Cloning of a *Brucella melitensis* Group 3 Antigen Gene Encoding Omp28, a Protein Recognized by the Humoral Immune Response during Human Brucellosis

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Received 2 August 1995/Returned for modification 11 September 1995/Accepted 22 April 1996

***Brucella* group 3 antigens (Ags) are outer membrane proteins (OMPs) with a molecular mass ranging from 25 to 30 kDa. The OMPs are of interest partially because of their potential use as vaccine and diagnostic reagents. We used human convalescent antibody (Ab) to clone a gene that encoded a 28-kDa protein from a λ gt11 library of *Brucella melitensis* 16M genomic DNA. DNA sequence analysis revealed a single open reading frame that would encode a protein of 26,552 Da. The 28-kDa protein had a primary amino acid sequence that was 43% similar to a previously described *Brucella abortus* group 3 Ag, Omp25 (P. de Wergifosse, P. Lintermans, J. N. Limet, and A. Cloeckart, J. Bacteriol. 177:1911–1914, 1995). The similarity to a known group 3 OMP, immunoreactivity with Ab prepared against *B. abortus* group 3 Ags, immunolabeling of whole cells, and Southern hybridization led to our conclusion that the *B. melitensis* 28-kDa protein was a group 3 protein distinct from *B. abortus* Omp25. We designated the *B. melitensis* protein Omp28. Human convalescent sera from patients infected with *B. abortus* and *Brucella suis* as well as rabbit antisera prepared against killed *B. abortus* whole cells recognized *B. melitensis* Omp28 on Western blots (immunoblots). Furthermore, mice and goats infected with smooth strains of *B. melitensis* produced Abs against Omp28. Our results may begin to explain the variability in molecular weight seen in *Brucella* group 3 Ags and point toward their possible use in vaccination against infection as well as diagnosis of the disease.**

Brucella spp. are a group of facultative intracellular bacteria which parasitize humans as well as several other animal species (27, 43). The best-characterized immune response during brucellosis is to *Brucella abortus* in the mouse model. Resistance to infection by *B. abortus* requires both cellular and humoral immunity (1, 2, 20, 34, 40, 41, 44). Humoral immunity in brucellosis is primarily due to antibodies (Abs) which react with the O region of the lipopolysaccharide (LPS) (20, 28, 40); however, this is not true in all individuals (41). Also, the use of LPS as a general vaccine for brucellosis is further compromised by the fact that *Brucella ovis* and *Brucella canis* are rough (R) and therefore lack the protective O polysaccharide of the LPS (9).

One non-LPS group of immunogens focused on for vaccine and diagnostic purposes is the outer membrane proteins (OMPs) of *Brucella* spp., also known as group antigens (Ags) (8, 11, 14, 19, 39, 42, 45). The *Brucella* group 3 Ags are OMPs which are categorized according to their molecular weight. Group 1 Ags have an approximate molecular mass of 94 kDa, group 2 Ags are approximately 41 to 43 kDa, and group 3 proteins have an approximate molecular mass of 30 kDa (38). *Brucella* group 2 proteins are outer membrane porins (12). Characterization of the group 2 Ag locus at the molecular level has been initiated (14, 15, 26). In contrast, the *Brucella* group 3 Ags have been proposed to be similar to *Escherichia coli*

OmpA (38). Recently, the nucleotide sequence of a *B. abortus* group 3 Ag gene designated *omp25* has been reported (11). *B. abortus* Omp25 was found to have an amino acid composition similar to that of *E. coli* OmpA and to have short regions of protein homology with several different OmpA-like proteins. However, no further molecular information on *Brucella* group 3 Ags is currently available. Although OMPs may not be very useful for full immunization against smooth (S) species of *Brucella* through Ab stimulation (28, 29, 40, 41), they may induce a protective humoral immune response against infection with R species (19). Accordingly, further characterization of the *Brucella* OMPs is warranted.

We are interested in the human immune response elicited by protein Ags during *Brucella melitensis* infection. With this in mind, we have used purified human convalescent serum to screen a genomic library of *B. melitensis* 16M DNA. Here, we report the isolation of a 28-kDa immunoreactive protein. The 28-kDa *B. melitensis* Ag is a group 3 protein distinct from the previously reported Omp25 (11) polypeptide on the basis of nucleotide sequence, Southern blotting, predicted protein sequence, and immunological reactivity. The *B. melitensis* group 3 protein described in this report has been designated Omp28. The isolation and characterization of *omp28* are further steps toward understanding the immune response during infection with *Brucella* spp. and the development of subunit vaccines as well as protein-based diagnostic reagents.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and media. Bacteria, plasmids, and phage used in this study are listed in Table 1. *B. melitensis* was routinely cultured in brucella broth (Difco Laboratories, Detroit, Mich.). *E. coli* was routinely grown in Lennox broth (Gibco BRL). When solid medium was required, the above media were supplemented with 1.5% (wt/vol) agar (Difco). When antibiotic

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TABLE 1. Bacterial strains, plasmids and bacteriophage

Strain, plasmid, or bacteriophage	Relevant comment(s)	Source or reference
Strain		
<i>B. melitensis</i> 16M	Wild type	G. Schurig ^a
<i>B. melitensis</i> Rev1	R vaccine strain	G. Schurig
<i>B. melitensis</i> RM1	Tn5-induced R mutant of strain 16M	G. Schurig
<i>B. melitensis</i> 201	<i>purE</i> mutant of strain 16M	13
<i>B. abortus</i> 2308	Wild type	G. Schurig
<i>B. abortus</i> S19	Vaccine strain	G. Schurig
<i>B. suis</i>	Wild type	ATCC ^b 4312
<i>B. ovis</i>	Wild type	ATCC 25840
<i>B. canis</i>	Wild type	ATCC 23365
<i>Brucella neotomae</i>	Wild type	ATCC 23459
<i>E. coli</i> DH5 α	ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169, <i>recA1</i> ; recombinant DNA host	GIBCO/BRL
<i>E. coli</i> LE392	<i>supE44 supF58 hsdR514</i> ; host for bacteriophage DNA preparation	Promega
<i>E. coli</i> Y1090	pMC9 (Ap ^r); Δ <i>lacU169</i> Δ <i>lon</i> ; host for screening λ gt11 plaques	Promega
<i>E. coli</i> K165(pGP1-2)	<i>htpR</i> mutant containing T7 RNA polymerase expression plasmid	23
Plasmid		
pSK+	Ap ^r , <i>lacZ</i> α -complementing cloning vector	Stratagene
pBM4D1	5.4-kb <i>EcoRI</i> -to- <i>KpnI</i> fragment cloned from λ 4D recombinant phage into pSK+	This study
pBM4D2	<i>PstI</i> deletion of pBM4D1 in pSK	This study
pBM4D2	<i>PstI</i> deletion of pBM4D1 in pSK+	This study
pBM4D3	1.8-kb <i>Erase-a-Base</i> deletion beginning at the <i>EcoRI</i> site of pBM4D1	This study
pBM4D4	2.3-kb <i>PstI</i> fragment cloned from pBM4D1 into pSK+	This study
pBM4D6	pET23b T7 expression plasmid encoding Omp28	This study
pET23b	Ap ^r , T7 promoter expression plasmid for <i>E. coli</i>	Novagen
pGP1-2	Km ^r , T7 RNA polymerase encoding plasmid	Stanley
pHC79	Cosmid vector	Tabor ^c
pC4H	<i>omp28</i> -containing cosmid	GIBCO/BRL
pG5A	<i>omp28</i> -containing cosmid	This study
Bacteriophage		
λ gt11	<i>lacZ</i> λ cloning vector with single <i>EcoRI</i> site	Promega
λ 4D	Immunoreactive λ recombinant encoding <i>B. melitensis omp28</i>	This study

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selection was appropriate, the above media were supplemented with 100 μ g of ampicillin per ml. Propagation and storage of bacteriophage λ were as described by Silhavy et al. (32).

Chimera pBM4D1 contained the 5.4-kb *EcoRI*-to-*KpnI* fragment of recombinant bacteriophage λ 4D in similarly cleaved pSK+ (Stratagene, La Jolla, Calif.). Recombinant pBM4D2 was constructed by digestion of pBM4D1 with *PstI* followed by self-ligation. Recombinant pBM4D3 was generated by exonuclease III and S1 nuclease digestion of *EcoRI*- and *SacI*-cleaved pBM4D1 followed by blunt-end self-ligation (*Erase-a-Base*; Promega, Madison, Wis.). Plasmid pBM4D4 was constructed by digestion of pBM4D1 with *PstI* and isolation of the 2.3-kb insert DNA fragment. The purified 2.3-kb *PstI* fragment then was ligated into similarly digested pSK+.

Genomic, plasmid, or bacteriophage DNA preparation; recombinant techniques; and DNA sequencing. Total genomic DNA was prepared from *Brucella* spp. as described previously (25). Plasmid DNA was prepared with either the Wizard Mini-preps or Maxi-preps DNA purification kit (Promega) according to the manufacturer's instructions. Bacteriophage DNA was prepared from 50-ml lysates with the Lambda Midi kit (Qiagen, Chatsworth, Calif.).

Restriction digestion, cloning of DNA fragments into plasmid vectors, and fractionation of DNA on agarose gels were performed as described by Sambrook et al. (30). Maximum-efficiency *E. coli* DH5 α competent cells were purchased from Gibco BRL. Nucleotide sequences were determined with an Applied Biosystems Incorporated (Foster City, Calif.) model 370A DNA sequencer. Sequencing reactions were with the ABI Prism Cycle sequencing kit. DNA or protein sequences were analyzed by using either PC/Gene (Intelligenetics Corporation, Mountain View, Calif.) software, Genetics Computer Group (Madison, Wis.) software running on a VAX computer (10), or Sequencher software (Gene Codes Corp., Ann Arbor, Mich.) for the Macintosh. Oligonucleotide primers were prepared with an ABI model 394 synthesizer.

PCR was performed with Hot Tub DNA polymerase (Amersham, Arlington Heights, Ill.). Reaction mixtures of 100 μ l contained 10 ng of plasmid template, 50 pmol of each primer, 200 μ M deoxynucleoside triphosphates, and reaction

buffer supplied by the manufacturer. All reactions were optimized for the various primer pairs used in a particular experiment. PCR products were purified with the Wizard PCR purification kit (Promega).

Southern blotting and hybridization. *Brucella omp28*- and *omp25*-specific probes were generated by PCR. A *B. melitensis omp28*-specific probe was generated by PCR of 100 ng of strain 16M chromosomal DNA with the primers GAACACTCGTGCTAGCAATT and TCACGGCGGAGGGATTATCA. PCR conditions were 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and polymerization at 72°C for 30 s. A *B. abortus omp25*-specific probe was generated by PCR of 100 ng of strain 2308 chromosomal DNA with the primers TCTCTCCTAATCGTCTCGGCTGCGC and TTGTTGCGAACAGT CGTACCGGCC. PCR conditions were the same as those above except that annealing was at 55°C.

Fragments generated by restriction enzyme digestion of 1.5 μ g of *Brucella* spp. chromosomal DNA fractionated on 0.7% agarose gels were transferred to maximum-strength Nytran membranes (Schleicher and Schuell, Keene, N.H.) and hybridized at 42°C as suggested by the manufacturer. The membranes were washed under high-stringency conditions at 65°C as follows: two 15-min washes in 1 $\times$ Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin [BSA])–2 $\times$ SSC (20 $\times$ SSC is 3 M NaCl, 0.3 M sodium citrate), three 15-min washes in 2 $\times$ SSC–0.1% sodium dodecyl sulfate (SDS), and three 30-min washes in 0.1 $\times$ SSC–0.1% SDS. The membranes were dried and autoradiographed on X-Omat AR film (Kodak, Rochester, N.Y.) at –80°C.

Expression plasmid construction, antiserum production, and immunoelectron microscopy. A T7 promoter-based expression clone of the *omp28* coding region was constructed in the pET23b vector (Novagen, Madison, Wis.). The coding region was amplified by PCR. The 5' primer was GGTCGGATCCTCAGGAG AATCAGATGACGACGC, and the 3' primer was GATCGTCGACCTTGATT TCAAAAACGACATTGACCG. When the amplified DNA was digested with *Bam*HI and *Sal*I and ligated with similarly cleaved pET23b, the resulting plasmid encoded amino acids 28 through 250 of Omp28. Residues encoded by the vector or oligonucleotides added during cloning were MASMTGGQQMGRDP and

VDKLAALAEHHHHHHH at the amino and carboxyl termini, respectively. The DNA sequence of the *omp28* expression plasmid was confirmed, and the plasmid was designated pBM4D6. For overexpression in *E. coli*, pBM4D6 was used to transform strain K165 containing the T7 RNA polymerase-encoding plasmid, pGP1-2, to ampicillin resistance (23). Growth of bacteria for expression and purification of recombinant Omp28 (rOmp28) was as described previously (23). rOmp28 was purified from crude lysates by Ni^{2+} binding chromatography with the His-Bind system (Novagen) according to the manufacturer's instructions. Purity was evaluated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining. Purified rOmp28 was greater than 95% pure by this method as judged by scanning densitometry. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) with BSA as standard.

Polyclonal rOmp28 antiserum was prepared with New Zealand White rabbits (Biotech Research Laboratories, Rockville, Md.). Rabbits 294, 295, and 296 were initially injected with 1 mg of purified rOmp28 mixed with Freund's complete adjuvant. At 2-week intervals, the rabbits were boosted with 1 mg of purified rOmp28 mixed with Freund's incomplete adjuvant. Two weeks after the final boost, production serum was collected from each rabbit. The final titer of the serum collected from all the rabbits was greater than 1:6,400 when tested against purified rOmp28 by enzyme-linked immunosorbent assay.

Ab preparation and purification. We used several sources of human antisera. First, human Ab was obtained from James Murphy at the United States Naval Medical Research Unit 3 in Cairo, Egypt. This Ab preparation did not represent individual patients but rather a pool of persons who had positive *B. melitensis* cultures. This antiserum was designated pooled human serum (PHS). PHS was prepared by purifying the immunoglobulin G (IgG) with a protein A/G column (Pierce). Second, we obtained human sera from individuals who had been infected with *Brucella* spp. Ab from individual patients was obtained from Arnold Kaufmann, Division of Bacterial and Mycotic Infections, Centers for Disease Control and Prevention, Atlanta, Ga. These patients included two who were culture positive for *Brucella suis* infection (samples 8093 and 8089), two who were culture positive for *B. abortus* infection (samples 8755 and 8855), and four who were not culture positive (samples 7445, 7468, 8150, and 8160). However, all of the patients who were not culture positive had anti-*B. abortus* titers equal to 1:320 or greater by the standard tube agglutination test. Third, a single serum sample was obtained from David Taylor, Naval Medical Research Institute Detachment, Lima, Peru. This latter patient had an anti-*B. abortus* titer of 1:1,280; however, the infection was not confirmed by culture of the organism.

Rabbit Ab against *B. abortus* S1119-3 killed whole cells was obtained from Difco Laboratories (product number 2871-47-7). The mouse antiserum was obtained from two individual BALB/cHSD mice which had been infected with 10^5 CFU of *B. melitensis* 16M by intraperitoneal injection 42 or 56 days earlier. Goat Ab was obtained from animals which had been infected with 10^{10} CFU of attenuated *B. melitensis* $\Delta\text{purE201}$ (13) 14 weeks before the serum sample was taken. Sera from swine naturally infected with *B. suis* and cattle experimentally infected with either *B. abortus* 2308 or RB51 were obtained from Steven Olsen, National Animal Disease Center, Ames, Iowa. Antisera directed against *B. abortus* 2308 group 2 and group 3 OMPs (39) were obtained from A. J. Winter, Cornell University, Ithaca, N.Y.

All antisera were absorbed with an *E. coli* Y1090 lysate column (Pierce) to remove Abs which recognized cloning host proteins. Briefly, 0.1 ml of antiserum was passed over a 5-ml lysate column as suggested by the manufacturer. All dilutions are given in relation to the original volume of serum before purification.

Library construction and screening. *B. melitensis* 16M chromosomal DNA was partially digested (30) with *Hae*III to yield blunt end fragments with an average size of 2.5 kb. The DNA fragments then were treated with *Eco*RI methylase (Promega) and ligated with the Promega *Eco*RI linkers that had CCGGAAT TCCGG as the nucleotide sequence. Ligation and packaging were performed as described by the manufacturer (Promega). The packaged mixture was used to infect *E. coli* Y1090 (32). The average size of DNA insert in the library was determined by PCR of isolated plaques with the λgt11 forward and reverse primers (Promega) followed by agarose electrophoresis.

Screening for recombinant phage which encoded *B. melitensis* immunoreactive protein was performed as described previously (30) with Nytran maximum-strength filters (Schleicher and Schuell). The filters were blocked with filler buffer, which was phosphate-buffered saline (PBS) (NaCl, 8.5 g/liter; KH_2PO_4 , 0.3 g/liter; Na_2HPO_4 , 0.6 g/liter [pH 7.4]) containing 1% casein, 1% BSA, 0.01% phenol red, and 0.02% sodium azide. PHS was diluted 1:1,000 in TNT buffer (0.01 M Tris, 0.15 M NaCl, 0.05% Tween 20 [pH 8.0]) and incubated with the filters for 3 h. The filters were washed and allowed to incubate with goat anti-human alkaline phosphatase-conjugated Ab diluted 1:7,500 (Promega) for 1 h 30 min. For development, the filters were washed and incubated with Western Blue substrate (Promega). Positive plaques were purified to homogeneity by several subsequent rounds of screening as described previously (30).

A cosmid library of *B. melitensis* 16M was constructed by partial digestion of genomic DNA with *Eco*RI to yield fragments of approximately 40 kb (30). The cosmid library was constructed by ligation of these chromosomal fragments into similarly cleaved pHC79 (30) followed by packaging (Promega) and infection of *E. coli* LE392. The number of recombinant phage in the cosmid library was determined by titrating the concentration of Ap^r transducing units. The library was screened by colony hybridization on nitrocellulose filters (Schleicher and

Schuell) with a *B. melitensis omp28*-specific probe as described by the manufacturer.

Radioactive labeling of proteins, protein electrophoresis, and Western blotting (immunoblotting). In vitro transcription and translation of linear DNA templates were performed with *E. coli* S30 extracts for linear templates (Promega). Template DNA was generated by PCR of various plasmids with the M13 forward and reverse universal primers. In vitro transcription and translation products were labeled with [^{35}S]methionine and separated on 4 to 20% denaturing polyacrylamide gels (SDS-PAGE) according to the method of Laemmli (21). SDS-PAGE gels then were treated with Enlightening (New England Nuclear, Boston, Mass.), dried, and fluorographed with X-Omat AR film (Kodak) at -70°C . Protein sizes were determined by comparison with ^{14}C -labeled Rainbow molecular weight markers (Amersham).

Western blotting was performed according to the method of Towbin et al. (36). Nitrocellulose membranes were processed as described above for plaque lifts with the following exception: in some experiments with human sera, IgM-specific Ab (Jackson Laboratories, West Grove, Pa.) was used for the secondary incubation. For elution of Abs bound to purified plaques, *E. coli* Y1090 was infected with 10^5 recombinant phage and plated with top agar onto a 90-mm NZYCM agar plate. A plaque lift was performed as described above for the immunoscreening. PHS was diluted 1:1,000, allowed to react with the filter for 6 h, and then washed five times with 10 ml of TNT. The Abs bound to the proteins produced by the recombinant phage were eluted as described previously (33).

Fluorescence-activated cell sorting (FACS). *B. melitensis* RM1 and 16M were grown overnight, washed twice in 0.9% NaCl, and suspended at 10^7 bacteria per ml in Ca- and Mg-free Dulbecco's PBS (Gibco BRL). Bacterial suspensions (100 μl) were treated with 10 μl of diluted preimmune or rOmp28-immune rabbit serum or a 1/10 dilution of rabbit anti-*B. abortus* S1119-3 serum (Difco) in PBS. After incubation at 4°C for 30 min, bacteria were washed twice in PBS and treated with 1/100 fluorescein isothiocyanate-labeled goat anti-rabbit IgG (GAR-FITC) (Sigma, St. Louis, Mo.). After a further 30-min incubation, bacteria were washed twice more in PBS and treated with isotonic 4% paraformaldehyde in PBS, pH 7.0. Bacteria were acquired on a FACScan (Becton Dickinson, San Jose, Calif.), and the data were analyzed with CellQuest software (Becton Dickinson). Controls included unstained bacteria and bacteria treated with GAR-FITC to determine nonspecific binding.

Electron microscopy (EM). *B. melitensis* 16M was grown for 48 h at 37°C , and 1.5 ml of cells was harvested by centrifugation at $15,800 \times g$ for 10 min in a refrigerated microcentrifuge (Eppendorf, Westbury, N.Y.). The supernatant was removed, and the cells were suspended in 500 μl of fresh buffered glutaraldehyde (2.5% glutaraldehyde, pH 7.2). The killed cells were dehydrated in graded ethanol washes and then embedded in LR White medium (Polysciences, Inc., Warrington, Pa.). Embedding was by successive suspensions of the cells in mixtures of LR White and ethanol at ratios of 33%:67%, 50%:50%, and 75%:25%, respectively. The final embedding step was in 100% LR White for 2 h at 4°C . The cells were pelleted, and fresh LR White was used to embed the processed cells in gelatin capsules. The capsule was baked at 65°C overnight. Thick and thin sections were cut for examination. Thin sections were placed on 200-mesh nickel grids for EM.

Immunogold staining of thin sections was by placing the grid sample side down onto a drop of solution at room temperature. The samples were blocked by incubation with filler buffer for 30 min. The grids were placed with dilutions of rabbit primary Ab for 30 min. All Abs were diluted in filler buffer. The grids were washed three times by placing them on drops of PBS for 5 min. Secondary goat anti-rabbit labeled with 10-nm gold particles (DAKO) was added at a 1:10 dilution and then incubated for 30 min. The grids were washed as described above and blotted dry. Primary Ab was either rabbit preimmune serum, polyclonal anti-rOmp28 (see above), anti-group 3 Ag (A. J. Winter), or anti-*B. abortus* S1119-3 (Difco). Grids containing bacterial sections were examined with a Philips 400 HM transmission electron microscope at an acceleration voltage of 80 kV.

Nucleotide sequence accession number. The *B. melitensis* 16M *omp28* DNA sequence was submitted to GenBank and assigned accession number U30815.

RESULTS

Cloning of the *B. melitensis* 16M Omp28 immunoreactive protein in *E. coli*. PHS was used as primary Ab on Western blots of crude extracts of *B. melitensis* RM1 and *E. coli* Y1090. At the 1:1,000 concentration used here, this Ab recognized at least eight proteins produced by *B. melitensis* RM1 that were 80, 57, 43, 36, 34, 28, 22, and 15 kDa in size (22). Purified PHS did not react with any *E. coli* proteins. PHS was used to screen our λgt11 library of *B. melitensis* 16M genomic DNA. Ninety-four percent of the phage in the nonamplified library contained insert DNA with an average insert size of 0.6 kb. Accordingly, the entire genome of *B. melitensis* 16M was included in 22,000 recombinant phage (30). Our entire library consisted of $1.65 \times$

FIG. 1. Identification of immunoreactive protein encoded by the λ 4D clone. PHS was allowed to bind to purified recombinant clone λ 4D and washed, and then the bound Ab that recognized the protein encoded by the *B. melitensis* insert DNA was eluted (see Materials and Methods). The eluted Abs then were used to probe a Western blot of crude extracts of the R mutant *B. melitensis* RM1 and *E. coli* Y1090. Each lane contained approximately 10^6 CFU. The sizes of protein molecular mass markers are given in kilodaltons on the left. The position of the 28-kDa immunoreactive protein encoded by λ 4D is denoted by the arrowhead.

10^6 recombinant phage. To ensure complete coverage of the genome, we screened ca. 80,000 phage from our library without amplification. We isolated five recombinant phage that were recognized by the PHS. None of the reactive phage required the presence of isopropyl- β -D-thiogalactopyranoside to synthesize the *B. melitensis* Ag, i.e., none of the cloned genes encoding the Ags were fusions with β -galactosidase.

One of the immunoreactive phage described above was chosen for further characterization and was designated clone λ 4D. Ab eluted from purified λ 4D plaques recognized a 28-kDa protein present in whole-cell extracts of *B. melitensis* RM1 as shown in Fig. 1. The immunoreactive doublet shown in Fig. 1 is most likely due to processing of the protein during insertion into the outer membrane of *B. melitensis* RM1 or cross-reactivity with another *Brucella* protein, possibly Omp25 (see below). Initial characterization of the insert DNA contained within cloned 4D revealed that, during library construction or cloning, one of the two *Eco*RI sites normally present within λ gt11 recombinant phage was absent. By performing various restriction enzyme digestions, we determined that the missing *Eco*RI site was the one that would be downstream relative to *lacZ* in the λ gt11 vector (30). The *B. melitensis* insert DNA along with some vector DNA was excised from clone λ 4D on a single 5.4-kb *Eco*RI-to-*Kpn*I fragment, and we ligated it into similarly cleaved pSK+. The resultant plasmid was designated pBM4D1.

Genetic characterization of the *omp28* locus. We determined a restriction map of pBM4D1 (Fig. 2, top). Since an *Eco*RI site was missing from the original λ 4D clone, we were unsure where the *B. melitensis* DNA ended in pBM4D1. However, there were no *Cla*I sites near the *Eco*RI cloning site in λ gt11. This fact indicated that at least the DNA encoded by pBM4D1 between the *Eco*RI and *Cla*I sites shown in Fig. 2 was donated by *B. melitensis* 16M.

We prepared several subclones of pBM4D1 (Fig. 2). Insert DNA of the various clones was amplified by PCR and used as template for in vitro transcription and translation reactions. The insert DNA encoded by pBM4D1 was the only clone that produced a 28-kDa protein (Fig. 2, bottom panel). Subclones pBM4D2 and pBM4D3 insert DNAs did not produce any detectable proteins, suggesting that the initiation of transcription and translation of *omp28* was between the *Eco*RI and *Pst*I sites within pBM4D1 (Fig. 2, top). Subclone pBM4D4 appeared to produce a truncated protein of 21 kDa (Fig. 2, lower panel). The truncated 21-kDa protein encoded by pBM4D4 did not react with the purified human convalescent Ab. The 18-kDa protein produced by pBM4D4 was most likely a degradation product of the truncated 21-kDa polypeptide. Taken together, the results of in vitro transcription and translation of the various plasmid subclones allowed us to assign the direction of transcription and approximate location of *omp28* as shown in the top panel of Fig. 2.

Nucleotide sequence of *B. melitensis* 16M *omp28*. We determined the nucleotide sequence of a 1.025-kb region of *B.*

FIG. 2. Subcloning and in vitro transcription and translation of *B. melitensis* *omp28*. Regions of λ 4D insert DNA included in the plasmid clones are indicated by black lines in the top portion of the figure. The position of the *omp28* locus is represented by the arrow and is labeled above the restriction map of pBM4D1. Sizes in kilobases are given below the map of pBM4D1. Restriction enzyme symbols are as follows: C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I. The reactivity of bacteria containing the various plasmid constructs on Western blots is indicated at the top right of the figure. The bottom panel shows the in vitro transcription and translation products produced by the various fragments of *B. melitensis* DNA and separated by SDS-PAGE followed by fluorography. The plasmid used as template in the PCR that produced the polypeptides shown in the fluorogram is indicated above each lane. The lane labeled control contained protein synthesized by positive control linear DNA (Promega). The negative control lane was from a reaction that did not contain template DNA. The numbers to the right of the fluorogram indicate the size of the protein in kilodaltons.

FIG. 3. Nucleotide sequence of *B. melitensis* *omp28*. The nucleotide sequence is numbered at the left. The predicted protein sequence is shown in single-letter amino acid code below the DNA sequence. The underlined amino acids represent the predicted secretion signal peptide at the amino terminus. Nucleotide residues that are overlined indicate the position of the *Pst*I site shown in Fig. 2. The underlined nucleotides labeled IR indicate an inverted repeat. The DNA sequence homology with the previously reported *Brucella* repeat sequence (17) is labeled and underlined.

melitensis 16M DNA around the *Pst*I site within pBM4D1 (Fig. 2). A single open reading frame (ORF) was identified, one encoding a protein predicted to have a molecular mass of 26,552 Da (Fig. 3). The predicted molecular weight for this ORF agreed with the observed molecular mass of the 28-kDa immunoreactive polypeptide. Furthermore, the direction of transcription and the location of the ORF were in agreement with the experimental evidence presented in Fig. 2. We designated the ORF shown in Fig. 3 as *omp28*. The amino acid sequence of *omp28* included a putative secretion signal of 28 residues beginning at the amino terminus of the predicted protein (Fig. 3). The nucleotide sequence of the DNA downstream from *omp28* included an inverted repeat with a short stem loop of 5 bp followed by a cluster of seven dTMP residues (Fig. 3). This structure may represent a transcriptional terminator encoded by the DNA sequence. Further downstream from this putative terminator (Fig. 3), we found a region of nucleotides with 98% homology to a *B. abortus* palindromic repeat element (17). The GC content of the *omp28* ORF was

55%, which is in close agreement with the published value of 58% for *B. melitensis* 16M (18).

We searched the current Swiss Protein and GenBank databases with the *B. melitensis* *omp28* protein predicted by translation of the nucleotide sequence. The only significant protein similarity was found with the *Omp25* group 3 protein of *B. abortus* (11). The predicted mature polypeptides of *B. melitensis* 16M *Omp28* and *B. abortus* 544 *Omp25* were 43% similar (Fig. 4). The Pcompare protein comparison routine of PC/ Gene produced an alignment score of 4.5 for *Omp28* and *Omp25*. Pcompare alignment scores above 3 are considered significantly similar.

It was possible that *Omp28* and *Omp25* were products of alleles of the same gene in two different *Brucella* species. To investigate this possibility and to determine if certain epitopes were shared between these two proteins, we performed Western blotting with previously characterized monoclonal Abs (MAbs). We obtained MAbs A59/01E11/D11 (11) and A59/05F01/C09 (7), which are known to react with *B. abortus* *Omp25*, and MAb A59/10F09/G10 (7), which reacts with a group of 31- to 34-kDa *B. abortus* proteins (A. Cloeckert, Institut National de la Recherche Agronomique, Nouzilly, France). Western blots of whole-cell extracts prepared from *E. coli* cells containing pBM4D1 and the pSK+ cloning vector revealed that *Omp28* and *Omp25* do not share the epitopes recognized by these MAbs.

Hybridization of *omp28* and *omp25* to *Brucella* spp. genomic DNAs. MAbs recognize only one epitope present on an Ag; therefore, results obtained through Western blotting with MAbs did not eliminate the possibility that *Omp25* and *Omp28* were products of alleles in different species. To address this question more fully and to determine the molecular relationship between *omp28* and *omp25*, we performed Southern blot hybridizations with specific gene probes. The gene probes were generated by PCR (see Materials and Methods) and were completely internal to the ORF that encoded the respective proteins. *B. melitensis* *omp28* and *B. abortus* *omp25* were both present in all *Brucella* species examined (Fig. 5). The 510-bp *B. melitensis* *omp28* probe hybridized with a 8.5-kb *Eco*RI fragment in the chromosome of *Brucella* spp. (Fig. 5A). In contrast, the 580-bp *B. abortus* *omp25*-specific probe hybridized with a 3.3-kb *Eco*RI fragment within the genome of all *Brucella* species examined. To further investigate the location of *omp25* and *omp28* within the *Brucella* spp. genome, we

FIG. 4. Alignment of *B. melitensis* *Omp28* and *B. abortus* *Omp25* (11) after removal of predicted secretion signal peptides. The sequences were aligned with the Genetics Computer Group Bestfit program with the amino acid similarity table and threshold at the default setting. Residue numbers are shown at the left and are relative to the complete protein including signal peptide. Amino acid identity is indicated by the vertical dashes. Amino acids that the program considers to be highly similar are shown as a colon. Similar amino acids are indicated by a single dot.

FIG. 5. Southern hybridization of *Brucella* DNAs with *omp28*- and *omp25*-specific nucleotide probes. (A) Hybridization of various *Brucella* spp. chromosomal DNAs with the *B. melitensis* 16M *omp28* probe. (B) Hybridization of *Brucella* DNAs with the *B. abortus* 2308 *omp25* probe. Approximately 1.5 μ g of each chromosomal DNA was digested with *Eco*RI. DNAs in each lane were as follows: 1, *B. melitensis* 16M; 2, *B. melitensis* Rev1; 3, *B. abortus* 2308; 4, *B. abortus* S19; 5, *B. suis*; 6, *Brucella neotomae*; 7, *B. canis*; 8, *B. ovis*.

repeated the Southern hybridizations with *Hind*III-digested DNAs. The *omp25* probe hybridized with a 4.1-kb fragment in all *Brucella* DNAs examined. In contrast, the *omp28* probe hybridized with an 11-kb *Hind*III fragment in the DNA of all species of *Brucella* tested. Therefore, *omp28* and *omp25* were located on different *Eco*RI and *Hind*III fragments that did not vary in size between the chromosomes of different *Brucella* spp. However, we did observe variation in the genome of the different species of *Brucella* with respect to *Eco*RV and *Cla*I fragments that hybridized to both the *omp25*- and *omp28*-specific probes (22). Our finding that the sizes of the DNA fragments were mostly constant between *Brucella* spp. was not surprising since this genus has been shown to be highly homologous at the genomic level (37).

We constructed a cosmid library of *B. melitensis* 16M genomic DNA in order to investigate the possible linkage of *omp28* and *omp25*. Our cosmid library contained a total of 1.3×10^4 recombinant bacteria. After screening approximately 672 isolates, we obtained two cosmids which hybridized with the *omp28*-specific probe. Both of the cosmids contained 8.5-kb *Eco*RI fragments that hybridized with the *omp28* probe (22). The *B. abortus* *omp25*-specific probe did not hybridize with either of these cosmids. It appeared that *omp28* and *omp25* are not closely linked on the *Brucella* chromosome. Taken together, the Southern hybridization and cosmid cloning experiments conclusively demonstrate that *omp28* and *omp25* are not alleles of the same gene in different *Brucella* species.

Surface localization of *B. melitensis* Omp28. In order to determine the cellular location of Omp28, we performed immunogold labeling and EM on thin sections of *B. melitensis* 16M. Typical gram-negative bacterial cell wall morphology in-

cluding a distinct peptidoglycan layer internal to an extensive outer membrane layer was observed (Fig. 6B). All three Ab preparations bound to the surface of the cells. Anti-rOmp28 was found at the cell surface (Fig. 6A). Binding of the anti-rOmp28 was sparse and appeared to cluster in patches at the cell surface. Some binding of the immunogold particles can be seen away from the cell surface. This binding most likely represents Omp28 located in membrane fragments or blebs (see inset, Fig. 6A) that have dissociated from the cell surface since the preimmune serum did not label the grids (Fig. 6B). The low level of Ab binding seen in Fig. 6A even in the presence of high titers of anti-Omp28 was most likely due to S LPS on the surface of strain 16M blocking access to Omp28 (6, 7, 19, 41) (see also Fig. 7). We noted a large number of cells which appeared to have membrane blebs that reacted strongly with the polyclonal anti-rOmp28 (Fig. 6A, inset). In contrast, the sections labeled with preimmune rabbit serum were not labeled by the immunogold secondary Ab (Fig. 6B). Use of anti-group 3 polyclonal rabbit serum (A. J. Winter) caused significant labeling of the surface of *B. melitensis* 16M that was evenly distributed over the surface of the cells as seen in Fig. 6C. Similarly, commercial anti-*B. abortus* (Difco) bound to the surface of the cells (Fig. 6D). It should be noted that both the Difco and the anti-group 3 sera contained anti-*Brucella* LPS.

EM experiments suggested that Omp28 was located within the cell wall of *B. melitensis* 16M. In order to confirm these observations and to better define the location of Omp28, we performed FACS on whole bacteria with polyclonal rabbit anti-rOmp28. Flow cytometric analysis demonstrated that IgG from anti-rOmp28 serum bound to the surface of RM1 in a dose-related manner, while preimmune serum did not bind (Fig. 7). In contrast, we could not detect the binding of preimmune or anti-rOmp28 serum to RM1's S parent, 16M, by FACS (Fig. 7A). Anti-*B. abortus* serum (Difco), which primarily recognizes the O-polysaccharide side chain of LPS, bound 16M but not RM1 (Fig. 7B). These studies verified the absence of O polysaccharide on the surface of RM1. They also indicated that the presence of O polysaccharide on the S parent strain interfered with binding of anti-Omp28 to the surface of the organism, presumably by steric hindrance. Most importantly, the EM and flow cytometric data demonstrate that Omp28 is located on the surface of the bacteria.

Immunoreactivity of *B. melitensis* Omp28. We tested various sera for Abs that recognized Omp28 by Western blotting. Figure 8B, C, D, and E show that sera from eight of nine humans tested at a 1:1,000 dilution or greater reacted with Omp28. The one remaining human serum which did not react at the 1:1,000 concentration did react with Omp28 at a dilution of 1:250. Patient 8755 (Fig. 8D) had culture-confirmed brucellosis caused by *B. abortus*. Patient sera (7445, 8150, 8160) had anti-*B. abortus* agglutination titers of at least 1:320. However, the latter three patients were not confirmed by culture; therefore, we did not know which *Brucella* spp. induced the Ab which recognized Omp28. Most individual human sera contained both IgM and IgG that reacted with the *B. melitensis* group 3 protein. A few patients' sera had either IgM or IgG but not both. Rabbits which had been immunized with killed *S. B. abortus* whole cells produced Abs which recognized Omp28 although the response appeared weak compared with that of infected mice, goats, or most humans (Fig. 8). Omp28 elicited a humoral immune response in goats and mice that were experimentally infected with *B. melitensis* 201 or *B. melitensis* 16M, respectively (Fig. 8G and H). In our experience, Omp28 was not recognized by sera obtained from *B. abortus* 2308-infected cattle or swine naturally infected with *B. suis* (22). Normal human, mouse,

FIG. 6. EM of *B. melitensis* 16M stained with Abs directed against various cell surface components. Single arrowheads indicate areas where primary Ab was recognized by the gold-labeled goat anti-rabbit serum. Double arrowheads indicate cell surface. (A) Polyclonal rabbit anti-rOmp28 reactivity. The inset shows labeling of a putative *B. melitensis* 16M membrane bleb. Primary Ab was diluted 1:200. (B) Reactivity of preimmune serum obtained before immunization of the rabbit shown in panel A. The primary Ab dilution was as in panel A. (C) *B. melitensis* labeled with anti-group 3 Ag serum (A. J. Winter). The primary Ab was diluted 1:10. (D) Representative labeling observed with Difco commercial anti-*B. abortus* serum. The primary Ab was diluted 1:10 before addition to the grids containing thin sections. Bar (A, B, and C), 0.25 μm ; bar (A [inset] and D), 0.125 μm .

rabbit, and goat sera did not react with extracts derived from cloned *B. melitensis* Omp28 on Western blots (22).

We obtained rabbit immune sera that were prepared separately against *B. abortus* 2308 group 2 and group 3 proteins from Alexander Winter (39). Western blots of crude extracts of *E. coli* DH5 α containing pBM4D1 compared with those for cells containing the pSK+ cloning vector revealed that Omp28 reacted with both the anti-group 2 and anti-group 3 sera (22).

DISCUSSION

Brucella group Ags were originally described over 14 years ago according to their molecular weight, heat modification characteristics, and presence in outer membrane preparations (38). Group 3 Ags were designated as having a molecular mass of approximately 30 kDa that was unchanged by heating of the samples before electrophoresis. The first *B. abortus* group 3 Ag that has been described was designated Omp25 (11). By the

FIG. 7. Detection of rabbit anti-*B. abortus* and anti-rOmp28 on R (RM1) and S (16M) *B. melitensis* by FACS. (A) Pelleted, suspended bacteria (100 μ l) were treated with 10 μ l of diluted serum from nonimmunized (PRE) or Omp28-immunized (POST) rabbits. Dilution was 1/10 except as noted. Surface binding of Ab was detected with GAR-FITC. (B) Bacteria were treated with a 1/10 dilution of rabbit anti-*B. abortus* S1119-3 (Difco), and binding was detected with GAR-FITC. Nonstained blank was cells reacted with anti-rOmp28 but no secondary GAR-FITC Ab (not shown for clarity). Nonspecific binding (NSB) (bacteria treated with GAR-FITC alone) was similar to the nonstained blank.

criteria described above (38), our results strongly indicate that Omp28 is a group 3 Ag of *B. melitensis* 16M that is different from Omp25 of *B. abortus*. First, *B. melitensis* Omp28 was located on the cell surface as demonstrated by EM (Fig. 6)

and flow cytometry (Fig. 7). Second, the molecular weight of Omp28 was within the range of *Brucella* group 3 Ags described previously (38, 39). Furthermore, Omp28 is significantly similar to a previously described group 3 Ag, Omp25 (Fig. 4). The

FIG. 8. Reactivity of cloned *B. melitensis* Omp28 with various sera by Western blot. The absorbed Ab which was used on the Western blot is indicated below each panel (see Materials and Methods). The immunoreactive protein is indicated by the labeled arrowhead. Each lane contained approximately 10^6 CFU of *E. coli* DH5 α containing either pBM4D1 (lanes 1) or the pSK+ cloning vector (lanes 2). The dilution of all the primary Abs was 1:1,000.

migration of Omp28 did not change upon heating of the samples before SDS-PAGE, i.e., the protein was not heat modifiable. Third, Omp28 was recognized by immune rabbit serum (39) prepared against purified group 3 Ags. Fourth, Omp28 and Omp25 were encoded on different *Eco*RI fragments within the *Brucella* genome and have different DNA sequences (Fig. 5 and Results). Accordingly, they are certainly encoded by two different genes. Taken together, these data indicate that *Brucella* DNA encodes the potential to synthesize at least two different group 3 Ags.

The fact that *B. melitensis* Omp28 and *B. abortus* Omp25 are both group 3 Ags which are distinct from each other could explain the variability seen previously in group Ag molecular weight (31, 39). Since *Brucella* spp. do encode two group 3 Ag loci, this may be similar to *B. abortus* group 2 porin genes (15). The *omp2* locus of *B. abortus* encodes two closely linked homologous proteins (14). However, so far only one of the ORFs is known to be expressed in *B. abortus* (26). It would appear that both *B. melitensis* Omp28 (Fig. 8) and *B. abortus* Omp25 (7, 11) are expressed by their respective species at least in vivo. It remains to be determined whether both of the group 3 Ags are expressed in a single *Brucella* species and if so what benefits to the host differential expression of OMPs might have for this pathogen.

Our results demonstrate that infection with *B. melitensis*, which is a S LPS-producing species, can elicit an Ab response to a group 3 protein. This finding was not surprising (29). However, production of Ab directed against Omp28 was host specific (Fig. 8 and results) since infected cattle and swine do not appear to produce a humoral immune response directed against this Ag. Accordingly, our results emphasize the need to study the immune response elicited by infection of various animals with *Brucella* spp. Ab recognition of *B. abortus* surface proteins such as OMPs may offer some protection against brucellosis caused by S LPS-producing species (20, 34). Humoral immune recognition following S *Brucella* infection is

only partially protective, and the highest level of protection is achieved with serum plus T cells (1, 2). In contrast, immunity to infection with R species of *Brucella* is most likely quite different from that necessary for protection against challenge with S species. Jimenez de Bagues et al. (19) have shown that passive transfer of serum is approximately four to six times more effective than transfer of T cells at preventing colonization of mice following exposure to *B. ovis*. Animals immunized with hot saline extracts of *B. ovis* containing predominately group 3 Ag (16) but depleted of R LPS were highly protected against infection with the homologous species (19). Furthermore, no saline extracts of *B. abortus* strongly stimulate T cells following infection of mice (45). Taken together, these observations suggest that the group 3 Ags may be a good candidate for inducing cellular immunity to infection by S strains and humoral immunity to R strains of *Brucella*. Experiments are currently under way in our laboratory to evaluate this hypothesis.

Our data support the concept that OMPs are more accessible on the surface of R *Brucella* strains compared with S LPS-producing strains (6, 7, 19, 41). *B. melitensis* Omp28 was detected only on the surface of the RM1 R strain by FACS (Fig. 7). Furthermore, the immunogold labeling of Omp28 on the 16M S strain detected by EM was specific but at a low level (Fig. 6), suggesting that Omp28 is not highly exposed on the surface of these cells. Omp28 was also seen on the surface of membrane blebs (Fig. 6A). Gamazo et al. (16) noted that group 3 Ag was present in membrane blebs of both *B. ovis* and *B. melitensis*. Our observation that Omp28 was present on similar blebs confirms the previous findings and lends credence to the identification of Omp28 as a group 3 Ag.

The development of subcellular protein-based vaccines to prevent brucellosis has several advantages. One of the major advantages is the possible ability to differentiate vaccinated animals from diseased animals. The current live attenuated vaccines against animal brucellosis induce high-titer Abs against the O polysaccharide of the LPS, which interferes with the serological diagnosis of the disease (3–5, 24, 35). Accordingly, a protein component of the *Brucella* cell which elicits an Ab response that cross-reacts among all or most members of the genus would improve the specificity of diagnostic reagents for brucellosis. Our results suggest that Omp28 may be a good candidate for such diagnostic purposes. We are only now, after over 14 years, beginning to understand the molecular genetics of OMP production by *Brucella* spp. Further characterization of group 3 OMP genes, gene products, and expression is required before we will understand more fully how these proteins and loci contribute to pathogenesis. Studies are currently under way to characterize the expression of *omp28* in *Brucella* spp. and define the epitopes on the protein which may be of use in vaccine development as well as diagnosis of the disease.

ACKNOWLEDGMENTS

Antisera directed against group 2 and group 3 *B. abortus* OMPs were kindly provided by A. J. Winter (Cornell University, Ithaca, N.Y.). We thank S. M. Boyle and G. G. Schurig (Virginia Polytechnic Institute and State University, Blacksburg) for providing *B. melitensis* RM1, and we thank Steven C. Olsen and N. F. Cheville (National Animal Disease Center, Ames, Iowa) for providing sera from infected goats, cattle, and swine. MAb to *B. abortus* surface proteins were kindly provided by Axel Cloeckart (Institut National de la Recherche Agronomique, Nouzilly, France). We thank Aamena Khan and James T. Harris for their technical assistance. We thank Nancy Saunders and Stuart Cohen for oligonucleotide synthesis.

This work was supported by the United States Army Medical Research and Materiel Command.

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