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NOTES

The Cryptic *ospC* Gene of *Borrelia burgdorferi* B31 Is Located on a Circular Plasmid

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***Borrelia burgdorferi* B31 cells lacking all linear plasmids or all but the 49-kb linear plasmid expressed the otherwise silent gene for the outer membrane protein OspC. In the first demonstration of a function for a circular plasmid of *Borrelia* spp., *ospC* was located on a 27-kb circular plasmid of B31.**

Genus *Borrelia* spirochetes have a linear chromosome, linear plasmids, and circular plasmids (1, 2, 4, 5, 12). These replicons are present in approximately equal copy numbers (7, 11), and the linear plasmids have hairpin telomeres (3, 6). In *Borrelia burgdorferi*, the cause of Lyme disease, and *Borrelia hermsii*, an agent of relapsing fever, linear plasmids encode the surface-exposed lipoproteins that determine serotype (3, 10). In strain B31 (ATCC 35210) *B. burgdorferi*, a North American isolate, the operon for the lipoproteins OspA and OspB is located on a 49-kb linear plasmid, lp49 (2). The gene for another outer membrane lipoprotein, OspD, was found on a 38-kb linear plasmid, lp38, in low-passage B31 (14). First lp38 and then lp29, a 29-kb linear plasmid, were lost from this lineage during serial passage in broth medium (1, 7). The location of the gene for a fourth outer membrane protein, OspC, has not been known (20). Under laboratory conditions, the *ospC* gene is commonly expressed in European strains of *B. burgdorferi* but not in most North American strains, including B31 (20).

No function has been demonstrated for any of the circular plasmids of *Borrelia* species. Supercoiled plasmids of about 8 kb are commonly found in initial isolates of North American Strains of *B. burgdorferi* but are soon lost during in vitro cultivation (9, 17, 18). More persistent in cultivated strains have been larger circular plasmids (2, 17, 18). High-passage isolates of the B31 lineage still had circular plasmids of 27 kb (cp27) and 30 kb (cp30) (1, 2, 7). Our previous research focused on the linear replicons of *Borrelia* spp.; for the present study, attention was also focused on the circular plasmids.

The *B. burgdorferi* isolates used for this study were of the B31 lineage (Table 1) and were grown in BSKII broth or solid medium as described before (6). The plasmid complements of high-passage isolates B311, B312, and B313 had been determined previously but under different designations (1, 2, 7, 16). The low-passage infectious isolate for this lineage retains the original strain designation (1). With the exception of B31, the isolates were derived from single-cell clones.

Our first intent was to isolate cells without any of the known linear plasmids, and for this purpose we started with B312, which had only lp49. We had previously used antibodies to OspA or OspB to select B313 from a population of B311 cells (16). For this purpose again, we used, at 1:100 dilutions, ascitic fluids of murine monoclonal antibody H5332, which is specific for OspA, together with antibodies H6831 and H614, which recognize different OspB epitopes, for selection of mutants in sealed microtiter plates containing broth medium (16). Mutants resistant to this antibody mixture were present in the population at a frequency of 2×10^{-7} to 5×10^{-7}, as calculated from Poisson distribution tables (16). Mutants were examined by Western immunoblot analysis with the aforementioned antibodies to identify those lacking OspA and OspB by using alkaline phosphatase-conjugated recombinant protein A/G (Pierce Chemical Co., Rockford, Ill.) and blot conditions described previously (16).

Another characteristic of these $OspA^-$ $OspB^-$ derivatives of B312 was their failure to grow on solid medium. The efficiency of plating was 0.0001% instead of the expected 50 to 100% for B311, B312, and B313 (7, 16). Consequently, a second clonal population of one of these mutants, B314, was obtained by limiting dilutions in broth medium (16). The plasmid content of B314 was compared with that of B313 by DNA extraction with diethylpyrocarbonate and sodium deoxycholate followed by low-voltage constant-field electrophoresis in a 0.2% agarose gel as described before (2, 7). The locations of lp49, lp16, and cp27 in such gels had been identified with DNA probes specific for these replicons (7) and by use of CsCl gradient centrifugation to isolate cp27 and cp30 (2, 4). B314 was found to have the smallest genome

TABLE 1. Isolates of the B31 lineage of *B. burgdorferi* and their plasmid contents

Isolate	Plasmid[a]						Reference
	lp16	lp29	lp38	lp49	cp27	cp30	
B31	+	+	+	+	+	+	2
B311	+	−	−	+	+	+	7
B312	−	−	−	+	+	+	7
B313	+	−	−	−	+	+	15
B314	−	−	−	−	+	+	This study

[a] Presence (+) or absence (−) of given plasmid in isolate.

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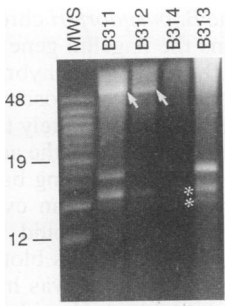

FIG. 1. Ethidium bromide-stained 0.2% agarose gel of plasmid-enriched DNA from *B. burgdorferi* isolates B311, B312, B313, and B314. Arrows point to lp49, and the two asterisks indicate the locations of cp27 and cp30 in the DNA from each isolate. Sizes (in kilobases) are shown to the left. MWS, size standards.

of the B31 lineage strains; it lacked all discernible linear plasmids. Like the other isolates, B314 retained the two circular plasmids, which varied in relative amounts (Fig. 1). The absence of *ospA* in B314 but not B312 was confirmed by Southern blot analysis with *Eco*RI-digested total DNA and the *ospA*- and lp49-specific DNA probe pTRH43 (8) as described before (2, 16) (data not shown).

To further characterize B314 and the other isolates, whole-cell lysates were subjected to polyacrylamide gel electrophoresis (12.5% acrylamide). Figure 2 (panel CB) shows, as expected, that B313 and B314 lacked OspA and OspB; these major proteins had apparent sizes of 31 and 33 kDa, respectively, in B311. The slightly greater apparent size of the OspB from low-passage B31 in comparison to that of OspB from higher-passage isolates has been noted before (3). The figure also shows the presence in B312 and B314 but not in B31, B311, or B313 of an abundant protein with an

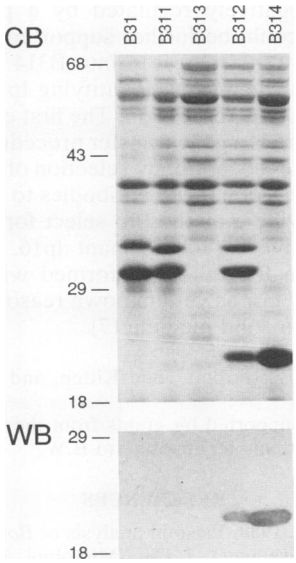

FIG. 2. Coomassie blue-stained polyacrylamide gel (CB) and Western blot analysis (WB) of whole-cell lysates of *B. burgdorferi* isolates B31, B311, B312, B313, and B314. The OspC-specific monoclonal antibody L22 1F8 was used for the Western blot (20). The size standards were bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18 kDa).

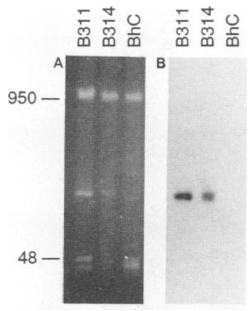

FIG. 3. Ethidium bromide-stained agarose gel (A) and Southern blot analysis (B) of total DNA from isolates B311 and B314 of *B. burgdorferi* and serotype C *B. hermsii* HS1 (BhC). Borrelias were lysed in situ in agarose blocks, and the DNA was separated by transverse alternating-field electrophoresis as described in the text. The blot was probed with the recombinant plasmid pUC-B31/ospCL+, containing the *ospC* gene of B31 (20). *S. cerevisiae* chromosome (950 kb) and bacteriophage lambda (48 kb) were used as size markers.

apparent size of 22 kDa. Inasmuch as B31 was known to carry a gene for OspC, albeit silent (20), we asked whether it was now detectably expressed in B312 and B314. Accordingly, we used the OspC-specific monoclonal antibodies L22 1F8 and L22 1C3 as hybridoma supernatants at 1:10 dilutions in Western blot analyses (20). Figure 2 (panel WB) shows the binding of L22 1F8 to the new 22-kDa protein in B312 and B314 but not to proteins in B31, B311, or B313; the same results were obtained with antibody L22 1C3.

The usually cryptic *ospC* gene was expressed in detectable amounts when lp16 was absent from the B31 lineage. The production of OspC by B314, which lacked all known linear plasmids, indicated that *ospC* was present either on the chromosome or on one of the remaining circular plasmids. This proposition was examined by pulsed-field gel electrophoresis. Preparation of agarose blocks and in situ cell lysis were done as described before (5, 11). Transverse-field alternating gel electrophoresis (TAFE) with the Beckman Geneline system (Fremont, Calif.) was carried out under conditions (60-s pulse time at 180 mA constant current in 1% agarose) in which cp27 and cp30 migrate equidistantly from the 1-megabase linear chromosome and the linear plasmids of 16 to 49 kb (5) (Fig. 3A). B311 and B314 were examined; *B. hermsii* HS1 (ATCC 35209), which was also grown in BSKII medium, served as a negative control. Size markers were *Saccharomyces cerevisiae* chromosomes obtained from Beckman and high-molecular-weight standards obtained from Bethesda Research Laboratories (Gaithersburg, Md.). The gel was probed in a Southern blot analysis under conditions described before (7) with plasmid pUC-B31/ospCL+, which contains the *ospC* gene of strain B31 (20). This and subsequent probes were labeled by random priming with a commercial kit (Boehringer-Mannheim, Indianapolis, Ind.). Figure 3B shows the hybridization of the *ospC* probe to bands of B311 and B314 but not of *B. hermsii*. The locations of the only hybridizing bands were characteristic of the circular plasmids but not the chromosome (5).

To determine which of the two circular plasmids had the *ospC* gene, we used low-percentage agarose electrophoresis of plasmid-enriched B314 DNA under the same conditions as for the experiments shown in Fig. 1. A blot of the gel was hybridized with the aforementioned *ospC* probe and recombinant plasmid pBC27a, which had been shown to be specific

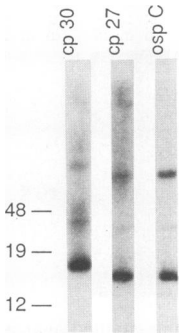

FIG. 4. Southern blot analysis of plasmid-enriched DNA from *B. burgdorferi* B314 with radiolabeled probes for *ospC*, cp27, and cp30. The probes for *ospC* and cp27 were recombinant plasmids pUC-B31/ospCL+ (20) and pBC27a (7), respectively. The probe for cp30 was total cp30 DNA extracted from a gel. Sizes are shown in kilobases.

for cp27 (7). As a probe for cp30, we extracted plasmid DNA directly from duplicate 0.2% agarose gels and labeled it by random priming as described before (5). Figure 4 shows that probes for *ospC* and cp27 bound to bands with the same migration rate; cp30, as expected, migrated more slowly in the gel. The more faintly hybridizing bands above the major bands in the blot probably represent open circular forms of cp27 and cp30 (5).

To confirm the circularity of the *ospC*-bearing plasmid, we used two-dimensional agarose gel electrophoresis of DNA from B312; the retardation of circular plasmids in relation to linear plasmids in two-dimensional gels has been demonstrated (5, 14). For the first dimension, TAFE was performed in 1% agarose at a constant current of 180 mA with pulse times of 1 s for 9 h and then 5 s for 9 h. For the second dimension, constant-field electrophoresis at 6 V/cm was carried out for 30 min. The blot was probed with pTRH43, which contains the *ospA* gene of strain B31 (8), to locate lp49 and with pUC-B31/ospCL+ to locate the *ospC*-bearing plasmid. As a probe for the *B. burgdorferi* chromosome, we used pVF18, which contains the flagellin gene (13, 15).

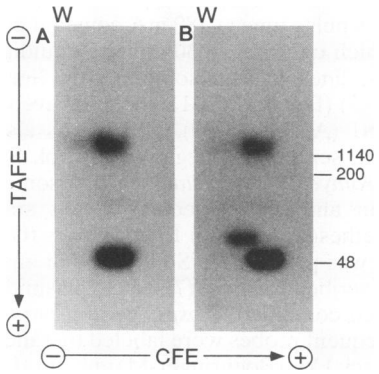

FIG. 5. Two-dimensional gel electrophoresis and Southern blot analysis of total DNA of *B. burgdorferi* B312. DNA from in situ-lysed cells was separated in the first dimension by transverse alternating-field electrophoresis (TAFE) and in the second dimension by constant-field electrophoresis (CFE) as described in the text. The location of the well (W) is shown. (A) The blot was first hybridized with probes for the chromosome (pVF18 [13]) (upper band) and for lp49 (pTRH43 [8]) (lower band). (B) The same blot was next probed with pUC-B31/ospCL+ for the *ospC* gene (20). Two *S. cerevisiae* chromosomes (1,140 and 200 kb) and bacteriophage lambda (48 kb) were used as size markers.

Figure 5A shows the blot after hybridization with the flagellin and *ospA* probes; the chromosome (upper) and lp49 (lower) bands migrated at approximately the same rate in the second dimension. Figure 5B shows the addition of the *ospC* probe to the blot; the new hybridizing band migrated more slowly in the second dimension than even the megabase-sized linear chromosome. This would be expected of a supercoiled circle (5, 14). When this blot was stripped and reprobed, the same retarded band was hybridized with the cp27 probe (not shown). Additional evidence of the circularity of the *ospC*-bearing plasmid was the different migrations of this plasmid relative to those of the linear molecules when the pulse conditions in the first dimension were changed between the gels for Fig. 4 and 5 (5).

The present study demonstrated that the usually silent or largely inactive *ospC* gene of a North American strain of *B. burgdorferi* can be expressed in vitro when one of the linear plasmids, lp16, is absent. While it is possible that a B31 cell with a full complement of plasmids expresses *ospC* at higher levels when present in a mammalian or tick host, the present findings at least allow further examination of *ospC* regulation under laboratory conditions.

In B31, *ospC* is located on circular plasmid cp27, which may be homologous to the 26-kb circular plasmid in strain Sh-2-82 described by Simpson et al. (19). This is the first outer membrane protein gene to be located on a circular plasmid, and as far as is known, this is the first function ascribed to a circular plasmid of a *Borrelia* species. Both cp27 and cp30 appear to be either highly stable or necessary for in vitro growth.

Further circumstantial evidence implicating lp16 in regulation of *ospC* was the finding that the only *B. burgdorferi* strains with sequences hybridized by total lp16 DNA were those that did not produce major proteins of the size of OspC (6, 15). European strains had this characteristic more often than North American strains (6). The proposal that *ospC* expression is negatively regulated by a protein or RNA product of lp16 could be further supported first by demonstrating that the transfer of lp16 into B314 reduced expression of *ospC* and second by identifying lp16 mutants deficient in putative *ospC* repression. The first experiment is not possible until there is a gene transfer procedure for borrelias. When that is achieved, antibody selection of OspC-less cells, similar to what was done with antibodies to OspA and OspB here, would provide a method to select for borrelias transformed with native or recombinant lp16. Another useful positive selection for cells transformed with lp16 may be growth on plates. For as yet unknown reasons, B313 but not B314 can grow on solid medium (7).

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