

# Repetition, Conservation, and Variation: the Multiple cp32 Plasmids of *Borrelia* Species

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## Abstract

Members of the spirochete genus *Borrelia* contain large numbers of extrachromosomal DNAs. Sequence analysis of the *B. burgdorferi* strain B31 genome indicated that its many plasmids contain large quantities of repeated sequences, the most obvious of which are the cp32 plasmid family. Individual spirochetes may carry nine or more different, but homologous, cp32 plasmids. Every other species of *Borrelia* examined thus far also contains multiple plasmids related to the *B. burgdorferi* cp32s. These plasmids are arguably the best characterized of all the borrelial plasmids, and epitomize the apparent redundancy evident in the many plasmids carried by these bacteria. Despite their extensive similarities, cp32 plasmids contain some open reading frames whose sequences often vary between plasmids, and which encode proteins synthesized by the bacteria during vertebrate infection. In this review, we analyze the hypervariable and conserved regions of the cp32 plasmid family, and discuss possible reasons why borreliae harbor multiple gene paralogs.

## Introduction

*Borrelia* species persist in nature through infectious cycles involving warm-blooded animals and blood-feeding arthropods. Many important diseases of humans and domestic animals are caused by members of this genus, including Lyme disease (*B. burgdorferi* and other closely related genospecies), tick-borne relapsing fever (*B. hermsii*, *B. duttonii*, *B. turicatae*, *B. parkeri*, and others), louse-borne relapsing fever (*B. recurrentis*), and avian borreliosis (*B. anserina*) (Schwan *et al.*, 1999). Studies of different *Borrelia* species have indicated similarities in their general biology and the mechanisms by which these bacteria interact with their hosts (for examples, see Barbour and Hayes, 1986; Marconi *et al.*, 1993b; Schwan, 1996; Zhang *et al.*, 1997;

Schwan and Hinnebusch, 1998; Stevenson *et al.*, 2000).

*Borreliae* contain linear chromosomes of approximately 1 megabase (Fraser *et al.*, 1997; Schwan *et al.*, 1999), reflecting the limited metabolic capacities of these obligately parasitic bacteria (Fulton and Smith, 1960; Kelly, 1971; Wyss and Ermert, 1996). The chromosome of *B. burgdorferi* strain B31 has been sequenced and found to be very compact, with over 93% devoted to open reading frames (ORFs) (Fraser *et al.*, 1997). This tendency toward compactness is exemplified by the finding that a single ORF encodes both the GyrA subunit of DNA gyrase and a smaller protein with HU-like activity (Knight and Samuels, 1999).

All examined *Borrelia* species contain numerous small circular and linear extrachromosomal DNA molecules that, in total, may exceed half the size of the chromosome (*e.g.* Plasterk *et al.*, 1985; Barbour, 1988; Hayes *et al.*, 1988; Perng and LeFebvre, 1990; Xu and Johnson, 1995; Cutler *et al.*, 1997; Hinnebusch *et al.*, 1998; Casjens *et al.*, 2000b). While these additional DNA molecules are generally described as plasmids, many contain genes that are probably essential for bacterial survival in nature. As examples, plasmids carried by *Borrelia* species encode guanine biosynthetic enzymes (Margolis *et al.*, 1994), and proteins synthesized during vertebrate and tick infections (*e.g.*, Barbour and Garon, 1988; Hayes *et al.*, 1988; Barbour, 1990; Marconi *et al.*, 1993a; Sadziene *et al.*, 1993; Skare *et al.*, 1999). But, in striking contrast to the tight restrictions apparently imposed upon the *B. burgdorferi* chromosome, a large proportion of these plasmids are occupied by seemingly redundant genes. Analysis of 21 sequenced plasmids from *B. burgdorferi* strain B31 revealed 107 families of paralogous ORFs, one of which contains 41 identifiable members (Casjens *et al.*, 2000b).

What are the reasons for the paradox of the *B. burgdorferi* genome? Why are these bacteria driven toward a minimalist chromosome, while at the same time maintaining large numbers of extrachromosomal elements that contain apparently superfluous DNA sequences? A closer examination of these plasmids and the proteins they encode provides clues toward understanding benefits they provide the bacteria. In this review, we focus on the most obvious example of sequence repetition found in *Borrelia* species, the cp32 plasmid family.

## The *B. burgdorferi* cp32 Plasmids

*B. burgdorferi* was first described in 1982 (Burgdorfer *et al.*, 1982), and the cp32 plasmids were independently discovered by a number of researchers during the early and mid-1990s. Initially described as "a circular plasmid ubiquitous among the Lyme disease agents" (Amouriaux *et al.*, 1993) and "plasmid associated repeated DNA sequences" (Simpson *et al.*, 1990; Zückert *et al.*, 1994), a picture emerged of a group of related plasmids, many of which could be maintained simultaneously by an individual bacterium. Analyses of restriction fragments led to an

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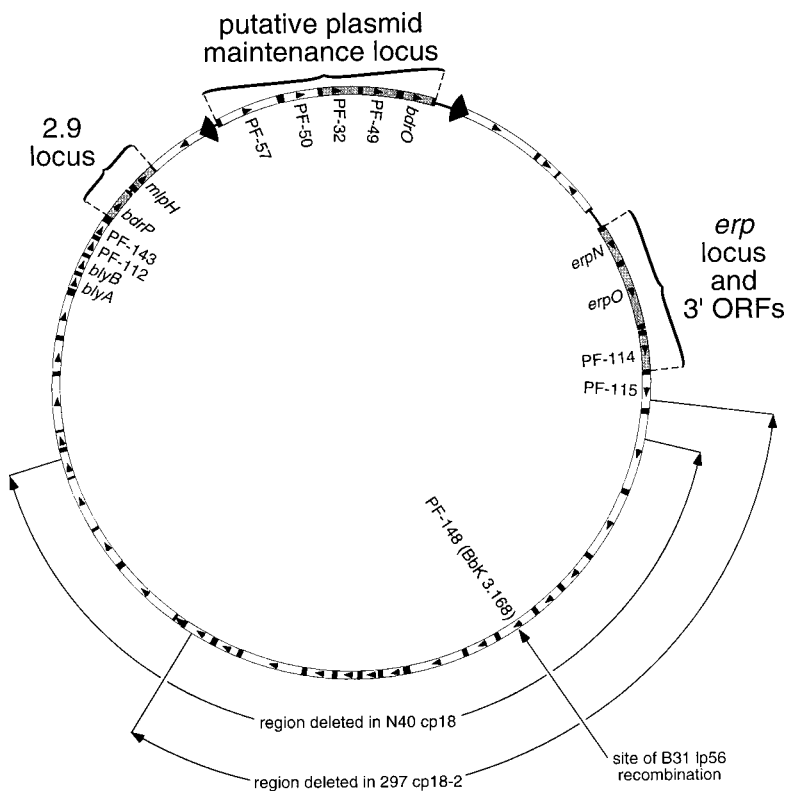


Figure 1. Map of the strain B31 cp32-8, a "typical" plasmid that contains all genes found on the majority of analyzed *B. burgdorferi* cp32s. ORFs are illustrated as boxes, with arrowheads indicating direction of transcription, and those discussed in the text are labeled with the gene name or paralog family (PF) number. Shaded boxes indicate those ORFs that vary significantly among different cp32s. Large arrowheads indicate the locations of the "inverted repeats" that flank the putative maintenance locus.

estimated size of 32 kb (Stevenson *et al.*, 1996; Casjens *et al.*, 1997), hence the name "cp32" (because borreliae can contain different plasmid forms, the prefixes "cp" and "lp" are used to denote circular and linear plasmids, respectively). Recent sequencing of seven such plasmids revealed actual sizes that ranged between 29.8 and 30.9 kb (Casjens *et al.*, 2000b). All analyzed Lyme disease spirochetes contain multiple cp32 plasmids (one clonal *B. burgdorferi* contains nine cp32 family members), and recent studies have demonstrated that other members of the genus also contain multiple cp32 family members (Simpson *et al.*, 1990; Amouriaux *et al.*, 1993; Casjens and Huang, 1993; Lam *et al.*, 1994; Zückert *et al.*, 1994; Akins *et al.*, 1995; Suk *et al.*, 1995; Wallich *et al.*, 1995; Marconi *et al.*, 1996; Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Theisen, 1996; Zückert and Meyer, 1996; Casjens *et al.*, 1997; Stevenson *et al.*, 1997; Sung *et al.*, 1998; Akins *et al.*, 1999; El-Hage *et al.*, 1999; Iyer *et al.*, 1999; Marconi *et al.*, 1999; Yang *et al.*, 1999; Zückert *et al.*, 1999; Caimano *et al.*, 2000; Carlyon *et al.*, 2000a; Carlyon *et al.*, 2000b; Casjens *et al.*, 2000b; Miller *et al.*, 2000; Stevenson *et al.*, 2000).

Early sequencing of plasmid fragments and mapping of restriction endonuclease cleavage sites demonstrated that cp32s contain homologous sequences throughout their lengths (Akins *et al.*, 1995; Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Zückert and Meyer, 1996; Casjens *et al.*, 1997; Stevenson *et al.*, 1997). When the Institute for Genomic Research (TIGR) initially published results from their sequencing of the *B. burgdorferi* B31 genome, the cp32s were not included, since the plasmids' similarities were too extensive for TIGR's computer assembly program to resolve (Fraser *et al.*, 1997). The TIGR Assembler program has since been modified to facilitate resolution of the B31 cp32s, which confirmed the homologies predicted for this

family (Casjens *et al.*, 2000b). Figure 1 illustrates the ORFs and other characteristics of the *B. burgdorferi* B31 cp32-8, a "typical" plasmid that contains all ORFs located on the majority of Lyme disease spirochete cp32s studied to date. Many of the cp32 genes are as yet unnamed, and are referred to according to their assigned paralog family (PF) number (Casjens *et al.*, 2000b). Despite the apparent redundancy of the cp32 plasmids, there are three regions of these plasmids that, although homologous, can differ considerably in their DNA sequences.

### cp32 Hypervariable Region 1: Putative Plasmid Maintenance Genes

Most borrelial plasmids are maintained at approximately one copy per chromosome (Hinnebusch and Barbour, 1992; Kitten and Barbour, 1992; Casjens and Huang, 1993), suggesting the plasmids have mechanisms to ensure efficient segregation to each daughter cell following division. In all studied bacteria, plasmids that utilize the same replication and segregation proteins are incompatible, and cannot be maintained in the same cell (Novick, 1987). Since clonal cultures have been isolated that contain nine cp32 family members (Casjens *et al.*, 2000b), one might well ask how so many homologous plasmids manage to coexist in a single organism?

Almost all sequenced borrelial plasmids contain four adjacent genes from PFs 32, 49, 50, and 57, suggesting that the products of these genes perform essential plasmid functions, such as replication and segregation (Barbour *et al.*, 1996; Stevenson *et al.*, 1998b; Casjens *et al.*, 2000b). PF-32 proteins are homologous to the ATPases of other bacterial plasmids that are required for plasmid segregation (Zückert and Meyer, 1996; Stevenson *et al.*, 1998b). With

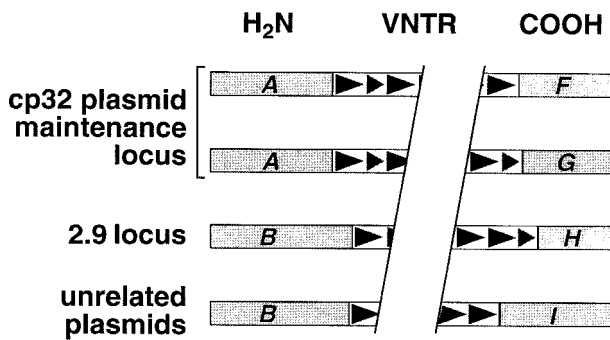


Figure 2. Variation in *B. burgdorferi* *bdr* loci. Paralogs vary in their combinations of N-, and C-terminal domains (A, B, and F, G, H, and I, respectively), which correspond with their respective plasmid loci (cp32 plasmid maintenance and 2.9 loci on members of the cp32 family, and loci on unrelated plasmids). Variation is more pronounced in the cp32 plasmid maintenance locus. Size variation between paralogs is due to the variable numbers of 7- and 11-amino acid repeat units in the central VNTR domain (for a more detailed analysis see Zückert *et al.*, 1999 and Zückert and Barbour, 2000).

one exception, cp32 plasmids of *B. burgdorferi* strain B31 contain PF-32 and PF-49 genes (previously referred to as ORF-C and ORF-3, respectively) (Figure 1) encoding proteins with less than 65% amino acid sequence identity (Stevenson *et al.*, 1998b; Casjens *et al.*, 2000b). If the products of these two genes are involved in plasmid maintenance, then their sequence differences may permit compatibility of the numerous plasmids carried by individual borrelial organisms. Two B31 plasmids, cp32-2 and cp32-7, both encode identical PF-32 and PF-49 proteins, and, consistent with our hypothesis, we have been unable to isolate a clonal population of B31 containing both cp32-2 and cp32-7 (Casjens *et al.*, 1997; Stevenson *et al.*, 1998b). Plasmid content analyses of clonal cultures generated from other isolates, such as the original uncloned 297 strain, will further test this hypothesis.

PCR and Southern blot analyses demonstrated PF-32 / PF-49 gene pairs similar to those of B31 in 13 different *B. burgdorferi* isolated from Lyme disease patients (Iyer *et al.*, 1999), indicating that the cp32s of many different bacteria are closely related at their putative plasmid maintenance loci. Additionally, cp18, a naturally truncated cp32 family member of strain N40, encodes PF-32 and PF-49 proteins identical to those of the B31 cp32-2 and cp32-7 (Stevenson *et al.*, 1997; Stevenson *et al.*, 1998b), as does a plasmid from strain Sh-2-82 (B. Stevenson, unpublished results), so it can be concluded that all four of these plasmids are closely related at this locus. Likewise, a cp32 family member of strain 297, cp18-2, encodes PF-32 and PF-49 proteins nearly identical to those of the B31 cp32-9 (Akins *et al.*, 1999; Caimano *et al.*, 2000), indicating a close relationship between those two plasmids. This widespread conservation suggests that, while all Lyme disease spirochetes probably contain multiple cp32s, there is only a limited number of different cp32s in nature. For this reason, it is recommended that any newly described cp32 plasmid from another strain be named according to the relationship of its PF-32 / PF-49 locus to the defined B31 plasmids. Any plasmids encoding novel PF-32 and PF-49 proteins would be distantly related to the known B31 cp32s, and should be given new names, such as cp32-10,

cp32-11, etc.

The putative cp32 maintenance locus contains a fifth gene, designated *bdr* (*Borrelia* direct repeat, Figures 1 and 2) (Zückert and Meyer, 1996; Zückert *et al.*, 1999). For unknown reasons, almost all cp32s contain a second, different *bdr* gene in another hypervariable region, the 2.9 locus (Porcella *et al.*, 1996; Yang *et al.*, 1999; Zückert *et al.*, 1999; Casjens *et al.*, 2000b), to be discussed later in this review. *Bdr* paralogs are also found on linear plasmids unrelated to the cp32 family, although some of those genes are probably non-functional pseudogenes (Zückert *et al.*, 1999). All *Bdr* proteins have a characteristic central domain consisting of various numbers of tandem 7- and 11-amino acid repeat units (VNTR domain), and thereby vary in size from about 20 to 30 kDa. Another common feature is a variable, yet structurally conserved, C-terminal putative transmembrane domain (Zückert *et al.*, 1999). This domain, as well as a variable N-terminal domain, differentiate three major *Bdr* homology groups, which also correlate with their respective plasmid loci (Figure 2). The variability and plasmid specificity of the paralogs suggests that each might perform a duty specific to its cp32. There are several indications that they are not under selective pressure by mammalian immune systems: the proteins are antigenically crossreactive, appear to be located in the bacterial inner membrane (Zückert *et al.*, 1999), and are concurrently synthesized at constant low levels under all conditions tested so far (Carlyon and Marconi, 1998; Zückert *et al.*, 1999). Furthermore, sequences of *bdr* genes are stable throughout mammalian infection, and during prolonged *in vitro* cultivation (Zückert and Barbour, 2000).

The cp32 putative maintenance locus is flanked by sequences that are nearly identical to each other, and oriented in opposite directions (Figure 1) (Zückert and Meyer, 1996; Casjens *et al.*, 2000b). These "inverted repeats" have been proposed to be insertion sequences (IS) (Dunn *et al.*, 1994; Carlyon *et al.*, 1998), although they lack the target site duplications characteristic of IS elements (Dunn *et al.*, 1994; Zückert and Meyer, 1996). Since the repeated sequences overlap the probable promoters and translation initiation sites of two divergently transcribed genes, it is more likely that these sequences function to coordinate expression of those genes (Dunn *et al.*, 1994; Casjens *et al.*, 2000a; Casjens *et al.*, 2000b).

### cp32 Hypervariable Region 2: the *erp* Locus and 3' ORFs

All cp32 family members contain an *erp* locus (Figure 1), encoding either one or two lipoproteins of unknown function. The *ospEF* operon of *B. burgdorferi* strain N40 was the first locus identified, but since individual bacteria contain many such loci, the general term *erp* has been applied to describe these *OspE/F*-related protein genes. A variety of other names have also been used to describe *erp* genes, including *p21*, *pG*, *elpA*, *elpB*, *bbk2.10*, *bbk2.11* and "upstream homology box" genes (Lam *et al.*, 1994; Akins *et al.*, 1995; Suk *et al.*, 1995; Wallich *et al.*, 1995; Marconi *et al.*, 1996; Akins *et al.*, 1999). There can be significant diversity among the *erp* genes of different isolates, and even within a single strain. Furthermore, recent analyses of known *Erp* proteins (Stevenson *et al.*, 1998b; Akins *et al.*, 1999) revealed that the mature portions of these proteins fall into at least three evolutionarily distinct

families that appear to have arisen from gene fusion events joining a common N-terminus with unrelated sequences. Therefore an alternative nomenclature has been proposed that divides the *erp* genes into three groups: *ospE* (genes similar to N40 *ospE*), *ospF* (those similar to N40 *ospF*), and *elp* (genes unlike either N40 *ospE* or *ospF* but containing *ospEF*-like leader peptide sequences) (Akins *et al.*, 1999). Nevertheless, *erp* loci hold several features in common: (i) they are preceded by nearly identical 5' non-coding regions, which presumably include the transcriptional promoter and regulatory elements, (ii) they encode lipoproteins with similar leader peptide sequences, (iii) some apparently divergent Erp proteins share other sequence motifs, and (iv) Erp proteins are highly charged, containing significant numbers of lysine and glutamate residues that could serve similar functions despite different primary structures (Lam *et al.*, 1994; Akins *et al.*, 1995; Wallich *et al.*, 1995; Marconi *et al.*, 1996; Stevenson *et al.*, 1996; Casjens *et al.*, 1997; Stevenson *et al.*, 1998a; Stevenson *et al.*, 1998b; Sung *et al.*, 1998; Casjens *et al.*, 2000b).

The *erp* genes of only two *B. burgdorferi* strains, North American strains B31 and 297, have been well characterized (Figure 3). Bicistronic *erp* loci are cotranscribed (Stevenson *et al.*, 1998a, and S. Hefty and D. Akins, unpublished results), although there is evidence suggesting that the downstream gene of such operons can also be transcribed independently (Stevenson *et al.*, 1998a). The B31 *erpG* and 297 *bbk2.10* loci each contain an unrelated gene, *bapA* (Akins *et al.*, 1995; Wallich *et al.*, 1995; Stevenson *et al.*, 1996), and northern blot analyses found that the B31 *bapA* is cotranscribed with *erpG* (Stevenson *et al.*, 1998a). The B31 *erpH* gene of cp32-4 was naturally disrupted by insertion of an unrelated gene fragment, and consists of a short ORF containing the signal peptide (*erpH'*), and an in-frame fusion between the inserted DNA and the remainder of *erpH* (ORF::'*erpH*') (Stevenson *et al.*, 1996; Stevenson *et al.*, 1998b; Casjens *et al.*, 2000b). It is unknown whether either of these "genes" is transcribed or translated, but the downstream *erpY* gene does produce a protein (J.A. Carroll and B. Stevenson, unpublished results). The mutation of *erpH* appears to have been a recent occurrence, since none of the *erp* loci of strain 297 contain a similar insertion (Akins *et al.*, 1999).

A recent report named a gene on plasmid lp28-1 *erpT*, based upon short stretches (<15 amino acids) of predicted protein similarity with the B31 ErpD protein (Fikrig *et al.*, 1999). However, since the DNA located immediately 5' of *erpT* bears no homology with the cp32 *erp* promoters, and the predicted ErpT protein lacks the conserved leader polypeptide / lipidation sequences of cp32 Erp proteins, this gene is probably not a member of the *erp* family.

Comparative analyses of *erp* genes and their proteins indicate both extreme diversity and absolute identity among their sequences (Casjens *et al.*, 1997; Stevenson *et al.*, 1998b; Akins *et al.*, 1999; Casjens *et al.*, 2000b). As examples, the B31 *erpA* and *erpB* genes and proteins share 35% and 19% identical nucleotides and amino acids, respectively, but the *erpA*, *erpI* and *erpN* genes are identical to each other, and share greater than 80% identity with *erpC* and *erpP*. The B31 *erpB*, *erpJ* and *erpO* genes are also identical, and are quite similar to *erpD*, *erpM*, *erpQ* and *erpX* (Casjens *et al.*, 1997; Stevenson *et al.*, 1998b; Akins *et al.*, 1999; Casjens *et al.*, 2000b). The *erp* genes

of strain 297 also exhibit similarities and diversity, although no duplicated *erp* loci have been identified (Akins *et al.*, 1999). Interestingly, 100% identity has been observed between *erp* genes of different strains, with the *erpG* gene of the North American strain B31 being identical to the *pG* gene of European strain ZS7 (Wallich *et al.*, 1995; Stevenson *et al.*, 1996).

As noted above, Erp proteins are postulated to be lipoproteins, based on their predicted amino acid sequences. Those proteins examined were all lipidated *in vivo*, either in *B. burgdorferi* (Lam *et al.*, 1994) or as recombinant proteins expressed in *E. coli* (Akins *et al.*, 1995; Wallich *et al.*, 1995). Initial characterization of Erp proteins based on immunofluorescence analysis of fixed bacteria suggested that they are surface exposed (Lam *et al.*, 1994). We have since used several other techniques that confirmed Erp proteins as being located primarily in the outer membrane and exposed to the external environment (Unpublished results from the laboratories of B. Stevenson and D. Akins). However, it appears that not all organisms contain surface-exposed forms of these proteins, since all bacteria of strain 297 synthesize OspE during *in vitro* cultivation, but only a fraction express OspE on their surface (S. Hefty and D. Akins, unpublished results). Therefore, it may be that those organisms without OspE on their surface are able to evade the host's immune response during a natural infection. Further studies are needed to determine if this is a common theme for all of the various Erps, but the above observation is consistent with the fact that none of the Erp proteins tested to date fully protected animals from borrelial infection (Nguyen *et al.*, 1994; Wallich *et al.*, 1995).

Several studies have demonstrated that infected humans and laboratory animals produce antibodies directed against Erp proteins within the first 2-4 weeks of infection, indicative of Erp synthesis during the initial stages of vertebrate infection (Nguyen *et al.*, 1994; Akins *et al.*, 1995; Stevenson *et al.*, 1995; Suk *et al.*, 1995; Wallich *et al.*, 1995; Das *et al.*, 1997; Stevenson *et al.*, 1998a; Miller *et al.*, 2000; Sung *et al.*, 2000). Reverse transcriptase-PCR analyses of *erp* mRNAs also indicated synthesis early in mammalian infection (Das *et al.*, 1997; Anguita *et al.*, 2000). These data indicate that surface-exposed Erp proteins could facilitate interactions with host tissues during the establishment of vertebrate infection.

Many infectious bacteria utilize environmental temperature as a signal to determine their location, synthesizing vector-specific proteins at cool temperatures and mammal-specific proteins in warmer environments (e.g., blood temperature) (Miller *et al.*, 1989). In culture, *B. burgdorferi* increase the synthesis of Erp proteins following a shift in culture temperature from 23°C to 35°C (Stevenson *et al.*, 1995; Akins *et al.*, 1998; Stevenson *et al.*, 1998a), a temperature change similar to that experienced by bacteria within the midgut of a feeding tick. The change of temperature from 23° to 35°C also causes a 3 to 4-fold increase in bacterial growth rate (Stevenson *et al.*, 1995), which in turn may provide internal signals affecting Erp protein production. Temperature and/or growth rate are not the only signal(s) directing Erp protein synthesis, since recent studies found that temperature shift does not alter the Erp protein levels of bacteria grown in some formulations of culture medium (N. El-Hage, K. Babb, J.C. Miller, and B. Stevenson, unpublished results). These and

other data indicate that *B. burgdorferi* regulates synthesis of Erp proteins (and possibly other infection-associated proteins) in response to sensing chemicals present in blood, tick saliva or other tissues encountered during the natural infection cycle (Akins *et al.*, 1995; Suk *et al.*, 1995; Wallich *et al.*, 1995; Das *et al.*, 1997; Akins *et al.*, 1998).

As noted above, all *erp* loci are preceded by nearly identical 5' noncoding regions, suggesting that they are all regulated by the same *trans*-acting factors (K. Babb, N. El-Hage, J. Miller, J. Carroll, and B. Stevenson, unpublished results). Consistent with this prediction, our studies indicate that all *erp* loci are transcribed simultaneously, although not necessarily at identical levels (Stevenson *et al.*, 1998, and S. Hefty and D. Akins, unpublished results). Differences in *erp* promoter regions, perhaps as subtle as single nucleotide changes, might alter affinity for *trans* acting factors, resulting in greater or lesser mRNA levels from some loci. The 3' non-coding sequences of some *erp* loci are also different, which might affect mRNA stability and, thus, cellular protein levels. Additionally, some *erp* genes exhibit evidence of post-transcriptional regulation, with proteins being made only when unknown signals in the mammalian host environment induce translation (S. Hefty and D. Akins, unpublished results). In some bacteria, growth at different temperatures influences DNA supercoiling (Drlica, 1992), which in turn alters gene expression (Pruss and Drlica, 1989), so it is possible that some conditions affecting *erp* expression do so through changes in DNA supercoiling (Samuels and Garon, 1993). There is also evidence that some Erp proteins may be synthesized at different times during the vertebrate-tick infection cycle (Das *et al.*, 1997). Thus many mechanisms may be utilized by this organism to alter expression patterns of these proteins as the spirochete goes through its complex enzootic life cycle. It should be noted that a recent study indicated that the ability of *B. burgdorferi* to regulate gene expression (including *erp* genes) is critical for bacterial dissemination throughout the vertebrate host and for Lyme disease pathogenesis (Anguita *et al.*, 2000).

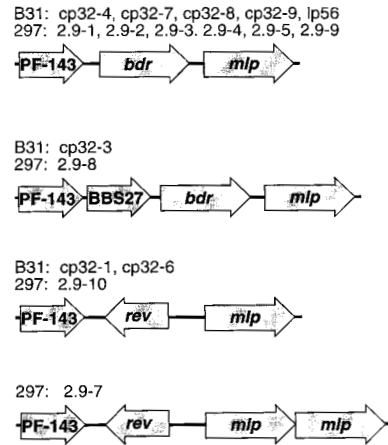


Figure 4. Orientation of genes in the 2.9 loci of *B. burgdorferi* strains B31 and 297.

Extensive diversity among cp32s has also been observed in the region immediately 3' of the *erp* genes (Figures 1 and 3). A well-conserved ORF, PF-114, occupies this space in many cp32 family members (Stevenson *et al.*, 1996; Casjens *et al.*, 1997; Stevenson *et al.*, 1997; Stevenson *et al.*, 1998a; Akins *et al.*, 1999; Casjens *et al.*, 2000b). However, other plasmids lack this ORF, where it is replaced with unrelated sequences that can include ORFs oriented in directions opposite to the *erp* locus (Akins *et al.*, 1995; Wallich *et al.*, 1995; Stevenson *et al.*, 1996; Stevenson *et al.*, 1998a; Akins *et al.*, 1999; Casjens *et al.*, 2000b). In one known example, this region contains a *mip* gene (Caimano *et al.*, 2000), which is normally located elsewhere on cp32 plasmids (see below). Northern blot analyses indicated that, with the exception of those loci containing a *bapA* gene, transcripts terminate short distances beyond the *erp* genes, and so would not contain any of these other downstream ORFs (Stevenson *et al.*,

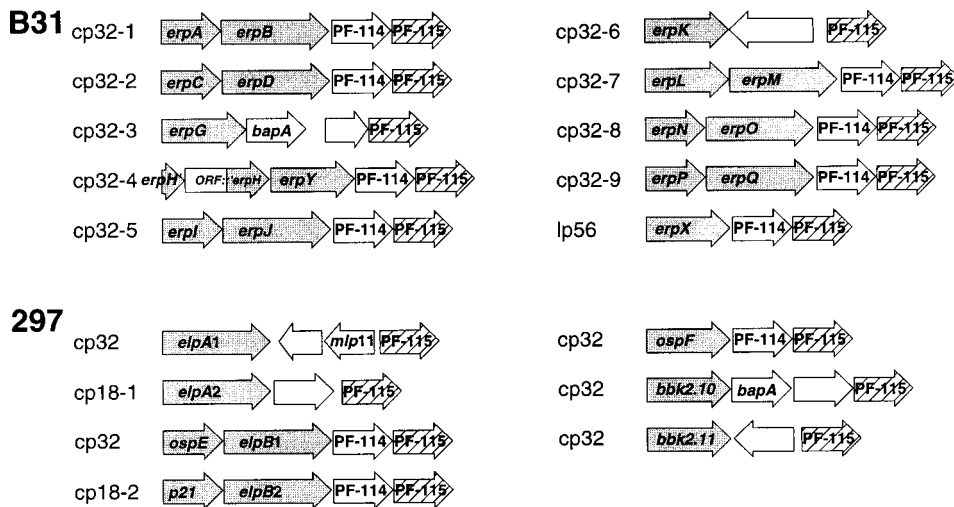


Figure 3. The *erp* loci of *B. burgdorferi* strains B31 and 297, and the plasmids on which each is located. *erp* genes are shaded, and conserved PF-115 genes are hatched. Other hypervariable genes are unshaded, and ORFs without paralogs in the *B. burgdorferi* genome are unnamed. The 297 cp32 plasmids are not numbered here, since the relationships of their PF-32 / PF-49 loci with the defined B31 cp32s are unknown.

1998a). It is not presently known whether any of the 3' ORFs are transcribed or translated, but the diversity of this region suggests that PF-114 is not required for cp32 maintenance or *B. burgdorferi* infectivity.

### cp32 Hypervariable Region 3: the 2.9 Locus

Named after the original recombinant clone analyzed, the 2.9 locus contains a lipoprotein gene, *mlp* (also called *nlpH*), which in most cases occurs as a single gene, although one locus of strain 297 contains two different *mlp* genes (Figures 1 and 4) (Porcella *et al.*, 1996; Theisen, 1996; Akins *et al.*, 1998; Yang *et al.*, 1999). As with the *erp* genes, *mlp* genes often display considerable diversity, but their proteins can be divided into two classes based upon size, hydrophilicity profiles, and antibody crossreactivity. Of the ten known 297 Mlp proteins, five fall into Class I and five into Class II, while of the known B31 Mlp proteins, seven are Class I and only one is Class II (Porcella *et al.*, 1996; Yang *et al.*, 1999; Casjens *et al.*, 2000b). Human Lyme disease patients and infected laboratory animals produce Mlp-directed antibodies within the first 2-4 weeks of infection, indicative of protein synthesis during that time period (Yang *et al.*, 1999). Mlp proteins appear to be located on the outer surface of *B. burgdorferi* (Theisen, 1996), and may thus function to assist interactions with host tissues.

All but two 2.9 loci from both B31 and 297 contain a second *bdr* gene, as described above. The loci without *bdr* genes instead contain an unrelated gene, *rev* (so named because it is oriented in the reverse direction of *mlp*). The two B31 cp32 *rev* genes are identical to each other, whereas the two 297 *rev* gene sequences differ

somewhat from each other and the B31 alleles. Rev proteins are synthesized by *B. burgdorferi* during mammalian infection, since infected humans and laboratory animals produced antibodies directed against this protein (Gilmore and Mbow, 1998). The Rev proteins have not been well studied, but appear to be located on the outer surface of *B. burgdorferi* (N. El-Hage, J.A. Carroll, K. Babb, J.C. Miller, R.D. Gilmore, M.L. Mbow and B. Stevenson, unpublished results). This is consistent with sequence analyses suggesting that Rev proteins contain leader peptides (Porcella *et al.*, 1996) that could be cleaved and result in the transport of Rev proteins to the outer membrane of *B. burgdorferi*.

Two known 2.9 loci, one each from B31 and 297, contain an additional ORF, given the reference nomenclature BBS27 by TIGR (Yang *et al.*, 1999; Casjens *et al.*, 2000b). There is no published information as to whether this ORF is either transcribed or translated by *B. burgdorferi*, and its sequence provides no clues as to possible function.

### Genetic Recombination Leads to cp32 Variation

Using the conserved PF-32 and PF-49 gene pairs as physically linked markers, it was demonstrated that *erp* loci undergo recombination events (Stevenson *et al.*, 1998b). The two B31 cp32 *rev* genes are identical to each other, but are located on plasmids with unrelated PF-32 / PF-49 gene pairs (Casjens *et al.*, 2000b), indicating that the *rev* locus can also undergo recombination. Since the B31 cp32-9 and the cp18-2 of strain 297 are closely related at their putative plasmid maintenance loci (see above), the

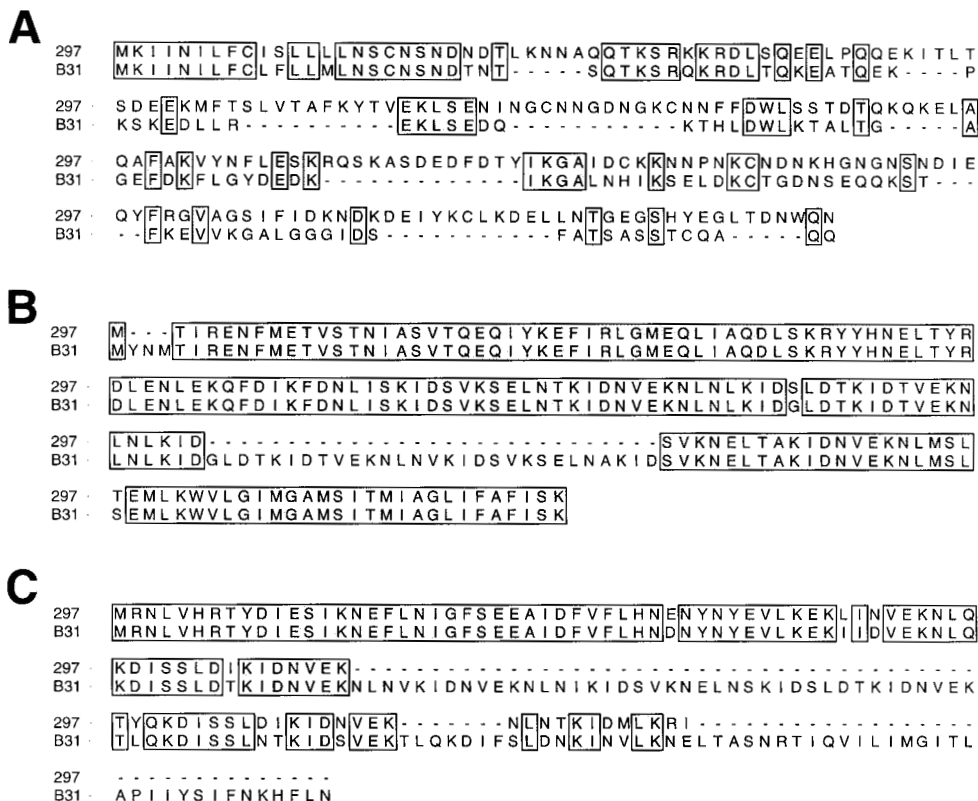


Figure 5. Evidence of past cp32 recombination events. Alignment of Mlp and Bdr predicted proteins encoded by two plasmids with nearly identical PF-32 / PF-49 loci, the 297 cp18-2 and B31 cp32-9 (GenBank accession numbers AF023852 and AF046999 {cp18-2} and AE001581 {cp32-9}). (A) Mlp proteins, (B) 2.9 locus Bdr proteins, and (C) maintenance locus Bdr proteins.

recent sequencing of the 297 cp18-2 (Akins *et al.*, 1999; Caimano *et al.*, 2000) now makes it possible to extend such analyses to the *mlp* and *bdr* genes. Alignment of the 297 cp18-2 and B31 cp32-9 *mlp* genes demonstrated considerable variation among these two genes and their predicted proteins (Figure 5A), consistent with past recombination events. The *bdr* genes show signs of evolution by recombination while maintaining their overall structure, with changes in the VNTR domain consisting of multiples of 7- and/or 11-amino acid repeat units. For example, the 297 cp18-2 and B31 cp32-9 2.9 *bdr* genes are nearly identical, although there is evidence of insertion and/or deletion events, including one of 29 amino acids in the VNTR domain (Figure 5B). Recombinational events are also evident in the different N- and C-terminal domain combinations (see Figure 2). While the *bdr* genes in the 2.9 loci of both B31 and 297 are quite conserved, the *bdr* sequences in the plasmid maintenance loci are more heterogeneous (Zückert *et al.*, 1999), suggesting a higher frequency of recombination that may have deleterious effects. For example, recombination within the 297 cp18-2 *bdr* paralog lead to a truncated, likely defective protein that lacks a C-terminal putative transmembrane domain (Figure 5C) (Zückert *et al.*, 1999; Caimano *et al.*, 2000). A similar truncation is also evident in the *bdrG* gene located on the B31 cp32-4 (Zückert *et al.*, 1999).

### cp32 Constant Regions

Other than in the three hypervariable regions just described, the cp32 plasmids are highly conserved (Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Zückert and Meyer, 1996; Casjens *et al.*, 1997; Stevenson *et al.*, 1997; Stevenson *et al.*, 1998b; Akins *et al.*, 1999; Caimano *et al.*, 2000; Casjens *et al.*, 2000b). Few studies have focused on these conserved regions, so little is known of the included ORFs or their putative proteins.

Two small ORFs located 5' of the 2.9 locus, *blyA* and *blyB*, were postulated to be hemolysins (Guina and Oliver, 1997). Further investigation indicated that the proteins are not actually hemolytic, but may be involved in the cell lysis step of *B. burgdorferi* bacteriophage production (C. Damman and D. Oliver, personal communication).

Outer surface lipoproteins and secreted proteins are of special interest to many researchers, due to their potential roles in the interactions between the bacteria and their hosts, as well as for their potential utility as novel serodiagnostics and vaccinogens for Lyme disease. Two small genes immediately 5' of the 2.9 locus, PF-112 and PF-143, probably encode lipoproteins, based on predicted protein sequences (Porcella *et al.*, 1996; Caimano *et al.*, 2000; Casjens *et al.*, 2000b), and a PF-143::PhoA fusion was processed when expressed as a recombinant protein in *E. coli* (Porcella *et al.*, 1996). Recombinant product of a conserved PF-148 cp32 gene, identified as clone BbK3.168 (GenBank accession number L31425), was localized to the periplasm of *E. coli*; however, further studies have not been performed to characterize this protein in *B. burgdorferi* (D. Akins and J. Radolf, unpublished results).

### Variant *B. burgdorferi* cp32 Family Members

Some *B. burgdorferi* strains, such as N40 and 297, contain circular cp32 family members with extensive deletions,

named cp18s for their approximate sizes (Figure 1) (Simpson *et al.*, 1990; Casjens and Huang, 1993; Zückert *et al.*, 1994; Porcella *et al.*, 1996; Zückert and Meyer, 1996; Casjens *et al.*, 1997; Stevenson *et al.*, 1997; Akins *et al.*, 1999; Caimano *et al.*, 2000). The N40 cp18 has an estimated size of 18.3 kb, and based on PF-32 / PF-49 similarities probably arose from a cp32-2/cp32-7-like plasmid via a single deletion event (Stevenson *et al.*, 1997; Stevenson *et al.*, 1998b). The 297 cp18-2 was recently sequenced and contains 21,170 bp (Caimano *et al.*, 2000), and presumably evolved from a cp32-9-like plasmid. These relationships indicate the potential for smaller plasmids to develop independently from different cp32s, as is also evident from the different cp32 regions deleted to produce these two plasmids (Figure 1). All cp32 variants identified to date contain 2.9, *erp*, and putative plasmid maintenance loci, suggesting the proteins encoded by those genes are important for either the borrelial life cycle or plasmid maintenance (Stevenson *et al.*, 1997; Akins *et al.*, 1999; Caimano *et al.*, 2000; Casjens *et al.*, 2000b). The mutations that produced the two characterized cp18s further indicate that a large portion of the cp32 plasmids is unnecessary for plasmid function, although it is possible that some required gene products are supplied *in trans* from complete cp32s within the same bacterial cell.

A small plasmid of European strain Ip21, cp8.3, contains ORFs with considerable similarity to those of the cp32s (Dunn *et al.*, 1994), and so this plasmid may have evolved from a cp32 via several deletion and inversion mutations (Casjens *et al.*, 2000b). Strain B31 contains two 9 kb plasmids, cp9-1 and cp9-2, that appear to be close relatives of cp8.3 (Champion, *et al.*, 1994; Fraser *et al.*, 1997; and J.C. Miller, J.L. Bono, N. El-Hage, K. Babb, S. Casjens, and B. Stevenson, unpublished results), and similarly sized circular plasmids from other isolates hybridized with cp32-derived probes (Simpson *et al.*, 1990; Zückert *et al.*, 1994; Porcella *et al.*, 1996; Zückert and Meyer, 1996; Casjens *et al.*, 1997). The relationship between cp32s and these smaller plasmids is further strengthened by the fact that the B31 cp9-1 contains paralogs of *bapA* and *rev* (Champion *et al.*, 1994; Fraser *et al.*, 1997).

Other Lyme disease spirochetes contain linear cp32 family members, with sizes of approximately 50 to 56 kb (Simpson *et al.*, 1990; Zückert *et al.*, 1994; Zückert and Meyer, 1996; Casjens *et al.*, 1997; Palmer *et al.*, 2000). The best characterized of these, the B31 Ip56, is approximately 53 kb in size (the sequence of one telomere remains undetermined), and contains an entire, linearized cp32 (Casjens *et al.*, 2000b). The ancestral cp32 apparently recombined with an unrelated linear plasmid, with the cp32 recombination site coincidentally located in the PF-148 gene identified in clone BbK3.168 (Figure 1). The two parts of PF-148 in the B31 Ip56 do not contain any deletions or frame shift mutations, suggesting that this was a relatively recent recombination event (Casjens *et al.*, 2000b). Linear cp32 family members of other *B. burgdorferi* isolates have not been studied in detail, so it is not known to what degree such plasmids resemble the B31 Ip56.

The B31 Ip54 plasmid, which contains the *ospAB* and *dbpAB* loci, also shares a large number of ORFs with the cp32s, and probably arose from fusion between a cp32 and a linear plasmid, similar to the history proposed for Ip56 (Casjens *et al.*, 2000b). Many differences are apparent

when comparing lp54 with cp32s, so the recombination event probably happened at a fairly distant time in the evolution of the strain B31 genome.

### cp32s in Non-Lyme Disease *Borrelia* Species

Most, if not all, other *Borrelia* species also contain cp32-related plasmids. Orthologs of *bdr* have been cloned from *B. turicatae* and *B. hermsii*, and probes based on these sequences and on other *B. hermsii* cp32 ORFs hybridized with DNAs from *B. parkeri* and *B. anserina* (Carlyon and Marconi, 1998; Carlyon *et al.*, 2000a; Carlyon *et al.*, 2000b; Roberts *et al.*, 2000; Stevenson *et al.*, 2000)

Analysis of a recombinant library of *B. hermsii* circular plasmid fragments enabled assembly of a composite circular DNA sequence homologous to the *B. burgdorferi* cp32s (Stevenson *et al.*, 2000). The assembled *B. hermsii* cp32 had a size of 30.3 kb, similar to the *B. burgdorferi* B31 cp32 plasmids. Several different overlapping cp32 fragments were identified, indicating that these bacteria also contain multiple plasmids of this family. The *B. hermsii* plasmids encode orthologs of most *B. burgdorferi* cp32 proteins, including Mlp and Bdr, and, as with *B. burgdorferi*, the relapsing fever Mlp orthologs are synthesized during mammalian infection (Stevenson *et al.*, 2000). Genes homologous to the *erp* family were not detected in this relapsing fever *Borrelia*, suggesting that Erp proteins perform functions specific to the Lyme disease-associated spirochetes.

While Lyme disease borreliae are generally difficult to observe during mammalian infection, relapsing fever bacteria replicate to high levels in the bloodstream (Schwan *et al.*, 1999). Thus it should be possible to use bacteria such as *B. hermsii* in future studies to analyze the synthesis and function of their cp32-encoded proteins during vertebrate infection.

### Models Explaining cp32 Repetition, Conservation, and Variation

Erp, Mlp, Bdr, Rev, and perhaps other cp32-encoded proteins, are synthesized during mammalian infection, and presumably perform necessary functions for the bacteria during that time. But why do *B. burgdorferi* and, apparently, all other species of the genus contain multiple cp32 plasmids, with large stretches of seemingly redundant DNA interspersed with regions of variability? If these plasmids serve functions for the bacteria that harbor them, should not one version of a cp32 be sufficient? Since bacterial plasmids commonly encode proteins that confer adaptations to specific local conditions (Eberhard, 1989), it is likely that each *B. burgdorferi* cp32 provides benefits to the bacterium. In the following section, we discuss several models that attempt to explain the presence of multiple cp32s and other repetitive DNAs found in *B. burgdorferi* and other members of this genus.

### Conservation is Required for Bacteriophage Function?

The seven sequenced B31 cp32 plasmids all have sizes between 29.8 and 30.9 kb, and all other Lyme disease-associated borreliae also contain cp32 family members within this approximate size range. Several years ago, it was suggested that the apparently tight size constraint could be due to their being prophages, since a

bacteriophage particle will have an optimal packaging size (Casjens *et al.*, 1997). Recently, bacteriophage-like particles associated with *B. burgdorferi* cultures were demonstrated to contain cp32 DNA molecules (Eggers and Samuels, 1999), indicating that the phage model may be correct. Based on studies of PF-32 and PF-49 relatedness, it appears that there are a finite number of *B. burgdorferi* cp32s, and that most bacteria contain one of each type (see above). Transfer of cp32 DNA via phage particles could be one mechanism utilized to ensure that all bacteria contain a full repertoire of these plasmids. Additionally, if the bacteriophage model holds true, phages may be exploitable for development of recombinant genetic tools for *Borrelia* species.

If cp32s are indeed prophages, then the widespread sequence conservation among these plasmids is explained by their need to synthesize structural and assembly proteins required for phage particle production. Some cp32s, such as the B31 cp32-9, contain numerous point mutations that may render them unable to produce phage particles. Alternatively, this plasmid may parasitize other cp32s for the products of those defective genes. It is also possible that smaller variant plasmids, such as the cp18s or cp9s, could usurp cp32 phage proteins for their packaging, similar to the P4 bacteriophage of *E. coli* (Six and Klug, 1973). Analysis of borrelial bacteriophages is only just beginning, but the ability of the B31 cp32-9 and other mutated DNAs to be packaged into phage particles is an obvious question to be addressed in future studies.

### Variation Ensures Plasmid Compatibility?

The PF-32, PF-49, PF-50 and PF-57 gene products are likely to be involved with plasmid maintenance, with differences in PF-32 and PF-49 possibly enabling compatibility of the many cp32s in each bacterium. As noted above, the B31 cp32-2 and cp32-7 encode identical PF-32 and PF-49 proteins, suggesting that these two plasmids may be incompatible. Recombinant genetic tools for *B. burgdorferi*, including selectable markers, are rapidly being developed (Rosa *et al.*, 1999; Bono *et al.*, 2000), so issues regarding borrelial plasmid compatibility will soon be testable.

### Variation Allows Persistent Vertebrate Infection?

Relapsing fever borreliae contain multiple copies of genes encoding surface proteins designated variable membrane proteins (VMPs) (Plasterk *et al.*, 1985; Barbour, 1990; Pennington *et al.*, 1999). Each bacterium contains a single VMP-encoding gene in an expression locus on a linear plasmid, and a large number of promoterless, silent VMP genes at other plasmid loci. During vertebrate infection, VMP-encoding genes continually recombine into the expression locus, and thus bacteria constantly produce new VMPs that are unrecognized by the host immune system, allowing for persistent infection. Lyme disease spirochetes contain a similar genetic system, wherein silent *vls* (VMP-like sequence) genes recombine with the *vlsE* expression locus to generate bacteria with variant VIs surface proteins (Zhang *et al.*, 1997).

The multiplicity of cp32s could also facilitate rearrangement of antigen-encoding genes during vertebrate infection to avoid recognition by the host's immune system, although there is evidence arguing against this hypothesis. Related cp32 genes contain virtually

identical promoters, and most genes examined are expressed simultaneously, suggesting that there are no silent cp32 loci. The sheer number of *erp*, *mlp*, *rev* and *bdr* genes contained by individual bacteria makes immune evasion an unlikely reason for their recombination, since all those genes would need to constantly rearrange into novel sequences throughout infection. Strain B31 contains three identical *erp* loci and two identical *rev* loci (Stevenson *et al.*, 1998a; Casjens *et al.*, 2000b), indicating a tendency toward genetic similarity, contrary to the predictions of this hypothesis. *Bdr* proteins are located in the bacterial inner membrane, where they should be protected from host antibodies and would not be expected to vary in sequence (Zückert *et al.*, 1999).

Yet variation of cp32 genes can occur during vertebrate infection: in a recent study, some *B. burgdorferi* reisolated from mice infected with a clonal culture encoded *Erp* proteins that were antigenically different from the inoculant bacteria (Sung *et al.*, 2000). But *erp* genetic variation is clearly not essential for persistent infection, since reisolated bacteria in the above study, and in another similar study, contained *erp* genes identical to the inoculant (El-Hage *et al.*, 1999; Sung *et al.*, 2000). It was likewise demonstrated that *bdr* genes also remain stable during persistent mammalian infection (Zückert and Barbour, 2000). Additionally, *erp*, *rev*, *mlp* and *bdr* genes from many different cultures of strain B31 have been sequenced, some of which were independently passaged through numerous laboratory mice, and only rarely have sequence differences been observed (Stevenson *et al.*, 1996; Zückert and Meyer, 1996; Casjens *et al.*, 1997; Stevenson *et al.*, 1998a; Casjens *et al.*, 2000b; Sung *et al.*, 2000). These data indicate that immune system evasion during persistent infection, although a possible reason for some heterogeneity, is probably not a major driving force behind cp32 genetic variation.

#### Variation Permits Vertebrate Re-Infection?

We noted above that mammals produce antibodies against cp32-encoded proteins during infection. Laboratory animals that have been cured of infection are generally resistant to reinfection by that same strain of bacteria, yet cured animals are often susceptible to infection by heterologous strains, so genetic variation might permit reinfection of previously exposed vertebrate hosts (Piesman *et al.*, 1997; Barthold, 1999). This selective force may play some part in variation of cp32 genes, but it cannot be the only evolutionary pressure. As above, the multiplicity of these genes in individual bacteria would require that numerous changes occur to develop bacteria with unique antigenic properties. The presence of multiple identical *erp* and *rev* loci in B31, and the similarities of some *erp* genes in different strains (Wallich *et al.*, 1995; Stevenson *et al.*, 1996; Marconi *et al.*, 1999) also argues against this hypothesis. Additionally, antibodies directed against tested *Erp* proteins were not very protective (Nguyen *et al.*, 1994; Wallich *et al.*, 1995), suggesting that variation is unlikely to greatly influence a bacterium's ability to infect previously infected hosts.

#### Variation Permits Broad Vertebrate Host Ranges?

Several of the cp32-encoded proteins are surface-exposed and are synthesized during vertebrate infection, and thus might function to interact with host tissues. Many infectious bacteria have limited host ranges, due to their encoding

proteins capable of interacting with tissues of only certain hosts (*e.g.* Eberhard, 1989; Bäumlner *et al.*, 1998). However, for survival in nature, Lyme disease-associated borreliae must be able to infect many different kinds of vertebrate hosts, since many of their tick vectors will feed upon a variety of mammals and birds (*e.g.* Brown and Lane, 1992; Olsen *et al.*, 1993; Hubalek *et al.*, 1998; Humair *et al.*, 1998; Oliver *et al.*, 1999). Since an infected tick feeds only a limited number of times during its life, a bacterium must be able to seize each opportunity for transmission and, ultimately, survival of its lineage. By encoding multiple alleles of infection-associated proteins, each capable of interacting with different potential hosts, there will be an increased chance of efficiently infecting whichever vertebrate the tick vector feeds upon. Therefore, *B. burgdorferi* may contain multiple cp32s to increase the range of mammalian and avian hosts it can infect.

This hypothesis can also explain variations among other borrelial genes. For example, Lyme disease spirochetes contain a single gene for the vertebrate infection-associated *OspC* protein, and *ospC* gene sequences often vary widely among different bacteria. While this variation may permit infection of previously exposed hosts (Piesman *et al.*, 1997; Hofmeister *et al.*, 1999), there are also indications that it can contribute to host specificity. Bacteria isolated from ticks can show extensive diversity in their *ospC* genes (Wang *et al.*, 1999), yet only a few *ospC* variants tend to be isolated from humans with Lyme disease (Seinost *et al.*, 1999). These data suggest that certain *OspC* proteins enable more efficient human infection than do others. Other genetic differences have been noted when comparing Lyme disease spirochetes isolated from humans and ticks (Picken *et al.*, 1996; Mathiesen *et al.*, 1997).

Additionally, certain types of these bacteria tend to be isolated from patients with different clinical manifestations of Lyme disease, suggesting that genetic differences influence a bacterium's ability to infect various tissues (van Dam *et al.*, 1993; Anthonissen *et al.*, 1994; Wienecke *et al.*, 1994; Balmelli and Piffaretti, 1995; Liveris *et al.*, 1996; Eiffert *et al.*, 1998; Ryffel *et al.*, 1999; Wormser *et al.*, 1999). This is not limited to only Lyme disease borreliae, since in *B. turicatae* the sequence of the expressed VMP surface protein apparently determines the course of mammalian infection (Pennington *et al.*, 1997; Pennington *et al.*, 1999).

The hypothesis that multiple, paralogous genes confer a broad host range can also be used to explain many of the other plasmids found in *B. burgdorferi*. For example, the gene for P35, a protein associated with mammalian infection, was mapped to plasmid lp54 (Gilmore *et al.*, 1997), but strain B31 contains over a dozen paralogs of this gene on several other plasmids (Casjens *et al.*, 2000b), each of which might confer an advantageous interaction with a different vertebrate host. Sequence analyses of the cp32s and other plasmids of human isolates may identify a common subset of proteins that preferentially assist in human infections, and could direct studies toward improved prophylactic, diagnostic and therapeutic tools.

#### Concluding Remarks

*B. burgdorferi* and all other members of the genus *Borrelia* contain numerous extrachromosomal DNAs, some of which encode proteins associated with host infection that can be

selectively regulated by temperature increase or other mammalian host signals. Among these many plasmids are members of the cp32 family, and individual bacteria have been identified that contain up to nine different cp32s. The cp32 plasmids, although similar throughout much of their sequence, harbor three regions that are hypervariable among plasmids. One hypervariable region appears to encode partitioning proteins that allow for compatibility of several different cp32 plasmids in a single organism. The other hypervariable loci (*erp* and 2.9) encode lipoproteins, some of which are surface-exposed, that are possibly integral to mammalian infection and Lyme disease pathogenesis. Although the variation observed in the lipoproteins encoded by the *erp* and 2.9 loci does not appear to be essential for escaping immune system recognition, further studies are needed to fully determine the role(s) of these lipoproteins in *B. burgdorferi* immune evasion and persistent mammalian infection. Among other possible explanations for the diversity seen in the lipoproteins encoded on these hypervariable loci, long-term vertebrate infection/re-infection and expansion of the borrelial host range must also be considered. The observation that cp32 plasmids contain many well-conserved ORFs may be explained by the recent finding that they may be prophages, an intriguing discovery that could lead to new tools for genetic manipulation of *Borrelia* species. Finally, many questions remain as to how the cp32 genes and their encoded proteins relate to *Borrelia* species physiology, virulence, and disease pathogenesis, in addition to the relationships of the cp32-related plasmids found in so many different *Borrelia* species, making this an area that deserves much attention.

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