INFECTION AND IMMUNITY, Sept. 1995, p. 3327–3335 Vol. 63, No. 9
0019-9567/95/$04.00+0


# Molecular Cloning and Immunological Characterization of a Novel Linear-Plasmid-Encoded Gene, *pG*, of *Borrelia burgdorferi* Expressed Only In Vivo

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Received 6 February 1995/Returned for modification 6 April 1995/Accepted 13 June 1995

**Previously we have found that sera from immunocompetent mice infected either naturally by ticks or experimentally with low numbers of *Borrelia burgdorferi* ZS7 bacteria lack OspA- and OspB-specific antibodies but confer optimal protection on severe combined immunodeficiency mice against challenge with spirochetes (U. E. Schaible, L. Gern, R. Wallich, M. D. Kramer, M. Prester, and M. M. Simon, Immunol. Lett. 36:219–226, 1993). We have now used the latter immune sera to identify new spirochetal structures with relevance for protection from an expression library of the virulent European strain *B. burgdorferi* ZS7. Here we report the cloning and characterization of a novel lipoprotein, designated pG, the gene for which is located on a 48-kb linear plasmid. Sequence analysis of the *pG* gene revealed an open reading frame encoding a putative lipoprotein of 196 amino acids with a calculated molecular mass of 22 kDa and a consensus cleavage sequence (Leu-X-Y-Z-Cys) recognized by signal peptidase II. Restriction fragment length polymorphism analyses of *pG* derived from independent *B. burgdorferi* isolates from different geographic areas revealed that the gene is species specific, with, however, extensive genotypic heterogeneity. Comparison of the protein sequence of pG with those of other known *B. burgdorferi* outer surface lipoproteins (OspA to OspF and P27) demonstrated that pG is most related to OspF. Furthermore, the upstream region of *pG* exhibited extensive sequence homology (>94%) with the *ospEF* promoter region. Mouse immune sera to recombinant pG did not recognize a corresponding molecule in lysates of in vitro-propagated ZS7 spirochetes. However, experimental or natural infection of mice with ZS7 resulted in the induction of antibodies with reactivity for pG and the potential to delay the development of clinical arthritis. Together with the finding that sera from Lyme disease patients also contain antibodies to pG, our data suggest that the *pG* gene is preferentially expressed in the mammal environment.**

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and represents the most common tick-borne infection in North America and Europe. The infection may lead to a multisystem illness with manifestations of the skin, heart, central nervous system, and musculoskeletal system (7, 43). The lack of reliable diagnostic and therapeutic tools for identification and control of this infection is due mainly to our incomplete knowledge of spirochetal structures eliciting specific and nonspecific host responses (10, 11). However, it is clear that spirochetal outer surface proteins (Osps) play a major role in both protection from and pathogenesis of Lyme disease (13, 15, 37, 40). All Osps (OspA to OspF and P27) have been shown to be lipoproteins with hydrophobic N-terminal domains and putative cleavage sites (Leu-X-Y-Z-Cys) presumably recognized by a lipoprotein signal peptidase (5, 49, 50). The respective *osp* genes are localized on extrachromosomal plasmids: those encoding the two major membrane antigens OspA and OspB, as well as OspD and P27, are associated with linear plasmids of 49, 38, and 62 kb, respectively (3, 28, 32, 33), whereas the *ospC* gene is localized on a 26-kb circular plasmid (30). The two most recently described genes, designated *ospE* and *ospF*, are structurally arranged in a single operon on a 45-kb plasmid (22).

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In the search for a vaccine against Lyme disease, OspA has been found to be the most promising candidate (12, 13, 37, 39, 40, 41). However, partial protection against *B. burgdorferi* infection was also obtained upon active immunization with OspB, OspC, and OspF or passive administration of corresponding antibodies (13, 27, 31, 40). The role of OspD, OspE, and P27 in protection is less clear (27, 28, 32).

Previously it has been shown that antigenic variants and mutants of *B. burgdorferi* lacking OspA and/or OspB are suitable vaccines against challenge with virulent wild-type spirochetes (18). Sera from these recipients contained antibodies not to OspA and OspB but to other structures, including OspC and the 39-kDa protein (18, 42). In an independent study, it was found that sera of mice previously inoculated with low numbers ($\leq 10^4$) of *B. burgdorferi* organisms or infected by experimentally engorged ticks also lacked antibodies to OspA and OspB but conferred full protection on severe combined immunodeficiency (SCID) mice against subsequent challenge with spirochetes (16, 36). These reports provide ample evidence for the existence of still other spirochetal structures with protective potential.

Here we describe the cloning and expression of a novel *B. burgdorferi* antigen, termed pG, by using an OspA-OspB-deficient protective mouse immune serum (36). We show that pG is a lipoprotein which seems to be expressed by *B. burgdorferi* ZS7 during infection in mice and humans but not during in vitro cultivation of spirochetes.

## MATERIALS AND METHODS

**Preparation and screening of a *B. burgdorferi* expression library.** Genomic DNA was prepared from *B. burgdorferi* ZS7 by the lysozyme-sodium dodecyl sulfate (SDS) method, and DNA fragments were generated by sonication (45). Blunt-ended DNA was inserted into the pUEX1 vector by using an adaptor cloning strategy (6). The ligated DNA was transformed into *Escherichia coli* MC1061; this was followed by expression screening with immune serum preparations derived from DBA/2 mice previously inoculated subcutaneously via the tail with $10^3$ *B. burgdorferi* ZS7 spirochetes (anti-$10^3$) (16, 36).

**Bacterial strains.** The following strains of *Borrelia* genospecies were used in this study: B31, ZS7, 19535, 21305, 26815, and 28691, which are strains of *B. burgdorferi* sensu stricto; ZQ1, 20047, NE11H, NE58, NE4, and S90, which are strains of *B. garinii*; ACA-1, MMS, and NE40, which are strains of *B. afzelii*; HO14, which is an isolate of *B. japonica*; and unclassified strain 21038 (24, 44; Table 1). Borreliae were grown in modified Barbour-Stoenner-Kelly II medium (2) at 33°C. Spirochetes were harvested by centrifugation at 10,000 × *g* and 4°C for 20 min, washed twice in phosphate-buffered saline, and enumerated by dark-field microscopy.

**Pulse-field gel electrophoresis (PFGE) and Southern blot analysis.** Total genomic DNA was extracted from *Borrelia* organisms as described previously (19, 45). Borrelia DNA was enclosed in agarose and loaded into wells of a 1% agarose gel. PFGE was performed with the Bio-Rad (Munich, Germany) CHEF Mapper system in 0.5× Tris-borate-EDTA at 14°C with buffer recirculation. The gels were run for 17 h at 200 V and at a fixed angle of 120°; the initial pulse time was 0.5 s, and the final pulse time was 3.5 s. After PFGE, DNA was transferred to Hybond-N nylon membranes (Amersham) and then subjected to UV cross-linking and hybridization as previously described (9, 47). In brief, membranes were hybridized overnight at 65°C in 0.5 M $NaHPO_4$–7% SDS (pH 7.2) with $^{32}$P-labeled gene probes. The gene fragment of interest was recovered from low-melting-temperature agarose gels, precipitated with ethanol, and radiolabeled in random priming reactions as previously described (44). After being washed in 40 mM $NaHPO_4$–1% SDS (pH 7.2) at room temperature for 30 min, the dry membrane was autoradiographed on Kodak XAR-5 film with intensifying screens at −80°C for 1 to 12 h.

**Gel electrophoresis.** For electrophoresis on one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) slab gels, 40 μl of each lysate (equivalent to ≈$10^9$ organisms) or 1 μg of recombinant proteins was mixed with 10 μl of 5× reducing sample buffer by the method of Laemmli (21).

Two-dimensional SDS-PAGE was carried out as described by O'Farrel (29), by using isoelectric focusing (Pharmacia/LKB ampholytes: 1.45%, pHs 3.5 to 10; 0.1%, pHs 2.5 to 4.0; 0.2%, pHs 4 to 6; 0.2%, pHs 9 to 11) in the first dimension. Equivalent amounts of lysate were analyzed as for one-dimensional SDS-PAGE. Gels were stained with Coomassie blue or processed for Western blotting (immunoblotting) as previously described (35, 47).

**Northern (RNA) blot analysis.** Total cellular RNA was isolated from *Borrelia* strains ZS7, ZQ1, and MMS by using the RNA Isolation kit (TRIzol reagent) in accordance with the manufacturer's (GIBCO-BRL, Eggenstein, Germany) recommendations. Approximately 10 μg of RNA from each sample was electrophoresed in duplicate through a 1.5% agarose–formaldehyde gel and transferred to nitrocellulose as previously described (47). The filters were probed separately with DNA fragments of either the *ospA* or *pG* gene radiolabeled with [α-$^{32}$P]dATP by using the Random Primer DNA Labeling System (GIBCO-BRL). Hybridization was conducted as described above for Southern blot analysis.

**Western blotting.** Following SDS-PAGE, proteins were electroblotted for 1 h at constant current (60 mA) onto Hybond-C nitrocellulose membranes (Amersham) employing a semidry electroblotting chamber (Bio-Rad) in accordance with the manufacturer's recommendations. Following overnight incubation in blocking buffer (50 mM Tris-HCl, 150 mM NaCl, 5% nonfat dry milk), immunoblots were incubated for 2 h at room temperature with mouse (1:500) or human (1:100) antiserum diluted in 50 mM Tris-HCl–150 mM NaCl–1% dried milk–0.2% Tween 20 (dilution buffer). Nitrocellulose filters were washed five times in dilution buffer and incubated for an additional hour with alkaline phosphatase-conjugated goat anti-mouse or anti-human serum (1:400 [vol/vol]; Dianova, Hamburg, Germany). Filters were washed four times in dilution buffer and twice in Tris-buffered saline, and immunoreactive bands were then visualized by addition of 20 ml of DEA buffer (0.1 M diethanolamine [Sigma], 0.02% $NaN_3$, 5 mM $MgCl_2$ [pH 9.0]) supplemented with 5-bromo-4-chloro-3-indoylphosphate (BCIP; 165 μg/ml; Sigma) and nitroblue tetrazolium (NBT; 330 μg/ml; Sigma) as the substrate. The reaction was stopped by washing the membrane in 50 mM Tris-HCl–150 mM NaCl–5 mM EDTA.

**DNA sequencing.** *B. burgdorferi* genomic DNA fragments cloned in pUEX1 plasmid derivatives were sequenced by using a T7 sequencing kit (Pharmacia) in accordance with the manufacturer's recommendation (45).

**DNA and protein sequence analyses.** Analysis of DNA and protein sequences was performed by using the HUSAR software program (Heidelberg Unix Sequence Analysis Resources, Release 3.0; German Cancer Research Center). Simultaneous alignment for DNA or protein sequences and calculations to determine the degree of divergence were performed by using the GAP algorithm (26).

**PCR amplification of a portion of the *pG* gene.** The *pG* gene (plasmid pΔG) lacking the sequence encoding the hydrophobic leader peptide was PCR amplified with oligonucleotide primers 5′-GTGGATCCAAGATTGATGCGAGTAG TG-3′ (corresponds to nucleotides 61 to 79) and 5′-GTGAATTCTATTTTTTA TCTTCTATATTTTGAGGCTCTG-3′ (corresponds to nucleotides 560 to 590). Plasmid lp77, containing the putative protein-encoding region of *pG*, was subjected to 30 cycles of PCR in a DNA Thermal Cycler (Bio-Med60). We carried out denaturation at 94°C for 60 s, annealing at 48°C for 90 s, and extension at 72°C for 90 s. The amplified DNA fragments were ligated in frame with the glutathione *S*-transferase (GST) gene into the pGEX-2T vector after digestion with *Bam*HI and *Eco*RI and used for transformation of DH5α host cells.

**Expression and purification of recombinant pG (rpG).** Expression of the GST-rpG fusion protein in *E. coli* DH5α and affinity purification and endoproteinase thrombin cleavage of the fusion protein were performed as recommended by the manufacturer (Pharmacia).

**[$^3$H]palmitate labeling and Triton X-114 phase partitioning.** *E. coli* organisms transformed with plasmids carrying full-length or amino-terminally truncated versions of the *pG* gene from *B. burgdorferi* ZS7 were grown in the presence of [9,10-(*n*)-$^3$H]palmitic acid (specific activity, ≈50 Ci/mmol; Amersham), and radiolabeled lipoproteins were extracted by Triton X-114 phase partitioning as described previously (47).

**Patient sera.** Serum specimens used in Western blotting were from a clinically characterized serum bank. All of the positive sera had optical densities of ≥0.3 in an enzyme-linked immunosorbent assay (ELISA) with either an immunoglobulin M (IgM) or an IgG conjugate and were reactive with certain immunodominant *B. burgdorferi* proteins (p41, p60, p20, etc.) in Western blotting on whole-cell lysates. Eighteen serum specimens were from patients with either clinically verified acrodermatitis chronica atrophicans or erythema chronicum migrans or were obtained from forestry workers who had been exposed to *B. burgdorferi* as evidenced by seroconversion. Nine negative control serum specimens were selected from healthy blood donors (courtesy of the Heidelberg Blood Bank).

**Generation of immune sera and serology.** Immune sera were taken from mice previously inoculated in the tail with either $10^8$ (C.B-17 [*H-2$^d$*]; anti-$10^8$) or $10^3$ (DBA/2 [*H-2$^d$*]; anti-$10^3$) viable *B. burgdorferi* ZS7 spirochetes or primed with 5 to 10 μg of either recombinant lipOspA (BALB/c [*H-2$^d$*]; anti-lipOspA) or rpG (BALB/c; anti-rpG) subcutaneously in adjuvant (ABM2; Sebac, Aidenbach, Germany) and boosted after 10 and 20 days. All sera were analyzed by ELISA for the amount of spirochete-specific Ig on either whole spirochetal lysate (*B. burgdorferi* Ig), lipOspA, or rpG as described previously (39).

**Protection experiments.** SCID (*H-2$^d$*) mice were reconstituted with either pooled immune sera or normal mouse serum or left untreated. The amount of spirochete-specific Ig (determined by ELISA on whole *B. burgdorferi* cell lysates) transferred with the individual immune sera was as follows: anti-$10^8$, 4.4 μg of Ig per mouse; anti-$10^3$, 4.5 μg per mouse; anti-lipOspA, 5 μg per mouse; anti-rpG, 72 ng per mouse. When tested on rpG, the amount of specific Ig was approximately 10- to 20-fold higher in anti-rpG than in anti-$10^3$. Immune sera were given intraperitoneally, and 1 h later, the recipients were challenged subcutaneously with $10^5$ *B. burgdorferi* ZS7 spirochetes (36). Mice were investigated for development of clinical arthritis under blinded conditions and for the presence of spirochetes by cultivating ear biopsies in Barbour-Stoenner-Kelly II medium after 27 days as described previously (38, 39). The development of clinical arthritis in the tibiotarsal joints was scored by using swelling and/or reddening as criteria as follows: ++, severe; +, prominent; (+), moderate; ±, mild; (±), mainly reddening without significant swelling; −, no clinical signs of arthritis.

**Nucleotide sequence accession numbers.** The *pG* gene sequence reported here has been deposited in the EMBL/GenBank databases under accession number X82409.

## RESULTS

**Cloning of the *pG* gene and DNA sequence analysis.** A *B. burgdorferi* ZS7 genomic DNA expression library was screened with a protective hyperimmune serum from mice previously infected with $10^3$ spirochetes (36). This immune serum was shown before to lack antibodies to OspA and OspB but to

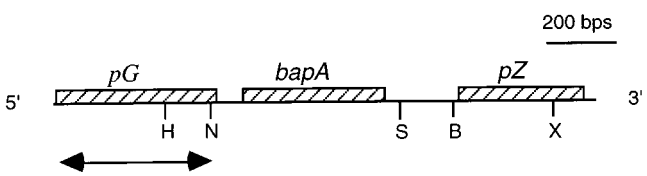


FIG. 1. Restriction endonuclease map of the cloned *Borrelia* DNA (strain ZS7) in pZS77. Unique *Hin*dIII (H), *Nhe*I (N), *Bgl*II (B), *Spe*I (S), and *Xba*I (X) restriction sites are shown. The arrow below the restriction map corresponds to the PCR-amplified DNA fragment (plasmid lp77) that hybridized with the probe (see Fig. 3, 4, and 7).

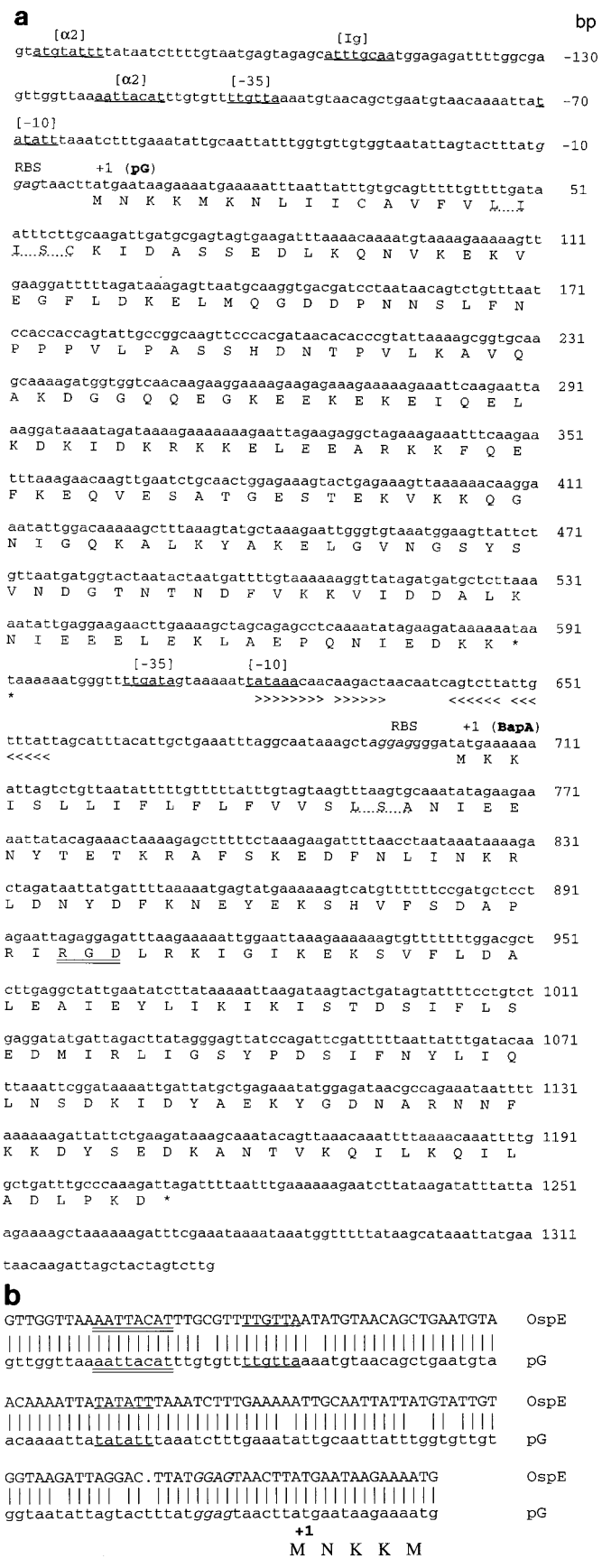


FIG. 2. (a) DNA and deduced amino acid sequences of the *pG* and *bapA* genes of *B. burgdorferi* ZS7. Nucleotides are numbered relative to the putative translational initiation ATG start codon (+1) of *pG*. Significant features of the DNA sequence include the following: putative active −35 and −10 promoter sequences (underlined) and consensus ribosome-binding sites (RBS) upstream of the putative pG-encoding regions. Termination codons (asterisks) at the 3′ ends of the open reading frames and a complementary region consistent with hairpin loop formation (>>> and <<<) between these two open reading frames are shown. Furthermore, two highly conserved regulatory *S. cerevisiae*-like (α2) and Ig-like (Ig) motifs (underlined) are located upstream of the putative *pG* promoter. The *pG* open reading frame encodes a 196-amino-acid polypeptide with a hydrophobic leader sequence (amino acids 1 to 19) and a consensus signal peptidase II cleavage site (dotted underlining). The *bapA* open reading frame encodes a 169-amino-acid protein (BapA) with a leader peptidase I cleavage site (dotted underlining) and the 3-amino-acid sequence motif RGD (double underlining). (b) Alignment of upstream regulatory regions of the *pG* (strain ZS7) and *ospE* genes. The sequence of the control region of the *ospE-ospF* operon is derived from strain N40 (22). A single-nucleotide gap (indicated by the dot) was introduced for optimal alignment. Identical *ospE* and *pG* promoter nucleotides at a given position are indicated by vertical lines. The proteins encoded by these DNA sequences are shown at the bottom in the single-letter code. The putative active −35 and −10 promoter sequences (underlined) and ribosome-binding sites are indicated. A sequence identical to the MATα2 protein-binding site of the *BAR1* gene of *S. cerevisiae* (α2 element) is doubly underlined (4). (c) Alignment of BapA (strain ZS7) with EppA (strain N40). Identical amino acid residues at a specific position are indicated by vertical lines. The RGD tripeptide is underlined. (d) Alignment of pG with OspF of *B. burgdorferi* ZS7 (43a). Identical amino acid residues at a specific position are indicated by vertical lines.

react with four distinct proteins in whole spirochetal lysates with relative molecular masses of 19 to 20 kDa and two proteins of ≈40 kDa when tested by two-dimensional gel electrophoresis (data not shown). Approximately 20 clones were identified, of which 1, designated pZS77, was particularly reactive with the immune sera. Plasmid pZS77 was subjected to restriction analysis, subcloning, and sequencing. The DNA analysis of clone pZS77 revealed three open reading frames, designated *pG*, *bapA*, and *pZ* (Fig. 1). The nucleotide sequences of *pG* and *bapA*, together with their deduced amino acid sequences, are shown in Fig. 2a. A consensus ribosome-binding site (GGAG) is located 10 bp upstream of the putative ATG start codon of the *pG* gene. Further upstream of this translational initiation

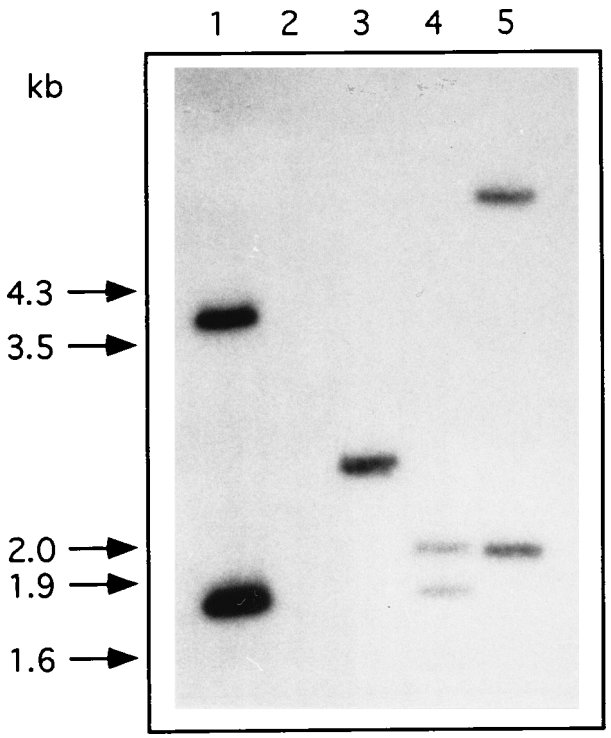


FIG. 3. Southern blot analysis of the *pG* gene in geographically diverse *B. burgdorferi* isolates (44). Total genomic DNAs from *B. burgdorferi* ZS7 (lane 1), *B. garinii* ZQ1 (lane 2), and *B. afzelii* ACA-1 (lane 3), MMS (lane 4), and NE40 (lane 5) were digested with endonuclease *Hin*dIII, electrophoretically separated, transferred to nylon membranes, and hybridized with the $^{32}$P-labeled *pG* probe.

sequence are the −10 region (TATATT), at positions −70 to −64, and the −35 region (TTGTTA), at positions −105 to −100. The open reading frame of 588 nucleotides encodes a polypeptide of 196 amino acids with a predicted molecular mass of 22,049 Da. The 3′ end of the open reading frame is marked by two termination codons followed by an imperfect inverted repeat consistent with a transcriptional termination. At 111 bp downstream of the first stop codon of the *pG* gene, a further open reading frame of 507 nucleotides, designated *bapA* (*B. burgdorferi*-associated protein A), is located. The end of the *bapA* gene (TAG at positions 1210 to 1212) is separated from another open reading frame (*pZ*) of 444 nucleotides. Control regions of transcription and translation that closely resemble those found in *E. coli* and *B. burgdorferi* could not be identified in the upstream region of *pZ*. We therefore concluded that the *pZ* gene is not expressed. None of the remaining reading frames could be translated to give proteins of any significant length. Alignment of the DNA sequence upstream of the ATG start codon of the *pG* gene with the recently reported promoter region of the *ospEF* operon revealed 94% identity as determined by the GAP algorithm (Fig. 2b; reference 26).

**Amino acid sequence analysis of pG and BapA.** The hydropathy profile of pG suggests that the protein is largely hydrophilic with one hydrophobic domain of about 20 amino acids in the amino-terminal portion (data not shown). This N-terminal region reveals sequence similarity to leader signal peptides present in typical prokaryotic lipoproteins (49, 50). At the COOH-terminal end of the signal sequence is a putative signal peptidase II recognition motif, Leu-X-Y-Z-Cys (Fig. 2a). The potential cleavage site in pG is located between the serine at position 19 and the cysteine at position 20. The calculated isoelectric point is at pI 5.2. Comparison of the amino acid sequence of pG with those of the known *B. burgdorferi* outer surface proteins, OspA to OspF and P27, revealed that pG exhibits the highest level of amino acid sequence similarity (61%) to OspF (Fig. 2d; reference 43a). Furthermore, the basic N-terminal peptide motif M-N-K-K-M of pG is identical to those observed for OspE and OspF (only shown for OspE in Fig. 2b).

The *bapA* gene encodes a precursor protein of 169 amino acids, including a signal peptide of 19 amino acids and a type I signal peptidase cleavage site. The mature BapA protein of 150 amino acids has a calculated molecular weight of 17,620. At amino acid position 66 is located the sequence Arg-Gly-Asp (RGD), which has been shown in some other proteins to be crucial for interaction with respective cell surface receptors, e.g., integrins (9a, 9b, 34). The hydrophilicity plot indicates that besides the large hydrophobic region in the amino terminus, BapA contains two hydrophilic domains separated by a hydrophobic segment (amino acids 77 to 120) (data not shown). Interestingly, BapA exhibits 80% amino acid sequence similarity to the previously reported EppA protein (Fig. 2c; reference 8).

**Restriction fragment length polymorphism analysis and mapping of *pG*.** Restriction fragment length polymorphism analysis of *pG* with endonuclease *Hin*dIII revealed at least seven distinct hybridization patterns among the *B. burgdorferi* isolates tested: the majority of *B. burgdorferi* sensu stricto isolates are characterized by two hybridization fragments of 1.8 and 3.8 kb (Fig. 3, lane 1); four of six *B. garinii* isolates tested did not hybridize with the *pG* probe (lane 2). Two *B. garinii* strains, 20047 and S90, exhibited fragments of 1.8 kb and 1.7 and 3 kb, respectively (data not shown). Among strains of the species *B. afzelii*, at least three different hybridization patterns

TABLE 1. Reactivity of DNAs prepared from different *Borrelia* isolates with the *pG* probe (strain ZS7) evaluated by restriction fragment length polymorphism analyses

| Genospecies and strain | Origin | Geographic location | *pG* reactivity |
|---|---|---|---|
| I, *B. burgdorferi* sensu stricto | | | |
| ZS7 | *Ixodes ricinus* | Germany | + |
| B31 | *Ixodes dammini* | United States | + |
| 26815 | Chipmunk | United States | − |
| 19535 | Mouse | United States | + |
| 28691 | *Ixodes dammini* | United States | − |
| 21305 | Mouse | United States | − |
| II, *B. garinii* | | | |
| ZQ1 | *Ixodes ricinus* | Germany | − |
| 20047 | *Ixodes ricinus* | France | + |
| S90 | *Ixodes ricinus* | Germany | + |
| NE11H | *Ixodes ricinus* | Switzerland | − |
| NE58 | *Ixodes ricinus* | Switzerland | − |
| NE4 | *Ixodes ricinus* | Switzerland | − |
| III, *B. afzelii* | | | |
| ACA-1 | Human skin | Sweden | + |
| MMS | Human skin | Germany | + |
| NE40 | *Ixodes ricinus* | Switzerland | + |
| IV, *B. japonica* HO14 | *Ixodes ovatus* | Japan | − |
| Unclassified strain 21038 | *Ixodes dentatus* | United States | + |

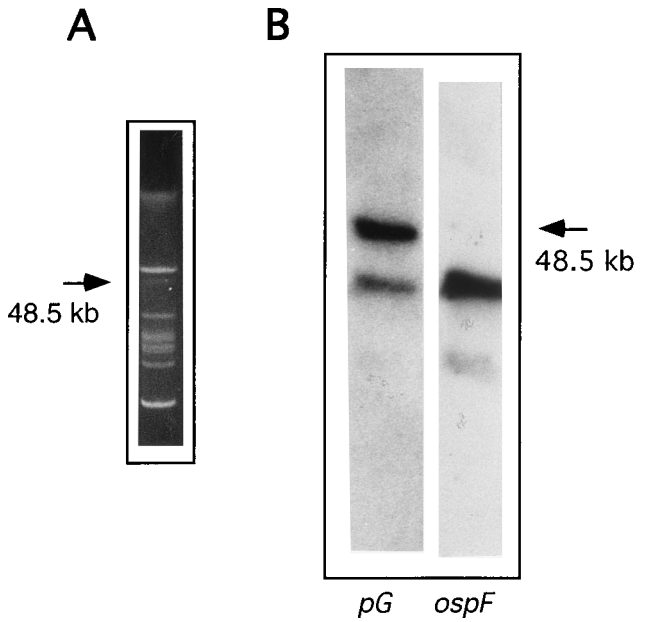


FIG. 4. PFGE separating the chromosomal and plasmid DNAs of *B. burgdorferi* ZS7. (A) The gel was visualized by staining with ethidium bromide. (B) The Southern blot of the PFGE gel was hybridized separately with $^{32}$P-labeled *pG* and *ospF* DNA probes (43a).

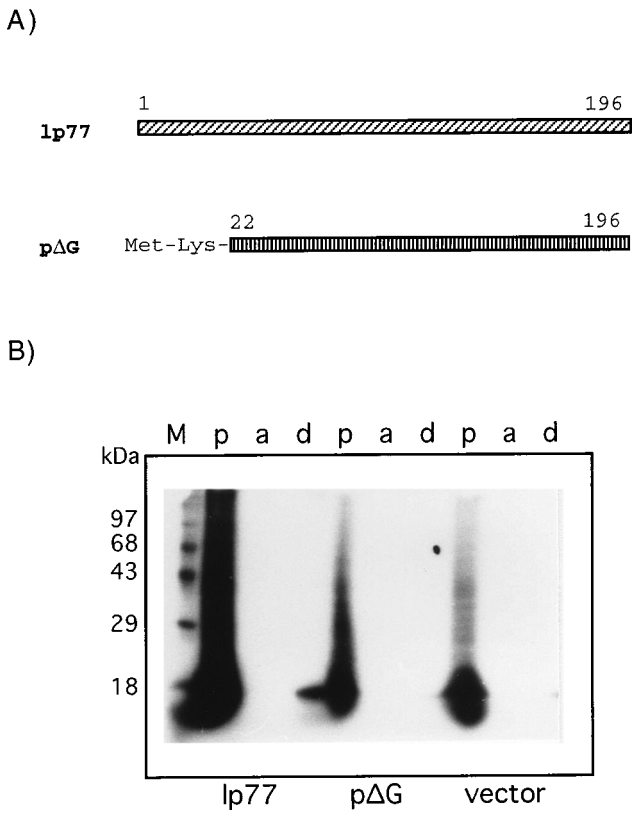


FIG. 5. (A) Diagrammatic representation of expressed recombinant *B. burgdorferi* proteins. The positions of the amino- and carboxy-terminal amino acids of the expressed recombinant proteins are indicated to the left and right of the bars. (B) Triton X-114 fractionation of recombinant proteins expressed in *E. coli* DH5α and biosynthetically labeled with [$^3$H]palmitate. Lanes: p, insoluble proteins (pellet); a and d, proteins in the aqueous and detergent phases, respectively, of *E. coli* cells expressing the complete (lp77) or truncated (pΔG) *pG* gene or the vector alone. Proteins were separated by SDS-PAGE and visualized by fluorography. Molecular masses of marker proteins (lane M) are indicated on the left.

were observed: one band of 2.4 kb for strain ACA-1 (lane 3) and two bands of either 1.9 and 2 kb for strain MMS (lane 4) or 2 and 5 kb for strain NE40 (lane 5 and Table 1).

Plasmid and chromosomal DNAs of *B. burgdorferi* ZS7 were separated by PFGE (Fig. 4A) and hybridized to the *pG*-specific gene probe (Fig. 4B). A prominent band was seen with a plasmid of approximately 48 kb, and cross-hybridization to a 45-kb plasmid was observed. Following a high-stringency wash, the 45-kb hybridizing band was not detected. Control hybridization studies with the cloned *ospF* gene of *B. burgdorferi* ZS7 revealed that the gene loci for *ospF* and *pG* are located on different linear plasmids (Fig. 4; reference 43a). As indicated by the processively shortened 48-kb hybridizing band following exonuclease *Bal* 31 treatment, the linear structure of the *pG*-harboring plasmid was demonstrated. Conversely, a circular plasmid harboring the *ospC* gene locus was not affected by *Bal* 31 treatment (data not shown). DNAs isolated from other *Borrelia* species, such as *B. coriaceae* Co53, *B. hermsii*, and *B. turicatae*, and from *Treponema pallidum* did not hybridize to the *pG* probe (data not shown).

**Expression of rpG in *E. coli*.** To amplify *pG* by PCR, primers were selected in such a way that the final recombinant product would lack the amino acid residues composing the leader peptide (47). The amplified products were inserted in frame with the carrier protein of expression vector pGEX-2T (Pharmacia, Freiburg, Germany), and after induction with IPTG, an approximately 44-kDa GST-rpG fusion protein was obtained and enriched from an *E. coli* lysate by use of glutathione-agarose beads. The rpG was obtained by digestion of the bound GST-rpG fusion protein with a site-specific protease (data not shown).

To determine whether rpG is expressed as a lipoprotein in *E. coli*, DH5α cells were transformed with either plasmid lp77 or plasmid pΔG and labeled with [$^3$H]palmitate. Plasmid lp77 encodes the full-length pG precursor protein, including the N-terminal signal sequence, whereas pΔG specifies a protein in which the first 21 residues of the pG precursor are replaced with the sequence Met-Lys (Fig. 5A). After extraction by detergent phase partitioning and separation by SDS-PAGE, radioactive products were visualized by fluorography. *E. coli* cells containing the full-length *pG* gene (plasmid lp77) expressed a 20-kDa lipoprotein that partitioned into the detergent phase, whereas lipoproteins were not detected in the detergent phase of *E. coli* DH5α cells containing the truncated *pΔG* gene or the vector alone (Fig. 5B). The radiolabeled 20-kDa lipoprotein was identified as pG by immunoprecipitation with anti-rpG serum (data not shown).

**Protective capacity of anti-rpG immune serum in SCID mice.** To test the protective capacity of anti-rpG immune serum, SCID mice were treated with the following *B. burgdorferi*-specific Igs from pooled immune serum preparations: C.B-17 anti-10$^8$, DBA/2 anti-10$^3$, BALB/c anti-lipOspA, and BALB/c anti-rpG. SCID mice were injected intraperitoneally with one of the indicated immune sera and subsequently challenged with 10$^5$ *B. burgdorferi* ZS7 organisms. Development of clinical arthritis (reddening and swelling) and recovery of spirochetes from ear biopsies were monitored (Table 2). Inoculated but otherwise untreated or normal mouse serum-treated SCID mice developed clinical arthritis starting at day 6 postchallenge, with prominent swelling on day 13 and severe swelling of the tibiotarsal joints developing between days 13 and 24 (end point). As described before, SCID mice passively immunized

TABLE 2. Development of clinical arthritis in individual SCID mice not treated or pretreated intraperitoneally with immune sera and subsequently inoculated with $10^5$ *B. burgdorferi* ZS7 spirochetes

| Serum transferred, amt/mouse | Mouse no. | Clinical arthritis score[a] at postinfection day: | | | | | Recultivation[b] from ear biopsy |
|---|---|---|---|---|---|---|---|
| | | 6 | 13 | 18 | 20 | 27 | |
| None | 1 | − | + | ++ | ++ | ++ | + |
| | 2 | (±) | + | ++ | ++ | ++ | + |
| Normal mouse serum, 5 μg | 1 | (±) | + | ++ | ++ | ++ | + |
| | 2 | ± | + | ++ | ++ | ++ | + |
| | 3 | (±) | − | ++ | ++ | ++ | + |
| Anti-$10^3$ (DBA/2), 4.5 μg of specific Ig | 1 | − | − | − | − | − | − |
| | 2 | − | − | − | − | − | − |
| | 3 | ± | − | − | − | (±) | − |
| | 4 | − | − | − | (±) | ± | − |
| Anti-$10^8$ (C.B-17), 4.4 μg of specific Ig | 1 | ± | ± | − | − | − | − |
| | 2 | − | − | − | − | − | − |
| | 3 | − | − | − | − | − | − |
| | 4 | ± | − | − | − | ± | − |
| Anti-lipOspA (BALB/c), 5 μg of specific Ig | 1 | (±) | − | − | − | (±) | − |
| | 2 | ± | − | − | − | − | − |
| | 3 | − | − | − | − | − | − |
| | 4 | − | − | − | − | − | − |
| Anti-rpG (BALB/c), 72 ng of specific Ig | 1 | − | (+) | + | ++ | ++ | + |
| | 2 | − | (+) | ± | (+) | ++ | + |
| | 3 | − | (+) | ± | ++ | ++ | + |
| | 4 | − | (+) | + | ++ | ++ | + |

[a] Scoring: ++, severe; +, prominent; (+), moderate; ±, mild; (±), mainly reddening without significant swelling; −, no clinical signs in the left or right tibiotarsal joint.
[b] After 27 days.

with anti-$10^8$, anti-$10^3$, or anti-lipOspA immune sera showed none or only marginal signs of clinical arthritis (Table 2; references 39 and 40). On the other hand, development of arthritis in SCID mice passively immunized with anti-rpG immune sera was delayed as indicated by the development of only mild swelling between days 6 and 18. At later time points, these mice developed more severe forms of arthritis, indicating that the anti-rpG immune serum is less efficient than anti-$10^3$, anti-$10^8$, and anti-lipOspA immune sera for control of infection. Although the anti-rpG serum contained >10-fold more rpG-specific antibodies than did the anti-$10^3$ immune serum, it was much less protective, suggesting that the anti-$10^3$ immune serum contains additional protective antibodies with specificities distinct from those for rpG.

**Absence of pG in *B. burgdorferi* ZS7 cultivated in vitro.** To identify pG in intact organisms, spirochetes were analyzed by two-dimensional SDS-PAGE and immunoblotting with hyperimmune murine anti-rpG serum. Although the anti-rpG serum readily reacted with rpG (Fig. 6B), this antiserum did not react with a pG-related protein or any other structure from in vitro-cultured strain ZS7 spirochetes (Fig. 6A). In some experiments, we observed weak reactivity with an ≈40-kDa polypeptide (Fig. 6A) distinct from flagellin and P39 (data not shown) that may represent either a variant of pG or a *B. burgdorferi* protein that cross-reacts with anti-rpG immune sera. As positive controls, identical nitrocellulose filters were probed with a monoclonal antibody directed to OspA, OspB, and flagellin (data not shown; 47).

To assess whether *pG* is transcribed in spirochetes during in vitro culture, total RNAs from *B. burgdorferi* ZS7, *B. garinii* ZQ1, and *B. afzelii* MMS were isolated and analyzed for the presence of a *pG* transcript by Northern blot hybridization. As a control for in vitro expression and RNA degradation, an *ospA* gene probe was used. In contrast to the *ospA* probe that bound to a single transcript of approximately 2 kb, the *pG* probe failed to detect a corresponding *pG* transcript (Fig. 7). These results suggest that the deficiency in *pG* expression during in vitro cultivation appears to be either due to (i) a block of transcription, (ii) rapid mRNA degradation, or (iii) suboptimal levels of specific transcripts which escape detection.

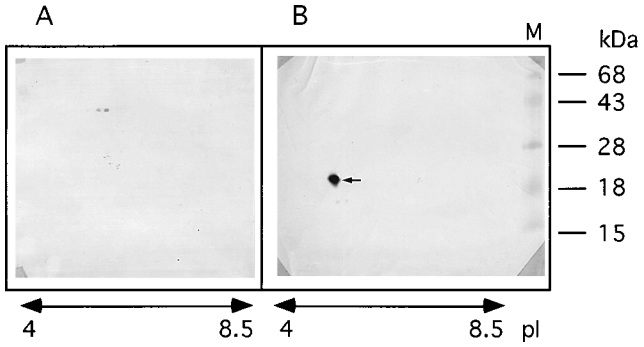


FIG. 6. Absence of pG from in vitro-cultivated *B. burgdorferi*. Two-dimensional gels (first dimension, pHs 4 to 8.5; second dimension, SDS–13% PAGE) of *B. burgdorferi* ZS7 whole-cell lysates (A) or rpG (1 μg) (B) were electrophoretically transferred to nitrocellulose membranes and reacted with murine anti-rpG sera (diluted 1:500). The position of rpG is indicated by the arrow. Molecular masses are indicated on the right, and pI values are indicated across the bottom.

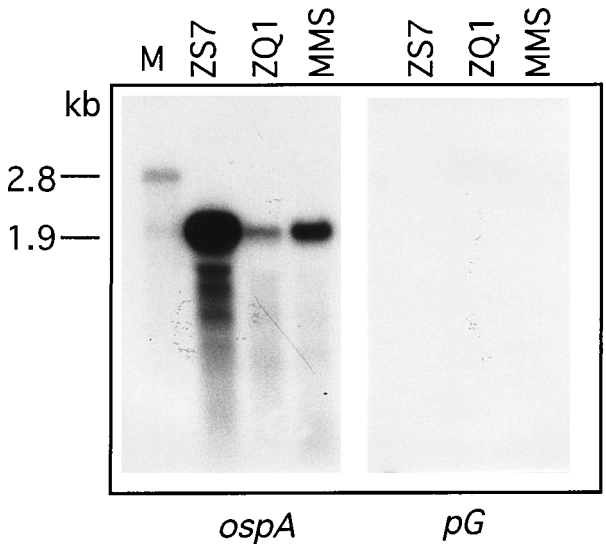


FIG. 7. Northern blot analysis of *pG* transcription. Ten-microgram samples of total cellular RNAs isolated from *B. burgdorferi* ZS7, *B. garinii* ZQ1, and *B. afzelii* MMS were separated by electrophoresis and transferred to nitrocellulose. The membrane was hybridized with radiolabeled DNA probes specific for *ospA* (46) and *pG*. Molecular mass marker (lane M) are indicated on the left.

However, pG seems to be expressed in mammalian hosts, since serum samples from experimentally infected mice, as well as from 7 of 18 Lyme disease patients, reacted with rpG (Fig. 8). In contrast, none of the sera from nine healthy donors contained antibodies to rpG (Fig. 8). These findings suggest that pG is expressed during the course of infection but not during in vitro cultivation of spirochetes.

## DISCUSSION

In the past, a number of spirochetal outer surface lipoproteins, such as OspA, OspB, OspC, OspE, and OspF, have been shown to induce protective immunity in mice against *B. burgdorferi* infection, although to various degrees (13, 27, 31, 40). Among these structures, OspA seems to be the most promising candidate as a vaccine against Lyme borreliosis (13, 41). However, recent studies on active and passive immunization in mice with either OspA-OspB-defective spirochetal strains (14, 18) or immune sera lacking OspA-OspB-specific antibodies (36) indicate the existence of yet other target structures involved in antibody-mediated elimination of spirochetes. By using protective immune sera (anti-$10^3$) that lack OspA-OspB-specific antibodies but contain antibodies specific for at least four *B. burgdorferi* antigens with $M_r$s of approximately 20,000 and two with $M_r$s of about 40,000 (36), we cloned and characterized a novel outer surface lipoprotein of *B. burgdorferi* termed pG. It was shown that pG is (i) encoded by a gene associated with a linear plasmid, (ii) expressed in spirochetes during infection of humans and mice but not during in vitro cultivation, and (iii) able to induce antibodies with the potential to delay the onset of clinical arthritis in SCID mice.

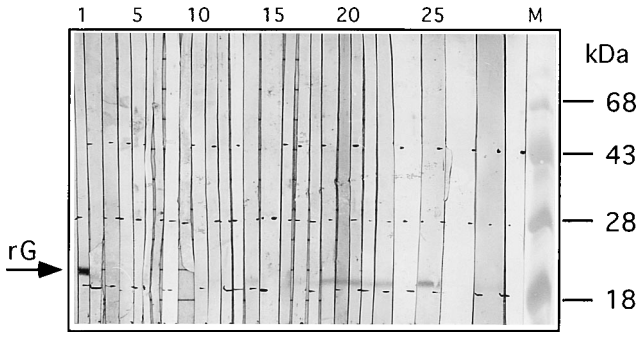


FIG. 8. Detection of anti-rpG antibodies in Lyme disease sera. Antigen strips containing 1 μg of rpG were reacted against sera obtained from Lyme disease patients (lanes 11 to 28) and healthy individuals (lanes 2 to 10) at a 1:100 dilution. A control strip (lane 1) was reacted against murine anti-$10^3$ sera (diluted 1:500). Molecular masses of prestained protein size markers (lane M) are indicated. The position of rpG (rG) is indicated by the arrow.

pG is a lipoprotein according to its amphiphilic character and its capacity to incorporate [$^3$H]palmitate in *E. coli*. The deduced pG sequence is consistent with those of typical bacterial lipoproteins in containing a hydrophobic leader sequence and a potential signal peptidase II cleavage site, Leu-X-Y-Z-Cys (49, 50). The remainder of the pG sequence does not show any long segment of hydrophobicity, indicating that the protein is tethered to the membrane surface by its lipidated N terminus. The amino-terminal M-N-K-K-M sequence of pG is identical to those of the recently described OspE and OspF proteins (22), and the overall levels of protein sequence similarity to OspE and OspF are approximately 53 and 65%, respectively. The *pG* gene is associated with a 48-kb linear plasmid that is distinct from two other linear plasmids of 53 and 45 kb that carry the *ospA* and *ospF* genes of *B. burgdorferi* ZS7, respectively (43a). The finding that the *pG* probe cross-hybridized with the 45-kb plasmid under low-stringency conditions could be expected on the basis of its extensive similarity to *ospF*.

When isolates of distinct *Borrelia* genospecies were tested for the presence of the *pG* gene by using the gene probe from strain ZS7 (*B. burgdorferi* sensu stricto), most strains of the species *B. afzelii* and *B. burgdorferi* sensu stricto were positive whereas the majority of *B. garinii* strains, as well as *B. japonica* isolate HO14, were negative (24, 44). Whether the *pG* gene is absent from the latter isolates or exhibits only little nucleotide sequence identity with the ZS7 gene is not known but will be determined in future studies (48).

Evidence for the expression of pG, at least temporarily, in spirochetes growing or persisting in a mammalian host is based on the detection of anti-pG antibodies in sera derived from experimentally inoculated mice and humans suffering from Lyme disease. The fact that immune sera to rpG partially protect SCID mice by delaying the onset of arthritis indicates that pG is exposed, at least partially, on the outer membrane of spirochetes. Further analysis is necessary to confirm the exact subcellular localization of pG in spirochetes. The lack of optimal protection of SCID mice by anti-rpG immune serum may also be due to the possibility that antibodies generated to rpG are less protective than those to native pG expressed by *B. burgdorferi*. Alternatively, antibodies to pG may play only a minor role in conferring protection on SCID mice against subsequent challenge with spirochetes. Most probably, other spirochetal proteins recognized by the anti-$10^3$ immune serum are target structures for protective antibodies.

The lack of pG expression in spirochetes during in vitro cultivation may be due to its downregulation induced by environmental factors. This could correspond to reduced pG transcription, instability of the *pG* transcript, or both. In fact, it was shown before that outer surface lipoproteins in *B. burgdorferi* are differentially expressed in the tick environment versus the mammalian environment and may also change their expression patterns in vitro (17, 17a, 20, 23). A notable feature of the DNA region upstream of the *pG* open reading frame is the

observed high level of similarity (94%) to the sequence of the *ospEF* operon, which is localized on a different linear plasmid in *B. burgdorferi* ZS7 (22, 43a). It is tempting to speculate that *pG* and *ospF* belong to a polygene family whose members may undergo recombination, as observed for *vmp* gene switches in *B. hermsii* (1). Additionally, the putative *pG* promoter contains two highly conserved octamer DNA motifs, ATGTATTT and AATTACAT, which have been shown before to be associated with protein-binding sites for a molecule, named MATα2, known to regulate gene expression during *Saccharomyces cerevisiae* differentiation (4, 25). Between those *S. cerevisiae*-like motifs is located a further Ig octamer-like sequence, ATTTG CAA, that differs from the Ig octamer motif by only one transition substitution (underlined). However, it is unclear which of the proposed mechanisms, if any, is utilized by *B. burgdorferi* to adapt to environmental changes during complex zoonotic transmission cycles and to evade the immune defense of mammalian hosts (25a).

Future studies need to reveal the precise biological roles of the different regulatory elements and DNA sequence motifs associated with *pG* in altering gene expression. The findings reported here provide the basis for these investigations, as well as for studies on the potential role of pG in the immunodiagnosis and pathogenesis of Lyme disease. pG may represent a virulence factor or may be a critical factor for spirochete infectivity. The idea that pG expression or repression is dependent upon environmental signals, as previously documented for many other pathogenic bacteria, is attractive and is being pursued (25a).

## ACKNOWLEDGMENTS

We thank Juri Habicht, Gudrun Prehn, Marlot Prester, and Manuel Stein for excellent technical assistance. We are indebted to Heidelore Hofmann for providing sera from Lyme disease patients.

This work was supported in part by the Bundesministerium für Forschung und Technologie (01 KI 9104) and by the Commission of the European Communities (P920321).

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