjusted to 7.4 with NaOH) and prehybridized in buffer containing 1.5× SSPE, 0.1% (wt/vol) SDS, 0.5% (wt/vol) nonfat dry milk, and 0.1 mg of single-stranded herring sperm DNA per ml for 4 to 16 h at 68°C (40). Hybridization was performed with labeled probes prepared as described previously for 16 h with 50,000 to 100,000 cpm/cm^2 of membrane. The membranes were washed in two changes of 2× SSPE–0.1% SDS at room temperature for 15 min each, followed by washes in 1× SSPE–0.1% SDS and 0.1× SSPE–0.1% SDS under the same conditions. The final wash was performed twice in 0.1× SSPE–0.1% SDS for 60 min at 50°C. Higher-temperature washes did not increase the specificity of hybridization but did significantly reduce the strength of the signal. Hybridization with oligonucleotide probes used UV irradiation to nick the DNA fragments prior to transfer, which was determined empirically. These filters were prehybridized as described above in 6× SSPE–0.1% SDS–0.1% sodium pyrophosphate–0.1 mg of single-stranded herring sperm DNA per ml–0.1 mg of *E. coli* tRNA per ml. Hybridization was performed for 2 to 4 h at 40°C in 6× SSPE–0.1% SDS with 20×10^6 cpm of labeled oligonucleotide at a specific activity of 5×10^8 cpm/μg prepared by using T4 polynucleotide kinase as described by Meinkoth et al. (40). The filter was subsequently washed twice in the same buffer at room temperature followed by successive washes at increasing temperatures up to 45°C. Washes above 45°C significantly reduced the strength of the signal without altering the specificity of hybridization. Hybridization and washing conditions for oligonucleotide probes were determined as described by Meinkoth et al. (40).

RESULTS

Selection of recombinants from an expression vector library constructed from *B. abortus* genomic DNA. Expression vector libraries were constructed in λgt11 by using random fragments of *B. abortus* DNA generated by partial DNase I digestion of S19 (vaccine strain) and S2308 (virulent strain) genomes (Materials and Methods). Using λgt11 as a cloning vehicle, gene fusions between *B. abortus* genomic DNA fragments and the *E. coli lacZ* gene were generated and expression of the fused gene products was placed under *lac*-PO control. The libraries generated were screened with polyclonal antibodies raised against SDS-PAGE-purified cell envelope proteins, including a 36-kDa protein which is a major component of these fractions (13, 49, 61, 62; Sowa, unpublished; see Fig. 3). Separation of the cell envelope fraction into outer and cytoplasmic membranes was performed by detergent stripping of cell envelopes prepared as described in Materials and Methods (61). The 36-kDa protein was not solubilized by deoxycholate but was solubilized by Triton X-100 and SDS. This is consistent with an outer membrane location for the 36-kDa protein (61; Sowa, unpublished). Eleven recombinant bacteriophage expressing *lacZ-B. abortus* gene fusions were independently selected by using the polyclonal anti-36-kDa-protein antisera. *E. coli* MC 4100 recombinant lysogens were grown, crude cell lysates were prepared, and samples were electrophoresed as described in Materials and Methods. The recombinant phage produced fusion products with decreased mobility relative to wild-type β-galactosidase (120 to 130 kDa versus 116 kDa) (Fig. 1; recombinants: lane 1, 2.11; lane 2, 2.12; lane 3, 2.33; lane 5, 2.63; and lane 6, 2.64; λgt11, lane 7). Lane 4 contains molecular weight markers described in the legend to Fig. 1. In the absence of IPTG (isopropyl-β-D-thiogalactopyranoside) induction, fusion product and β-galactosidase synthesis was not observed (data not shown).

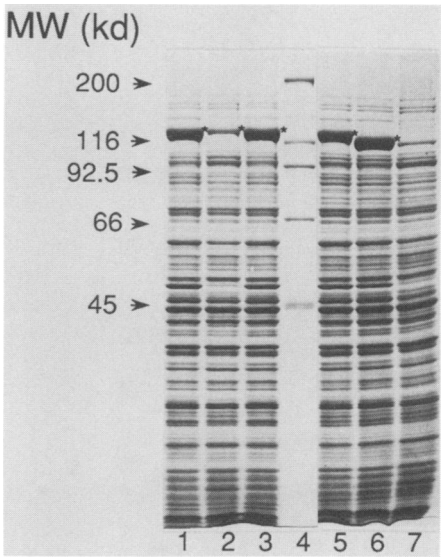

FIG. 1. SDS-PAGE of λgt11 recombinant fusion products. Recombinant lysogens were grown and protein samples were prepared for SDS-PAGE as described in the text. Lanes: 1 to 3 and 5 to 6, λgt11 recombinant-infected *E. coli* MC4100 expressing *B. abortus* 36-kDa-protein antigens; 7, λgt11-infected *E. coli* MC4100; 4, molecular weight markers myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), and ovalbumin (45,000). The relative positions of the fusion products which migrate more slowly than wild-type β-galactosidase are indicated by asterisks. MW, Molecular mass in kilodaltons (kd).

Analysis of recombinant fusion products via Western blotting and antibody selection. Lysates from recombinant lysogens which produced high levels of fusion product (Fig. 1) were examined by Western blot analysis to demonstrate the expression of the 36-kDa antigens fused to β-galactosidase. Crude cell extracts were prepared from recombinant lysogens 2.11, 2.12, 2.33, and 2.63 (lanes 1, 2, 3, and 5 in Fig. 1) and electrophoresed as described in the previous section. Following transfer, the Western blot was incubated with the original anti-36-kDa-protein antisera used to select the recombinants. The results shown in Fig. 2 (lane 1) illustrate the absence of antibody activity directed against β-galactosidase in anti-36-kDa-protein antisera following preadsorption with extracts from λgt11-infected cells as described in Materials and Methods. In contrast, the recombinant fusion products (lanes 2 to 5) reacted with the antisera, which indicated that the 36-kDa antigens were expressed under the control of *lac*-PO. Additional bands present in lane 4 were presumably caused by proteolysis of the fusion product and were visible in this lane because of sample overloading. To provide further evidence that a portion of *B. abortus* gene encoding a 36-kDa major cell envelope protein had been cloned, fusion products expressed by recombinant phage were used to select antibody from the original rabbit antisera raised against the 36-kDa protein. If the recombinants were selected because of minor contaminating antibody activities in the original sera, then the selected antibody should react

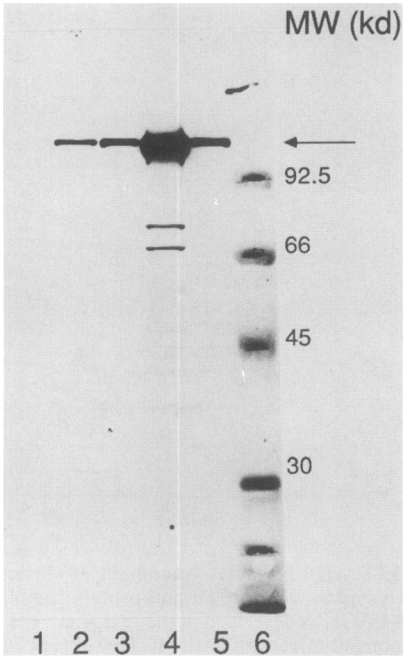

FIG. 2. Western blot analysis of *lacZ* fusion products by using anti-36-kDa-protein antisera. Selected recombinants shown in Fig. 1 (lanes 1 to 3 and 5) were prepared and electrophoresed as described in the text. The proteins were electrophoretically transferred to nitrocellulose, and fusion products were visualized by Western blotting as described in Materials and Methods. Lanes: 1, λgt11-infected *E. coli* MC4100; 2 to 5, recombinants 2.11, 2.12, 2.33, and 2.63, respectively; 6, molecular weight markers as shown in Fig. 1. MW, Molecular mass in kilodaltons (kd).

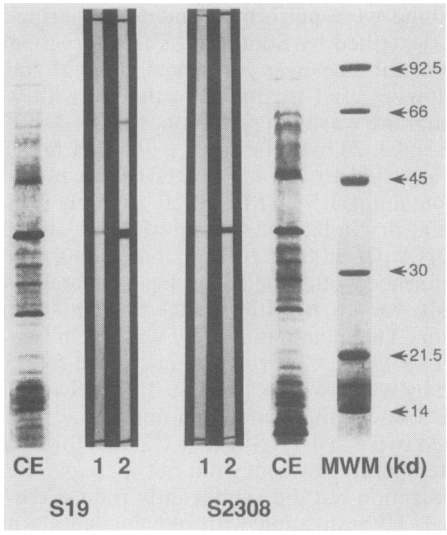

FIG. 3. Specificity of antibody selectively adsorbed by recombinant fusion products. *B. abortus* cell envelopes were prepared and SDS-PAGE and Western blotting were performed as described in Materials and Methods. Lanes CE, Coomassie-stained gels of SDS-PAGE-separated cell envelope proteins prepared from S19 and S2308. Western blot strips prepared from cell envelopes and reacted with antibody selected as described in the text are shown: lanes 1, λgt11-selected antibody; lanes 2, λgt11-recombinant (2.33 and 2.63)-selected antibody. Top and bottom of the blotted gel are indicated by the black lines present at the origin and end of strips (lanes 1 and 2). MWM, Molecular mass markers in kilodaltons (kd).

with a band other than the 36-kDa protein. Antibody selection was performed as described in Materials and Methods by using either λgt11 nonrecombinant phage expressing only β-galactosidase or recombinant phage expressing fusion products 2.33 and 2.63. Nitrocellulose strips excised from a Western blot of cell envelopes derived from *B. abortus* 19 and 2308 and reacted with selected antibody are shown in Fig. 3 (lanes 1 and 2). Coomassie-stained gels of cell envelope proteins (lanes CE) are shown for comparison. The results demonstrate the selection of antibodies by recombinant phage-encoded protein which reacted predominantly with a 36-kDa protein and to a lesser extent with a 66-kDa protein present in the cell envelopes of both strains 19 and 2308 of *B. abortus* (Fig. 3, lanes 2). In contrast, antibody selected by the nonrecombinant phage-encoded protein produced a low-level reaction with the 36-kDa protein (Fig. 3, lanes 1). Extensive preadsorption of cross-reacting antibodies is achieved by preincubation of antisera with nonrecombinant phage lysates (Materials and Methods); the low-level activity observed in Fig. 3, lanes 1 indicates a failure to completely preadsorb cross-reacting antibodies which are present in rabbit sera. The identity of the 66-kDa protein (lanes 2) has not been determined, and the basis for the recombinant-selected antibody activity with this protein is not known. Antigenic cross-reactivity has been observed to occur between *B. abortus* membrane proteins, most notably the group 2 and group 3 proteins (49, 61, 62). However, this has been interpreted to represent a common mucopolysaccharide determinant, which should not be present in fusion products produced in *E. coli*. The data presented here suggest that cross-reacting determinants between *B. abortus* cell envelope proteins may, in fact, be peptides. The 66-kDa protein does not correspond to either group 2 or group 3 proteins; however, it may represent another antigenically related species or an incompletely denatured form of the group 3 protein (49), although no reactivity was observed at a position corresponding to the completely denatured form of group 3 at 25 to 30 kDa. It might be argued that the recombinants are, in fact, expressing 66-kDa antigens, and antibodies selected by these fusion products cross-react with the 36-kDa protein. This is less likely since (i) less activity is observed with the S2308 strip in the 66-kDa region despite the increased amount of protein present which should preferentially bind the selected antibody and (ii) the original antisera were raised against a 36-kDa protein band excised from SDS-PAGE. Finally, it must be pointed out that the selected antibody does not cross-react with LPS present on the Western blot strips, prepared from smooth strains 19 and 2308, shown in Fig. 3. This activity would appear as a heterogeneous mixture of bands of various molecular weights distributed along the entire length of the gel (54). These results are consistent with the isolation of the gene encoding a 36-kDa protein which is a major component of the cell envelope of *B. abortus*.

Analysis of the *B. abortus* DNA inserts cloned into λgt11. Positive clones selected for the expression of *lacZ* fusion products were grown to confluent lysis on solid media at 42°C. The bacteriophage were harvested, and the DNA was extracted and analyzed by restriction enzyme analysis as described in Materials and Methods. Purified λgt11 DNA was digested with *Eco*RI, end labeled with [α-^{32}P]dATP by using DNA polymerase I (Klenow fragment), and electrophoresed on a 1.5% (wt/vol) agarose gel (Fig. 4). Labeled wild-type λgt11 DNA which lacks an insert at the *Eco*RI site is shown in lane 2. Lanes 3 to 12 contain DNAs extracted

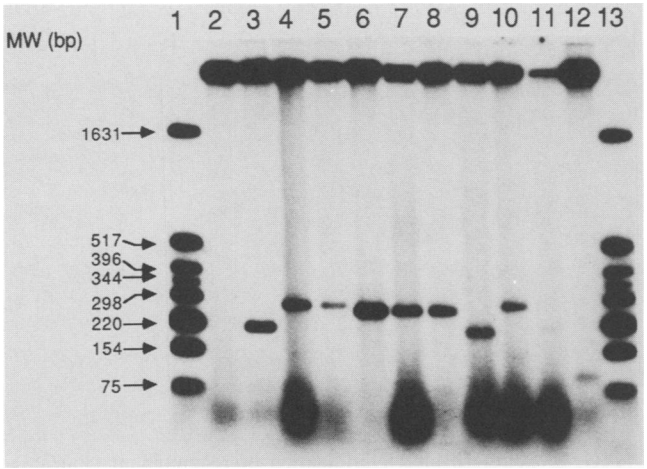

FIG. 4. Agarose gel electrophoresis of *Eco*RI-digested λgt11 recombinants. Recombinant DNAs were extracted from bacteriophage and digested with *Eco*RI as described in Materials and Methods. The fragments were end labeled, electrophoresed, and exposed to X-ray film (Kodak XAR-5) as described in the text. Lanes: 3 to 12, *B. abortus* λgt11 recombinants (2.11, 2.12, 2.21, 2.22, 2.31, 2.33, 2.43, 2.51, 2.63, 2.64, respectively); 2, λgt11; 1 and 13, *Hin*fI-digested pBR322. MW, Molecular size.

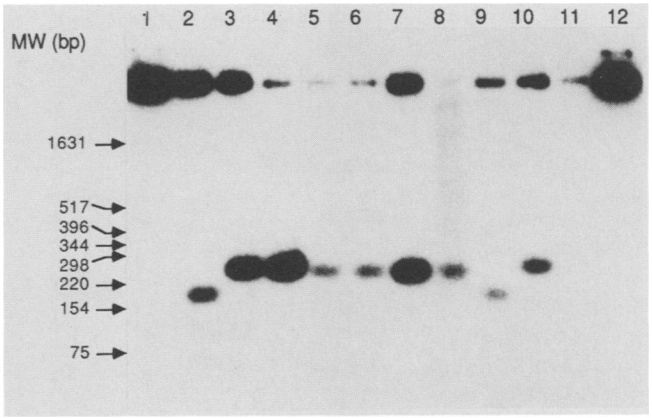

FIG. 5. Sequence homology between recombinants as observed by cross-hybridization on Southern blots. Recombinant λgt11 DNA minipreps were digested with *Eco*RI, electrophoresed, and transferred to nylon membrane as described in Materials and Methods. The insert from recombinant 2.51 (Fig. 4, lane 10) was subcloned into M13mp18 and labeled by primer extension as described in the text. Hybridization was performed under high-stringency conditions as described in Materials and Methods. Lanes: 1, λgt11; 2 to 12, recombinants 2.11, 2.12, 2.21, 2.22, 2.31, 2.33, 2.42, 2.43, 2.51, 2.63, and 2.64, respectively. MW, Molecular size.

from recombinant bacteriophage (2.11, 2.12, 2.21, 2.22, 2.31, 2.33, 2.43, 2.51, 2.63, and 2.64, respectively) selected for expression of 36-kDa antigens. The size of the inserts varies over a restricted range of between 100 and 300 bp and is consistent with the small size of the fusion products previously observed (Fig. 1 and 2). The sizes of the inserts contrast with the sizes of inserts selected at random, which range from 500 to 3,000 bp (data not shown). The exact cause of this discrepancy is not known but may reflect either an instability or the lethal nature of fusion products of larger size (6, 7, 22; Fig. 2). Polyacrylamide gel electrophoresis did not reveal the presence of additional *Eco*RI fragments (data not shown). Bands present at the bottoms of the lanes migrating faster than the 75-bp marker are presumably degraded DNAs or unincorporated ^{32}P-labeled nucleotides. Further characterization of these DNA inserts was performed by Southern blot analysis. The largest *Eco*RI insert contained in recombinant 2.51 shown in Fig. 4 (lane 10) was subcloned into the unique *Eco*RI site of M13mp18, labeled according to the method of Hu et al. (20), and used as a hybridization probe under conditions of high stringency as described in Materials and Methods. The results shown in Fig. 5 indicate that all of the recombinants selected cross-hybridize with the 2.51 probe under these conditions. Similar results were obtained by using recombinant inserts from 2.33 and 2.63 as hybridization probes (data not shown). Samples 2.63 and 2.64, visualized in lanes 11 and 12, did not digest well in these miniprep samples but were visible following a longer exposure of this blot and corresponded in size to the insert fragment sizes shown in Fig. 4, lanes 11 and 12. These results indicate a preferential selection of a restricted region of the gene encoding the 36-kDa cell envelope protein. The basis for this selection is not known, and speculation as to probable causes will be reserved for Discussion.

Sequence analysis of the cloned *B. abortus* DNA fragments. Of the original 11 recombinants, 3, designated 2.33, 2.51 and 2.63, have been subcloned into M13mp18 and sequenced (Fig. 6). These recombinants are homologous over 100% of their shared length and represent a nested set with one end in common (3′ end as shown) and the opposite end shortened by various lengths. It is not clear which end represents the 5′ or 3′ end of the insert in λgt11, as the original orientation was lost during subcloning into M13. All three insert fragments contain identical open reading frames in both orientations when fused to *lacZ* in λgt11. Sequence analysis of the termini suggests that the common end is followed by the sequence GGAATT and may represent an internal *Eco*RI site present in the gene since it does not have a sequence characteristic of the linker used during formation of the library (12-mer CCGGAATTCCGG). This result may be explained in either of three ways; an additional fragment representing the opposite end of the insert contains the

```
2.51   TATGACCGAGTCATAGGCAACAACACCAGCGATCGAACCCCAGCCGCCAG      50 bp
2.33   ---------------GGCAACAACACCAGCGATCGAACCCCAGCCGCCAG
2.63   ----------------------------------------------------

2.51   CATACTTCAGGCCGCCAACAACGTCAGGCATGTAGCCGTCGATGTGGTAG     100 bp
2.33   CATACTTCAGGCCGCCAACAACGTCAGGCATGTAGCCGTCGATGTGGTAG
2.63   ----------------AACAACGTCAGGCATGTAGCCGTCGATGTGGTAG

2.51   TTGGTCGTGCCAGTGTAACCACCGTCGTTGTCGCCACCCTGTTCGAGAGC     150 bp
2.33   TTGGTCGTGCCAGTGTAACCACCGTCGTTGTCGCCACCCTGTTCGAGAGC
2.63   TTGGTCGTGCCAGTGTAACCACCGTCGTTGTCGCCACCCTGTTCGAGAGC

2.51   GATCACAGCCGAGAAGCCGTTTCCGCCAGTGAAGGTGTACGAGATCTTGC     200 bp
2.33   GATCACAGCCGAGAAGCCGTTTCCGCCAGTGAAGGTGTACGAGATCTTGC
2.63   GATCACAGCCGAGAAGCCGTTTCCGCCAGTGAAGGTGTACGAGATCTTGC

2.51   CGGTGCGGTAGGAGCCAGCCGAGATCACGTCATCGTTGATGACATCGCCG     250 bp
2.33   CGGTGCGGTAGGAGCCAGCCGAGATCACGTCATCGTTGATGACATCGCCG
2.63   CGGTGCGGTAGGAGCCAGCCGAGATCACGTCATCGTTGATGACATCGCCG

2.51   AGGTAACCGGTGAAGGTAT                                    269 bp
2.33   AGGTAACCGGTGAAGGTAT
2.63   AGGTAACCGGTGAAGGTAT
```

FIG. 6. Partial DNA sequence of *B. abortus* inserts. Inserts from λgt11 recombinants 2.33, 2.51, and 2.63 (Fig. 4, lanes 8, 10, and 11, respectively) were subcloned into the *Eco*RI site of M13mp18 and sequenced by primer extension as described in Materials and Methods. The figure illustrates the alignment of common sequences contained in the cross-hybridizing fragments. *Eco*RI sites found at the ends of all fragments are not shown.

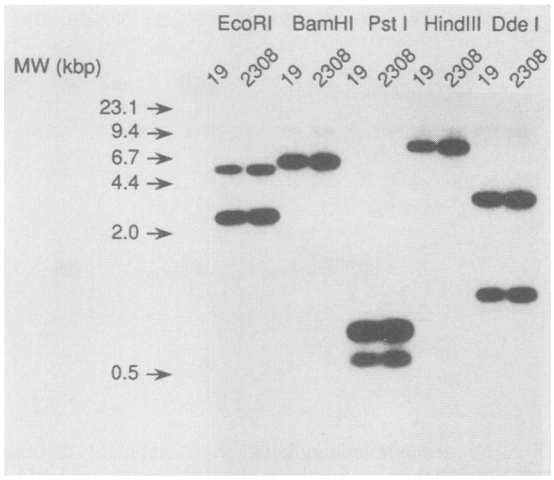

FIG. 7. Southern blot hybridization of genomic DNAs extracted from *B. abortus*. Genomic DNA was extracted from freshly grown *B. abortus* strains 19 (vaccine) and 2308 (virulent) as described in Materials and Methods. Restriction digests were performed as described in the text. The samples were electrophoresed on a 0.8% (wt/vol) agarose gel and transferred to nylon membrane as described in Materials and Methods. Hybridization was performed at high stringency by using recombinant insert 2.51 (Fig. 6) cloned into M13mp18 as a probe. MW, Molecular size.

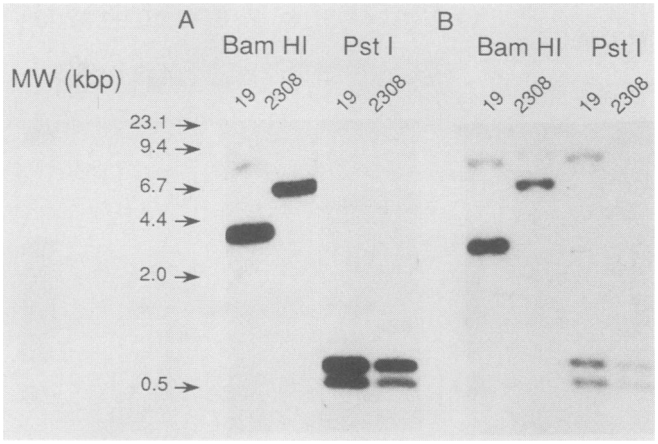

FIG. 8. Southern blot hybridization of recombinant clones selected from a λ2001 library. Recombinant clones were selected from genomic libraries constructed as described in Materials and Methods. Purified recombinant bacteriophage were amplified and the DNA was extracted as described in Materials and Methods. Digestion with restriction endonucleases, electrophoresis, and Southern blotting were performed as described in the legend to Fig. 7. Restriction digests were performed by using the enzymes indicated except that the S19 clones required the presence of *Xho*I in the *Bam*HI digests to remove the flanking λ2001 arm (26). Blots were probed at high stringency with the recombinant insert 2.51 cloned into M13mp18 (A) or a synthetic oligonucleotide, GAPuCCNGAPuGCNGT (B), prepared as described in the text. Hybridization of the oligonucleotide probe was performed as described in Materials and Methods. MW, Molecular size.

appropriate linker, the linkers used were not pure and contained some oligomers of shorter length, or methylation of *B. abortus* DNA was incomplete and digestion following linker ligation resulted in the restriction of *Eco*RI sites present in *B. abortus* DNA. PAGE of the end-labeled *Eco*RI restriction digests did not reveal additional smaller fragments which may have been missed on the agarose gels. The presence of shorter-length linkers cannot be ruled out completely. The most probable explanation, however, concerns the relatively short time used for the methylation of *B. abortus* DNA prior to linker ligation. We have noted that under optimal conditions restriction digestion of *B. abortus* genomic DNAs purified in our laboratory requires overnight incubation with a minimum of 5 U of enzyme per μg of DNA. Contamination of the DNAs by cellular components or as yet undetermined structural features of *B. abortus* DNAs may interfere with the activity of methylation enzymes (47).

Identification of 36-kDa gene in restriction digests of genomic DNA. Restriction digests of genomic DNAs were performed as described in Materials and Methods. The digested DNAs were electrophoresed on a 0.8% (wt/vol) agarose gel and transferred to nylon membrane (Zeta-Bind; AMF-CUNO). These blots were hybridized at high stringency with the *B. abortus* DNA fragments cloned into M13mp18 and labeled as described in Materials and Methods. The result with recombinant 2.51 as a hybridization probe is shown in Fig. 7. Identical results were obtained when 2.33 and 2.63 were used as hybridization probes (data not shown). The probes hybridized to single *Bam*HI and *Hin*dIII fragments of approximately 6.5 and 9 kbp, respectively, in digests of both strain 19 and 2308 genomic DNAs (Fig. 7). In addition, identical patterns were obtained with DNAs isolated from both strains in restriction digests by using *Eco*RI, *Pst*I, and *Dde*I, each of which generated two fragments which hybridized to the probe. This latter result was surprising since these sites are not present within the *B. abortus* DNA fragments used as hybridization probes (Fig. 6) and suggested that sequences complementary to the cloned fragments or portions thereof are present in more than one copy in the genomes of strains 19 and 2308. These results suggest that there are two closely related genes present in *B. abortus* which encode the 36-kDa protein and a related protein. Alternatively, the 36-kDa protein may be composed of repeated epitopes encoded by the duplicated DNA sequences.

Genomic organization of 36-kDa-protein genes. Genomic libraries of *B. abortus* DNAs were constructed from strains 19 and 2308 in the vector λ2001. Recombinants containing the 36-kDa-protein gene were selected by hybridization with the 2.51 probe labeled as described in Materials and Methods. Recombinant DNAs were extracted from a single confluently lysed agarose plate (15 cm) and mapped by restriction enzyme analysis. Four recombinants were selected from each library and digested with *Bam*HI and *Pst*I endonucleases. The structure of the recombinants from both libraries were similar, and those selected within a library (e.g., S19 or S2308) were identical (data not shown). Hybridization with the 2.51 probe revealed that all copies of the gene identified in the genomic digests (described above) were present within the 20-kb inserts cloned into λ2001 (Fig. 8A). The single *Bam*HI restriction fragment of 6.5 kb present in genomic digests (Fig. 7) was shortened to less than 4 kbp in the S19 clones but appears to be unchanged in the S2308 clones. This difference is a cloning artifact which has deleted one end of the *Bam*HI fragment from the strain 19 recombinants; as a result, this fragment cannot be excised from the λ2001 vector unless a double digestion with *Bam*HI and *Xho*I is performed (Fig. 8A, lane 1, and data not shown) (27). In contrast, excision of the fragment from the S2308 recombinants requires only *Bam*HI, as both sites have been conserved during cloning (Fig. 8A, lane 2). The *Pst*I frag-

ments of 900 and 600 bp present in the genomic digests were present in the recombinants derived from both strains and were unaffected by the shortened length of the S19 recombinants. These data suggest that the repeated portions of the 36-kDa-protein gene are located within a limited stretch of approximately 4 kbp of genomic DNA. To determine whether this repetition of sequences represented a portion or all of the gene, an oligonucleotide probe was synthesized on the basis of the amino-terminal sequence of the mature gene product excised from polyacrylamide gels and sequenced out to amino acid residue 55 Sowa, unpublished. This 55-amino-acid sequence corresponds to 165 nucleotides at the 5′ end of the gene which exhibits no homology with the λgt11 DNA inserts. The oligonucleotide constructed, GA-PuCCNGAPuGCNGT, represents the amino acid sequence Glu-Pro-Glu-Ala-Val present at approximately 8 to 12 residues from the amino-terminal end of the mature protein. Since the codon usage in the *Brucella* spp. was unknown, the synthesized probe contained a mixture of oligonucleotides (64 combinations) because of variability at the wobble base position in the degenerate codons. The oligonucleotide mixture was labeled and hybridized as described in Materials and Methods. The Southern blot shown in Fig. 8B demonstrates the hybridization of an oligonucleotide to the single *Bam*HI fragments of less than 4 and 6.5 kbp derived from S19 and S2308 genomic DNAs, respectively, which were identical in size to those which hybridized to the 2.51 probe (Fig. 8A). In addition, the oligonucleotide hybridized to *Pst*I fragments of 900 and 600 bp which were identical in size to those hybridizing to the 2.51 probe. The difference in the size of the *Pst*I fragments suggests that the duplicated regions are not identical. We believe that the duplication of sequences corresponding to the oligonucleotide probe and the λgt11 insert, which are separated by at least 165 bp, is best explained by the presence of two closely related but nonidentical copies of the gene within a 4-kbp region of *B. abortus* genomic DNA. Alternatively, these sequences may encode repeated epitopes within a single gene product. This latter conclusion is consistent with the predicted function of the gene product which as an OMP must span the lipid bilayer and, presumably, does so repeatedly (6, 7, 63). Whether this arrangement of DNA does, in fact, represent two complete gene copies encoding similar or related genes or repeated regions within a single gene will be the subject of our future research.

DISCUSSION

The cloning of a *B. abortus* gene encoding a major cell envelope protein has been performed by using antibody directed against a 36-kDa protein purified by preparative SDS-PAGE of isolated cell envelopes (49, 61, 62). The identity of the encoded protein has not been established. However, the protein partitions with the cell envelope fraction and is resistant to solubilization by several detergents, suggesting that it is associated with the outer membrane (61; Sowa, unpublished). Taken together with the apparent molecular weight, the evidence suggests the identification of the gene or genes encoding a group 2 OMP. Sequence analysis of three DNA inserts of slightly different lengths subcloned into M13mp18 identified open reading frames on both strands of all three inserts. Identification of the polypeptides encoded by the gene fusions was impossible since knowledge of the orientation in λgt11 was lost following subcloning into M13. The predicted polypeptide encoded by one strand (the complement of the strand shown in Fig. 6) does have an extensive amount of β-sheet structure which is consistent with its preliminary identification as an OMP (13, 61, 62). Comparison of the partial amino acid sequence encoded by either strand of the *B. abortus* gene with the known sequences of the *E. coli* OMPs and hybridization with the cloned *E. coli* genes did not detect any similarities (43).

Southern blot analysis of the DNA inserts in λgt11 demonstrated a preferential selection of a restricted region of the gene encoding the 36-kDa cell envelope protein. Although this region may encode an immunodominant epitope which favors selection of these recombinants, this does not explain the small sizes of the inserts selected. The preferential selection of these sequences and the small sizes of the inserts may be linked and might reflect the hydrophobic nature of the cloned gene products. That is, the addition of flanking sequences or the cloning of additional regions may produce unstable or lethal fusion products (6, 7, 22). Examination of the recombinants selected may provide some clue for the basis of this restricted selection. For example, of the 11 independent recombinants originally selected, only 7 demonstrated detectable expression of *lacZ* fusion products expressed under the control of *lac*-PO (data not shown). The recombinants which did not produce a detectable fusion product in the lysogens may have contained sequences which resulted in their instability in the cytoplasm of *E. coli* (6, 7, 22). This is best illustrated by comparison of recombinants 2.33, 2.63, and 2.51 (Fig. 6). Although all three were selected by antibody screening of plaques produced by recombinant phage and contain over 200 nucleotides in common, the recombinant containing the longest insert, 2.51, did not produce any detectable fusion product in either *E. coli* MC4100 or Y1089. Presumably the polypeptide encoded by the larger insert contained in recombinant 2.51 resulted in an instability of this fusion product. Evidence of decreased stability of fusion products may be observed in Fig. 3; in this case recombinant 2.33 shows several lower-molecular-weight bands not present in the other samples. Problems of this kind are frequently encountered when fusing membrane proteins with cytoplasmic proteins such as β-galactosidase (6, 22). Fusion of signal peptide-encoding regions of *omp* genes to *lacZ* produce hybrid proteins which are unable to cross the plasma membrane and, when expressed at high levels, are frequently lethal to the cells.

Southern blot analysis of genomic DNAs extracted from two strains of *B. abortus*, S19 (vaccine strain) and S2308 (virulent strain), indicated the presence of a single *Bam*HI fragment and multiple *Eco*RI, *Pst*I, and *Dde*I fragments which hybridized to the cloned gene probes. Recombinants selected from a λ2001 genomic library were also found to contain the same number of *Bam*HI and *Pst*I fragments, which is consistent with a clustered arrangement of the repeated sequences. In an attempt to distinguish between repeated regions within a single gene and multiple gene copies, an oligonucleotide probe was synthesized on the basis of the amino-terminal amino acid sequence of the mature gene product (Sowa, unpublished). The distance between the mature amino terminus and the portion encoded by the λgt11 inserts is not known. A minimum estimate of 165 nucleotides was made on the basis of the first 55 amino-terminal amino acids of the mature gene product, which shows no homology with the DNA sequence of the λgt11 inserts. Hybridization of this probe to the same *Bam*HI and *Pst*I fragments in restriction digests of the genomic clones indicated that a duplication of these sequences has also occurred. Assuming that these sequences

are not independently duplicated, this suggests that a region including the oligonucleotide sequences and the region cloned into λgt11 of no less than 400 nucleotides is repeated. Since the 36-kDa protein is encoded by a gene of 1,100 bp, these results suggest that the genes share as much as 40 to 50% sequence conservation. Furthermore, as demonstrated in Fig. 8, the organization of repeated sequences is conserved in the vaccine strain (S19) as well as in a model virulent strain (S2308). It is also clear from the data presented that other envelope protein genes present in the *B. abortus* genome do not share extensive sequence homology, since they are not detected by Southern blot analysis of genomic DNA under high-stringency conditions. Whether the repeats identified here represent two functional genes cannot be stated with any certainty. One possibility is that the two genes encode proteins which have vastly different molecular masses but which cross-react antigenically because of similarities in sequence, such as the 36-kDa and the 66-kDa proteins (Fig. 3). The DNA sequence of this region in the *Brucella* genome is being determined to elucidate the structure and arrangement of these repeated regions and the sizes of any open reading frames.

Group 2 has been identified as a cluster of proteins, 1 to 3 bands, which vary in molecular mass (35 to 40 kDa) but which are structurally and antigenically related (13). The uniformity observed in the 36-kDa protein present in these studies presumably stems from the isolation procedure used; in this case, no previous lysozyme treatment is used. Previous work by Douglas et al. (13) concluded that the multiple bands of group 2 represented modified forms of a single gene product. It is possible that the modified forms arise because of covalent attachment to the peptidoglycan layer and are released by lysozyme treatment. Although the results presented here cannot rule out the possibility of modified forms arising posttranslationally from single gene products, the multiple products previously reported may be explained, in part, by repeated gene copies encoding variant proteins. This is similar to the situation in *E. coli* K-12, in which the *ompF* and *ompC* genes are conserved over 69% of their nucleotides and *ompC* and *phoE* genes share 60% nucleotide homology (43). The close proximity of the putative genes in the *B. abortus* genome is quite different from the organization of porin genes in *E. coli* K-12 and *Salmonella typhimurium*, in which *ompF*, *ompC*, and *phoE* are separated by more than 40 map units or approximately 2×10^6 bp (43). Preliminary mapping results (T. A. Ficht, unpublished results) suggest that the 5′ ends of the *B. abortus* genes, which hybridize to the oligonucleotide probes, are adjacent. Such an arrangement suggests the potential for coordinate expression from this locus via bidirectional transcription. An analogous arrangement has been identified at the *ompC* locus of *E. coli* (1, 42). In this case, divergent transcription results in the production of *ompC* RNA and *micF* RNA; the latter is an antisense RNA which apparently regulates porin expression by inactivating *ompF* RNA. Sequence comparison between the potential polypeptides encoded by the *B. abortus* λgt11 inserts described here and the amino-terminal end (55 amino acids) of the mature gene product (Sowa, unpublished) shows no regions of overlap and places these fragments downstream an unknown distance from amino acid position 55 which corresponds to a nucleotide position greater than 165. The homology between the two *B. abortus* genes thus extends beyond the point of complementarity between *ompF* and *micF* which lies between positions 99 and 174 in the 174-nucleotide-long *micF* transcript. Whether the locus described in *B. abortus* represents an efficient method for the regulation of divergent transcripts or a regulatory mechanism related to that described in *E. coli* will require complete knowledge of the DNA sequence of this region and the nature of the encoded products.

Speculation regarding the close proximity of the *B. abortus* genes suggests the possibility for efficient regulation of their expression (1, 42). This increased efficiency may be required of an organism capable of survival within host macrophages (31). Little is known about expression by *B. abortus* during growth in vivo or following macrophage engulfment. It is possible that environmental factors which accompany the shift from extracellular to intracellular location stimulate the appropriate shift in expression as is observed with the membrane proteins of other bacteria, including the iron-binding proteins (11, 16, 35). Although in vitro model systems of macrophage engulfment are available, they require further development to test this hypothesis. Alternatively, regulation of expression of the *Brucella* gene products may mimic that of the *E. coli* porins shifting expression from OmpF to OmpC under various conditions of growth (34, 35) simply by alternating the direction of transcription. Previous work has suggested the presence of more than one species of porin in *B. abortus* and the apparent transition in expression at certain stages of growth in vitro (13, 49). Coordinate expression of the two gene copies identified here may be invoked to explain the observations with regard to the altered expression of *B. abortus* proteins under various growth conditions (49). Correlation of this shift in expression with osmotic strength or other factors known to regulate the expression of *E. coli* proteins has not been examined and will be the subject of our future research.

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