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Borrelia burgdorferi Supercoiled Plasmids Encode Multicopy Tandem Open Reading Frames and a Lipoprotein Gene Family

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DNA sequencing and Southern blot analyses of a *Borrelia burgdorferi* DNA fragment encoding a signal sequence led to the discovery of a genetic locus, designated 2.9, which appears to be present in at least seven copies in virulent *B. burgdorferi* 297. DNA sequence analysis of these regions revealed that each 2.9 locus contained an operon of four genes (*ABCD*) and open reading frames designated *rep*$^+$ (positive strand) and *rep*$^-$ (negative strand) which encoded multiple repeat motifs. Downstream of the *rep*$^+$ gene(s) in six of the completely cloned and sequenced 2.9 loci also were lipoprotein (*LP*) genes possessing highly similar signal sequences but encoding variable mature polypeptides. The lipoproteins could be separated into two classes on the basis of hydrophilicity profiles, sequence similarities, and reactivity with specific antibodies. The 2.9 loci were localized to two (20- and 30-kb) supercoiled plasmids in *B. burgdorferi* 297. Northern (RNA) blot analysis established that the 2.9 *ABCD* operon was only minimally expressed, whereas the *rep*$^-$ gene(s) and at least three of the seven *LP* genes were expressed by *B. burgdorferi* in vitro. A single putative promoter element was identified by RNA primer extension analysis upstream of the *ABCD* operon, whereas a number of potential promoter regions existed upstream of the *LP* genes. The combined data indicate that the *ABCD* operon, *rep*$^+$ and *rep*$^-$ genes, and *LP* genes are separately transcribed during in vitro growth. The 2.9 loci possess a repetitiveness, diversity, and complexity not previously described for *B. burgdorferi*; differential expression of these genes may facilitate the spirochete's ability to survive in diverse host environments.

Borrelia burgdorferi is the etiological agent of Lyme disease, a tick-borne spirochetosis. Now recognized as the most common arthropod-borne infection in the United States (10), Lyme disease typically proceeds in stages characterized by a wide range of clinical manifestations (47). When left untreated, the disease may become chronic, culminating in debilitating arthritis and various neurologic disorders (17, 27, 47). During the development of these clinical manifestations, there are vigorous cellular and humoral immune responses which, for unclear reasons, fail to entirely eradicate the spirochetes.

The ability of *B. burgdorferi* to cause persistent infection has prompted efforts to define the mechanisms underlying the bacterium's remarkable immunoevasiveness. Of note, *B. burgdorferi* generally is perceived as an extracellular pathogen with minimal or transitory intracellular existence (19, 23, 43). In efforts to identify potential virulence factors and molecules that may promote host dissemination and/or immune evasion, research thus far has focused upon the characterization of surface-exposed proteins and the genetic elements which encode them (40). All *B. burgdorferi* strains isolated to date contain an unusual linear chromosome and numerous extrachromosomal elements in the form of linear and supercoiled circular (SC) plasmids (15, 40). Most notably, the genes encoding all of the outer surface lipoproteins (Osps) characterized thus far have been localized to linear or circular plasmids (7, 16, 24, 32, 38). However, it has been recognized that prolonged in vitro cultivation of *B. burgdorferi* results in the concomitant loss of plasmids and virulence expression (5, 29, 31, 41). While a direct correlation between the loss of any one *B. burgdorferi* plasmid and infectivity has yet to be established, it is plausible that *B. burgdorferi* plasmids encode key virulence determinants and/or proteins involved in immune evasion. This speculation is supported by the demonstration that *B. burgdorferi* contains plasmid-encoded genes which are selectively expressed during infection of mammalian hosts (2, 11, 49, 52).

Circular plasmid-specific DNA sequences containing conserved regions and regions of variability in *B. burgdorferi* were first described by Simpson et al. (45). In their study, repetitive sequences on circular plasmids were identified by Southern hybridization (45). Zuckert et al. (53) also have described repeated DNA sequences which were localized to both 29-kb circular and 50-kb linear plasmids of *B. burgdorferi* B31. During our attempts to clone and characterize novel *B. burgdorferi* membrane-associated proteins, we discovered a gene for a presumptive cytoplasmic membrane protein (designated *2.9orfD*). Southern hybridization of restricted genomic DNA revealed multiple copies of *2.9orfD* that also were subsequently localized to two large, supercoiled plasmids. In the process of further characterizing these closely related genes, we discovered that *2.9orfD* was part of a locus consisting of four tandem open reading frames (ORFs) (*2.9orfD* being the fourth) followed by an unusual ORF containing a number of repeated motifs and, most commonly, a gene encoding a lipoprotein. Surprisingly, we found that at least seven copies of this locus could be identified on supercoiled plasmids in *B. burgdorferi* 297 and that the encoded lipoproteins could be separated into two distinct but related classes. Moreover, Northern (RNA) blot analysis revealed that there were marked differences in the levels of in vitro expression of the genes comprising the 2.9 loci, including the lipoprotein (*LP*) genes. The 2.9 loci possess a repetitiveness, diversity, and complexity not previously described for *B. burgdorferi*; differential expression of these genes

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may facilitate the spirochete's ability to survive in diverse host environments.

MATERIALS AND METHODS

Bacterial strains and plasmids. Low-passage-number *B. burgdorferi* 297, B31, and N40 were obtained from Russell Johnson (Minneapolis, Minn.), Alan Barbour (San Antonio, Tex.), and Stephen Barthold (New Haven, Conn.); they were passaged in BSKII medium (4). Virulence was confirmed by induction of carditis and arthritis following intradermal syringe inoculation of 2-week-old C3H/HeJ mice with 10^4 bacteria and/or by recovery from ear punch biopsy samples (46). *B. burgdorferi* clones, designated 297-1, N40-1, and B31-1, were generated from the respective uncloned parental strains by three rounds of limiting dilution performed as described by Bundoc and Barbour (9). All isolates were passaged not more than seven times before experimental manipulations. *Escherichia coli* DH5α (Gibco/BRL, Gaithersburg, Md.) and XL1-Blue (Stratagene, La Jolla, Calif.) were used as cloning hosts and were cultivated either in yeast-tryptone broth or on yeast-tryptone agar supplemented with the appropriate antibiotic(s). The cloning vector was either pGEX-2T (Pharmacia LKB Biotechnology, Piscataway, N.J.), pGEX-4T-2 (Pharmacia), pBluescript II KS− (Stratagene), or pBluescript II SK+ (Stratagene).

Gene library construction. *B. burgdorferi* 297 genomic libraries were constructed in plasmids pGEX-2T (Pharmacia) and pKSII/pho as described previously (2). To create pKSII/pho, the *E. coli phoA* gene lacking the signal sequence-encoding region was amplified by PCR, digested to completion with *Hin*dIII and *Pst*I, and ligated into the corresponding restriction sites of pBluescript II KS− (2). Plasmid pKSI/pho was identical to pKSII/pho except that it was constructed in an alternative reading frame. To construct plasmid pKSI/pho, a DNA fragment containing the *E. coli phoA* gene lacking the signal sequence (i.e., nucleotides 361 to 1765 of *E. coli phoA* [20]) was generated by PCR using the following primers: 5′-GGTACTGCAGCCTGTTCTGGAAAACCGGGCT-3′ and 5′-CCCAAGCTTCCATTAAGTCTGGTTGCTAAC-3′. The PCR-generated fragment was digested with *Hin*dIII and *Pst*I and then ligated into the corresponding restriction sites within the polylinker of pBluescript KS− (Stratagene). A separate genomic library was constructed in lambda-ZapII (Stratagene) by using randomly sheared total genomic DNA from uncloned low-passage-number *B. burgdorferi* 297.

Identification of *E. coli* clones harboring *B. burgdorferi* 2.9 genetic loci. *B. burgdorferi* 297 genomic libraries were screened by using two probes, designated 2.9orfD-phoA, and Lib, respectively, that were generated by PCR (Table 1). The probes were gel purified and labeled with [α-^{32}P]dCTP by using a Boehringer Mannheim (Indianapolis, Ind.) random DNA primer labeling kit according to the manufacturer's instructions.

Construction of *phoA* fusions. PCR primers, containing restriction sites (*Bam*HI and *Pst*I) for in-frame ligation to the pKSI/pho fusion vector (Table 1), were derived from the *2.9-1LP* gene such that the resulting product encoded a putative promoter element, a ribosomal binding site, and the first 84 amino acids encoded by the structural gene. The construct and resultant *phoA* fusion (designated *2.9-1LP-phoA*) was verified by nucleotide sequence analysis (see below).

Labeling of PhoA$^+$ clones with [^{3}H]palmitate. *E. coli* DH5α clones harboring *phoA* gene fusions were inoculated into 10 ml of minimal medium containing 100 μg of ampicillin per ml. The cultures were incubated overnight at 37°C with shaking. A 50-μl volume of the overnight culture was inoculated into 5 ml of minimal medium, and the culture was incubated at 37°C with agitation until the optical density at 600 nm reached 0.2. Approximately 100 μCi of [9,10(n)-^{3}H] palmitate was then added, and cultures were incubated until the optical density at 600 nm reached approximately 1.0. Labeled bacteria were collected by centrifugation and washed twice in phosphate-buffered saline (PBS; pH 7.4), and pellets were suspended in final sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 0.001% [vol/vol] bromophenol blue) for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and fluorography.

Generation of fusion proteins and polyclonal antisera. Glutathione *S*-transferase (GST) fusions of the lipoproteins (designated 2.9-1LP, 2.9-2LP, 2.9-3LP, and 2.9-4LP) were generated by PCR amplification of the DNA encoding the predicted mature portion of each protein (see Table 1 for PCR primer pairs). PCR products were then ligated into the appropriate polylinker sites of pGEX-4T-2. The resultant fusions were purified by affinity chromatography on an agarose-glutathione matrix as instructed by the manufacturer (Pharmacia). To generate polyclonal antisera, 4- to 6-week-old Sprague-Dawley rats were primed by intraperitoneal injection with 100 μg of purified fusion protein in a 1:1 mixture of PBS and Freund's complete adjuvant. This was followed at 2-week intervals by intraperitoneal booster injections with 25 μg of purified protein in a 1:1 mixture of PBS and incomplete Freund's adjuvant.

Processing experiments in *E. coli* minicells. *E. coli* ORN 103 was transformed with a pKSII/pho plasmid construct encoding 65 amino acids (signal peptide and partial mature protein) encoded by the gene designated *2.9orfD* (see Results and Discussion). Processing experiments were performed in *E. coli* minicells as previously described (50, 51).

SDS-PAGE and immunoblotting. Samples for protein analysis were boiled for 5 min in final sample buffer prior to electrophoresis through 2.4% stacking and 12.5% polyacrylamide separating gels. Gels were then either stained with Coomassie brilliant blue (prior to fluorography) or transferred electrophoretically to a 0.2-μm-pore-size nitrocellulose filter (Schleicher & Schuell, Keene, N.H.) for immunoblotting. Immunoblots were incubated with 1:100 dilutions of rat polyclonal antisera or a 1:500 dilution of purified murine anti-alkaline phosphatase monoclonal antibody (Caltag Laboratories, San Francisco, Calif.) and then sequentially incubated with 1:1,000 dilutions of either rabbit anti-rat or goat anti-mouse immunoglobulin G–horseradish perioxidase conjugates and goat anti-rabbit or rabbit anti-goat immunoglobulin G–horseradish peroxidase conjugates (Zymed, San Francisco, Calif.). Immunoblots were developed by using 4-chloro-1-naphthol as the substrate.

Southern hybridization analysis. Probes for *ospB*, *ospC*, *ospF*, *fla*, *bbk2.10*, *orfA*, *orfD*, *2.9-1LP*, *2.9-2LP*, *2.9-3LP*, *2.9-4LP*, *2.9-5LP*, *2.9-7LPA*, *2.9-7LPB*, *rep*$^+$, and *rev*$^-$ for use in either DNA or RNA hybridization experiments were generated by PCR using the primer pairs listed in Table 1. The resulting products were gel purified and labeled with [α-^{32}P]dCTP by using a Boehringer Mannheim random DNA primer labeling kit. Individual oligonucleotide probes (Table 1) also were 5′ end labeled with [γ-^{32}P]ATP by using a Boehringer Mannheim 5′ end labeling kit. *B. burgdorferi* supercoiled plasmids were isolated by a previously described CsCl density gradient purification method (45); for electrophoretic analysis, 20 ng was loaded per well in 0.4% agarose gels. *B. burgdorferi* total DNA was isolated by using a DNA extraction kit (Stratagene) as recommended by the manufacturer. Total DNAs from *B. burgdorferi* 297-1, N40-1, and B31-1 were digested to completion with either *Eco*RI, *Bam*HI, or *Xba*I; 8-μg quantities of each sample were then loaded into wells of 1% (26-well) agarose gels. After electrophoresis, gels were stained with ethidium bromide, photographed, and destained. Gels were partially depurinated by soaking in 0.25 M HCl with shaking for 30 min at room temperature. The gels were subsequently denatured for 30 min in 1.5 M NaCl–0.5 M NaOH with constant agitation, rinsed in water, and neutralized for 1 h with 1 M Tris (pH 7.4)–1.5 M NaCl. DNA in the gels was then transferred to nylon membranes. Individual nylon strips containing the purified SC plasmids and the restricted total genomic DNAs were hybridized overnight in 2× Denhardt's solution–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–1 mM EDTA–100 μg of sonicated salmon sperm DNA per ml at 65°C, using a rotating hybridization oven (Robbin's Scientific Corp., Sunnyvale, Calif.). After hybridization, nitrocellulose filters were washed twice for 15 min each time at 65°C in 2× SSC containing 0.1% SDS and then washed four times at room temperature in 0.1× SSC containing 0.1% SDS. Filters then were subjected to autoradiography at −70°C for 4 to 48 h. To enable precise comparisons of identical Southern blot gels (hybridized with different probes), all filters subsequently were stripped multiple times in 50% formamide–2× SSPE (1× SSPE is 0.6 M NaCl, 0.04 M monobasic sodium phosphate, and 4 mM EDTA) for 4 h at 65°C, rinsed briefly with 0.1× SSPE, and analyzed on a PhosphorImager to ensure removal of the radioactive probes before reprobing.

The specificities of the probes used to detect the 2.9 *LP* genes in *B. burgdorferi* were confirmed by performing standard dot blot Southern analyses using plasmid DNAs from the respective *E. coli* clones. In these assays, purified plasmid DNAs were cleaved with *Eco*RI and *Bam*HI, loaded into a 96-well vacuum (transfer) device, and cross-linked to nylon filters by using standard protocols. Nylon filters then were cut into strips such that each strip contained the entire panel of the cloned 2.9 loci (all containing different *LP* genes). The strips were hybridized with individual gene probes specific for each *LP* gene (see Fig. 2), using hybridization conditions identical to those described above.

Pulsed-field gel electrophoresis analysis. Agarose blocks containing cloned 297-1 were prepared from spirochetes grown to a density of 10^8 organisms per ml as described by Ferdows and Barbour (15). Solidified plugs were recovered from the molds, transferred to petri dishes, and then incubated for 18 to 24 h at 37°C in lysis buffer containing 1 mg of proteinase K per ml and 1% (wt/vol) sodium lauroyl sarcosinate. Lysis buffer was decanted from the plugs and replaced with a solution of 10 mM Tris (pH 8.0)–1 mM EDTA (TE); plugs were then stored at 4°C. Prior to pulsed-field gel electrophoresis, plugs containing approximately 5×10^6 spirochetes were washed twice for 1 h each time in TE buffer and loaded into wells of 1.2% agarose gels immersed in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA [pH 8.0]). Molten 1.2% agarose was overlaid to seal the plugs into the wells. Constant homogeneous electric field electrophoresis was carried out in a DRIII apparatus (Bio-Rad, Palo Alto, Calif.) at 9°C in 0.5× TBE with continuous buffer recirculation. Gels were subjected to electrophoresis at 6 V/cm with 5-s pulses for 2 h followed by 0.5 to 1.5 s of ramping for an additional 18 h. For two-dimensional gel analyses, gels were rotated 90° and subjected to constant field electrophoresis (6 V/cm) for an additional 2 h. Gels were then stained for 30 min in 1 μg of ethidium bromide per ml and irradiated with 60 mJ of UV light in a Stratalinker 1800 apparatus (Stratagene) to enhance capillary transfer of large DNA fragments. Separated DNAs were then transferred to 0.2-μm-pore-size nylon membranes (Hoefer Scientific, San Francisco, Calif.) and were UV cross-linked for subsequent Southern hybridizations. Probes were labeled and hybridized to membranes as described above for 16 h in a rotating oven (Robbin's Scientific Corp.). Washed membranes were covered in plastic wrap and exposed to X-ray film at −70°C. Lambda bacteriophage multimers (Clontech, Palo Alto, Calif.) and high-molecular-weight DNA (Gibco/BRL) were used as molecular weight standards.

Northern blot analysis. RNA was extracted from 10^{11} clone 297-1 organisms harvested at mid-log phase and collected by centrifugation. The resulting pellet was washed once with PBS and then extracted with RNAzol B as recommended

TABLE 1. Oligonucleotide primers and probes used in this study

Designation	Sequence	Purpose	Source; complementary nucleotides
phoA-seq	5′-GTGCAGTAATATCGCCCTGAG-3′	pKSII/pho sequencing primer	*E. coli phoA*; 386–402
ospF-270	5′-GGCTAAAAAAACTTCTCTTAAAACATATTCTGAGTATGAAG-3′	*ospF* probe	*ospF*; 270–310
pGEX-seq	5′-CCTTTGCAGGGCTGGCAAGC-3′	pGEX-2T sequencing primer	pGEX-4T; 861–880
2.10-3 spec	5′-AAACACCTTGAGCCACTTGCTC-3′	*bbk2.10* probe	2.10; 713–692
2.10-5 spec	5′-AAAAAAGAAGAGTTGGTTGGTGGTTT-3′	*bbk2.10* probe	2.10; 590–615
ospB 3′	5′-CTAGCTGATGCCTTGTAGGG-3′	*ospB* probe	*ospB*; 2006–1887
ospB 5′	5′-TGTGCACAAAAAGGTGCTGAG-3′	*ospB* probe	*ospB*; 1027–1047
ospC 3′E	5′-ATAGAATTCCTTATTAAGGTTTTTTTGGACTTTCTGC-3′	*ospC* probe	*ospC*; 751–778
ospC 5′B	5′-ATAGGATCCAATAATTCAGGGAAAGGTGGGGAT-3′	*ospC* probe	*ospC*; 187–216
fla 3′	5′-AAACTCCTCAATAAGCCTGC-3′	*fla* probe	*fla*; 1161–1142
fla 5′	5′-ATGATTATCAATCATAATAC-3′	*fla* probe	*fla*; 1–20
2.9orfD-phoA 3′	5′-TGGCAATAGTGGTGCAACATAG-3′	*orfD* signal sequence probe	2.9-1; 1046–1067
2.9orfD-phoA 5′	5′-ATATCAAAATACTTAAAAGAGG-3′	*orfD* signal sequence probe	2.9-1; 980–1001
orfD 3′	5′-ATTATAGACTTTTTCCGCTATTGG-3′	*orfD* probe	2.9-1; 1313–1336
orfD 5′	5′-TTGCTTTGCTACTATGTTGCAC-3′	*orfD* probe	2.9-1; 1035–1056
rep 3′	5′-CGGAATTCATGTTTTAAGTGAGGTAGTAG-3′	*rep* probe	2.9-1; 2020–2048
rep 5′	5′-CGGGATCCGAAACAGTGTCAACAAATATTGCAAG-3′	*rep* probe	2.9-1; 1468–1493
LIB 3′	5′-CATAACATACTCTGAAAGTTGTG-3′	Library probe	2.9-1; 1131–1153
LIB 5′	5′-TATAAGCACACTAAAATCTATGG-3′	Library probe	2.9-1; 813–835
AB 3′	5′-TTATTCTTTCCTTATAGATAGG-3′	*orfAB* probe	2.9-1; 654–675
AB 5′	5′-CTTCTCAAGCCTTTATTAAAAG-3′	*orfAB* probe	2.9-1; 237–258
Intg 3′	5′-GTGTCATTATCATTAGAATTAC-3′	Intergenic region probe	2.9-1; 2176–2198
Intg 5′	5′-ATTATGGGAGCAATGTCTATAAC-3′	Intergenic region probe	2.9-1; 1957–1979
2.9-1LP-phoA 3′	5′-TTCCAATGCATTGGCTGCAGGCTACACTCTTGCATATCATCGTATAG-3′	2.9-1LP-PhoA fusion	2.9-1; 2350–2400
2.9-1LP-phoA 5′	5′-CGGGATCCGCCAAAATAGACAATGTGGAAAAG-3′	2.9-1LP-PhoA fusion	2.9-1; 1891–1914
2.9-1LP 3′ spec	5′-TATATCACCAGCGACACCTC-3′	2.9-1LP probe	2.9-1; 2624–2643
2.9-1LP 5′ spec	5′-AGTGTAGCAATGGAAATAAG-3′	2.9-1LP probe	2.9-1; 2369–2388
2.9-1LP 3′ Eco	5′-CGGAATTCGACCAGCATAATGGTTGTTTTC-3′	2.9-1LP-GST fusion	2.9-1; 2701–2722
2.9-1LP 5′ Bam	5′-CGGGATCCTTAAGCCAAGAAGAGTTGCCAC-3′	2.9-1LP-GST fusion	2.9-1; 2239–2266
rev 3′	5′-GAAGAAAAGAAACAAATAGATTCATTG-3′	*rev* probe	2.9-7; 1881–1907
rev 5′	5′-TCTTGCCCTTTGCCAGCAGTATTG-3′	*rev* probe	2.9-7; 1564–1587
2.9-7LPA 3′ spec	5′-CATGTAGTAGTTGCTTGAGCCGTG-3′	2.9-7LPA probe	2.9-7; 2549–2572
2.9-7LPA 5′ spec	5′-CGCTTGACCACATAAAAAGTGAAC-3′	2.9-7LPA probe	2.9-7; 2431–2454
2.9-7LPB 3′ spec	5′-ATTGCTCTATATCGTTGCTACCATTTC-3′	2.9-7LPB probe	2.9-7; 3073–3099
2.9-7LPB 5′ spec	5′-CTCAAAAACAAAAAGAATTAGCTCAGG-3′	2.9-7LPB probe	2.9-7; 2914–2940
2.9-2LP 3′ spec	5′-CCGTTGCAGGTACTAACTGCTC-3′	2.9-2LP probe	2.9-2; 1883–1904
2.9-2LP 5′ spec	5′-AATGTAATGGAAATGATGAGGG-3′	2.9-2LP probe	2.9-2; 1793–1814
2.9-2LP 3′ Eco	5′-CGGAATTCTGCATTGTAAAAAAAGAAAGAC-3′	2.9-2LP-GST fusion	2.9-2; 1974–2003
2.9-2LP 5′ Bam	5′-CGGGATCCCAAGAAAAACCTAAATCTAAAG-3′	2.9-2LP-GST fusion	2.9-2; 1611–1636
2.9-3LP 3′ spec	5′-CGCAGGTAGTAACTGCATTATTTG-3′	2.9-3LP probe	2.9-3; 1780–1803
2.9-3LP 5′ spec	5′-ATGCCAATGAACAAAAAACCAC-3′	2.9-3LP probe	2.9-3; 1705–1726
2.9-3LP 3′ Eco	5′-CGGAATTCTGCATGTTGAAAAAAAGAAAGG-3′	2.9-3LP-GST fusion	2.9-3; 1884–1908
2.9-3LP 5′ Bam	5′-CGGGATCCCAAGAAAAACCTAAATCTAAAG-3′	2.9-3LP-GST fusion	2.9-3; 1509–1533
2.9-4LP 3′ spec	5′-GTGCTTTTGATTTTAAGAAGTTG-3′	2.9-4LP probe	2.9-4; 1766–1788
2.9-4LP 5′ spec	5′-AAATACAAGGGTGCAATAATGGG-3′	2.9-4LP probe	2.9-4; 1654–1676

Continued on following page

TABLE 1—*Continued*

Designation	Sequence	Purpose	Source; complementary nucleotides
2.9-4LP 3′ Eco	5′-CGGAATTCAAGGTTTTTATTTTTGATTGAG-3′	2.9-4LP-GST fusion	2.9-4; 2039–2067
2.9-4LP 5′ Bam	5′-CGGGATCCATTACTTTAACATCCGACGAAG-3′	2.9-4LP-GST fusion	2.9-4; 1572–1593
2.9-5LP 3′ spec	5′-TGCTCTATTTCGTTAGTACCTTTTC-3′	2.9-5LP probe	2.9-5; 2678–2702
2.9-5LP 5′ spec	5′-TGGCTTTCTACAGATATTCAAAAAC-3′	2.9-5LP probe	2.9-5; 2500–2524
ABCD PE	5′-CAAAAATCATTACGGCTATAAGTTTAATTTC-3′	*ABCD* primer extension	2.9-1; 177–207
2.9orfD PE	5′-TGGCGGTTTTGGCTCTTCTGGTAGG-3′	*orfD* primer extension	2.9-1; 1069–1093
Rep+ North Prim.	5′-AAATGCTTAAATGGGTATTGGGAATTATGGGAG-3′	2.9-1 *rep*$^+$ probe	2.9-1; 1934–1966
Rep− North Prim.	5′-TCCCAATACCCATTTAAGCATTTCTGAAAGAGAC-3′	2.9-1 *rep*$^-$ probe	2.9-1; 1923–1956

by the manufacturer (Cinna/Biotecx Laboratories Inc., Houston, Tex.). RNA was dissolved in sterile deionized water treated with 0.1% diethyl pyrocarbonate. Twenty micrograms of RNA, denatured under formaldehyde-formamide conditions in the presence of 1× MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer (0.2 M MOPS, 0.5 M sodium acetate, 0.01 M EDTA [pH 7.0]), was mixed, incubated for 15 min at 65°C, and then immediately chilled on ice. Ten microliters of formaldehyde loading buffer (1 mM EDTA, 50% [vol/vol] glycerol, 25% [wt/vol] bromophenol blue) was added, and the sample was loaded onto a gel of 1% (wt/vol) electrophoretic-grade agarose in 88.2 ml of diethyl pyrocarbonate-treated H_2O, 10 ml of 10× MOPS buffer, and 1.8 ml of formaldehyde (0.666% [vol/vol], final concentration). The gel was subjected to electrophoresis in 1× MOPS–0.666% formaldehyde. A lane containing RNA Ladder molecular weight markers (0.24 to 9.5 kb; Gibco-BRL) was trimmed away from the gel, stained, and photographed for estimation of molecular weights of hybridizing borrelial RNA transcripts. RNA was subsequently transferred from the gel to positively charged nylon membranes and cross-linked to the membrane by using a Stratalinker 1800 apparatus (Stratagene). Individual nylon strips were hybridized with probes (see description of Southern hybridization analysis) overnight at 42°C in 5× Denhardt's solution–6× SSC–1 mM EDTA–1% SDS–50% formamide (deionized)–and 100 μg of sonicated salmon sperm DNA per ml, using a rotating hybridization oven (Robbin's Scientific Corp.). After hybridization, the membranes were washed twice for 15 min each time at room temperature in 2× SSC containing 0.1% SDS and then four times at room temperature in 0.1× SSC containing 0.1% SDS.

Transcriptional initiation studies. RNA for primer extension assays was isolated either from *B. burgdorferi* (as described above) or from approximately 10^{10} *E. coli* cells, using RNAzol B as recommended by the manufacturer (Cinna/Biotecx Laboratories). RNA was dissolved in sterile deionized water treated with 0.1% diethyl pyrocarbonate. Primers corresponding to the initiation regions of the *2.9orfD-phoA* and *ABCD* genes (designated 2.9orfD PE and ABCD PE, respectively [Table 1]) were prepared. Primer extension reactions were performed with an avian myeloblastosis virus reverse transcriptase primer extension system (Promega, Madison, Wis.) as recommended by the manufacturer. Sequencing reactions were performed with the same primers in conjunction with their respective DNA templates (Table 1), using an Amplicycle sequencing kit (Perkin-Elmer, Foster City, Calif.) as suggested by the manufacturer.

DNA sequencing and computer analyses. Nucleotide sequencing was performed with an Applied Biosystems Inc. model 373A automated DNA sequencer and PRISM ready reaction DyeDeoxy terminator cycle sequencing kits as specified by the manufacturer (Applied Biosystems Inc., Foster City, Calif.). Nucleotide and deduced amino acid sequences were analyzed and manipulated by using the University of Wisconsin Computer Genetics Group version 7.3 (GenBank database release 82.0) (12), Lasergene (DNASTAR, Madison, Wis.) and MacVector version 4.1.1 (International Biotechnologies Inc.-Kodak, New Haven, Conn.) software packages. Potential promoter sites within DNA sequences were searched and assigned similarity scores based on known *E. coli* promoter sequences, using the MACTARGSEARCH algorithm (30).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper, totaling approximately 21 kb, were submitted to the GenBank database and were assigned the following accession numbers: U45421 (2.9-1), U45422 (2.9-2), U45423 (2.9-3), U45424 (2.9-4), U45425 (2.9-5), U45426 (2.9-6), and U45427 (2.9-7).

RESULTS AND DISCUSSION

Identification of *2.9orfD*. Genes encoding proteins with export signals were identified by cloning small fragments of total genomic DNA from *B. burgdorferi* 297 into pKSII/pho as previously described (2). DNA sequencing of the borrelial DNA insert of one PhoA$^+$ clone (subsequently designated *2.9orfD-phoA*; Fig. 1 and 2) revealed an ORF encoding 65 amino acids fused in frame to PhoA (not shown); this construct was distal to a possible promoter element and a ribosomal binding site. Encoded within the 250 nucleotides adjacent to and upstream of the ATG start codon of this ORF was another partial ORF subsequently designated *orfC* (Fig. 1 and 2). The designation of the putative *orfD* translational initiation site, derived from the DNA sequence, was consistent with the 51-kDa apparent molecular mass of the entire fusion protein (according to mobility upon SDS-PAGE). The first 16 residues of OrfD contained a putative prokaryotic signal peptide terminated by sequences consistent with either a signal peptidase I cleavage site (T-I-A) or a lipoprotein processing and modification site (L-L-L-C-C) (35) (Fig. 1). Processing experiments performed with *E. coli* minicells (50, 51) and in vivo radiolabeling with [^{3}H]palmitate were performed to clarify the type of N-terminal export signal; neither processing nor lipidation of BbK2.9-PhoA was demonstrable (data not shown). This result suggested that, at least in *E. coli*, the export signal is an uncleaved signal sequence and that the native OrfD polypeptide is a cytoplasmic membrane-associated protein.

Southern blot analysis of *2.9orfD*. Southern blot analysis was performed to further characterize the *2.9orfD* gene in *B. burgdorferi*; the borrelial DNA portion of the *2.9orfD-phoA* fusion (Table 1 and Fig. 2 [2.9-1]) was hybridized against uncloned and cloned low-passage-number *B. burgdorferi* 297 total DNAs digested to completion with *Xba*I, *Bam*HI, and *Eco*RI. This probe hybridized strongly with five fragments of the DNAs digested with *Eco*RI and *Xba*I and two large fragments of the *Bam*HI-digested DNA (Fig. 3a). This hybridization pattern was not due to incomplete digestion of the genomic DNAs, inasmuch as hybridization of the DNAs with a probe corresponding to the *B. burgdorferi* B31 flagellin structural gene (*fla*) (which lacks restriction sites for *Eco*RI and *Bam*HI) yielded only a single hybridizing fragment of 1.8 kb for *Xba*I, 3.7 kb for *Bam*HI, or 3.1 kb for *Eco*RI, as predicted (18) (data not shown). The combined results suggested that *orfD* was either a multicopy gene or one of a number of closely related genes. Furthermore, the Southern blot patterns observed for DNAs of the uncloned and cloned *B. burgdorferi* isolates were identical (Fig. 3a), indicating that the results were not reflective of heterogeneity among the population of uncloned *B. burgdorferi* 297.

Localization of *2.9orfD* to supercoiled plasmids. The Southern blot findings for *orfD* prompted a determination of wheth-

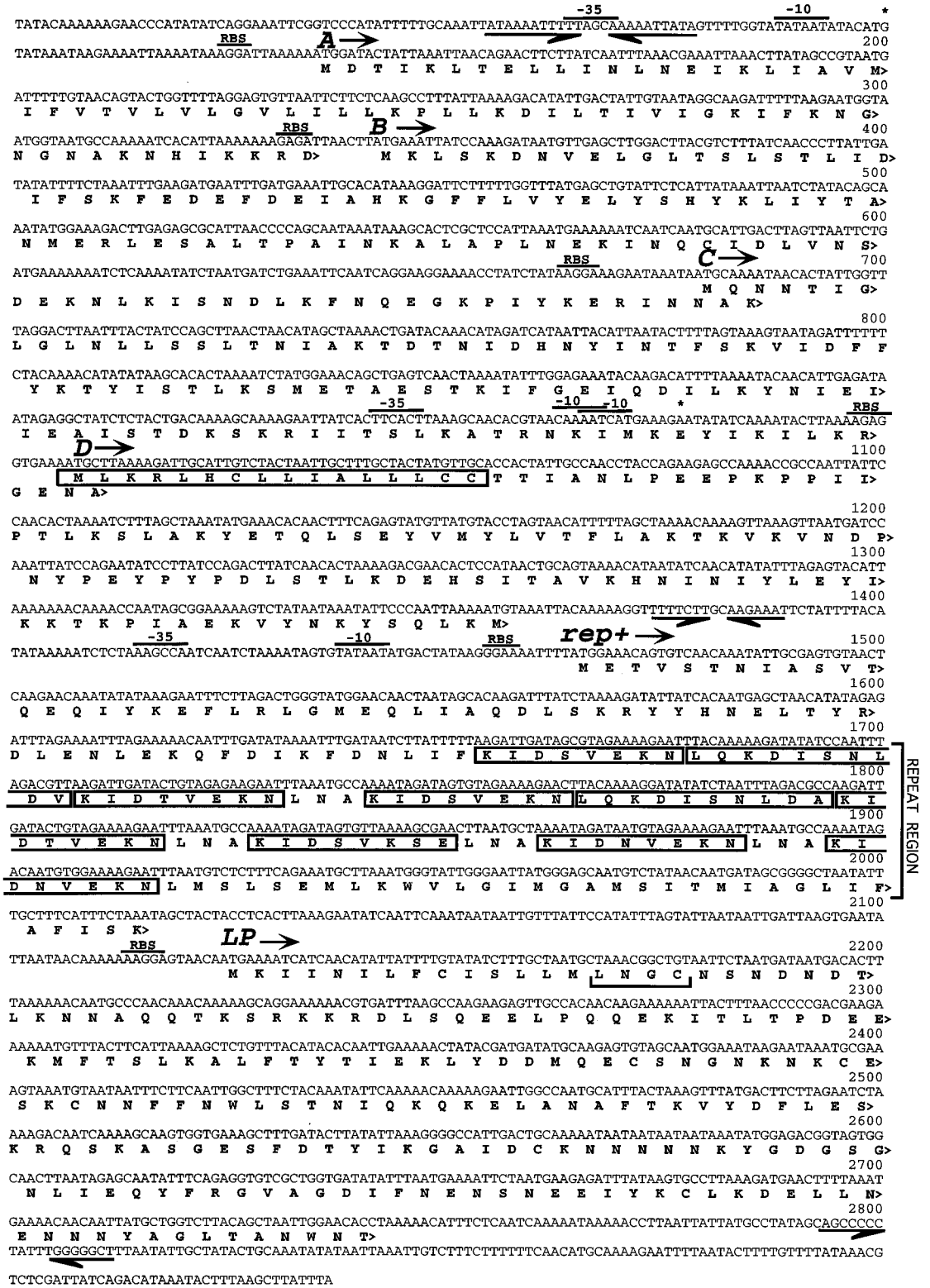

FIG. 1. Complete DNA sequence of the 2.9-1 locus of *B. burgdorferi* 297. Putative −35 and −10 sites and ribosomal binding sites (RBS) are shown, as are mRNA start sites (asterisks). A stem-loop (putative rho-independent terminator) structure is shown as inverted half-arrows. The first 16 amino acids of the putative signal-like sequence of the *orfD* product are boxed. The portion of *rep*$^+$ containing the repeated motifs is shown and its repetitive motifs are boxed. The consensus tetrapeptide for lipid modification and processing in *LP* is designated by a bracket below the sequence. Promoters were corroborated on the basis of primer extension analyses.

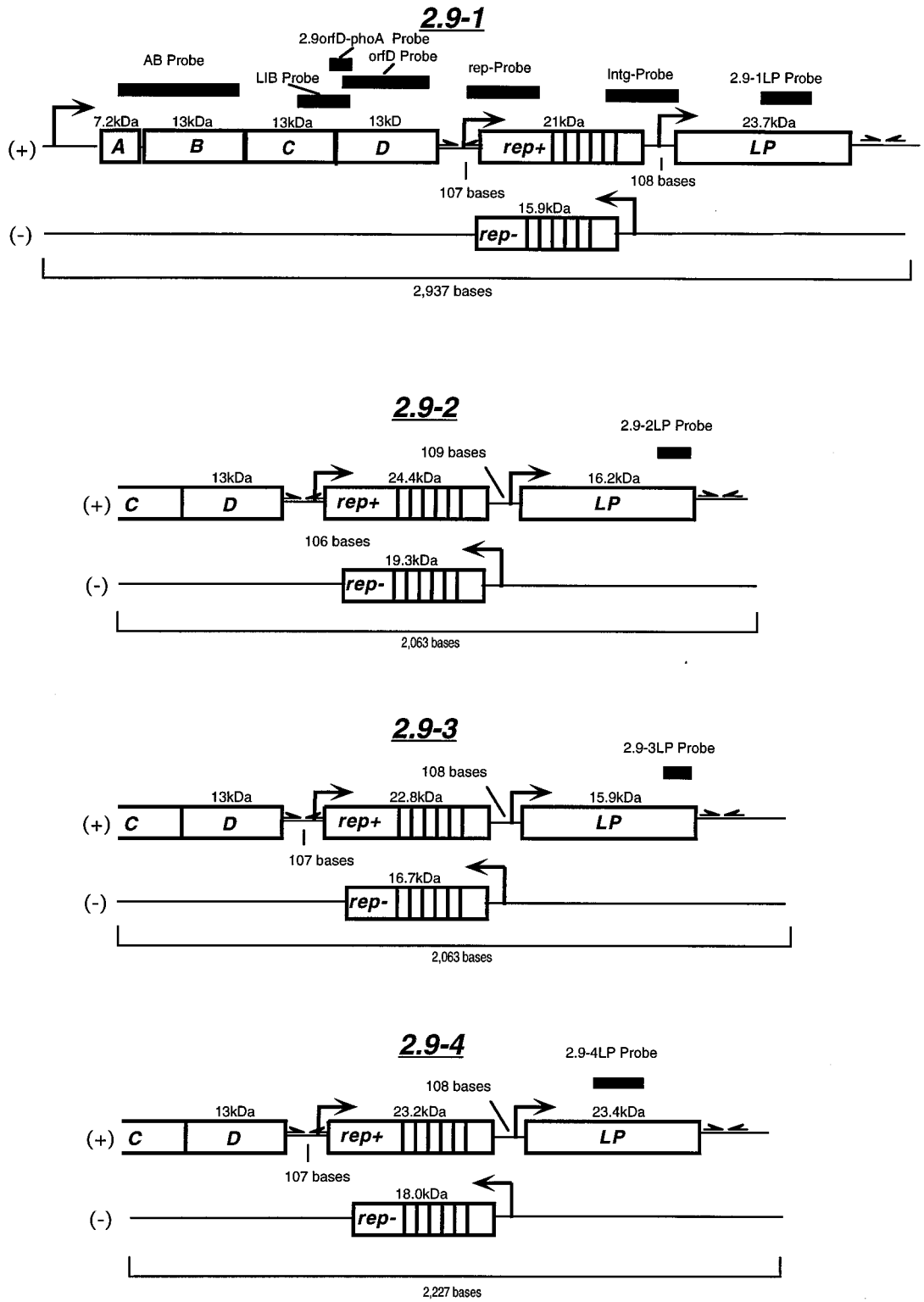

FIG. 2. Schematic representation of the seven 2.9 loci present in *B. burgdorferi* 297-1. ORFs are shown as boxed regions, and the number above each box indicates the size of the polypeptide encoded by each ORF. Promoter-like elements are delineated as arrows in the direction of transcription; a stem-loop (putative rho-independent terminator) structure is shown as inverted half arrows. Probes used for Southern and Northern blot analyses are shown above the ORFs. The 2.9-6 locus is truncated as described in the text. Areas of close vertical lines represent repeat motifs. Positive and negative DNA strands are designated by + and −, respectively. rep, repeat-containing gene; LP, lipoprotein; rev, an ORF encoded on the negative strand of 2.9-7.

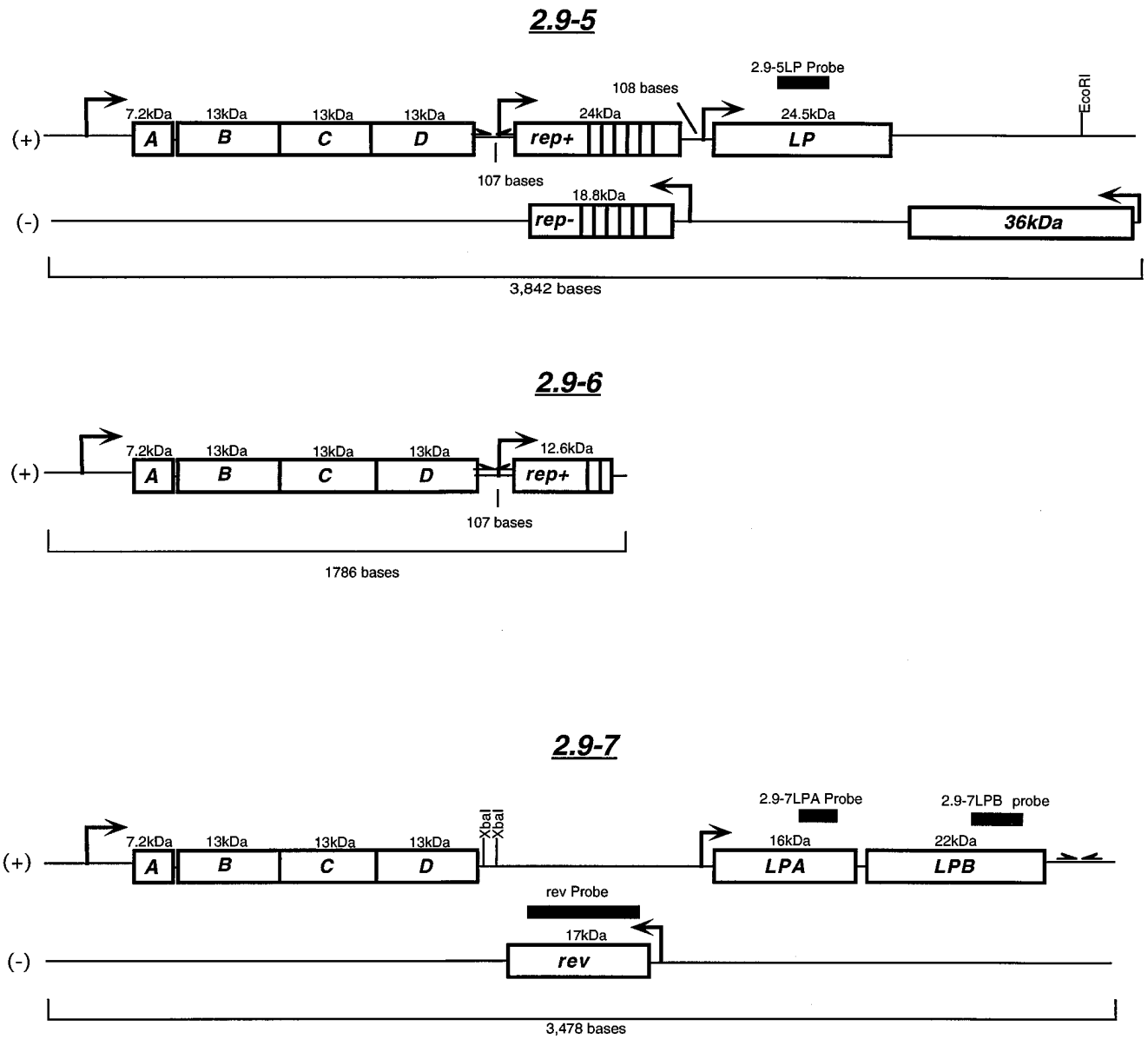

FIG. 2—*Continued.*

er this multicopy gene was either dispersed throughout the *B. burgdorferi* 297 genome or present on a particular replicon(s). Of note, Simpson et al. (45) reported that certain repetitive DNA fragments of *B. burgdorferi* are located exclusively on SC plasmids. To determine whether a similar situation existed for *2.9orfD*, the same probe was hybridized against undigested 297-1 DNA separated by two-dimensional pulsed-field gel electrophoresis. As shown in Fig. 3b, the hybridizing sequences were localized exclusively to one or more (i.e., comigrating) circular plasmids which migrated between the *B. burgdorferi* linear chromosome and the 49-kb *ospAB*-encoding linear plasmid. This pulsed-field gel migration pattern for *B. burgdorferi* has been noted previously for *B. burgdorferi* SC plasmids of approximately 30 kb (2, 39).

Cloning and sequence analysis of the 2.9 genetic loci. The complete sequence for one 2.9 locus, 2.9-1, is presented in Fig. 1; diagrammatic representation of all seven of the 2.9 loci is shown in Fig. 2.

The 2.9orfD-phoA probe was used to screen a *B. burgdorferi* genomic library (in pGEX-2T) to obtain and characterize the complete gene and flanking sequences. Eight clones with inserts ranging in size from 2.0 to 2.2 kb were sequenced using outward-facing primers. The complete *orfD* genes, found in all eight clones, encoded polypeptides of 13 kDa (Fig. 1) which were not homologous to any sequences in the protein and DNA databases searched. Interestingly, among the eight clones were four distinct *orfD* sequences which differed by approximately 1% at the nucleotide level; these clones were designated 2.9-1, 2.9-2, 2.9-3, and 2.9-4 (Fig. 2). The remaining four clones were duplicates of either the 2.9-1, 2.9-2, or 2.9-4 clone. The presence of these duplicate clones was taken as evidence that (i) DNA libraries were constructed appropriately, (ii) a level of gene redundancy was achieved during the screening of clones, and (iii) DNA sequencing methods were accurate. Also noteworthy was that none of the four complete *orfD* sequences was an exact match for the sequence of

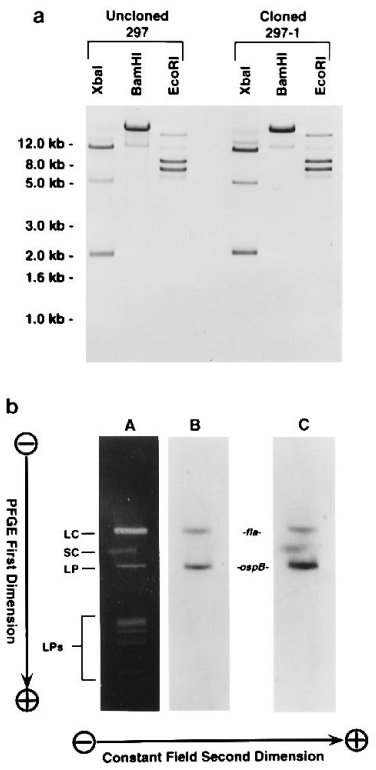

FIG. 3. Identification of multiple copies of *orfD* in *B. burgdorferi* and its localization to supercoiled plasmid(s). (a) DNAs from uncloned and cloned low-passage-number *B. burgdorferi* 297, digested to completion with *Xba*I, *Bam*HI, and *Eco*RI, were probed with a portion of the *orfD* gene (2.9orfD-phoA probe [Fig. 2]). Molecular weight markers are shown at the left. (b) Undigested total DNA from the low-passage-number 297-1 clone was subjected to pulsed-field gel electrophoresis (PFGE) in the first dimension and constant field electrophoresis in the second dimension. After staining with ethidium bromide (lane A), plasmids resolved in this system were hybridized to the 2.9orfD-phoA probe (lane C only) in conjunction with *fla* and *ospB* control probes for genes located on the *B. burgdorferi* linear chromosome (LC) and 49-kb linear plasmid (LP), respectively (lanes B and C). SC, supercoiled plasmid(s).

2.9orfD-phoA. These results indicated that at least five different *orfD* genes (inclusive of the original *2.9orfD-phoA*) are present in *B. burgdorferi* 297.

Immediately upstream of each *orfD* in all four 2.9 clones was another ORF, designated *orfC* (Fig. 1 and 2), whose 3′ end overlapped with the first two codons of each *orfD* structural gene. Minor differences (ca. 1% nucleotide divergence) also were noted in portions of the *orfC* sequences contained within these clones (not shown). Because none of the four pGEX-2T clones contained complete *orfC* genes, a lambda-ZapII genomic library with larger (4- to 10-kb) inserts was screened with a 400-bp probe, designated LIB (Table 1 and Fig. 2 [2.9-1]), encompassing approximately equal portions of the 2.9-1 *orfC* and *orfD* genes (derived from the 2.9-1 clone). As with the pGEX-2T DNA library, duplicate clones again were identified. One of the lambda clones was identical to, though a larger version of, the pGEX-2T clone designated 2.9-1; this clone was instrumental in completing the sequence of the *orfC* and other genes of the 2.9-1 locus (Fig. 1 and 2). Three other additional unique clones were designated 2.9-5, 2.9-6, and 2.9-7 (Fig. 2). In addition to the complete *orfC* and *orfD* genes, inserts for these three clones contained two other upstream ORFs (designated *orfA* and *orfB*) as well as downstream ORFs (described further below). This cluster of *ABCD* genes was preceded by a potential promoter element and a ribosomal binding site; downstream of the *ABCD* locus was a rho-independent, stem-loop terminator-like structure, suggesting that these genes comprise an operon. *orfA*, *-B*, and *-C* encoded polypeptides of 7, 13, and 13 kDa, respectively, all of which lacked N-terminal export signals; database searches failed to identify proteins or DNA sequences homologous to these ORFs. Similar to the situation with *orfD*, minor sequence differences (ca. 1%) were observed among the four clones containing complete *orfA*, *-B*, *-C*, and *-D* genes. None of the *orfD* genes in the four lambda-ZapII clones matched the three clones obtained from the pGEX-2T library or the portion of *orfD* within the original PhoA clone (*2.9orfD-phoA*), indicating that *B. burgdorferi* 297 must contain at least eight distinct copies of the *orfD* gene and, by implication, eight copies of the entire *ABCD* operon.

Sequences downstream of the *orfD* genes also were obtained for all seven clones (three in pGEX-2T and four in lambda-ZapII); in comparison with the *orfA* to *-D* genes, much greater sequence divergence was noted for these downstream regions. In five of the seven clones, *orfD* was followed by a potential promoter, a putative ribosomal binding site, and an ORF encoding a polypeptide which ranged in size from 12.6 to 24.4 kDa (Fig. 1 and 2). These ORFs, designated *rep*$^+$, contained nearly identical 5′ regions followed by variable numbers and arrangements of two particular motifs (KIDXVEKN and LQKDISNLDX) often separated by an LNA motif (Fig. 1) and then highly similar 3′ termini. Computer searches revealed that the *rep*$^+$ gene was homologous (BLAST score of 219) to an ORF E gene encoded by a 30.5-kb circular plasmid of *B. burgdorferi* B31 (GenBank number X87127); additional details of this gene have not yet been published. Downstream of the *rep*$^+$ genes in these same five clones were intergenic spacers of either 108 or 109 nucleotides. These spacers contained three promoter-like elements and putative ribosomal binding sites upstream of ORFs encoding polypeptides ranging in molecular mass from 15.9 to 24.5 kDa (designated *LP*). Each of these LP polypeptides contained virtually identical putative signal sequences terminated by a tetrapeptide (L-N-G/S-C) consistent with the consensus motif for lipoprotein modification and processing (35, 36) (Fig. 1 and 4A). None of these polypeptides showed sequence homology to other proteins in the databases. The sixth clone (designated 2.9-6) lacked the conserved 3′ terminus of *rep*$^+$ (including a majority of the repeats) as well as an *LP* gene (Fig. 2). In their place was a 3-kb fragment encoding a homolog of the phosphoenolpyruvate-dependent sugar phosphotransferase system enzyme III of *E. coli* (*crr* gene) and an ORF encoding a protein homologous to *Haemophilus influenzae* HTPG, an ATP-binding chaperone of the HSP90 family of heat shock proteins. Inasmuch as the latter previously was reported to be located on the *B. burgdorferi* chromosome (33) (a finding confirmed here by Southern analysis of pulsed-field gels [not shown]), we concluded that the cloned 2.9-6 locus was truncated by a cloning artifact. Given the extremely close relatedness between the cloned portion of the 2.9-6 locus and the other 2.9 loci, it was deemed infeasible to design a specific probe which could be used to clone the remainder of the 2.9-6 locus. For this reason, subsequent work focused on the six other fully sequenced loci. Downstream of the *orfD* gene in clone 2.9-7 was 737 bp of noncoding, positive-strand DNA (in place of *rep*) followed by two closely linked putative lipoprotein genes (designated *LPA* and *LPB* [Fig. 2]) which appeared to be under the control of a single promoter-like element.

For all seven 2.9 loci, the large majority of negative-strand DNAs were noncoding; however, in almost all cases, putative ORFs with upstream promoter-like elements and putative ribosomal binding sites were found on the opposite strands (Fig.

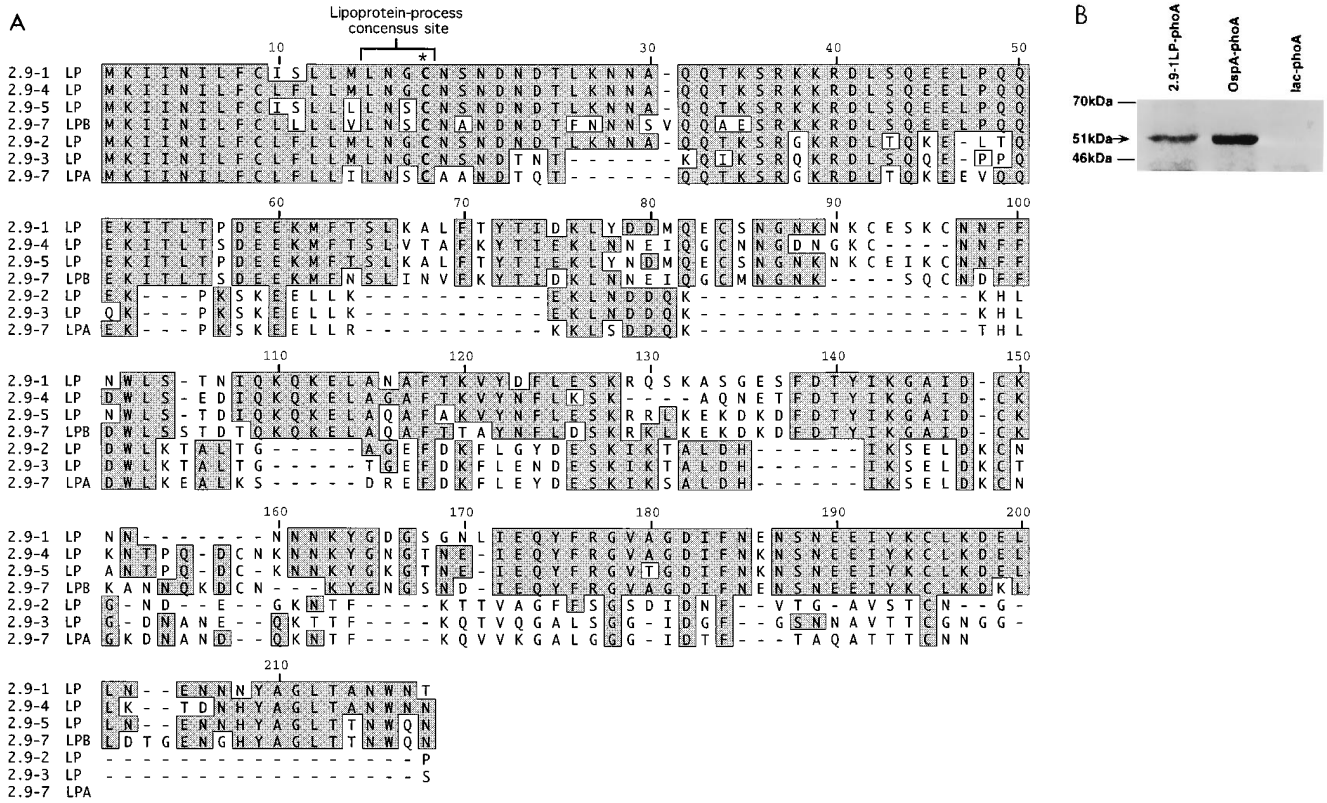

FIG. 4. Consensus alignments of the 2.9 lipoproteins and confirmation of acylation for one (2.9-1LP) representative lipoprotein. (A) The sequences of seven 2.9 lipoproteins were aligned by using the Clustal (PAM250) algorithm. Shaded residues represent exact matches. Shown in the bracket is the consensus lipid modification and processing site(s). (B) *E. coli* clones expressing PhoA fusions with OspA, LacZ, and 2.9-1LP were incubated with [^{3}H]palmitate and then analyzed by SDS-PAGE and fluorography. Molecular mass markers are shown at the left.

2). With the exception of the 2.9-6 and 2.9-7 loci, ORFs designated *rep*$^-$ were complementary to the *rep*$^+$ genes; the *rep*$^-$ genes encoded highly similar polypeptides ranging from 15.9 to 19.3 kDa which contained repeat motifs consisting of KFF-STXSILX (not shown). All five of the *rep*$^-$ genes encoded putative ribosome binding sites, potential promoter elements, and N-terminal hydrophobic amino acid stretches consistent with either cleaved or uncleaved signal sequences (not shown). Of note, downstream of the *rep*$^+$ and *LP* genes in the 2.9-5 locus was an ORF on the negative strand which encoded a polypeptide of 36-kDa (Fig. 2). A BLAST search indicated that this ORF had homology with both ORF 4 of the 8.3-kb circular plasmid of *B. burgdorferi* Ip21 (BLAST score of 476) (13) and ORF G encoded on a 30.5-kb circular plasmid of *B. burgdorferi* B31 (BLAST score of 424) (GenBank number X87127); further details of ORF G have not yet been published, and therefore the relevance of these homologies remains unclear. For the 2.9-7 locus, the negative strand encompassing the region referred to as *rep*$^-$ in the other 2.9 loci contained an unrelated ORF (designated *rev*) encoding a 17-kDa protein with a presumptive leader peptide proximal to a putative signal peptidase I cleavage site (Fig. 2). The *rev* gene and its product failed to show sequence homologies with genes and proteins in the databases searched.

Evidence that the 2.9 *LP* gene products are lipoproteins. Given that each of the LP polypeptides contained consensus motifs potentially indicative of lipid modification (Fig. 4A), studies were conducted to garner evidence that the proteins encoded by these genes were, in fact, lipid modified. Because the predicted signal sequences for all seven proteins were virtually identical (Fig. 4A), one lipoprotein (2.9-1LP) was selected for further study. Using PCR, we generated a gene fusion in which the predicted borrelial control elements and ribosomal binding site along with the first 84 amino acids (the signal sequence plus 66 amino acids beyond the predicted signal peptidase II cleavage site) of the lipoprotein were placed proximal to and in frame with the nonsecreted PhoA reporter in pKSI/pho (Fig. 1). When incubated with [^{3}H]palmitate, this clone expressed a lipid-modified fusion protein of the appropriate molecular mass (51 kDa) (Fig. 4B) which reacted with a monoclonal antibody directed against *E. coli* PhoA (not shown). As expected, under these same conditions, an OspA-PhoA fusion incorporated radiolabeled palmitate, whereas a LacZ-PhoA fusion (48 kDa) did not (Fig. 4B) (2). Although we have not shown that the native proteins are lipidated, we are unaware of any instances in which spirochetal proteins are lipidated in *E. coli* but not in *B. burgdorferi*. From this finding, we have concluded that the 2.9 *LP* genes encode a family of lipoproteins.

The 2.9 lipoproteins segregate into two distinct classes. The consensus alignment (Fig. 4A) of the 2.9 lipoprotein sequences revealed that there was a high degree of conservation among the seven lipoproteins through their first 50 amino acids but that thereafter the sequences diverged into two distinct classes (class 1, 2.9-1LP, 2.9-5LP, 2.9-4LP, and 2.9-7LPB; class 2, 2.9-7LPA, 2.9-2LP, and 2.9-3LP). This partitioning of the lipoproteins into two classes also was supported by size (Fig. 2), weighted dendogram analysis (not shown), and hydrophilicity

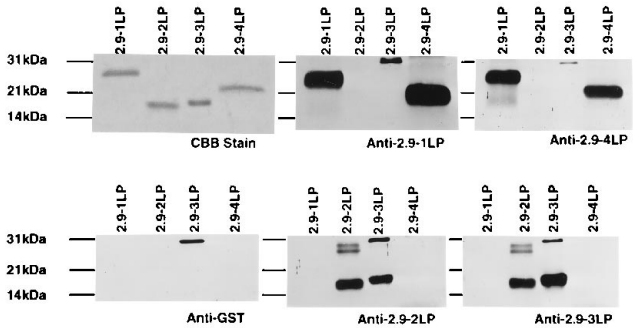

FIG. 5. The *B. burgdorferi* 2.9 lipoproteins segregate into two classes. Recombinant, nonacylated 2.9 lipoproteins (mature portions of 2.9-1LP, 2.9-2LP, 2.9-3LP, and 2.9-4LP) were cleaved from their GST fusion partners and immunoblotted against rat polyclonal antisera directed against either GST or the individual lipoproteins. Molecular mass standards are shown on the left. CBB, Coomassie brilliant blue.

analysis (not shown). All seven proteins were extremely hydrophilic (not shown), a characteristic shared with other borrelial lipoproteins (8, 40). Interestingly, although 2.9-7LPA and 2.9-7LPB were encoded by contiguous genetic loci (Fig. 2), they partitioned into classes 2 and 1, respectively.

From the foregoing analyses, it was hypothesized that lipoproteins within each class might be immunologically cross-reactive but that antibodies directed against one class of lipoproteins would fail to cross-react with those in the other class. To investigate this, two lipoproteins from each class (2.9-1LP and 2.9-4LP from class I; 2.9-2LP and 2.9-3LP from class II) were expressed as fusions with GST and used to generate monospecific, polyclonal antisera in rats. As shown in Fig. 5, antibodies against lipoproteins from each class were cross-reactive within the class but were not reactive with lipoproteins from the other class. Thus, computer-derived structural similarities and differences between the two classes of lipoproteins appeared to be corroborated using antibody probes.

Southern blotting for genomic characterization of the 2.9 loci in *B. burgdorferi*. Results of prior cloning, DNA sequencing, plasmid localization, and preliminary Southern blot experiments indicated that at least seven distinct 2.9 loci were encoded on a small number of supercoiled plasmids in *B. burgdorferi* 297. To obtain additional information regarding the number of 2.9 genes, their genomic organization, and their linkage patterns in *B. burgdorferi*, more extensive Southern blotting experiments were performed. In addition to clone 297-1, these analyses included DNAs from low-passage-number, virulent *B. burgdorferi* clones B31-1 and N40-1 to determine whether the 2.9 locus (or loci) is common to other *B. burgdorferi* sensu stricto isolates. All borrelial DNAs were digested with *Eco*RI and *Xba*I because restriction sites for both enzymes are extremely rare in the 2.9 loci from strain 297 (Fig. 2).

Nine probes for these analyses were designed to be specific for different regions of the 2.9 loci (Table 1 and Fig. 2). Two of the probes (designated AB and orfD) were modeled after the highly conserved, multicopy *orfA* to *-D* genes (Fig. 2). A third intergenic probe (designated Intg) extended from the highly conserved 3′ region of rep^+ to the highly conserved 5′ region of *2.9-1LP* (Fig. 2). This region was chosen for analysis because it represented an area which was present in many of the 2.9 loci but lacking in at least two of the 2.9 loci (Fig. 2). Five other probes, ranging in length from 88 to 300 nucleotides, were designed to hybridize specifically with individual *LP* genes (Fig. 2). The ninth probe, based upon the unique *rev* gene located centrally within the 2.9-7 locus (Fig. 2), was used to localize the 2.9-7 locus. Two approaches were used to improve the precision with which the *B. burgdorferi* DNA fragments hybridizing to the various probes could be compared. First, multiple samples of DNA from the three *B. burgdorferi* strains were separated simultaneously on the same large (26-well) gel. Second, each nylon membrane was stripped and reprobed (using different probes) up to three times.

It was predicted that probes to regions having high DNA similarities (i.e., the *ABCD* operons and the intergenic regions between *ref* and *LP*) should hybridize to multiple restriction fragments, whereas probes against unique sequences (i.e., the *rev* and *LP* genes) should yield only single, dominant hybridizing fragments. Therefore, a restriction fragment identified by a unique probe (e.g., sequences derived from the *rev* and *LP* genes) should, in general, correspond to one of the multiple fragments which hybridize with a probe directed against the highly repeated *ABCD* gene clusters. Correlation of these fragments would confirm the linkage (i.e., *ABCD* to *LP*) inferred from the sequence data (Fig. 2) and enable us to identify the genomic fragment which contains each particular 2.9 locus.

Results for Southern blot analyses are presented in Fig. 6. First, it was essential to confirm that the DNAs used for these experiments were digested to completion by *Eco*RI and *Xba*I. A probe consisting of the *B. burgdorferi* flagellin structural gene (*fla*) hybridized to single fragments in all three restricted genomic DNAs (sites for *Eco*RI and *Xba*I are absent within the *fla* gene), as predicted (18). As hypothesized above, the AB probe hybridized to multiple bands in *B. burgdorferi* 297-1 DNA, some of which appeared to be composed of comigrating fragments (as judged from the broadness of the bands and the intensities of their radioactive signals) (Fig. 6, AB probe). The DNA fragments, therefore, in the *Eco*RI digests of 297-1 appeared to include (i) single bands of 10, 5.3, and 4.3 kb and (ii) doublet fragments of 15, 8, and 6.5 kb. Thus, the total amount of *B. burgdorferi* DNA encoded on these fragments is about 78 kb, a figure comparable to the sum of the fragments obtained by *Xba*I digestion (total of about 77 kb, assuming that the bands of 10 and 5 kb are triplets) of strain 297-1. (The existence of doublet and/or triplet comigrating fragments was supported by obtaining comparable Southern hybridization data by using probes [see below] specific for the individual 2.9 lipoprotein genes [not shown].) Interestingly, multiple fragments also were obtained for the N40-1 and B31-1 *B. burgdorferi* clones, indicating that these two strains also contained multiple copies of the putative *ABCD* operon. For all three borrelial strains, the orfD probe yielded hybridization patterns identical to those obtained with the AB probe (data not shown), thereby confirming linkage of the *A*, *B*, and *D* genes in all three *B. burgdorferi* sensu stricto strains. Of note, although the Southern blot banding patterns for the AB and orfD probes differed among the three *Borrelia* strains (Fig. 6 and data not shown for the orfD probe), there appeared to be more similarity between the DNA patterns of strains 297 and N40 than between those of strain B31 and the other two *B. burgdorferi* strains. This interpretation is consistent with the conclusion by Liveris et al. (26), based on PCR-restriction fragment length polymorphism typing, that strain B31 is a member of a less common taxonomic subgroup of *B. burgdorferi* sensu stricto, whereas strains 297 and N40 are more closely related. The Intg probe also hybridized to multiple restriction fragments in all three borrelial DNAs, albeit to fewer than those hybridizing with the AB probe (Fig. 6), consistent with the absence of the intergenic region in two (2.9-6 and 2.9-7) of the seven cloned 2.9 loci (Fig. 2). Also noteworthy is the presence of weakly hybridizing restriction fragments detected by both

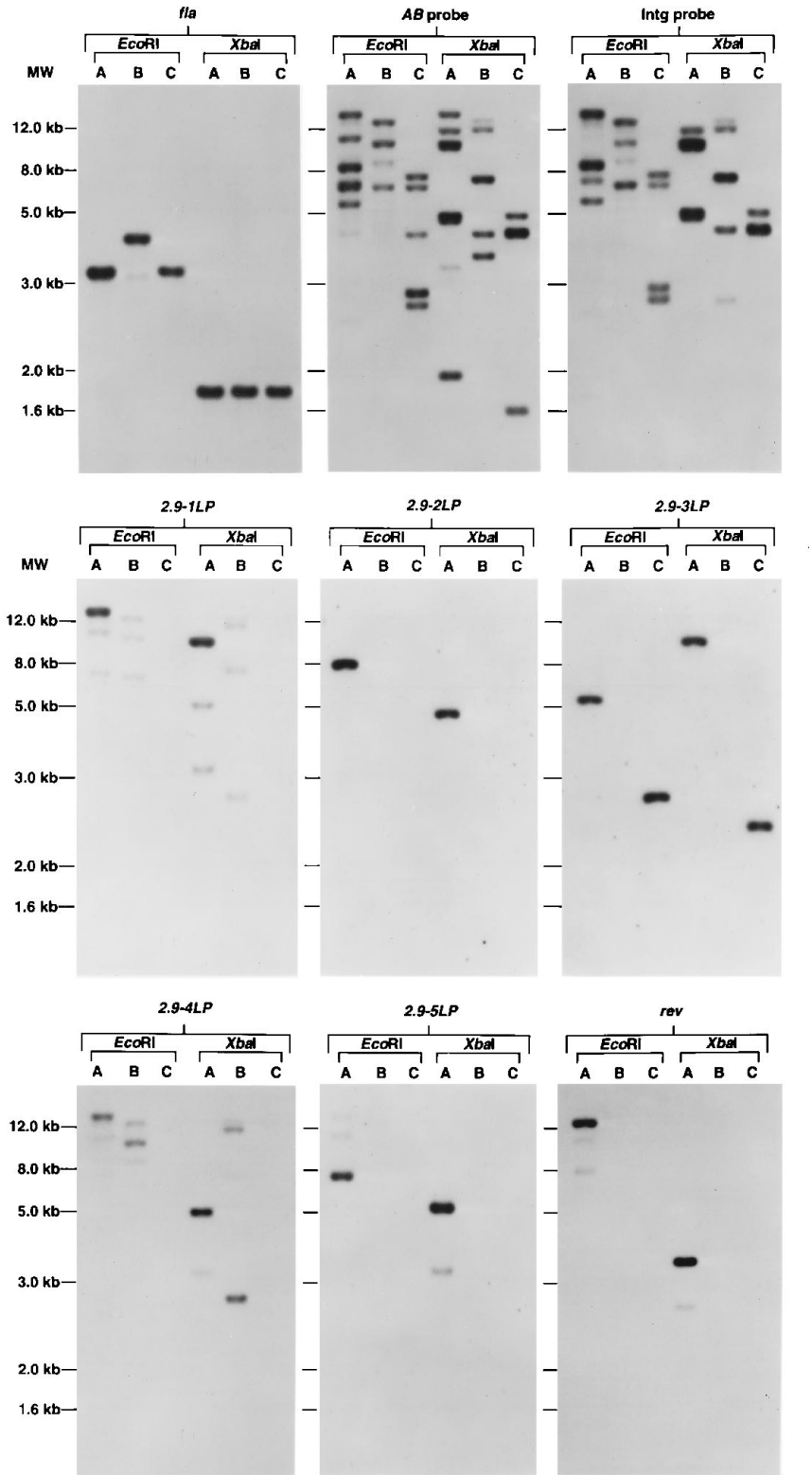

FIG. 6. Southern blot analyses of the 2.9 loci. DNAs from low-passage-number clones of *B. burgdorferi* 297 (lanes A), N40 (lanes B), and B31 (lanes C), digested to completion with *Eco*RI and *Xba*I, were hybridized with probes for *fla* and various portions of the 2.9 loci which are shown diagrammatically in Fig. 2. The probe (designated AB) was modeled after the highly conserved, multicopy *orfA* to *D* genes. The Intg probe extended from the highly conserved 3′ region of the *rep* genes to the highly conserved 5′ region of *2.9-1LP*. Five other probes, ranging in length from 88 to 300 nucleotides, were specific for the *2.9-1LP*, *2.9-2LP*, *2.9-3LP*, *2.9-4LP*, and *2.9-5LP* genes. The probe for the unique *rev* gene was used to localize the 2.9-7 locus. Molecular size standards are shown at the left.

the AB and Intg probes (Fig. 6) which we believe is evidence of more distantly related sequences not yet identified.

Each LP probe hybridized predominantly with a single, unique DNA fragment of clone 297-1 (Fig. 6) which in every case corresponded to one of the strongly hybridizing fragments detected by the AB probe. In addition, fragments recognized by the 2.9-2LP 2.9-3LP, and 2.9-4LP probes corresponded to fragments recognized by the AB and D probes, supporting the inference that these three loci also contain complete *ABCD* operons. Thus, at least seven complete 2.9 loci appear to be present in *B. burgdorferi* 297.

While some of the LP probes (e.g., 2.9-2LP and 2.9-5LP) appeared to hybridize strongly with the same restriction fragments in 297-1, careful alignments of the autoradiographs revealed that the bands were, in fact, dissimilar. The uniqueness of these restriction fragment hybridization patterns was further substantiated by cross-hybridization experiments in which each LP probe hybridized only with its respective cloned 2.9 locus under conditions identical to those used for the *B. burgdorferi* DNA blots shown in Fig. 6 (not shown). Fragments which hybridized weakly with several of the LP probes (e.g., 2.9-1LP, 2.9-4LP, and 2.9-5LP) also were observed. On the basis of the knowledge that the LP probes hybridized only with their respective 2.9 loci, we have concluded that these additional fragments in the 297-1 clone contain related sequences, most likely lipoprotein homologs, which are yet to be discovered. Also noteworthy was that putative homologs for some *LP* genes were identified in the B31 (2.9-3) and N40 (2.9-1 and 2.9-4) clones which also corresponded to fragments recognized by the AB and D probes for these same strains, whereas other *LP* genes (e.g., 2.9-2 and 2.9-5) were unique to strain 297. Interestingly, no LP probe hybridized with DNAs from all three strains. Thus, *B. burgdorferi* strains other than 297 appear to contain multiple copies of genes closely related to the *ABCD* operon, at least some of which appear to be linked to the *LP* genes.

Lastly, the rev probe, a probe specific for the 2.9-7 locus (Fig. 2), hybridized principally to a single fragment of clone 297-1 DNA but not with the DNA of strain B31-1 or N40-1 (Fig. 6). In addition, the presence of weakly hybridizing fragments, analogous to results with the LP probes, suggested the presence of one or more *rev* homologs in this clone.

Distribution of 2.9 genes among supercoiled plasmids. As noted earlier (Fig. 3b), pulsed-field gel hybridization experiments with a *2.9orfD* probe localized this gene to one or more SC plasmids. To more precisely characterize the SC plasmid(s) containing the 2.9 loci, SC and linear plasmids from 297-1 were separated on CsCl density gradients (45) and electrophoresed separately on 0.4% agarose gels (5, 45). Three SC plasmids were resolved electrophoretically and designated 20-, 26-, and 30-kb plasmids on the basis of their putative open circular forms relative to linear DNA standards (Fig. 7) (28, 39). Consistent with prior findings for the B31 strain (28, 39), the *ospC* structural gene hybridized to the 26-kb SC plasmid and its more slowly migrating open circular form (Fig. 7). In contrast, the *orfD* probe hybridized strongly with the other two (30- and 20-kb) SC plasmids (Fig. 7) as well as to the small amounts of the open circular forms present in the gels (Fig. 7). Four of the LP probes (2.9-1LP, 2.9-2LP, 2.9-4LP, and 2.9-5LP) and the rev probe (unique to the 2.9-7 locus) hybridized to the 30-kb plasmid, while the 2.9-3LP probe hybridized exclusively with the 20-kb plasmid. The 2.9-1LP, 2.9-4LP, and 2.9-5LP probes also hybridized weakly with the 20-kb plasmid (Fig. 7); this low level of hybridization was consistent with the Southern blot analyses (Fig. 6) which suggested the existence of other as yet uncharacterized lipoprotein gene homologs. No hybridization

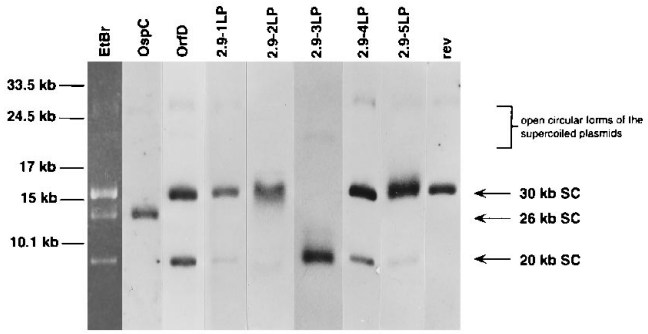

FIG. 7. Distribution of the 2.9 loci among *B. burgdorferi* 297 SC plasmids. SC plasmids isolated from clone 297-1 were separated by electrophoresis on 0.4% agarose gels and hybridized with probes for *ospC* (control for 26-kb SC plasmid) and regions of the 2.9 loci (see Fig. 2). The ethidium bromide (EtBr)-stained plasmid profile is shown at the far left alongside molecular size markers. Open circular forms of these SC plasmids are denoted by the bracket at the right.

was observed between any of these probes and the linear plasmids, whereas an *ospA* control probe hybridized, as expected, to the 49-kb linear plasmid (not shown). Our failure to identify 2.9 loci on linear plasmids of *B. burgdorferi* 297 contrasted with the findings by Zuckert et al. (53), who found that other repetitive sequences were present on both supercoiled (29-kb) and linear (50-kb) plasmids of *B. burgdorferi* B31.

An important issue concerns the discrepancy between the amount of DNA comprising the seven 2.9 loci (approximately 77 to 78 kb) and the combined coding capacity of the 20- and 30-kb SC plasmids. This potential discrepancy is compounded by the fact that at least five of the seven 2.9 loci were localized to a 30-kb plasmid. A potential solution to this paradox was provided by the observation that the 30-kb SC plasmid stained more intensely with ethidium bromide than did the other two SC plasmids (Fig. 7), implying either greater copy number or the presence of comigrating plasmids. We propose, therefore, that the 30-kb SC plasmid band actually consists of multiple, highly similar, comigrating plasmids. In support of this view, Zuckert and Meyer (54) found that *B. burgdorferi* B31 contains comigrating 29- and 30.5-kb supercoiled plasmids, while Stevenson et al. (48) also have determined that *B. burgdorferi* B31 contains at least four comigrating 32-kb SC plasmids which contain highly similar sequences.

Northern blot analysis of the 2.9 loci. Northern blot analyses next were performed to (i) analyze native borrelial mRNA transcripts specific for the various 2.9 loci, (ii) compare actual transcript sizes with 2.9 gene organization predictions derived from computer-assisted ORF assignments, and (iii) assess levels of expression of the various genes or operons within the 2.9 locus (on the basis of abundances of the relevant transcripts). The probes described earlier for Southern blot analyses were used, along with two additional probes specific for *2.9-7LPA* and *2.9-7LPB* and appropriate positive and negative controls (Fig. 2).

The *orfD* probe hybridized faintly with a 1.3-kb transcript, the size predicted if the *orfA* to *-D* genes were cotranscribed (Fig. 8). The rep probe recognized a broad band, presumably containing several transcripts ranging in size from 0.4 to 0.6 kb, consistent with the expression of several individual genes. Additional Northern blot analyses using oligonucleotides specific to the conserved regions of rep^+ or rep^- revealed that the transcript was predominantly from the rep^- gene(s) (data not shown). Transcripts also were detected for the *2.9-1LP*, *2.9-2LP*, and *2.9-4LP* genes, while none were detected for *2.9-3LP*,

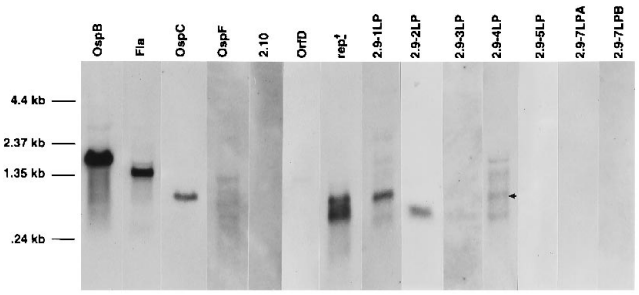

FIG. 8. Northern blot analysis of 2.9 loci. RNAs from clone 297-1 were hybridized with probes directed against selected regions of the 2.9 loci (see Fig. 2) along with control probes directed against *ospB*, *ospC*, *fla*, *ospF*, and *bbk2.10*. The *ospF* probe was included because it results in a banding pattern which reflects the transcription of two closely related *ospF* homologs, while *bbk2.10* is not expressed by in vitro-cultivated spirochetes. The arrow denotes the 0.7-kb transcript which is believed to correspond to that for *2.9-4LP*. Molecular size markers are shown at the left.

2.9-5LP, *2.9-7LPA*, or *2.9-7LPB*. The sizes of the *2.9-2LP* transcript and the predominant RNA species detected by the *2.9-1LP* probe (0.5 and 0.7 kb, respectively) were consistent with the respective sizes of the two ORFs (0.5 and 0.7 kb). Multiple, less intensely hybridizing transcripts also were detected for *2.9-4LP*; although no band was predominant, the 0.7-kb transcript corresponded in size to that predicted by DNA sequence analysis of the respective ORF. This was confirmed in a separate Northern analysis in which only this same transcript was detected with an oligonucleotide probe specific for *2.9-4LP* (data not shown). Of note, the patterns of multiple transcripts detected with the *2.9-1LP* and *2.9-4LP* probes resembled those detected with a probe directed against *ospF*, which we have shown has an unusual Northern blot banding pattern believed to emanate from the expression of two or more OspF homologs, each of which might have a single site of transcriptional initiation (1, 2). Thus, the banding patterns for *2.9-1LP* and *2.9-4LP* are consistent with the genomic Southern blot data which indicated that there may be additional unidentified homologs of these genes.

Transcriptional initiation studies. Results of earlier DNA sequencing, computer analyses, and Northern blotting indicated that the *ABCD*, *rep*, and *LP* genes of the 2.9 locus represented independent transcriptional units. To confirm these findings and aid in the identification of active promoters, primer extension analyses were performed.

The 5′ terminus of the *ABCD* operon transcript was mapped by primer extension using a primer (designated ABCD PE [Table 1]) located in the *orfA* gene; the detection of a primer extension product (not shown) was consistent with the presence of a specific transcript detected previously by Northern blot hybridization (Fig. 8). Six nucleotides upstream of this initiation site (Fig. 1), a putative promoter element was identified; this promoter had a similarity score of 63.9% which denotes a relatively strong promoter, on the basis of information available for *E. coli* consensus promoters (see reference 30 for a discussion of promoter similarity scores). The validity of applying *E. coli* promoter parameters to evaluate *B. burgdorferi* promoters is supported by the fact that the *B. burgdorferi* OspA to -E promoters tend to follow *E. coli* σ^{70} consensus rules (24).

The finding that the *ABCD* gene cluster is an operon seemed incongruous with the observation that the original *2.9orfD-phoA* fusion contained only 250 nucleotides of *orfC* (i.e., lacked the promoter in front of *orfA*) but yet was expressed in *E. coli*. To resolve this discrepancy, primer extension analysis was performed using RNAs from both the *E. coli 2.9orfD-phoA* clone and *B. burgdorferi* 297-1 in conjunction with a primer specific for *orfD* (2.9orfD PE primer [Table 1]). No primer extension product was detected from the borrelial RNA. In contrast, a primer extension product was obtained from the RNA of the relevant *E. coli* clone (data not shown). Inspection of the DNA sequence immediately upstream of the transcriptional initiation site (Fig. 1) identified a weak promoter-like element in the 3′ portion of *orfC* which does not appear to be active in *B. burgdorferi.*

Concerning primer extension analysis of the expressed 2.9 *LP* genes, it was not possible to design transcriptional initiation primers specific for each of the individual *LP* genes because (i) the DNA sequences of the 5′ regions for all of the 2.9 *LP* genes were virtually identical and (ii) the unique regions in the various 2.9 *LP* genes were too distal (300 to 420 bases) from the predicted sites of transcriptional initiation for the design of primers which could provide interpretable results (optimal distance is ca. 70 bases). However, it is worth noting that the regions upstream of the translational starts for the *LP* genes are highly similar and include a number of potential promoter elements. Thus, the question arises as to how the individual *LP* genes are differentially expressed. Two possible explanations can be envisioned. First, minor DNA sequence differences could affect promoter strengths; studies of promoters regulating sporulation genes in *Bacillus subtilis* have shown that single nucleotide substitutions in the −35, −10, −9, and −5 positions, as well as minor variations in spacer lengths, can appreciably affect promoter activities (22, 37). An alternative explanation is that *trans*-acting elements sensitive to the minor nucleotide differences in these upstream sequences are involved in regulating the expression of the various native *LP* genes. Support for this notion is provided by (i) the finding by Sadziene et al. (39) that *ospC* expression is negatively regulated by a linear 16-kb plasmid, (ii) the report by Jonsson and Bergstrom (21) that *ospAB* operons with nearly identical control regions are differentially regulated in *Borrelia* strains, and (iii) our own observation that an OspF homolog with a consensus upstream promoter-like element is expressed by *E. coli* but not by *B. burgdorferi* in vitro (2). Either of these possibilities would contrast with the recombination (gene conversion) events involved in the differential expression of variable surface lipoprotein genes in relapsing fever borreliae (6).

Conclusions. Simpson et al. (45) described repeated DNA sequences which were associated exclusively with supercoiled plasmids in *B. burgdorferi*, and they hypothesized that these fragments encoded closely related genes that encode functionally similar but antigenically distinct proteins. Unfortunately, because sequence information was not obtained, the significance of their finding has not been evident. Subsequently, other gene redundancies which involve sequence variants of *LP* genes have been documented in *B. burgdorferi*. P39, a lipoprotein antigen which elicits an antibody response in the early stages of Lyme disease (14), is now known to be one of four closely related, tandemly arranged chromosomal genes (3, 34, 44). More recently, Suk et al. (49) and Akins et al. (2) reported that *B. burgdorferi* contains at least two OspE and three OspF homologs among which are genes selectively expressed in the infected mammalian host. Wallich et al. (52) have shown that a *B. burgdorferi* 22-kDa lipoprotein (designated pG) which is preferentially expressed in the mammalian host also has homology with OspF. The discovery that *ospA* and *ospB* are closely related genes (7), in retrospect, may be regarded as discovery of the first example of *B. burgdorferi* gene redundancy. Because such redundancy runs counter to the notion that prokaryotes tend to parsimoniously allocate se-

quence information within their genomes, one must presume that this type of gene redundancy is driven by powerful selective pressures, perhaps related to the need for *B. burgdorferi* to exist in diverse host environments (25, 28, 42). Our finding of a multigene, multicopy locus which is associated exclusively with supercoiled plasmids represents the most extensive example of genetic redundancy in *B. burgdorferi* characterized thus far. While the functions of the individual 2.9 genes and the reasons for this extraordinary redundancy have yet to be determined, several features of these loci are worthy of comment. First, the 2.9 loci include genes with and without export signals, suggesting that the polypeptides segregate to different cellular compartments. This view is supported further by the fact that all three classes of export signals (i.e., uncleaved transmembrane domain and signal peptidase I- and signal peptidase II-cleaved leader peptides) appear to be represented. Second, these genes are organized into several independently transcribed units, a finding which, in conjunction with their sequence diversity, could lead to the conclusion that the 2.9 gene products are functionally unrelated. That the 2.9 lipoproteins also fall into two antigenically distinct classes may be a reflection of some level of functional heterogeneity. On the other hand, functional interrelationships among these transcriptional units could be inferred from the observation that their overall genetic organization is preserved among the majority of 2.9 loci. If this is the case, then the minor sequence variations noted among highly conserved 2.9 genes (i.e., *ABCD* operons), as well as the presence of genes containing variable numbers of repeat motifs (i.e., *rep* genes) in association with a unique *LP* gene at each locus, could provide some level of functional diversity. Lastly, a body of evidence which supports the notion that *B. burgdorferi* genes can be selectively expressed either in vitro or in infected mammalian hosts has arisen (2, 11, 42, 49, 52). Given our findings that (i) not all *2.9LP* genes appear to be expressed, (ii) the multicopy *ABCD* operons are transcribed at relatively low levels, and (iii) transcripts from the *rep* regions appear to be derived from only one of two possible genes, it is our working hypothesis that the patterns of expression of individual 2.9 genes, and perhaps even entire 2.9 loci, during infection may differ from those delineated herein in in vitro-cultivated organisms.

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