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A Family of Genes Located on Four Separate 32-Kilobase Circular Plasmids in *Borrelia burgdorferi* B31

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We have identified four loci in *Borrelia burgdorferi* B31 that contain open reading frames capable of encoding six proteins that are related to the antigenic proteins OspE and OspF. We have designated these proteins Erp, for OspEF-related protein, and named their respective genes *erp*. The *erpA* and *erpB* genes are linked, as are *erpC* and *erpD*, and the pairs probably constitute two operons. The *erpG* and *erpH* genes appear to be monocistronic. The ErpA and ErpC proteins are expressed by *B. burgdorferi* B31 in culture and are recognized by a polyclonal antiserum raised against the OspE protein of *B. burgdorferi* N40. The four *erp* loci are each located on different 32-kb circular plasmids that contain additional DNA sequences that are homologous to each other and to an 8.3-kb circular plasmid of *B. burgdorferi* sensu lato Ip21. All four 32-kb plasmids can be maintained within a single bacterium, which may provide a model for the study of plasmid replication and segregation in *B. burgdorferi*.

Borrelia burgdorferi is a member of the order *Spirochaetales*, the spirochetes, an order that is phylogenetically and morphologically distinct from such well-characterized bacteria as *Escherichia coli* and *Bacillus subtilis* (8, 24, 34, 35, 53). *B. burgdorferi* bacteria naturally contain a wide variety of plasmids (4, 13, 25, 40, 42, 45, 47, 54), yet nothing is known about the mechanisms by which plasmids are maintained in these bacteria. Several observations suggest that the small DNA species of *B. burgdorferi* and other spirochetes may be fundamentally different from the plasmids of other bacteria, the most significant being that there are linear plasmids in *B. burgdorferi* (4, 5, 7, 13, 22, 23, 42). Also, two *B. burgdorferi* genes involved in purine biosynthesis are located on a circular plasmid (29), which appears to be inconsistent with the classical definition of a plasmid as a nonessential extrachromosomal element. Other plasmids of *B. burgdorferi* appear to be capable of undergoing recombination to form multimeric plasmids (25, 31), and there have been reports of DNA sequences located on several different plasmids within the same bacterium (46, 47, 54–56).

B. burgdorferi is the causative agent of Lyme disease, an increasingly common ailment of humans and several other mammals (15, 48). Infection of a mammal by *B. burgdorferi* is generally accompanied by the production of antibodies directed against a limited number of bacterial antigens (17, 19, 41, 52), several of which have been identified as surface-exposed lipoproteins. At least three of these lipoproteins, OspC, OspE, and OspF, are differentially expressed by *B. burgdorferi*, being produced in greater quantities by cultured bacteria that are shifted from 23 to 32°C than by those maintained at the lower temperature (43, 49). For OspC, at least, this appears to be related to induction of specific protein synthesis by the spirochete in ticks following feeding upon a warm-blooded animal (43). Temperature-dependent differential expression of bacterial proteins that are involved in host infection has been observed in a number of other pathogenic bacteria (30). We sought to clone and characterize the *ospE* and *ospF* genes of *B. burgdorferi* B31 to better understand the genetic elements responsible for the temperature-induced differential expression of these and other genes. In so doing, we have identified genes that could encode six different proteins that are related to OspE and OspF. These genes are located in four separate loci, each preceded by an extended DNA sequence almost identical to that found upstream of the *ospEF* operon of *B. burgdorferi* N40 (26). We have mapped each of these loci to a separate circular plasmid of approximately 32 kb, and all four plasmids can be maintained within a single *B. burgdorferi* bacterium.

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MATERIALS AND METHODS

Bacteria and DNA. *B. burgdorferi* B31 is a wild-type isolate, originally obtained from a tick collected on Shelter Island, N.Y. (15), that was cloned by limiting dilution (6) and has been passaged continuously in culture medium for over 10 years. *B. burgdorferi* B31 clones A1.4 and A3H.3 were derived from individual colonies of isolate B31 plated on solid Barbour-Stoener-Kelly medium (37) and cultured for no more than three passages. *B. burgdorferi* N40 (obtained from S. Barthold, Yale University, New Haven, Conn.) is a wild-type isolate, originally obtained from a tick in Westchester, N.Y. (10), that has been cloned by limiting dilution (9) and has been passaged in culture fewer than five times. *B. burgdorferi* cells were grown at 34°C in BSK-H medium (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma).

Total DNA was isolated from *B. burgdorferi* as previously described (38). Supercoiled circular plasmid DNA was isolated from *B. burgdorferi* by ethidium bromide-$CsCl_2$ gradient centrifugation essentially as described by Maniatis et al. (27).

Cloning and sequencing. Unless otherwise noted, PCR conditions consisted of 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. Plasmid DNA was sequenced by using Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio) in accordance with the manufacturer's instructions. The CLUSTAL V program (21) was used to determine relatedness of DNA and protein sequences. The oligonucleotides used for gene cloning and DNA probe synthesis are listed in Table 1.

Total *B. burgdorferi* B31 DNA was subjected to PCR amplification with oligonucleotides 1 and 2. The PCR products were then cloned directly into pCRII (Invitrogen, San Diego, Calif.) and transformed into *E. coli* InvαF^- (Invitrogen). Transformants were assayed for a plasmid insert that was PCR amplifiable by oligonucleotides 1 and 2 together but not by either oligonucleotide separately. The insert of one such plasmid was amplified by two rounds of PCR with oligonucleotides 1 and 2, diluted into 2 ml of water, and concentrated to a final volume of approximately 60 μl with a Centricon-100 microconcentrator (Amicon, Beverly, Mass.). The purified PCR product was radiolabelled with [α-^{32}P] dATP (Du Pont, Boston, Mass.) by random priming (Life Technology, Gaithersburg, Md.) and used to screen a *B. burgdorferi* B31 DNA library in Lambda

TABLE 1. Oligonucleotides (5′ to 3′) used in the course of this work

Oligonucleotide	Sequence	Use in this work
M13-Forward	GTAAAACGACGGCCAGT	λBLS434 subcloning
M13-Reverse	AAACAGCTATGACCATGA	λBLS434 subcloning
1	ATGTAACAGCTGAATG	*erpG* cloning
2	CATATAAGCTTTCTCC	*erpG* cloning, *ospE* probe
3	GACCTTTACCTCTCC	λBLS434 subcloning
4	GTGCTGTTTTTATAC	λBLS434 subcloning, *ospE* probe
5	CTTAAATTATGTCTAGTATCACTC	Cloning of DNA 5′ of *erpAB*
6	TAATAGATCTGCACC	Cloning of DNA 5′ of *erpAB* and *ospEF*
7	ATAGATTAAAGGGACTAC	Cloning of DNA 5′ of *erpAB* and *ospEF*
8	GGACTTGGATATATTAAGAGC	Cloning of DNA 5′ of *erpAB* and *ospEF*
9	CTGCTTTAGCCCTAGCTTC	Cloning of DNA 5′ of *erpCD*, *erpD* probe
10	AATATGTTGATTGTG	Cloning of DNA 5′ of *erpCD*
11	GTGCTGTTTTTATAC	*erpA* probe
12	CAGTTATTAATTTTATCTCC	*erpA* probe
13	AGAATTATGCAATTAAAGATTTAG	*erpB* probe
14	GATTCTTCTACTTTTTTCACTTTC	*erpB* probe
15	ATCATACTTTATATGATGGGC	*erpC* probe
16	CAAAATCATCAATTTTTTCTATTTTTG	*erpC* probe
17	GATTTAAAACAAAATCCAGAAGGG	*erpD* probe
18	TGCAAGATTGATGCG	*erpG* probe
19	ATTTTGAGGCTCTGC	*erpG* probe
20	AATATTGCAATTATTAGCTGTTG	*erpH* probe
21	ATTCATTCTTAGGGTTTTCATATC	*erpH* probe
22	TAGCTAGTCCTGGGCCGGGA	*guaA* probe
23	ATCTCTTTTATTTTCAGAAC	*guaA* probe
24	AAGCTTAATTAGAACCAAAC	*ospA* probe
25	GTTCCTTCTTTAACCACCAA	*ospA* probe

ZAPII (Stratagene, La Jolla, Calif.) (29). LambdaZAPII clones that hybridized with this probe were excised in vivo to produce plasmids by following the manufacturer's instructions, and the inserts were sequenced.

The insert in one of the LambdaZAPII phages, λBLS434, was not excised in vivo and was subcloned as follows. A suspension of intact phage particles was lysed by heating to 94°C. The lysate was subjected to PCR amplification with oligonucleotides 1 and 2, and the PCR product was cycle sequenced (Life Technology) with these same two oligonucleotides. On the basis of this sequence, the inserted region of λBLS434 was PCR amplified in two pieces with oligonucleotides 3 and M13-Reverse and oligonucleotides 4 and M13-Forward. The resulting PCR products were cloned into pCRII and sequenced with Sequenase 2.0.

DNA sequences located 5′ of the *erpAB* genes of *B. burgdorferi* B31 and the *ospE* gene of *B. burgdorferi* N40 were PCR amplified from total bacterial DNA with oligonucleotide 5 or 2, respectively, and oligonucleotides 6, 7, and 8. DNA located 5′ of the *erpCD* genes of *B. burgdorferi* B31 was PCR amplified with oligonucleotides 9 and 10. PCR products were cloned into pCRII, and the inserts were sequenced from both ends.

Radiolabelled DNA probes for Southern blotting. Probes for the *erp* loci were generated by PCR amplification from plasmid clones containing the appropriate regions of DNA. Probes were produced for *erpA* (oligonucleotides 11 and 12), *erpB* (oligonucleotides 13 and 14), *erpC* (oligonucleotides 15 and 16), *erpD* (oligonucleotides 9 and 17), *erpG* (oligonucleotides 18 and 19), and *erpH* (oligonucleotides 20 and 21). A probe for the *ospE* gene of *B. burgdorferi* N40 was generated by PCR amplification from a plasmid containing this gene (see above) by using oligonucleotides 2 and 4. Probes for the *guaA* and *ospA* genes of *B. burgdorferi* B31 were generated by PCR amplification of total bacterial DNA with oligonucleotides 22 and 23 and oligonucleotides 24 and 25, respectively. PCR conditions for probe production consisted of 25 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 2 min. Each PCR amplification was assayed for a single product by agarose gel electrophoresis and visualization with ethidium bromide. Probes were purified and labelled as described above for the library screen.

Restriction endonuclease mapping of plasmids carrying *erp* genes. All restriction endonucleases were obtained from New England Biolabs (Beverly, Mass.). The digested DNAs were separated on a 0.7% agarose gel by pulsed-field electrophoresis for 18 h at 7 V/cm with program 3 of a PPI-200 programmable power inverter (MJ Research, Watertown, Mass.). DNAs were transferred bidirectionally to nylon membranes (ICN, Irvine, Calif.) and hybridized (29) at 55°C with a probe specific for each *erp* locus. Filters were washed with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 55°C, and specifically bound DNAs were detected by autoradiography. Filters were also hybridized at 45°C with a probe specific for the *ospE* gene of *B. burgdorferi* N40 and washed in 2× SSC–0.1% SDS at 45°C.

Two-dimensional gel electrophoresis. The conformations of *B. burgdorferi* DNAs were determined by two-dimensional chloroquine gel electrophoresis essentially as described by Samuels and Garon (40). Briefly, DNA was electrophoresed for 16 h at a constant voltage of 1.3 V/cm on a 0.35% agarose gel in 0.5× TBE buffer (1× TBE contains 0.089 M Tris [pH 8.0], 0.089 M boric acid, and 0.002 M EDTA) (27). The gel was next rotated 90°, soaked for 4 h in 0.5× TBE containing 100 μM chloroquine, and electrophoresed for an additional 16 h at 1.3 V/cm in this same buffer. Following electrophoresis in the second dimension, the gel was soaked in several changes of water, and DNA was visualized by ethidium bromide staining. DNA was transferred to nylon membranes (ICN) and hybridized with radiolabelled DNA probes at 55°C as previously described (29). Filters were washed with 0.2× SSC–0.1% SDS at 55°C, and specifically bound DNAs were detected by autoradiography.

Immunoblotting. Immunoblotting of *B. burgdorferi* lysates was performed as described previously (49). ErpA and ErpC proteins were detected with a 1:100 dilution of a polyclonal rabbit serum directed against the OspE protein of *B. burgdorferi* N40 (32, 49) (provided by E. Fikrig, Yale University).

Nucleotide sequence accession numbers. The sequences of the *erpAB*, *erpCD*, *erpG*, and *erpH* loci and the surrounding DNA have been deposited in GenBank and given accession numbers U44912, U44914, U42598, and U44913, respectively. The DNA sequence located 5′ of the *ospE* gene of *B. burgdorferi* N40 has been given accession number U42599.

RESULTS

Cloning of genes related to *ospE* and *ospF*. We attempted to clone an *ospE* gene from *B. burgdorferi* B31 by PCR amplification with oligonucleotides complementary to sequences located immediately upstream and downstream of the *B. burgdorferi* N40 *ospE* gene (26). A PCR product of approximately 500 bp, which contained a truncated open reading frame that could encode a protein with homology to the *ospE* gene of *B. burgdorferi* N40, was used as a probe to screen a *B. burgdorferi* B31 LambdaZAPII library. Four separate loci (containing a total of six *ospEF*-related open reading frames) were identified, each preceded by at least 2 kb of DNA that is similar to that found 5′ of the *ospEF* genes of *B. burgdorferi* N40 (Fig. 1). The region of DNA immediately upstream of each of these loci, which presumably includes transcriptional regulatory elements, is nearly the same (Fig. 2). In addition, the open reading frames could encode proteins with hydrophobic amino termini and potential lipidation sites that are similar to those found in OspE and OspF of *B. burgdorferi* N40 (Fig. 3) (26). These loci are similar to each other and to the *ospEF* locus of *B. burgdorferi* N40, yet none of the six genes can be clearly identified as the *B. burgdorferi* B31 equivalent of *ospE* or *ospF*. We have therefore designated these proteins Erp, for OspEF-related protein, and named their respective genes *erp*.

Sequence analysis of the *erp* loci. Two of the loci, *erpAB* and *erpCD*, appear to be bicistronic operons, as is the *ospEF* locus of *B. burgdorferi* N40 (Fig. 1) (26, 49). The first open reading frames of these two loci, *erpA* and *erpC*, share 85 and 88% identical nucleotides, respectively, with the *ospE* gene of *B. burgdorferi* N40 and could encode proteins very similar to OspE (Fig. 3A). The second open reading frames, *erpB* and *erpD*, would encode proteins with very little similarity to the OspF protein of *B. burgdorferi* N40 (Fig. 3B). The nucleotide sequences of *erpB* and *erpD* share only 36 and 38% identical nucleotides, respectively, with the *ospF* gene of *B. burgdorferi*

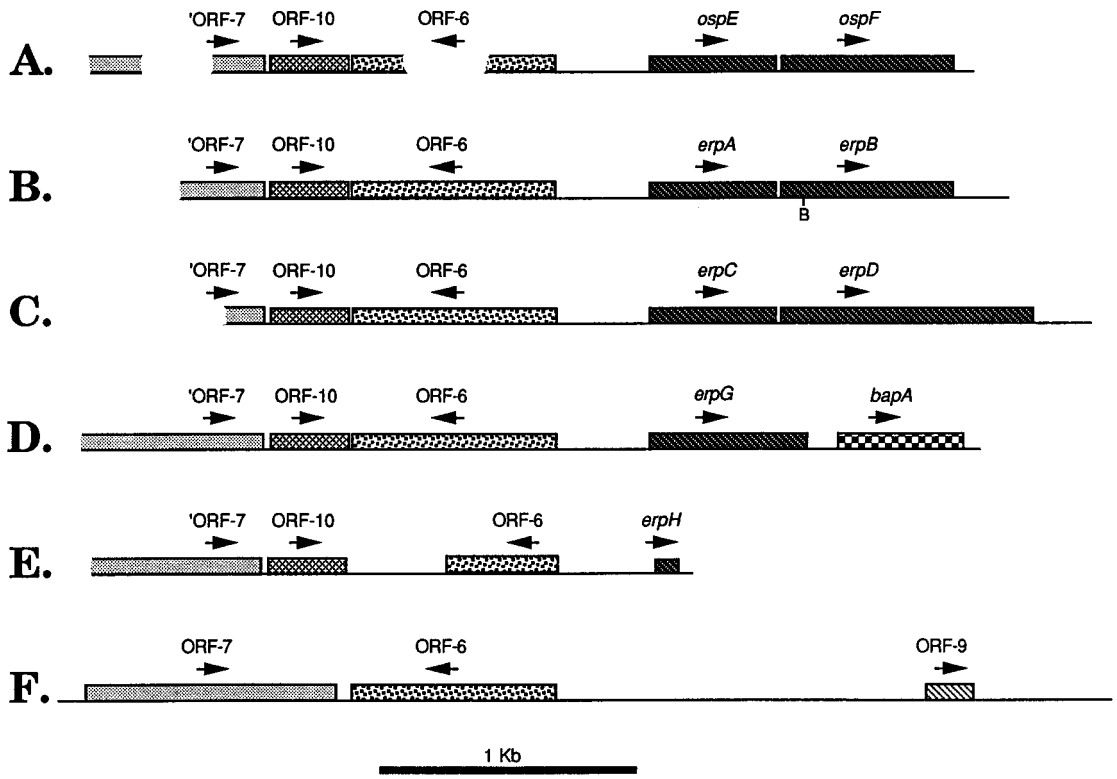

FIG. 1. Alignment of genes for OspEF-related proteins and flanking DNAs. Arrows indicate the 5′-to-3′ orientation of each open reading frame. (A) *ospE* and *ospF* of *B. burgdorferi* N40. The complete sequence of the DNA located 5′ of the *B. burgdorferi* N40 *ospEF* locus was not determined. (B) *erpA* and *erpB* (cp32-1) of *B. burgdorferi* B31. The *Bam*HI site within *erpB* is indicated by the letter B below this open reading frame. (C) *erpC* and *erpD* (cp32-2) of *B. burgdorferi* B31. (D) *erpG* (cp32-3) of *B. burgdorferi* B31. (E) *erpH* (cp32-4) of *B. burgdorferi* B31. (F) cp8.3 of *B. burgdorferi* sensu lato Ip21.

N40, which suggests that neither *erpB* nor *erpD* is the *B. burgdorferi* B31 allele of *ospF*. The noncoding regions located between *erpA* and *erpB* and between *erpC* and *erpD* are of the same length, and the sequences are nearly identical to that found between the *ospE* and *ospF* genes of *B. burgdorferi* N40

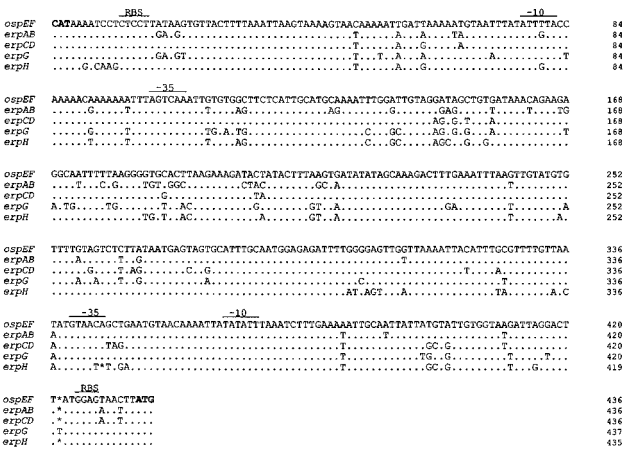

FIG. 2. Comparison of the DNA sequences located 5′ of the *ospEF* locus of *B. burgdorferi* N40 and the *erpAB*, *erpCD*, *erpG*, and *erpH* loci of *B. burgdorferi* B31. Nucleotides identical to those 5′ of the *B. burgdorferi* N40 *ospEF* locus are represented by dots. Asterisks indicate spaces introduced into the sequences for optimal alignment. The presumed initiation codons of each ORF-6 gene (CAT) and the first gene of each locus (ATG) are in boldface. Potential ribosome-binding sites (RBS) and promoters (−10 and −35) are overlined.

(26). The predicted molecular masses of ErpA (19.6 kDa) and ErpC (20.2 kDa) are similar to that of OspE (19.2 kDa) (26), and that of ErpB (25.3 kDa) is similar to that of OspF (26.1 kDa) (26), while ErpD (38.9 kDa) is predicted to be much larger than OspF. The DNA sequences located 3′ of the *erpB* and *erpD* stop codons are not homologous to that found 3′ of the *ospF* gene of *B. burgdorferi* N40, further indicating that neither *erpAB* nor *erpCD* is allelic to *ospEF*.

A third locus, *erpG* (Fig. 1), could encode a single protein with an approximate molecular mass of 22 kDa that is homologous to the OspE and OspF proteins of *B. burgdorferi* N40 at the amino terminus but has only limited regions of similarity for the remainder of the protein (Fig. 3B). The *erpG* gene shares 32 and 48% identical nucleotides with the *ospE* and *ospF* genes of *B. burgdorferi* N40, respectively. Our original 500-bp PCR clone contains a 5′ portion of *erpG* and a short stretch of the DNA that precedes the gene. The 75 bp of DNA located immediately 3′ of *erpG* shares 75% identical nucleotides with the 74-bp region 3′ of the *ospF* gene of *B. burgdorferi* N40 (26); no homology between the two was observed beyond that point. The nucleotide sequence of *erpG* is identical to that of the *pG* gene that was cloned independently from *B. burgdorferi* ZS7 by Wallich et al. (51). The *erpG* gene shares 67 and 52% identical nucleotides with the *bbk2.10* and *bbk2.11* genes, respectively, of *B. burgdorferi* 297 (1). Located 114 bp downstream of the *erpG* gene is an additional open reading frame, oriented in the same direction but lacking similarity to any OspEF-related protein. This gene is similar to *eppA*, which has been reported to be located on a 9-kb circular plasmid in *B. burgdorferi* B31 (16). An identical open reading frame was

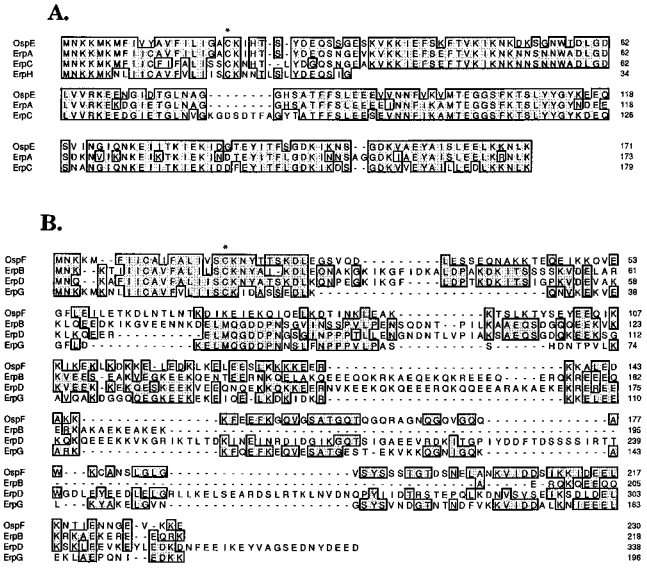

FIG. 3. Alignment of predicted amino acid sequences of OspEF-related proteins of *B. burgdorferi*. The signal peptidase II cleavage-lipidation sites of OspE and OspF (26) are indicated by asterisks. (A) Alignment of the predicted amino acid sequences of the OspE protein of *B. burgdorferi* N40 (26) and the ErpA, ErpC, and ErpH proteins of *B. burgdorferi* B31. (B) Alignment of the predicted amino acid sequence of the OspF protein of *B. burgdorferi* N40 (26) with those of the ErpB, ErpD, and ErpG proteins of *B. burgdorferi* B31.

identified 3′ of *pG* in *B. burgdorferi* ZS7 (51) and was called *bapA* by those researchers. A similar open reading frame was also found 3′ of the *bbk2.10* gene of *B. burgdorferi* 297 (1).

A fourth locus, *erpH*, contains a single, short open reading frame (Fig. 1) capable of encoding a polypeptide of only 34 residues but with a predicted amino acid sequence that is similar to the amino terminus of the *B. burgdorferi* N40 OspE protein (Fig. 3A). The DNA 3′ of *erpH* is not homologous to that found immediately downstream of either *erpAB*, *erpCD*, *erpG*, or the *ospEF* locus of *B. burgdorferi* N40.

The amino termini of the proteins encoded by the six *erp* genes are all predicted to contain motifs similar to those seen in lipidated membrane proteins such as OspA, OspB, OspE, and OspF (14, 26), suggesting that the Erp proteins may also be membrane-bound lipoproteins. ErpG homologs of *B. burgdorferi* ZS7 (pG) (51) and *B. burgdorferi* 297 (Bbk2.10) (1) have been shown to be lipidated when expressed as recombinant proteins in *E. coli*.

Recognition of ErpA and ErpC by antiserum directed against OspE. We have previously reported that polyclonal antibodies directed against the OspE protein of *B. burgdorferi* N40 also recognized a 19- to 20-kDa protein in *B. burgdorferi* B31 (49). We further examined *B. burgdorferi* B31 to determine if this protein was ErpA and/or ErpC or possibly a third protein. Low-stringency Southern blot analysis with a probe made from the *B. burgdorferi* N40 *ospE* gene hybridized with *B. burgdorferi* B31 clone A1.4 DNA fragments (Fig. 4A), which also hybridized with a probe for either *erpA* or *erpC* (data not shown), as was expected from the similarity in nucleotide sequences of these three genes. The DNA fragments that contain the *erpA* or *erpC* gene were the only ones that hybridized with the *ospE* probe, indicating that *erpA* and *erpC* are the only genes in *B. burgdorferi* B31 clone A1.4 that could encode proteins closely related to OspE.

Immunoblotting of a lysate of *B. burgdorferi* B31 clone A1.4 with a polyclonal anti-OspE serum indicated that two proteins with approximate molecular masses of 19 and 20 kDa were recognized by this serum (Fig. 5A). These results suggest that ErpA and ErpC (predicted molecular masses, 19.6 and 20.2 kDa, respectively) are produced by *B. burgdorferi* B31 clone A1.4 grown in culture and are recognized by the polyclonal serum directed against the *B. burgdorferi* N40 OspE protein.

In Southern blot analysis of DNA from *B. burgdorferi* B31 clone A3H.3, the *ospE* probe hybridized only with DNA fragments consistent with those containing the *erpA* gene (data not shown). The anti-OspE serum recognized an approximately

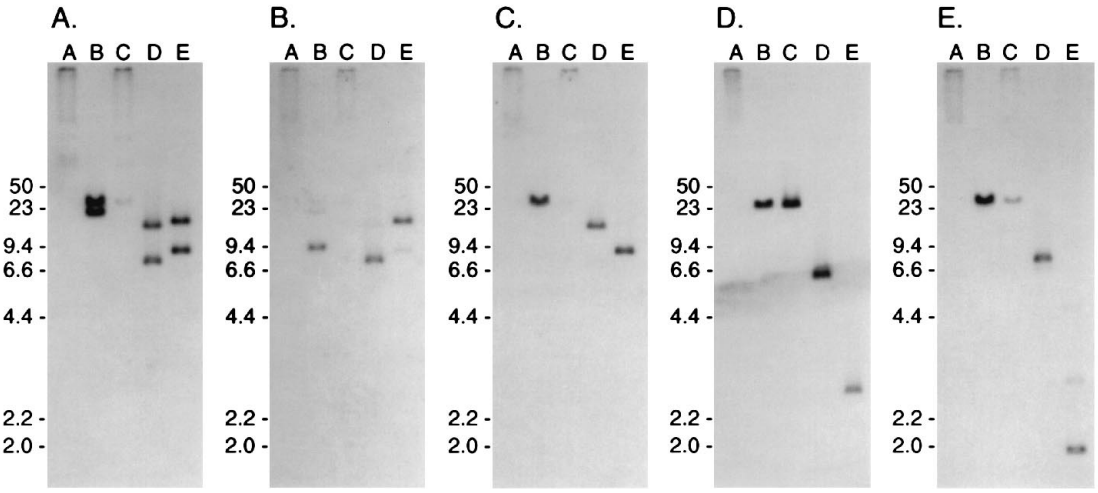

FIG. 4. Southern blot analysis of DNA from *B. burgdorferi* B31 clone A1.4 digested with restriction endonucleases and separated by pulsed-field electrophoresis. All blots contained undigested DNA (lane A) and DNA digested with *Bam*HI (lane B), *Kpn*I (lane C), *Cla*I (lane D), and *Eco*RV (lane E). (A) DNA hybridized with the *ospE* probe of *B. burgdorferi* N40. (B) DNA hybridized with the *erpB* probe. There is a *Bam*HI site within *erpB* (Fig. 1), resulting in the hybridization of this probe to a *Bam*HI fragment different from that to which the probe for *erpA* hybridized. The *erpA* probe hybridized with a *Bam*HI fragment of the same size as the smaller band seen in lane B of panel A but otherwise with fragments of the same size as those to which the *erpB* probe hybridized (data not shown). (C) DNA hybridized with the *erpD* probe. The *erpC* probe hybridized to fragments of the same size as did the *erpD* probe (data not shown), as predicted from the nucleotide sequence. (D) DNA hybridized with the *erpG* probe. (E) DNA hybridized with the *erpH* probe. Circular DNAs migrated near the origin as broad bands under the pulsed-field electrophoresis conditions used in this work. The numbers to the left of each panel are molecular sizes in kilobase.

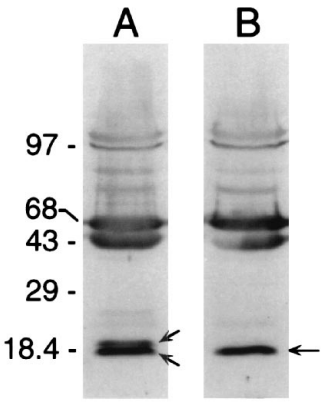

FIG. 5. Immunoblotting of *B. burgdorferi* B31 clone A1.4 (A) and A3H.3 (B) with a polyclonal serum directed against a recombinant *B. burgdorferi* N40 OspE protein (26) (obtained from E. Fikrig). Arrows indicate antigens of approximately 19 (lanes 1 and 2) and 20 (lane 1) kDa. The identities of the antigens with sizes of approximately 43 and 68 kDa are unknown. Polyclonal rabbit serum raised against a recombinant OspF protein (26) (obtained from E. Fikrig) also recognized these proteins but did not detect any 19- and 20-kDa proteins in *B. burgdorferi* B31 lysates (our unpublished results). The numbers to the left are molecular sizes in kilodaltons.

19-kDa protein, but not a 20-kDa protein, in a *B. burgdorferi* B31 clone A3H.3 lysate (Fig. 5B), suggesting that ErpA, but not ErpC, is produced by these cloned bacteria.

The OspE-directed antiserum also recognized antigens of approximately 43 and 68 kDa in the lysates of *B. burgdorferi* B31 clone A1.4 and A3H.3 (Fig. 5). Antiserum directed against the OspF protein of *B. burgdorferi* N40 (obtained from E. Fikrig) also recognized these antigens but not the 19- and 20-kDa proteins (data not shown). The identities of these *B. burgdorferi* proteins are unknown.

Conservation of DNA sequences located 5′ of the *erp* genes. The sequences located 5′ of the *erp* loci were found to be very similar, and oligonucleotides complementary to the upstream DNA of the *erpG* locus were used to PCR amplify and clone sequences 5′ of *erpAB* and *erpCD*. Located within all of these 5′ regions are open reading frames homologous to the potential genes ORF-6 and ORF-7 found on an 8.3-kb circular plasmid (cp8.3) of *B. burgdorferi* sensu lato Ip21 (18) (Fig. 1). The ORF-6 gene located 5′ of *erpH* contains a 1-bp deletion compared with the other ORF-6 genes, so it would encode a truncated protein.

TABLE 2. Comparison of ORF-6, ORF-7, and ORF-10 homologs located on 32-kb circular plasmids of *B. burgdorferi* B31 and circular plasmid cp8.3 of *B. burgdorferi* sensu lato Ip21 (18)

Plasmid	% Identical nucleotides (% identical predicted amino acids)			
	cp32-1	cp32-2	cp32-3	cp32-4
ORF-6				
cp32-2	83 (80)			
cp32-3	83 (79)	88 (88)		
cp32-4	81 (67)	85 (68)	95 (81)	
cp8.3	69 (62)	74 (67)	71 (65)	68 (52)
ORF-7[a]				
cp32-2	96 (86)			
cp32-3	98 (98)	96 (86)		
cp32-4	97 (95)	98 (93)	99 (98)	
cp8.3	73 (65)	67 (57)	74 (69)	72 (64)
ORF-10				
cp32-2	95 (97)			
cp32-3	95 (95)	96 (96)		
cp32-4	93 (97)	95 (97)	93 (95)	
cp8.3	NA[b]	NA	NA	NA

[a] The complete sequences of the ORF-7 homologs of cp32-1, cp32-2, cp32-3, and cp32-4 are unknown. The percentages indicated are for the homologous regions of each ORF-7 that have been sequenced.

[b] NA, not applicable (cp8.3 does not contain an ORF-10 homolog).

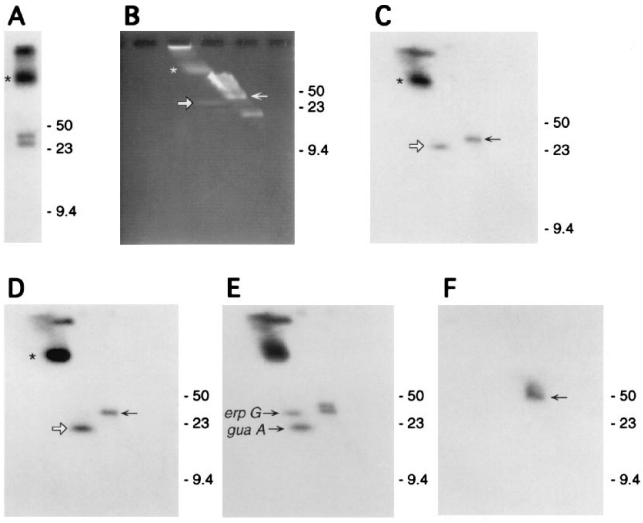

FIG. 6. Southern blot analysis of intact *B. burgdorferi* B31 clone A3H.3 DNA. (A) DNA separated by one-dimensional electrophoresis and hybridized with the *erpG* probe. (B) Example of DNA separated by two-dimensional electrophoresis of DNA and stained with ethidium bromide. The axis on which linear DNAs migrated is indicated by the small left-facing arrow. The axis on which circular DNAs migrated is indicated by the larger, right-facing arrow. (C) DNA separated by two-dimensional electrophoresis and hybridized with the *erpG* probe. Supercoiled and linear forms of cp32-3 are indicated by open and closed arrows, respectively. (D) DNA separated by two-dimensional electrophoresis and hybridized with the *guaA* probe. Supercoiled and linear forms of the 26-kb plasmid are indicated by open and closed arrows, respectively. (E) Panels C and D superimposed. The supercoiled DNAs hybridizing with the *erpG* and *guaA* probes are indicated. (F) DNA separated by two-dimensional electrophoresis and hybridized with the *ospA* probe. The 49-kb linear plasmid is indicated by the arrow. Asterisks in panels A to D indicate very large, circular DNA species that hybridized with both the *erpG* and *guaA* probes and were probably either relaxed circles or concatemers of the plasmids containing these genes. The numbers to the right of each panel are molecular sizes in kilobases.

An additional open reading frame, which we have called ORF-10, was found between each of the ORF-6 and ORF-7 genes linked to the *erp* loci (Fig. 1). Each ORF-10 gene is preceded by a consensus ribosome-binding site, AGGAGG (44), and could encode a protein with a calculated molecular mass of 17 kDa. The translational reading frame of each ORF-10 is oriented in the same direction as ORF-7 and opposite to that of ORF-6 (Fig. 1). The ATG translational start codons of the ORF-10 genes are each located 56 bp from the translational stop codons of the ORF-7 genes. The intervening DNA sequences are very A-T rich and may contain a separate transcriptional promoter for ORF-10, or the two genes may be cotranscribed. In all cases, five nucleotides separate the translational stop codons of ORF-6 and ORF-10. The ORF-10 genes are not homologous to any part of cp8.3, and BLAST (2) searches indicated no homology to any protein or nucleotide sequence in the GenBank or EMBL database. All of the ORF-10s were found to be very similar to one another in both nucleotide and predicted amino acid sequences, as were the ORF-6s and ORF-7s (Table 2).

TABLE 3. Characteristics of the 32-kb circular plasmids of *B. burgdorferi* B31 and their relevant genes

Plasmid	Gene(s)	Predicted protein(s)	Predicted protein size(s) (kDa)	No. of restriction endonuclease sites		Presence in *B. burgdorferi* B31 clone:	
				*Bam*HI	*Kpn*I	A1.4	A3H.3
cp32-1	*erpA*, *erpB*	ErpA, ErpB	19.6, 25.3	2	0	Yes	Yes
cp32-2	*erpC*, *erpD*	ErpC, ErpD	20.2, 38.9	1	0	Yes	?[a]
cp32-3	*erpG*	ErpG	22.0	1	1	Yes	Yes
cp32-4	*erpH*	ErpH	3.8	1	0	Yes	Yes

[a] *B. burgdorferi* B31 clone A3H.3 lacks the *erpCD* locus. It cannot be determined whether cp32-2 is lacking or contains a deletion of *erpCD* in this strain.

The sequence and arrangement similarities of the ORF-6, -7, and -10 genes on these plasmids indicate that they also constitute a gene family within *B. burgdorferi*. Furthermore, the conserved juxtaposition of the ORF-6, -7, and -10 genes with the *erp* loci suggests that there are functional interactions between these genes or their protein products. A small open reading frame, ORF-9, is located near ORF-6 on plasmid cp8.3 (Fig. 1) and contains a potential signal peptidase II-lipidation site (18) analogous to those of the Erp proteins, suggesting a relationship between cp8.3 and the 32-kb plasmids of *B. burgdorferi* B31.

Location of the *erp* loci on circular plasmids. Finding sequences so similar to those seen on a *B. burgdorferi* circular plasmid led us to question whether the *erp* genes are also located on circular plasmids. Southern blot analysis of DNA isolated from *B. burgdorferi* B31 clone A3H.3 separated by constant-field gel electrophoresis indicated that a probe specific for *erpG* hybridized to two DNA species with mobilities consistent with molecular sizes of 30 and 32 kb, in addition to a more slowly migrating species (Fig. 6A). These data suggested that *erpG* is located on a circular plasmid and that the different bands correspond to circular and linearized forms of the plasmid.

We used two-dimensional electrophoresis to examine the conformation of the DNA species that hybridized with the *erpG* probe. The second dimension was run in the presence of chloroquine, which intercalates into DNA and alters the supercoiling of circular molecules. The migration of linear DNA molecules is not significantly affected by chloroquine, and they migrate on a diagonal in two-dimensional gels. Circular molecules, on the other hand, are retarded in mobility by the change in supercoiling and migrate off the linear DNA axis in the second dimension (Fig. 6B) (40). Southern blot analysis of DNA from *B. burgdorferi* B31 clone A3H.3 with the *erpG*-specific probe indicated that the DNA with an apparent size of 30 kb and the very large species both migrated off the linear DNA axis (Fig. 6C) and therefore represent circular DNAs. The DNA with an approximate size of 32 kb did, however, migrate on the diagonal with the linear DNA, suggesting that it was the linearized version of a circular plasmid. Identical results were obtained when probes specific for *erpB* and *erpH* were used, but hybridization was not detected when a probe specific for *erpD* was used (data not shown), indicating that *B. burgdorferi* B31 clone A3H.3 contains *erpB*, *erpG*, and *erpH* on circular DNA but lacks *erpD*. Southern blot analysis of *B. burgdorferi* B31 clone A1.4 DNA separated by two-dimensional electrophoresis indicated that *erpB*, *erpD*, *erpG*, and *erpH* were all present on circular DNA in this isolate (data not shown).

In control experiments, we used probes specific for genes known to be located on either circular or linear plasmids to demonstrate that two-dimensional electrophoresis can accurately discriminate between these different forms of DNA. The filter was stripped and rehybridized with a probe specific for *guaA*, which is located on the same 26-kb circular plasmid as *ospC* (28, 29, 39). This probe hybridized to a linear molecule of approximately 26 kb and to two circular DNAs (Fig. 6D). Alignment of the autoradiographs of Southern blots with the *erpG* and *guaA* probes indicated that *erpG* is located on a larger circular plasmid than is *guaA* (Fig. 5E). The filter was again stripped and rehybridized with a probe specific for *ospA*, which is located on a 49-kb linear plasmid (7). This probe hybridized to a single DNA species that migrated with an approximate size of 50 kb on the same axis as the linear forms of the *erpG*- and *guaA*-containing plasmids (Fig. 6F).

Location of *erpAB*, *erpCD*, *erpG* and *erpH* on separate circular plasmids. To further study the genomic arrangements of the *erp* genes, we digested both total DNA from *B. burgdorferi* B31 clone A1.4 and circular DNA from *B. burgdorferi* B31 with a number of restriction endonucleases. Southern blot analysis of these DNAs with individual probes specific for *erpB*, *erpD*, *erpG*, and *erpH* indicated hybridization to DNA fragments of identical sizes in the digests of both DNA preparations (data not shown). These data indicated that *erpB*, *erpD*, *erpG*, and *erpH* are located only on circular DNA, as different restriction patterns would be expected when comparing the digested circular and total bacterial DNAs if the *erp* loci were located on additional DNA species.

Southern blot analysis of restriction endonuclease-digested DNA from *B. burgdorferi* B31 clone A1.4 indicated that each of the four *erp* loci is located on a separate circular plasmid, each with a unique restriction pattern (summarized in Table 3). The *erpAB* locus is located on a plasmid that is cut twice by *Bam*HI and not at all by *Kpn*I (Fig. 4B). The *erpCD* locus is located on a plasmid that is cut once by *Bam*HI and not at all by *Kpn*I (Fig. 4C). The *erpG* locus is located on a plasmid that is cut once each by *Bam*HI and *Kpn*I (Fig. 4D). The *erpH* locus is located on a plasmid that is cut once by *Bam*HI and not by *Kpn*I (Fig. 4E). Southern blot analysis of DNA from *B. burgdorferi* B31 clone A3H.3 indicated identical restriction patterns, with the exception that neither the *erpC* nor the *erpD* probe hybridized with DNA from this strain (data not shown). Further restriction endonuclease mapping of the *erp* loci has confirmed that each locus is indeed located on a separate circular plasmid of approximately 32 kb (15a). We have designated the circular plasmids that contain *erpAB*, *erpCD*, *erpG*, and *erpH* cp32-1, cp32-2, cp32-3, and cp32-4, respectively (Table 3). *B. burgdorferi* B31 clone A1.4 contains all four 32-kb plasmids, while *B. burgdorferi* B31 clone A3H.3 either lacks cp32-2 or contains a deletion of the *erpCD* locus of this plasmid.

Several other reports have observed 32-kb circular plasmids in *B. burgdorferi* B31. Zückert and coworkers (55, 56) have found conserved DNA sequences on at least two circular plasmids of approximately 30 kb. Amouriaux et al. (3) noted that *B. burgdorferi* B31 contains a circular plasmid of approximately 30 kb and that this plasmid could be cut twice by *Bam*HI to

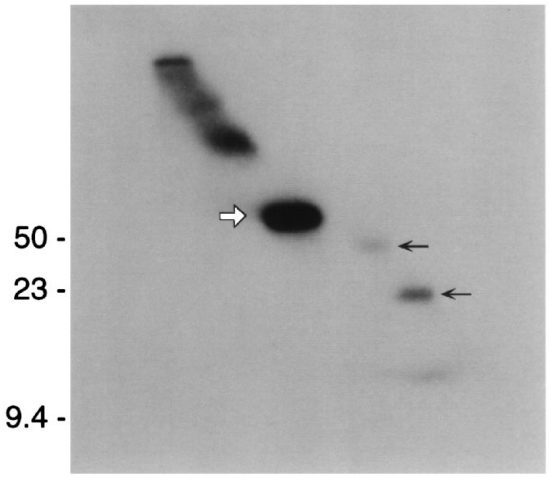

FIG. 7. Southern blot analysis of DNA from *B. burgdorferi* N40 separated by two-dimensional electrophoresis and hybridized with an *ospE* probe. The supercoiled and linear DNAs hybridizing to this probe are indicated by the open arrow and the two closed arrows, respectively. The numbers to the left are molecular sizes in kilobases.

produce fragments of approximately 20 and 9 kb. This plasmid might have been cp32-1, which contains the *erpAB* locus and was cut by *Bam*HI to produce fragments of approximately 22 and 10 kb. Samuels and coworkers (28, 40) have also observed a circular plasmid in *B. burgdorferi* B31 that migrated in two-dimensional gels with a mobility slightly slower than that of the 26-kb *ospC-guaA* plasmid, as was found for the 32-kb plasmids described in this work. In addition, Porcella et al. (36) have cloned several related copies of a sequence from *B. burgdorferi* 297 that hybridize to an approximately 30-kb circular plasmid, suggesting that this isolate may also contain multiple circular plasmids of this size.

Location of the *ospE* gene of *B. burgdorferi* N40 on a circular plasmid. The similarities between the *ospEF* genes of *B. burgdorferi* N40 and the *erp* loci of *B. burgdorferi* B31 led us to re-examine whether the *ospEF* operon is also located on a circular plasmid. We isolated total DNA from the same clone of *B. burgdorferi* N40 as used by Lam et al. (9, 26). The DNA was subjected to two-dimensional electrophoresis and analyzed by Southern blotting with a probe specific for *ospE*, resulting in very strong hybridization to a circular DNA molecule (Fig. 7). Weaker signals were detected from hybridization of the probe to two linear DNAs with approximate sizes of 32 and 18 kb. Lam et al. (26) separated *B. burgdorferi* N40 DNA by one-dimensional pulsed-field gel electrophoresis and Southern blotted with an *ospF* probe, from which they concluded that *ospEF* is located on a 45-kb linear plasmid in *B. burgdorferi* N40. In our experiences, linear DNAs migrate as discrete bands in pulsed-field electrophoresis while circular molecules form broad smears and are often difficult to detect (for example, see lane A of Fig. 4A to E). It is possible, then, that Lam et al. detected the linearized form of the circular plasmid to which we have mapped *ospE*.

Our mapping of the *ospE* gene of *B. burgdorferi* N40 to a circular plasmid led us to examine the DNA located 5′ of *ospEF* to see if it is also homologous to the 32-kb circular plasmid sequences. Total bacterial DNA from *B. burgdorferi* N40 was PCR amplified with an oligonucleotide complementary to a sequence located 3′ of *ospE* and oligonucleotides complementary to sequences within the ORF-7 or ORF-10 gene of cp32-3. Sequencing demonstrated that all of the PCR products contained an *ospE* gene sequence identical to that reported by Lam et al. (26). Sequencing of these PCR products also indicated the presence of sequences homologous to the ORF-6, -7, and -10 genes found on the 32-kb circular plasmids of *B. burgdorferi* B31 (Fig. 1 and 2).

DISCUSSION

We have isolated four loci from *B. burgdorferi* B31 that contain genes related to *ospE* and *ospF*, which we have designated *erp*. This novel gene name reflects our conclusion that these loci are members of a closely related gene family and are very different from other *B. burgdorferi* genes that have been designated *osp*, such as *ospA*, *ospC*, and *ospD* (12, 20, 33). Two of the loci, *erpAB* and *erpCD*, contain two open reading frames in tandem, suggesting that these genes may form operons, as has been suggested for the *ospEF* locus of *B. burgdorferi* N40 (26, 49). The *ospE*, *erpA*, and *erpC* genes are all very similar to each other, while the *ospF*, *erpB*, and *erpD* gene sequences are divergent. Further studies on the expression of these genes and the recognition of the proteins they encode by mammalian host immune systems may help explain the reason for this pattern.

B. burgdorferi B31 clone A1.4 was recently cloned from a single bacterium, indicating that all four of the *erp* loci we have described can be maintained within a single bacterial cell. The parent of this strain, *B. burgdorferi* B31, was originally derived from a clone (6, 15) but has since been passaged continuously in the laboratory for several years. It is possible that additional *erp* genes present in the original *B. burgdorferi* B31 clone may have been lost during cultivation. The "*B. burgdorferi* B31" present in other laboratories may therefore contain more, or fewer, *erp* genes than those reported here. For example, *B. burgdorferi* B31 clone A3H.3 lacks the *erpCD* locus.

Recently, other OspEF-related proteins have been identified in several isolates of *B. burgdorferi*. The reported sequence of the *p21* gene of *B. burgdorferi* N40 (50) indicates that it is followed closely by a second open reading frame. Both of the encoded proteins appear to be related to OspE and OspF, and the genes may constitute a bicistronic locus. Akins et al. (1) have cloned a third locus from *B. burgdorferi* N40 that appears to contain a single gene that may be a homolog of *erpG*. Three OspEF-related loci (*bbk2.10*, *bbk2.11*, and *ospF*) have been identified in *B. burgdorferi* isolate 297 (1). The *bbk2.10* locus contains a gene homologous to *erpG*, which is followed by a gene similar to *bapA*. The other two genes of *B. burgdorferi* 297, *ospF* and *bbk2.11*, encode proteins predicted to share amino acid identities of 88 and 70%, respectively, with the OspF protein of *B. burgdorferi* N40. All three of these loci contain only a single gene, and each is preceded by a DNA sequence similar to those found 5′ of *ospEF* and the *erp* loci. Wallich et al. (51) have cloned a locus, *pG*, from a European isolate of *B. burgdorferi*, ZS7, that has a nucleotide sequence identical to those of the *erpG* and *bapA* genes of *B. burgdorferi* B31.

These data indicate that different *B. burgdorferi* isolates can encode a number of OspEF-related proteins, the genes for which appear to vary in both sequence and arrangement, and perhaps in genomic location. This variability may be an indication of recombination among these related genes, either between the genes of a single bacterium or during horizontal transfer of genetic material between bacteria. Such rearrangements may serve to prevent the bacteria from being immediately recognized and cleared by the immune systems of previously infected mammals. Several reports indicate that at least some of the OspEF-related proteins are antigenic in infected humans and laboratory animals (1, 26, 32, 49–51). The pres-

ence within a single bacterium of a number of different genes encoding OspEF-related proteins may also allow sequential expression of the proteins during the course of mammalian infection. Further studies are necessary to characterize the genes encoding OspEF-related proteins in different isolates of *B. burgdorferi* and the immune responses directed against these proteins to understand the biological significance of this multigene family.

The variation exhibited among the OspEF-related proteins of different isolates of *B. burgdorferi* indicates that these proteins are unlikely candidates for serodiagnostics and vaccines. Nguyen et al. (32) observed that only a small percentage of Lyme disease patients produced antibodies to recombinant *B. burgdorferi* N40 OspE and OspF proteins. Likewise, Wallich et al. (51) found that sera from 11 of 18 Lyme disease patients failed to recognize a recombinant *B. burgdorferi* ZS7 pG protein. It is quite likely that the patients who lacked an immune response were infected with a *B. burgdorferi* strain that did not produce proteins closely related to OspE, OspF, or pG. Additionally, Suk et al. (50) discriminated between antibodies directed against the OspE and p21 proteins of *B. burgdorferi* N40 by using an 11-amino-acid polypeptide found only in p21. This amino acid sequence is not found in any of the *B. burgdorferi* B31 Erp proteins, which raises doubts as to whether it would be useful in screening sera from patients with Lyme disease.

The presence of all four *erp* loci on similar plasmids within a single bacterium raises interesting questions as to the mechanisms of plasmid replication and maintenance in *B. burgdorferi*. Plasmids of bacteria such as *E. coli* exhibit incompatibility, in which two plasmids with the same mechanisms of replication and partitioning cannot be stably maintained in a single bacterium (11). Each 32-kb circular plasmid of *B. burgdorferi* must, therefore, possess a different replication mechanism, or else these plasmids are maintained significantly differently from plasmids of *E. coli*. Further studies on the composition of the 32-kb plasmids may shed light upon the processes of DNA replication and segregation in *B. burgdorferi.*

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