

Stability of *Borrelia burgdorferi* *bdr* Loci In Vitro and In Vivo

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The Lyme disease spirochete *Borrelia burgdorferi* expresses diverse subsurface yet antigenically cross-reactive Bdr protein paralogs from distinct circular- and linear-plasmid loci. We assessed the possible effects of in vitro and in vivo growth on *bdr* locus structure, searching for recombinational events leading to either deletions or insertions of central repeat units or novel amino- and carboxy-terminus combinations. Our data indicate that, apart from plasmid loss during in vitro cultivation, the *bdr* paralog loci of strain B31 are stable. This suggests that recombinatorial variation of *bdr* genes is not essential for persistent mammalian infection.

The genomic sequence of the spirochete *Borrelia burgdorferi*, the etiologic agent of Lyme disease, revealed a host of paralogous gene families (9, 13). In a previous study, we characterized the Bdr (*Borrelia* direct repeat) protein family (paralogous gene family 80 [9, 13, 40]). *bdr* paralogs are carried on multiple circular plasmids (cp's) and linear plasmids (lp's) of *Borrelia burgdorferi* sensu lato isolates (1, 2, 9, 13, 20, 29, 31, 39; see also the *Borrelia burgdorferi* Genome Database at <http://www.tigr.org>). Orthologs have been found on lp's and cp's of the causative agents of relapsing fever *Borrelia hermsii* (B. Stevenson, personal communication) and *Borrelia turicatae* (8).

B. burgdorferi Bdr proteins are expressed at low levels in a temperature-independent manner and are not surface exposed. Yet they are recognized by the humoral immune response during Lyme disease (40). Protein sizes range from approximately 20 to 30 kDa, depending on the number of short direct repeats in a central "variable number of tandem repeat" (VNTR) (17, 33) domain. Different combinations of N and C termini of Bdrs carried by distinct plasmid loci yield several distinct homology groups. Nevertheless, the proteins give rise to cross-reacting antibodies, likely due to the more conserved VNTR domains. We were therefore able to detect Bdr proteins in a variety of *B. burgdorferi* sensu lato isolates as well as *B. turicatae* and *B. hermsii* using an antibody against *B. burgdorferi* B31 BdrA. Each of the isolates had a specific reactive protein pattern (40).

On the multiple 32-kb circular plasmids (cp32's) and the related 56-kb linear plasmid lp56 of *B. burgdorferi* strain B31, *bdr* paralogs are in two distinct loci (2.9-like [20] and ORF-1-2-C-3-E [10, 38, 39]) separated by approximately 5 kb. Single *bdr* loci are on lp28-1, lp28-2, lp28-3, lp36, and lp38, some of which carry *bdr* pseudogenes unlikely to encode functional proteins (9, 13). There is evidence that the different paralog loci have undergone recombination in the past. (i) In two of the 2.9-like cp32 loci, on cp32-1 and cp32-6, a *bdr* paralog has been replaced by an unrelated *rev* paralog (20). (ii) The varying numbers of repeats in the *bdr* VNTR domains appear to indicate that the different paralogs have evolved through deletions and/or insertions of repeat units. This could be the result of recombination between multiple loci or of slipped-strand mispairing in combination with inadequate DNA mismatch re-

pair pathways (33). (iii) Different combinations of N- and C-terminal domains suggest recent recombination events in the VNTR domain as well (40). However, it is unknown whether these rearrangements are the result of recent recombinatorial events and whether they occur in vivo. To address these questions, we analyzed the effect of prolonged in vitro or in vivo growth on the *bdr* loci. The assays were designed to detect (i) recombinations within the *bdr* VNTR domains leading to insertions and/or deletions of repeat units and/or novel N- and C-terminus combinations and (ii) variations in *bdr*-flanking regions. The results indicate that, in the absence of plasmid loss, *bdr* loci are stable during both in vitro and in vivo growth.

In vitro and in vivo growth of *B. burgdorferi* B31 strains. Three *B. burgdorferi* strain B31 clones were used. B31-ATCC and B313 are in vitro-passaged clones and have been analyzed in earlier studies (23, 40). Frozen stocks of B31-5A3, a low-passage-number infectious clone (18), and B31-5A3-derived mouse isolates were a gift from S. J. Norris (University of Texas Medical School, Houston). These isolates had been produced in the course of previous studies by Zhang et al. (35–37) and had revealed extensive segmental recombinations in the *vlsE* locus on lp28-1 during in vivo growth. Briefly, eight immunocompetent C3H/HeN mice were infected with B31-5A3, which had previously undergone no more than three in vitro passages since cloning. One year postinfection, samples of heart, bladder, and skin were inoculated into BSKII medium (4), the *B. burgdorferi* cultures were grown at 34°C, and aliquots were frozen in 15% glycerol at –70°C. A total of 17 isolates were obtained: 8 heart isolates (5A3-1487 to 5A3-1494), 7 bladder isolates (5A3-1495 to 5A3-1501) and 2 skin isolates (5A3-1502 and 5A3-1503) (35–37). After thawing, the *B. burgdorferi* isolates were passaged once in BSKII at 34°C and total bacterial DNA was isolated using the DNeasy tissue kit (Qiagen).

Computer predictions of PCR amplicon and restriction fragment length polymorphism (RFLP) fragment sizes. *B. burgdorferi* B31 DNA sequences were downloaded from the Institute for Genomic Research *B. burgdorferi* genome server (<http://www.tigr.org>). Based on alignments of *bdr* paralog sequences using ClustalX version 1.62b (32), degenerate and compatible PCR primers were designed with the help of the OLIGO primer analysis software version 4.04 (National Biosciences, Inc.) (22) and amplicon sizes were calculated (Table 1). Restriction endonuclease fragments of individual circular and linear plasmids (Table 1) were predicted using the MacDNASIS Pro program suite version 3.7 (Hitachi Software Engineering).

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TABLE 1. Predicted sizes of *B. burgdorferi* B31 *bdr* paralog PCR amplicons and Southern blot restriction fragments

| Paralog | Locus | Locus presence in B31 clone ^a | | | PCR primer ^b | | PCR amplicon size (bp) | <i>Xba</i> I restriction fragment size (bp) |
|----------------------------|----------|--|------|------|-------------------------|----------------|------------------------|---|
| | | 5A3 | ATCC | B313 | Fwd | Rev | | |
| <i>bdrK</i> | cp32-6 | + | – | – | A | F | 571 | 8,650 |
| <i>bdrC/M</i> ^c | cp32-2/7 | + | + | + | A | F | 475 | 3,048 |
| <i>bdrQ</i> | cp32-9 | + | + | – | A | F | 442 | 9,368 |
| <i>bdrG</i> | cp32-4 | + | + | + | A | F | 289 | 3,074 |
| <i>bdrA</i> | cp32-1 | + | + | + | A | G | 519 | 5,788 |
| <i>bdrE</i> | cp32-3 | – | + | + | A | G | 477 | 8,499 |
| <i>bdrO</i> | cp32-8 | + | – | – | A | G | 465 | 5,946 |
| <i>bdrV</i> | lp56 | – | – | – | A | G | 423 | 7,927 |
| <i>bdrW</i> | lp56 | – | – | – | B | H | 581 | 9,869 |
| <i>bdrF</i> | cp32-3 | – | + | + | B | H | 494 | 5,257 |
| <i>bdrD/N</i> ^c | cp32-2/7 | + | + | + | B | H | 473 | 4,762 |
| <i>bdrP</i> | cp32-8 | + | – | – | B | H | 440 | 4,743 |
| <i>bdrR</i> | cp32-9 | + | + | – | B | H | 440 | 4,717 |
| <i>bdrH</i> | cp32-4 | + | + | + | B | H | 386 | 4,659 |
| <i>bdrT</i> | lp28-2 | – | + | – | B | I | 665 | 4,165 |
| <i>bdrU</i> | lp28-3 | + | + | – | B | I | 534 | 8,841 |
| <i>bdrS</i> ^d | lp28-1 | + | + | – | B | I ^d | 450 ^d | 1,422 |
| <i>bdrI</i> ^e | cp32-5 | ND ^f | + | – | ND | ND | ND | ND |
| <i>bdrJ</i> ^e | cp32-5 | ND | + | – | ND | ND | ND | ND |

^a +, presence; –, absence. For B31-ATCC and B313, plasmid profiles were determined independently in a previous study using plasmid-specific probes and PCR primers (40). The plasmid profile of B31-5A3 was deduced from the observed *bdr* PCR amplicon sizes.

^b As described in the legend to Fig. 1A.

^c B31-ATCC and B313 carry cp32-2 and not cp32-7 (40). *bdrC* and *bdrD* sequences are not in the database, since cp32-2 has not been sequenced in its entirety. The available sequences, however, indicate that cp32-2 and cp32-7 are almost identical (and in fact may be incompatible [28]). We therefore assume that their *bdr* paralogs are very similar if not identical.

^d *bdrS* is considered a nonfunctional pseudogene, since it does not possess a consensus Shine-Dalgarno ribosomal binding site upstream of the start codon (9, 13, 40). *bdrS* and *bdrY*, a pseudogene on lp38, were not considered at the point of primer design. Although primer B-fwd is complementary to the *bdrS* locus, I-rev is complementary for only 7 bases at the 3' end, making PCR amplification unlikely under the conditions used. *bdrS* is, however, detected by Southern hybridization (Fig. 2). *bdrY* and a distant relative of the *bdr* family, *bdrX* on lp36, are neither amplified by PCR nor detected by Southern hybridizations.

^e *bdrM* and *bdrN* sequences are not in the database, since cp32-5 was not sequenced in its entirety. Amplicon and fragment sizes can therefore not be predicted. However, PCR amplicons and RFLP fragments likely corresponding to the cp32-5 paralogs can be seen in B31-ATCC (see the text and Fig. 1B and 2).

^f ND, not done.

Stability of the *bdr* VNTR domain. We assessed possible variations in the VNTR domains by multiplex PCR amplification of *bdr* paralogs followed by separation on high-resolution polyacrylamide gels. As mentioned above, Bdr protein sequences differ mainly in their N and C termini, which has led to their division into several homology groups. *B. burgdorferi* B31 carries *bdr* paralogs which belong to homology groups I (N- and C-terminal group A and groups F and G, respectively), II (B and H), and III (B and I) (40). Oligonucleotide primers specific for N- and C-terminal groups A and B and groups F, G, H, and I were designed for the separate or combined amplification of *B. burgdorferi* B31 *bdr* homology groups I, II, and III (see above) (Fig. 1A). *bdr* paralogs were amplified from total bacterial DNA using *Taq* polymerase (Boehringer Mannheim). PCR conditions in a Hybaid DNA thermal cycler consisted of 30 cycles of 94°C for 15 s, 49°C for 30 s, and 72°C for 45 s. Amplification products were separated in a nondenaturing 5% polyacrylamide gel using 1× Tris-borate-EDTA (TBE) as running buffer and visualized with ethidium bromide (3). This allowed separation of fragments which differ by less than 20 bp, thereby permitting the detection of possible differences in the number of short (21-bp) or long (33-bp) direct repeat units.

Plasmid loss during in vitro passage has been observed repeatedly (6, 16, 18, 19, 24, 34) and has led to different plasmid profiles of the three B31 clones used in this work (Table 1). As Fig. 1B shows, there are differences in the amplicon pattern between B31-5A3, B31-ATCC, and B313. Yet the product sizes match those of the expected *bdr* paralogs on plasmids carried by the individual clone. For example, compared to

B31-ATCC, B313 lacks lp28-2 (23) and thus the *bdrT* amplicon. Please note that the unsequenced cp32-5 paralogs *bdrI* and *bdrJ* likely account for two of the additional amplicons seen with B31-ATCC, e.g., the approximately 620-bp B-fwd/H-rev/I-rev product. In contrast, amplicon patterns of the mouse tissue isolates after 1 year of infection were identical to those of the original B31-5A3 clone used for infection (Fig. 1C). This indicates that the *bdr* VNTR domains are stable after even prolonged in vivo growth.

Assaying all isolates by PCR using B-fwd, F-rev, and G-rev primers, we were also unable to detect intraplasmidic recombinations of the *bdr* paralogs carried by the two cp32 loci (not shown). This kind of recombinational event would likely be subject to negative selection: it would delete the putative plasmid replication-partitioning gene locus (ORF-1-2-C-3) (10, 28, 38, 39) and might lead to plasmid loss.

Stability of flanking regions. To assess possible variations in *bdr*-flanking regions we used an RFLP-based approach. Total bacterial DNA was digested separately with *Hind*III and *Xba*I, and the resulting fragments were separated on 0.7 to 1.0% agarose gels in 0.5× TBE (3). Subsequently, the DNA was transferred to positively charged nylon membranes (Immobilon-Ny+; Millipore) by alkaline transfer (21). *bdr* PCR amplicons (see above) were pooled at equivalent concentrations, labeled with horseradish peroxidase by using the ECL direct labeling kit (Amersham Pharmacia), and used as probes. Southern blot hybridizations and subsequent washes were carried out in glass tubes in a Autoblott mini-hybridization oven (Bellco) at 42°C under low-stringency conditions (0.5× SSC in

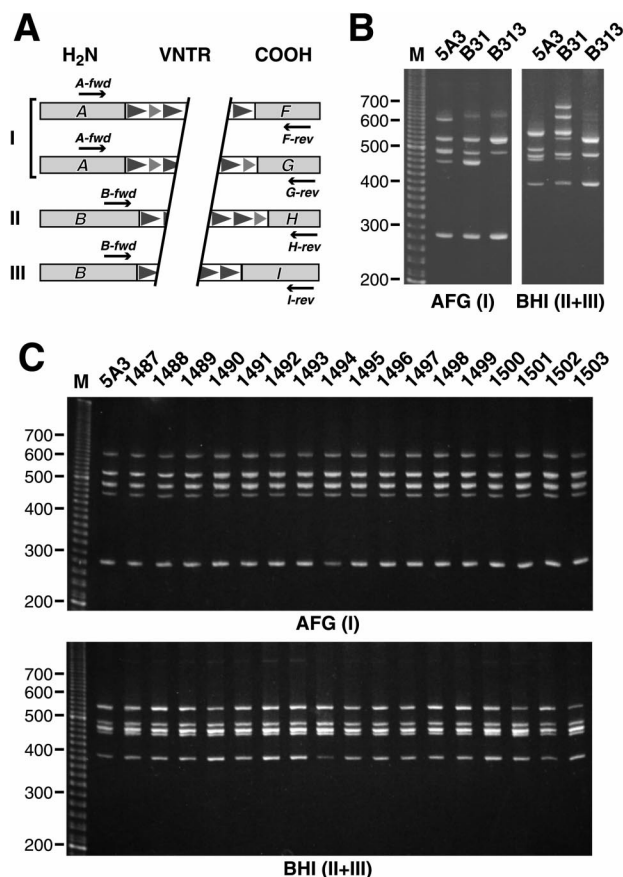


FIG. 1. Multiplex PCR assay indicating stability of the *bdr* VNTR domains. (A) Amplification scheme. *bdr* VNTR domains were amplified by using oligonucleotide primers specific for the N- and C-terminal homology groups A or B and F, G, H, or I (40), respectively. Primer sequences (5' to 3') are as follows: A-fwd, WRGGRTTGTAGYRARGARGCAATAGAT; F-rev, ATTGGRGCAA RYGTATTCCCATTTAT; G-rev, GAATYCTGCTGTAWTATCATAAAA ATGTA; B-fwd, CWCAAGATTTTCWAAAAGATATTATCACAATGA; H-rev, CCKGCTATCATTGKATAGACATTGC; I-rev, ATATAGCARTGGMACWAYTAYTACTG. Degenerate nucleotides are indicated according to International Union of Pure and Applied Chemistry nomenclature as follows: R = A + G, Y = C + T, W = A + T, K = G + T, M = A + C. (B and C) PCR amplicons obtained with primer combinations A-fwd/F-rev/G-rev (AFG) or B-fwd/H-rev/I-rev (BHI) by using either three B31 clones (B) or 17 isolates obtained 1 year after infection of mice with B31-5A3 (C) were separated on a 5% nondenaturing polyacrylamide gel. A 20-bp ladder (Gibco) served as the size marker.

primary wash; 20× SSC is 0.3 M sodium citrate plus 3 M NaCl, pH 7).

Figure 2 shows the results obtained from the *Xba*I digest. As expected from their different plasmid profiles (Table 1), the three B31 clones obtained before or after prolonged in vitro passage differ in their RFLP pattern. As was the case for the PCR assay described above, the hybridizing restriction fragments match the predicted *bdr*-carrying restriction fragments. For example, B313 lacks the *bdrS* (lp28-1) and *bdrT* (lp28-2) fragments. The additional fragments in B31-ATCC, e.g., a 6-kb fragment, are likely to originate from the cp32-5 paralog(s) that has yet to be sequenced. In contrast, the mouse tissue B31-5A3-derived isolates have an RFLP pattern identical to that of the original B31-5A3 clone. Note that the upper band of 8- to 9-kb doublets apparently missing in the original clone is readily detected in longer film exposures (not shown). To-

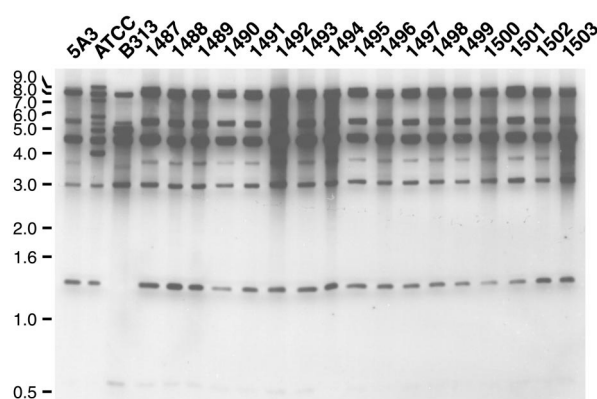


FIG. 2. RFLP analysis indicating stability of the *bdr*-flanking regions. Southern blots of total *B. burgdorferi* DNA digested with *Hind*III (data not shown) and *Xba*I were probed with a pool of PCR-derived *bdr* probes. Three in vitro-cultured B31 clones (5A3, ATCC, and B313) and 17 isolates obtained 1 year after infection of mice with B31-5A3 (1487 to 1503) are shown. A 1-kb ladder (Gibco) served as the size marker.

gether, these data indicate that the *bdr* loci and their flanking sequences are stable.

Potential applications in strain typing. The fact that Bdr proteins have sequence heterogeneity but also genus-wide antigenic cross-reactivity (40) is remarkable and potentially useful. We have previously speculated that the VNTR domains with their more-conserved direct repeat units are mainly responsible for this property. It is interesting that in another type of plasmid-encoded *B. burgdorferi* antigen with short direct repeats, *VraA* (26), the repetitive region has been determined to be the antigenic domain.

Originally used as markers for human genetic mapping (17), VNTR loci have since been identified in a number of bacterial species (33). Recently, they have been used to study the genetic diversity of *Mycobacterium tuberculosis* (14). Since all *bdr* paralogs so far identified are plasmid encoded, we surmise that Bdr protein and plasmid profiles will correspond closely. The stability of *bdr* loci in vivo and the previously shown cross-reactivity of antibodies could make anti-Bdr-based immunoblots or *bdr*-based PCR assays alternative tools for plasmid profiling and strain typing. They might be especially useful in quickly assessing possible variations of complex *Borrelia* populations present in vector ticks or mouse reservoirs.

Conclusion. Several previous studies have shown that *B. burgdorferi* adapts to different environments by regulating its surface protein expression. The majority of the involved proteins are plasmid encoded. One example of adaptation is the differential expression of outer surface proteins *OspA* and *OspC* in the tick vector and the mammalian host (25, 30). The extensive recombinatorial variation of the *B. burgdorferi vlsE* genes in vivo, notably shown with clones from the isolates used here (35–37), is another example. This phenomenon is similar to the variation of variable major protein gene families (*vsp* and *vlp*) in relapsing fever *Borrelia* spp. (5, 7, 15).

The Bdr protein paralogs appear to play a different role in *Borrelia*. In an earlier characterization we concluded that the proteins are targeted to the spirochete's cytoplasmic membrane and expressed concurrently at low levels under a variety of conditions (40). This suggested that the Bdr proteins are not under selective pressure during infection of vertebrates. The stability of *bdr* loci in vivo demonstrated here supports this. In comparison to the extensive recombination in the *vlsE* expression locus on lp28-1 (35, 36), which can be detected as early as

4 days after infection (37), the *bdr* loci remain stable over a year in a natural environment. Studies of the Erp proteins, another protein family encoded on the cp32's, have yielded similar results. Although the Erps are antigenic surface lipoproteins (27) and *erp* loci show evidence of past recombination (28), they were stable during in vivo experiments similar to the ones presented here (12). The biological function of the majority of the cp32-encoded proteins, including the Bdrs, remains unknown. The recent demonstration that the cp32's indeed represent prophage DNA of polyhedral tailed bacteriophages (11) will certainly encourage further studies of these molecules as well as the proteins they encode.

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