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Cloning, Characterization, and Expression of *Bartonella henselae p26*

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In order to identify immunoreactive *Bartonella henselae* proteins, *B. henselae* antiserum from an experimentally infected cat was used to screen a *B. henselae* genomic DNA expression library. One immunoreactive phage clone contained a gene (*p26*) with significant nucleotide identity with orthologs in brucellae, bartonellae, and several plant-associated bacteria. *p26* gene sequences from four *B. henselae* strains, one *B. koehlerae* strain, and one *B. clarridgeiae* strain were cloned. Comparative nucleotide sequence analysis showed that *p26* is a potential marker for molecular diagnosis of infection, as well as for identification to species level and genotyping of *Bartonella* sp. isolates. Alignment of the predicted amino acid sequences illustrated conserved putative protein features including a hydrophobic transmembrane region, a peptide cleavage site, and four dominant antigenic sites. Expression of *p26* in *Escherichia coli* produced two proteins (26 and 27.5 kDa), both of which were reactive with feline anti-*B. henselae* antisera. Furthermore, murine hyperimmune serum raised against either recombinant protein reacted with both proteins. No reactivity to either recombinant protein was detected in nonimmune serum, and reactivity persisted as long as 20 weeks for one cat. The *p26* protein product is an immunodominant antigen that is expressed during infection in cats as a preprotein and is subsequently cleaved to form mature P26.

Bartonella henselae is a gram-negative bacterial pathogen of humans and animals. Like other members of the alphaproteobacteria subdivision, *B. henselae* is capable of establishing an intracellular lifestyle within eukaryotic cells (3). Infection of immunocompetent humans with *B. henselae* results in "cat scratch disease," a regional lymphadenopathy that is typically associated with wounds of feline origin (12, 22). In most cases, the lymph node lesions are self-limiting and antibiotic therapy is not indicated (32). However, in 5 to 14% of infected individuals, atypical manifestations can occur, including prolonged fever, malaise, fatigue, myalgia, arthralgia, weight loss, splenomegaly, and Parinaud's oculoglandular syndrome (32). In contrast, infection of immunosuppressed humans can result in a variety of severe and life-threatening disease syndromes, such as bacillary angiomatosis, peliosis hepatis, endocarditis, and bacteremia (22). Even in cases of severe disease, antibiotic therapy is often effective, but relapses can occur (32).

Domestic and wild felids are the hosts for *B. henselae*, which is transmitted by the cat flea (*Ctenocephalides felis*) (8, 13). Unlike humans, the majority of experimentally and naturally infected cats are asymptomatic. The most widely reported clinical signs in experimentally infected cats are mild and transient fever, lethargy, and anorexia (18, 21, 27, 37). Experimental infection leads to bacteremia that typically resolves in 8 to 12 weeks; however, persistent infection lasting longer than 1 year has been reported (1, 28, 30, 37, 38). Despite a robust immune response, relapsing bacteremia is commonly encountered, most frequently following infection with strains of feline origin (18, 21, 37, 38). The lack of clinical signs, prolonged bacteremia, and relapsing bacteremia can complicate the accurate clinical diagnosis of infection. Furthermore, epidemiologic studies have shown that, depending on the geographic location, seroprevalence can be as high as 80% and up to 55% of cats are bacteremic (8). With more than 60 million cats in U.S. households, the potential reservoir for zoonotic transmission remains a serious concern.

Culture of feline blood is typically a reliable means for diagnosis, but both molecular and serologic tests are often used for confirmation, identification to species level, and characterization of isolates. Molecularly based assays include PCR amplification followed by either partial sequencing or restriction fragment length polymorphism (RFLP) (6, 7, 15, 23, 31). The most widely used serologic test for diagnosis is the indirect fluorescence assay to detect antibodies against *B. henselae* whole cells (14, 35). Additional serologic tests include Western blot analysis and enzyme-linked immunosorbent assays (5, 25). Sources of antigens for serodiagnostic assays are whole-cell lysates, outer membrane protein preparations, and, more recently, recombinant proteins (5, 20, 25, 26).

Here we describe the cloning, characterization, and expression of the *B. henselae* strain F1 *p26* gene, which encodes a major immunodominant antigen (P26) recognized by feline antiserum. This description of P26 adds to the growing list of immunoreactive proteins recognized by the feline humoral immune system during infection with *B. henselae*. Finally, we propose that *B. henselae p26* and reactivity to its recombinant protein product are potential diagnostic markers of infection.

MATERIALS AND METHODS

Animals. Six 8- to 12-month-old specific-pathogen-free (SPF) cats were obtained from the Feline Nutrition Laboratory, School of Veterinary Medicine, University of California, Davis (UC Davis) (courtesy of James Morris and Quinton Rogers). Before experimental infection, all six cats were confirmed to be

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Bartonella sp. negative by serology and culture. The cats were maintained in a controlled flea-free environment and were examined every day for the first 2 weeks of infection and every week thereafter. For production of feline antiserum, experimental inoculation of *B. henselae* F1 was performed as previously described (38). Briefly, cultured *B. henselae* F1 colonies were suspended in sterile saline, and 6.64×10^6 to 2.26×10^8 CFU per ml was injected intradermally at three to five different sites.

Eight adult female SPF CD-1 mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA) for production of recombinant P26 (rP26) hyperimmune serum. Mice were maintained in a pathogen-free room with restricted access on a 12-h light, 12-h dark cycle. They were fed irradiated Pico Lab Mouse Diet 20 (PMI Nutrition International, Inc., Brentwood, MO). Mice were euthanized with CO_2.

The University of California laboratory animal care program is fully AAALAC accredited, and this study was reviewed and approved by the institutional animal care and use committee. All procedures and treatments of cats and mice were in compliance with the *Guide for the Care and Use of Laboratory Animals* (27a).

Bacterial culturing and isolation. The *Bartonella* species and strains used in this study were as follows: *B. henselae* Houston I (H1; ATCC 49882), *B. henselae* U4-11 (UC Davis), *B. henselae* F1 (UC Davis), *B. henselae* JK-47 (kindly provided by J. Koehler), *B. koehlerae* (ATCC 700693), and *B. clarridgeiae* (ATCC 51734). *B. henselae* strains are most often isolated from humans or felines, and strains can be divided into two genotypes based on partial 16S ribosomal DNA sequencing (4). Therefore, we chose human and feline strains from both genotypes: strains F1 and H1 are genotype I, strains U4-11 and JK-47 are genotype II, strains H1 and JK-47 are of human origin, and strains F1 and U4-11 are of feline origin (10, 19). Bacteria were grown on either brain heart infusion agar plates supplemented with 5% rabbit red blood cells (*B. henselae* and *B. clarridgeiae*) or chocolate agar (*B. koehlerae*) and were incubated at 35°C under 5% CO_2. After 5 to 7 days of growth, the bacterial colonies were harvested by suspension in sterile phosphate-buffered saline (PBS) and washed three times in sterile PBS. Bacterial cultures were confirmed to be *B. henselae*, *B. clarridgeiae*, or *B. koehlerae* by PCR/RFLP analysis (16, 19, 29).

Screening of a *B. henselae* genomic DNA expression library. *B. henselae* F1 was grown and harvested as described above, and the washed bacterial pellet was shipped to BBI BioTech Research Laboratories, Gaithersburg, MD, for construction of the λ ZAP genomic library. Briefly, genomic DNA was isolated from whole cells using the Puregene DNA purification kit according to the manufacturer's instructions (Gentra, Minneapolis, MN). Approximately 8 to 10 μg of genomic DNA was digested with ApoI, and fragments ranging from 1 to 9 kb were purified through an agarose gel using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). Purified genomic DNA fragments were ligated to EcoRI-digested λ ZAP II vector arms (Stratagene, La Jolla, CA). The recombinant phage was in vitro packaged using Gigapack III packaging extracts per the manufacturer's instructions (Stratagene).

Prior to screening, 150-μl aliquots of *B. henselae* F1 antiserum taken at weeks 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 postinfection from a single experimentally infected cat were combined and absorbed with *Escherichia coli* phage lysates (Stratagene) to remove background reactivity. The λ ZAP II *B. henselae* F1 genomic expression library was screened with the picoBlue immunoscreening kit (Stratagene) as previously described (17). The λ ZAP II phage contained pBluescript, which was excised and transformed directly into *E. coli* with ExAssist helper phage (Stratagene). Purified pBluescript containing a portion of *B. henselae* genomic DNA was submitted to the W. M. Keck Foundation Biotechnology Resource Laboratory at the Yale University School of Medicine for sequencing by primer walking. The *B. henselae* F1 genomic DNA sequence was analyzed using MacVector (Kodak, New Haven, CT).

Expression and purification of rP26. *B. henselae* F1 *p26* was amplified by PCR using oligonucleotide primers based on the phage insert DNA sequence. The primers used in the amplification reaction correspond to nucleotides 1 to 33 and 712 to 738 of *B. henselae* F1 *p26*. The forward primer and reverse primer contain EcoRI and HindIII restriction enzyme sites, respectively, that allow for in-frame insertion into the pMX protein expression vector, a modified pGEX-2T vector (Pharmacia, Piscataway, NJ). The amplification reaction included 5 ng of the pBluescript DNA from the original reactive clone as a DNA template and HotStarTaq master mix (QIAGEN). Amplification conditions included an initial *Taq* polymerase enzyme activation step at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Following digestion of the PCR product with the restriction enzymes EcoRI and HindIII (Roche, Indianapolis, IN) and purification using the Rapid PCR purification system (Marligen Biosciences, Ijamsville, MD), *B. henselae* F1 *p26* was ligated in frame with the glutathione *S*-transferase (GST) gene in pMX. The inserted DNA sequence was confirmed to be *B. henselae* F1 *p26* by sequencing of both strands (Davis Sequencing, Davis, CA).

Expression of rP26 utilized *E. coli* DH5α cells transformed with recombinant pMX. After cells reached the log phase of growth, protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 nM. Recombinant proteins were purified by affinity chromatography using glutathione Sepharose 4B columns (General Electric, Piscataway, NJ) and freed of their GST fusion partners by thrombin cleavage as previously described (17). After elution, the protein preparation was concentrated using a CP10 Centriplus centrifugal filter column (Millipore, Bedford, MA), and protein concentrations were determined by the Bradford protein assay.

Sequence analysis of *Bartonella p26*. To amplify *p26* from additional *Bartonella* spp. and strains, we designed primers based on *p26* flanking DNA sequences that are highly conserved between the original phage clone and published paralogous sequences in the *B. henselae* H1 and *Bartonella quintana* Toulouse genomes (GenBank accession no. BX897699 and BX897700, respectively) (2). Amplification of *p26* from four *B. henselae* strains and *B. koehlerae* used the forward primer F1 (5′-GATAGTCAATCAACAAAAAAAAGGAAGAGATATG-3′) and reverse primer R1 (5′-GAGATTATTTTACTCGGTGATTGATAATATTATATG-3′). Due to sequence divergence in *B. clarridgeiae p26*, amplification used forward primer F2 (5′-TGGCTCTAACCAATTGAGCTACAGG-3′), which was derived from a conserved DNA sequence within an upstream gene (BH11500), and reverse primer R2 (TTCTTTGTGAAGGCCGGTGATG), which was derived from a separate site of conserved DNA sequence in the downstream untranslated region of *p26*. Genomic DNAs from all *Bartonella* spp. and strains were isolated from cultured bacteria using a DNeasy tissue kit according to the manufacturer's instructions for gram-negative bacteria (QIAGEN) and used as templates. PCR amplification for all primer sets was performed using HotStarTaq DNA polymerase (QIAGEN). Amplification conditions for the *B. henselae* strains and the *B. koehlerae* strain included an initial *Taq* polymerase enzyme activation step at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The same reaction conditions were used for the *B. clarridgeiae* strain, except that annealing was performed at 65°C. PCR products were purified using the Rapid PCR purification system (Marligen Biosciences, Ijamsville, MD) and submitted for direct sequencing (Davis Sequencing). Sequences were analyzed with MacVector (Kodak).

SDS-PAGE and Western blot analysis. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 1 μg of purified *B. henselae* F1 rP26 protein was mixed with loading buffer, heated to 100°C, and resolved on precast 15% denaturing polyacrylamide Tris-HCl gels (Bio-Rad, Hercules, CA), followed by staining with Bio-Safe Coomassie blue (Bio-Rad). For Western blot analysis, 1 μg of purified rP26 or 5 μg of *B. henselae* F1, *B. koehlerae*, or *B. clarridgeiae* whole-cell lysates was electrophoretically separated as described above. The resolved proteins were transferred to nitrocellulose membranes (Bio-Rad), cut into strips, and incubated in blocking buffer (8% [wt/vol] whole milk, 50 mM Tris, 250 mM NaCl, and 0.2% [vol/vol] Tween) for 1 h at room temperature. The strips were incubated overnight at 4°C with a feline *B. henselae* F1 antiserum or a murine rP26 hyperimmune serum diluted in blocking buffer to a final concentration of 1:500 or 1:1,000, respectively. Appropriate positive and negative controls were included. After a wash in Tris-buffered saline containing 0.05% Tween 20 (Sigma), the strips were incubated in a 1:3,000 dilution of alkaline phosphatase-conjugated goat anti-feline immunoglobulin G (Kirkegaard & Perry Laboratories) for 2 h at 25°C. The strips were washed three times, and bound antibodies were detected by color development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Stratagene).

Production of murine rP26 hyperimmune serum. For production of murine *B. henselae* F1 rP26 hyperimmune serum, purified rP26 was resolved by SDS-PAGE and stained with Coomassie blue (Bio-Rad). Distinct and dominant protein bands that migrated to the expected size range for the rP26 preprotein and mature rP26 were excised separately from the acrylamide gel using a sterile blade. The gel was homogenized in sterile PBS and frozen until use. CD-1 mice were divided into two groups of four for hyperimmunization with either the rP26 preprotein or mature rP26. In this study, the acrylamide gel served as an adjuvant to boost antibody production. Each mouse was injected subcutaneously with approximately 0.5 μg of protein and boosted at 14 and 28 days with the same amount. Mice were bled 2 weeks after the last boost to collect the hyperimmune serum, and reactivity to the rP26 preprotein and mature rP26 was confirmed by Western blotting.

Nucleotide sequence accession numbers. The UniProt accession numbers for the orthologs of *B. henselae* P26 are as follows: *B. quintana*, Q6FZ72; *B. henselae* strain H1, Q6G2N4; *Brucella abortus*, Q6YA76; *Brucella suis*, Q540I8; *Brucella melitensis*, Q6GV67; *Brucella ovis*, Q6YA70; *Brucella cetaceae*, Q71T34; *Brucella*

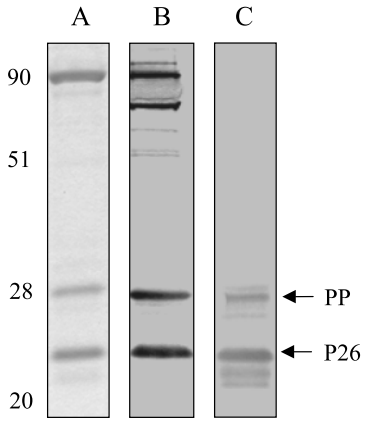

FIG. 1. (A) Coomassie blue-stained SDS-polyacrylamide gel of *B. henselae* rP26 protein preparation. (B and C) Western blots of *B. henselae* rP26 protein preparation probed with a feline antiserum (B) or with a murine rP26 hyperimmune serum (C). Arrows on the right show the locations of the preprotein (PP) and the mature rP26 (P26). Molecular mass markers (in kilodaltons) are shown on the left.

pinnipediae, Q71T35; *Agrobacterium tumefaciens*, Q8UDG4; and *E. coli* O157:H7, Q7ADC9.

The *p26* sequences determined in this study have been submitted to the GenBank nucleotide sequence database. The accession numbers for sequences from *B. henselae* strains U4-11, F1, and JK-47, *B. koehlerae*, and *B. clarridgeiae* are DQ270026 to DQ270030, respectively.

RESULTS

Screening of the *B. henselae* F1 genomic expression library. The *B. henselae* F1 genomic expression library was probed with a feline *B. henselae* F1 antiserum to detect immunoreactive proteins. In the initial screening, 75 positive phage plaques were obtained, and a clonal population of one phage was derived by two successive screenings. Using helper phage, the associated pBluescript plasmid was excised from the clonal phage population. The *B. henselae* F1 genomic DNA insert was 1,402 bp, with 100% nucleotide identity to a portion of the published *B. henselae* strain H1 sequence (bp 1269403 to bp 1270804), and contained two full-length *B. henselae* open reading frames: an outer membrane protein (BH11510; *p26*) and a hypothetical protein (BH11520). The predicted molecular masses of the outer membrane protein and the hypothetical protein were 26.7 and 18.3 kDa, respectively.

Expression of recombinant *B. henselae* genes. *B. henselae* F1 *p26* and BH11520 were inserted into expression vector pMX and synthesized as recombinant proteins. The fusion proteins were released from their GST partners, purified, concentrated to a final concentration of 1 μg/μl, and evaluated by SDS-PAGE. Following resolution of rP26 on an acrylamide gel, Coomassie blue staining revealed two dominant protein bands with approximate molecular masses of 27.5 and 26 kDa (Fig. 1A). The 18-kDa *B. henselae* F1 hypothetical protein encoded by BH11520 was not reactive with the feline antiserum (data not shown). Therefore, BH11520 was not further characterized. In both protein preparations, additional proteins of *E. coli* origin were present in the higher-molecular-weight region of the gel.

Characterization of *Bartonella* sp. *p26*. Primers were designed for amplification of *p26* from four *B. henselae* strains (H1, F1, U4-11, and JK-47), one *B. koehlerae* strain, and one *B. clarridgeiae* strain. Sequence analysis of *p26* showed open reading frames of 738 bp for all four *B. henselae* strains, 744 bp for

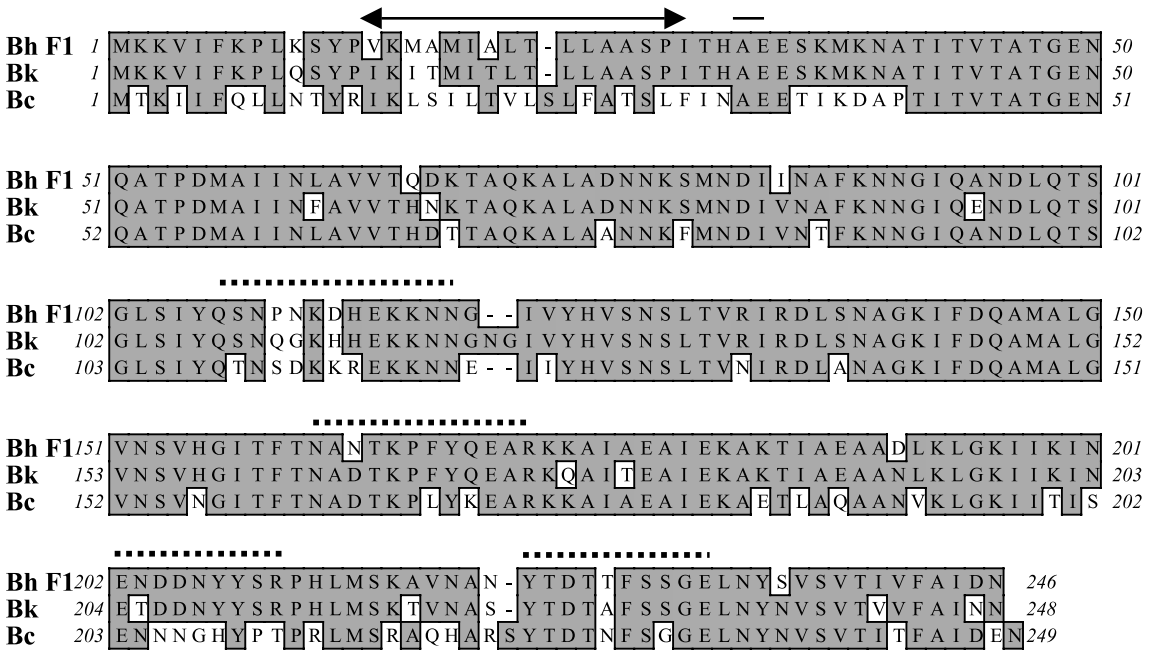

FIG. 2. Alignment of the predicted amino acid sequences of P26 from *B. henselae* strain F1 (Bh F1), *B. koehlerae* (Bk), and *B. clarridgeiae* (Bc). The putative hydrophobic transmembrane region (double-headed arrow), peptidase cleavage site (overline), and four dominant antigenic sites (dashed overlines) are highlighted.

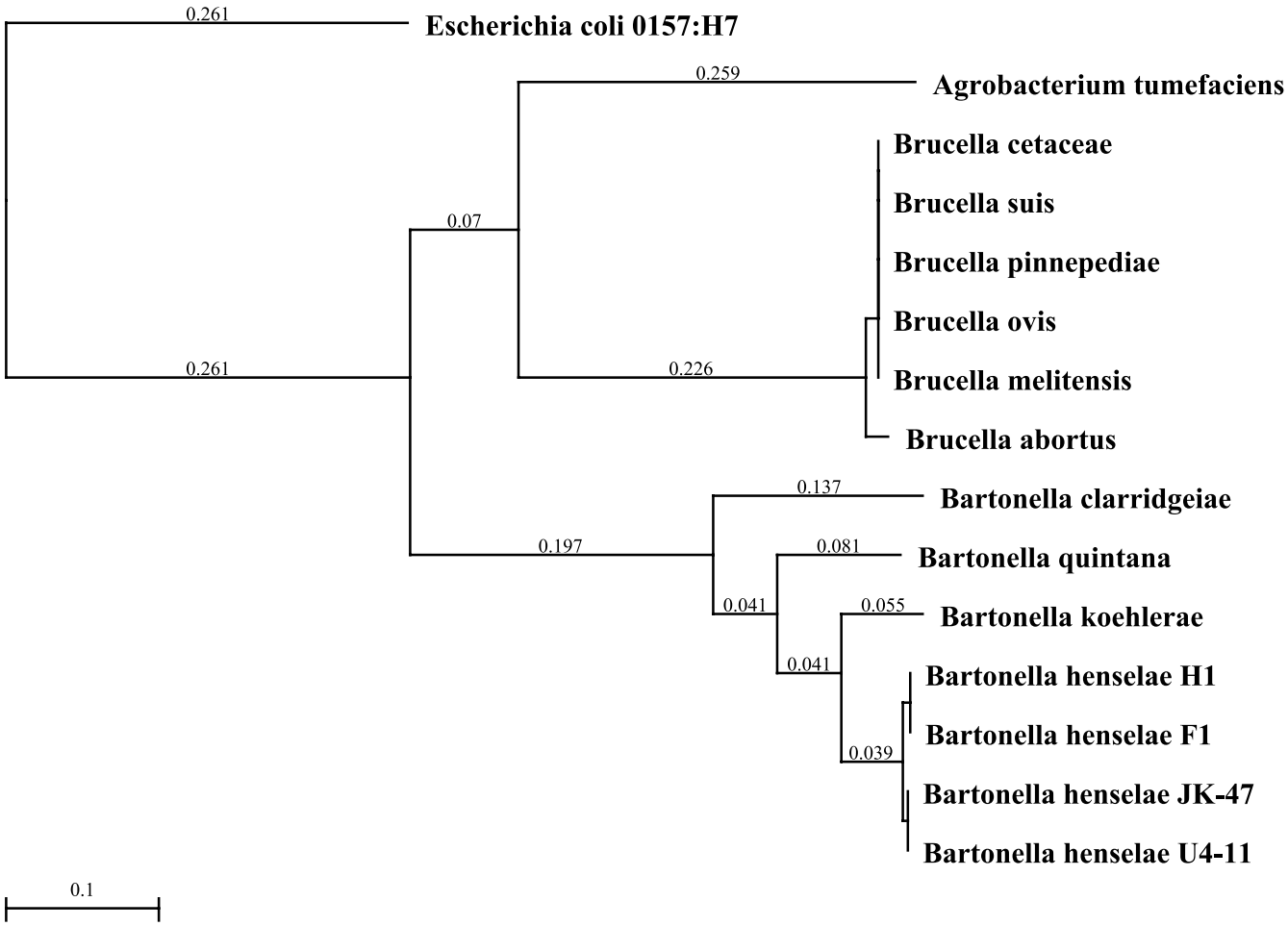

FIG. 3. Phylogenetic tree obtained from neighbor-joining analysis of P26 from related members of the alphaproteobacteria subdivision. Uniprot accession numbers for the sequences are provided in Materials and Methods. The branch distance represents the fraction of amino acid changes between nodes. The distantly related P26 ortholog from *E. coli* O157:H7 is provided to root the tree.

B. koehlerae, and 747 bp for *B. clarridgeiae*. The four *B. henselae* strains and the *B. koehlerae* isolate have a TAA termination codon, whereas *B. clarridgeiae* has a TAG stop codon followed by three in-frame TAA stop codons 15, 18, and 22 bp downstream.

Among the four *B. henselae* strains examined, two *p26* nucleotide sequences were obtained that shared >99% nucleotide sequence identity. The genotype I strains (H1 and F1) have identical *p26* nucleotide sequences that differ by 3 nucleotides (positions 130, 575 and 733) from identical *p26* sequences in the genotype II strains (U4-11 and JK-47). The later two nucleotide differences result in changes at amino acid positions 194 and 245. At nucleotide position 733, the presence of a G · C pair in the genotype I strains creates enzyme restriction sites for BsiXI, ClaI, and TacI. As expected, the percentages of nucleotide sequence identity between *B. henselae* F1 *p26* and orthologs in other bacteria examined here were lower and are listed in decreasing order as follows: *B. koehlerae*, 92%; *B quintana*, 86%; *B. clarridgeiae*, 76%; all brucellae, 49% to 50%; *A. tumefaciens*, 47%; and *E. coli* O157:H7, 37%.

Alignment of the deduced amino acid sequences of P26 orthologs in the feline-adapted species, *B. henselae* F1, *B. koehlerae*, and *B. clarridgeiae*, illustrated regional variation in amino acid similarity (Fig. 2). The highest amino acid sequence variation occurs in the first 40 amino acids and in the first and third putative antigenic sites. Hydrophilicity, antigenicity, and transmembrane profiles for *B. henselae* P26 demonstrated several important features. A large hydrophobic region (positions 14 to 29) was identified in the amino-terminal portion of P26. Based on the transmembrane profile, this hydrophobic region was predicted to have a helical tertiary structure that associates with lipid membranes. Antigenicity profiles of P26 showed multiple putative antigenic sites, with four appearing dominant at amino acid positions 107 to 119, 161 to 172, 201 to 210, and 222 to 232. The profiles of *B. koehlerae* and *B. clarridgeiae* had the same features as those described above. Based on sequence comparison with the *Brucella abortus* BP26 amino acid sequence, *B. henselae* P26 has a putative signal peptide cleavage site between amino acids alanine and glutamate at positions 32 and 33.

A phylogenetic tree was constructed using the neighbor joining method to illustrate the phylogenetic relationship between the P26 orthologs (Fig. 3). Orthologs from closely related alphaproteobacteria, including *Brucella* spp., *Bartonella* spp., and

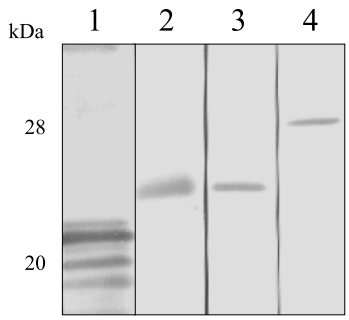

FIG. 4. Western blot of in vitro P26 expression in *B. henselae* (lanes 1 and 2), *B. koehlerae* (lane 3), and *B. clarridgeiae* (lane 4). A feline antiserum (lane 1) or a murine recombinant P26 hyperimmune serum (lanes 2 to 4) was used to probe the lysates. Molecular mass markers are shown on the left.

A. tumefaciens, were included. The members of the genus *Bartonella* formed a clade that is closely related to a clade formed by members of the genus *Brucella* and the phytopathogen *A. tumefaciens*. The ortholog from the distantly related *E. coli* O157:H7 was included to root the tree.

Evaluation of feline serum reactivity to *B. henselae* F1 rP26. Western blot analysis of preinfection and week-8 postinfection sera from six SPF cats experimentally infected with *B. henselae* F1 showed that only infected cats produce rP26-specific antibodies. Reactivity to both the rP26 preprotein and mature rP26 was detected in each feline antiserum examined, and an example of serum reactivity is provided (Fig. 1B). For each serum sample, variable levels of reactivity to high-molecular-mass contaminant proteins of *E. coli* origin within the rP26 protein preparation were also noted. For one experimentally infected cat, the first week of detectable reactivity to the mature protein occurred at week 3 postinfection, and reactivity persisted for at least 20 weeks postinfection (data not shown).

Generation and evaluation of murine rP26 hyperimmune sera. Two groups of four mice were hyperimmunized with homogenized acrylamide gel containing either rP26 preprotein or mature rP26. Sera from both groups of mice recognized both the preprotein and the mature protein (Fig. 1C). There was no reactivity with the additional protein bands within the high-molecular-mass region of the gel, suggesting that these proteins are most likely contaminants of *E. coli* origin.

Figure 4 shows a Western blot with cultured *Bartonella* sp. whole-cell lysates probed with either a feline *B. henselae* F1 antiserum or a murine rP26 hyperimmune serum. Each feline antiserum consistently reacted with four unidentified proteins in *B. henselae* whole-cell lysates in the molecular mass range of 18 to 22 kDa. The murine rP26 hyperimmune serum reacts with a single protein band in lysates from *B. henselae*, *B. koehlerae*, and *B. clarridgeiae*. Based on migration rates in acrylamide gels, *B. henselae* and *B. koehlerae* P26 have similar molecular masses (approximately 26 kDa), whereas the *B. clarridgeiae* P26 is slightly larger (approximately 28 kDa). The murine rP26 hyperimmune serum showed no immunoreactivity with either *E. coli* DH5α lysates or *Brucella abortus* phenolized antigen (data not shown).

DISCUSSION

B. henselae F1 P26 is a member of a family of proteins (Uniprot accession no. IPR007497) with unknown functions. Orthologs of *p26* are present in several closely related bacterial species in the taxonomic families *Bradyrhizobiaceae*, *Phyllobacteriaceae*, and *Rhizobiaceae*, most of which are soil-borne organisms associated with nitrogen fixation. Orthologs of *B. henselae* F1 *p26* are also present in mammalian bacterial pathogens within the families *Brucellaceae* and *Bartonellaceae*, and there is a high percentage of nucleotide sequence identity with orthologs in the recently published *B. henselae* strain H1 and *B. quintana* strain Toulouse genomes (2). Perhaps the best-characterized orthologs are from members of the genus *Brucella*, in which the protein is highly conserved (36). The genes in *Brucella abortus* and *Brucella melitensis*, designated *bp26*, were initially described by three separate research groups and were characterized as immunodominant antigens in infected cattle, sheep, goats, and humans (24, 33, 34).

The evidence presented here suggests that P26 is expressed as a preprotein that is subsequently cleaved at a putative peptide cleavage site to form the mature protein. Following expression in *E. coli* and electrophoresis of the purified rP26 protein preparation, two dominant protein bands with approximate molecular masses of 26 and 27.5 kDa are observed. Both proteins are reactive with sera from cats experimentally infected with *B. henselae*. Moreover, murine hyperimmune sera raised against either the 27.5-kDa preprotein or mature rP26 react with both protein bands. Two immunoreactive proteins with similar molecular masses have been described in protein preparations of *Brucella abortus* and *Brucella melitensis* BP26 expressed in *E. coli* host systems, which is believed to be the result of decreased efficiency of BP26 processing by *E. coli* (24, 33, 34). A single reactive protein was detected with murine rP26 hyperimmune serum in each of the *Bartonella* sp. lysates examined. In *B. henselae* and *B. koehlerae*, the molecular mass of this protein was consistent with mature P26. The absence of a detectable preprotein in the lysates suggests that cleavage of the P26 preprotein is highly efficient in these *Bartonella* species. The single reactive protein in *B. clarridgeiae* lysates has a larger molecular mass (approximately 28 kDa), but the cause for this difference was not determined.

Hydrophilicity profiles of P26 from *B. henselae*, *B. koehlerae*, and *B. clarridgeiae* revealed a hydrophobic region near the amino terminus of the protein. Based on Rao and Argos transmembrane profiles, this hydrophobic region is predicted to form a helical structure that associates with cellular membranes. Immediately adjacent to the hydrophobic region is a putative peptide cleavage site, which was identified based on sequence similarity to *Brucella abortus* BP26 (33). In *Brucella abortus* BP26, cleavage of the preprotein was shown to occur between the amino acids alanine and glutamine, located at positions 28 and 29, respectively (33). Identical peptide cleavage sites are present in all *Brucella* BP26 sequences and in the ortholog from *A. tumefaciens*. In all of the *Bartonella* P26 sequences, the putative cleavage site occurs between the amino acids alanine and glutamate (located at positions 32 and 33 of *B. henselae* P26, respectively). Substitution of glutamate for glutamine in the *Bartonella* P26 sequences may have little effect

on the tertiary structure of the cleavage site, since both are polar hydrophilic amino acids that tend to reside in contact with the aqueous phase. Furthermore, in all of the alphaproteobacteria examined here, there is a conserved glutamate next to the carboxy side of the putative cleavage site (position 34 in *B. henselae* P26). As expected, cleavage of the *B. henselae* P26 preprotein at the putative cleavage site would produce the observed difference in molecular mass between the preprotein and the mature P26.

The high percentage of nucleotide identity between orthologs in the four *B. henselae* strains and the nucleotide sequence divergence from orthologs in other bacteria make molecular techniques based on *p26*, such as partial DNA sequencing or RFLP analysis, viable options for diagnosis of human and feline infection and for identification of isolates to species level. Based on our limited results, similar molecular techniques may also be sufficient to differentiate between genotypes of *B. henselae*. In order to ensure the accuracy of future molecularly based diagnostic assays, *p26* from additional *Bartonella* spp. and strains should be sequenced and analyzed. It is noteworthy that the percentage of nucleotide identity with the ortholog in *B. quintana*, a human-adapted species, is significantly higher than that with the ortholog in *B. clarridgeiae*, a feline-adapted species. Similar phylogenetic relationships have been found for other genes (9, 31, 39, 40).

In this study, 6/6 SPF cats experimentally infected with *B. henselae* developed antibodies that reacted with *B. henselae* rP26 within 3 weeks postinfection. No reactivity was present before inoculation in this group of cats, and reactive antibodies persisted for at least 20 weeks in one cat that was examined. Importantly, *B. koehlerae* and *B. clarridgeiae p26* orthologs are expressed in vitro, and the protein products of both react with a murine rP26 hyperimmune serum. This raises concerns regarding the species specificity of a serodiagnostic assay based on detection of feline antibodies to *B. henselae* rP26.

Western blot analysis of cultured *B. henselae* whole-cell lysates using a murine rP26 hyperimmune serum identified a single protein with the expected molecular mass for mature P26. However, a reactive protein with a similar molecular mass was never detected by using antisera from cats experimentally infected with *B. henselae*. Although these data show that P26 is expressed in vitro, they also suggest that the amount of P26 present in cultured cell lysates may be insufficient for detectable reactivity with a feline antiserum by routine immunoblotting techniques. This may explain the lack of detection of P26 in a previous study (11). Reactivity of feline antiserum to expressed and concentrated rP26 was detected for all experimentally infected cats examined. Therefore, if in vitro P26 expression is indeed low, this suggests either that P26 in small amounts is highly antigenic or that it is up-regulated during infection.

In this study, screening of a genomic expression library with a feline antiserum led to the identification of *B. henselae* F1 *p26*. Comparative analysis of *p26* orthologs showed that the gene is a potential marker for infection with *Bartonella* spp. and may be useful for the identification of feline carriers and the diagnosis of *Bartonella*-associated diseases in humans. Moreover, this gene may be useful for identification to species level and genotyping of *Bartonella* isolates. *p26* encodes an immunodominant antigen that is expressed during feline infection, and a feline antiserum reacted strongly with the recombinant protein product. These results suggest that an rP26-based feline serodiagnostic assay may be feasible. In the future, the ability of P26 subunit vaccines to protect cats against infection should be evaluated.

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