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Cloning and Sequence Analysis of a Newly Identified *Brucella abortus* Gene and Serological Evaluation of the 17-Kilodalton Antigen That It Encodes

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A thus far unknown gene encoding a *Brucella abortus* protein has been isolated from a lambda *gt*11 expression library probed with sera from *Brucella*-infected sheep. Sequence analysis of the cloned gene revealed the presence of an open reading frame of 158 amino acids encoding a protein of 17.3 kDa (calculated molecular mass). The recombinant *B. abortus* protein, expressed in *Escherichia coli*, and the corresponding *Brucella melitensis* protein migrated at the same apparent molecular masses as shown by Western blotting (immunoblotting). Among a series of serum samples from *B. melitensis*- or *B. abortus*-infected sheep and cows, 51 and 39%, respectively, showed a signal at 17 kDa on Western blot analysis of total protein extract from *Brucella* bacteria. These figures amount to 70 and 61% for sheep and cattle, respectively, in a competitive enzyme-linked immunosorbent assay with a specific monoclonal antibody. These data indicate that the 17-kDa antigen may be useful for serological diagnosis of *Brucella* infection.

Brucellosis is an infection caused by a small intracellular gram-negative bacterium which is pathogenic for humans as well as for domestic animals. This infection induces abortions in livestock animals leading to severe economic losses. Within the genus *Brucella*, six closely related species have been described (5, 21, 22), the most important of which are *B. abortus* and *B. melitensis*. Humans and ruminants (sheep, goats, and cows) are predominantly infected by these two *Brucella* strains.

Serological tests currently used for the diagnosis of brucellosis infection are based on the detection of antilipopolysaccharide antibodies (1). These tests do not permit the differentiation between vaccinated and infected animals and fail to reveal some infected animals which test positive in an intradermic test. Moreover, important cross-reactions with other gram-negative bacteria have been reported (3, 15, 17). Diagnostic tests with higher specificities are based on the isolation of *Brucella* bacteria or on the intradermic injection of a protein preparation from *Brucella* bacteria, Brucellergen (6), leading to a delayed-type hypersensitivity (DTH) reaction. However, classical bacteriology is time-consuming, and it is not always easy to produce Brucellergen preparations that are free of lipopolysaccharide. The latter can cause seroconversion of animals upon DTH testing, precluding its use for distinguishing infected animals serologically. The identification of specific antigens for the diagnosis of brucellosis is therefore a matter of a great interest for the development of a specific serological test.

In our effort to identify such antigens, we screened an expression library with sera from sheep infected in the field. The DNA insert from a positive recombinant phage was analyzed in detail. Sequence analysis revealed a hitherto unknown gene able to encode a 17-kDa *Brucella* protein. The antigenicity of the encoded protein was analyzed by Western blotting (immunoblotting) and in a competitive enzyme-linked immunosorbent assay (ELISA) with sera from infected sheep or cows to evaluate its significance for serological diagnostic purposes.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany), Sigma (St. Louis, Mo.), or Bio-Rad Laboratories (Richmond, Calif.). Restriction enzymes and DNA-modifying enzymes were purchased from Boehringer Mannheim (Brussels, Belgium) and were used according to the manufacturer's instructions. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, Ill.).

Bacteria and vectors. *Escherichia coli* Y1090, *E. coli* MC1061, and *E. coli* DH5α F′-competent cells and pBluescript SK+ vector were from Stratagene (La Jolla, Calif.).

MAbs. The monoclonal antibodies (MAbs) A66/05H01/E09 and A68/04G01/C06 were prepared by the fusion of splenocytes from mice infected with *B. abortus* 45/20 together with myeloma cells as described previously (2). Ascitic fluids or hybridoma culture supernatants were used.

Sera. Sheep sera were obtained from J. Blasco (Servicio de Investigacion Agraria, Zaragoza, Spain), and cow sera were obtained from the Faculté Universitaire Notre-Dame de la Paix (FUNDP; Namur, Belgium). One hundred serum samples from naturally *B. melitensis*-infected sheep and 36 serum samples from naturally *B. abortus*-infected cows with a positive classical serology for *Brucella* infection (1) were used. Twenty serum samples from infected sheep which had a negative serology but a positive DTH reaction were also included. Sera from experimentally infected cows were obtained as follows. Pregnant heifers (10 animals) were vaccinated subcutaneously with *B. abortus* B19 (150×10^6 CFU). At 88 days postvaccination, the heifers were infected in the conjunctiva with 16.6×10^6 *B. abortus* 544. Animals were bled at 135 days postinfection. Fifteen serum samples from healthy sheep and 14 serum samples from healthy cows were used as controls. Successful vaccination was proved by the absence of infection in the heifers and the newborn calves.

Preparation of bacterial protein extracts. Total protein extract from *B. melitensis* H38R (depleted of high-molecular-mass lipopolysaccharide) was kindly provided by G. Dubray (Institut National de la Recherche Agronomique, Nouzilly, France). Briefly, the bacteria were grown on solid agar, collected by washing, and heated at 95°C for 1 h in the buffer described by Laemmli (11) containing 2% sodium dodecyl sulfate (SDS). To obtain total protein extract from the *B. abortus* 45/20 rough strain, which was grown in liquid medium, the cells were pelleted by centrifugation and the cell pellet was treated as described above. Total protein extract from recombinant *E. coli* was obtained from 5 ml of an overnight culture (optical density at 600 nm, 1) in Luria broth medium (LB) (12) supplemented with 0.1 g of ampicillin per liter and 1% glucose. Bacteria were centrifuged at $5,000 \times g$ for 10 min. The pellet was resuspended in 1 ml of electrophoresis sample buffer (11), heated at 100°C for 5 min, and centrifuged.

Recombinant *B. abortus* minor OMP. The outer membrane protein (OMP) OMP16.5 was expressed in *E. coli* as a fusion protein with a murine tumor

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necrosis factor (mTNF) leader peptide (25 amino acids) (20), and between the mTNF and the OMP, a cluster of six histidine residues was inserted to allow purification of the fusion protein by immobilized metal ion affinity chromatography (10). The fusion protein was purified to at least 99% homogeneity, as determined by gel electrophoresis and then silver staining (unpublished data). Because of this fusion, this recombinant protein migrated at an apparent molecular mass of 20 kDa in SDS-polyacrylamide gels.

Construction of a *B. abortus* genomic library. The lambda *gt*11 *B. abortus* genomic library was generously provided by P. de Wergifosse, Université Catholique de Louvain, Brussels, Belgium (4). It was prepared by *Sau*3A digestion of *B. abortus* chromosomal DNA. The sticky ends were filled in, and *Eco*RI linkers were added to insert the DNA fragments into the lambda *gt*11 *Eco*RI cloning site. General molecular biological techniques were performed as described by Maniatis et al. (12).

Library plating and immunoscreening. A bacteriophage suspension (10 μl) in phage dilution buffer (20 mM Tris, 100 mM NaCl, 10 mM magnesium sulfate [pH 7.4]) was incubated (20 min at 37°C) with an overnight culture (0.6 ml) of *E. coli* Y1090 grown at 37°C in LB and supplemented with 0.1 g of ampicillin per liter, 10 mM magnesium sulfate, and 2% maltose. Thirty milliliters of top agar (LB, 0.7% agar, 0.1 g of ampicillin per liter, 10 mM magnesium sulfate) prewarmed at 48°C was then added to the mixture, and the mixture was immediately plated onto a petri dish (220 by 220 mm) containing 150 ml of solid medium (LB containing 1.2% agar). After plating, the dishes were left for 5 h at 37°C and were subsequently covered with a nitrocellulose membrane (Hybond-C; Amersham, Brussels, Belgium), wetted in a 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) solution, and blotted dry between two sheets of Whatman 3MM paper. After overnight incubation (37°C), the membrane was peeled off and was saturated for 1 h at room temperature with 5% fat-free milk in a Tris salt buffer (TBS [TBS is 10 mM Tris and 150 mM NaCl]; pH 7.4) containing 0.05% Nonidet P-40 (NP-40). All subsequent incubations were also performed at room temperature. Sera from *B. melitensis*-infected sheep were diluted 1/40 in TBS–NP-40 buffer containing 5% fat-free milk and *E. coli* MC1061 lysate (diluted to 1 mg of protein content per ml), and the mixture was incubated with the membrane for 90 min. After three washes in TBS–NP-40, a rabbit anti-sheep alkaline phosphatase conjugate (Jackson, West Grove, Penn.) diluted 1/5,000 in TBS–NP-40 was added to the membrane for a 1-h incubation. After three more washes, bound antibody was revealed with Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) (0.37 mM each) in a Tris buffer (0.1 M Tris, 0.1 M NaCl, 5 mM magnesium sulfate; pH 9.5) for 10 min. A positive plaque was removed from the top agar with a sterile tip and was suspended in 100 μl of phage dilution buffer. The positive bacteriophage was replated several times until plaque lifts showed more than 99% immunoreactive plaques.

Gel electrophoresis and Western blotting. The total bacterial protein extracts (40 μg/well) were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) in the presence of β-mercaptoethanol as described by Laemmli (11). Proteins were transferred by semi-dry Western blotting (19) (the transfer buffer was 25 mM Tris, 192 mM glycine, and 20% methanol) to nitrocellulose for 40 min at 0.8 mA/cm^2. The membrane was saturated with 5% fat-free milk as describe above and the mixture was incubated overnight with MAbs or sera. Ascites were diluted 1/1,000 in TBS–NP-40 containing 5% fat-free milk, and sera were diluted 1/40 in TBS–NP-40 containing 5% fat-free milk and *E. coli* lysate (final *E. coli* protein concentration, 1 mg/ml). After three washes, the bands were revealed with rabbit anti-mouse immunoglobulin G conjugate (Dako, Glostrup, Denmark), rabbit anti-sheep conjugate (as described above), or rabbit anti-cow conjugate (1/5,000; Sigma).

Competitive ELISA. A sonicated cell extract of *B. abortus* 45/20 was coated on microplates (69620; Nunc) at 20 μg/ml in buffer (0.17 M NaCl, 0.1 M glycine, 6 mM NaN_3; pH 9.2 [GBS]) diluted fivefold. After saturation with casein hydrolysate, twofold-diluted sera and MAb ascites (1/10,000) in a GBS buffer supplemented with 50 mM EDTA and 0.1% Tween 20 (GBS-EDTA-Tw) were incubated for 1 h at room temperature in the microwells. The binding of the MAb was revealed by incubation for 1 h at room temperature with a sheep anti-mouse peroxidase conjugate (Amersham, Brussels, Belgium) diluted 1/1,000 in GBS-EDTA-Tw containing 4% casein hydrolysate. Reagents in excess were removed between each step by six washings with a 0.15 M NaCl solution containing 0.01% Tween 20. *o*-Phenylenediamine (0.4%; wt/vol) with 2 mM H_2O_2 in a citrate-phosphate buffer (0.05 M Na_2HPO_4, 0.025 M citric acid [pH 5]) was used to develop the assay. The signal was read at 490 and 630 nm, and the difference was recorded with a BIO kinetics Reader EL-340 (Bio-tek Instruments, Inc., Winooski, Vt.). A reduction in the signal of more than 30% relative to that of the serum-free control was considered a positive competition reaction.

Colony blotting. Recombinant *E. coli* colonies grown on solid medium were transferred by colony blotting to a nitrocellulose disk prewetted in phosphate-buffered saline (PBS; 135 mM NaCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 5 mM KCl [pH 7.4]). The filter was gently shaken in a solution containing PBS with 0.5% Tween 20 for 30 min at room temperature to lyse the bacteria. The membrane was subsequently rinsed twice with TBS–NP-40, and the immunoscreening was performed as described above.

PCR. PCR was performed as described previously (14). The *B. abortus* DNA insert was recovered from the purified bacteriophage by PCR directly on the isolated bacteriophage. The lambda *gt*11 primers (Stratagene, La Jolla, Calif.) were used under the following conditions. The reaction mixture (50 μl) contained 0.1×10^6 PFU of recombinant phage, 100 pmol of each primer, 200 mM (each) deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), and 1 U of *Taq* polymerase (Cetus, Emeryville, Calif.) with the appropriate buffer. The reaction mixture was heated for 5 min at 95°C, and then 40 cycles (95, 55, and 72°C for 1 min each) were performed in a thermocycler (Perkin-Elmer Cetus, Emeryville, Calif.). To terminate the reaction, a 10-min elongation step at 72°C was added.

Cloning. The DNA fragments obtained by *Eco*RI or *Hin*dIII digestion were cloned into a pBluescript SK+ vector (SK+; Stratagene), and plasmids were transformed and propagated in *E. coli* DH5α F′. Plasmid purification was performed on a QIAGEN matrix (Diagen, Hilden, Germany) as described by the manufacturer.

Nucleic acid sequencing. Sequence analysis of the DNA fragments cloned in SK+ plasmids was performed by the chain termination procedure (16), which was adapted to allow analysis on an automated DNA sequencer (Applied Biosystems, Foster City, Calif.). Sequencing reactions were carried out by using the dye-terminator technology, as described by the manufacturer, with the universal or reverse M13 primers. In order to obtain the complete sequence of the fragment, two internal oligonucleotides were custom synthesized (Pharmacia) to enable internal priming. Sequence manipulations were performed by using the Intelligenetics (Mountain View, Calif.) software package.

Nucleotide sequence accession number. The sequence of the new gene was deposited in the EMBL database under accession number Z46864.

RESULTS

Identification of a new gene. In a first screening, about 35,000 *gt*11 plaques were probed with a pool of field sera from *B. melitensis*-infected sheep. One positive plaque was identified and purified by repeated plating. The purified bacteriophage was again plated and the plaques were probed with different MAbs directed to *B. abortus* proteins (2). One MAb (MAb A66/05H01/E09), described as reactive with a protein in the 16- to 17-kDa range as defined by Western blot analysis, clearly yielded a strong signal. To recover the DNA fragment, the purified bacteriophage was used as a template in a PCR with primers flanking the cloning site. A 1,500-bp DNA fragment was obtained and purified via agarose gel electrophoresis (11). After *Eco*RI digestion, two bands of about 800 and 700 bp, respectively, appeared on an agarose gel (data not shown). Both fragments were cloned separately in the SK+ vector. The recombinant bacteria were then analyzed for the presence of an antigen reactive with the MAb cited above. Only the 800-bp insert produced a protein which was immunologically reactive with the MAb A66/05H01/E09, as shown by colony blotting. The protein was produced irrespective of IPTG induction of the culture.

Sequence analysis. By treatment of the 800-bp insert fragment with *Hin*dIII, two fragments of 425 and 375 bp, respectively, were obtained. The fragments were cloned in an SK+ vector as *Eco*RI-*Hin*dIII fragments, and both strands were sequenced. The sequence crossing the *Hin*dIII site was obtained by using internal primers on the parental 800-bp fragment. On one strand, an 463-bp open reading frame (ORF) was identified (Fig. 1). The calculated molecular mass of the encoded protein (17.3 kDa) matched very well the size observed on Western blots (Fig. 2). A Shine-Dalgarno consensus sequence (GAGGA) was found upstream from the putative initiation codon (position −8). A consensus promoter sequence, as described previously for *Brucella* genes (13), was not found in the 290 bp upstream from the ORF which is present in the cloned fragment.

Serology. Western blot analysis of the recombinant *E. coli* bacterial lysate with MAb A66/05H01/E09 showed that the immunoreactive protein migrated as a 17-kDa molecule (Fig. 2C, lane 3). When this MAb was used to develop Western blots prepared with lysates from *B. melitensis* or the recombinant *E. coli* strain described above, in both cases a protein of 16.5 to 17 kDa was revealed (Fig. 2A and C, lanes 3). Another MAb (MAb A68/04G01/C06) known to reveal a 16.5-kDa OMP

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1   GAATTCCGATCAGTGCATAGTTTCCGCGTGCTCGCGCAATGGTGCGCGGGCTTGTTCTC
60  GGGGCGGGGTGAAACTCCCCACCGGCGGTATGAAAAGCAATTTTCAAGCCCGCGAGCGC
119 CTGAAATGGAAGCCGATTCGCATGCCATTTCAGGGTCAGCAGATCCGGTGAGATGCCGG
178 AGCCGACGGTTAAAGTCCGGATGGAAGAGAGCGAATGAGCGTCACGATTGCGCCTTCCG
237 GCGTCGTTCTTGCGTTCTTTTGTGCGCCCTGATTCTAGTTTCGTGAGGAACCTATGAAC
                                                    MetAsn
296 CAAAGCTGTCCGAACAAGACATCCTTTAAAATCGCATTCATTCAGGCCCGCTGGCACGCC
    GlnSerCysProAspLysThrSerPheLysIleAlaPheIleGlnAlaArgTrpHisAla
356 GACATCGTTGACGAAGCGCGCAAAAGCTTTGTCGCCGAACTGGCCGCAAAGACGGGTGGC
    AspIleValAspGluAlaArgLysSerPheValAlaGluLeuAlaAlaLysThrGlyGly
416 AGCGTCGAGGTAGAGATATTCGACGTGCCGGGTGCATATGAAATTCCCCTTCACGCCAAG
    SerValGluValGluIlePheAspValProGlyAlaTyrGluIleProLeuHisAlaLys
476 ACATTGGCCAGAACCGGGCGCTATGCAGCCATCGTCGGTGCGGCCTTCGTGATCGACGGC
    ThrLeuAlaArgThrGlyArgTyrAlaAlaIleValGlyAlaAlaPhrValIleAspGly
536 GGCATCTATCGTCATGATTTCGTGGCGACGGCCGTTATCAACGGCATGATGCAGGTGCAG
    GlyIleTyrAspHisAspPheValAlaThrAlaValIleAsnGlyMetMetGlnValGln
596 CTTGAAACGGAAGTGCCGGTGCTGAGCGTCGTGCTGACGCCGCACCATTTCCATGAAAGC
    LeuGluThrGluValProValLeuSerValValLeuThrProHisHisPheHisGluSer
656 AAGGAGCATCACGACTTCTTCCATGCTCATTTCAAGGTGAAGGGCGTGGAAGCGGCCCAT
    LysGluHisHisAspPhePheHisAlaHisPheLysValLysGlyValGluAlaAlaHis
716 GCCGCCTTGCAGATCGTGAGCGAGCGCAGCCGCATCGCCGCGCTTGTCTGACTAACCCTC
    AlaAlaLeuGlnIleValSerGluArgSerArgIleAlaAlaLeuVal***.........
776 TATAATACGCCCGCAATGGGTATAAATGTCGAATTC
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FIG. 1. Nucleotide sequence of the 811-bp fragment encoding the 17-kDa *B. abortus* protein. The predicted amino acid sequence is shown below the nucleotide sequence, and the peptides mentioned by Goldbaum et al. (9) are underlined. The putative Shine-Dalgarno sequence and the stop codon are shown in boldface type.

(OMP16.5) from *B. abortus* was also used to probe Western blot strips containing the new recombinant 17-kDa antigen as well as a recombinantly produced fusion of OMP16.5 migrating at 20 kDa (unpublished data) and *B. melitensis* extract. This experiment indicated that the latter MAb does not recognize the new 17-kDa antigen (Fig. 2C, lane 2), whereas good reactivity with the recombinant OMP as well as with a 15.5-kDa protein in the *Brucella* extract was observed (Fig. 2A and B, lanes 2). Both MAbs used here had been previously described

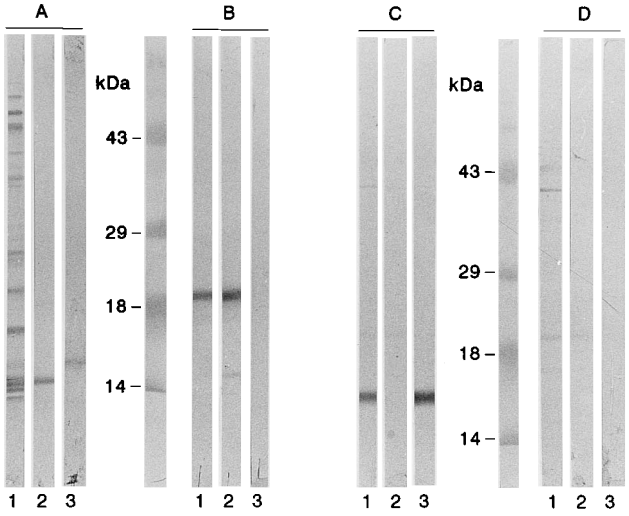

FIG. 2. Western blot analysis of *B. melitensis* proteins. Blots were probed with a serum sample from a sheep (lane 1) or with MAb directed against OMP16.5 MAb A68/04G01/C06 (lane 2) or against the 17-kDa protein (MAb A66/05H01/E09 (lane 3). (A) Total protein extract from *B. melitensis* 38 (rough strain; 40 μg per well); (B) purified recombinant OMP16.5 (0.1 μg per well); (C) total protein extract from recombinant *E. coli* expressing the 17-kDa antigen (40 μg per well); (D) total protein extract from negative control *E. coli* loaded as described for panel C.

as being directed to the OMP16.5 antigen (2). The present experiments show that these MAbs are directed to two different antigens, both of which migrate in the 15- to 17-kDa region when *Brucella* extracts are analyzed by SDS-PAGE and Western blotting. Furthermore, upon Western blot analysis of other bacterial species (*E. coli* O:157, *Yersinia enterocolitica* O:9, *Salmonella urbana*, *Pseudomonas maltophilia*, and *Pseudomonas multocida*) no reactivity with the anti-p17 MAbs could be demonstrated (data not shown), adding to the specificity of this *Brucella* antigen.

From *B. melitensis*-infected sheep, 100 serum samples which were positive by classic standard serology and 20 serum samples from animals with a negative serology but a positive DTH reaction were analyzed by Western blotting on total protein extract from *B. melitensis* H38 (rough strain). Each serum sample identified up to 14 bands in the range of 10 to 97 kDa (data not shown). In general, more bands with higher intensities were revealed in the case of the serologically positive sera than in the case of the serologically negative samples. Of the 100 and 20 serum samples mentioned above, 51 and 20%, respectively, showed a band at 17 kDa (Table 1). On the other hand, 36 serum samples from *B. abortus*-infected cows in the field were analyzed by Western blotting on total protein extract from *B. abortus* 45/20 (rough strain). Each serum sample was reactive with up to 12 bands in the range of 9 to 97 kDa (data not shown). Thirty-nine percent of these 36 serum samples showed a band at 17 kDa (Table 1). None of the 15 serum samples from healthy sheep and none of the 14 serum samples from healthy cows gave a signal in the 17-kDa region upon Western blot analysis.

To assess the reaction with the 17-kDa protein (ensuring that the reactivity was not due to OMP16.5), 20 serum samples from sheep and 4 serum samples from cows, which were randomly chosen from those with a positive serology and which reacted at 17 kDa in Western blots of *Brucella* extracts, were retested by Western blotting on a total protein extract from a recombinant *E. coli* strain expressing the 17-kDa antigen. Seventy percent (14 serum samples) of the serum samples from sheep and 75% (3 serum samples) of the serum samples from cows were positive on the recombinant *E. coli*-produced protein, indicating that most of the reactivity seen at the 17-kDa position can be attributed to reaction with this newly identified protein (data not shown). In addition, of 10 control serum samples from sheep and 6 control serum samples from cows, none were reactive with the *E. coli*-produced protein.

Fifty serum samples from sheep infected in the field with a positive serology, including 23 which did not react with the 17-kDa protein on Western blots, were tested in a competition ELISA with MAb A66/05H01/E09. Seventy percent (35 serum samples) were positive in this test, including 12 samples which showed no reactivity in the 17-kDa position on Western blotting. Of the 20 serum samples from infected sheep with a negative serology but with a positive DTH reaction, 20% could still be detected by this test; however, these serum samples were not the same as those that reacted in the Western blot analysis mentioned earlier. Under the same conditions, the 36 serum samples from cows infected in the field were tested: 61% were positive in the competition ELISA, even though only 39% showed a band at 17 kDa (Table 1). Of the 10 vaccinated animals, 6 were protected from subsequent infection, but only 3 of these produced healthy calves. The sera from the three latter heifers did not react in the competition ELISA, whereas sera from the other heifers (seven animals) were positive in the competition ELISA after the experimental infection (Table 1), but all serum samples were negative between vaccination and infection. The specificity of the reaction

TABLE 1. Western blot and competition ELISA performed on sera from sheep and cattle

Serum sample	Western blot analysis		Competition ELISA	
	No. of serum samples tested	No. (%) of serum samples positive[a]	No. of serum samples tested	No. (%) of serum samples positive[a]
Sheep				
Field infected, positive serology	100	51 (51)	50	35 (70)
Field infected, DTH positive	20	4 (20)	20	4 (20)
Healthy	15	0 (0)	10	0 (0)
Cattle				
Field infected, positive serology	36	14 (39)	36	22 (61)
Experimentally infected, P[b]	3	0 (0)	3	0 (0)
Experimentally infected, NP[c]	7	5 (71)	7	7 (100)
Healthy	6	0	6	0
Other infections				
Y. enterocolitica			2	0
S. urbana			2	0

[a] Number (percent) of positive sera (a band at 17 kDa in Western blots or at least 30% inhibition in the competition ELISA).
[b] Complete protection upon vaccination.
[c] No protection upon vaccination.

was assessed with 10 serum samples from healthy sheep and 6 serum samples from healthy cows, all of which were negative. Furthermore, two serum samples from *Y. enterocolitica* O:9-infected cows and two serum samples from *S. urbana*-infected cows were also negative in the competition ELISA (Table 1).

DISCUSSION

In order to identify protein antigens which provoke a serological immune response in animals infected with *Brucella* bacteria, an expression library was screened with sera from *B. melitensis* sheep infected in the field. This resulted in the identification of a new gene capable of encoding a protein of 17 kDa. The predicted size of the protein agreed very well with the size of the protein observed on Western blots of the recombinant *E. coli* strain containing the gene fragment. Moreover, upon screening of a set of MAbs elicited by injection of mice with *B. abortus* (2), a MAb (MAb A66/05H01/E09) reactive with this 17-kDa protein was identified. This MAb was used to probe a Western blot of *B. melitensis* extract, and a 17-kDa protein was revealed, indicating that the natural *Brucella* antigen and the recombinant *E. coli*-produced protein are very similar. Comparison of the newly identified gene sequence or the deduced 17-kDa protein sequence with the nucleic acid and amino acid sequences in the sequence libraries (Swiss-Prot, A-GeneSeq, N-GeneSeq, EMBL, or GenBank) did not reveal significant similarity with known sequences. Upon inspection of the cloned gene fragment, another ORF encoded on the complementary DNA strand was identified. However, no in-frame stop codon was found in the cloned gene fragment, suggesting that the complete gene is not present in this fragment. The putative protein fragment derived from this ORF was calculated to have a molecular mass of 25 kDa, indicating the minimal size of the protein. A similar situation has been observed for other *Brucella* genes (7, 8), in which the complementary strand also contains an ORF of significant length. Whether or not these proteins are expressed in *Brucella* spp. still has to be investigated.

Upon computer analysis with PCGENE software (Intelligenetics), no consensus promoter sequence (13) was found upstream from the putative start codon for each of these genes, and no signal sequences or transmembrane domains were found. However, on the DNA strand encoding the 17-kDa antigen, a Shine-Dalgarno sequence (GAGGA) was found 8 bp upstream from the first ATG.

Recently, Goldbaum et al. (9) described a new 18-kDa cytoplasmic protein from *Brucella* bacteria present in *B. abortus*, *B. melitensis*, *B. canis*, *B. suis*, and *B. ovis* but not in *Y. enterocolitica* O9. Those investigators sequenced three peptides, the sequences of which are very close to the sequences found in the protein described here (Fig. 1). We consequently conclude that the protein described by Goldbaum et al. (9) is probably identical to the 17-kDa protein described in this report. Another cytoplasmic protein with an apparent molecular mass of 20-kDa has already been described by Zygmunt et al. (23). This immunogenic protein is different from the 17-kDa protein, as deduced from its N-terminal amino acid sequence (22a).

To evaluate the significance of this 17-kDa antigen for diagnostic purposes, Western blot experiments were performed with sera from animals infected in the field. These sera from sheep and cattle, which were positive by classical serology, were tested on total protein extract from *B. melitensis* or *B. abortus*, respectively, by Western blot analysis. A band migrating at about 17 kDa was specifically revealed in 51 of 100 serum samples from sheep (51%) and by 14 of 36 serum samples from cows (39%). The corresponding antigen in *B. melitensis* and *B. abortus* seemed to be very similar because of their molecular masses and antigenicities. A serologically reactive minor OMP (OMP16.5) was also present in the 17-kDa region. This protein is distinct from the 17-kDa antigen described here, as shown by its reactivity toward specific MAbs (2) as well as by its primary sequence (18). The serological prevalence of the 17-kDa antigen was also studied with a MAb in a competition ELISA. Nearly all serum samples (23 of 27) showing a band at 17 kDa were positive in this competition ELISA. Moreover, some serum samples which were negative by Western blotting scored positive in this ELISA, allowing detection of 70% of the infected sheep and 61% of the infected cows. This indicates that the competition ELISA is more sensitive than Western blotting in detecting antibodies to the 17-kDa protein, even though the sensitivity of the latter might still be improved when purified recombinant antigen is available. The specificity of the test was assessed with sera from healthy animals, which were negative in both assays. Furthermore, this antigen which is present in *B. melitensis* and *B. abortus* is not recognized by sera from animals infected with *E. coli*, *Y. enterocolitica*, or *S. urbana*, suggesting

that this antigen might be specific for *Brucella* species. This conclusion is substantiated by the results from Western blot analysis of other bacterial species with MAbs directed to the 17-kDa antigen, since no cross-reaction could be demonstrated.

The specificity obtained with the new antigen described here seems acceptable, and although the sensitivity is not sufficient to allow detection of all infected animals, the serological detection of some animals (20%) negative by classical serology but with a positive DTH reaction may enlarge the window for the serological detection of brucellosis. Complementation with other antigens will, however, be necessary to enhance the overall sensitivity.

From experiments with vaccinated animals, preliminary results seem to indicate that the serological response toward this new 17-kDa antigen may be absent in B19-vaccinated and protected animals, whereas it is present in animals infected upon challenge. This is an important property which enables the use of the antigen in differential serology.

The availability of the gene encoding this 17-kDa antigen now allows for the large-scale production and purification of this protein, which can then be used to further analyze the use of this protein in *Brucella* serology.

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