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Borrelia burgdorferi erp (*ospE*-Related) Gene Sequences Remain Stable during Mammalian Infection

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A number of studies have indicated that *Borrelia burgdorferi erp* genes need not vary during vertebrate infection. However, it was recently reported that a *B. burgdorferi* bacterium reisolated from an infected mouse evidenced mutation and recombination events in several *erp* genes. Reexamination of that reisolate indicates that the previously reported changes were no doubt artifacts of the PCR processes originally used to clone those DNAs. Thus, no evidence has been found of *erp* gene variation during mammalian infection.

All examined isolates of the Lyme disease spirochete, *Borrelia burgdorferi*, contain numerous different circular plasmids of approximately 32 kb in size, the cp32 family (30). As many as 10 different cp32 plasmid family members have been identified in clonal populations of *B. burgdorferi* (30). These plasmids are largely homologous, varying significantly at only three loci: two separate lipoprotein-encoding loci (the *erp* and 2.9 loci) and the plasmid segregation protein-encoding genes (30). Apparently, it is differences among the segregation proteins that account for the ability of so many different cp32 plasmids to reside within a single bacterial cell, since plasmids with similar segregation genes are incompatible (6, 20).

Erp proteins are surface-exposed outer membrane proteins that are produced during the initial stages of mammalian infection, and a single bacterium can express its entire Erp repertoire simultaneously (7, 9, 11, 16, 30). The cp32 *erp* loci have been given various other names by different researchers, including *ospE*, *ospF*, *elp*, *p21*, *bbk2.10*, *bbk2.11*, *pG*, and "upstream homology box genes" (30). The many *erp* genes found within an individual bacterium often exhibit a considerable range of sequence variation. For example, the *B. burgdorferi* type strain B31 is known to contain 17 *erp* genes, arranged in 10 separate loci, that encode proteins having primary sequences that range between 16 and 100% identity (3, 4, 26). This sequence variability has arisen, at least in part, from recombination among *erp* genes, although it is not yet known at which point(s) in the infection cycle such recombination occurs (27).

Lyme disease spirochetes can persistently infect immunocompetent warm-blooded animals for extensive periods of time. The mechanism(s) by which *B. burgdorferi* avoids clearance by the host immune system is yet to be fully elucidated. A number of pathogenic microorganisms, such as the relapsing fever borreliae and the malaria plasmodia, utilize genetic variation mechanisms that constantly produce novel surface proteins, permitting the pathogen to remain a step ahead of host antibody production (15, 23, 31). Lyme disease borreliae possess at least one such genetic variation mechanism, the *vlsE* system (35).

When the *erp* genes were first discovered in *B. burgdorferi*, the multiplicity of these genes, coupled with the variations observed between different strains, suggested that they may also constitute an antigenic variation mechanism that facilitates chronic vertebrate infection. However, three separate studies have demonstrated that *erp* variation is not required for persistent infection of immunocompetent animals. In each of those studies, laboratory animals were infected with a clonal *B. burgdorferi* culture and then bacteria were reisolated from infected tissues after up to 1 year of infection. *erp* gene sequences of these reisolates were identical to those of each clonal inoculant strain (8, 10, 18, 32). Other experimental evidence adds to the results of those studies. *erp* genes have been sequenced from at least five different cultures of strain B31, all of which had been passaged through different immunocompetent mice at least once prior to analysis, and in all but one case, *erp* gene sequences have been absolutely identical. The only known difference is a single point mutation in the *erpB* gene of a high-passage-number, noninfectious culture that has lost many plasmids and acquired several other mutations (3, 4, 26, 29). Together, these data indicate that variation among *erp* genes serves a function other than evasion of host immune responses during persistent vertebrate infection.

It was of great interest when a recent report announced evidence of recombination and other mutational events in *erp* genes during mammalian infection (32). In that study, mice were infected with a clonal culture of *B. burgdorferi* strain B31-MI (referred to as B31G in that report), and 3 months later, bacteria were reisolated, cultured, and cloned by plating. Oligonucleotides designed to amplify three of the *erp* genes were then used in PCR of each clonal reisolate, following which the amplicons were cloned into plasmids in *Escherichia coli* and sequenced. The PCR products of one such reisolate, clone 53, contained sequences different from those of the original B31-MI inoculant (32). The *erp* genes examined in that study all share greater than 80% sequence identity to the *ospE* gene of strain N40 and were therefore referred to as *ospE* genes (32). Strain B31-MI contains three such genes, with the formal designations *erpA* (BBP38, on

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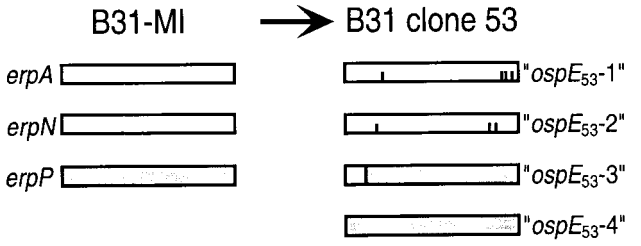

FIG. 1. Schematic of the *ospE*-like *erp* genes of *B. burgdorferi* strain B31-MI (3) and those reported for reisolate clone 53 (32). The B31-MI *erpA* and *erpN* genes are identical, while the sequence of *erpP* is somewhat different. The reported clone 53 genes $ospE_{53}$-1 and $ospE_{53}$-2 are identical to *erpA* and *erpN* with the exception of four and three single-nucleotide substitutions, respectively (indicated by vertical lines). The reported clone 53 gene $ospE_{53}$-3 is a chimera of either *erpA* or *erpN* with *erpP*. The clone 53 gene $ospE_{53}$-4 is identical to the B31-MI *erpP* gene.

cp32-1), *erpN* (BBL39, on cp32-8), and *erpP* (BBN38, on cp32-9) (3, 4, 26, 29). The *erpA* and *erpN* genes are identical, while *erpP* shares approximately 87% identical nucleotides with the other two genes (3). Reisolate clone 53 appeared to be especially remarkable, in that this bacterium was reported to contain four *ospE*-like genes (Fig. 1) (32). One gene was identical to *erpP*, and two differed from *erpA* and *erpN* by either 3- or 4-bp changes, while the fourth was a chimera of *erpA* or *-N* and *erpP*. As noted above, plasmids with identical segregation genes are incompatible. Thus, it appeared that clone 53 contains not only mutant *erp* genes but that one of the original genes must have been duplicated, mutated, and moved to a novel location. Richard Marconi (Virginia Commonwealth University, Richmond) kindly provided a low-passage-number culture of B31 clone 53 to permit further characterization of this bacterium.

Confirmation of this unique genetic phenomenon was first addressed by Southern blot analyses of digested DNAs from both B31-MI and clone 53. Bacteria were grown to densities of approximately 10^8 bacteria per ml in modified Barbour-Stoenner-Kelly medium (BSK-H; Sigma, St. Louis, Mo.). Cultures were harvested by centrifugation, and plasmids were purified using Qiagen mini kits (Qiagen, Valencia, Calif.). Due to the extensive homologies among cp32 plasmids, very few restriction endonucleases generate unique digestion patterns from multiple plasmids (4). Analysis of the sequences of B31-MI cp32 plasmid family members (GenBank accession numbers AE001575 through AE001581 and AE001584) revealed that digestion with either *Hpa*II or *Pst*I will yield uniquely sized DNA fragments that each contain one of that strain's *erp* loci. None of the *erp* genes examined in this study contains *Hpa*II or *Pst*I sites. Total plasmid DNAs from B31-MI and B31 clone 53 were digested with either of those two enzymes, separated by pulsed-field agarose gel electrophoresis, and blotted to a nylon membrane (8). A DNA probe was produced by PCR from an *E. coli* recombinant plasmid clone of *erpP* (pBLS538), using oligonucleotides AINP5′ and ACINP3′ (Table 1), with reaction conditions consisting of 20 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The amplicon was diluted 100-fold in water and amplified a second time, and an aliquot was analyzed by agarose gel electrophoresis for purity. The resulting

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)
AINP5′	ATGCTCGAGAAGATTCATACTTCATATGATGAG
ACINP3′	TATGGATCCTCTCTCCTATATTTCTAACTTC-3′
CL53-1	AGCAAAGCAATGGAGAGGTAAAGG
CL53-2	CTTTGTTTTGGATACCATTTGCAC
E-142	CTAGTGATATTGCATATTCAG
D-1	ACGATAGGGTAATATCAAAAAAGG
CP8-1	GAAGATTTAAACAAAAAAATTGCG

DNA was purified through a Centricon-100 microconcentrator (Amicon, Beverly, Mass.) and was labeled with [α-^{32}P]dATP by random priming (Life Technologies, Gaithersburg, Md.). This probe was hybridized with the membrane overnight at 55°C in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate–5 g of nonfat dried milk per liter. The following day the blot was washed extensively with 0.2× SSC–0.1% sodium dodecyl sulfate at 55°C (high-stringency wash conditions). Hybridized bands were detected by autoradiography. Both B31-MI and clone 53 exhibited identical *Hpa*II and *Pst*I restriction patterns, with three autoradiography bands of the predicted sizes detected from each (Fig. 2).

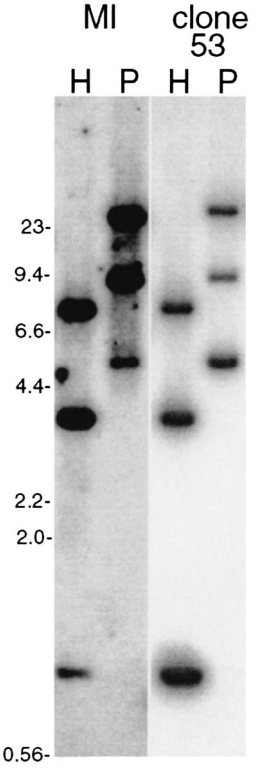

FIG. 2. Southern blot analysis of B31-MI and clone 53 plasmid DNAs digested with either *Hpa*II or *Pst*I (lanes H or P, respectively) and hybridized with a radiolabeled probe derived from the B31-MI *erpP* gene. DNA fragment sizes were as predicted from analysis of the B31-MI genome (see text). In order of descending size, *Hpa*II fragments correspond with *erpA* (cp32-1, 7,540 bp), *erpN* (cp32-8, 3,631 bp), and *erpP* (cp32-9, 1,014 bp), while *Pst*I fragments correspond with *erpN* (24,725 bp), *erpA* (9,452 bp), and *erpP* (5,073 bp). DNA size standards are indicated to the left (in kilobases).

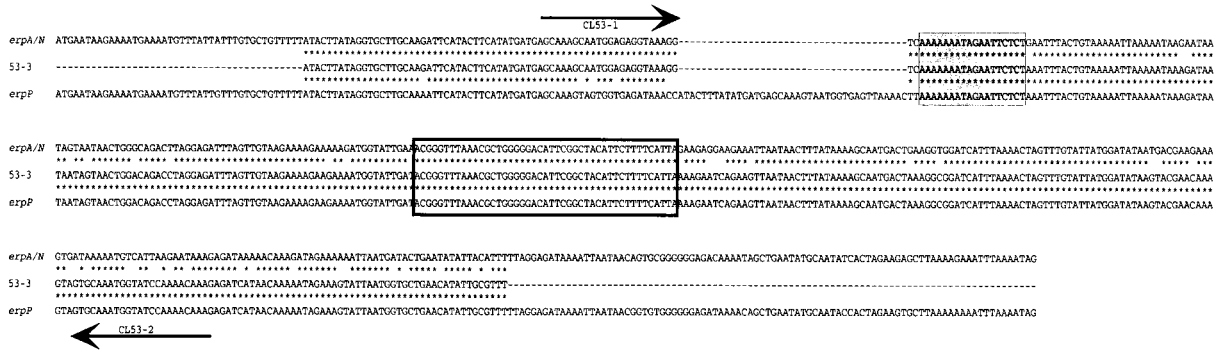

FIG. 3. Alignment of the B31-MI *erpA*/*N* and *erpP* genes with the reported sequence of the clone 53 $ospE_{53}$-3 gene (labeled 53-3). Identical nucleotides are indicated by asterisks. The site of the DNA strand exchange event that would have given rise to $ospE_{53}$-3 is indicated by a shaded box. $ospE_{53}$-3 is identical to *erpA*/*N* 5′ of this location and identical to *erpP* 3′ of this site. The location of the DNA strand exchange event that gave rise to the novel PCR artifactual chimera generated during the present studies is indicated by an unshaded box. The DNA sequences complementary to PCR oligonucleotides are indicated by arrows above or below the sequence, with arrowheads indicating their 5′-to-3′ direction.

These data indicate that either the conclusion of four *ospE*-like genes in clone 53 was incorrect or that the postulated duplication-mutation-translocation event occurred in a DNA segment having final *Hpa*II and *Pst*I restriction patterns identical to those of a parental *erp* locus.

The *erp* genes of B31 clone 53 were further analyzed by PCR using gene-specific primers. The sequence reported as gene $ospE_{53}$-3 is a chimera of either *erpA* or *erpN* with *erpP* (Fig. 1 and 3). Unique sequences found in this chimera enabled production of $ospE_{53}$-3-specific PCR oligonucleotide primers CL53-1, complementary to the *erpA*/*erpN* 5′ end, and CL53-2, complementary to the *erpP* 3′ end (Table 1; Fig. 3). Both B31-MI and B31 clone 53 plasmid DNAs were subjected to PCR, with conditions consisting of 20 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 1 min. Repeated attempts all failed to produce an amplicon from clone 53, although these reaction conditions are used routinely in our laboratory to specifically amplify other *B. burgdorferi* DNA fragments (data not shown). Less stringent PCR conditions were also utilized, consisting of 25 cycles of 94°C for 1 min, 50°C for 1 min, and 65°C for 1 min. Five separate reactions yielded products from clone 53 DNA only three times. Additionally, PCR of B31-MI, which lacks an *erpA*/*N*::*erpP* chimera (3), yielded amplicons from two of four separate reactions.

The nature of the above-described amplification products was addressed by DNA sequencing of both uncloned and cloned amplicons. Most products were determined to have resulted from misprimed PCR, with one oligonucleotide or the other having primed from an incompletely matched DNA site. Six of 9 analyzed B31-MI amplicons contained *erpA*/*N* genes, one contained an *erpP* gene, and two were chromosomal fragments. Sequencing of uncloned amplicons from clone 53 identified only nonmutated *erpA*/*N* or *erpP* genes, with no evidence of a chimera. Examination of five cloned amplicons from this bacterium revealed that three consisted of nonmutated *erpA*/*N* genes and that one consisted of a nonmutated *erpP* gene, while the fifth clone contained a chimeric gene. However, this chimera was unlike that reported as $ospE_{53}$-3, with the apparent strand-crossing event having occurred at a different location (Fig. 3).

The above data indicate that the earlier conclusions regarding $ospE_{53}$-3 were most likely incorrect. Southern blot analysis indicated identical restriction endonuclease digestion patterns of both the parent and reisolated clone, and PCR failed to amplify an $ospE_{53}$-3 sequence from clone 53 DNA despite repeated attempts. The only other possible explanation for these results is that every bacterium in the studied clone 53 culture has lost the $ospE_{53}$-3 locus. While *B. burgdorferi* may lose plasmids during in vitro cultivation, the analyzed clone 53 was grown in culture for a very limited number of generations since reisolation. Unless another culture of clone 53 can be found and conclusively demonstrated to contain an $ospE_{53}$-3 sequence, it is most reasonable to conclude that the previously reported sequence was a PCR artifact.

PCR is notorious for producing such artifacts, which arise from strand exchange during amplification of a mixture of similar DNA templates (1, 19, 21, 22, 24, 25, 33). As one example, rRNA gene PCR analysis of a mixture of defined bacterial species yielded at least 32% aberrant amplicons (34). Strand exchange during PCR also best explains the novel *erp* chimera obtained in the present study: as with $ospE_{53}$-3, it is extremely unlikely that this previously undetected chimera arose from a recombination event accompanied by duplication and translocation into a DNA segment that has a restriction pattern identical to that of a parental *erp* locus and that can only rarely be amplified from clone 53 DNA.

Sequencing of the misprimed clone 53 amplicons indicated that, in addition to the nonmutated *erpP* gene previously reported as being present (32), these bacteria also contain at least one nonmutated *erpA*/*erpN* gene. Yet the earlier report stated that the two genes in clone 53 most similar to *erpA* and *erpN*, $ospE_{53}$-1 and $ospE_{53}$-2, both contain point mutations (32). To clarify this discrepancy, both the *erpA* and *erpN* loci of clone 53 were specifically analyzed. While the *erpA* and *erpN* genes of the starting strain, B31-MI, are identical, they are carried by different plasmids, each of which possesses a unique set of plasmid segregation genes (3, 27). Thus, each of these two *erp* loci can be specifically PCR amplified using an oligonucleotide complementary to the shared DNA sequences 3′ of the *erp* locus (E-142 [Table 1]), and a specific oligonucleotide

complementary to the appropriate plasmid segregation locus (D-1 and CP8-1, for cp32-1 and cp32-8, respectively) (3, 4, 27). PCR conditions consisted of 10 cycles of 94°C for 10 s, 50°C for 30 s, and 68°C for 8 min, followed by 20 cycles starting with 94°C for 10 s, 50°C for 30 s, and 68°C for 8 min with successive extension steps increased by 20 s each, using an Expand PCR amplification system (Boehringer-Mannheim, Indianapolis, Ind.). Amplicons were purified using Centricon-100 microconcentrators and were partially sequenced. Both amplification reactions were separately performed two times. Both the *erpA* and *erpN* genes of clone 53 proved to be identical to those of the parent B31-MI. The PCR process is well known for introducing nucleotide substitutions, especially when using a nonproofreading DNA polymerase such as the *Taq* enzyme utilized in the earlier study (2, 5, 13, 17). It was for this reason that the present study examined the sequences of multiple, independently produced amplicons, rather than a single, cloned PCR product as with the previous report. These data indicate that the clone 53 DNA sequences reported as $ospE_{53}$-1 and $ospE_{53}$-2 were also likely to have been PCR artifacts.

It is therefore concluded that all of the "mutant" *erp* genes reported to be present in clone 53 can be discounted as PCR artifacts and that they are not legitimate genetic variants that arose during mammalian infection. The same report described four parallel reisolate clones containing point mutations in either the *erpA*, *erpN*, or *erpP* gene, each also identified through sequence analysis of single PCR amplicons (32). While those four reisolates were not studied here, the results of the present analysis of clone 53 strongly suggest that those other reported variants may also be artifactual.

In summary, these and other studies indicate that *B. burgdorferi erp* genes remain stable during vertebrate infection. Many, if not all, Erp proteins bind mammalian complement inhibitor factor H and thus likely protect the bacteria against complement-mediated killing in vertebrate hosts (12, 28). Erp protein amino acid sequence variations affect their relative affinities for the complement factor H of different potential vertebrate hosts, which is proposed to contribute to the broad host range of Lyme disease spirochetes (14, 28). This function of Erp proteins suggests a possible reason for *erp* gene stability during vertebrate infection: mutation of a gene encoding an essential factor H-binding Erp might be lethal to the bacterium.

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