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R. L. Chirhart-Gilleland  
*LSU Medical Center at Shreveport*

M. E. Kovach  
*LSU Medical Center at Shreveport*

P. H. Elzer  
*LSU Medical Center at Shreveport*

S. R. Jennings  
*LSU Medical Center at Shreveport*

R. M. Roop  
*LSU Medical Center at Shreveport*

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## Identification and Characterization of a 14-Kilodalton *Brucella abortus* Protein Reactive with Antibodies from Naturally and Experimentally Infected Hosts and T Lymphocytes from Experimentally Infected BALB/c Mice

REBECCA L. CHIRHART-GILLELAND,<sup>†</sup> MICHAEL E. KOVACH, PHILIP H. ELZER,<sup>‡</sup>  
STEPHEN R. JENNINGS, AND R. MARTIN ROOP II\*

Department of Microbiology and Immunology, Louisiana State University Medical Center,  
Shreveport, Louisiana 71130-3932

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**A low-molecular-weight recombinant *Brucella abortus* protein reactive with antibodies from a variety of naturally and experimentally infected hosts and T lymphocytes from experimentally infected mice was identified and given the designation BA14K. The gene encoding BA14K was cloned and characterized, and the predicted amino acid sequence of this immunoreactive protein showed no significant homology with previously described proteins. Sequences homologous to the cloned fragment encoding BA14K were identified by Southern blot analysis of genomic DNAs from representatives of all of the currently recognized *Brucella* species. Studies employing BA14K should contribute to our efforts to better understand the antigenic specificity of protective immunity to brucellosis.**

*Brucella* spp. are gram-negative, facultative intracellular bacterial pathogens which cause abortion and infertility in numerous domestic and wild mammals, as well as a disease known as undulant fever in humans (1). People become infected through direct contact with infected animals or animal products (16). Consequently, brucellosis in animals used for food is not only a serious economic problem but also a potential public health hazard. Attenuated live vaccines, such as *Brucella abortus* S19, which until recently was the vaccine used in cattle in the United States, and *Brucella melitensis* Rev1, which is used in sheep and goats in other parts of the world, have been used successfully in eradication and control programs (1). Although these vaccines have been invaluable components of eradication programs, there are significant problems associated with their use. These include the virulence of S19 and Rev1 for humans (24), the potential for abortion when these strains are used in pregnant animals (2, 17), and the development of agglutinating antibodies in animals vaccinated as adults which are indistinguishable from those elicited by natural infection (17). Clearly, the construction of brucellosis vaccines lacking these undesirable properties would be of great benefit to both veterinary medicine and human medicine.

Similarly to other infections caused by facultative intracellular pathogens, the induction of specific cell-mediated immunity is required for effective clearance of *Brucella* infections (4, 22). Unfortunately, the nature and antigenic specificity of protective cellular immunity against brucellosis are unclear (13). Therefore, the identification of *Brucella* cellular components

which contribute to the induction of protective responses in the host will be an important step in designing improved vaccines. The cloning and characterization of genes encoding immunoreactive *Brucella* proteins will provide a useful source of antigen for immunologic assays and subunit immunization studies. It will also facilitate the construction of replicating antigen delivery systems such as those based on salmonellae (7) and vaccinia virus (15). Studies employing both purified subunit preparations and live, recombinant antigen delivery systems should allow a comprehensive evaluation of the relative importance of specific *Brucella* proteins in eliciting protective immunity.

In an attempt to identify *Brucella* proteins capable of inducing protective immune responses, a collection of recombinant *Escherichia coli* clones expressing *Brucella* proteins reactive in immunoassays with sera from a variety of experimentally and naturally infected hosts was assembled (18). One of these clones, which was designated IV-4, produced a recombinant *Brucella* protein with an apparent molecular mass of 14 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Restriction enzyme analysis of the plasmid encoding the recombinant 14-kDa protein, which was designated pBA44, revealed the presence of a 1.8-kb insert. For ease of communication, the recombinant *Brucella* protein produced by clone IV-4 was designated BA14K.

To further examine the extent of the reactivity of BA14K with sera from naturally and experimentally infected hosts, cell lysates of recombinant *E. coli* DH5 $\alpha$  [F<sup>−</sup>  $\phi$ 80dlacZM15 $\Delta$  (*lacZYA-argF*)U169 *deoR recA1 endA1 phoA hsdR17* (r<sub>K</sub><sup>−</sup> m<sub>K</sub><sup>+</sup>) *supE44*  $\lambda$ <sup>−</sup> *thi-1 gyrA96 relA1*] carrying pBA44 and pUC9 were prepared and subjected to SDS-PAGE and immunoblot analysis by previously described methods (19, 20). Affinity-purified, species-specific anti-immunoglobulin G (IgG) horseradish peroxidase conjugates (Sigma Chemical Co., St. Louis, Mo.) were employed in these experiments. IgG-type antibodies reactive with BA14K were detected in sera from naturally infected dogs (Fig. 1), cattle (data not shown), and humans

\* Corresponding author. Mailing address: Department of Microbiology and Immunology, Louisiana State University Medical Center, P.O. Box 33932, 1501 Kings Highway, Shreveport, LA 71130-3932. Phone: (318) 675-5771. Fax: (318) 675-5764. E-mail: rroop@lsu.mc.edu.

<sup>†</sup> Present address: Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294.

<sup>‡</sup> Present address: Department of Veterinary Science, Louisiana State University Agricultural Center, Baton Rouge, LA 70803.

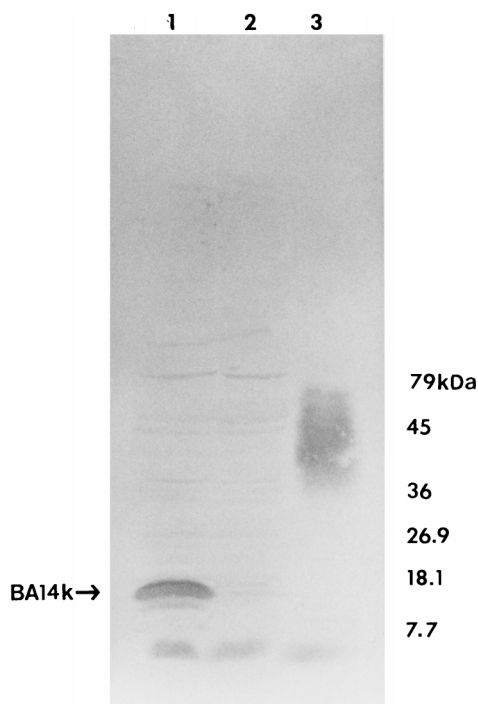


FIG. 1. Reaction of recombinant *E. coli* DH5 $\alpha$  producing BA14K in Western blots with serum from a dog naturally infected with *Brucella canis*. Lanes: 1, DH5 $\alpha$ /pBA44; 2, DH5 $\alpha$ /pUC9; and 3, *B. abortus* 2308 cell lysate.

(data not shown) and also in sera obtained from BALB/c mice and goats experimentally infected with *B. abortus* 2308 (data not shown). The detection of IgG-type antibodies specific for BA14K in sera from these hosts is relevant for several reasons. First, cattle, goats, dogs, and humans are important natural hosts for *Brucella* infections (1, 16), and mice represent a well-established model for both human (6, 25) and ruminant (14) brucellosis. Second, levels of IgG in infected hosts are directly correlated with the presence of active infections (8). Third, protective antibodies in the mouse model appear to be predominantly of the IgG class (9).

Lymphocyte proliferation assays were used to determine if BA14K-specific T lymphocytes were present in mice with chronic *Brucella* infections. Female BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) that were 8 to 10 weeks of age were infected with  $5 \times 10^4$  CFU of *B. abortus* 2308, *B. melitensis* 16M, or *B. abortus* S19 via the intravenous route by previously described procedures (10, 14). Between 28 and 30 weeks postinfection, five mice from each experimental group were euthanized with a halothane overdose. Their spleens were aseptically removed, and lymphocyte transformation assays were performed on pooled, single-cell suspensions of T-lymphocyte-enriched splenocytes by previously described methods (13). Test antigens were added to the splenocytes at concentrations of 0.5, 1, and 2  $\mu$ g of protein per well. A commercial T7 polymerase-based expression system (RSET; Invitrogen, San Diego, Calif.) was used for enhanced production of BA14K in recombinant *E. coli*. An overexpression plasmid (pRS44.8) was constructed by cloning the 1.8-kb fragment encoding BA14K into pRSETB (Invitrogen). This plasmid and pRSETB were independently introduced into *E. coli* BL21 ( $F^-$  *ompT*  $r_B^-$   $m_B^-$ ) (DE3) by the procedures described by Hanahan (11). *E. coli* cell lysates and *B. abortus* 2308 whole killed cells were prepared as previously described (20). Concanavalin

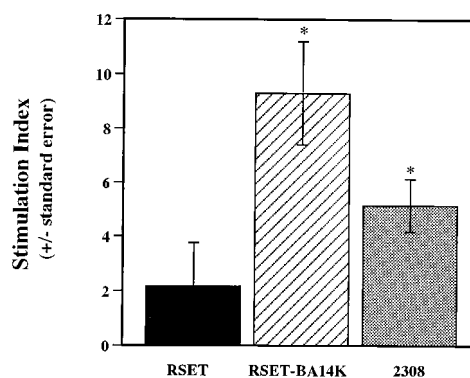


FIG. 2. Proliferative responses of T lymphocyte-enriched splenocyte cultures from *B. melitensis* 16M-infected mice at 28 weeks postinfection. Lymphocytes were stimulated with antigen (1- $\mu$ g protein concentration) for 96 h and pulsed with [ $^3$ H]thymidine for 18 h. Antigens: RSET, *E. coli* BL21 (DE3)/pRSETB cell lysate; RSET-BA14K, *E. coli* BL21 (DE3)/pRS44.8; 2308, *B. abortus* 2308 whole killed cells. Stimulation indices were calculated as described in the text. \*,  $P < 0.05$  compared to RSET.

A (ConA) (Sigma Chemical Co.) was used as a positive control. Splenocytes were incubated with antigen or ConA for 48, 72, 96, or 120 h and pulsed with [ $^3$ H]thymidine 18 h prior to harvesting. The results of the lymphocyte transformation assays were expressed as stimulation indices, which were calculated by dividing the counts per minute obtained with cells treated with antigen or mitogen by the counts per minute obtained with untreated cells. A stimulation index of  $\geq 3$  was considered a positive response. Statistical comparisons between experimental groups were performed by the two-tailed Student *t* test (23). At least four separate lymphocyte transformation assays were performed with T-cell-enriched splenocytes from mice infected with each of the *Brucella* strains used as immunogens. T lymphocytes reactive with BA14K were detected in BALB/c mice infected with virulent *B. melitensis* 16M (Fig. 2), *B. abortus* 2308 (data not shown), or the attenuated live vaccine strain 19 (data not shown) at 28 to 30 weeks postinfection. Because of the intracellular nature of *Brucella* infections, the induction of a cellular immune response is critical for effective host clearance of infection. Thus, detection of BA14K-reactive T lymphocytes in mice infected with both virulent and vaccine strains suggests that further investigation of the immunoreactive nature of this protein along with its capacity to induce protective immune responses is justified.

Western blot analysis showed that expression of BA14K was isopropyl- $\beta$ -D-thiogalactopyranoside inducible in *E. coli* JM109 [ $\Delta$ (*lac-proAB*) ( $F'$  *traD36 proAB lacI<sup>q</sup>*  $\Delta$ M15) *e14-(mcrA) recA1 endA1 hsdR17* ( $r_K^-$   $m_K^+$ ) *supE44 thi-1 gyrA96 relA1*], suggesting that the cloned gene encoding BA14K was expressed from the *lac* promoter resident in pUC9 (data not shown). By employing the dideoxynucleotide-based procedures described by Sanger et al. (21), nucleotide sequence analysis of the 1.8-kb insert in pBA44 confirmed that BA14K was produced in *E. coli* as a fusion composed of the C-terminal 133 amino acids of a native *Brucella* protein fused to the N-terminal 13 amino acids of the  $\alpha$ -subunit of  $\beta$ -galactosidase encoded by pUC9.

The complete gene encoding the native *Brucella* protein was cloned in the following manner. Southern blot analysis of *B. abortus* 2308 genomic DNA employing a 1.5-kb *Hind*III fragment from pBA44 which encompassed the BA14K coding region and the *Hind*III site from the multiple cloning site of pUC9 was used to identify a *Hind*III fragment of approxi-

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GGGCGACGGCTTCAGAGGCGCTGGTGGATGATGCTGCTGCGACGCCATGGGCGGTTCTATGACACATCAGCGCATGACCA 90
R H G L P E G L G A I H S L S H P I G A F Y D T H G M T

ATCGCGTCTTACGCGCTATGCTGCTGACACACACCGGGATGCCATGAGAAAGCGCATTCGCGCGCTGGCGATTATCTCGGCATGAG 180
N A V F M P Y V L H H N R D A I E K R I A R L A D Y L G I E

GCGGGTTGAGCGCTTTCGCGATGATCATGAAGCTGCGACGAACTGGCGTGGCGGCACTGCGCGCATCATCAAGAACTTC 270
G G F D G F A D A I M K L R T E L G V P R T L P D L I K N L

AGATGACCAATGCGCGAGGAGCTGATGCGGNAATGGCACTGGCAGATCCACCGCAGCGGAAACCGCGTTGACATTACAAAAGG 360
Q M D N A R K E L I A E M A V A D P T A G G R E V E L T K N

ACGCGCTAAAGTTGCTCAATGACGAGCTGGAGTAGTAATTCGCTGGCGACGCGTAAATCAGGTGCGCGTGGCGCGCATGGTGG 450
D A L K L L N A A L E G R *

GGCGGATGATGAGGGGCGATGTTCTTGGGAGGAGCGCTGCCCGCTTCATTCTTCTCTGCGCGAATGCCGCGAATAATGATA 540
+ + + + +

TTTCAAGCGTTTGACCTGGAGTTTGAATAGGAGCTTGACATGAACGCTTCAGGAAACTTCGCGCGCGCGCTTGGCTTTGATCTTT 620
M N S F R K T C A G A L A L I F 16

GGTGAACCTTCATCTGCTCCGACCGTATGATGAATGATGATGCGCGCGGATCAACCGAGAGCTGATGACGCGCGCGCGCAT 720
G A T S I V P T V A A P M N M D R P A I N Q N V I Q A R A H 46
A

TATCGTCCGCGAAGTAAATGATGCGCGCATGCGCGCGCTTACTGCGCGCGGATGCGCGCATGATGCGCGCATGATGCGCGCAT 810
Y R P Q N Y N R G H R P G Y M H G H R G Y R H Y R H G Y R R 76

CAGATGAGCGCTGGTATGCGCTGCGCGCTTGGTGGCGCGGATCATTCGCGCGCGGATCAACCGAGAGCTGATGACGCGCGCGCAT 900
H N D G W Y P L A A F G A G A I I G G A I S Q P R P V Y R 106

GCGCGCGCGAGTTGCGCGCATGCGCGATGCGCTGATAGCGCGTAAATGCTGATGCGCGCATGACGCGCATGATGCGCGCATGATA 990
A P A G S P H V Q W C Y S R Y K S Y R A S D N T F Q P Y N G 136

CAGCGAGCGAGTGGCTGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1080
P R K Q C R S P Y S R * 147

TGGTCTGATTGCGCATTTGCGATCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1170

ACAGTACGAGCGGTTCTGCTGCGAATGGCGCATGCGCGCGCGGAAATTAAGATGCGCGCGTGGCGCGCATTAATAATACATTT 1260

TTTAGGATCAGCTGATGAGGCGGTATATCTGCTGCTGCGCGATGCGCGCATGAGGCTTGAAGATTGCGCGCGCGGAAACGG 1350

TAATCATTCCTGCGACCATGCTGCGAGCGCGCGCGGATGCGCGTACGCGAGCGGTGCGCATGAAATGCGCGTCTGCGAATCTGCG 1440

CATACCGCATTCGACCGCGCGCTTCTGCTGCTGCGCGATGCGCGTGGCGCATGCGCGTATGCGCGCATGCGCGCGGCGATT 1515

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FIG. 3. Nucleotide sequence of the gene encoding the *B. abortus* immunoreactive protein BA14K and the deduced amino acid sequence of this protein. Underlining, putative ribosome-binding site; asterisks, stop codons; double dagger, fusion site between the *Brucella* DNA sequence and pUC9 in pBA44; open triangle, the predicted signal peptidase cleavage site; dashed arrows, inverted repeat, which may serve as a rho-independent terminator. A dot above every 10th nucleotide is present.

mately 4.6 kb in the genomic DNA preparation which likely contained the complete gene. Following NaCl gradient fractionation (12), *Hind*III fragments of 2308 genomic DNA ranging in size from 4 to 10 kb were cloned into the *Hind*III site of pBC/SK<sup>+</sup> (Stratagene, La Jolla, Calif.), and the resulting recombinant plasmid bank was used to transform *E. coli* DH5 $\alpha$ . Transformants were screened by colony blot hybridization with the 1.5-kb *Hind*III fragment from pBA44, and six reactive clones were identified. All six of these transformants contained recombinant plasmids containing 4.6-kb *Hind*III fragments that hybridized with the 1.5-kb *Hind*III probe derived from pBA44 by Southern blot analysis. One of these recombinant plasmids was selected for further evaluation and was given the designation pRLF6. Nucleotide sequence analysis with primers specific for the partial BA14K coding region in pBA44 revealed that the open reading frame encoding the complete *Brucella* protein was present in pRLF6 (Fig. 3). Computer-assisted analysis of this sequence indicated that it encoded a polypeptide of 147 amino acids with a predicted molecular weight of 16,822 and an isoelectric point of 11.47. An inverted repeat which may act as a rho-independent terminator is present 18 nucleotides downstream of the predicted stop codon for this coding region, and an open reading frame encoding the C-terminal 132 amino acids of a protein showing

limited homology with the lactaldehyde reductase protein FucO of *E. coli* (5) (GenBank accession no. M31059) ends approximately 172 nucleotides upstream of the predicted start codon of the BA14K coding sequence, with no detectable consensus promoter elements in the intervening sequences. This organization suggests that the gene encoding the immunoreactive *Brucella* protein is the last component of an operon. Further characterization of the upstream nucleotide sequence in pRLF6 has not been performed.

Although the unprocessed form of the immunoreactive *Brucella* protein has a predicted molecular mass of approximately 17 kDa, computer-assisted analysis of its amino acid sequence employing the Protein Analysis Toolbox suite of programs (MacVector 6.0; Oxford Molecular Group, Campbell, Calif.) suggests that the first 26 amino acids of this polypeptide form a potential signal sequence for export. Removal of the predicted leader sequence would result in the production of a protein of approximately 14,200 Da and with a pI of 11.25, which is consistent with the mobility of BA14K detected by SDS-PAGE and Western blot analysis. Therefore, we have retained the designation BA14K for this protein. Amino acids 83 to 98 of the unprocessed form of BA14K form a potential transmembrane domain; thus, it appears likely that this protein is associated with the bacterial cell envelope; however, further biochemical characterization will be required to confirm this subcellular localization. No homology to previously deposited protein sequences in the SWISS-PROT database could be detected when the predicted BA14K amino acid sequence was evaluated with the FSTPSCAN program from PCGENE (Intelligenetics, Mountain View, Calif.) or when this sequence was compared with multiple protein sequence databases by using the BLAST algorithm (3). This latter service was provided by the National Center for Biotechnology Information.

The results presented here indicate that the *B. abortus* protein BA14K is strongly immunoreactive, inducing both humoral and cellular immune responses in hosts during the course of infection. BA14K-specific humoral immune responses were detected in a variety of relevant natural and experimental hosts, which is consistent with the observation that nucleotide sequences homologous to the 1.8-kb cloned fragment encoding BA14K were detected by Southern blot analysis in genomic DNAs from strains representing all six of the currently recognized *Brucella* species (data not shown). BA14K-reactive T lymphocytes were also detected in experimentally infected BALB/c mice, which are an established model for both human (6, 25) and ruminant (14) brucellosis. Based on these findings, it appears that further investigation of the biological significance of BA14K-specific immune responses is warranted. For example, it would be interesting to determine if BA14K-reactive lymphocytes are present in naturally infected hosts such as cattle and goats or in infected humans. Information obtained from such studies may provide the basis for the design of improved vaccines and diagnostic reagents for brucellosis in humans and animals. The construction of relevant mutants may also provide insight into the biological function of BA14K.

**Nucleotide sequence accession number.** The nucleotide sequence of the BA14K coding region has been deposited in GenBank under accession no. U62541.

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