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Intra- and Interbacterial Genetic Exchange of Lyme Disease Spirochete *erp* Genes Generates Sequence Identity Amidst Diversity

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Abstract. All isolates of the spirochete *Borrelia burgdorferi* contain multiple, different plasmids of the cp32 family, each of which contains a locus encoding Erp surface proteins. Many of these proteins are known to bind host complement regulatory factor H, enabling the bacteria to avoid killing by the alternative complement pathway during vertebrate infection. In the present study, we characterized the *erp* loci and cp32 plasmids of strains N40, Sh-2-82, and 297 and compared them to the previously determined cp32 sequences of type strain B31. Bacteria of strain N40 contain 6 different cp32s, those of Sh-2-82 contain 10, and 297 bacteria contain 9 cp32s. Significant conservation between all strains was noted for the cp32 loci responsible for plasmid maintenance, indicating close relationships that appear to correspond with incompatibility groups. In contrast, considerable diversity was found between *erp* gene sequences, both within individual bacteria and between different strains. However, examples of identities among *erp* loci were found, with strains Sh-2-82, 297, and B31 each containing three identical loci that likely arose through intrabacterial genetic rearrangements. These studies also found the first evidence of large-scale genetic exchanges between Lyme disease spirochetes in nature, including the apparent transfer of an entire cp32 plasmid between two different bacteria.

Key words: *Borrelia burgdorferi* — Factor H — OspE-related protein — cp32 — Plasmid — Lyme disease

Introduction

One unusual feature of the Lyme disease spirochete, *Borrelia burgdorferi*, is the abundance of extrachromosomal DNAs maintained by all natural isolates. The type strain, B31, is known to contain 24 different linear and circular plasmids, with a combined size in excess of 675 kb (Casjens et al. 1997, 2000; Fraser et al. 1997; Miller et al. 2000a). In contrast, the chromosome has a size of only 910 kb (Fraser et al. 1997). It is also remarkable that much of this plasmid DNA appears to be redundant. Strain B31 contains approximately 160 paralog families, that is, multiple, closely related genes carried within an individual bacterium (Casjens et al. 2000).

Perhaps the best characterized of the *B. burgdorferi* repetitive DNAs are the cp32s, a family of circular plasmids having sizes of approximately 30–32 kb (Stevenson et al. 2001). All examined Lyme disease spirochetes contain multiple cp32s, with strain B31 possessing 10 family members (Casjens et al. 1997, 2000). The different cp32s within a bacterium are virtually identical to each other, varying at only a few loci (Fig. 1). The conserved sequences and sizes among the cp32 family are probably due to their being prophages, those features likely being required for bacteriophage particle production and appropriate packaging of the phage genome into the capsid

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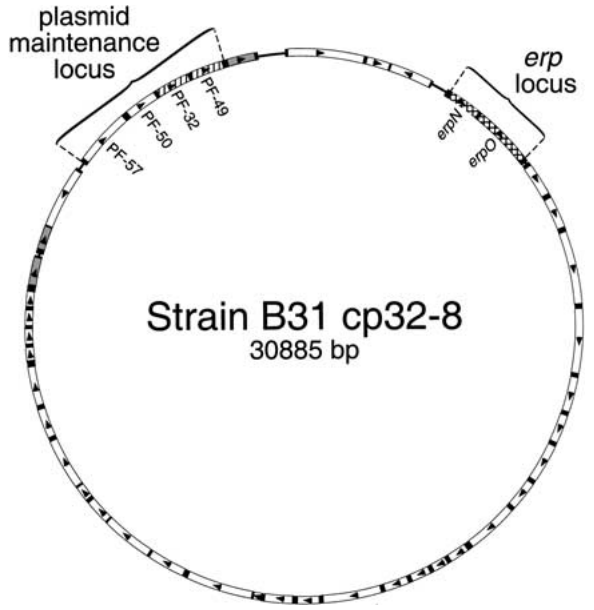


Fig. 1. Open reading frame map of plasmid cp32-8 of *B. burgdorferi* strain B31, a typical member of this family. *Arrows* indicate direction of gene transcription. Genes having extensive conservation between different cp32 plasmids are indicated as *white boxes*. Genes of the *erp* locus, which may be either mono- for bicistronic depending upon the plasmid, are *cross-hatched*. The plasmid maintenance locus consists of four genes, two of which are well conserved among different plasmids. The two other maintenance genes, members of paralog families (PF) 32 and 49, differ considerably between plasmids, confer compatibility, and define each plasmid group. Other variable cp32 loci not discussed in this report are shaded *gray*.

(Eggers and Samuels 1999; Damman et al. 2000; Eggers et al. 2001a). Transduction of cp32 DNA has been observed in the laboratory (Eggers et al. 2001b), and such bacteriophage-mediated exchanges between bacteria may account for the ubiquity of these plasmids in nature. Multiple members of the cp32 family are also maintained by *Borrelia* species that cause relapsing fever, indicating that the evolution of these plasmids/phages most likely predated the divergence of Lyme disease and relapsing fever spirochetes (Carlyon et al. 2000; Stevenson et al. 2000).

Some variant cp32 family members have been observed, which probably cannot produce phage particles. The lp56 plasmid of strain B31 contains an entire cp32 integrated into an unrelated linear plasmid (this work; Casjens et al. 2000). The plasmids cp18 of strain N40 and cp18-2 of strain 297 each arose independently through large deletion events affecting two different cp32s (Stevenson et al. 1997; Caimano et al. 2000). Several other *B. burgdorferi* plasmids appear to have evolved from cp32s via various genetic deletions, insertions and rearrangements (Casjens et al. 2000).

Two genes in the cp32 plasmid maintenance locus, members of paralog families 32 and 49 (PF-32 and PF-49; Fig. 1), differ considerably between plasmids (Stevenson et al. 1998b). PF-32 proteins are homologous to those encoded by other bacterial plasmids that are responsible for plasmid partition, such as ParA and SopA (Zückert and Meyer 1996; Stevenson et al. 1998b; Eggers et al. 2002). Apparently, it is differences in their PF-32 and PF-49 proteins that account for the coexistence of the numerous cp32 plasmids maintained by individual bacteria, since plasmids sharing similar maintenance loci are incompatible (Novick 1987; Eggers et al. 2002). Comparisons of PF-32 and PF-49 genes of cp32 plasmids from different strains often indicate high degrees of similarities, which appear to correlate with incompatibility groups (Stevenson et al. 1998b, 2001; Eggers et al. 2002; Miller and Stevenson 2003). Moreover, the PF-32/PF-49 gene pairing of each group is maintained in different strains, suggestive of specific interactions between the protein products of these two genes (Stevenson et al. 1998b; Miller and Stevenson 2003).

Members of the cp32 family contain another genetic region, the *erp* locus, that also tends to vary in sequence between different plasmids. These genes have been given various other names by different researchers, including *ospE*, *ospF*, *elp*, *p21*, *bbk2.10*, *bbk2.11*, and *pG* (Stevenson et al. 2001). Members of this diverse gene family hold many features in common, suggesting that all are alleles of one another. All Erp proteins are surface-exposed outer membrane lipoproteins that are produced during the initial stages of mammalian infection (Lam et al. 1994; Stevenson et al. 1995; Wallich et al. 1995; Miller et al. 2000b; El-Hage et al. 2001; Hefty et al. 2002a). *erp* loci are all preceded by essentially identical promoter sequences, and, as would be expected, a single bacterium can express its entire Erp repertoire simultaneously (Lam et al. 1994; Akins et al. 1999; Babb et al. 2001; Hefty et al 2001, 2002b; Stevenson et al. 2001; El-Hage and Stevenson 2002). Many Erp proteins have been demonstrated to bind host complement regulator factor H, which helps protect the bacteria from complement-mediated killing in their vertebrate hosts (Alitalo et al. 2001, 2002; Hellwage et al. 2001; Kraiczy et al. 2001a, b; Stevenson et al. 2002). Amino acid variations among Erp proteins influence their affinities for factor H proteins of different animals, suggesting that these differences contribute to the broad host ranges of Lyme disease spirochetes (Kurtenbach et al. 1998, 2002a, b; Stevenson et al. 2002). Thus, it appears that the Erp proteins serve as virulence factors to enhance the fitness of the host bacteria and, ultimately, the cp32 prophages themselves (Hendrix et al. 2000).

Prior to the present study, *erp* genes of only *B. burgdorferi* strains B31 and 297 had been analyzed in detail (Akins et al. 1995, 1999; Stevenson et al. 1996, 1998a, b; Casjens et al. 1997, 2000; Caimano, et al.

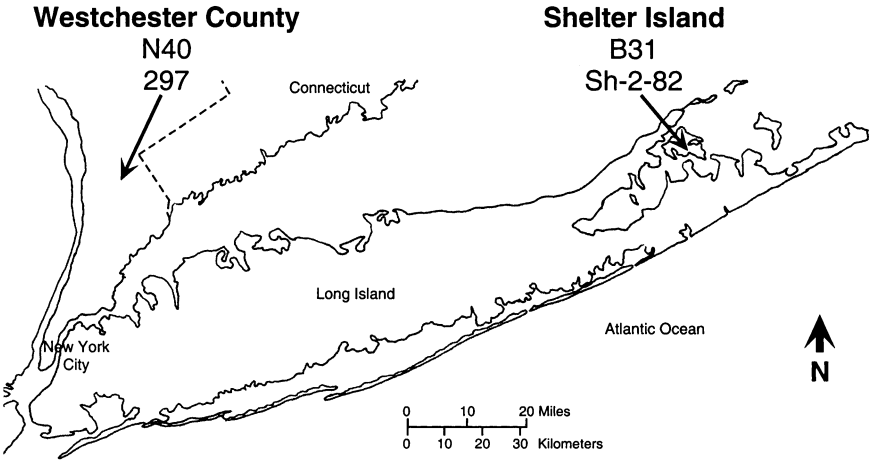


Fig. 2. Map of coastal New York State indicating the sites at which strains Sh-2-82, N40, B31, and 297 were isolated.

2000). Only in the B31 strain was the linkage between *erp* and plasmid maintenance loci known. To obtain a broader view of the degree of diversity among *erp* genes, we cloned and sequenced the *erp* loci of two additional isolates of *B. burgdorferi*, strains N40 and Sh-2-82. In addition, the plasmid maintenance loci linked to the *erp* loci of those two new strains and of strain 297 were analyzed, enabling determination of relationships among the cp32 plasmids.

Materials and Methods

Bacterial Strains and Culture Conditions

The cultures of *B. burgdorferi* strains B31, Sh-2-82, 297, and N40 are all infectious to both mammals and ticks and have low in vitro passage histories. Bacteria were cultured at 34°C in modified Barbour–Stoener–Kelly medium (BSK-H) containing 6% rabbit serum (Sigma).

Strain B31 was originally isolated from an infected *Ixodes scapularis* tick collected on Shelter Island, NY (Fig. 2) (Burgdorfer et al. 1982). The genome of a subculture of B31 was recently sequenced (Fraser et al. 1997; Casjens et al. 2000). Strain B31 contains all nine previously identified cp32 plasmid maintenance locus types (Stevenson et al. 2001). Different cultures of this strain can contain either of two different plasmids with identical maintenance loci, cp32-2 and cp32-7 (Stevenson et al. 1998b). However, a clonal culture of strain B31 has yet to be identified that carries both cp32-2 and cp32-7, consistent with the plasmids being incompatible and suggesting that strain B31 was actually derived from at least two different but closely related bacteria (Saint Girons and Davidson 1992; Casjens et al. 2000).

Strain Sh-2-82 was also isolated from an infected *I. scapularis* tick collected on Shelter Island, and has been cloned by limiting dilution (Schwan et al. 1988). Strain 297 was isolated from the cerebrospinal fluid of a human Lyme disease patient, with the infection reportedly acquired in Westchester County, NY (A. Steere, personal communication; Steere et al. 1983). Strain N40 was originally isolated from an infected tick collected in Westchester County, NY, and was cloned by limiting dilution (Barthold et al. 1988, 1993).

In addition, maintenance loci of select cp32 plasmids from five additional *B. burgdorferi* strains were analyzed. Strain BL206 was isolated from the blood of a human Lyme disease patient in New York (Wang et al. 2001). Strains 90-1654 and 93-0107 were isolated from biopsies taken from infected humans in Germany and Wisconsin, respectively (Miller et al. 2000b). Strains CA15 and DN127cl9-2 were isolated from infected *Ixodes pacificus* ticks collected in California (Bissett and Hill 1987; Schwan et al. 1993; Miller and Stevenson 2003).

Purification and Sequencing of B. burgdorferi *DNA*

B. burgdorferi were grown to late exponential phase, and harvested by centrifugation. Total plasmid DNAs were purified using Qiagen Minikits (Qiagen). DNAs were resuspended in TE (10 m*M* Tris, pH 8.0, 1 m*M* EDTA) and stored at 4°C. Specific DNA fragments were obtained by PCR as described below, using oligonucleotide primers listed in Table 1. Amplicons were either cloned into the *E. coli* vector pCR2.1 (Invitrogen) or sequenced directly without cloning. Plasmid DNA was purified from *E. coli* using Qiagen Midikits. Uncloned DNAs were purified by dilution in 2 ml of water followed by centrifugation through a Centricon-100 microconcentrator (Ambion) (El-Hage et al. 1999). Automated DNA sequencing was performed by Davis Sequencing (Davis, CA). For each novel gene, at least two independently produced PCR amplicons were sequenced. Gene and predicted protein alignments were performed using Clustal X (Jeanmougin et al. 1998) set at default parameters (gap penalty, 10.0; gap extension penalty, 0.20). Phylograms were constructed by the neighbor-joining method using PAUP* version 4.0b10 software (Swofford 2000).

Cloning and Sequencing of erp *Genes*

The first approach utilized to identify novel *erp* loci involved PCR amplification using oligonucleotide primers complementary to sequences found in previously identified *erp* loci. PCR primer pairs consisted of an oligonucleotide complementary to the well-conserved promoter region found 5′ of every *erp* locus, coupled with an oligonucleotide either complementary to a conserved sequence located 3′ of each known *erp* locus or complementary to a sequence found in a previously identified *erp* gene. The 5′ oligonucleotides used were either E-1 or E-203, and the 3′ oligonucleotides were either CP-0, E-24, E-174, or E-604. PCR conditions consisted of 25 cycles at 94°C for 1 min, 50°C for 1 min, and 65°C for 2 min. Amplicons were cloned into pCR2.1 and sequenced.

The second approach used an oligonucleotide specific to a cp32 plasmid maintenance locus paired with oligonucleotide CP-0, which is complementary to a sequence found approximately 1 kb downstream of every known *erp* locus (Amouriaux et al. 1993; Casjens et al. 1997; Stevenson et al. 1997). The Expand PCR amplification system (Boehringer–Mannheim) was used for these long

Table 1. Oligonucleotides used in this study

Designation	Sequence (5′ to 3′)	Use
E-1	ATGTAACAGCTGAATG	Amplify *erp* loci
E-24	TTTTTTCAAATTAAAATC	Amplify *erp* loci
E-174	GTGTATACAAAATTTAGTTATATC	Amplify *erp* loci
E-203	GGACTTATGGAGAAATTTATG	Amplify *erp* loci
E-604	AATAACAATTGAAATTCCTGAGAG	Amplify *erp* loci
B-1	CAACAAAGTTTTATTTAGTATG	Amplify maintenance loci
B-3	TTTATGGGAAAAAATACCCGG	Amplify maintenance loci
CP-0	GAAAAAGATAACATGCAAGATACG	Amplify *erp* loci
SH-701	CAATGGAGAGATTTTGGGGAGTTG	Amplify *erp* loci
D-1	ACGATAGGGTAATATCAAAAAAGG	Test for presence of cp32-1, amplify associated *erp*
D-2	AGTTCATCTAATAAAAATCCCGTG	Test for presence of cp32-1
E-311	GGAATGTATTAATTGATAATTCAG	Test for presence of cp32-2/7, amplify associated *erp*
E-328	GCGAAATAAATAGTGCCTTATGGG	Test for presence of cp32-2/7, amplify cp18-1
CP3-1	TTACGAAAAAATAGAAAAACTAGG	Test for presence of cp32-3, amplify associated *erp*
CP3-2	TTTCCACTGCCCACTTTTCAGCCG	Test for presence of cp32-3
CP4-1	AGATCCTCAAAATAGTTTAACCAG	Test for presence of cp32-4, amplify associated *erp*
CP4-2	TTAATATTGGCAGAGAGTCTACAG	Test for presence of cp32-4
D-3	ACTGATAATGATGTTATGGTTAGG	Test for presence of cp32-5, amplify associated *erp*
D-2	AGTTCATCTAATAAAAATCCCGTG	Test for presence of cp32-5
CP6-1	GACTTTACATAGTATAAATGCTTTTGG	Test for presence of cp32-6, amplify associated *erp*
CP6-2	TCTCGTTATTATAAAATAAGTAGG	Test for presence of cp32-6
CP8-1	GAAGATTTAAACAAAAAAATTGCG	Test for presence of cp32-8, amplify associated *erp*
CP8-2	GTAATCACTTCTTTTTTACCATCG	Test for presence of cp32-8
CP9-1	TATCAAAAAAGTGCTGTTTTATAG	Test for presence of cp32-9, amplify associated *erp*
CP9-2	TAATCTCAAATATTCTTCTTTATG	Test for presence of cp32-9
D-4	TTTCTTAGGCTGAAATCTTAGGGG	Test for presence of cp32-10
D-5	TTACCAAAGAGGAGATATTTGCTC	Test for presence of cp32-10, amplify associated *erp*
CP11-1	ATTTAAGCTTACATATGCTTAACG	Test for presence of cp32-11, amplify associated *erp*
CP11-4	CGTTGTCCTTTTCTTCCAAATTTC	Test for presence of cp32-11
CP12-4	GAATTCCCTCTTGCAAAGCAGCTG	Test for presence of cp32-12
CP12-7	ATTCTTTAAAGGATAGATTGAGGG	Test for presence of cp32-12, amplify associated *erp*
CP13-1	AGCTTGAAAGATGGTCAGTGGAAG	Test for presence of cp32-13, amplify associated *erp*
CP13-2	ACTCCTTATTTCAAAGTACGTTAG	Test for presence of cp32-13
SH-3	TGCCAGTAGTAGTAAAACCAGTAG	Amplify cp18-1
PCFwd	TGCAAAGAAAAATTGTTGGAC	Amplify *ospC*
PCRev	CCAGTTACTTTTTTAAAACAA	Amplify *ospC*
N2	CAACAAGACTGGTTGTTAGCCTTG	Test N40 for *bbk2,10* gene

amplifications. PCR conditions consisted of 10 cycles at 94°C for 10 s, 50°C for 30 s, and 68°C for 10 min, followed by 20 cycles starting at 94°C for 10 s, 50°C for 30 s, and 68°C for 10 min, with successive extension steps increased by 20 s each. The resultant amplicon (product 1) was purified by dilution in 2 ml of water followed by centrifugation through a Centricon-100 microconcentrator, as described above. Product 1 was then used as template for PCR using Taq polymerase and oligonucleotides CP-0 and SH-701, which is complementary to a well-conserved sequence of *erp* transcriptional promoter regions. These PCR conditions consisted of 25 cycles at 94°C for 1 min, 50°C for 1 min, and 65°C for 2 min. The resulting amplicon, product 2, was cloned into pCR2.1 and subsequently sequenced.

Identification of cp32 Maintenance Loci

The maintenance locus physically linked to each *erp* locus of strains N40, Sh-2-82, and 297 was confirmed by gene-specific PCR, essentially as previously described (Stevenson et al. 1998b). Primer pairs consisted of an oligonucleotide specific to the *erp* locus under investigation coupled with either oligonucleotide B-1 or B-3, which are complementary to well-conserved sequences located in the PF-50 gene of every known cp32. PCR utilized the Expand system with the same conditions as described above. Amplicons were purified in Centricon-100 microconcentrators, and sequenced directly. Each amplification was performed at least two independent times.

Each *B. burgdorferi* strain was also PCR screened for plasmids containing each of the known types of cp32 maintenance locus. PCR conditions consisted of 25 cycles at 94°C for 1 min, 50°C for 1 min, and 65°C for 1 min. Reaction products were subjected to agarose gel electrophoresis and DNAs visualized by ethidium bromide staining.

Additional Characterization of the Strain 297 and Sh-2-82 cp18-1 Plasmids

The truncated plasmids of group cp32-2/7 carried by strains 297, Sh-2-82, and N40 were compared to ascertain their relatedness. These plasmids' reduced sizes are adequate to permit PCR of plasmid regions from 5′ of the plasmid maintenance locus to 3′ of the *erp* locus, a distance of approximately 11 to 14 kb (Stevenson et al. 1997). These long PCRs used the Expand amplification system (Boehringer–Mannheim). PCR conditions consisted of 10 cycles at 94°C for 10 s, 50°C for 30 s, and 68°C for 15 min, followed by 20 cycles starting at 94°C for 10 s, 50°C for 30 s, and 68°C for 15 min, with successive extension steps increased by 20 s each. PCR of Sh-2-

Table 2. *B. burgdorferi* cp32 plasmids and their associated *erp* loci

Plasmid group	B31	N40	Sh-2-82	297
cp32-1	*erpAB*	—	*erp41-1,42-1*	*ospE-1*, *elpB1-1*
cp32-2/7	*erpCD*, *erpLM*[a]	*ospEF*[b]	*erp43*[c]	*elpA2*[d]
cp32-3	*erpG*, *bapA*	—	*erp44*	*ospF*
cp32-4	*erpHY*	*erp23,24*	*erp45*	*elpA1*
cp32-5	*erpIJ*	*erp25*	*erp41-5,42-5*	*ospE-5*, *elpB1-5*
cp32-6	*erpK*	—	*erp46*, *bapA*	*bbk2.10*, *bapA*
cp32-8	*erpNO*	—	*erp50,51*	—
cp32-9	*erpPQ*	*p21*, *erp22*	*erp47,48*[e]	*p21*, *elpB2*[f]
cp32-10	*erpX*[g]	*erp26*	—	—
cp32-11	—	—	*erp49*	*bbk2.11*
cp32-12	—	*erp27*	*erp41-12,42-12*	*ospE-12*, *elpB1-12*
cp32-13	—	—	—	—

Note: —Not detectecd.

[a] Two different plasmids of this cp32 group have been identified in various cultures of strain B31.

[b] The N40 member of group cp32-2/7 is truncated, forming plasmid cp18.

[c] The Sh-2-82 member of group cp32-2/7 is truncated, forming a plasmid of the same size as cp18-1 of strain 297.

[d] The 297 member of group cp32-2/7 is truncated, forming plasmid cp18-1.

[e] The size of the Sh-2-82 cp32-9 group plasmid was not investigated.

[f] The 297 member of group cp32-9 is truncated, forming plasmid cp18-2.

[g] The B31 member of group cp32-10 is integrated into an unrelated linear plasmid, forming plasmid lp56.

82 and 297 DNAs used oligonucleotides E-328 and SH-3 (Table 1), which are specific for sequences within the plasmid maintenance and *erp* loci, respectively, of these strain's cp32-2/7 group members. Reaction products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Two-Dimensional Agarose Gel Electrophoresis

The conformations of *B. burgdorferi* DNAs were determined by two-dimensional chloroquine gel electrophoresis (Samuels and Garon 1993; Stevenson et al. 1996). 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 is 0.089 *M* Tris [pH 8.0], 0.089 *M* boric acid, 0.002 *M* EDTA). The gel was next rotated 90°, soaked for 4 h in 0.5× TBE containing 100 μ*M* 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 a nylon membrane (ICN). A probe specific for the strain B31 lp56 maintenance locus was generated by PCR from recombinant plasmid clone pOMB14 (Zückert et al. 1994; Zückert and Meyer 1996; Casjens et al. 1997). PCR conditions consisted of 20 cycles at 94°C for 1 min, 50°C for 30 s, and 72°C for 2 min. The reaction product was then diluted 1:100 in water and used as the template for a second round of PCR. Each PCR amplification was assayed for a single product by agarose gel electrophoresis and visualization with ethidium bromide. The final PCR product was purified through a Centricon-100 column as described above, radiolabeled with α-^{32}P-dATP (DuPont) by random priming (Life Technology), and hybridized with the nylon membrane overnight at 55°C. The membrane was then washed with 0.2× SSC, 0.1% SDS at 55°C and bound DNAs were detected by autoradiography.

Nucleotide Sequence Accession Numbers

Sequence data from this article have been deposited with the EMBL/GenBank libraries under accession numbers AF500203, AY090887–AY090889, and AY142077–AY142106.

Results

cp32 Plasmids and erp *Loci*

Two different approaches were employed simultaneously to clone the *erp* and plasmid maintenance loci of strains N40 and Sh-2-82. Both techniques used PCR and DNA sequencing to identify novel *erp* loci, followed by additional PCR to link that locus with other loci on the same plasmid. All PCR were independently performed at least twice, with identical results from each. Used in combination, these techniques identified 6 cp32 plasmids and associated *erp* loci of strain N40 and 10 such plasmids and *erp* loci of strain Sh-2-82 (Table 2). In addition, we identified that strain 297 contains nine *erp* loci rather than the seven previously reported (Akins et al. 1999; Caimano et al. 2000), and we linked each of those to a specific cp32 family plasmid.

For all the strains examined in this work, each cp32 was found to contain both a maintenance locus and an *erp* locus. As discussed above, many previous studies indicate that *erp* genes are allelic to one another (Marconi et al. 1996; Stevenson et al. 1996, 1997, 1998a, 2002; Casjens et al. 1997, 2000; Akins et al. 1999; Babb et al. 2001; El-Hage et al. 2001; Hefty et al. 2001; Alitalo et al. 2002; El-Hage and Stevenson 2002). We therefore assigned allele number designations to the newly described genes of strains N40 and Sh-2-82 (Table 2). For historical reasons, however, we have retained the earlier nomenclatures of previously characterized genes. As has been observed previously for other strains, the *erp* loci of strains N40 and Sh-2-82 exhibit a wide range of similarities, each containing both distantly and closely related genes (Figs. 3 and 4).

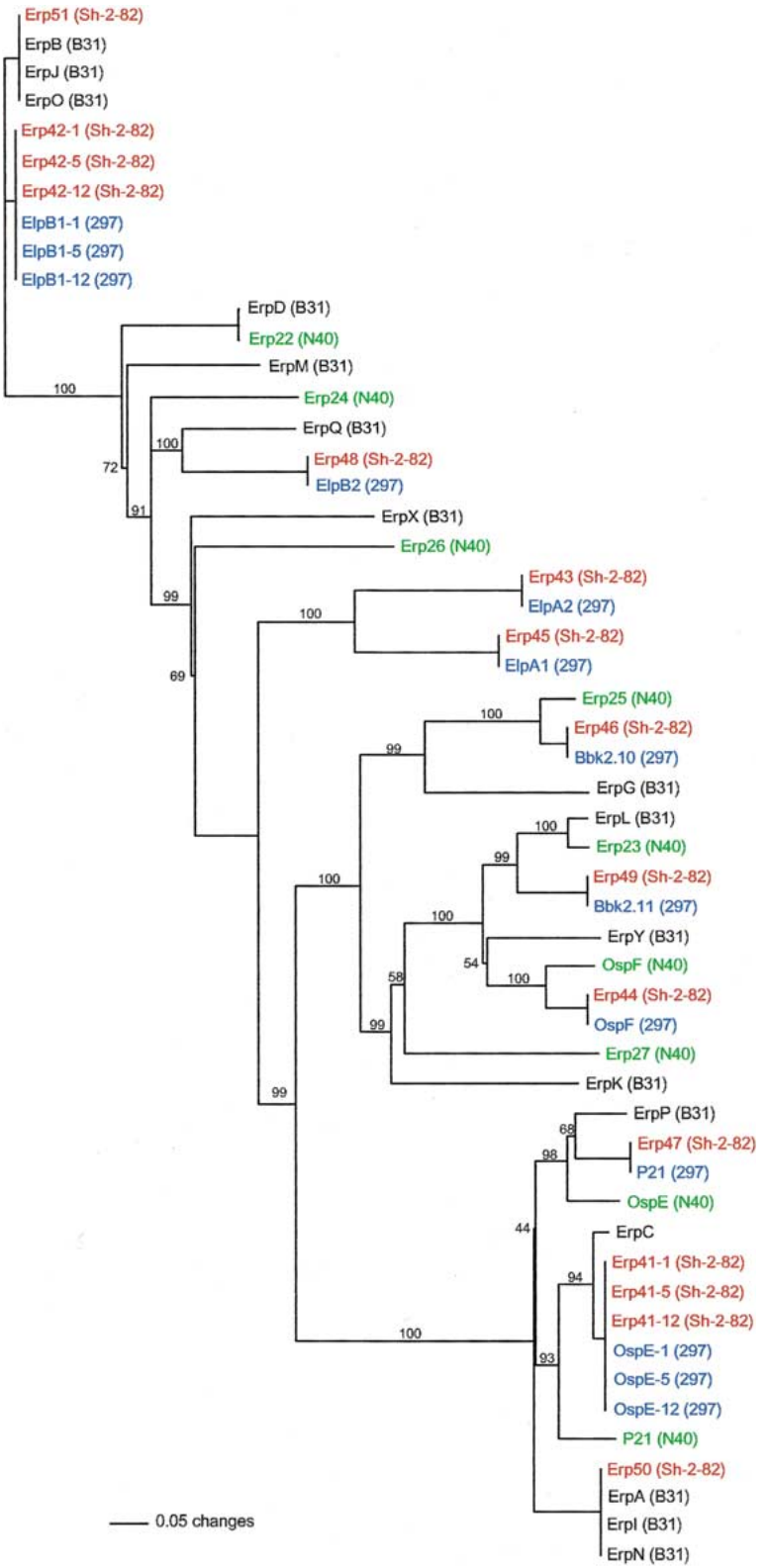


Fig. 3. Phylogenetic tree of the Erp proteins encoded by strains Sh-2-82 (*red*), N40 (*green*), B31 (*black*), and 297 (*blue*). Bootstrap confidence levels (in percentages) are indicated for each branch. Complete protein sequences were utilized for construction of this phylogram. An indistinguishable tree was also determined from comparisons of the mature proteins (sans leader polypeptides). Based on sequence comparisons such as those shown in this figure, it has been suggested that the *erp* gene family should be divided into three separate groups, each bearing a unique name (Akins et al. 1999). Depending upon where the dividing lines are drawn, one could instead divide the *erp* family into four or five groups or interpret these data as illustrative of diversity within a single family, as we have done. In further support of the unified nomenclature, members of all three of the proposed *erp* groups have been demonstrated to perform identical functions (Alitalo et al. 2002; Stevenson, et al. 2002), suggesting a common ancestry for all *erp* genes.

The cp32 plasmid family of *B. burgdorferi* was first characterized in strain B31, and plasmids were named cp32-1, cp32-2, etc., in the order in which they were discovered (Stevenson et al. 1996; Casjens et al. 1997, 2000). It was shortly thereafter recognized that each cp32 contains a unique pair of genes from paralog families 32 and 49 and that cp32 family members from different bacterial strains often share identical PF-32 and PF-49 genes (Stevenson et al. 1997, 1998b; Caimano et al. 2000; Eggers et al. 2002). Phylogenetic

analysis of cp32 maintenance loci indicates tight clusters located on deep branches, indicating high degrees of conservation among the different groups (Fig. 5). Acknowledging these relationships, we and other researchers in the field have recommended that newly identified cp32 plasmids be given names according to the similarities of their maintenance loci to the well-defined cp32s of strain B31 (Stevenson et al. 2001). In this work, we ascribed a cp32 to a particular group if its maintenance locus shared greater than 95% identity with that of a previously described plasmid. The majority of cp32 plasmid family members carried by strain N40, Sh-2-82, and 297 were of such types. However, two new groups were discovered in these strains that contain maintenance loci with significantly less than 85% identities with B31 plasmids, which were named groups cp32-11 and cp32-12. Members of these two new groups appear to be compatible with each of the other defined groups (Table 2).

Additional studies in our laboratory have identified another novel cp32 group, cp32-13, in strain CA15, a California isolate of *B. burgdorferi* (Miller and Stevenson, 2003). Neither strain N40, strain Sh-2-82, strain B31, nor strain 297 was found to contain a member of the cp32-13 group (Table 2).

These data indicate that there is great conservation of cp32 plasmid maintenance loci among distinct *B. burgdorferi* strains and that there appears to be a limited number of cp32 groups in nature. Additionally, only one member of each cp32 group was found in each clonal strain, in agreement with the roles of PF-32 and PF-49 in plasmid compatibility.

B. burgdorferi *Strain N40* erp *Loci*

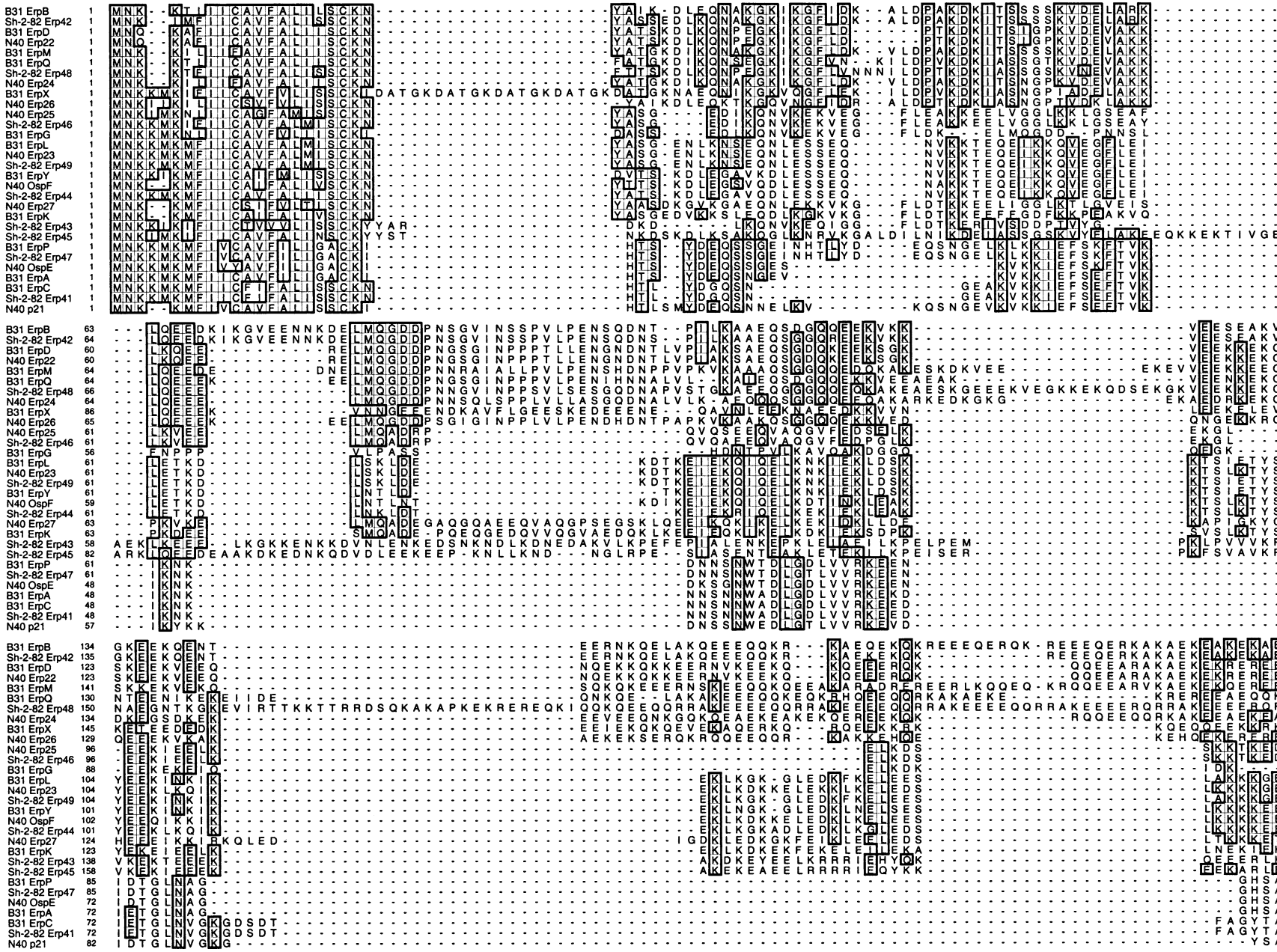
Previously, two *erp* loci had been identified in the clonal culture of strain N40 used in the current study (Lam et al. 1994; Suk et al. 1995). We discovered four additional *erp* loci and one novel cp32 type (Table 2). No plasmid group duplications were found in this strain, consistent with it being a clonal culture. No evidence was found for multiple copies of identical *erp* loci within strain N40, as has been found in other strains (see below; Stevenson et al. 1998a; Casjens et al. 2000).

The *ospEF* locus of strain N40 was the first *B. burgdorferi erp* locus to be identified (Lam et al. 1994) and was again cloned during our efforts. This locus is carried by a truncated circular plasmid, cp18, that we have previously described in detail, and which contains a group cp32-2/7 plasmid maintenance locus (Stevenson et al. 1997, 1998b).

A second *erp* locus of strain N40, including an *erp* gene named *p21*, was previously only partially sequenced (Suk et al. 1995). This locus was reisolated through the current work, completely sequenced, and found to contain a second gene, designated *erp22*. Curiously, the 1017-bp *erp22* gene is 100% identical to the *erpD* gene of strain B31, whereas *p21* shares only 79% identity with the B31 *erpC* (Table 2, Fig. 3). The N40 *p21*,*erp22* locus is located on a plasmid with a cp32-9 group maintenance locus, while the B31 *erpCD* locus is found on a cp32-2/7 group plasmid (Stevenson et al. 1998b) (Table 2).

The four novel N40 loci consist of the bicistronic *erp23*,*24* locus and three monocistronic loci, *erp25*, *erp26*, and *erp27* (Table 2). The *erp23*,*24*, *erp25*, and *erp26* loci mapped to plasmids containing maintenance loci of groups also found in strain B31, those of groups cp32-4, cp32-5, and cp32-10, respectively. The *erp27* locus is located on a plasmid of the previously undescribed cp32-12 group. PCR analysis did not find evidence for the presence of plasmids of group cp32-l, cp32-3, cp32-6, cp32-8, cp32-11, or cp32-13 in strain N40.

The strain B31 *erpX* gene is located on a mutant plasmid, lp56, that consists of an entire cp32 integrated into an unrelated linear plasmid. Analysis of the B31 lp56 sequence indicated that this recombination event probably occurred fairly recently, as the cp32 sequences at the point of recombination are still clearly recognizable and do not contain any additional mutations (Casjens et al. 2000). Since the N40 *erp26* gene is located on a plasmid having the same type of maintenance locus as the B31 lp56 plasmid, we examined the conformation of the N40 plasmid. To do so, we used two-dimensional agarose gel electrophoresis, which permits discrimination between linear and covalently closed circular plasmids (Samuels and Garon 1993; Stevenson et al. 1996). In this technique, DNA is subjected to electrophoresis in standard buffer, then electrophoresed again at a 90° angle to the first run in buffer containing chloroquine. The chloroquine intercalates with DNA, changing the relative mobility of supercoiled circular DNAs. Linear and nicked circular DNAs, however, are free to relax any twists introduced by chloroquine, and so do not change in their electrophoretic mobilities (Samuels and Garon 1993). Electrophoresis was followed by Southern blotting and hybridization with a probe derived from the B31 lp56 maintenance locus. As expected, the B31 blot indicated a 56-kb linear plasmid (Fig. 6). The N40 blot demonstrated a pattern indicative of a 32-kb circular plasmid, consisting of a supercoiled species that was electrophoretically retarded by chloroquine, an open circular form, and an approximately 32-kb linear, sheared form. Thus, it appears that strain N40 contains a close relative of the circular precursor of the strain B31 lp56. We therefore named the type of plasmid that contains a


B31 ErpB
Sh-2-82 Erp42
B31 ErpD
N40 Erp22
B31 ErpM
B31 ErpQ
Sh-2-82 Erp48
N40 Erp24
B31 ErpX
N40 Erp26
N40 Erp25
Sh-2-82 Erp46
B31 ErpG
B31 ErpL
N40 Erp23
Sh-2-82 Erp49
B31 ErpY
N40 OspF
Sh-2-82 Erp44
N40 Erp27
B31 ErpK
Sh-2-82 Erp43
Sh-2-82 Erp45
B31 ErpP
Sh-2-82 Erp47
N40 OspE
B31 ErpA
B31 ErpC
Sh-2-82 Erp41
N40 p21

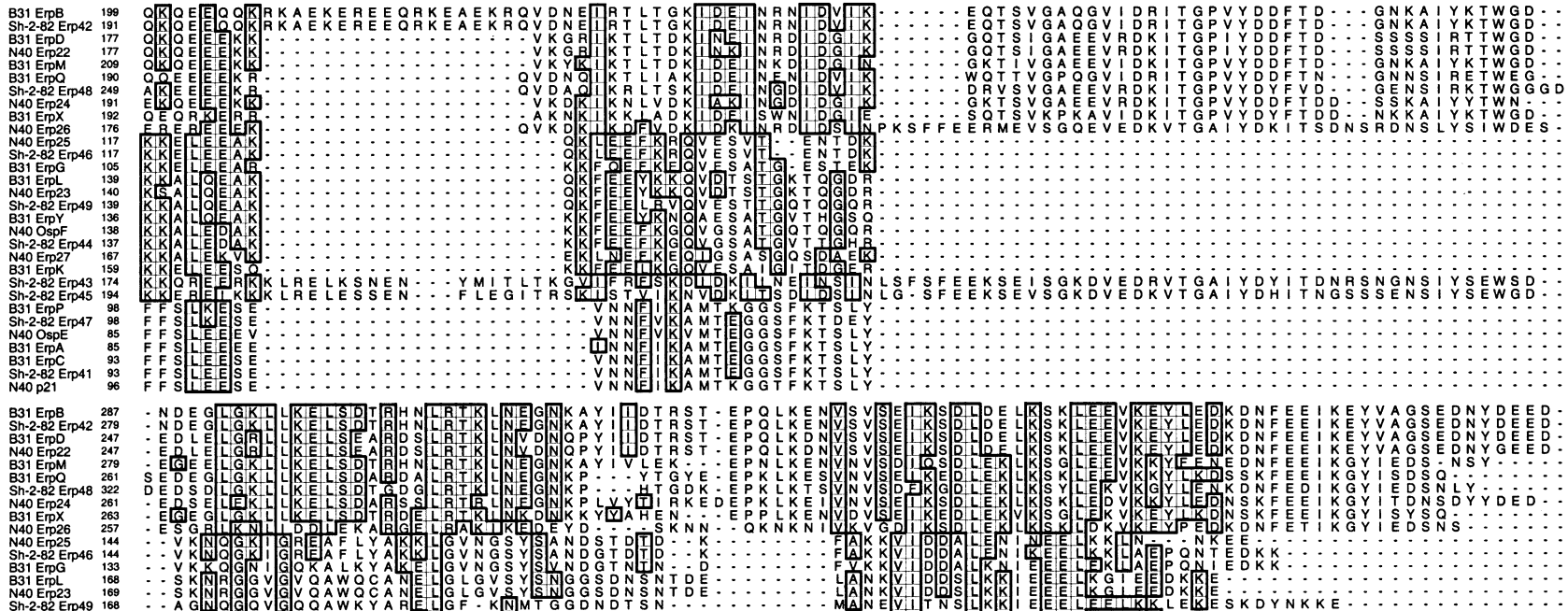


Fig. 4. Alignment of Erp proteins of strains Sh-2-82, N40, and B31. Only unique Erp proteins are included. Strains Sh-2-82 and B31 each contain triplicated *erp* loci (see text), and proteins encoded by only one of those loci is included in the alignment. All identified *erp* loci of strain 297 are identical to those found in strain Sh-2-82 and so were not included in this analysis.

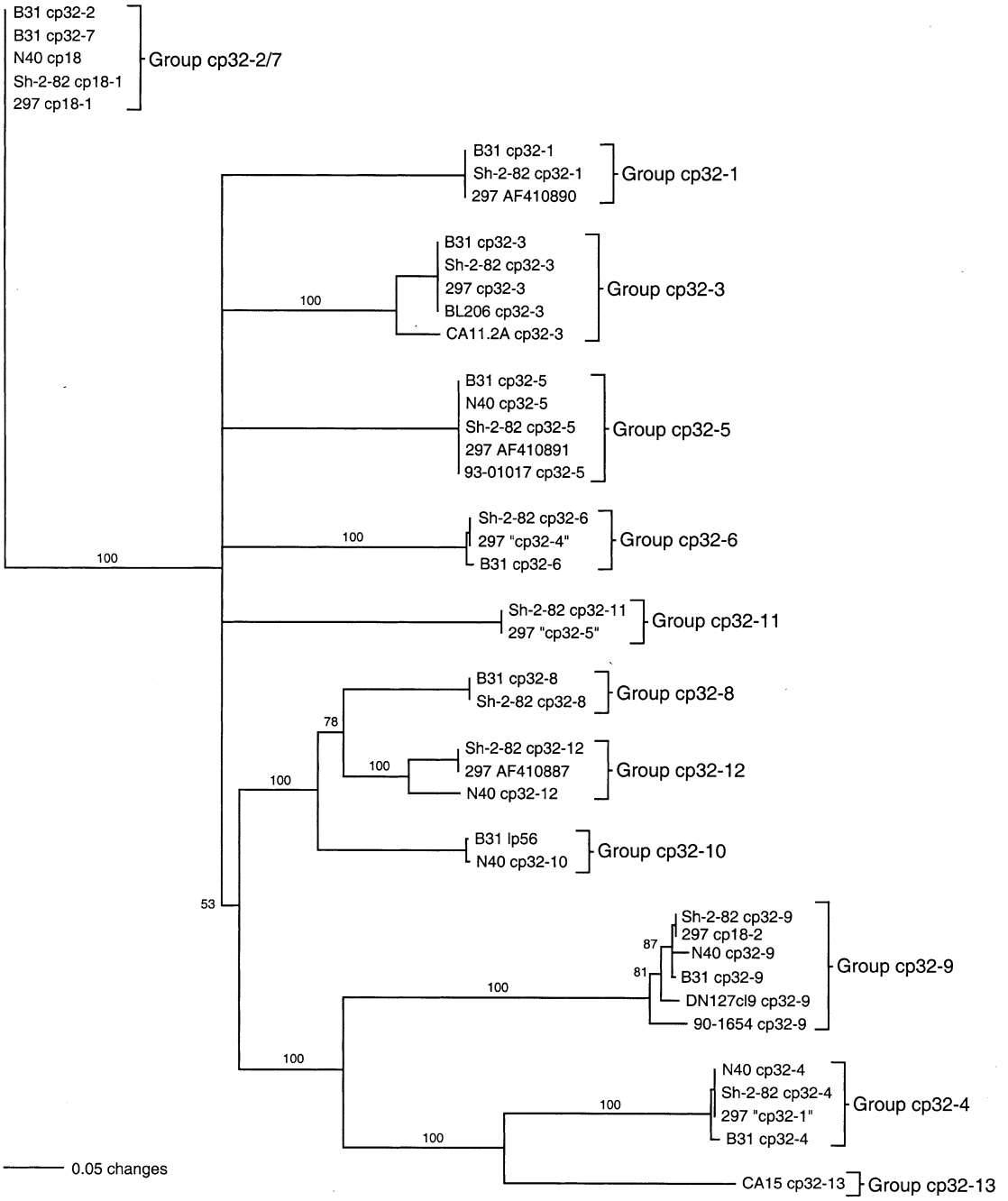


Fig. 5. Phylogenetic tree of all the known cp32 PF-32 proteins of strains Sh-2-82, N40, B31, and 297 and homologs found in other strains. Complete protein sequences were utilized for construction of this phylogram. A similar tree was obtained from analysis of proteins encoded by the physically linked PF-49 genes. The deep branches appear to correspond with incompatibility groups (Eggers et al. 2002). Several of the plasmids found in strain 297 have previously been given names that do not correspond with their cp32 group, and are indicated by quotation marks. The strain 297 *ospE*, *elpB1* locus was previously thought to be located on a single plasmid named "cp32-2" (Akins et al. 1999; Caimano et al. 2000). The present study found that this locus is actually present in three copies in strain 297, as is an identical locus in Sh-2-82, which are located on plasmids of groups cp32-1, cp32-5, and cp32-12. For this reason, only the GenBank accession numbers for those 297 PF-32 proteins are noted in the figure (Eggers et al. 2002). Bootstrap confidence levels (in percentages) are indicated for each branch.

maintenance locus such as found on the N40 cp32 and the B31 lp56 plasmids as "group cp32-10," to reflect that the ancestral form of this plasmid type is most likely a 32-kb circle (Table 2). An earlier survey that examined several *B. burgdorferi* strains for the linear plasmids of strain B31 failed to identify an lp56 homolog in strain N40 (Palmer et al. 2000). However, as that study utilized contour-clamped homogeneous electric field (CHEF) electrophoresis to separate plasmids, it is probable that the circular

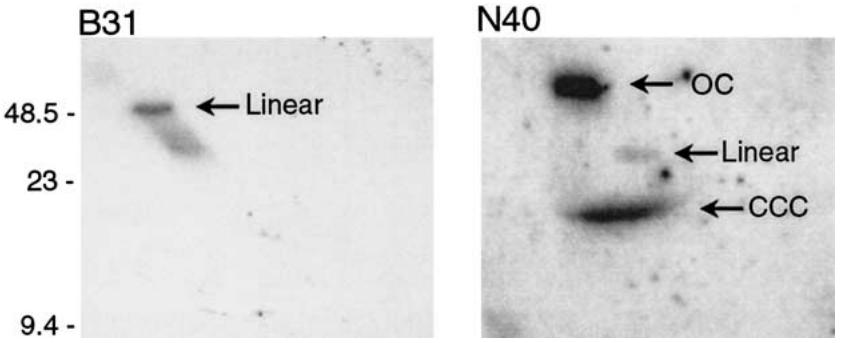


Fig. 6. Two-dimensional Southern blot analysis of the cp32-10 group plasmids of strains B31 and N40. Hybridization with a probe specific for the plasmid maintenance locus revealed hybridization with the linear plasmid lp56 of strain B31 and three forms of the circular plasmid cp32-10 of strain N40. OC, open circular; CCC, covalently closed circular.

cp32-10 of strain N40 would not have resolved well and was therefore missed.

One additional *erp* locus has been reported as being present in a nonclonal culture of strain N40, designated *bbk2.10* (Akins et al. 1995). This locus was not isolated during the analyses that resulted in cloning of the six loci described above, nor could it be PCR amplified using oligonucleotides specifically designed from its reported sequence. We conclude that this locus is absent from the clonal culture of strain N40 used in the present study, and therefore could not include it in our characterization of this strain.

B. burgdorferi *Strains Sh-2-82 and 297* erp *Loci*

Prior to the present study, almost nothing was known about the cp32s of strain Sh-2-82. Although one of the first reports of multiple, 32-kb circular plasmids in *B. burgdorferi* came from Southern blot analyses with three cp32 DNA fragments of this strain (Simpson et al. 1990), none of them were sequenced at that time. We now report that two of the clones isolated by Simpson and coworkers, pSPR13 and pSPR14, each include a portion of a different *erp* locus, while the third clone contains highly conserved genes from another region of a cp32.

Our analysis of Sh-2-82 revealed a total of 10 *erp* loci (Table 2, Fig. 3), all but one of which is identical to a locus previously identified in strain 297 (Akins et al. 1995, 1999; Caimano et al. 2000). The strain 297 *erp* locus, designated *elpA2*, is located on a truncated circular plasmid (Akins et al. 1999), and the identical locus of strain Sh-2-82, *erp43*, is also located on a small plasmid that appears to be identical in size and sequence to that of 297 (data not shown). During the course of this work, sequences of the cp32 PF-32 genes found in strain 297 were published (Eggers et al. 2002), all of which are also identical to those identified in Sh-2-82. However, since the linkages of the 297 PF-32 and *erp* loci were not included in that publication, we undertook those analyses. Each of the identical Sh-2-82 and 297 *erp* loci mapped to plasmids of the same cp32 group. Evidence that we had not confused bacterial strains was provided by sequencing of the Sh-2-82 cp32 fragments cloned by Simpson and colleagues in 1990 (Simpson et al. 1990): pSPR13 contains the 5′ terminus of the gene designated *erp41*, and pSPR14 contains the 5′ end of *erp47*.

As with strain N40, most of the cp32 plasmids of strains Sh-2-82 and 297 are members of groups previously identified in strain B31 (Table 2), although both contain plasmids of the new groups cp32-11 and cp32-12. Unfortunately, several of the plasmids of strain 297 have previously been given other names that conflict with the plasmid group to which they belong (Akins et al. 1999). Those names are in quotation marks in Fig. 5 but are referred to by their cp32 group nomenclature throughout the remainder of this report.

Strain Sh-2-82 contains three identical *erp41,42* loci, one each on a cp32-1, a cp32-5, and a cp32-12 group plasmid (Table 2). This observation prompted us to examine strain 297 more closely, which revealed that the *ospE,elpB1* locus of that strain, which is identical to *erp41,42*, is also triplicated. To differentiate among the identical *erp* loci of Sh-2-82 and 297, each was assigned an additional allele number of 1, 5, or 12, depending upon the plasmid on which it is located (Table 2).

The extensive identities among the cp32 plasmids of strains Sh-2-82 and 297 suggest that these bacteria are closely related. This possibility was further examined by characterization of both strains at another, unlinked locus that also tends to vary considerably between different *B. burgdorferi*. The *ospC* gene encodes a surface protein expressed by *B. burgdorferi* during transmission from ticks to vertebrate hosts and is located on a 26-kb circular plasmid that is not part of the cp32 family. While the *ospC* sequences of strains 297, B31, and N40 each share less than 70% identical nucleotide sequences, the *ospC* genes of Sh-2-82 and 297 proved to be identical (data not shown; Stevenson and Barthold 1994; Livey et al. 1995). Clearly, strains Sh-2-82 and 297 shared a very recent common ancestor.

One difference was detected between strain Sh-2-82 and strain 297, indicating that they are not quite clones of the same organism. Both strains hold nine *erp* loci in common, while a tenth *erp* locus, *erp50,51*,

was identified in Sh-2-82 and mapped to a cp32-8 group plasmid (Table 2). PCR using oligonucleotides specific for either the *erp50*,*51* locus or the cp32-8 maintenance locus failed to yield amplicons from strain 297 DNA, so we conclude that the plasmid is absent from that strain. It is impossible to determine whether Sh-2-82 recently acquired the cp32-8 plasmid or if 297 lost that DNA species. However, we were surprised to note that the Sh-2-82 *erp50*,*51* locus is absolutely identical to an *erp* locus carried by strain B31. As with strains Sh-2-82 and 297, B31 contains three identical *erp* loci, designated *erpAB*, *erpIJ*, and *erpNO*, and the Sh-2-82 *erp50*,*51* locus is identical to these. Additionally, the B31 *erpNO* locus is carried on a cp32-8, as is *erp50*,*51*, and the Sh-2-82 and B31 cp32-8 plasmid maintenance genes are completely identical to each other. These observations strongly suggest that there has been a recent transfer of a complete cp32-8 plasmid between the precursor organisms of strains Sh-2-82 and B31.

Many *B. burgdorferi* strains, including B31 and 297, contain *erp* loci that also include an unrelated gene, *bapA* (Akins et al. 1995; Wallich et al. 1995; Stevenson et al. 1996; Miller and Stevenson 2003). Although the sequence of this gene is quite stable in nature, its plasmid location varies from strain to strain (Miller and Stevenson 2003). The B31 *bapA* gene is located on a cp32-3 plasmid, while the Sh-2-82 and 297 alleles are found on members of the cp32-6 group. The function of the BapA protein is unknown, but we note that strain N40 lacks a *bapA* gene, suggesting that it either does not perform a crucial role or can be complemented by another gene located elsewhere in the genome.

The naturally truncated plasmids cp18 of strain N40 and cp18-2 of strain 297 have been well characterized, with sequence analyses indicating that they arose independently from different cp32 precursors (Stevenson et al. 1997, 1998b; Caimano et al. 2000). Our finding that the strain 297 cp18-1 and the homologous truncated plasmid of strain Sh-2-82 are of the same plasmid group as the strain N40 cp18 led us to further investigate the relationships between these small plasmids. The deleted region of the N40 cp18 is known (Stevenson et al. 1997). The homologous regions of the 297 and Sh-2-82 cp32-2/7 group plasmids were PCR amplified and partially sequenced, which revealed that both contain sequences deleted from the N40 plasmid. Thus, we conclude that the N40 and the 297/Sh-2-82 truncated plasmids most likely evolved through independent deletion events of cp32-2/7 plasmids.

Discussion

It was previously known that bacteria of strain B31 contain three identical *erp* loci (Casjens et al. 1997, 2000). The present studies indicate that this characteristic is not uncommon, as strains Sh-2-82 and 297 also contain three identical loci. The most plausible origin of these phenomena are multiple allelic exchange events among the plasmids within ancestral bacteria. A possible reason for these triplications is suggested by a recently elucidated function of Erp proteins (Hellwage et al. 2001; Stevenson et al. 2002). These outer surface proteins bind complement factor H, an important inhibitor of the alternative pathway of complement-mediated killing. Furthermore, the amino acid differences among Erp proteins affect their affinities for the factor H proteins of different vertebrates (Stevenson et al. 2002). Since the vector ticks of *B. burgdorferi* often feed on a variety of mammals, birds, and reptiles, it is hypothesized that *B. burgdorferi* produce numerous different Erp proteins to protect against killing by complement in a wide range of potential hosts (Kurtenbach et al. 2002b; Stevenson et al. 2002). As a corollary to that hypothesis, it is also expected that a bacterium which cycles between ticks and a single vertebrate host species will accumulate *erp* genes that encode proteins with high affinities for the factor H of that particular host. Perhaps the triplicated *erp* genes of strains B31, Sh-2-82, and 297 conferred an advantage to those bacteria in nature by enabling more efficient infection of their predominant host vertebrate. On the other hand, strain N40 does not appear to contain any such triplicated *erp* loci, which may reflect a more diverse host range of that bacterium's ancestors. Analyses of additional Lyme disease spirochetes having either wide or narrow natural host ranges will help unravel the mystery behind seemingly redundant *erp* sequences.

Identities were also detected between otherwise unrelated strains, indications of naturally occurring DNA exchange between spirochetes. The *erp22* gene of strain N40 is identical to the B31 *erpD* gene, although each is physically linked to a second, unique *erp* gene and is located on a different type of cp32. The most reasonable explanation for this is that the ancestors of these two strains recently exchanged at least 1 kb of DNA which recombined into a heterologous *erp* locus. The cp32-8 plasmids of strains Sh-2-82 and B31 bear extensive identity to each other, suggesting the recent transfer of an entire plasmid between different organisms. It has been argued that *B. burgdorferi* is largely a clonal organism with very limited DNA exchange that, when it does occur, involves only small DNA fragments (Dykhuizen et al. 1993; Dykhuizen and Baranton 2001). The evidence of genetic exchange observed in our study challenges that hypothesis, at least in regards to the cp32 plasmids and their associated genes. As all evidence indicates that these plasmids are actually bacteriophage genomes, transduction of cp32 DNA may

represent a unique exception to the rule of limited genetic exchange among Lyme disease spirochetes.

When *erp* genes were first discovered, it was suggested that they may constitute a mechanism of antigenic variation, wherein these genes constantly rearrange throughout mammalian infection to create surface proteins with novel amino acid compositions. Several studies have since disproved that hypothesis, demonstrating that *erp* sequences remain constant throughout vertebrate infection (El-Hage et al. 1999; Sung et al. 2000; McDowell et al. 2001; Hefty et al. 2002b; Stevenson 2002). While one study did suggest that genetic variation can occur in *erp* genes during mammalian infection (Sung et al. 2000), it has since been demonstrated that the evidence presented in that report was most likely artifactual (Stevenson 2002). Results of the present study provide further evidence for the conservation of *erp* gene sequences during vertebrate infection, since nine of the *erp* loci of strains Sh-2-82 and 297 are completely identical, even though the ancestors of each had infected different vertebrate hosts. Moreover, the identities of these strains indicate that there is quite a strong tendency toward maintaining constant *erp* gene sequences in nature.

Considerable sequence identity was also obvious among the cp32 plasmid maintenance loci of the analyzed strains. The 35 plasmids of the four well-characterized strains fell into only 11 groups. Additionally, analysis of plasmids from six other strains, including isolates originating in California, Wisconsin, New York, and Germany, indicated that all but one fell into those 11 groups. These data indicate that there is a limited number of different cp32 plasmid types in nature. A recent study indicated that cp32 groups most likely correspond with plasmid incompatibility groups. In that work, a recombinant plasmid capable of replicating in *B. burgdorferi* was constructed using as its origin of replication the maintenance locus of the cp32-3 plasmid of *B. burgdorferi* strain CA11.2A (Eggers et al. 2002). Transformation of this plasmid into strain 297 resulted in the loss of the native cp32-3 group plasmid (Eggers et al. 2002). In light of that study and with the identification that cp32 plasmids fall into discrete groups, it is strongly urged that researchers utilize a standardized nomenclature for these plasmids indicative of their relationships. Newly identified cp32 plasmids should be named according to the relationships of their PF49/PF32 loci to the previously defined cp32 plasmids. Any plasmids encoding novel PF49 and PF32 proteins would be distantly related to the previously known plasmids, and should be given new names, such as cp32-14, cp32-15, etc. (Stevenson et al. 2001).

Earlier studies found that Lyme disease spirochetes isolated from different geographic areas may be identical to each other at certain loci (Wang et al. 1999; Qiu et al. 2002). The unexpected discovery that Sh-2-82 and 297 are almost clonal to each other is a further indication that similar bacteria can be spread over a wide range. Both these strains were isolated in the early 1980s, but Sh-2-82 was cultured from an infected tick collected on Shelter Island, while 297 was cultured from the cerebrospinal fluid of a human who was infected in Westchester County. Those locations are over 100 km distant to each other and are separated by two large bodies of water (Fig. 2). The *Ixodes scapularis* ticks that vector *B. burgdorferi* in the northeastern United States often feed on birds (Keirans et al. 1996), and it is likely that such birds were responsible for the spread of the ancestors of Sh-2-82 and 297 across coastal New York. Since strains N40 and B31 were also isolated in Westchester and Shelter Island, respectively, birds were probably likewise involved with the spread of the identical *erp22* and *erpD* genes between the ancestors of those strains.

In conclusion, the cp32 plasmids of four different *B. burgdorferi* strains have now been characterized. Considerable diversity was observed among *erp* genes, both within and between strains. However, high degrees of similarity, and even identity, were also found. That conservation may reflect evolutionary forces felt by all four of these isolates, such as the need to produce Erp surface proteins capable of binding the complement factor H proteins of shared reservoir hosts. Conservation was also noted among the maintenance loci of these plasmids, with only 12 cp32 groups having been identified in the 41 different plasmids analyzed to date. Evidence of naturally occurring genetic exchange between bacteria was also revealed, with at least 1 kb of DNA having recently been moved between the ancestors of strains N40 and B31, while an entire cp32 appears to have been exchanged between the parents of strains Sh-2-82 and B31. As there is strong evidence that cp32 plasmids are bacteriophage genomes, transduction between different bacteria may be an important mechanism for horizontal gene transmission in *B. burgdorferi*. Continued study of the paradoxically diverse yet conserved cp32 plasmids will no doubt provide further insight into the genetics and pathogenic properties of these intriguing bacteria.

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