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Complete Nucleotide Sequence of a Circular Plasmid from the Lyme Disease Spirochete, *Borrelia burgdorferi*

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We have determined the complete nucleotide sequence of a small circular plasmid from the spirochete *Borrelia burgdorferi* Ip21, the agent of Lyme disease. The plasmid (cp8.3/Ip21) is 8,303 bp long, has a 76.6% A+T content, and is unstable upon passage of cells in vitro. An analysis of the sequence revealed the presence of two nearly perfect copies of a 184-bp inverted repeat sequence separated by 2,675 bp containing three closely spaced, but nonoverlapping, open reading frames (ORFs). Each inverted repeat ends in sequences that may function as signals for the initiation of transcription and translation of flanking plasmid sequences. A unique oligonucleotide probe based on the repeated sequence showed that the DNA between the repeats is present predominantly in a single orientation. Additional copies of the repeat were not detected elsewhere in the Ip21 genome. An analysis for potential ORFs indicates that the plasmid has nine highly probable protein-coding ORFs and one that is less probable; together, they occupy almost 71% of the nucleotide sequence. Analysis of the deduced amino acid sequences of the ORFs revealed one (ORF-9) with features in common with *Borrelia* lipoproteins and another (ORF-2) having limited homology with a replication protein, RepC, from a gram-positive plasmid that replicates by a rolling circle (RC) mechanism. Known collectively as RC plasmids, such plasmids require a double-stranded origin at which the Rep protein nicks the DNA to generate a single-stranded replication intermediate. cp8.3/Ip21 has three copies of the heptameric motif characteristically found at a nick site of most RC plasmids. These observations suggest that cp8.3/Ip21 may replicate by an RC mechanism.

Lyme disease, a tick-borne infection caused by the spirochete *Borrelia burgdorferi*, has become a serious health threat in North America, Europe, Eurasia, and Japan (31). The causative agent was first isolated and cultured in 1982 from the midgut of an *Ixodes scapularis* (*dammini*) deer tick harvested on Shelter Island, N.Y. (4, 12). Subsequent research identified what appeared to be identical spirochetes in *Ixodes ricinus* ticks in Europe and *Ixodes persulcatus* ticks in Eurasia (11).

All *Borrelia* spp. are distinguished by their characteristic morphology and motility, as well as a very high (≥70%) A+T content in their DNA (7, 32). One of the most notable features of this genus is the finding that borrelias possess a linear chromosome of about 1,000 kb rather than circular chromosomes (13, 15, 21, 45). In addition to this "maxichromosome" (10), all *Borrelia* isolates have a complex mixture of extrachromosomal elements: covalently closed linear and circular plasmids, some of which are thought to carry genes coding for factors that contribute to virulence (5, 6, 20, 24, 29, 48, 51). The linear plasmids, or minichromosomes, range in size from 16 to about 50 kb (6). Genes for the abundant outer membrane lipoproteins OspA and OspB are encoded by tandemly arrayed genes that are part of a single transcription unit on the largest of the linear plasmids, lp49 (9). OspD, another outer surface lipoprotein, is encoded on a 38-kb linear plasmid, lp38, present in low-passage B31 cells (41). Recently, two groups (37, 44) determined that the gene for OspC, an additional major outer surface lipoprotein (22, 55), is on a 27-kb covalently closed circular plasmid, cp27. Interestingly, under laboratory conditions all the linear plasmids seem to be completely dispensable, whereas cp27 and another circular plasmid, cp30, appear to be stable with respect to their replication and partitioning to daughter cells (28, 44).

The stability of cp27 and cp30 in vitro is in contrast to the instability of circular *Borrelia* plasmids of less than 10 kb. Several small 8- to 9-kb supercoiled circular plasmids have been detected in fresh, low-passage isolates of North American strains of *B. burgdorferi*; however, they are unstable and are lost completely after a maximum of 30 passages in vitro (24, 29, 47, 51). Their loss correlates with reduced infectivity in the white-footed mouse, *Peromyscus leucopus* (47, 51). Even though the presence of these smaller plasmids has been correlated with virulence and, therefore, they are of potential medical significance, not much is known about the genes of these plasmids or about their mechanisms of replication. Attempts to clone the smallest of the supercoiled circular plasmids, pBBC1, from the infectious strain Sh-2-82 were unsuccessful; only 1.6 kb of the starting 8.4-kb *Eco*RI-linearized DNA was recovered in *Escherichia coli* (47, 51).

During our studies on the phylogenetic relationship between geographically diverse *Borrelia* isolates (19), we compared the plasmid profiles from several low-passage North American and Eurasian isolates. One strain we examined, Ip21, which originated near Saint Petersburg, Russia, had a DNA band that migrated during agarose gel electrophoresis with the expected mobility of a circular plasmid of less than 10 kb that was recovered in diminishing amounts upon passage in vitro. To further characterize this plasmid, we decided to take a direct approach and determine its entire nucleotide sequence. Determining the nucleotide sequence of this small, unstable

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TABLE 1. *B. burgdorferi* strains and plasmids

Strain or plasmid	Source or description[a]	Reference
Strains		
B31	*I. scapularis*, Shelter Island, N.Y.	4, 10
Ip3	*I. persulcatus*, Leningrad province, Russia	34
Ip21	*I. persulcatus*, Leningrad province, Russia	34
Ip90	*I. persulcatus*, Khabarovsk territory, Russia	34
DK29	Skin of a patient with erythema migrans, Denmark	54
Plasmids		
cp8.3/Ip21	Native, cryptic plasmid	This work
pJD5851	*Hin*cII B fragment of cp8.3/Ip21 in the *Bam*HI site of pBR322, Ap^r	This work
pJD5852	*Hin*cII C fragment of cp8.3/Ip21 in the *Bam*HI site of pBR322, Ap^r	This work
pJD5853	*Hin*cII D fragment of cp8.3/Ip21 in the *Bam*HI site of pBR322, Ap^r	This work
pJD5854	Subfragment of *Hin*cII A of cp8.3/Ip21 from nt 259 to the *Hae*III site at nt 2147 in pCR II, Ap^r Km^r	This work
pJD5855	Subfragment of *Hin*cII A of cp8.3/Ip21 from the *Hae*III site at nt 2148 to nt 3776 in pCR II, Ap^r Km^r	This work
pJD5856	Subfragment of *Hin*cII A of cp8.3/Ip21 from the *Hae*III site at nt 2148 to nt 3776 in the *Bam*HI site of pBR322, Ap^r	This work

[a] Ap, ampicillin; Km, kanamycin.

plasmid and the larger, more stably maintained *Borrelia* plasmids would be first steps toward a thorough understanding of their genetic organization and should help us understand how they replicate. Prior to this work, no *Borrelia* plasmid has been sequenced in its entirety. In this article, we present the complete nucleotide sequence of this small plasmid and analysis of its open reading frames (ORFs) and other features in the sequence. A striking feature of the sequence is the presence of two long inverted repetitive elements having some features in common with transposable elements. These data suggest that this plasmid may eventually have use as a vector for gene transfer in borrelia.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Table 1 lists the *B. burgdorferi* strains used. Spirochetes were grown in BSK II medium at 32°C (4, 46). Commercial preparations of frozen, competent *E. coli* DH5α [F^- φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA96* Δ(*lacZYA-argF*)*U169*] (26) (GIBCO BRL, Gaithersburg, Md.) and One-Shot INVαF′ cells [F′ φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169 deoR*] (Invitrogen, San Diego, Calif.) cells were used as the recipients for recombinant plasmids according to the supplied protocols. *E. coli* Tap90 [*pro leuB6 rpsL lacY1 tonA1 supE44 supF58 hsd*(r_K^- m_K^+) *thi-1 recD1903*::mini-tet] (43) was used as the host for infection with the λ ZapII vector (Stratagene). It was grown in glucose-free medium supplemented with maltose (52), a condition known to stimulate synthesis of the λ receptor. Transformed *E. coli* strains were cultured at 37°C as previously described (52). For antibiotic selection, ampicillin and kanamycin were added at final concentrations of 40 and 50 μg/ml, respectively.

Recombinant DNA methods. Plasmid DNA was isolated from 25- to 250-ml samples of *Borrelia* cells taken at both early and late stages of logarithmic growth by using Qiagen-tip 100 cartridges (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's plasmid Midi protocol. This procedure, which involves alkaline-sodium dodecyl sulfate (SDS) lysis of cells in the presence of RNase followed by chromatography of a high-salt-concentration supernatant on a proprietary resin, gave reproducible preparations of linear and circular *Borrelia* plasmids, as judged by electrophoresis on 0.3% agarose gels in the presence 0.5 μg of ethidium bromide per ml. The same procedure was used to purify plasmid subclones from overnight *E. coli* cultures. Most other DNA manipulations were performed as described by either Maniatis et al. (36) or Ausubel et al. (2). DNA modification enzymes were obtained from several sources (GIBCO BRL; New England Biolabs, Beverly, Mass.; and Stratagene, La Jolla, Calif.) and were used according to the instructions of the manufacturers. *E. coli* single-stranded DNA-binding protein was purified by standard techniques after expression from the cloned gene in a bacteriophage T7-based expression system (52). Plasmid DNA was incubated with purified bacteriophage T7 gene 3 endonuclease, the gift of F. W. Studier, Brookhaven National Laboratory, at 37°C in an assay mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgSO_4$, 1 mM dithiothreitol, 50 μg of bovine serum albumin, and 5 μg of tRNA per ml. Under these conditions gene 3 endonuclease has a strong preference for supercoiled DNA over double-stranded linear DNA, and the enzyme converts supercoiled plasmids to a mixture of nicked circles and then to full-length linear double-stranded DNAs (17). Purified λ Zap arms and λ packaging extract were gifts of B. Burr (Brookhaven National Laboratory). *Borrelia* cells were embedded in low-melting-temperature agarose plugs as described elsewhere (21) by using cells harvested from early-stationary-phase cultures. Oligonucleotide primers were synthesized in a Milligen 7550 DNA synthesizer (Millipore) and purified by using Poly-Pak purification cartridges (Glen Research Corp., Herndon, Va.) according to the manufacturers' specifications. Hexamer primers were purchased from Research Genetics (Huntsville, Ala.).

PCR amplifications were carried out in 100-μl reaction volumes containing 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), each primer at 0.5 μM and ≈0.1 μg of template DNA. The reaction mix also contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.28 mM dATP, 0.28 mM dTTP, 0.12 mM dCTP, and 0.12 mM dGTP (the deoxynucleoside triphosphate concentrations used reflect the known DNA base content of ≈29% G+C for *B. burgdorferi* [32]). Reactions were carried out for 32 cycles in a 9600 DNA thermal cycler (Perkin-Elmer Cetus), with each cycle consisting of 1 min at 94°C, 1 min at 47°C, and 4.5 min at 72°C. Amplification was completed by a final incubation at 72°C for 10 min. The amplified products were purified by electrophoresis on 0.7% low-melting-temperature agarose gels (Bethesda Research Laboratories).

DNA sequencing. The complete nucleotide sequence of cp8.3/Ip21 was determined on both strands by the chain

termination method using Sequenase 2.0 essentially according to the manufacturer's instructions (United States Biochemicals); however, the ratios of dATP and dTTP to their corresponding dideoxy analogs were doubled to balance the signal in all four lanes and prevent loss of signal in the A and T lanes on the AT-rich *Borrelia* templates. The chemical method of Maxam and Gilbert (38) was used to sequence short stretches of nucleotide sequence near the *Eco*RI and *Sau*3A sites of the plasmid. Subclones in pBR322 or TA vectors (pCR II; Invitrogen) were sequenced directly after heat denaturation of the double-stranded DNA and annealing with suitably positioned forward and reverse primers for each vector. Direct sequencing of PCR products in the presence of 25 μg of *E. coli* SSB per ml was routinely performed with primers that anneal to the ends of the amplicons or to internal sites (primer walking). Before sequencing, the PCR-amplified templates were purified from low-melting-temperature agarose gels by using β-agarase I to digest the melted agarose and release the DNA according to the suppliers' instructions. New primers, typically 15 to 24 nucleotides (nt) long, based on cp8.3/Ip21 sequence information, were synthesized to extend the sequence into unknown regions of the DNA. Primer walking with strings of contiguous hexamers in the presence of saturating amounts of *E. coli* SSB protein also was employed under conditions essentially similar to those described previously (33). The determination of the relative orientation of each subclone and the junction sequence between each pair was determined by PCR analysis and sequencing of the amplified DNAs. No discrepancies between sequences determined on cloned DNA and those determined on PCR-generated templates were found.

Hybridization. Southern hybridization was performed in situ (2) in 0.7% agarose gels that had been run in Tris-acetate-EDTA buffer at 4°C for 14 to 16 h at 1 V/cm. DNA was denatured by gently rocking the gel in 2.5 volumes of 0.5 M NaOH–1 M NaCl for 20 min at room temperature. After the denaturation step was repeated, the gel was washed briefly with two changes of water, neutralized twice with 2.5 volumes of 0.5 M Tris-HCl (pH 7.2)–3 M NaCl for 30 min, and then washed in distilled water for 20 min. The gel was dried on a vacuum apparatus at 60°C for 1 h and then rehydrated overnight at 42°C in a hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 20 mM NaH_2PO_4 [pH 7.4], 5× Denhart's solution, 250 μg of sonicated calf thymus DNA per ml, and 0.1% SDS). Hybridization with 5′-^{32}P-labeled oligonucleotide probe (≈10^6 cpm/ml) was carried out in the fresh hybridization buffer for 18 h at 42°C. The unhybridized probe was removed by washing the gel three times in 6× SSC–0.1% SDS and once with 2× SSC–0.1% SDS and then exposed to X-ray film. Oligonucleotide probes radiolabeled with [γ-^{32}P]ATP (specific activity of 6,000 Ci/mmol) and T4 polynucleotide kinase were passed through a 1-ml Sephadex G-25 spin column equilibrated with Tris-EDTA buffer to remove free ATP beforehand. To differentiate between double- and single-stranded DNAs, electrophoretically resolved DNAs were transferred to nitrocellulose with or without denaturation with NaOH prior to transfer by diffusion with 20× SSC (53). For some experiments, 0.5% low-melting-temperature agar plugs (250 μl) containing ≈10^7 *Borrelia* cells were used as the source of total genomic DNA. Cells were lysed (21), and the plugs were thoroughly washed with 10 mM BisTris-HCl (pH 6.5)–100 mM NaCl, melted at 68°C, cooled to 40°C, and then incubated with β-agarase I (2 U/100 μl) overnight. *Hin*dIII (20 U/100 μl) and 1/10 volume of 10× *Hin*dIII restriction buffer (New England BioLabs) were added, and after a 2-h incubation at 37°C, the digests were fractionated by electrophoresis on 0.7% agarose gels. Blots were exposed to X-ray film, or images were obtained by using a Molecular Dynamics PhosphorImager system and ImageQuant software.

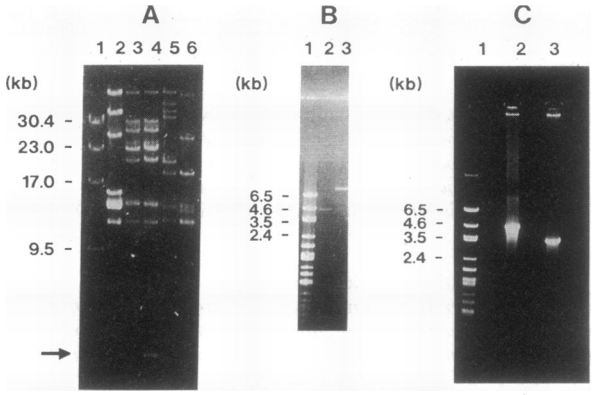

FIG. 1. (A) Agarose gel electrophoretic profiles of *Borrelia* plasmid preparations of different isolates. Lane 1, T7 *Mlu*I and *Nhe*I restriction fragment size markers (18); lanes 2 to 6, plasmids from isolates B31, Ip3, Ip21, Ip90, and DK29, respectively. Samples were analyzed on a 0.3% agarose gel. Arrow, cp8.3/Ip21 DNA band. (B) Restriction enzyme digestion of cp8.3/Ip21 analyzed on a 0.7% agarose gel. Lane 1, T7 *Hae*II digest (18); lanes 2 and 3, purified cp8.3/Ip21 DNA before and after digestion with *Eco*RI, respectively. (C) Agarose gel analysis of PCR-amplified cp8.3/Ip21. Lane 1, T7 *Hae*II digest; lanes 2 and 3, amplification products using primers pairs 1801 plus 1649 and 1802 plus 1648, respectively. Gels were stained with ethidium bromide and photographed with UV illumination. The positions and sizes of marker bands are indicated on the left of each panel.

Computer analysis of the DNA sequence. DNA sequence data were assembled by using software packages developed by S. Zhang at Brookhaven National Laboratory. The homologies of DNA and protein sequences were analyzed by using Fasta and the BLAST service of the National Center for Biotechnology Information (1).

Nucleotide sequence accession number. The cp8.3/Ip21 nucleotide sequence data reported here have been deposited in the GenBank data base under accession number U03641.

RESULTS AND DISCUSSION

Plasmid analysis of *Borrelia* spp. We compared several methods for preparing endogenous linear and circular plasmids from *Borrelia* sp. cultures and found that the Qiagen Midi kit procedure invariably gave excellent recovery and highly reproducible results. As expected, each *Borrelia* strain listed in Table 1 harbors a minimum of six plasmids ranging from about 16 kb to more than 40 kb, on the basis of their electrophoretic mobility relative to linear bacteriophage T7 marker DNAs (Fig. 1A). Although each strain has a unique plasmid profile, some strains have patterns that are very similar. In particular, the plasmid patterns of strains Ip3 and Ip21 are nearly identical except for the presence in Ip21 of a much smaller plasmid that migrates on agarose gels near the position of a linear 4.6-kb marker. This small plasmid was not seen in the plasmid pattern of a B31 strain that has been passaged repeatedly in our laboratory. After three passages in BSK II medium the small Ip21 plasmid was no longer visible in plasmid preparations, and the Ip21 and Ip3 plasmid patterns became essentially indistinguishable (data not shown).

Cloning and sequencing strategy. Initially, we surveyed several restriction endonucleases for their ability to cleave preparations of the plasmid after it had been purified from

agarose gels. No sites were found for *Acc*I, *Apa*I, *Ava*I, *Bam*HI, *Bcl*I, *Bgl*II, *Bst*YI, *Kpn*I, *Pst*I, *Sma*I, or several other restriction enzymes which should cut infrequently a high-A+T-content DNA. However, the plasmid appeared to have one recognition site for *Eco*RI, producing a band with a mobility approximately equal to that of an 8.3-kb linear DNA (Fig. 1B). When the purified plasmid was incubated with T7 gene 3 endonuclease, an enzyme known to convert covalently closed supercoiled circles to double-stranded linear molecules (17), a band with the same mobility as that of the *Eco*RI digestion product was produced. From these data and the observation that before cleavage the plasmid did not enter a 5% polyacrylamide gel, we concluded that this DNA is a double-stranded circular plasmid; we have named it cp8.3/lp21.

Our first approach was to attempt to clone the *Eco*RI-linearized DNA into λ ZapII with *E. coli* Tap90, a strain believed to minimize loss of sequences from genomic libraries (43), as the host strain. However, two attempts to identify phage containing an insert in the expected 8-kb size range were unsuccessful. Very few plaques were positive when screened with a randomly primed, ^{32}P-labeled cp8.3/lp21 whole-DNA probe, and the resulting lysates they produced either had inserts that were considerably smaller than the starting DNA or appeared to have lost the insert entirely (data not shown). These results are very similar to those reported by Simpson et al. (51), who also were unsuccessful in cloning a similar-sized plasmid from the North American *Borrelia* strain Sh-2-82 in a λ Zap vector. In all likelihood, this failure was due to the presence of sequences in each plasmid that prevent stable cloning in *E. coli*. Therefore, we decided to try two approaches to circumvent this problem: direct sequencing of PCR-amplified cp8.3/lp21 DNA and subcloning of individual restriction fragments of the plasmid.

Before primer pairs could be designed to amplify the cp8.3/lp21 sequence, limited sequence information had to be obtained. This was accomplished by chemical sequencing in both directions from the single *Eco*RI site in cp8.3/lp21 and from a *Sau*3A site situated about 40% around the plasmid. Two sets of primer pairs—primers 1801 and 1649 (GATAC AATAGTCGCTGTAATTCAC and GGTTTATATAATAT ACTACGTC) and primers 1802 and 1648 (GTGAATTAC AGCGACTATTGTATC and GAGCGATATCCTTAAAA TTGCC)—which, subsequent analysis showed, were able to amplify fragments of 4,772 and 3,555 bp, respectively (Fig. 1C), were synthesized on the basis of the known sequences. Together, the two amplified fragments represent the entire cp8.3/lp21 DNA molecule with a 24-bp region of overlapping sequence.

Since cloning PCR fragments in this size range could conceivably result in selecting DNA molecules with amplification errors, we used several approaches to subclone cp8.3/lp21 DNA that had been purified from low-melting-temperature agarose gels. Cutting cp8.3/lp21 with *Hin*cII generated four fragments (A to D in order of decreasing size from approximately 3.3 kb to just under 1 kb) which could be resolved on agarose gels. Three of the *Hin*cII sites are also sites for *Hpa*I. After *Bam*HI linkers were added and cut with *Bam*HI to generate sticky ends, *Hin*cII fragments B to D could be subcloned directly into the *Bam*HI site of pBR322 and propagated in the *E. coli* host strain, DH5α. Repeated attempts to subclone *Hin*cII fragment A using this strategy were unsuccessful. Further analysis indicated that this region of the DNA contained a single recognition site for *Hae*III situated nearly in the middle of the fragment. Two related approaches were used to try to subclone these *Hae*III subfragments. The first involved digesting agarose gel-purified *Hin*cII A with *Hae*III, adding *Bam*HI linkers, and ligating with *Bam*HI-digested pBR322. The second approach took advantage of the template-independent terminal transferase activity of *Taq* DNA polymerase to add an adenosine to the 3′ end of DNA fragments (14). Such molecules are conveniently cloned into so-called T vectors by virtue of a 1-nucleotide complementary overhang. *Hae*III-digested *Hin*cII A was incubated with *Taq* DNA polymerase in the presence of dATP and then ligated to pCR II. Each ligation mixture was used to transform competent INVαF′ cells, and the resulting colonies were screened for the presence of inserts by digesting the plasmid preparations with *Bam*HI or *Eco*RI, respectively. Clones of each *Hin*cII-*Hae*III subfragment were obtained when the T vector was used, but for unknown reasons, clones of the slightly larger subfragment were not recovered in the pBR322 vector (Table 1).

Complete sequence of cp8.3/lp21. Figure 2 shows the complete nucleotide sequence of cp8.3/lp21, and a physical map of the plasmid is presented in Fig. 3. The total length is 8,303 bp, in close agreement with the size predicted from the mobility of the linearized DNA on agarose gel electrophoresis. The overall G+C content is 23.4%, a range typical for *Borrelia* DNA. The longest uninterrupted run of A's is 10, beginning at nt 4960; the longest run of uninterrupted C's is 7, beginning at nt 1353. Direct sequencing of PCR-amplified DNA revealed two closely spaced *Hpa*I sites, 10 bp apart, between fragments D and A, that had been overlooked during initial construction of the physical map. All of the restriction endonuclease cleavage sites shown in Fig. 3 have been confirmed, and there are no recognition sites in the nucleotide sequence for enzymes that are known not to cut the DNA. *Sau*3A cleaves the two sites in cp8.3/lp21 nonrandomly. The *Sau*3A site at nt 7163 is cleaved more slowly than the site at nt 3537; this difference is not due to methylation of the C residue in the GATC recognition sequence, since both sites are followed by the same 3′-bordering sequence, TAA. Interestingly, the 5′ border of the GATC recognition sequence at nt 7163 is GAT. Perhaps the *Sau*3A binds to the 5′ trinucleotide GAT sequence and, in the process, occludes recognition of the adjacent 4-base site.

Comparison of the long inverted repeats of cp8.3/lp21. One of the most striking features of the sequence is the presence of two long, nearly perfect, inverted elements, IR-A and IR-B (Fig. 2 to 4). These elements also contain a putative promoter similar to the *E. coli* promoter consensus sequence (27). The −35 sequence in the putative promoter (TTGTAA) differs by 2 bases from the *E. coli* consensus sequence (TTGACA), and the −10 sequence (TATATT) differs by 1 base from the consensus sequence (TATAAT). If active, each promoter would direct transcription outward into flanking unique sequences of the plasmid. The resulting transcripts each would contain a Shine-Dalgarno sequence (AGGAG) capable of uninterrupted pairing with nucleotides near the 3′ end of *Borrelia* 16S rRNA (49) upstream of a suitably positioned AUG initiation codon that could initiate translation for polypeptides 266 (ORF-4) or 88 (ORF-8) amino acids long. In each case, the inverted repeat includes sequences 12 codons past the start codon of the ORF. Therefore, the deduced amino acid sequences at the N termini of polypeptides 4 and 8 are nearly identical for the first 13 residues; codon 10 of ORF-4 is AAA (Lys), and in ORF-8 it is GAA (Glu). The portions of IR-A and IR-B that may function in translation seem to have little potential to form stem-and-loop structures, and therefore any RNA derived from the putative promoters would have a high probability of serving as a functional mRNA. In contrast, the other end of each inverted repeat has the potential to form several long stem-and-loop structures (Fig. 5). Inspection of the sequence immediately adjacent to the inverted repeats

FIG. 2. Complete nucleotide sequence of cp8.3/Ip21 beginning with the first T residue of the single *Eco*RI site. Every 10th residue is marked (.). The deduced amino acid sequences of the predicted ORFs are shown above or below the DNA sequence, depending on their orientation. The Met residue for the potential start of ORF-7b is underlined. The major inverted repeats IR-A and IR-B are indicated (open arrows). Possible locations for plus origins of replication are boxed. Potential −35 and −10 promoter regions (asterisks) and regions with potential to form stem-loop structures (arrows over the sequence) are indicated.

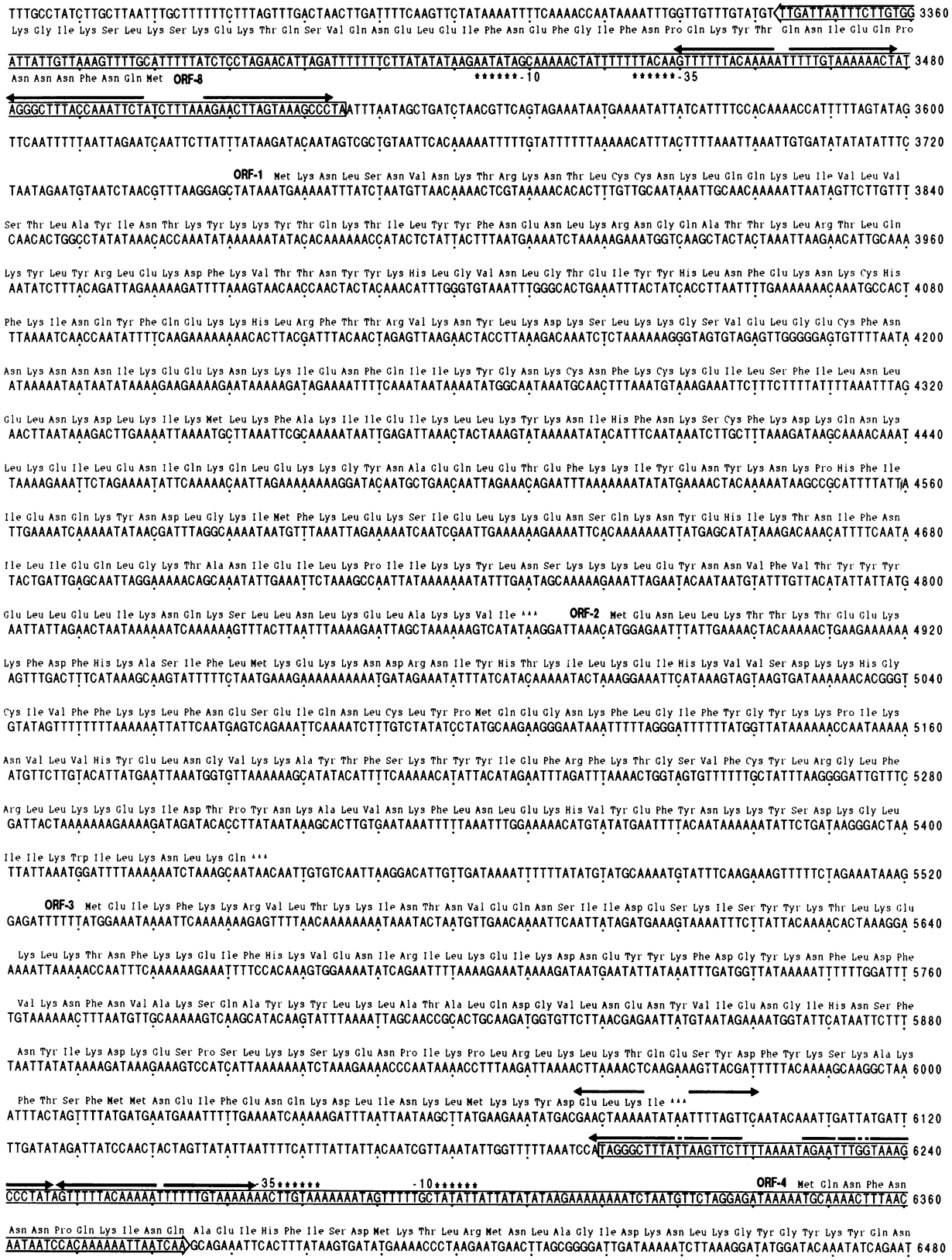

FIG. 2—*Continued.*

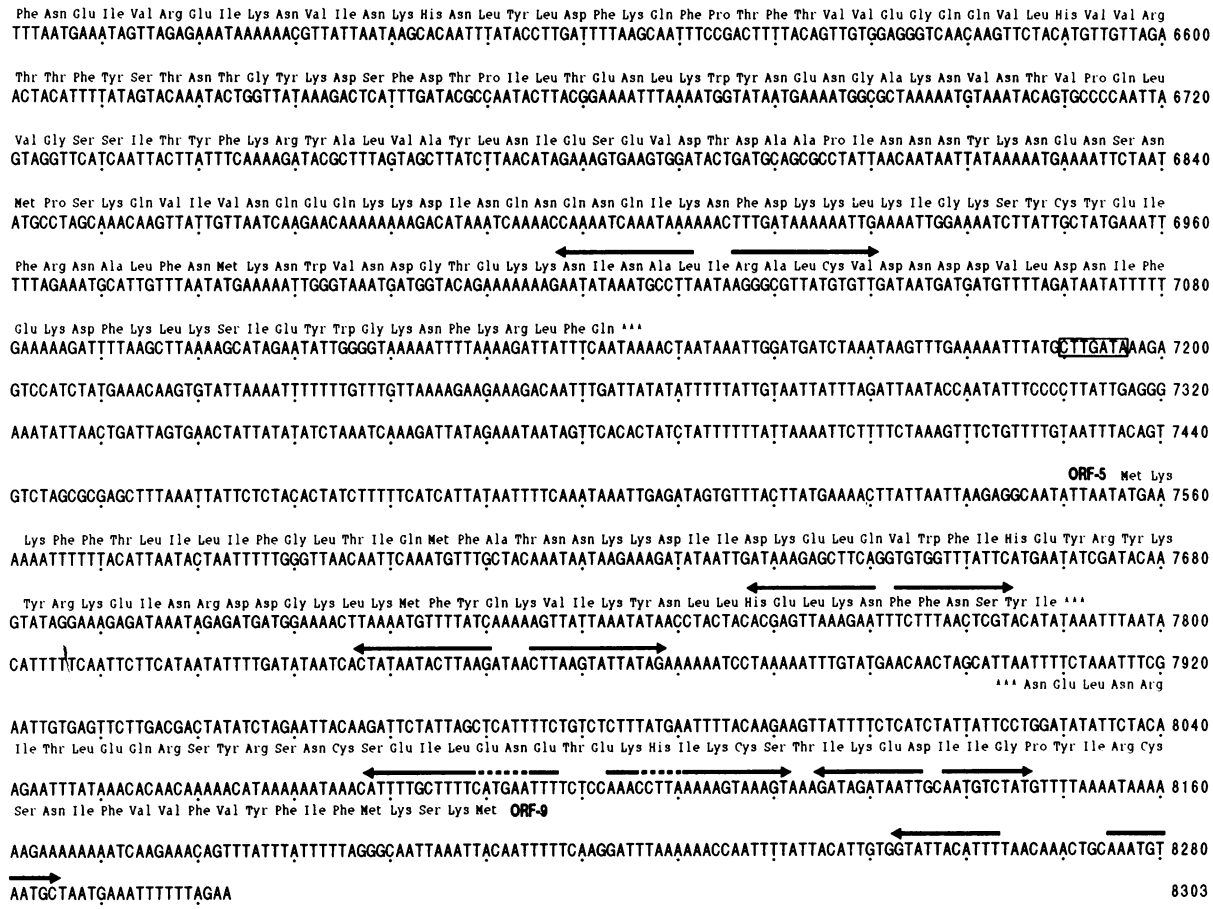

FIG. 2 —*Continued.*

failed to reveal the presence of direct repeats which could have served as target sequences for insertion of the DNA segment bounded by IR-A and IR-B.

Hybridization analysis. Recombination between inverted repeats is a mechanism for inversion isomerization. The extent of isomerization can be quantified by cutting the DNA with a restriction enzyme(s) that cuts at least once on both sides of a repeat. In the orientation shown in Fig. 3, cutting cp8.3/Ip21 with *Hin*dIII would generate fragments 7,266 and 1,037 bp long; however, inversion via recombination between IR-A and IR-B would generate *Hin*dIII fragments 4,876 and 3,427 bp long, each having a single copy of the IR element. To test for inversion, we cut Ip21 total plasmid preparations, gel purified cp8.3/Ip21 with *Hin*dIII, and analyzed them by Southern hybridization using a radioactive oligonucleotide probe designed to detect both *Hin*dIII DNA fragments because it is complementary to positions 3477 to 3497 within IR-A as well as positions 6228 to 6248 within IR-B (Fig. 4). Figure 6 shows autoradiograms of dried gels containing Ip21 DNA. In lanes containing uncut DNA, most of the hybridization signal was at the position of covalently closed supercoiled cp8.3/Ip21 and relaxed cp8.3/Ip21. A small signal was associated with a band migrating slightly faster than covalently closed cp8.3/Ip21. Subsequent analysis demonstrated that this faster band contains irreversibly denatured forms of the cp8.3/Ip21 plasmid. In *Hin*dIII-digested samples, only two fragments hybridized with the probe, and they had mobilities consistent with their being

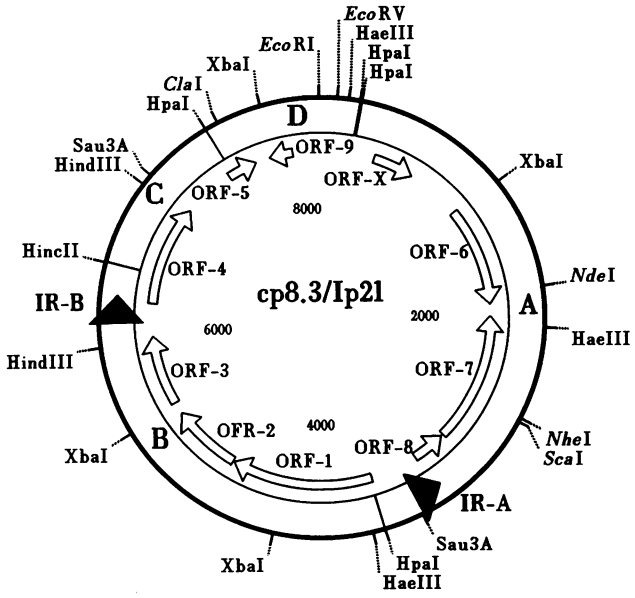

FIG. 3. Circular map of cp8.3/Ip21. The locations and orientations of the inverted repeats and ORFs are indicated (solid arrowheads and open arrows, respectively). Diagnostic restriction sites are marked along with the locations of *Hin*cII fragments A to D; the small 10-bp fragment between C and D has been omitted for clarity. Unique restriction sites are italicized.

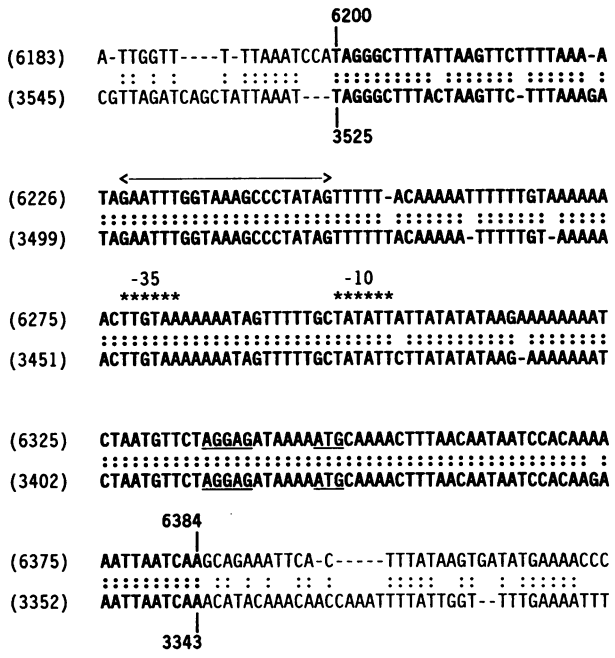

FIG. 4. Nucleotide alignment of IR-A (lower sequence) and IR-B (upper sequence). The regions of cp8.3/Ip21 DNA containing IR-A (nt 3525 to 3343) and IR-B (nt 6200 to 6384) are shown. Both sequences are written 5′ to 3′. The repeated sequences are boldfaced. :, identity; dashes, gaps. Disregarding the six single-nucleotide gaps (two in IR-B and four in IR-A), 178 of the remaining 181 nucleotide residues are identical. Ribosome binding sites and appropriately positioned ATG initiation triplets are underlined. Potential −35 and −10 promoter regions (asterisks) and the position of oligonucleotide 2443 (GAATTT GGTAAAGCCCTATAG), used for probing within the inverted repeats (double-headed arrow), are indicated.

7.3 and 1 kb, respectively. Thus, there was no indication of isomerization of cp8.3/Ip21. Because no additional bands were observed when total genomic DNA was analyzed by hybridization with this probe, we concluded that there are no additional copies of this particular IR element in any of Ip21's other plasmids or in its linear chromosome. We also surveyed the other strains in Table 1 for the presence of this particular repetitive DNA element. Southern blots of total genomic DNA that had been cut with *Hin*dIII in liquified agarose plugs gave no indication of the cp8.3/Ip21 repetitive element in any of the strains tested.

Polypeptides predicted by the most probable ORFs. Analysis of the sequence in all possible phases revealed 10 ORFs of more than 50 codons that seem likely to be expressed. All use ATG as the potential initiation triplet; none begin with GTG. Of these ORFs, nine appear to have a characteristic ribosome binding sequence capable of uninterrupted pairing with the 3′ end of 16S rRNA positioned a favorable distance in front of the putative translational start site (Table 2). ORF-7 may encode two overlapping polypeptides, 7a and 7b, which would begin at initiation sites 60 nucleotides apart and end at the same termination site. The 10th ORF (ORF-X) does not have an ideal ribosome binding sequence upstream of the initiation codon, and if the polypeptide is expressed, the mRNA may interact atypically with the ribosomes. Table 2 summarizes the major characteristics of these ORFs, and the deduced polypeptide sequences are shown in Fig. 2 with the DNA sequence. The putative polypeptides all have basic pI's, and together their coding sequences occupy 70.7% of the nucleotide sequence. The total percent G+C content is slightly higher in the potential coding regions than in the noncoding regions, 24.19 versus 21.41%.

Computer searches were performed to look for similarity between these ORFs and any known proteins in the GenPept, EMBL, and Swiss-Prot data bases. In addition, these sequences were examined by using a computer program devel-

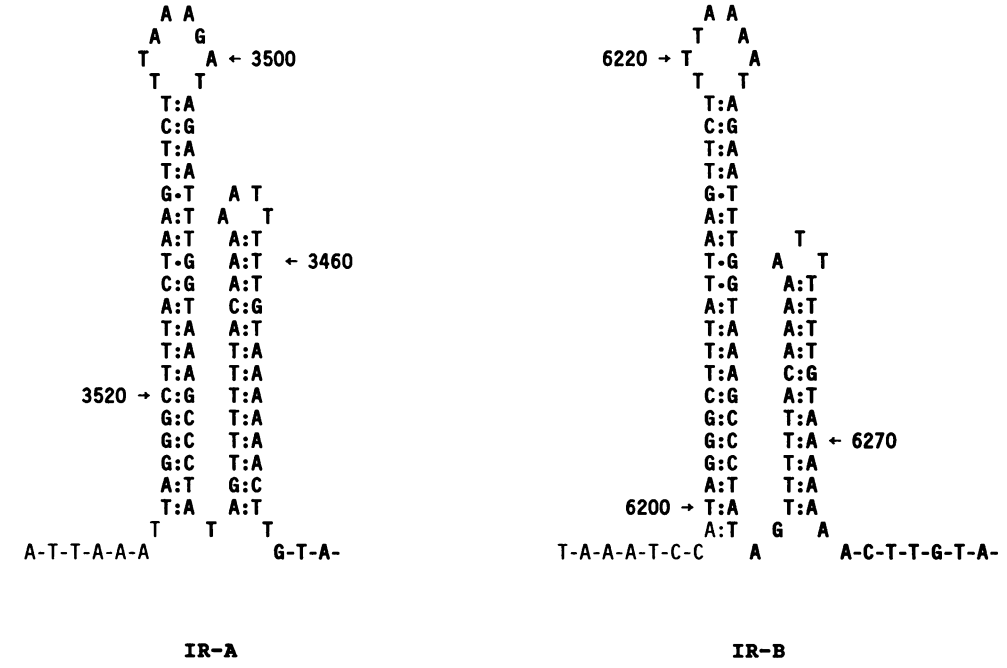

FIG. 5. Potential stem-and-loop structures at the ends of IR-A and IR-B. Both sequences are written 5′ to 3′, and the repeated sequences are boldfaced.

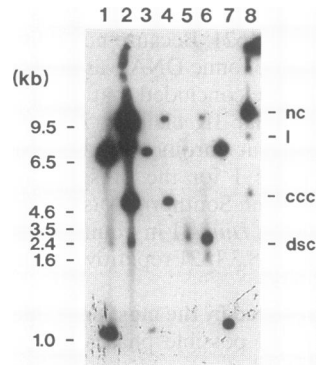

FIG. 6. In situ Southern blot of agarose gel-separated total and purified plasmid DNA from *B. burgdorferi* Ip21 DNA hybridized with oligonucleotide 2443 as the probe. Lanes: 1 and 2, purified total linear and circular plasmid preparations digested with *Hin*dIII or undigested, respectively; 3 and 4, gel-purified cp8.3/Ip21 digested with *Hin*dIII or undigested, respectively; 5 and 6, gel-purified denatured cp8.3/Ip21 digested with *Hin*dIII or undigested, respectively; 7 and 8, total genomic Ip21 DNA prepared in agarose plugs digested with *Hin*dIII or undigested, respectively. The sizes are indicated on the left; the forms of the DNA are indicated on the right. ccc, covalently closed supercoiled circle; nc, nicked circle; dsc, denatured supercoiled circle; l, linear plasmid.

oped by S. Zhang for known structural motifs in the Prosite data base (3). These comparisons revealed that the 62-amino-acid polypeptide predicted by ORF-9 begins with an N-terminal sequence that has several features in common with the signal sequences of bacterial lipoproteins. The initiating methionine is followed closely by two positively charged residues, a hydrophobic core, and a sequence (I-N-S-C) that resembles the canonical lipidation and proteolytic processing site for signal peptidase II (L-X-Y-C), where X and Y generally are amino acids with small, nonpolar side chains (56). Further work will show whether asparagine in the sequence (I-N-S-C) hinders lipidation or proteolytic processing of the nearby cysteine during translation of ORF-9. Cleavage at this cysteine residue by signal peptidase II would produce a polypeptide 45 amino acids long that has no sequence homology with the known *Borrelia* lipoproteins, OspA, OspB, OspC, and OspD, or the variable major proteins of *B. hermsii*.

Recently, Giladi et al. (24) screened a recombinant *Borrelia* library for sequences that could facilitate export of an alkaline phosphatase fusion protein to the periplasmic space of *E. coli*. They identified a new gene, pBb244, that is present on a small circular plasmid, cp9, found in a low-passage, virulent *B. burgdorferi* B31 strain. DNA sequence analysis of pBb244 indicates that the gene product should be present in or between the *Borrelia* cell's inner and outer membranes, since the encoded protein begins with an amino acid sequence that has features characteristic of bacterial leader peptidase I signal sequences. None of cp8.3/Ip21's ORFs shows homology with pBb244, and except for ORF-2, none of the predicted polypeptides showed significant homology with known proteins. ORF-2 encodes a 184-amino-acid polypeptide with limited homology to the replication protein, RepC, of pCB101, a plasmid found in the gram-positive bacterium *Clostridium butyricum* (accession number X62684 in GenBank). This homology (Fig. 7) is most pronounced at the putative active site of RepC, which itself shows significant conservation of amino acid sequence with the active site present in Rep proteins of the pC194 group of plasmids (50), plasmids known to replicate by a rolling circle (RC) mechanism. Therefore, we searched the cp8.3/Ip21 sequence for the nick site sequence CTTGATA, which is found at the double-stranded origin of most members of the pC194 group of plasmids. Exact matches to this heptameric motif were found at three positions (Fig. 2): twice on one strand of the DNA (nt 1104 to 1110 and 7190 to 7196) and once on the other strand (nt 3159 to 3165). This result suggests that double-strand synthesis may be initiated on both strands of cp8.3/Ip21.

We attempted to detect single-stranded cp8.3/Ip21 plasmid DNA in our plasmid preparations by the method of te Riele et al. (53), in which DNA is electrophoresed on agarose gels, transferred to nitrocellulose without prior denaturation, and then probed with a radiolabeled oligonucleotide. In the absence of denaturation, only single-stranded nucleic acids bind to the filter. Two different complementary 24-nt oligonucleotides (1801 and 1802 [Fig. 1C]) were used in an attempt to determine the polarity of any positive signal. Equal amounts of hybridization signal were associated with a single band migrating faster than covalently closed cp8.3/Ip21 with each oligonucleotide probe (data not shown). Because the signal was in the same region of the gel to which irreversibly denatured forms of the cp8.3/Ip21 plasmid would migrate, we decided to look for a similar signal in total DNA preparations obtained by lysing cells embedded in agarose using conditions which should not denature supercoiled plasmids. The results (Fig. 6, lanes 7 and 8) indicate that such whole-cell DNA preparations do not

TABLE 2. Most-probable ORFs in cp8.3/Ip21

ORF	Potential ribosome binding site[a]	Product (amino acids)	Stop codon	Predicted mol of product	Predicted pI of product	Location (nt)	% G+C content
1	TTTAAGGAGCTATAAATG	371	TAA	44,769	10.67	3756→4871	21.56
2	ATATAAGGATTAAACATG	184	TAA	22,213	10.65	4881→5435	21.92
3	TAAAGGAGATTTTTTATG	186	TAA	22,350	10.32	5531→6091	20.97
4	TTCTAGGAGATAAAAATG	266	TAA	31,245	9.96	6346→7146	26.07
5	AGAGGCAATATTAATATG	78	TAA	9,780	10.53	7556→7792	23.93
6	AAGTGGAGTTAATTAATG	261	TAA	30,558	10.77	1193→1978	24.90
7a	GGGCTAAAAGATTTAATG	343	TAG	39,977	9.50	2069←3100	26.82
7b	TGAAGAGAATTTATAATG	323	TAG	37,543	9.51	2069←3040	26.93
8	TTCTAGGAGATAAAAATG	88	TAG	10,349	10.02	3115←3381	28.41
9	GTTTGGAGAAAATTCATG	62	TAA	7,425	9.43	7903←8091	24.19
X	TAAATTCTTTTTATAATG	108	TAA	12,466	10.63	431→757	24.69

[a] Nucleotides that would be expected to pair with the end of *B. burgdorferi's* 16S rRNA sequence (3′ OH-UUUCCUCCACUA · · · 5′) (49) and the initiation codon are underlined.

φX174	Ala Lys Tyr Val Asn Lys Lys Ser Asp
pTD1	Ala Lys Tyr Ser Val Lys Ala Thr Asp
pCB101	Phe Lys Tyr Met Thr Lys Val Thr Gly
cp8.3/Ip21	Lys Lys Tyr Ser Asp Lys Gly Leu Ile

FIG. 7. Alignment of ORF-2 with the amino acid sequences at the active site of Rep proteins of the pC194 group of plasmids. The consensus sequence at the active site is GluXXLysTyrXValLysXXAsp (50).

contain irreversibly denatured forms of cp8.3/Ip21 DNA; the majority of the cp8.3/Ip21 appears to have been converted to a band with the mobility of a relaxed circle. Furthermore, no signal was apparent from the region of the gel below the position of the supercoiled monomeric plasmid, a result which indicates that, if single-stranded plasmid DNA is present normally, it is below the level needed to give an easily detectable hybridization signal.

Other features of the nucleotide sequence. In addition to the putative promoters found in IR-A and IR-B, close matches to appropriately separated −35 and −10 consensus sequences were found at three other locations (Fig. 2). Positioned between ORF-6 and ORF-7 is a potential transcription terminator-like sequence with a GC-rich region of dyad symmetry capable of forming a 9-bp stem-loop structure which is preceded by and then followed by a string of T-rich residues. Interestingly, ORF-6 is encoded by the DNA strand opposite to that which encodes ORF-7. Termination at this site may be necessary to balance the expression of ORF-6 and ORF-7. Other regions of the sequence having the potential to form stem-loop structures are found mainly between bp 7710 and 250. Such structures are characteristic of minus origins of replication in plasmids which replicate by an RC-type mechanism.

Conclusion and discussion. We isolated and characterized at the nucleotide level a small cryptic plasmid, cp8.3/Ip21, found in *B. burgdorferi* Ip21, which shares several features with plasmid pBB1, studied by Schwan and coworkers (47, 51). In addition to having similar sizes, both plasmids contain a single *Eco*RI site and appear to be unclonable in λ vectors. We do not know if there is extensive homology between these plasmids; however, because there is no *Acc*I site in cp8.3/Ip21 while pBB1 contains at least two, these plasmids should be able to be readily differentiated in isolates. Both cp8.3/Ip21 and pBB1 are also similar in size to a circular plasmid, pCT1, isolated from *I. scapularis* (*dammini*) collected in northern Wisconsin (29). Restriction enzyme analysis indicates that pCT1 is a dimer composed of two tandem copies of a 4.6-kb DNA. Although no sequence information is available, the pCT1 restriction map is markedly different from those of cp8.3/Ip21 and pBB1. Further work will be needed to determine if these plasmids and the 9.0-kb plasmid cp9, recently reported for virulent *B. burgdorferi* B31 (24), are all part of a related family of plasmids sharing regions of sequence homology and function. Our analysis did not reveal significant homology between the pBb244 gene on cp9 and any ORF of cp8.3/Ip21. This result may portend significant differences in coding sequences carried by these plasmids in terms of geographic locations of the isolates and the tick species in which they were found. In this regard it should be noted that strains Ip21 and B31 belong to separate genospecies (8) and that strain Ip21 should be considered to be a member of the *B. burgdorferi* sensu lato complex of spirochetes.

Currently, there is no known mechanism for genetic transfer in borrelia, and plasmid shuttle vectors that could be used for gene transfer have not been developed. Presumably, integrative or replicating plasmids able to transform borrelia could be constructed by using as the DNA backbone all or part of one of borrelia's linear plasmids or cryptic circular plasmids. Such vectors would be extremely useful and allow the use of powerful molecular approaches to evaluate the contributions of individual genes to the physiology and pathogenicity of this organism. Although many plasmids from gram-negative and gram-positive bacteria have been sequenced and their mechanisms of replication have been elucidated, there has been only one reported complete sequence analysis of a plasmid from a spirochete. MacDougall et al. (35) determined the complete nucleotide sequence of a 2,647-bp cryptic plasmid, pTD1, from the oral spirochete *Treponema denticola* (30). They showed that pTD1 has several features in common with plasmids typically found in gram-positive cells which usually replicate by an RC mechanism via single-stranded intermediates rather than the theta-form mechanism most gram-negative plasmids use to replicate (16, 25, 50). However, whether pTD1 can be used to develop a vector for gene transfer to borrelia is problematic. Even if pTD1 encodes all the proteins needed to initiate its own replication, these proteins might not be expressed in borrelia, because the ribosome binding sites upstream of pTD1's two ORFs probably would not function in borrelia. The sequence at the 3′ end of the 16S rRNA of *T. denticola* (3′ OH-UUUGCAUGGA· · · 5′ [42]) is quite different from the sequence at the end of *B. burgdorferi* 16S rRNA (3′ OH-UUUCCUCCAC· · · 5′ [49]).

Ip21's cp8.3/Ip21 plasmid appears to be lost rather readily on routine passage in vitro, and before this plasmid can be used as a genetic tool, several obstacles will have to be overcome. If cp8.3/Ip21 does belong to the large family of double-stranded circular DNAs that replicate via a single-stranded DNA intermediate, it probably uses an RC pathway that first generates a circular plus-strand DNA which is then converted to double-stranded DNA by complementary-strand synthesis initiated at an origin located elsewhere on the single-stranded DNA circle. This RC mode of replication is known to lead to high frequencies of both homologous recombination and illegitimate recombination (39, 40). Furthermore, if two plus-strand origins are present on the same replicon, initiation can occur at one origin and termination can take place at the other, generating two smaller molecules that may or may not contain active signals for minus-strand synthesis or further rounds of plus-stand synthesis. This feature of RC plasmid replication may be responsible for loss of cp8.3/Ip21 in vitro in the absence of selective pressure. If regions leading to instability can be identified, perhaps they can be modified, thereby stabilizing the inheritance of the plasmid. Alternatively, an antibiotic resistance gene or other selectable marker can be introduced into the DNA to allow positive selection for cells that maintain the plasmid in culture. This may be feasible since the plasmid contains several unique restriction sites. A stabilization cassette, such as the *parB* locus of plasmid R1, also may have to be added to stabilize the plasmid (23). Eventually, it should be possible to use this plasmid as a vector for gene disruption or, alternatively, if it can be shown that the IR elements of cp8.3/Ip21 promote insertion or recombination of plasmid DNA with other molecules, as a vehicle to begin genetic mapping in borrelia. In ongoing experiments we are examining the mechanism by which cp8.3/Ip21 is lost on in vitro passage and attempting to devise techniques to select integrants in the resulting population of spirochetes.

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