

Surface exposure and protease insensitivity of *Borrelia burgdorferi* Erp (OspEF-related) lipoproteins

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***Borrelia burgdorferi* can encode numerous lipoproteins of the Erp family. Although initially described as outer surface proteins, the technique used in that earlier study has since been demonstrated to disrupt bacterial membranes and allow labelling of subsurface proteins. Data are now presented from additional analyses indicating that Erp proteins are indeed surface exposed in the outer membrane. Surface localization of these infection-associated proteins indicates the potential for interactions of Erp proteins with vertebrate tissues. Some Erp proteins were resistant to *in situ* digestion by certain proteases, suggesting that those proteins fold in manners which hide protease cleavage sites, or that they interact with other protective membrane components. Additionally, cultivation of *B. burgdorferi* in the presence of antibodies directed against Erp proteins inhibited bacterial growth.**

Keywords: spirochaete, immunofluorescence, Erp proteins, bacterial surface proteins, protease resistance, Lyme disease

INTRODUCTION

The causative agent of Lyme disease, the spirochaete *Borrelia burgdorferi*, has evolved mechanisms enabling infection of both warm-blooded and arthropod hosts, and permitting efficient transmission between these two very different types of hosts. When a tick feeds on an infected vertebrate, ingested spirochaetes colonize the tick's midgut and persist in that organ until the tick feeds again. After the tick attaches to another vertebrate host

and begins feeding, the *B. burgdorferi* penetrate the tick midgut wall, migrate through the haemolymph to the salivary glands and are transmitted to the vertebrate via the tick's saliva. Upon entering the new, warm-blooded, host, the bacteria presumably interact with the vertebrate's tissues to facilitate dissemination throughout its body (Schwan *et al.*, 1999). The different environments encountered by *B. burgdorferi* during this complex infectious cycle undoubtedly require that the bacteria sense their location and synthesize proteins appropriate for that time and place. Since proteins that interact with host tissues will most likely be located on the outer surface of the bacteria, identification of surface-exposed *B. burgdorferi* proteins can lead to increased understanding of the mechanisms underlying the ability of these bacteria to infect humans and other animals. In addition, surface proteins synthesized during

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Abbreviation: IFA, immunofluorescent antibody.

mammalian infection are potential candidates for vaccines to prevent Lyme disease.

B. burgdorferi contains many lipoproteins, and early studies of these and other spirochaetes utilized a variety of methods to study lipoprotein localization (Barbour *et al.*, 1983a; Bledsoe *et al.*, 1994; Cunningham *et al.*, 1988; Fuchs *et al.*, 1992; Jones *et al.*, 1995; Lam *et al.*, 1994; Luft *et al.*, 1989; Radolf, 1994; Radolf *et al.*, 1995). More recently, it has become apparent that outer membranes of many spirochaetes, including *B. burgdorferi*, are relatively fragile, and some previously utilized techniques may yield inaccurate results (Cox *et al.*, 1996). Re-evaluation of spirochaetal membrane proteins indicated that some lipoproteins initially described as 'outer surface proteins' appear to be primarily located in the inner membrane and/or inner leaflet of the outer membrane (Bledsoe *et al.*, 1994; Brusca *et al.*, 1991; Cox *et al.*, 1996; Radolf, 1994; Radolf *et al.*, 1994). It is important, then, that proteins designated as surface exposed by potentially flawed methods be examined further by other techniques (Haake, 2000).

All Lyme disease spirochaetes that have been examined contain large numbers of genes encoding members of the Erp protein family, encoded on multiple, homologous circular and linear plasmids (the cp32 family of plasmids). These proteins apparently perform functions that are unique to the infectious cycle of Lyme disease spirochaetes, since *erp* genes have not been identified in any other bacteria of the genus *Borrelia* (Stevenson *et al.*, 2000a). All *erp* genes hold several features in common, including well-conserved promoter DNA sequences, locations on a family of homologous plasmids and the encoding of highly charged lipoproteins with well conserved leader polypeptides (reviewed by Stevenson *et al.*, 2000b). Those Erp proteins examined were all found to be lipidated by bacteria (Akins *et al.*, 1995; Lam *et al.*, 1994; Wallich *et al.*, 1995). Laboratory animals infected by tick bites generally produce antibodies against Erp proteins within the first 2–4 weeks of infection (Akins *et al.*, 1995; Das *et al.*, 1997; Nguyen *et al.*, 1994; Stevenson *et al.*, 1998a; Suk *et al.*, 1995; Sung *et al.*, 2000; Wallich *et al.*, 1995), suggesting that Erp proteins play roles in the initial stages of mammalian infection. Reverse transcriptase-PCR studies have also demonstrated *erp* expression during this time period (Anguita *et al.*, 2000; Das *et al.*, 1997), as have analyses of bacteria grown in chambers implanted within the bodies of laboratory animals (Akins *et al.*, 1998). To date, 17 *erp* genes at 10 loci have been identified in *B. burgdorferi* strain B31. The coding regions of three bicistronic loci, *erpAB*, *erpIJ* and *erpNO*, are identical, so their protein products are indistinguishable and are referred to collectively as ErpA/I/N and ErpB/J/O. The other seven B31 loci encode Erp proteins with varying degrees of dissimilarity (Casjens *et al.*, 1997, 2000; Stevenson *et al.*, 1998a, b). It is assumed that the variations among Erp protein sequences confer some advantage to the bacteria, but the nature of that benefit remains to be elucidated (El-Hage *et al.*, 1999; Stevenson *et al.*, 2000b; Sung *et al.*, 2000).

The lipid moiety of Erp lipoproteins anchors them to a membrane, but prior to the current study it was not known with confidence to which membrane(s) or to which leaflet(s) the Erps are attached. One previous study examined the cellular localization of two proteins of the Erp family, the OspE and OspF proteins of strain N40, using immunofluorescent antibody (IFA) analyses of bacteria fixed with formaldehyde (Lam *et al.*, 1994). While that study detected antibody binding to the fixed bacteria, suggestive of surface exposure, it has since been observed that fixation can disrupt the outer membrane, allowing subsurface proteins to bind antibodies (Cox *et al.*, 1996). Additionally, the polyclonal antisera used in the earlier study contain antibodies that recognize other proteins besides OspE and OspF (Marconi *et al.*, 1996; Stevenson *et al.*, 1995), so it is possible that the proteins detected by those IFA analyses were neither OspE nor OspF. Other studies demonstrated that vaccination with some recombinant Erp proteins provided partial protection against *B. burgdorferi* infection, suggestive of exposure to the external environment, yet vaccination with other Erp proteins failed to protect against infection, arguing against surface localization (Nguyen *et al.*, 1994; Wallich *et al.*, 1995). Since the cellular location of the *B. burgdorferi* Erp proteins was unclear, we examined these proteins through a variety of independent techniques. Together, these data indicate that Erp proteins are exposed to the external environment in the *B. burgdorferi* outer membrane and can therefore facilitate interactions between these bacteria and their hosts.

METHODS

Bacteria and culture conditions. *B. burgdorferi* strain B31 was originally isolated from a tick collected on Shelter Island, NY, USA, and cloned by limiting dilution (Barbour *et al.*, 1983b; Burgdorfer *et al.*, 1982). The B31 culture used in these studies is infectious to both mice and ticks (Schwan *et al.*, 1995). These bacteria harbour all known B31 cp32 plasmid family members except cp32-2, and thus encode all known B31 Erp proteins except ErpC and ErpD (Casjens *et al.*, 1997, 2000; Miller *et al.*, 2000a; Stevenson *et al.*, 1998a). Two alleles of *erpB* have been identified in different cultures of strain B31: *erpB1*, which contains a premature stop codon and is carried by some non-infectious bacteria (Stevenson *et al.*, 1996), and *erpB2*, which encodes a much longer protein and is carried by infectious bacteria of this strain (Casjens *et al.*, 2000; Stevenson *et al.*, 1998a, b). Bacteria in the culture used in the present studies contain only the *erpB2* allele (Stevenson *et al.*, 1998a). As noted in the Introduction, the coding regions of the strain B31 *erpAB2*, *erpIJ* and *erpNO* loci are identical, and thus their proteins are indistinguishable and are collectively referred to as ErpA/I/N and ErpB/J/O to reflect this point.

In vitro temperature shift experiments were conducted as previously described (Stevenson *et al.*, 1995), using Barbour–Stoener–Kelly II (BSK-II) (Barbour, 1984) medium containing 6% rabbit serum (Sigma). Bacteria used for Erp protein localization analyses were grown at 35 °C to late-exponential phase (approx. 10⁸ bacteria ml⁻¹) in a commercially prepared BSK formulation, BSK-H (Sigma), from lot number 38H8425. For an as yet undetermined reason, cultivation in this particular lot of BSK-H medium causes *B. burgdorferi* strain

B31 to constitutively synthesize high levels of Erp proteins (K. Babb, N. El-Hage, J. C. Miller, J. A. Carroll and B. Stevenson, unpublished results).

Antibodies. All antibodies were generated in strict accordance with United States requirements for the experimental use of animals. Polyhistidine-tagged recombinant B31 Erp proteins were produced in *Escherichia coli* and purified as previously described (Miller *et al.*, 2000b; Stevenson *et al.*, 1998a). Polyclonal antisera were generated for the B31 ErpA/I/N, ErpB/J/O, ErpK, ErpL, ErpM and ErpX proteins by vaccinating New Zealand White rabbits with approximately 50 µg purified protein in Freund's complete adjuvant, followed by booster vaccinations 2 and 4 weeks later with the same dose of protein in Freund's incomplete adjuvant. Rabbit antisera directed against the B31 ErpP, ErpQ and ErpY proteins were similarly produced by Animal Pharm Services. All rabbits were exsanguinated 2 to 4 weeks after the final boost. For some studies, nonspecific antibodies were adsorbed from polyclonal antisera by incubating serum, diluted 1:200 in Tris-buffered saline/Tween 20 (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), for 1 h at 37 °C with lysates of *E. coli* expressing all other recombinant Erp proteins (Stevenson *et al.*, 1998a). Specificities of preadsorbed antisera were assessed by immunoblotting with all purified recombinant Erps.

A panel of monoclonal antibodies (mAbs) were produced from the spleen of a mouse infected with isolate B31 via tick bite, as previously described (Gilmore & Mbow, 1998, 1999; Mbow *et al.*, 1999). Briefly, *B. burgdorferi* B31-infected *Ixodes scapularis* nymphal ticks were fed to repletion upon Balb/cByJ mice, and mice were reinfested 1 month later with additional *B. burgdorferi* B31-infected ticks. Three weeks after tick feeding, spleen cells were fused with P3X63-Ag8.653 myeloma cells. Wells were screened by ELISA using a B31 lysate as antigen and cells from positive wells were cloned by limiting dilution. mAb specificities were determined by immunoblotting isolate B31 whole-cell lysates and recombinant Erp proteins. Unless otherwise stated, all mAbs used in the following experiments were undiluted hybridoma supernatants.

Two previously described mAbs that recognize other *B. burgdorferi* B31 proteins were used as experimental controls: B5, directed against the OspC protein (Gilmore & Mbow, 1999; Mbow *et al.*, 1999), and H9724, directed against the flagellar FlaB subunit (Barbour *et al.*, 1986) (provided by Tom Schwan, Rocky Mountain Laboratories, NIH, Hamilton, MT, USA). Two antibodies directed against non-borrelial proteins were used as additional controls in growth inhibition assays: mAb 83-12-5, directed against mouse CD8 (provided by Jerold Woodward, University of Kentucky, KY, USA), and polyclonal rabbit antiserum HTV, raised against the *Yersinia pestis* LcrV protein (provided by Susan Straley, University of Kentucky, KY, USA).

In situ protease treatment. *B. burgdorferi* was grown to mid-exponential phase in BSK-H, pelleted by centrifugation for 10 min at 1000 r.p.m. in a Beckman GPR centrifuge with a GH3.8 swinging-bucket rotor, washed once with PBS plus 10 mM MgCl₂ (PBS-Mg), and resuspended in the same buffer to a final concentration of 2×10^9 bacteria ml⁻¹. Examination of bacterial suspensions by phase-contrast light microscopy did not indicate detectable lysis of the bacteria. Bacteria were then incubated at room temperature with a protease for 30 min, 1 h or 2 h, whereupon digestion was terminated by addition of an appropriate inhibitor followed by sample boiling. One of three different proteases were used in each

experiment at the following final concentrations: 40 µg proteinase K ml⁻¹ (Sigma), 40 or 100 µg trypsin ml⁻¹ (Sigma) or 0.05 µg Pronase ml⁻¹ (Boehringer Mannheim). Proteinase K was inhibited by addition of PMSF to a final concentration of 1.6 mg ml⁻¹. Trypsin was inhibited by the addition of PMSF and pefabloc SC (Boehringer Mannheim) to final concentrations of 1.6 mg ml⁻¹ and 0.3 mg ml⁻¹, respectively. Pronase was inhibited by addition of PMSF, pefabloc SC and EDTA to final concentrations of 0.06 mg ml⁻¹, 0.3 mg ml⁻¹ and 0.5 mg ml⁻¹, respectively. Control aliquots of bacteria were incubated in buffer for 2 h at room temperature without added protease, followed by addition of inhibitor and boiling as with protease-treated bacteria. Equal volumes of bacterial lysates were subjected to SDS-PAGE and the proteins transferred to nitrocellulose membranes. Susceptibility of individual proteins to protease digestion was assessed by immunoblotting with appropriate monoclonal or polyclonal antibodies, followed by incubation with protein A-horseradish peroxidase conjugate (Amersham) and bound antibodies visualized by enhanced chemiluminescence (Amersham) (Miller *et al.*, 2000b). As experimental controls, lysates were also immunoblotted with mAbs directed against OspC [(located on the bacterial outer surface and thus susceptible to proteolysis (Fuchs *et al.*, 1992; Mathiesen *et al.*, 1998; Wilske *et al.*, 1993)] and FlaB [located in the periplasmic space and thus protected against protease digestion in intact bacteria (Bono *et al.*, 1998; Bunikis & Barbour, 1999; Holt, 1978)].

Additional control experiments were performed to assess the protease susceptibility of proteins when not *in situ*. Bacteria were treated with proteases as above, but with the addition of Triton X-100 to a final concentration of 0.1%, which generally disrupts *B. burgdorferi* outer membranes (Cox *et al.*, 1996), and lysates were immunoblotted with Erp- and FlaB-directed antibodies. Purified recombinant Erp proteins were incubated for 30 min in either of the three proteases and immunoblotted with an appropriate antibody.

Immunofluorescence analysis of intact *B. burgdorferi*. One millilitre of a mid-exponential phase culture, grown in BSK-H, was placed on a Biocoat poly-D-lysine-coated 12 mm round cover slip (Becton Dickinson) in the well of a 24-well culture plate (Bunikis & Barbour, 1999; Cox *et al.*, 1996). Bacteria were gently pelleted onto the cover slip by centrifugation for 10 min at 200 g in a Beckman GPC centrifuge holding a GH3.8 swinging-bucket rotor. Slides were then washed twice with PBS. Bacteria were incubated in 500 µl PBS containing 10% heat inactivated fetal bovine serum (Life Technologies) and 1% mouse serum (Sigma) for 1 h at room temperature. Cells were then washed with PBS, incubated overnight with 200 µl mAb B11 (anti-ErpA/I/N), B5 (anti-OspC) or H9724 (anti-FlaB), followed by incubation for 2 h with goat anti-mouse IgG-Oregon green conjugate (Molecular Probes) in PBS containing 1% mouse serum. The coverslips were washed with PBS, mounted to slides and viewed by epifluorescence microscopy. As controls for possible interactions with the secondary antibody, bacteria were also treated as above except without incubation with a primary antibody.

Electron microscopy. Samples were washed in Hank's Balanced Salt Solution (HBSS) (Life Technologies) and 5 µl droplets were allowed to adhere to carbon/collodion-coated nickel grids for 60 min at room temperature. The samples were blocked with 3% BSA in HBSS for 30 min prior to a 60 min incubation with either mAb B11 (anti-ErpA/I/N) or H9724 (anti-FlaB). After washing with the blocking agent, the samples were labelled with a secondary gold-conjugated antibody (Ted Pella) for 60 min. Cells were washed, fixed with

2.5% glutaraldehyde and stained with 1% ammonium molybdate prior to being viewed at 80 kV on a Hitachi 7500 transmission electron microscope.

Growth inhibition by antibodies. *B. burgdorferi* were grown in BSK-H to mid-exponential phase (approx. 10^7 bacteria ml^{-1}) and 100 μl aliquots were placed in each well of a 96-well tissue culture dish (Becton Dickinson). One hundred microlitres of each polyclonal rabbit antiserum or mAb hybridoma supernatant, either undiluted or serially diluted in BSK-H, was added to the bacterial cultures. Plates were covered with Breathe-Easy gas-permeable adhesive seals (Diversified Biotech) (Bono *et al.*, 2000) and incubated at 37 °C in a 5% CO_2 environment. After 72 h, growth was monitored visually for colour changes in the medium's phenol red indicator, since a change from red to yellow indicates acidification due to bacterial growth (Sadziene *et al.*, 1993). Culture aliquots were also examined at that time by phase-contrast light microscopy for absence of bacterial motility and the formation of immobile bacterial aggregates (Bunikis & Barbour, 1999; Cinco, 1992; Coleman *et al.*, 1992; Hanson *et al.*, 1998; Luke *et al.*, 2000; Pavia *et al.*, 1991; Sadziene *et al.*, 1993).

As controls, bacteria were also cultivated as above with the OspC-directed mAb B5, the FlaB-directed mAb H9724, non-borrelial mAb 83-12-5, hybridoma culture medium, non-borrelial polyclonal rabbit antiserum HTV, normal rabbit serum (all undiluted) or BSK-H without any additions.

RESULTS

Production of Erp-specific antibodies

Previously, a panel of five mAbs was generated from mice that had been infected with *B. burgdorferi* B31 via tick bite. One of these mAbs was directed against the OspC surface protein (Gilmore & Mbow, 1999; Mbow *et al.*, 1999). Two others recognized the Rev protein (Gilmore & Mbow, 1998, and our unpublished results), an uncharacterized 17 kDa protein encoded by a pair of identical genes, one each on the B31 cp32-1 and cp32-6 plasmids (Casjens *et al.*, 2000). The two remaining mAbs, B11 and B31.100, both specifically recognized B31 proteins with approximate molecular masses of 19 kDa. We and others have previously reported that a

small number of *B. burgdorferi* proteins, including the Erp proteins, are produced at low levels by bacteria cultured at 23 °C but are made at significantly higher levels following a shift from 23 to 35 °C, a temperature change that mimics that experienced by the bacteria within a tick vector before and during feeding on a warm blooded animal (air and blood temperatures, respectively) (Akins *et al.*, 1998; Schwan *et al.*, 1995; Stevenson *et al.*, 1995, 1998a). Since both the B11 and B31.100 mAbs recognized antigens of sizes similar to some strain B31 Erp proteins, lysates of strain B31 grown at 23 °C or shifted from 23 to 35 °C, as well as recombinant Erp proteins, were immunoblotted with these hybridoma supernatants. These studies revealed that both the B11 and B31.100 mAbs recognized ErpA/I/N and no other protein produced by the B31 culture used in the present studies (Fig. 1 and data not shown). The nature by which these mAbs were produced is additional evidence that *B. burgdorferi* synthesize ErpA/I/N during mammalian infection (Stevenson *et al.*, 1998a). Significantly, for the studies described below, the specificities of these two antibodies enabled analysis of ErpA/I/N in intact bacteria without the complications of cross-reactivity often found with polyclonal antiserum (Lam *et al.*, 1994; Marconi *et al.*, 1996; Stevenson *et al.*, 1995).

Polyclonal antisera were also generated by vaccination of rabbits with recombinant B31 Erp proteins. Since B31 Erps can share significant amino acid sequence identities (Stevenson *et al.*, 1998b), some antisera contained antibodies that also recognized other Erp proteins. For example, antisera from rabbits vaccinated with recombinant ErpB/J/O also bound the similar ErpM, ErpQ and ErpX proteins (data not shown). Preincubation of diluted antisera with non-targeted recombinant Erp proteins was found to adsorb all detectable cross-reactive antibodies. Such sera were used to confirm that proteins detected by the immunoblotting studies described below were indeed the Erp proteins in question. These sera also confirmed that strain B31 synthesizes the newly described ErpP, ErpQ and ErpY proteins (Casjens *et al.*, 2000) *in vitro* and that their synthesis is regulated by culture temperature shift in manners similar to the other, previously described B31 Erps (data not shown).

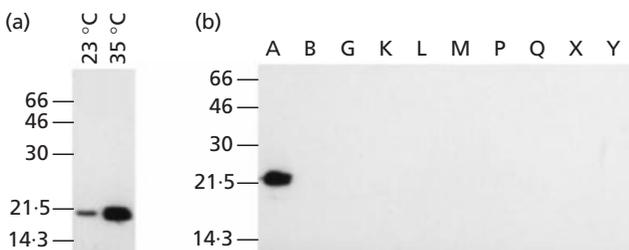


Fig. 1. Specificity of mAb B11 for ErpA/I/N. (a) Immunoblot of bacteria cultured at 23 °C or shifted from 23 to 35 °C (labelled 23 °C and 35 °C, respectively). Note that this mAb recognizes only a single protein in the bacterial lysates. (b) Immunoblot of recombinant B31 Erp proteins ErpA/I/N, ErpB/J/O, ErpG, ErpK, ErpL, ErpM, ErpP, ErpQ, ErpX and ErpY (labelled A, B, G, K, L, M, P, Q, X and Y, respectively). As previously described (Stevenson *et al.*, 1998a), native and recombinant ErpA/I/N exhibit different electrophoretic mobilities.

Protease sensitivity of Erp proteins *in situ*

Protein surface exposure was first examined by incubation of intact bacteria with proteases, on the premise that exposed proteins will be degraded, while subsurface proteins are protected against proteolysis. Three different proteases were used in these studies, since some *B. burgdorferi* surface proteins are known to be resistant to certain enzymes (Bunikis & Barbour, 1999; Dunn *et al.*, 1990; Zückert *et al.*, 2001). Some B31 Erp proteins, such as ErpA/I/N, were completely degraded after short exposure times to all examined proteases, indicative of exposure to the external environment (Fig. 2, Table 1). The known outer surface protein OspC was also degraded by protease (Fuchs *et al.*, 1992; Mathiesen *et al.*, 1998; Wilske *et al.*, 1993). On

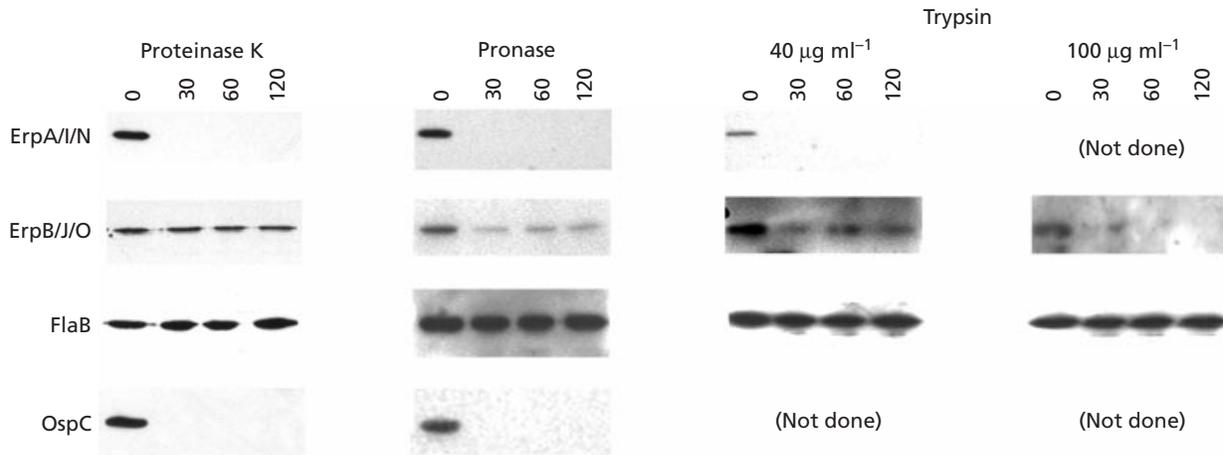


Fig. 2. Representative immunoblots of *in situ* protease treated *B. burgdorferi*. Bacteria were incubated with either proteinase K, Pronase or trypsin (at 40 or 100 µg ml⁻¹) for either 30, 60 or 120 min, or in buffer without any protease (labelled 0).

Table 1. Susceptibility of proteins to protease digestion in intact bacteria

Lysates of bacteria that had been incubated with proteases were immunoblotted with each antibody and scored for complete protein digestion (+), digestion of only some protein molecules (±) or no detectable digestion (-). ND, Not done.

Protein	Proteinase K	Pronase	Trypsin (µg ml ⁻¹)	
			40	100
ErpA/I/N	+	+	+	ND
ErpB/J/O	-	±	±	+
ErpK	-	±	ND	ND
ErpL	+	ND	ND	ND
ErpM	-	±	±	+
ErpP	+	+	+	ND
ErpQ	±	±	±	+
ErpX	-	±	±	±
ErpY	+	ND	ND	ND
OspC	+	ND	ND	ND
FlaB	-	-	-	-

the other hand, some proteins, such as ErpB/J/O, were not detectably degraded by proteinase K and only a portion of the molecules were digested by Pronase or 40 µg trypsin ml⁻¹. Complete degradation of ErpB/J/O was only observed after 60 min incubation with 100 µg trypsin ml⁻¹ (Fig. 2). Even a 2 h incubation with the highest tested concentration of trypsin did not completely digest all ErpX molecules, although some proteolysis was apparent (Table 1). The bacterial outer membranes remained intact during all *in situ* proteolysis treatments, since there was no detectable degradation of FlaB, a component of the periplasmic flagella (Fig. 2). We conclude that all tested B31 Erp proteins are exposed

to the environment and that some are resistant to degradation by certain proteases.

Additional experiments demonstrated that protease insensitivity of Erp proteins was not solely a consequence of amino acid composition. Incubation of cells with proteases in buffer containing 0.1% Triton X-100, which generally disrupts *B. burgdorferi* outer membranes (Cox *et al.*, 1996), resulted in complete digestion of all tested Erp proteins. FlaB was also completely degraded by proteases under these conditions. Furthermore, purified recombinant Erp proteins were completely digested after 30 min incubations with each of the examined proteases (data not shown).

IFA analysis and immunogold labelling of unfixed bacteria

Since fixation can disrupt *B. burgdorferi* membranes, unfixed bacteria were used for IFA studies to further assess Erp surface exposure. We observed that greater than 70% of examined bacteria bound ErpA/I/N-specific mAb B11, again indicating that this Erp protein is exposed on the outer surface of *B. burgdorferi* (Fig. 3). Bacteria did not fluoresce uniformly, but instead exhibited a punctate pattern, suggesting localization of ErpA/I/N at certain points along the bacterial cell. However, since *B. burgdorferi* B31 does not produce quantities of ErpA/I/N sufficient to be visualized by Coomassie brilliant blue staining of cell lysates (Stevenson *et al.*, 1995), the punctate fluorescence pattern may simply be a consequence of low ErpA/I/N protein concentration. This may also be the reason why we did not observe fluorescence from all of the bacteria. Alternatively, since membrane proteins are generally free to move laterally in the membrane, this pattern may have been due to aggregation of proteins by the antibodies (Barbour & Hayes, 1986; Barbour *et al.*, 1983a). Less than 1% of bacteria incubated with the

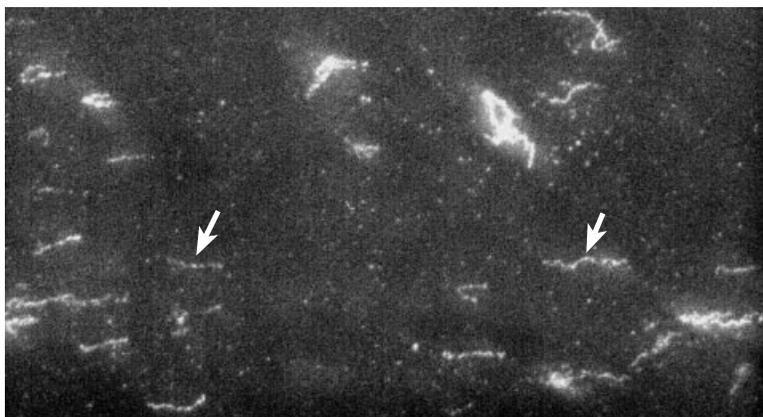


Fig. 3. Immunofluorescence analysis of unfixed *B. burgdorferi* with ErpA/I/N-specific mAb B11. The majority of bacteria did not label uniformly; two examples are indicated by arrows.

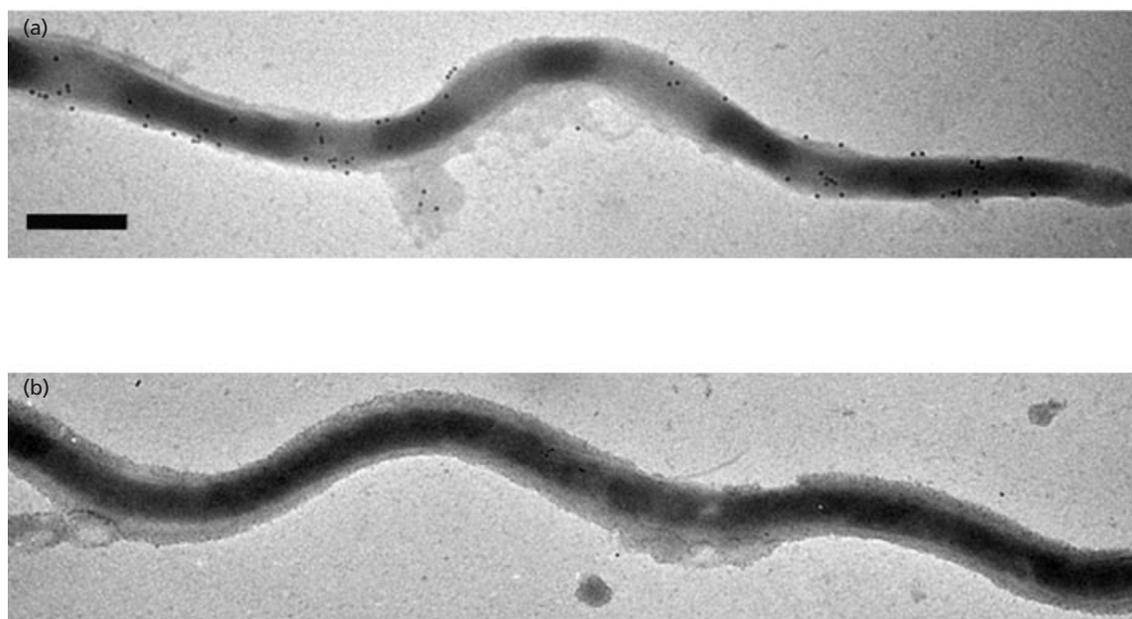


Fig. 4. Electron microscopic analyses of unfixed *B. burgdorferi* labelled with mAbs and gold bead-conjugated secondary antibody. (a) ErpA/I/N-specific mAb B11, (b) FlaB-specific mAb H9724. Bar, 0.5 μ m.

FlaB-specific mAb H9724 exhibited antibody binding, indicating that the outer membranes of the vast majority of examined bacteria remained intact during IFA processing. Additionally, no bacteria were detected by epifluorescence microscopy after incubation with only the fluorescence-tagged secondary antibody.

Similarly, electron microscopic analysis of unfixed bacteria incubated with mAb B11 and colloidal gold bead-conjugated secondary antibody demonstrated spirochaete labelling (Fig. 4). Control bacteria incubated with the anti-FlaB mAb H9724 were not labelled, indicating that the bacterial outer membranes were not damaged by this procedure, further proving that ErpA/I/N is exposed to the bacteria's outer environment.

Growth inhibition by Erp-directed antibodies

The IFA and immunogold experiments described above indicated that antibodies directed against Erp proteins can bind to intact bacteria. *B. burgdorferi* is often unable to grow in the presence of antibodies directed against surface-exposed proteins, which can cause the bacteria to form immobile clumps impaired for growth (Bunikis & Barbour, 1999; Cinco, 1992; Coleman *et al.*, 1992; Hanson *et al.*, 1998; Luke *et al.*, 2000; Pavia *et al.*, 1991; Sadziene *et al.*, 1993). The reasons behind antibody-mediated growth inhibition are not well understood, but may be related to impaired nutrient acquisition. Such inhibition is not necessarily bacteriocidal, and complement is not required for growth

Table 2. Growth inhibition by incubation with Erp-directed antibodies

Antibody*	Final medium colour†	Immobile aggregate formation‡
ErpA/I/N mAb	Red	+
ErpA/I/N mAb + rErpA/I/N	Yellow	-
ErpA/I/N pAb	Red	+
ErpB/J/O pAb	Red	+
ErpK pAb	Red	+
ErpL pAb	Red	+
ErpM pAb	Red	+
ErpP pAb	Red	+
ErpQ pAb	Red	+
ErpX pAb	Red	+
ErpY pAb	Red	+
FlaB mAb	Yellow	-
OspC mAb	Red	+
None	Yellow	-
Normal rabbit serum	Yellow	-
Unused hybridoma culture medium	Yellow	-
α -Y. pestis LcrV pAb	Yellow	-
α -Murine CD8 mAb	Yellow	-

* pAb, polyclonal antibody.

† Determined after 72 h incubation. A change of the medium's phenol red indicator from red to yellow indicates bacterial growth (Sadziene *et al.*, 1993).

‡ Cultures were examined microscopically and scored for presence (+) or absence (-) of immobile bacterial aggregates.

impairment (Pavia *et al.*, 1991; Sadziene *et al.*, 1993). Some *B. burgdorferi* proteins are able to elicit protective immune responses in mammals and antibodies directed against such proteins tend to also inhibit bacterial growth *in vitro* (Hanson *et al.*, 1998; Luke *et al.*, 2000; Pavia *et al.*, 1991; Sadziene *et al.*, 1993).

Cultivation of *B. burgdorferi* in medium containing Erp-directed antibodies indicated that such antibodies can indeed inhibit bacterial growth. Addition of mAb B11, directed against ErpA/I/N, caused bacterial aggregation and inhibited growth (Table 2). This effect was specifically due to ErpA/I/N binding, since growth inhibition could be prevented by addition of recombinant ErpA/I/N protein at concentrations as low as 1.5 ng ml⁻¹, the lowest level tested. Similar growth inhibition was also observed when cultivating bacteria in medium containing mAb B5, directed against the outer surface protein OspC. No inhibition of growth was detected in control experiments with the FlaB-directed mAb H9724, non-borrelial mAb 83-12-5 or unused hybridoma culture medium.

All polyclonal rabbit antisera also prevented bacterial growth, at dilutions up to 1:5 (Table 2). More dilute antisera did not appreciably affect growth. As noted above, some of the polyclonal antisera cross-reacted

with other Erp proteins, so it is possible that in some cases aggregate formation was enhanced by antibodies binding to Erp proteins in addition to, or other than, the one being targeted. For example, antibodies raised against ErpB/J/O may have also interacted with ErpM, ErpQ and ErpX proteins, thus augmenting the formation of bacterial aggregates. Control experiments showed no inhibition of growth by normal rabbit serum or undiluted polyclonal rabbit antisera raised against a non-borrelial protein.

DISCUSSION

Studies of *B. burgdorferi* have indicated that the outer membranes of these bacteria can be damaged by treatments commonly used to study surface proteins of more typical Gram-negative bacteria. Since an earlier study used IFA binding to formaldehyde-fixed bacteria to examine Erp protein localization (Lam *et al.*, 1994), we determined the cellular location of these proteins through several other methods that do not cause damage to the outer membrane. First, all examined B31 Erp proteins were susceptible to *in situ* digestion by proteases. Second, IFA binding to unfixed, unpermeabilized bacteria indicated that mAb B11 bound ErpA/I/N on the surface of *B. burgdorferi*. Third, similar results were obtained from immunogold labelling studies. Finally, Erp-directed antibodies, including an ErpA/I/N-directed mAb, caused formation of immobile bacterial aggregates. Taken together, these data greatly strengthen the earlier assertion that Erp proteins are located on the outer surface of *B. burgdorferi* (Lam *et al.*, 1994).

Since mammals produce antibodies against Erp proteins within the first few weeks of infection (Akins *et al.*, 1995; Das *et al.*, 1997; Miller *et al.*, 2000b; Nguyen *et al.*, 1994; Stevenson *et al.*, 1995, 1998a; Suk *et al.*, 1995; Sung *et al.*, 2000; Wallich *et al.*, 1995), it is clear that Erps are made by the bacteria during that time and presumably perform a function(s) for the bacteria. The surface exposure of Erp proteins indicates that these proteins are positioned to interact with host cells, extracellular matrices, or other substances encountered by the bacteria during the natural infection cycle. An individual bacterium may contain many different Erp proteins, often with extensively variable sequences, which may permit the bacteria to interact with numerous host tissues. Alternatively, there are also conserved characteristics among Erp proteins, such as a high percentage of charged amino acid residues, that might permit Erps with different primary structures to interact with similar ligands (Stevenson *et al.*, 2000b). Several *B. burgdorferi* proteins have been identified that bind specific host tissues (Coburn *et al.*, 1999; Guo *et al.*, 1995; Parveen & Leong, 2000) and similar techniques might be adapted to search for substances that bind Erp proteins.

The ability of Erp-directed antibodies to prevent *B. burgdorferi* growth *in vitro* suggests that similar antibodies could protect animals against *B. burgdorferi*

infection. Previous studies involving vaccination of mice with each of three Erp proteins, OspE or OspF of strain N40, or pG of strain ZS7, found either partial or no protection against bacterial challenges (Nguyen *et al.*, 1994; Wallich *et al.*, 1995). We have noted in the current study that antibodies from animals vaccinated with some Erp proteins also cross-reacted with other, similar Erp proteins, which could have contributed to the observed growth inhibition. Thus it may be possible that protection can be achieved by vaccination with an Erp that promotes cross-reactive antibody formation, or with a mixture of several different Erp proteins.

All B31 Erp proteins were sensitive to *in situ* treatment with at least one protease, with some Erps demonstrating resistance to digestion by certain enzymes (e.g. ErpB/J/O was uncleaved by proteinase K; Fig. 2). Since *B. burgdorferi* may be exposed to proteolytic enzymes during the bacterial infection cycle, resistance to proteases may be an important feature of borrelial surface proteins. There are several possible explanations for the ability of surface-exposed proteins to resist proteolysis. Although recombinant Erp proteins synthesized by *E. coli* and native proteins in Triton X-100-treated *B. burgdorferi* were susceptible to the tested proteases, proteins of intact *B. burgdorferi* might be folded in different manners, such that protease recognition sites are hidden. Some surface proteins of the related spirochaete *Borrelia turicatae* are resistant to certain proteases, apparently due to their secondary structures (Zückert *et al.*, 2001). Erp proteins in their native states may form multimers or interact with other membrane proteins that protect protease cleavage sites, similar to what has been postulated for the *B. burgdorferi* p66 (Oms66) and OspA proteins (Bunikis & Barbour, 1999; Exner *et al.*, 2000). While *B. burgdorferi* does not contain lipopolysaccharides identical to those of enteric Gram-negative bacteria (Takayama *et al.*, 1987), borreliae and other spirochaetes contain non-proteinaceous membrane constituents that might serve similar functions (Beck *et al.*, 1985; Cinco, 1992; Cinco *et al.*, 1991; Eiffert *et al.*, 1991; Schultz *et al.*, 1998; Wheeler *et al.*, 1993), and could interact with membrane proteins such as the Erps in manners that interfere with protease accessibility. Since a portion of ErpX molecules were not degraded by any of the protease treatments used in these studies, it is also possible that some Erps are located both on the bacterial outer surface and in the periplasmic space, as apparently are some other *B. burgdorferi* lipoproteins (Brusca *et al.*, 1991; Cox *et al.*, 1996; Radolf, 1994). Additionally, the insensitivity of ErpB/J/O and other proteins to digestion by proteolytic enzymes raises the possibility that the results of studies on other *B. burgdorferi* membrane proteins may be flawed in assigning subsurface localization based solely upon their inability to be degraded *in situ*.

While these studies demonstrated that Erp proteins are located on the *B. burgdorferi* outer surface, many additional questions about these proteins now must be answered. Why were some proteins, such as ErpQ and ErpX, resistant to *in situ* protease degradation? If Erps

are protected from proteolysis by interactions with other surface components, what are they? If the reason behind partial resistance is that a fraction of those Erp protein molecules had subsurface locations, why were those unequally distributed while others, such as ErpA/I/N and ErpY, were completely localized to the outer surface? Do Erp proteins interact with host components, and if so, what are they? We are continuing studies to address these and other questions about the intriguing Erp proteins and their roles in the biology of *B. burgdorferi* and the pathogenesis of Lyme disease.

NOTE ADDED IN PROOF

A possible function for Erp proteins is suggested by a recent finding that at least one Erp, the OspE protein of strain N40, can bind complement regulatory factor H (Hellwage *et al.*, 2001).

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