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Recommended Citation

Bryksin, A., Tomova, A., Godfrey, H., & Cabello, F. (2010). BmpA Is a Surface-Exposed Outer-Membrane Protein of *Borrelia Burgdorferi*. *FEMS Microbiology Letters*, 309 (1), 77-83. <https://doi.org/10.1111/j.1574-6968.2010.02020.x>

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Published in final edited form as:

FEMS Microbiol Lett. 2010 August 1; 309(1): 77–83. doi:10.1111/j.1574-6968.2010.02020.x.

BmpA is a surface-exposed outer membrane protein of *Borrelia burgdorferi*

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Abstract

BmpA is an immunodominant protein of *Borrelia burgdorferi* as well as an arthritogenic factor. Rabbit anti-recombinant BmpA (rBmpA) antibodies were raised, characterized by assaying their cross reactivity with rBmpB, rBmpC and rBmpD, then rendered monospecific by absorption with rBmpB. This monospecific reagent reacted only with rBmpA in dot immunobinding and detected a single 39-kDa, pI 5.0, spot on two-dimensional immunoblots. It was used to assess BmpA cellular location. BmpA was present in both detergent-soluble and -insoluble fractions of Triton X-114 phase-partitioned borrelial cells, suggesting it was a membrane lipoprotein. Immunoblots of proteinase K-treated intact and Triton X-100 permeabilized cells showed digestion of BmpA in intact cells, consistent with surface exposure. This exposure was confirmed by dual-label immunofluorescence microscopy of intact and permeabilized borrelial cells. Conservation and surface localization of BmpA in all *B. burgdorferi* sensu lato genospecies could point to its playing a key role in this organism's biology and pathobiology.

Keywords

Borrelia burgdorferi; Lyme disease; BmpA

Introduction

The *Borrelia burgdorferi* B31 genome contains many genes coding for putative lipoproteins (4.9% of the chromosomal genes, 14.5% of the plasmid genes) (Fraser, Casjens S, Huang WM *et al.*, 1997; Casjens, Palmer N, van Vugt R *et al.*, 2000). Lipoproteins are usually considered structural components of the cell, but surface-exposed lipoproteins of *B. burgdorferi* can also be involved in interactions with hosts (Fraser, Casjens S, Huang WM *et al.*, 1997; Casjens, Palmer N, van Vugt R *et al.*, 2000; Liang, Nelson FK, & Fikrig E, 2002; Xu, McShan K, & Liang FT, 2008). Sequential expression of these borrelial lipoproteins in infected ticks and mammals by tightly regulated global regulatory mechanisms also underlines their relevance for the successful life cycle of this pathogen (Revel, Talaat AM, & Norgard MV, 2002; He, Oman T, Xu H, Blevins J, Norgard MV, & Yang XF, 2008). Lipoproteins such as OspA and OspC are involved in the interaction of borrelia with

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Authors' contribution

A.V.B. and A.T. contributed equally to this work.

intestinal and salivary epithelia of ticks, respectively (Pal, de Silva AM, Montgomery RR, Fish D, Anguita J, Anderson JF, Lobet Y, & Fikrig E, 2000; Pal, Yang X, Chen M, Bockenstedt LK, Anderson JF, Flavell RA, Norgard MV, & Fikrig E, 2004; Strother, Hodzic E, Barthold SW, & de Silva AM, 2007; Radolf & Caimano MJ, 2008). VlsE plays a role in evading antibacterial effects of antibodies (Zhang, Hardham JM, Barbour AG, & Norris SJ, 1997; Zhang & Norris SJ, 1998; Xu, McShan K, & Liang FT, 2008). OspE and ErpA are involved in the ability of *B. burgdorferi* to evade complement by interacting with human factor H and plasminogen (Hellwage, Meri T, Heikkila T, Alitalo A, Panelius J, Lahdenne P, Seppala IJ, & Meri S, 2001; Stevenson, El Hage N, Hines MA, Miller JC, & Babb K, 2002). Many borrelial lipoproteins mediate the organism's adhesion to integrins and host extracellular matrix molecules (Cabello, Godfrey HP, & Newman SA, 2007). P66, BBB07 and DbpA/DbpB bind to α IIb β 3/ α v β 3, α 3 β 1 and decorin (Guo, Norris SJ, Rosenberg LC, & Höök M, 1995; Guo, Brown EL, Dorward DW, Rosenberg LC, & Höök M, 1998; Coburn & Cugini C, 2003; Behera, Durand E, Cugini C, Antonara S, Bourassa L, Hildebrand E, Hu LT, & Coburn J, 2008). Bgp, DbpA and DbpB bind to glycosaminoglycans, heparin and dermatan sulfate (Parveen & Leong JM, 2000; Parveen, Caimano M, Radolf JD, & Leong JM, 2003), and BBK32 and RevA bind to fibronectin (Seshu, Esteve-Gassent MD, Labandeira-Rey M, Kim JH, Trzeciakowski JP, Höök M, & Skare JT, 2006; Brissette, Bykowski T, Cooley AE, Bowman A, & Stevenson B, 2009).

Another lipoprotein, BmpA, is highly immunogenic in human beings and animals and is one of the antigens used in serodiagnostic tests for Lyme disease (Aguero-Rosenfeld, Wang G, Schwartz I, & Wormser GP, 2005; Bryksin, Godfrey HP, Carbonaro CA, Wormser GP, Aguero-Rosenfeld ME, & Cabello FC, 2005). It is a member of the chromosomally-located paralogous family 36 which also includes BmpB, BmpC and BmpD (Cabello, Dubytska L, Bryksin A, Bugrysheva J, & Godfrey HP, 2006; Simpson, Schruppf ME, & Schwan TG, 1990). Its expression is co-regulated with that of BmpC and BmpB and appears to be subject to global regulation (Dobrikova, Bugrysheva J, & Cabello FC, 2001; Revel, Talaat AM, & Norgard MV, 2002; Ramamoorthy, McClain NA, Gautam A, & Scholl-Meeker D, 2005). BmpA is also involved in borrelial pathogenicity, and participates in development of borrelial arthritis (Pal, Wang P, Bao F, Yang X, Samanta S, Schoen R, Wormser GP, Schwartz I, & Fikrig E, 2008).

Attempts at unequivocal demonstration of BmpA surface localization using monoclonal and polyclonal antibody reagents have produced conflicting results as a result of the incomplete characterization of their reactivities with all four Bmp proteins (Scriba, Ebrahim JS, Schlott T, & Eiffert H, 1993; Sullivan, Hechemy KE, Harris HL, Rudofsky UH, Samsonoff WA, Peterson AJ, Evans BD, & Balaban SL, 1994; Bunikis & Barbour AG, 1999; Pal, Wang P, Bao F, Yang X, Samanta S, Schoen R, Wormser GP, Schwartz I, & Fikrig E, 2008). Determination of the cellular localization of BmpA is important because of its involvement in diagnosis and virulence. For this reason, we have prepared a well-characterized monospecific anti-rBmpA reagent and have used it to provide definitive evidence for the display of BmpA on the outer surface of *B. burgdorferi*.

Materials and Methods

Production of anti-BmpA rabbit antisera

After amplification by PCR from *B. burgdorferi* B31 genomic DNA, *bmpA* was cloned in pQE40 (QIAGEN, Valencia, CA) and *bmpB*, *bmpC*, and *bmpD* were cloned in pET30 (NOVAGEN, EMD Chemicals Inc, NJ). We transformed, expressed and purified rBmpA from *Escherichia coli* M15 (pREP4) (Novagen, Madison, WI) and rBmpB, rBmpC and rBmpD from *E. coli* BL21 (RIL) (Sambrook & Russell DW, 2001). Cultures were grown at 32°C to 0.5 absorbance units (595 nm), induced with 1 mM isopropyl thiogalactoside

(Denville Scientific Inc., Metuchen, NJ), and grown for an additional 3 h. rBmpA was purified from bacterial sonicates using nitriloacetate-Ni²⁺ affinity chromatography (Qiagen) and Sephacryl S-300 gel filtration chromatography (GE Healthcare, Piscataway, NJ). rBmpA purification was monitored by SDS-PAGE and silver staining (Kovarik, Hlubinova K, Vrbenska A, & Prachar J, 1987; Harlow & Lane D, 1988). Anti-rBmpA antibodies were raised by intramuscular immunization of 2.5 ± 0.3 kg female New Zealand white rabbits (Millbrook Breeding Labs, Amherst, MA) with 70 µg of purified rBmpA emulsified in 50 µl of TiterMax Gold adjuvant (Sigma Chemical Corp., St. Louis, MO), boosted with 25 µg of rBmpA emulsified in 50 µl of TiterMax Gold 100 days after primary immunization, and exsanguinated by cardiac puncture under anesthesia 28 days later. Antibody content of sera was determined by dot immunobinding (Landowski, Godfrey HP, Bentley-Hibbert SI *et al.*, 2001). Immunoglobulin (Ig) was purified from sera by precipitation with 50% saturated ammonium sulfate, precipitates were extensively dialyzed against phosphate-buffered saline (PBS), pH 7.4, and stored in aliquots at -80°C (Harlow & Lane D, 1988). Protein content was determined by absorbance at 280 nm.

Antibodies

Mouse monoclonal anti-OspA antibodies were a gift from Dr. M. Gomez-Solecki. Rat polyclonal anti-FlaB was a gift from Drs. M. Caimano and J. D. Radolf to Dr. I. Schwartz.

SDS-PAGE, non-equilibrium pH gradient electrophoresis (2D-NEPHGE) and immunoblotting

For 2D-NEPHGE (O'Farrell, 1975; Nowalk, Nolder C, Clifton DR, & Carroll JA, 2006; Nowalk, Gilmore RD, Jr., & Carroll JA, 2006), *B. burgdorferi* B31 (2.5–5 × 10⁷ cells/ml) lysates were prepared by sonication of pellets resuspended in 0.05M Tris-HCl (pH 7.4), 0.01M EDTA and 0.3% SDS buffer followed by treatment with 9.5 M (5.045g) urea-2% (0.2 g) Nonidet P-40-5% (0.5 ml) 2-mercaptoethanol-2% ampholytes (pH 3.0 to 10.0) (Bio-Rad) (Cox, Akins DR, Bourell KW, Lahdenne P, Norgard MV, & Radolf JD, 1996; Carroll, Garon CF, & Schwan TG, 1999). For the first dimension of 2D-NEPHGE, a urea-ampholine isoelectric focusing tube gel was focused for a total of 2,000 Vh. For the second dimension in 2D-NEPHGE and for SDS-PAGE, 4.5 and 12% polyacrylamide gels were used for stacking and running gels, respectively. For immunoblotting, proteins were electrolytically transferred to PVDF membranes (Bio-Rad), developed using enhanced chemiluminescence technology (ECF Western blotting kit, GE Healthcare), and read using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Preparation of monospecific anti-rBmpA

rBmp proteins were induced in *E. coli* strains using the protocols described above, the bacteria were lysed by French press, and inclusion bodies obtained by ultracentrifugation. These inclusion bodies were solubilized in 6M guanidinium HCl-1mM 2-mercaptoethanol-20 mM HEPES, pH 8.0, and rBmp proteins isolated by immobilized metal ion affinity chromatography on HisTrap FF columns (GE Healthcare) following the manufacturer's instructions. Eluted rBmp proteins were renatured in 100 mM Bis-Tris-propane, pH 7.0-400 mM magnesium formate, concentrated using Amicon Ultra-4 PL-10 centrifugal filter devices (Millipore, Billerica, MA) and chromatographed on Sephacryl S-300 (GE Healthcare). Purification of Bmp proteins was monitored by SDS-PAGE and silver staining. Anti-rBmpA was adsorbed with rBmpB immobilized on Affigel15 (Bio-Rad, Hercules, CA). Monospecificity of adsorbed anti-rBmpA antibodies was confirmed by dot immunobinding against rBmp proteins and by immunoblotting of 2D-NEPHGE gels of *B. burgdorferi* lysates.

Triton X-114 phase partitioning

To localize BmpA in cell fractions, *B. burgdorferi* B31 were lysed with 1% (vol/vol) Triton X-114 (Brandt, Riley BS, Radolf JD, & Norgard MV, 1990; Skare, Shang ES, Foley DM, Blanco DR, Champion CI, Mirzabekov T, Sokolov Y, Kagan BL, Miller JN, & Lovett MA, 1995). Bacterial cells, 5×10^8 cells/ml, were washed with PBS once, resuspended to 5×10^9 cells/ml in 1% Triton X-114 in PBS, and incubated at 4°C on a rotating platform overnight (Brusca & Radolf JD, 1994). Isolation of the detergent-insoluble fraction (periplasmic core) was performed by centrifugation at $15,000 \times g$, 45 min (Skare, *et al.*, 1995). Phase partitioning of the detergent-soluble fraction with Triton X-114 was performed by centrifugation at $15,000 \times g$ for 1 h after an incubation at 37°C for 30 min (Skare, *et al.*, 1995). Phases were precipitated by 7 volumes of acetone (Cunningham, Walker EM, Miller JN, & Lovett MA, 1988). The presence of BmpA and FlaB in the different protein fractions was assessed by immunoblotting with monospecific anti-rBmpA and anti-FlaB, respectively.

Proteinase K digestion

To determine the *in situ* susceptibility of BmpA to proteolysis, mid-log-phase *B. burgdorferi* B31 (100 µl at a concentration 2×10^9 bacteria/ml) were incubated with soluble proteinase K at final concentrations of 40, 400, or 4,000 µg/ml for 45 min at 25°C in the absence or presence of 0.05% (vol/vol) Triton X-100 (Cox, Akins DR, Bourell KW, Lahdenne P, Norgard MV, & Radolf JD, 1996; Bunikis & Barbour AG, 1999; El-Hage, Babb K, Carroll JA, Lindstrom N, Fischer ER, Miller JC, Gilmore RD, Jr., Mbow ML, & Stevenson B, 2001). The reaction was stopped and proteolysis was inhibited by adding protease inhibitors (Pefabloc SC (AEBSF), Roche Diagnostics, Mannheim, Germany). Susceptibility of BmpA, OspA, and FlaB to proteolysis was assessed by immunoblotting.

Dual immunofluorescence labeling

To demonstrate surface exposure of BmpA, 5×10^7 *B. burgdorferi* B31 were resuspended in 100 µl of BSK-H media and incubated with optimal dilutions of monospecific anti-rBmpA (1/10 dilution) and mouse anti-OspA (1/50 dilution), with monospecific anti-rBmpA (1/10 dilution) and rat polyclonal anti-FlaB antibodies (1/100), or with similar dilutions of preimmunization rabbit Ig (Cox, Akins DR, Bourell KW, Lahdenne P, Norgard MV, & Radolf JD, 1996). Cells were incubated with primary antibodies or preimmunization rabbit Ig for 1 h at 37°C with gentle mixing, washed three times with 400 µl of PBS supplemented with 10% fetal calf serum (PBS-FCS). After the final centrifugation cells were resuspended in 100 µl of PBS-FCS and 15 µl of the washed cells were placed on a glass slide in a circle marked with wax pencil and allowed to dry at room temperature. Cells were fixed with 4% formaldehyde-PBS for 20 minutes at 4°C and subsequently washed three times with the washing buffer described above. Optimal dilutions (1/200 in PBS-FCS) of Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 anti-rat IgG and Alexa Fluor 594 anti-mouse IgG (Molecular Probes, Invitrogen) were used as secondary antibodies. Slides were incubated in a wet chamber in the dark at room temperature for 1 h, washed three times with PBS-FCS and once with PBS. They were then fixed a second time with 4% formaldehyde-PBS for 15 minutes at 4°C, mounted in VectaShield media containing DAPI (Vector Laboratories, Burlingame, CA), covered with a 1 mm coverslip and sealed with nail polish. A similar protocol was used for *B. burgdorferi* cells that had been fixed with 50 µl of 60% methanol for 10 minutes, before being washed and reacted with the primary and secondary antibodies as described above. Stained cells were visualized with a Zeiss Inverted Axiovert 200 motorized microscope with a 100X (PlanApo 1.4 oil PH3 objective) and Zeiss filter sets 31, 34, and 38 for AlexaFluor 594, 488 and DAPI respectively. The pictures were taken with a Zeiss AxioCam MRM cool CCD camera and were analyzed using Axiovision 4.3 software.

RESULTS AND DISCUSSION

Unabsorbed anti-rBmpA Ig had a dot immunobinding titer of 1:10,000 with 10 ng of rBmpA or rBmpB and reacted minimally with rBmpC or rBmpD. After absorption with rBmpB, anti-rBmpA Ig had a titer of 1:100 with 1 and 10 ng of rBmpA and did not react with similar quantities of rBmpB, rBmpC or rBmpD (Fig. 1A2). Absorbed anti-rBmpA at a 1:100 dilution detected a single immunoreactive spot consistent with BmpA at 39 kDa, pI 5.0, in 2D-NEPHGE gels of *B. burgdorferi* lysates (Fig. 1B). This dilution of this reagent was used for all subsequent immunoblotting.

Fractionation of intact *B. burgdorferi* cells with Triton X-114 showed that both immunoreactive BmpA and FlaB were present in the detergent-insoluble fraction containing periplasmic core proteins (Fig. 2A, lanes 2), while only BmpA was present in the detergent phase of the Triton X-114 soluble fraction containing the outer membrane proteins (Fig. 2A, lanes 4). A small amount of BmpA was also detected in the aqueous phase of the Triton X-114 soluble fraction (Fig. 2A, lanes 3). Detection of BmpA in the detergent phase of Triton X-114 fractionation is consistent with its being located in the outer membranes of *B. burgdorferi* (Brusca & Radolf JD, 1994; Skare, Shang ES, Foley DM, Blanco DR, Champion CI, Mirzabekov T, Sokolov Y, Kagan BL, Miller JN, & Lovett MA, 1995). While detection of immunoreactive BmpA in the Triton X-114-insoluble fraction might imply that some BmpA is associated with periplasmic cellular proteins and the cytoplasmic membrane, this fraction also includes intact cells with outer membranes still attached (Crother, Champion CI, Wu XY, Blanco DR, Miller JN, & Lovett MA, 2003). These data suggest that BmpA, unlike FlaB, is a lipoprotein, and most probably located in the outer membrane of *B. burgdorferi*.

To provide additional data on BmpA localization, intact *B. burgdorferi* cells were incubated with increasing concentrations of proteinase K in the absence or presence of Triton X-100. In the absence of proteinase K, BmpA, OspA, and FlaB were not affected by incubation of *B. burgdorferi* with Triton X-100 (Fig. 2B, lanes 1 and 2). In the presence of Triton X-100, all three proteins were completely digested by proteinase K at final concentrations of 40, 400 (Fig. 2B, lanes 4 and 6) and 4,000 (not shown) $\mu\text{g/ml}$. In the absence of Triton X-100, BmpA and OspA were digested by proteinase K at final concentrations of 40 and 400 $\mu\text{g/ml}$ (Fig. 2B, lanes 3 and 5); FlaB was not (Fig. 2B, lanes 3 and 5). The susceptibility of BmpA and OspA to proteinase K in intact *B. burgdorferi* indicates that BmpA, like OspA, is exposed on the surface of *B. burgdorferi*. The insensitivity of FlaB to proteinase K in intact organisms is consistent with its location in the periplasmic space below the surface membrane (Bunikis & Barbour AG, 1999).

The surface exposure of BmpA in *B. burgdorferi* B31 was further confirmed by dual-label indirect immunofluorescence. Intact borrelia cells were double labeled in solution with optimal dilutions of monospecific anti-rBmpA and anti-OspA antisera or anti-rBmpA and anti-FlaB antisera. Similar dilutions of preimmunization rabbit Ig were used as controls. Intact *B. burgdorferi* showed dual labeling of their surface with anti-rBmpA and anti-OspA antibodies (Fig. 3) but remained unlabeled by anti-FlaB (Fig. 3) or preimmunization Ig (Fig. 3). After permeabilization of the outer membrane by methanol fixation, *B. burgdorferi* cells were labeled by all three antibodies (Fig. 3) but not by preimmunization Ig (Fig. 3). These results confirm the results of cell fractionation and proteinase K treatment experiments and indicate that BmpA is exposed on the surface of *B. burgdorferi* cells (Cox, Akins DR, Bourell KW, Lahdenne P, Norgard MV, & Radolf JD, 1996).

Previous work with monoclonal anti-BmpA antibodies indicated that BmpA was resistant to treatment with proteinase K in intact borrelia and suggested its lack of exposure on the

surface of these cells (Bunikis & Barbour AG, 1999). However, it was not clear from this earlier study whether the epitopes recognized by this monoclonal antibody were potentially exposed on the surface of borrelial cells and whether the epitopes it recognized were only found on BmpA. Experiments with a different monoclonal anti-BmpA antibody and biotin-labeled intact borrelia suggested that BmpA was probably associated with the cytoplasmic membrane (Sullivan, Hechemy KE, Harris HL, Rudofsky UH, Samsonoff WA, Peterson AJ, Evans BD, & Balaban SL, 1994). Here again the epitope recognized was not identified and the reactivity of this antibody with the other Bmp proteins was not determined. A third series of experiments concluded that BmpA and FlaB were detected with rat antisera only when the outer membrane was disrupted but again the specificity of the antisera against other Bmp proteins was not examined (Cox & Radolf JD, 2001). The monospecificity of our anti-rBmpA reagent and its lack of reactivity with BmpB, BmpC and BmpD in dot immunobinding and 2D-NEPHGE is the probable explanation of the differences between our results and those of previous workers (Sullivan, Hechemy KE, Harris HL, Rudofsky UH, Samsonoff WA, Peterson AJ, Evans BD, & Balaban SL, 1994; Bunikis & Barbour AG, 1999; Cox & Radolf JD, 2001).

Our results with this well characterized monospecific reagent provide unequivocal evidence for exposure of BmpA on the surface of *B. burgdorferi* cells. They are also fully consistent with earlier suggestive evidence locating BmpA on the surface of borrelial cells (Roessler, Hauser U, & Wilske B, 1997; Bryksin, *et al.*, 2005) and with the ability of *B. burgdorferi* expressing BmpA to elicit proinflammatory cytokines from cultured human synovial cells and to bind to laminin (Yang, Izadi H, Coleman AS, Wang P, Ma Y, Fikrig E, Anguita J, & Pal U, 2008; Verma, Brissette CA, Bowman A, & Stevenson B, 2009). They also complement a recent report demonstrating the virulence activity of BmpA that was based on a less well-characterized monospecific anti-BmpA reagent (Pal, Wang P, Bao F, Yang X, Samanta S, Schoen R, Wormser GP, Schwartz I, & Fikrig E, 2008). The availability of monospecific anti-BmpA antibodies can be critical for future *in vitro* and *in vivo* studies of binding of *B. burgdorferi* to host molecules and its role in virulence.

Acknowledgments

We thank Dr. Maria Gomez-Solecki for the OspA monoclonal antibody and Drs. M. Caimano and J. Radolf for the rat polyclonal anti-FlaB that was provided to us by Dr. I. Schwartz. We thank Dr. Dana Mordue for advice with the immunological labeling of borrelia. We thank Ms. J. J. Shin for help in the preparation of the rabbit anti-rBmpA polyclonal sera as a part of her doctoral thesis. We thank Mrs. Harriett V. Harrison for help with the preparation of this manuscript. This work was supported by NIH grant R01 AI48856 to F. C. C.

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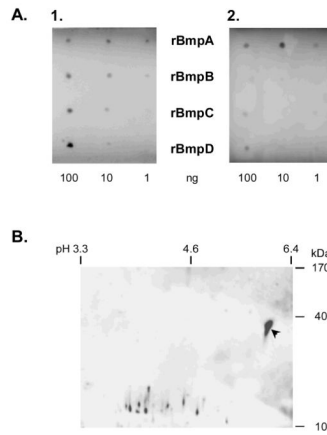
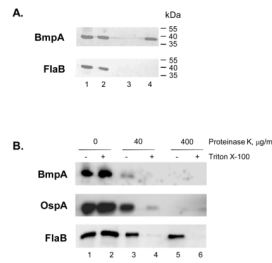


Fig. 1.

A. Dot immunobinding of rabbit anti-rBmpA before (1) and after (2) absorption with insolubilized rBmpB to generate a monospecific anti-BmpA reagent. This analysis was repeated a second time with similar results. B. Immunoblot of *B. burgdorferi* B31 lysate proteins separated by 2D-NEPHGE and developed with monospecific anti-rBmpA. A single reactive spot (arrowhead) consistent with the 39-kDa, pI 5.0, characteristics of BmpA is detected. This analysis was repeated a second time with similar results. The low molecular weight spots in the acidic range of the blot are artifacts due to locally high concentrations of the fluorescent detection agent ECF substrate. See Materials and Methods section for details.

**Fig. 2.**

A. Fractionation of BmpA and FlaB in 2×10^8 *B. burgdorferi* B31 cells treated with 1% Triton X-114, separated by SDS PAGE, and developed with anti-rBmpA or anti-FlaB antibodies. Lanes 1, whole *B. burgdorferi* B31 treated with Triton X-114; lanes 2, insoluble material from whole cells treated with Triton X-114; lanes 3, aqueous phase of Triton X-114-soluble fraction; lanes 4, detergent phase of Triton X-114-soluble fraction. See Materials and Methods section for details. B. Sensitivity of BmpA, OspA, and FlaB in 2×10^7 intact *B. burgdorferi* B31 to digestion by proteinase K in the absence (-) or presence (+) of 0.05% Triton X-100. Treated cells were lysed and BmpA, OspA and FlaB were analyzed by SDS-PAGE and immunoblotting using monospecific rabbit anti-rBmpA, monoclonal mouse anti-OspA, and rat anti-FlaB. See Materials and Methods section for details.

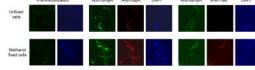


Fig. 3.

Dual-labeling indirect immunofluorescence staining of unfixed or methanol-fixed *B. burgdorferi* B31 with monospecific rabbit anti-BmpA and monoclonal mouse anti-OspA antibodies (center panels), or monospecific rabbit anti-rBmpA and rat anti-FlaB antibodies (right panels). Controls were incubated with preimmunization rabbit Ig and treated as the experimental slides with Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 anti-rat IgG and Alexa Fluor 594 anti-mouse IgG. Only controls reacted with anti rabbit AlexaFluor 488 and DAPI are displayed ((left panels). The DAPI fluorescent stain was used to monitor the presence of borreliae on a slide. See Materials and Methods section for details.