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The Nucleotide Sequence of a Linear Plasmid of *Borrelia burgdorferi* Reveals Similarities to Those of Circular Plasmids of Other Prokaryotes

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A linear plasmid of *Borrelia burgdorferi* had 16,927 bp, a G+C content of 23.1%, a relative deficiency of CpG dinucleotides, and open reading frames A to O. The OrfC and OrfE proteins were similar to hypothetical proteins encoded by circular plasmids of *B. burgdorferi*. The OrfM and OrfN proteins were similar to replication proteins of circular plasmids of other bacteria.

Members of the genus *Borrelia* cause a variety of diseases of humans and other animals (6). A feature of *Borrelia* spp. that distinguishes the genus from most other eubacteria is a genome that is largely linear (3). Double-stranded linear plasmids were first demonstrated in *B. hermsii* and *B. burgdorferi* (4, 30) and then *B. turicatae* (14). The linear plasmids range in size from 10 to 180 kb, and some encode the Osp or Vmp lipoproteins of *Borrelia* spp. (3). The chromosomes of *B. burgdorferi*, *B. hermsii*, *B. turicatae*, and *B. anserina* were also shown to be linear (9, 10, 13, 14). One chromosome and one copy each of the different types of plasmid make up a genome equivalent (18, 22). There are usually several varieties of linear plasmids and a few types of circular plasmid per genome (12, 21, 33, 39). Multiple genomes are distributed along the entire length of each cell (18, 22).

The structures of 16- and 49-kb linear plasmids of *B. burgdorferi* have been studied in detail (4, 5). The ends, or telomeres, of the linear plasmids are covalently closed hairpins; the left and right ends are inverted repeats with respect to each other (17, 19).

The sequence of a 8-kb circular plasmid (cp8.3) of *B. burgdorferi* sensu lato has been determined previously (12). Partial sequences of circular plasmids of about 30 kb of *B. burgdorferi* sensu lato have also been reported (34, 39). Some sequences of cp8.3 were similar to those of plasmids with a rolling-circle-type replication (15, 28). A circular plasmid of the spirochete *Treponema denticola* appears to use this method of replication (24).

DNA cloning, sequencing, and analysis. To extend our knowledge of these novel replicons, we determined the complete sequence of the 16-kb linear plasmid of *B. burgdorferi* B31 (ATCC 35210). An overview of the sequencing project is shown in the lower part of Fig. 1. We used recombinant plasmids containing the left (pTL16) and right (pTR16) ends of the plasmid; the sequences of 208 bp at the left end and 191 bp of the right end had been determined previously (19). Other recombinant clones were constructed with restriction fragments of purified plasmid, prepared as described previously (4). To minimize contamination of the DNA with other linear plasmids, the B31 strain derivative B313, which lacks all linear plasmid but the 16-kb one, was used (32). Plasmid DNA was recovered from agarose gels by electroelution as previously described (17), digested with *Bgl*II or *Hin*dIII, and ligated into pBR322. The ligation products were transformed into *Escherichia coli* DH5α or SURE (Stratagene, La Jolla, Calif.). The recombinant plasmids obtained were designated p31, p39, p63, p59, and p5 and are shown from left to right on the map of lp16 (Fig. 1).

Three gap sequences remained: A, between pTL16 and p31; B, between p59 and p5; and C, between p5 and pTR16. We used PCR to amplify the DNAs of the gap sequences with *Thermus aquaticus* polymerase as described previously (31) or with the Expand Long Template PCR system (Boehringer Mannheim, Indianapolis, Ind.). Primers for the gap sequences were designed on the basis of previously obtained sequence. The PCR products containing the gap A sequence were digested with *Ssp*I or *Dra*I to produce small overlapping fragments; these fragments were ligated into either pBR322 or pUC19, and the ligation products were transformed into *E. coli* JM109. PCR products containing gap C sequence were cloned into the plasmid pCRII (Invitrogen, La Jolla, Calif.) and were transformed into *E. coli* INVαF′. At least two clones of each PCR product inserted into a vector were used for sequencing.

PCR amplified all or part of gap B sequence, but the resultant products could not be cloned in different *E. coli* host-vector cloning systems, including vectors pCRII, pUC19, and pBR322 and hosts JM109, INVαF′, SURE, and STBL2 (Gibco-BRL, Gaithersburg, Md.). PCR was also used to overlap junctions of the fragments represented in the clones. The junction between p63 and p59 is an example for which an overlapping PCR product was produced (Fig. 1).

The DNA sequences of inserts of the recombinant plasmids were determined by double-stranded dideoxy sequencing with Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio) and custom oligonucleotide primers as described previously (31). Sequences of uncloned double-stranded PCR products were determined with [α-^{33}P]ATP and either the Cycle Sequencing System (Gibco-BRL) or the Easy Sides system (Dupont-New England Nuclear, New Bedford, Mass.). The sequences were assembled and analyzed by using the Wisconsin Package (version 8.1, Open VMS) of software programs from Genetics Computer Group, Inc. (Madison, Wis.).

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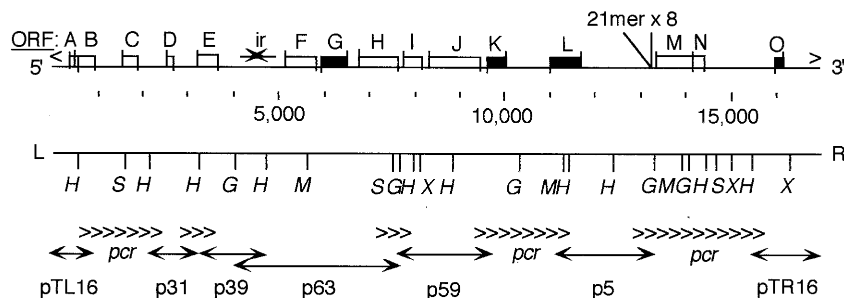


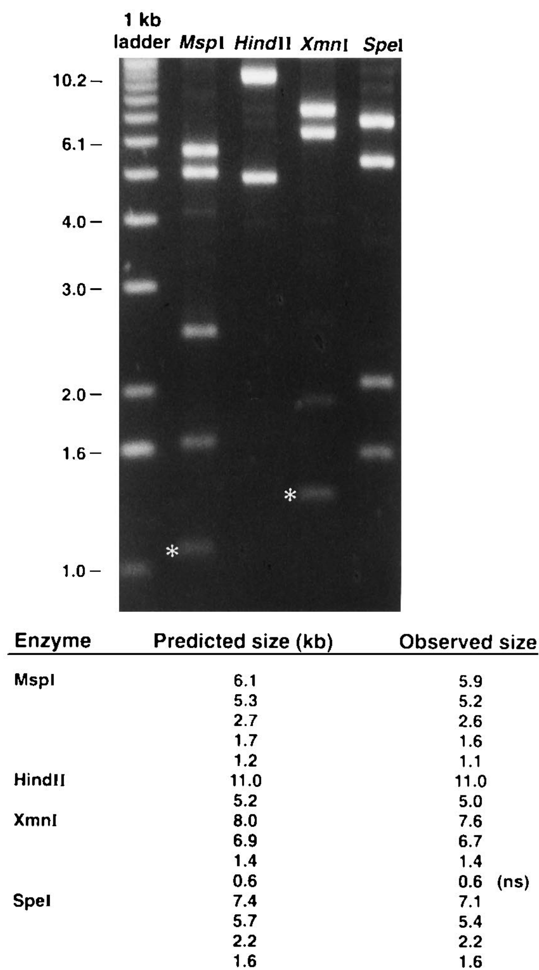
FIG. 1. Schematic representation of sequencing strategy for and the physical map, deduced ORFs, and selected features of the linear plasmid lp16.9 of *B. burgdorferi* B31. In the bottom third of the figure, the recombinant clones used for sequencing and the regions (>>>>>) subjected to PCR (*pcr*)-based sequencing are shown. The middle third of the figures shows the left (L) and right (R) ends of the plasmid and selected restriction sites for the following enzymes:*Hin*dIII (*H*), *Spe*I (*S*), *Bgl*II (*G*), *Msp*I (*M*), and *Xmn*I (*X*). The upper third of the figure shows the locations of ORFs A to O and the nucleotide positions (in base pairs). The location of the ORF by strand is indicated by the color of the box: white for the plus strand and black for the minus strand. The approximate positions of start and stop codons are indicated by a vertical line above and below the horizontal line, respectively. Other features indicated in the figure are the inverted repeat (ir) region and eight tandemly arrayed directed repeats of a 21-mer.

The organization of the assembled sequences was confirmed by digestion of purified native linear plasmid and comparison of the electrophoretic migration positions of the observed fragments of native plasmid DNA with the sizes of the fragments predicted by the assembled sequence. Native plasmid DNA was isolated as described above, digested with restriction enzymes *Hin*dIII, *Spe*I, and *Msp*I, and probed with radiolabeled plasmid DNA. The resultant Southern blot is shown in Fig. 2. The observed sizes of restriction fragments corresponded to fragment sizes predicted from the sequence.

Open reading frames (ORFs) were determined for start codons of ATG, GTG, and TTG. Possible ORFs were examined by using the CODONPREFERENCE algorithm of the Wisconsin Package to assess the similarity of their codon usages to a custom codon frequency table based upon the following *Borrelia* spp. sequences (with accession numbers in parentheses): *B. anserina* flagellin structural protein (*flaB*) gene (X75201); *B. crocidurae flaB* (X75204); *B. burgdorferi* DNA gyrase beta subunit (U04527), DnaA (L14948), elongation factor EF-Tu gene (L23125), flagellar distal rod protein (*flgE*) gene (U12870), GroEL gene (X54059), GMP synthetase (*guaA*) gene (L25883), OspA and OspB protein genes (X14407), OspC gene (X69596), OspD gene (M97452), OspE gene (L13924), OspF gene (L13925), P83 protein gene (X71398), P39 protein gene (L24194), Rho protein gene (L07656), GrpE gene, DnaK gene, and DnaJ gene (M96847); and *B. hermsii* Vmp genes 1 (L33870), 7 (X53926), 17 (L04788), 21 (M57256), 26 (L26497), and 33 (L24911).

By the above criteria, we identified proteins encoded by these ORFs that were at least 50 amino acids in length and that had mean CODONPREFERENCE statistic values (Wisconsin Package), using the *Borrelia* spp. codon table, of at least 1.1 over their lengths. Potential ribosomal binding sequences were defined as sequences that were capable of complete or partial pairing with nucleotides near the 3′ end of *Borrelia* spp. 16S rRNA (29); a completely base-paired sequence would be AG GAG. Promoter regions consistent with the σ^{70} promoter of *E. coli* (16), as well as with those of alternative sigma factors (26), were identified.

The deduced Orf proteins were submitted for BLASTP analysis with the nonredundant protein database at the National Center for Biotechnology Information (2). Possibly homologous proteins were then compared with the linear plasmid Orf proteins by GAP or BESTFIT algorithm (Wisconsin Package); the linear plasmid Orf sequence was randomized at least 20 times. The mean quality score with standard deviation of these alignments with the shuffled sequence was compared with the alignment with the unshuffled sequence. If the quality score of the unshuffled sequence was at least 3 standard deviations removed from the mean quality score of the shuffled sequences, then the Orf protein and the other protein were considered homologous.



Enzyme	Predicted size (kb)	Observed size
MspI	6.1	5.9
	5.3	5.2
	2.7	2.6
	1.7	1.6
	1.2	1.1
HindII	11.0	11.0
	5.2	5.0
XmnI	8.0	7.6
	6.9	6.7
	1.4	1.4
	0.6	0.6 (ns)
SpeI	7.4	7.1
	5.7	5.4
	2.2	2.2
	1.6	1.6

FIG. 2. Restriction digests of purified native linear plasmid lp16.9 of *B. burgdorferi* B31 and a comparison of the observed sizes with the sizes predicted from the assembled sequence. The purified plasmid was digested with *Msp*I, *Hin*dIII, *Xmn*I, or *Spe*I, and the products were analyzed by ethidium bromide staining of a 1.0% agarose gel. Fainter bands are indicated by a white asterisk. The predicted and observed sizes (in kilobases) of the fragments are shown below the gel. ns, not shown.

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TABLE 1. ORFs of lp16.9 of *B. burgdorferi* B31

ORF	Positions[a]	Sequence[b]	Polypeptide		
			Length	Weight	pI
A	241→432	AGGAGCTATAATATCC*ATG*	64	7,646	10.2
B	359→829	AGCGAATATCAAATAA*ATG*	157	17,971	7.8
C	1469→1792	AAAGAAAATATTAAGT*ATG*	108	13,116	10.3
D	2415→2567	CAAAACACTAGATCAT*ATG*	51	6,041	10.5
E	3169→3630	TTCTACACTAAGAACT*ATG*	154	18,629	10.4
F	5088→5768	TATAATAAGTTGTATA*ATG*	227	26,658	10.1
G	5903←6484	ACTAAGGGGGGTTTTA*ATG*	193	22,818	9.9
H	6711→7583	ATCTATAGGAGAAATC*GTG*	291	33,791	10.1
I	7815→8138	AAATTATGAGGTATTT*ATG*	108	12,693	10.6
J	8297→9406	AAGCCAGGATAAGATA*ATG*	370	44,197	10.8
K	9618←9917	TAGGGGAAAGAAATTT*ATG*	98	11,513	10.0
L	11011←11676	TTTTTAAAGGATAAAAA*ATG*	220	25,729	10.2
M	13369→14106	TTAAAAGGAAGTTTGT*ATG*	246	28,815	8.1
N	14100→14357	GATTGGGAGATGCTTT*ATG*	86	9,988	10.1
O	15918←16145	GGAGAATTAAAATTTTT*ATG*	76	9,112	9.5

[a] Positions refer to those used for GenBank sequence U43414.
[b] Ribosomal binding sequences are underlined. Start codons are italicized.

A schematic representation of the sequence of the linear plasmid is shown in Fig. 1. Figure 1 shows the putative coding sequences, certain features of the DNA, selected restriction enzyme sites, subclones, and the sequencing strategy. The left and right ends correspond to 5′ and 3′ ends, respectively, of one strand of the plasmid. Table 1 summarizes the deduced coding sequences A to O by location and predicted sizes and isoelectric points. The sequence of the region containing ORFs M and N is presented separately (Fig. 3).

Nucleotide composition. The linear plasmid contained 16,927 bp as well as 4 unpaired bases at each end. Henceforth, this plasmid is called lp16.9. The overall nucleotide composition was 41.9% A, 35.0% T, 11.2% C, and 11.9% G. At 23.1%, the G+C content of lp16.9 was similar to those of cp8.3 (23.4%) and the partial sequence of cp30 (25.1%) (12, 39). Of the dinucleotides, the greatest difference between the observed and expected frequencies was for CpG. Of a total of 16,926 dinucleotides, there were only 123 CpG dinucleotides, half of what would be expected ($0.112 \times 0.119 \times 16,926 = 226$) and almost threefold lower than the 355 GpC dinucleotides. In this characteristic of CpG dinucleotides occurring less frequently than chance would predict, lp16.9 is similar to eukaryotic DNA (36). In eukaryotes, the cytosine of this dinucleotide is often methylated. Some *Borrelia* spp. have a Dam-type methylase (20); it is not known whether methylation also occurs at CpG.

Although the G+C contents were near identical for the two halves of lp16.9, there were differences between the arms in the distribution of the individual nucleotides, evidence that the two ends of lp16.9 have different lineages. Whereas the nucleotide contents of the left half were 12.7% for C, 10.4% for G, 44.2% for A, and 32.8% for T, the corresponding values for the right half were 9.7, 13.4, 40.0, and 37.2%.

The left end of the plasmid. Comparison of the entire sequence of lp16.9 with cp8.3 and the partial sequence of cp30 showed that only a short region of the linear plasmid was similar to the circular plasmids. Sequence at positions 3069 to 3367 of lp16.9 was 70% identical to sequence from positions 3853 to 4152 of cp8.3 (12). A shorter stretch in the same region (positions 3069 to 3169) of lp16.9 was 69% identical to the sequence from positions 779 to 878 of cp30 (39). These homologous sequences contain ORFs as follows: ORF1 in cp8.3, ORF A in cp30, and ORF E in lp16.9 (Fig. 1 and Table 1). A shorter ORF of lp16.9, ORF C, was also similar to ORF 1 of cp8.3 and ORF A of cp30.

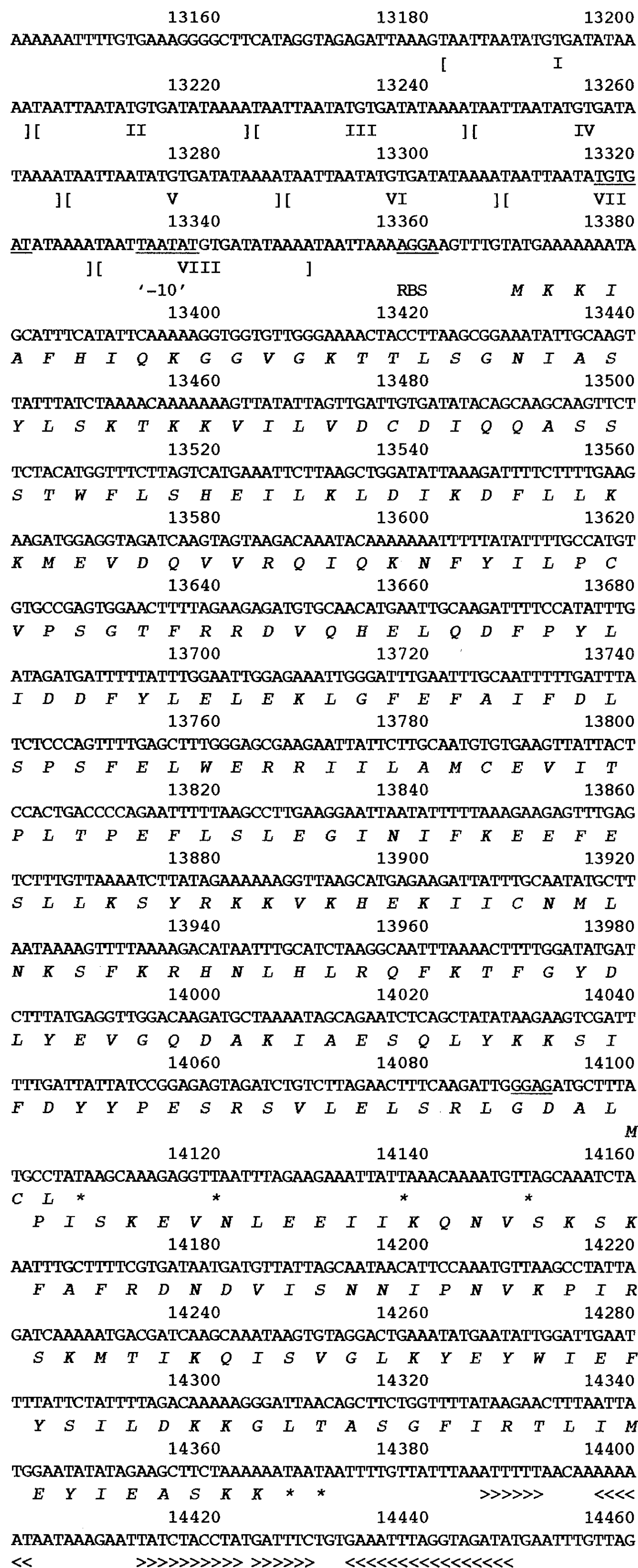

FIG. 3. DNA sequence and deduced amino acid sequence of two ORFs, ORFs M and N, of lp16.9 of *B. burgdorferi* B31 from nucleotide positions 13141 to 14460. Preceding the start of ORF M are eight direct repeats, which are indicated by Roman numerals I to VIII. Putative −35 and −10 boxes (underlined) of a σ^{70}-type promoter, a consensus ribosomal binding sequence (RBS), termination codons (asterisks), and complementary regions consistent with hairpin loop formation (>>>>> and <<<<<) are indicated.

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The finding of a conserved or repetitive sequence in the left arm of lp16.9 is consistent with results of previous studies. For at least 0.5 kb inward from their left telomeres, lp16.9 and lp49 are nearly identical (19). When pTL16 was used as a probe of plasmids from several different strains of *B. burgdorferi*, the probe bound to three different size ranges of linear plasmids: 16 or 17 kb, 25 to 30 kb, and 49 or 50 kb (18). In some isolates, three different plasmids were bound by the probe. In contrast, the right end of lp16.9, as represented by pTR16, bound only to linear plasmids of 16 or 17 kb among *B. burgdorferi* sensu stricto (18).

The fragment that hybridized to multiple linear plasmids was the first 570 bp of lp16.9, from the left telomere to the first *Hin*dIII site (Fig. 1). This sequence contains from positions 153 to 201 the imperfect palindrome described previously (19). The present study revealed in this fragment also contains two overlapping ORFs, ORFs A and B, and neither OrfA nor OrfB protein was discernibly similar to deposited protein sequences.

Analysis of ORFs F to N. Of the 16,927 nucleotides, 7,174 (42%) made up potential coding sequences. The greatest concentration of coding sequences was in the region from positions 5000 to 10000 (Fig. 1). In this region were six ORFs: ORFs F, G, H, I, J, and K (Table 1). ORFs H, I, and J were on the plus strand, and ORFs G and K were on the minus strand. Homologous proteins to these deduced proteins were not found in the databases. Residues 19 to 23 of the OrfK protein are ILGCD, which are consistent with a signal peptidase II site for the processing and modification at the cysteine (7, 8). Between ORFs E and F were 1.5 kb of sequence without any discernible coding sequence. In this region, sequence from positions 4155 to 4478 contained a large inverted repeat, which was not similar to the repeats found on circular plasmids of *B. burgdorferi* (12, 34, 39).

From positions 10010 to 13369 there was only one detectable ORF: L (Fig. 1 and Table 1). The coding sequence was on the minus strand and would encode a protein of 220 residues of unknown function.

The sequence from positions 13141 to 14460 is shown in Fig. 3. This sequence comprises ORFs M and N. If ORFs A and L were distinctive for the inverted repeats in their putative promoter regions, then ORF M was distinctive for a series of direct repeats preceding the coding region. Beginning at position 13182 was a series of eight perfect direct repeats of this 21-mer: 5′TAATTAATATGTGATATAAAA 3′. Tandemly arranged in this way, the repeats would provide several possible −10 (TATAAT) and −35 (TGTGAT) boxes of a σ^{70} promoter in front of ORF M. This feature of the upstream flanking region of lp16.9 resembles the multiple direct repeats preceding the gene for the outer membrane protein OspD of *B. burgdorferi* on the 38-kb linear plasmid of strain B31 (27).

There are consensus ribosomal binding sequences preceding ORFs M and N. The ORF N sequence overlaps the ORF M sequence by a few nucleotides. There was not a discernible promoter in front of ORF N. These characteristics suggest that ORFs M and N constitute a single transcriptional unit.

Of the 15 individual Orf proteins submitted for database searches, 4 Orf proteins, OrfC, OrfE, OrfM, and OrfN were homologous to one or more of the deposited sequences. Among these four, only OrfM and OrfN were found to be similar to proteins of known function. The greatest number of matches was with OrfM. Near its N terminus, the deduced protein had the sequence KGGVGKTT, which is the nucleotide-binding motif of several proteins, including nitrogenase reductase, with ATPase activity (11, 35, 37).

Other polypeptides with this motif and similarities to OrfM included proteins involved in cell or plasmid replication. MinD of *Bacillus subtilis* and *E. coli* are cytoplasmic membrane ATPases required for the correct placement of the division site (11, 37). ParA, also known as SopA, proteins are *trans*-acting proteins of large plasmids, like F and P1, of *E. coli* and other gram-negative bacteria; they are essential for the equal partitioning of newly replicated plasmids to daughter cells (1, 25). The RepB protein of *Enterococcus faecalis* controls the copy number of a pheromone-responsive plasmid (38).

The database search also revealed several proteins whose function was either unknown or only suspected. Notably, in this group were two Orf proteins of *Borrelia* spp.: OrfC of the circular plasmid cp30 of *B. burgdorferi* (39) and Orf1 of a linear plasmid of *B. hermsii* (GenBank accession number U63319).

OrfN is a hypothetical polypeptide with a size of about 10 kDa and a basic pI. The features and primary sequence of OrfN are similar to those of the CopB proteins, sometimes called RepB or RepA2 proteins, which are DNA-binding proteins that are involved in the determination of copy number in plasmid replication of gram-negative bacteria (23). They bind to the promoter for a gene, *repA*, that is directly upstream from the CopB protein in the same transcriptional unit. By this action CopB inhibits the synthesis of mRNA for RepA, a replication initiator protein. Although RepA and OrfM do not have discernible similarity, it is conceivable that the putative target sequence for OrfN is the short repeat sequence upstream of OrfM and that, in binding to this promoter region, OrfN regulates the expression of OrfM.

The right end of the plasmid. As discussed above, when the 1.5-kb *Hin*dIII fragment of the right end of lp16.9 was cloned and the resultant plasmid, pTR16, was used as a probe of different strains of *B. burgdorferi*, only linear plasmids of approximately 16 or 17 kb were hybridized. This finding suggested that the sequence at this end was unique to this replicon. Sequence analysis of the rightmost 2.5 kb of lp16.9 sequence confirmed that this region lacked sequences with any discernible similarity to those of other *Borrelia* spp. replicons. Indeed, OrfO, the only Orf protein of longer than 50 residues in the region, was unlike any other known protein. OrfO was remarkable only for a putative signal peptidase II recognition site (7, 8), LTMC, beginning at position 10 of the polypeptide, and for a pair of 19-mer inverted repeats (positions 16037 to 16082) in the middle.

The sequence further preceding the coding region for OrfO lacked distinguishing features, save the presence of 19- to 23-bp sequences that were direct repeats of sequences that occurred in other parts of the plasmid. These sequences were as follows: (i) 5′ TATACAAAAAGGAATGTTT 3′ starting at positions 15630 and 4795; (ii) 5′ TTTATAAAGAAATAAAAA 3′ starting at positions 15711 and 10562; (iii) 5′ ATGATCTTTGATTTAGAAATAGTA 3′ starting at positions 15847 and 10688. These direct repeats at multiples of approximately 5,000 suggests that the linear plasmid may have its origin in replicons of about 5 kb; the repeats may be the only remnants of a remote multimerization.

Possible origin of linear plasmids of *Borrelia* spp. The structures of extreme termini of the linear plasmids lp16.9 and lp49 of *B. burgdorferi* were similar to those of certain double-stranded linear DNA viruses of animals, most notably African swine fever virus (17). Given this similarity, we asked whether the linear plasmids of *Borrelia* spp. were in fact derived from an animal virus. The findings of the present study do not support that conclusion. Although most of the proteins encoded by the ORFs of lp16.9 could not be identified with proteins of known or hypothetical function, the few that could be identified were most similar to proteins of prokaryotic organisms, in particular to proteins involved in the replication and maintenance of

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some bacterial plasmids. The additional finding in lp16.9 of regions of identity to sequences of circular plasmids of *B. burgdorferi* indicates that circular and linear plasmids of this genus have a common origin (12, 39). The change of a linear plasmid to a stably maintained circular one in *B. hersmii* is evidence of a shared replication mechanism for circular and linear plasmids of this genus (14).

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been assigned GenBank accession number U43414. The deduced amino acid sequences of the ORFs described herein are also available under this accession number.

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