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# Heterogeneity of BmpA (P39) among European Isolates of *Borrelia burgdorferi* Sensu Lato and Influence of Interspecies Variability on Serodiagnosis

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**The molecular and antigenic variabilities of BmpA (P39) among European isolates of *Borrelia burgdorferi* were analyzed. The *bmpA* sequences of 12 isolates representing all three species of *B. burgdorferi* sensu lato pathogenic for humans were amplified by PCR, cloned, and sequenced. The BmpA protein of *Borrelia garinii* is heterogeneous, with an amino acid sequence identity ranging from 91 to 97%, whereas the BmpA proteins of *Borrelia afzelii* and *B. burgdorferi* sensu stricto strains appear to be highly conserved (>98.5% intraspecies identity). The interspecies identities ranged from 86 to 92%. Cluster analysis of BmpA reflected the subdivision of *B. burgdorferi* sensu lato isolates into the three species as well as a considerable heterogeneity among *B. garinii* strains. The BmpA protein of each species of *B. burgdorferi* sensu lato was recombinantly expressed in *Escherichia coli*, purified, and used to generate monoclonal antibodies. Seven BmpA-specific antibodies were identified; six of them recognized conserved epitopes of all three species, whereas one was specific for BmpA of *B. afzelii* and *B. garinii*. A monoclonal antibody (H1141) recommended by the Centers for Disease Control and Prevention for use in the standardization of immunoblots showed strong reactivity with BmpA of *B. burgdorferi* sensu stricto but no or only weak reactivity with BmpA of *B. garinii* and *B. afzelii*, respectively. Sera from 86 European patients with Lyme borreliosis in different stages and 73 controls were tested in immunoglobulin G (IgG) and IgM immunoblots with the recombinant BmpA proteins of the three species, revealing specificities of 98.6 to 100%. IgM antibodies against recombinant BmpA were only rarely detected (1.1 to 8.1%). With the BmpA proteins of *B. afzelii* and *B. garinii*, sensitivities for the IgG test (sera from stages I to III) were 36.0 and 34.9%, respectively, in contrast to 13.9% with BmpA of *B. burgdorferi* sensu stricto. Therefore, we recommend that recombinant BmpA of *B. afzelii* or *B. garinii* should be used solely, or in addition to *B. burgdorferi* sensu stricto BmpA, in serodiagnostic tests for Lyme borreliosis in Europe.**

In Europe, *Borrelia burgdorferi* sensu lato, the etiologic agent of Lyme borreliosis, comprises three species pathogenic for humans (4). Many antigens of *B. burgdorferi* sensu lato have been described to be heterogeneous between the three species *B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii*. The molecular variability was especially demonstrated for the plasmid-encoded antigens OspA (43, 47), OspC (19, 41, 48), and OspD (23), whereas chromosomally encoded antigens like p83/100 (32) and flagellin (19) are more conserved. Among different strains of *B. garinii* this antigenic variability of defined antigens is more pronounced, e.g., OspA types 3 to 8 (44). Several studies have shown differences in immunoblot reactivity patterns depending on the strain, serotype, or species used as the antigen (8, 16, 24, 26, 49, 50). Proper identification of reactive bands and thus comparison of the reactivities of defined borrelial proteins is difficult in immunoblots with ultrasonicated antigens (conventional immunoblotting). Recently, we showed differences in reactivity among homologous proteins derived from representatives of the three species pathogenic for humans using recombinant proteins for the proper identification of reactive proteins (45). Such differences were shown for OspA, OspC, p83/100, and truncated flagellin in the immunoblot (45) as well as by other investigators with enzyme-linked immunosorbent assay (ELISA) for truncated flagellin (6) and for OspC with conventional immunoblots (24).

Simpson et al. (38) described a specific and sensitive antigen for the serodiagnosis of Lyme disease in U.S. patients. Several studies (all done with *B. burgdorferi* sensu stricto antigen) confirmed the high value of P39 as a serodiagnostic antigen (9–11, 16, 22, 27, 35). Recently, the gene locus for P39 was identified in *B. burgdorferi* sensu stricto strain Sh-2-82 by Simpson and coworkers (37). The chromosomally encoded *bmpA* gene is located in a *bmp* gene cluster consisting of *bmpA* to *bmpD* (31). The *bmpA* gene is arranged tandemly with *bmpB* (37) downstream of the putative monocistronic transcribed *bmpD* or *bmpC* (31). The deduced translation products of all four *bmp* reading frames are of similar size and contain putative cleavage sites for signal peptidase II, and thus, their locations might be within the borrelial membrane, as shown for BmpA (40). Only BmpA (P39) of the *bmp* gene cluster is described as an important immunogenic protein for the serodiagnosis of Lyme borreliosis. So far, no data on the in vivo expression and immunogenicity of *bmpB*, *bmpC*, or *bmpD* are available. The molecular heterogeneity of BmpA (P39) among the three *B. burgdorferi* sensu lato species pathogenic for humans and its possible effects on the serodiagnosis of Lyme borreliosis in Europe have not been investigated yet.

Thus, we analyzed the molecular and immunological heterogeneities of BmpA proteins among *B. burgdorferi* sensu lato isolates. We found considerable heterogeneity among BmpA proteins and evaluated the influence of this heterogeneity on

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TABLE 1. *B. burgdorferi* sensu lato strains analyzed for molecular heterogeneity of *bmpA* and *bmpB*

| Strain[a] | Biological origin | Geographic origin | Species | OspA type[b] | *bmpA* + *bmpB* sequence EBI database accession nos. or reference |
|---|---|---|---|---|---|
| B31 | *Ixodes dammini* | United States | *B. burgdorferi* sensu stricto | 1 | X81515 + X81517 |
| T255 | *Ixodes ricinus* | Germany | *B. burgdorferi* sensu stricto | 1 | X97240 |
| PBre | Human skin | Germany | *B. burgdorferi* sensu stricto | 1 | X97235 |
| Sh-2-82 | *Ixodes dammini* | United States | *B. burgdorferi* sensu stricto | 1 | 37 |
| PKo | Human skin | Germany | *B. afzelii* | 2 | X81516 + X81519 |
| PWudl | Human skin | Germany | *B. afzelii* | 2 | X97241 |
| PLe | Human skin | Germany | *B. afzelii* | 2 | X97237 |
| PLj7 | Human skin | Slovenia | *B. afzelii* | 2 | X97239 |
| PBr | Human CSF | Germany | *B. garinii* | 3 | X97242 |
| PBi | Human CSF | Germany | *B. garinii* | 4 | X97244 + X81518 |
| PLi | Human CSF | Germany | *B. garinii* | 5 | X97238 |
| TN | *Ixodes ricinus* | Germany | *B. garinii* | 6 | X97243 |
| PKi | Human CSF | Germany | *B. garinii* | 8 | X97236 |

[a] Strains are described elsewhere (47, 48).
[b] OspA type was determined as described previously (43, 47).

serodiagnosis. Seven BmpA-specific monoclonal antibodies (MAbs) were produced with recombinantly expressed BmpA. They were tested for their reactivities with BmpA of *B. burgdorferi* sensu lato (OspA types 1 to 7). Recombinant BmpA was highly specific but a weak marker for immunoglobulin M (IgM), and the BmpA proteins of *B. afzelii* and *B. garinii* were more than twice as sensitive as *B. burgdorferi* sensu stricto BmpA at detecting IgG antibodies.

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## MATERIALS AND METHODS

**Patient sera.** Sera from the following groups of patients with Lyme borreliosis ($n$ = 86) and controls ($n$ = 73) were investigated. Sera from unselected, untreated patients ($n$ = 30) with erythema migrans (EM) were obtained by a dermatologist during a former study of therapy for Lyme borreliosis (42). The median of the time period between the onset of the EM and serum sampling was 2 weeks (range, 2 days to 19 weeks). The neuroborreliosis group ($n$ = 30) included untreated patients with typical signs of acute neuroborreliosis (NB): cerebrospinal fluid (CSF) pleocytosis and specific IgG CSF/serum indexes of ≥2.0. All serum specimens were obtained on the same day as the CSF specimens. The median time of onset of the neurological symptoms before the serum samples were obtained was 4 weeks (range, 2 to 18 weeks). The group with late Lyme borreliosis ($n$ = 26) comprised 16 patients with acrodermatitis chronica atrophicans (ACA) diagnosed by a dermatologist and 10 patients with Lyme arthritis (AT). Possible differential diagnoses had been excluded. Sera from 52 healthy blood donors, sera from 10 patients with syphillis in stage II or III, and 11 serum specimens with rheumatoid factor levels of ≥45 IU/ml served as controls. The actual healthy blood donors had no history of frequent tick bites, erythemas, neurological symptoms, or joint disorders.

***Borrelia* sp. strains and cultivation.** The *B. burgdorferi* strains used in this study (Table 1) were grown in modified Kelly medium at 33°C for 4 to 5 days as described previously (30). All strains have been described and classified previously (44, 47, 48).

**PCR, molecular cloning, and sequencing.** If not mentioned otherwise, standard techniques of molecular cloning were performed as described elsewhere (2). Total genomic *B. burgdorferi* DNA was extracted as described previously (21). The coding regions of *bmpA* and *bmpB* were amplified by PCR in a Perkin-Elmer 9600 thermal cycler under standard conditions (20 pmol of each of the 5′ and 3′ primers) by using AmpliTaq DNA polymerase (PE Applied Biosystems, Weiterstadt, Germany). After initial denaturation of chromosomal DNA for 3 min at 96°C, a total of 30 cycles (96°C for 1 min, 42°C for 1 min, 72°C for 2 min) were performed. The 5′ forward and 3′ reverse primers for PCR amplification as well as the sequencing primers for *bmpA* and *bmpB* are listed in Table 2. The primers were designed according to the *bmpAB* sequence published for strain Sh-2-82 (37) (GenBank accession no. L24194) and results obtained from sequence alignments. The primers used for amplification of the overall *bmpA* gene including the signal sequence were bmpA-A1 and bmpA-B1. For expression cloning of *bmpA*, leaving out the coding region of the signal peptide but with six additional histidine residues fused to the 3′ terminus, primer pair bmpA-A3 and bmpA-B2 was used. The resulting PCR amplicon was used to

TABLE 2. Oligonucleotides used for PCR amplification, cloning, and sequencing of *bmpA* and *bmpB*

| Primer | Primer sequence 5′-3′[a] | Primer direction | Target site position[b] | Sites for restriction enzymes |
|---|---|---|---|---|
| bmpA-A1 | ggggtaccggatcctatcATGAATAAAATATTGTTGTTGATT | Forward | 1–24 | *Kpn*I, *Bam*HI |
| bmpA-A3 | agcaaccatggtacctTTTTTATCTTGTAGTGGTAA | Forward | 43–62 | *Nco*I, *Kpn*I |
| bmpA-B1 | gtttcgtcgaccaagcttTTAAATAAATTCTTTAAG | Reverse | 1020–1003 | *Sal*I, *Hin*dIII |
| bmpA-B2 | aaagtcgaccaagcttaatggtgatggtgatggtgAATAAATTCTTTAAGGAATTT | Reverse[c] | 1017–997 | *Sal*I, *Hin*dIII |
| bmpA-C | TTATTTATCTGATCTTGAAG | Forward | 216–235 | |
| bmpA-D | GAAAGTATTAGTTTTTAAAT | Reverse | 834–815 | |
| bmpA-E | CTTCAAGATCAGATAAATAA | Reverse | 235–216 | |
| bmpA-F | ATTTAAAAACTAATACTTTC | Forward | 815–834 | |
| bmpB-A1 | ggggtaccggatcctatcATGAGAATTGTAATTTTTATA | Forward | 1–21 | *Kpn*I, *Bam*HI |
| bmpB-B1 | tttggtcgaccctgcagTTATAACTTTAATATTTG | Reverse | 1026–1009 | *Sal*I, *Pst*I |
| bmpB-C | CCATCTCTTAATCCCATTT | Reverse | 891–873 | |
| bmpB-D | CCAGAAAATATTGAAGAAGTTTT | Forward | 175–197 | |

[a] Unpaired nucleotides are indicated by lowercase letters.
[b] Position numbering refers to that in the open reading frames.
[c] The primer encodes an additional six histidine residues.

produce an in-frame construct of *bmpA* lacking its own 3′ signal sequence but containing the *Escherichia coli ompA* signal sequence of the secretory pASK40 expression vector (39). PCR amplification of the 3′-truncated *bmpA* gene was carried out with primer pair bmpA-A3 and bmpA-D. The overall *bmpB* coding region was amplified by PCR with primers bmpB-A1 and bmpB-B1.

For cloning of *bmpA* and *bmpB*, PCR fragments were either cloned directly into pCR II (Invitrogen, Leek, The Netherlands) or cut with a restriction enzyme (Boehringer Mannheim GmbH, Mannheim, Germany), purified by agarose gel electrophoresis (FMC NuSieve GTG agarose; Biozym, Hess. Oldendorf, Germany), and eluted (QIAquick Gel Extraction Kit; Qiagen, Hilden, Germany). About 60 fmol of the expression unit was ligated with 6 fmol of an appropriately prepared pUC18 (*Bam*HI, *Pst*I) or pASK40 (*Kpn*I, *Hin*dIII) expression vector by standard procedures. Competent *E. coli* JM83 cells were electroporated (Gene Pulser II; Bio-Rad, Munich, Germany) with a 10% aliquot of the ligation mixture (ethanol precipitated). Transformants were selected after restriction analysis, and subsequent DNA sequencing was performed on an ABI 377 DNA Sequencer (ABI Prism dye terminator cycle sequencing; Perkin-Elmer Applied Biosystems, Weiterstadt, Germany).

**Sequence comparison.** Sequence comparison was done as described previously (32).

**Expression of rBmpA, purification, and protein characterization.** The selected transformants (*E. coli* JM83) harboring the expression plasmids to produce ANSSSVP[F15-I339]6×H-BmpA in pASK40 were 1611 (PKo), 2615 (B31), and 3613 (PBi). Cells were grown, induced with isopropyl-β-D-thiogalactopyranoside, and harvested as described previously (6). *E. coli* cells were lysed (50 mM sodium phosphate buffer [pH 8.0], lysozyme [0.4 μg/ml], Benzonase [2.5 U/ml; Merck, Darmstadt, Germany]) for 30 min at room temperature and ultrasonicated, and the suspension was adjusted to contain 500 mM NaCl and 1 mM imidazole and centrifuged at 10,000 × *g* for 15 min. The supernatant containing soluble recombinant BmpA (rBmpA) was then filtered through a 0.2-μm-pore-size filter. For isolation of partial insoluble rBmpA of strains B31 (2615) and PBi (3613), urea was added to the cell lysate to a final concentration of 4 M before ultrasonication. For subsequent metal chelate chromatography, NaCl and imidazole were added so that the final concentrations were 500 mM NaCl, 5 mM imidazole, and 2 M urea in 50 mM sodium phosphate buffer (pH 8.0).

rBmpAs containing C-terminal hexahistidine residues were purified by immobilized metal chelate affinity chromatography (17) on Fractogel EMD-Chelat (S) (Merck) or Poros 20 MC (Boehringer Mannheim GmbH) columns charged with $Ni^{2+}$. rBmpA was loaded in buffer A (50 mM sodium phosphate [pH 8.0], 500 mM NaCl, 5 mM imidazole). For insoluble rBmpAs of strains PBi and B31, buffer A with 2 M urea was used. Elution was performed with a linear gradient of from 0 to 500 mM imidazole in buffer A on an FPLC system (Pharmacia Biotech, Freiburg, Germany). Detection of rBmpA was performed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and MAbs (see following sections). Fractions containing rBmpA were pooled, dialyzed against 20 mM Tris-OH/HCl (pH 8.0), and subsequently purified by anion-exchange chromatography on a MonoQ column with a linear gradient from 0 to 500 mM NaCl. rBmpA was analyzed for impurities by immunoblotting with a broadly reactive polyclonal *E. coli* antiserum (K69) derived from rabbits. N-terminal sequencing of rBmpA was performed with a gas-phase sequencer (ABI 473A; PE Applied Biosystems) by following the instructions of the manufacturer.

**MAbs.** MAbs were raised against purified rBmpA of strains PKo and B31 and were identified by ELISA with rBmpA or with conventional immunoblots by using *B. burgdorferi* whole-cell lysates by methods described previously (32). Hybridomas were produced by standard techniques (13). Cloning and determination of the immunoglobulin class and isotype were performed as described previously (46). An MAb against P39 (MAb H1141) was kindly provided by Tom Schwan (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.) and was used at a 1:500 dilution.

**SDS-PAGE and Western immunoblots.** The protein concentration was determined by the method of Bradford (5) or by measuring the optical density at 280 nm. Preparative discontinuous SDS-PAGE (12.5% polyacrylamide) was carried out by standard procedures (20) with gels that were 17 cm in width and 9 cm in length and that had 0.75-mm spacers. Four preparative SDS-PAGE runs, one for each rBmpA (3.6 μg of rBmpA/per gel) and one gel with a mixture of all three rBmpAs (equal amounts of B31, PKo, and PBi rBmpAs; total, 3.6 μg) were performed. After SDS-PAGE, zones containing rBmpA were cut out of the gels, and all four gel slices were subjected in parallel to Western blotting on one sheet of a Immobilon-P polyvinylidene difluoride membrane (Millipore, Eschborn, Germany) by the semidry technique described by the manufacturer (2-h blotting time). Membranes were blocked for 1 h at 37°C (50 mM Tris-OH/HCl [pH 7.4], 200 mM NaCl, 0.1% Tween 20, 5% nonfat dried milk) and cut into 68 strips (2.5 mm in width), and successful protein transfer onto the polyvinylidene difluoride membrane was analyzed by staining the two outer strips of each gel by using the colloidal gold enhancement kit (Bio-Rad). The strips were dried and stored for up to 2 weeks at 4°C. The estimated amount of each rBmpA or the rBmpA mixture per immunoblot strip was approximately 30 ng. MAb L39B5 and a positive patient serum sample served as positive controls.

Prior to IgM testing, sera were pretreated with reumathoid factor absorbent (Behringwerke AG, Marburg, Germany). Strips were incubated with sera at room temperature overnight with 1:200 and 1:100 dilutions (in 10 mM Tris-OH/HCl [pH 7.4], 150 mM NaCl, 0.2% Tween 20, 1% nonfat dried milk) for IgG and IgM immunoprints, respectively, washed, and incubated for 2 h with horseradish peroxidase-conjugated anti-human IgG and IgM antibodies (Dakopatts, Copenhagen, Denmark) at a dilution of 1:1,000 for IgG detection and a dilution of 1:500 for IgM detection. Color was developed by adding diaminobenzidine and $H_2O_2$. All sera were randomized before testing. Incubation and evaluation were performed in a blinded manner, and semiquantitative evaluation of the strips was independently done by two persons.

To verify the diagnostic value of rBmpA, results from this study were compared to the results obtained with natural BmpA (nBmpA) (derived from whole borrelial cell lysates and conventional immunoblotting) in a former standardization study (16) with the same 86 serum specimens from patients with Lyme borreliosis and *Borrelia* strains but not the same *B. burgdorferi* strains (strain PKa2 was used instead of B31).

**Nucleotide sequence accession numbers.** The newly identified *bmpA* and *bmpB* sequences of *B. burgdorferi* sensu lato strains were submitted to the EBI database and have the accession numbers presented in Table 1.

## RESULTS

**BmpA and BmpB sequence heterogeneity.** The coding regions (positions 43 to 1020) of *bmpA* were amplified from the 12 *B. burgdorferi* sensu lato strains listed in Table 1. Only *bmpA* of *B. garinii* PBr, TN, and PKi failed to give a full-length PCR product and were amplified as shorter products lacking approximately 180 to 230 bp at the 3′ terminus.

The deduced amino acid sequences showed a high degree of identity (86.4 to 99.7%) if single sequences were compared to the previously described BmpA of *B. burgdorferi* sensu stricto strain Sh-2-82. Alignment studies at the amino acid level (amino acid positions 15 to 257; numbering for strain Sh-2-82) showed highly conserved BmpA within *B. burgdorferi* sensu stricto (98.7 to 99.7% identity) and *B. afzelii* strains (98.5 to 99.4% identity), whereas *B. garinii* BmpA appeared to be more heterogeneous (91.4 to 97.5% identity). The interspecies identities between the three *B. burgdorferi* species pathogenic for humans ranged from 86.4 to 91.6%. Cluster analysis of the BmpA sequences (amino acid positions 15 to 257; numbering for strain Sh-2-82) showed three main clusters representing the three species and a higher variability within *B. garinii* BmpA compared to that within the BmpA proteins of *B. afzelii* and *B. garinii* (Fig. 1). The average identity of the BmpA sequences compared to that of the BmpB sequence is 57.7%. The theoretically determined isoelectric points of BmpAs (varying from pI 4.96 to pI 5.98) were confirmed by isoelectric focusing with the rBmpAs of strains B31, PKo, and PBi (data not shown).

The three amplified *bmpB* sequences of strains B31, PKo, and PBi were compared to the previously published sequence of strain Sh-2-82. The identities of the deduced amino acid sequences ranged from 97.9% between the two *B. burgdorferi* sensu stricto strains (B31 and Sh-2-82) to 83.8 and 85.6% compared to the BmpB sequence of *B. garinii* PBi and the BmpB sequence of *B. afzelii* PKo, respectively.

**N-terminal sequence of rBmpA.** For internal quality assurance and to test the integrity of the secreted and purified rBmpA, the material was subjected to N-terminal sequencing. As expected, the rBmpA of strain B31 revealed a homogeneous N terminus harboring the seven additional amino acid residues derived from the OmpA signal sequence linker peptide of pASK40 (ANSSSVP-[F15-I339]6×H-BmpA). Recombinant BmpA of strain PKo showed the expected N terminus (about 40%) as well as an N-terminal prolonged variant (60%) with an additional six amino acid residues (underlined), ATV AQAANSSSVP-[F15-I339]6×H-BmpA. rBmpA of strain PBi showed N-terminal truncated forms missing 4 (approximately 50%) or 11 amino acid residues resulting in SVP-[F15-I339] 6×H-BmpA or [S19-I339]6×H-BmpA of strain PBi.

**Isolation, characterization, and reactivities of MAbs.** After screening by ELISA with rBmpA as the antigen as well as by conventional immunoblotting with *B. burgdorferi* total cell ly-

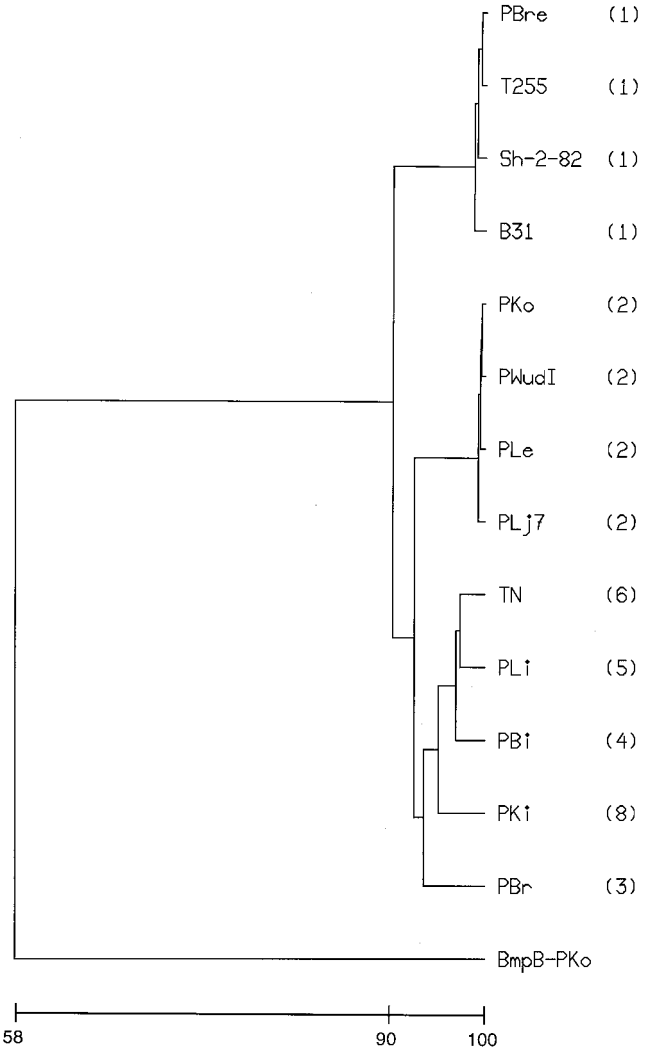


FIG. 1. Cluster analysis of the deduced BmpA protein sequences (amino acid positions 15 to 257; numbering for strain Sh-2-82) of various *B. burgdorferi* sensu lato strains and BmpB of *B. afzelii* PKo as the outgroup. The scale bar represents the average identity (in percent) for the branching points. Numbers in parentheses indicate OspA types.

sates (data not shown), seven different BmpA-specific MAbs (MAbs L39B1, L39B2, L39B3, L39B5, L39B7, L39B8, and L39B10) could be identified. All MAbs belong to the IgG1 subclass. One MAb (MAb L39B8) was reactive with *B. afzelii* (strains PKo and PGau; OspA type 2) and *B. garinii* (strains PBr, PBi, W12, TN, and T25; OspA types 3 to 7, respectively) but not with *B. burgdorferi* sensu stricto (strains PKa2 and B31; OspA type 1) by using conventional immunoblots. All other MAbs were reactive with all three species of *B. burgdorferi* sensu lato. None of the antibodies was reactive with whole-cell lysates of relapsing fever borreliae (*Borrelia recurrentis*, *Borrelia duttonii*, *Borrelia parkeri*, *Borrelia hermsii*, and *Borrelia turicatae*) or *Treponema pallidum*, *Treponema phagedenis*, *Treponema denticola*, *Leptospira grippotyphosa*, *Listeria monocytogenes*, *Legionella micdadei*, or *Salmonella typhimurium* (data not shown).

All MAbs were also tested for their reactivities with rBmpA of strains B31, PKo, and PBi. MAb L39B8 recognized only the rBmpA proteins of *B. garinii* PBi and *B. afzelii* PKo but not the

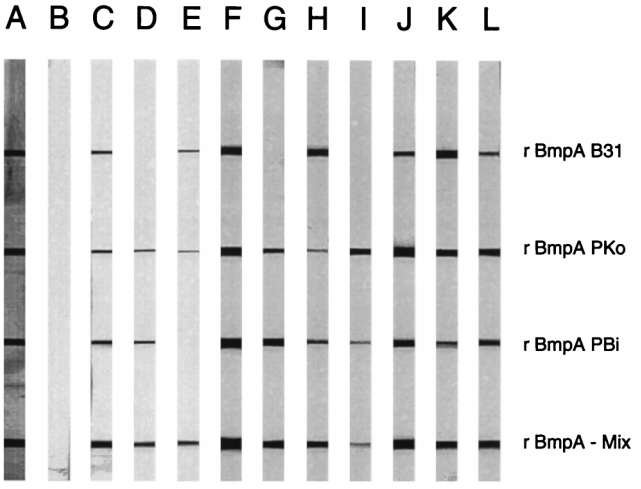


FIG. 2. Immunoblot strips with rBmpA of strains of the three *B. burgdorferi* sensu lato species pathogenic for humans. Lanes: A, transfer control (colloidal gold stain); B, purification control of BmpA with polyclonal anti-*E. coli* antisera (K69); C to E, MAbs L39B5, L39B8, and H1141, respectively; F, reference serum IgG; G to L, selection of IgG-positive sera from patients with Lyme borreliosis.

rBmpA of *B. burgdorferi* sensu stricto strain B31 (Fig. 2, lane D). All other MAbs were reactive with recombinant BmpAs from all three species (Fig. 2, lane C; MAb L39B5). A BmpA-specific monoclonal antibody (MAb H1141) recommended by the Centers for Disease Control and Prevention for the calibration of immunoblots in the United States showed weak reactivity with rBmpA of *B. afzelii* PKo, no reaction to rBmpA of *B. garinii* PBi, but a clear reaction with rBmpA of *B. burgdorferi* sensu stricto strain B31 (Fig. 2, lane E). The same results were obtained with MAb H1141, but by using *B. burgdorferi* sensu lato whole-cell lysates in conventional immunoblots (data not shown).

**Serological testing of rBmpA.** A panel of serum specimens from 86 patients and 73 controls was tested for the strain-specific reactivities of the sera with the rBmpAs of strains PKo, B31, and PBi in immunoblots and with a mixture of the three rBmpAs.

To determine the cutoff criteria for positive results, control serum specimens from 73 healthy control persons were examined. A five-grade criterion evaluation (strongly positive, positive, weakly positive but definitely positive, detectable "shading," and no reaction) was performed. Only 1 of the 73 control serum samples reacted weakly positive by IgG testing with rBmpA of strain B31. None of the other serum specimens were reactive (Table 3), thus giving specificities of 100% for rBmpA of PKo, PBi, or the rBmpA mixture and 98.6% for rBmpA of

TABLE 3. Reactivity of 73 control serum samples[a] with rBmpA proteins from various *B. burgdorferi* sensu lato strains

| Strain | Protein | Species | No. of serum specimens (% specificity) | |
|---|---|---|---|---|
| | | | IgG | IgM |
| B31 | rBmpA | *B. burgdorferi* sensu stricto | 1 (98.6) | 0 (100) |
| PKo | rBmpA | *B. afzelii* | 0 (100) | 0 (100) |
| PBi | rBmpA | *B. garinii* | 0 (100) | 1 (98.6) |
| Mixture[b] | rBmpA | | 0 (100) | 0 (100) |

[a] Sera were from 52 healthy blood donors, 10 syphilis-positive patients, and 11 rheumatoid factor-positive patients.
[b] The mixture included proteins from all three species.

TABLE 4. IgG reactivity of sera[a] from clinically diagnosed Lyme borreliosis patients

| Lyme borreliosis patients | | | No. of serum specimens (% sensitivity) | | | |
|---|---|---|---|---|---|---|
| Strain | Protein | Species | Stage I (EM; $n$ = 30) | Stage II (NB; $n$ = 30) | Stage III (ACA and AT; $n$ = 26) | Stage I to III ($n$ = 86) |
| B31 | rBmpA | *B. burgdorferi* sensu stricto | 0 (0) | 3 (10.0) | 9 (34.6) | 12 (13.9) |
| PKo | rBmpA | *B. afzelii* | 2 (6.6) | 12 (40.0) | 17 (65.4) | 31 (36.0) |
| PBi | rBmpA | *B. garinii* | 2 (6.6) | 10 (33.3) | 18 (69.2) | 30 (34.9) |
| Mixture[b] | rBmpA | | 2 (6.6) | 11 (36.6) | 17 (65.4) | 30 (34.9) |
| One of all rBmpA positive[c] | | | 2 (6.6) | 12 (40.0) | 18 (69.2) | 32 (37.2) |
| PKa2 | nBmpA | *B. burgdorferi* sensu stricto | 0 (0) | 4 (13.3) | 16 (61.5) | 20 (23.2) |
| PKo | nBmpA | *B. afzelii* | 2 (6.6) | 12 (40.0) | 22 (84.6) | 36 (41.8) |
| PBi | nBmpA | *B. garinii* | 2 (6.6) | 10 (33.3) | 23 (88.4) | 35 (40.7) |
| One of all nBmpA positive[c] | | | 2 (6.6) | 13 (43.3) | 23 (88.4) | 38 (44.2) |

[a] A total of 86 serum specimens were tested. Results for rBmpA from three different *B. burgdorferi* sensu lato strains and a mixture of rBmpA were compared to sensitivity data from conventional immunoblotting with nBmpA.
[b] The mixture included proteins from all three species.
[c] At least one from the three species was positive.

strain B31. Similar results were obtained in the IgM tests (Table 3).

IgM antibodies were detected in only 1.1 to 8.1% of the Lyme borreliosis patients. In contrast, IgG sensitivities for the three rBmpAs and the rBmpA mixture were significantly higher (Table 4). Recombinant BmpA of PKo or PBi and the rBmpA mixture reacted more than twice as often (34.9 to 36.0% overall sensitivity) in the IgG immunoblot than rBmpA of B31 (13.9%) alone. IgG antibodies were detected by the rBmpAs of strains PKo and PBi in 65 to 69% of the sera from patients with stage III of the disease (AT plus ACA), 33 to 40% of the sera from patients with stage II (NB), and only 6.6% of the sera from patients with the early (EM) stage (Table 4). In contrast, rBmpA of *B. burgdorferi* sensu stricto strain B31 was recognized by none of the sera from patients with stage I, only 10% of the sera from patients with stage II, and 34.6% of the sera from patients with stage III, resulting in this low overall sensitivity. The rBmpA mixture used in this study showed IgG sensitivities similar to that of rBmpA of strain PKo or strain PBi alone (Table 4). Results for a selection of various IgG-positive patient sera are presented in Fig. 2, lanes F to L.

## DISCUSSION

Many studies have indicated that strain heterogeneity has a considerable influence on the serodiagnosis of Lyme borreliosis, especially in Europe (16, 24, 45, 50). Therefore, we investigated by molecular and immunological methods BmpA (P39) from various *B. burgdorferi* sensu lato strains and evaluated the serological reactivity of the respective recombinantly expressed antigens with sera from Lyme borreliosis patients.

We found considerable heterogeneity among BmpAs derived from strains belonging to different species of *B. burgdorferi* sensu lato. This was also true for *B. garinii* strains, whereas BmpA was highly conserved among *B. afzelii* and *B. burgdorferi* sensu stricto strains. The topology of the BmpA cluster analysis is similar to that of the chromosomally encoded p83/100 (32) or to that of the plasmid-encoded OspA (43) and reflects heterogeneity among the three species as well as the intraspecies heterogeneity found solely among different strains of *B. garinii.*

Since BmpA is heterogeneous and variable regions are evenly distributed (only the region between amino acid positions 164 and 184 is highly conserved), this heterogeneity might influence serodiagnosis. Therefore, BmpAs from three different *B. burgdorferi* species were expressed recombinantly in *E. coli*. The expression of recombinant protein in a soluble form was most effective when an *E. coli* secretory system with the *E. coli* OmpA signal sequence instead of the BmpA signal sequence was used. Variations within the N terminus (linker peptide) of the expression products might be explained by an inaccurate endoproteolytic cleavage of the *E. coli* signal peptidase during secretion. Unfortunately, no data on the expression and purification procedure of recombinant P39 used by other investigators are available in the literature (11, 27, 34). Thus, the presence of the BmpA signal sequence within the recombinant protein and its possible influence on the sensitivity of serodiagnosis remain unclear.

The BmpA molecule has epitopes conserved among the three species, as shown by MAbs L39B1, L39B2, L39B3, L39B5, L39B7, and L39B10. These MAbs were specific for BmpA of *B. burgdorferi* sensu lato, did not show any reactivity with relapsing fever borreliae or other treponemes, and thus can possibly be used for the specific detection of *B. burgdorferi* sensu lato antigens. Whereas attempts to develop a borrelial vaccine solely with outer surface proteins appear to be successful (12, 29, 33), the importance of P39 for a protective immune response is still controversial (15, 18, 36). The potential bactericidal capability of the MAbs presented here must be evaluated in future experiments.

MAb H1141 recommended by the Centers for Disease Control and Prevention (7) for the calibration of immunoblots showed a preferential reactivity with BmpA of *B. burgdorferi* sensu stricto, weak reactivity with *B. afzelii*, but no reactivity with *B. garinii*, irrespective of the nature of BmpA (whole-cell lysate in conventional or single rBmpA in recombinant immunoblots). On the other hand, MAb L39B8 reacted with *B. afzelii* and *B. garinii* but failed to react with *B. burgdorferi* sensu stricto. Since in Europe all three species, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, have been isolated from patients (3, 47), we recommend the use of BmpA-specific MAbs recognizing conserved epitopes for the calibration of immunoblots.

The serological specificity of rBmpA is in good accordance with the results of other studies. Only a low specificity was described by Dressler et al. (9), who used conventional immunoblotting. Others reported specificities that ranged from 94 to 100% (1, 11, 16, 22, 25, 27, 34). Data for sensitivities of the P39 IgM test are controversial, depending on the assay system

used, and may also be influenced by the manifestation of the disease.

In our hands rBmpA was not a useful antigen for IgM antibody detection. Results from laboratories in the United States varied considerably, with the assays having from low (1, 9) to high (10, 22) sensitivities. Those studies used conventional blots with *B. burgdorferi* sensu stricto as the antigen, and it is not clear whether the band at 39 kDa identified in those blots truly represents BmpA. Very recent immunoprint data presented by Padula et al. (28) are in agreement with a general low IgM reactivity of this protein.

The diagnostic sensitivity of the rBmpA IgG test in this study is in accordance with most data from other investigators (1, 9–11, 22, 34). rBmpA and nBmpA in the conventional blot appear to be equally sensitive. Only Oksi and coworkers (27) found a very low sensitivity (14.6% for a *B. burgdorferi* sensu stricto-derived P39 ELISA), even though the sera investigated were defined to be from patients with late-stage disease. Since these patients were from Northern Europe (Finland), it appears reasonable that the lack of sensitivity is at least partially caused by the use of recombinant *B. burgdorferi* sensu stricto P39 as a single antigen. Our results indicate that rBmpAs from strains PKo and PBi are considerably more sensitive than rBmpA of *B. burgdorferi* sensu stricto alone for the serodiagnosis of Lyme borreliosis in European patients. Thus, a significant increase in sensitivity can be achieved by using rBmpA of *B. afzelii* and *B. garinii*, in addition to or instead of *B. burgdorferi* sensu stricto BmpA. None of the sera tested in this study showed an exclusive reaction with rBmpA of *B. burgdorferi* sensu stricto but lacked reactivity with rBmpA of the other two species. However, it cannot be excluded that infection with *B. burgdorferi* sensu stricto may lead to antibodies solely reactive with *B. burgdorferi* sensu stricto BmpA.

In our hands recombinant BmpA is as sensitive as nBmpA for the detection of IgG antibodies in patients with stage I and stage II manifestations. There are different explanations for a higher sensitivity of the blot with the whole-cell lysate late in the course of the disease. Late in the course of the disease a very broad panel of proteins is recognized by the sera. Thus, especially the use of conventional blots does not exclude the recognition of other overlying borrelial proteins with similar apparent molecular masses by SDS-PAGE. Recently, a flagellum-associated protein, FlaA, with an estimated size of approximately 38 kDa has been described (14). In addition, not all members of the *bmp* gene cluster have been examined for transcription and translation in vivo (31), and it has not been examined whether they are immunogenic. Since *bmpB*, *bmpC*, and *bmpD* are similar in size, their putative translation products might appear as one protein band in conventional immunoblots if they are expressed by the borreliae and recognized by patient sera. A reason for the lower sensitivity of rBmpA compared to that of nBmpA might also be the expression of rBmpA without its own signal sequence.

For the serodiagnosis of Lyme borreliosis in Europe, we recommend the use of rBmpA derived from all three species and the use of BmpA-specific MAbs recognizing conserved epitopes for the standardization of immunoblots.

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