INFECTION AND IMMUNITY, Nov. 1992, p. 4662–4672
0019-9567/92/114662-11$02.00/0

Vol. 60, No. 11

Low-Passage-Associated Proteins of *Borrelia burgdorferi* B31: Characterization and Molecular Cloning of OspD, a Surface-Exposed, Plasmid-Encoded Lipoprotein

STEVEN J. NORRIS,[1]* CAROL J. CARTER,[2] JERRILYN K. HOWELL,[1] AND ALAN G. BARBOUR[2]

Department of Pathology and Laboratory Medicine, University of Texas Medical School at Houston, P.O. Box 20708, Houston, Texas 77225,[1] and Departments of Microbiology and Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284[2]

Received 29 May 1992/Accepted 25 August 1992

Borrelia burgdorferi*, the causative agent of Lyme disease, loses its ability to infect and cause disease in mammalian hosts after repeated in vitro passage. To identify proteins preferentially expressed by the low-passage strain and thus representing potential virulence factors, the polypeptide profiles of virulent, low-passage and nonvirulent, high-passage forms of *B. burgdorferi* B31 were compared by nonequilibrium pH gradient two-dimensional gel electrophoresis. Four low-passage-associated proteins with relative molecular masses (M_rs) of 35,000, 28,000, 24,000, and 20,000 were identified. Of these, the 28- and 35-kDa polypeptides were not expressed in detectable quantities in the high-passage B31 strain, whereas the 24- and 20-kDa proteins were present in reduced quantities. All four of these proteins were lipoproteins, as determined by labelling with [^{3}H]palmitate. The abundant 28-kDa component, called outer surface protein D (OspD), is surface exposed on the basis of its proteolysis during treatment of intact organisms with proteinase K. The *ospD* gene is located on a 38-kb linear plasmid present in seven of nine low-passage strains of *B. burgdorferi* examined but absent in most high-passage, nonvirulent strains tested. Molecular cloning and sequence analysis of the *ospD* gene locus revealed an open reading frame encoding a 28,436-Da polypeptide with a putative signal peptidase II leader sequence. An unusual feature of the region upstream of the gene was the presence of seven contiguous, direct repeats of a 17-bp sequence that includes consensus −35 and −10 transcription initiation signals; however, only one transcription initiation site was active as determined by primer extension analysis. Further study of these and other polypeptides associated with low-passage strains may lead to identification of *B. burgdorferi* gene products required for infection and pathogenesis in mammalian hosts.

Lyme disease, caused by the spirochete *Borrelia burgdorferi*, is an arthropod-borne infection with local and systemic manifestations (for a review, see reference 58). The clinical course can be divided into three stages. Stage 1 is characterized by erythema migrans, an erythematous rash occurring days to weeks following the bite of the infected arthropod (usually a hard-bodied tick such as *Ixodes dammini* in the United States and *Ixodes ricinus* in Europe). Weeks to months after this local form of the disease, patients often develop constitutional symptoms including fatigue, malaise, myalgia, and lymphadenopathy, as well as more severe neurologic and cardiovascular manifestations such as Bell's palsy and atrioventricular block (stage 2). After months to years, a migrating, asymmetric polyarthritis, chronic neurologic disorders, and skin lesions called acrodermatitis chronica atrophicans may develop (stage 3). These stages may overlap to some extent.

The pathogenetic mechanisms that generate the clinical manifestations of Lyme disease are not clearly understood. Evidence to date indicates that *B. burgdorferi* is primarily an invasive pathogen with little, if any, toxigenic activity. Few organisms are present in Lyme disease lesions at any stage of infection; in fact, they are often difficult to detect by histologic examination or culture. Factors that may contribute to the invasiveness of *B. burgdorferi* are motility (35, 51), adherence to mammalian cells (20, 37, 60), and the penetration of endothelial barriers (19). Georgilis et al. (27) have also reported that infectious strains of *B. burgdorferi* are relatively resistant to phagocytosis. As in syphilis, another chronic spirochetal infection, the late manifestations of Lyme disease (especially the polyarthritis) appear to result in part from immunopathologic mechanisms (58, 59). It has been postulated that immunologic cross-reactivity between *B. burgdorferi* proteins and the related human proteins (such as the chaperonin proteins Hsp60 and DnaK) may elicit an autoimmune reaction, leading to chronic inflammation and tissue destruction (18, 36, 56). Lipoproteins of *B. burgdorferi* have also been shown to nonspecifically activate interleukin-1 and tumor necrosis factor expression, which may in turn promote inflammation (29, 47, 59).

B. burgdorferi contains a linear chromosome ~1,000 kb in size (10, 21, 23), along with a variable number of linear and circular plasmids. The original isolate of strain B31 from *I. dammini* ticks in Shelter Island, N.Y., has linear plasmids with sizes of 49, 38, 24, and 16 kb and a 27-kb circular plasmid (3, 31). The low-passage Sh-2-82 strain was shown by Simpson et al. (57) to contain six circular, supercoiled plasmids ranging in size from 51 to 8.6 kb, as well as linear plasmids. OspA and OspB are two major, surface-exposed lipoproteins found in most *B. burgdorferi* strains and are encoded in a single operon in the 49-kb linear plasmid (12). Passive and active immunization against OspA have been shown to protect mice against infection with *B. burgdorferi* (24, 25), although OspA heterogeneity among different isolates results in various degrees of cross-protection (26). Other than OspA and OspB, the gene products and functional activities of this wide array of plasmids have not been

* Corresponding author. Internet address: norr@casper.med.uth.tmc.edu.

characterized. Another surface-associated lipoprotein called OspC (previously identified as pC [63]) is expressed in different amounts by *B. burgdorferi* strains (62).

B. burgdorferi strains are a heterogeneous group because of both naturally occurring differences and changes occurring during in vitro culture. *B. burgdorferi* strains freshly cultured from infected ticks, humans, and other mammals exhibit differences in protein content, antigenicity, chromosomal DNA sequences (as detected by DNA-DNA hybridization, restriction fragment polymorphism, sequences of rRNA and other genes, and polymerase chain reactions), plasmid content, and multilocus enzyme electrophoresis profiles (1, 2, 4, 7, 8, 13, 46, 50, 54, 55, 57, 63). At least three distinct subgroups of *B. burgdorferi* can be identified by these means (1, 13, 46, 50). In addition to this naturally occurring heterogeneity, *B. burgdorferi* undergoes genetic and phenotypic changes during in vitro culture. Most notable of these changes is the loss of infectivity and virulence in mammalian hosts that usually occurs after 10 to 16 in vitro passages (34, 41, 54). This loss of virulence is associated with the loss of plasmids and concomitant changes in protein expression (4, 31, 41, 54, 57). In addition, some changes in individual proteins have been observed, as exemplified by variation in gel mobility and antigenic reactivity of OspB (8, 15, 54). DNA rearrangement has also been shown to result in the formation of OspA-OspB fusion proteins because of recombination between the OspA and OspB genes (49).

Variation and attenuation of *B. burgdorferi* occurring during in vitro culture interfere with the systematic study of this organism, much as phase variations in *Neisseria gonorrhoeae* have complicated gonorrhea research. However, the association of loss of virulence with changes in plasmid content and protein expression provides an opportunity to identify plasmids and proteins that are potentially important in the pathogenicity of *B. burgdorferi*. In this study, we have compared the protein expression and plasmid content of low-passage (virulent) and high-passage (avirulent) *B. burgdorferi* B31 as a starting point for the identification of low-passage-associated plasmids and proteins important in the pathogenesis and immunogenicity of the Lyme disease spirochete.

MATERIALS AND METHODS

Spirochetal strains. B31 (ATCC 35210), the type strain of *B. burgdorferi*, was isolated in 1981 from a pool of *I. dammini* ticks from Shelter Island, N.Y. (16). Low-passage B31 was stored at liquid N_2 temperatures and had undergone 4 to 10 in vitro passages; it has been shown to be infectious to laboratory rats and mice (11, 42). High-passage B31 had been passaged in vitro for several years (approximately 7,000 generations) and is noninfectious (41, 54). Additional *B. burgdorferi* strains examined for plasmid content have been described previously (4). *Borrelia hermsii*, strain HS1, serotype C (ATCC 35209), was used as a control in some portions of this study. Strains were cultured in BSK II medium with 7% normal rabbit serum (Pel-Freez Biologicals, Rogers, Ark.) as described previously (3).

Protein electrophoresis and characterization. Late-log-phase to early-stationary-phase cultures of *B. burgdorferi* were centrifuged, washed with phosphate-buffered saline (PBS; 135 mM NaCl, 9 mM Na_2HPO_4, 6 mM KH_2PO_4 [pH 7.2]), resuspended at a concentration of $\geq 10^{10}$ organisms per ml in PBS, and either used immediately or frozen at −20°C. Quantities equivalent to 2×10^8 organisms were subjected to nonequilibrium pH gradient electrophoresis (NEPHGE) as previously described (45) and then to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 8 to 20% linear gradient polyacrylamide gels (43). In the SDS-PAGE dimension, lanes containing low-passage B31 or low- and high-molecular-weight standards (Bio-Rad, Richmond, Calif.) were included on either side of the NEPHGE pattern as controls. The relative molecular masses (M_rs) of *B. burgdorferi* polypeptides in the two-dimensional pattern were estimated by using internal standard gels, in which molecular weight standards were added to the overlay agarose used to seal the NEPHGE gel to the SDS-polyacrylamide gel. Proteins were visualized by silver staining (26) or staining with Coomassie blue R. To minimize variation, sample preparation and gel electrophoresis were performed in parallel whenever samples were to be compared directly (e.g., Fig. 1).

Immunoperoxidase staining of two-dimensional electroblots was performed as described previously (43, 44), with 4-chloronaphthol as the substrate. The monoclonal antibodies H5332 (anti-OspA) (9), H6831 (anti-OspB) (8), and H9724 (anti-flagellin) (6) were used as culture supernatants at a 1:10 dilution in PBS with 0.05% Tween 20 (Sigma, St. Louis, Mo.). Polyclonal rabbit antiserum against a gel-purified 24-kDa *B. burgdorferi* protein was graciously provided by Tom G. Schwan, Rocky Mountain Laboratories, Hamilton, Mont., and was used at a 1:200 dilution in PBS-Tween. Monoclonal antibody L22 as culture supernatant was the kind gift of Bettina Wilske (Pettenkofer Institute, University of Munich) and was used at a dilution of 1:20.

Intrinsic radiolabelling with [9,10(*n*)-^{3}H]palmitic acid (TRK909; 40 to 60 Ci/mmol; Amersham, Arlington Heights, Ill.) was carried out according to the method of Brandt et al. (14), and the labelled polypeptides were separated by two-dimensional gel electrophoresis (2DGE) and visualized by autofluorography.

Surface proteolysis was performed as described previously (9) with minor modifications. Low-passage *B. burgdorferi* B31 grown to late log phase was centrifuged at 3,000 × *g* for 15 min and resuspended in PBS-Mg (PBS, 5 mM $MgCl_2$ [pH 7.5]) at a concentration of 2×10^9/ml. Fifty microliters of proteinase K solution (4 mg/ml; Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) was added to 450 μl of cell suspension and incubated at 24°C for 10, 20, and 40 min; controls were incubated under the same conditions without proteinase K. The reaction was stopped by addition of 10 μl of phenylmethylsulfonyl fluoride (50 mg/ml in isopropanol; Sigma), and the organisms were washed twice with PBS-Mg at 4°C. The spirochetes were motile before and after proteinase K treatment, as determined by dark-field microscopy. Samples (2×10^8 organisms) were prepared as described above and subjected to 2DGE and silver staining.

Protein purification and amino acid sequencing. OspD was purified from *B. burgdorferi* low-passage B31 strain by large-scale 2DGE as described previously (44). Gel slices containing 20 two-dimensional gel spots (equivalent to OspD from $\sim 10^{10}$ organisms) were emulsified and electrophoresed in a single well of a preparative SDS-polyacrylamide gel. The resulting protein band was electroeluted and dialyzed by using a model 1750 electrophoretic concentrator (Isco, Lincoln, Neb.) and precipitated twice with ethanol (33). The dried pellet was treated with cyanogen bromide in 70% formic acid as described by Matsudaira (38). Fragments were separated on an SDS–20% polyacrylamide gel, electroblotted to a polyvinyl difluoride membrane (Millipore, Bedford, Mass.), and stained with Coomassie blue R (39).

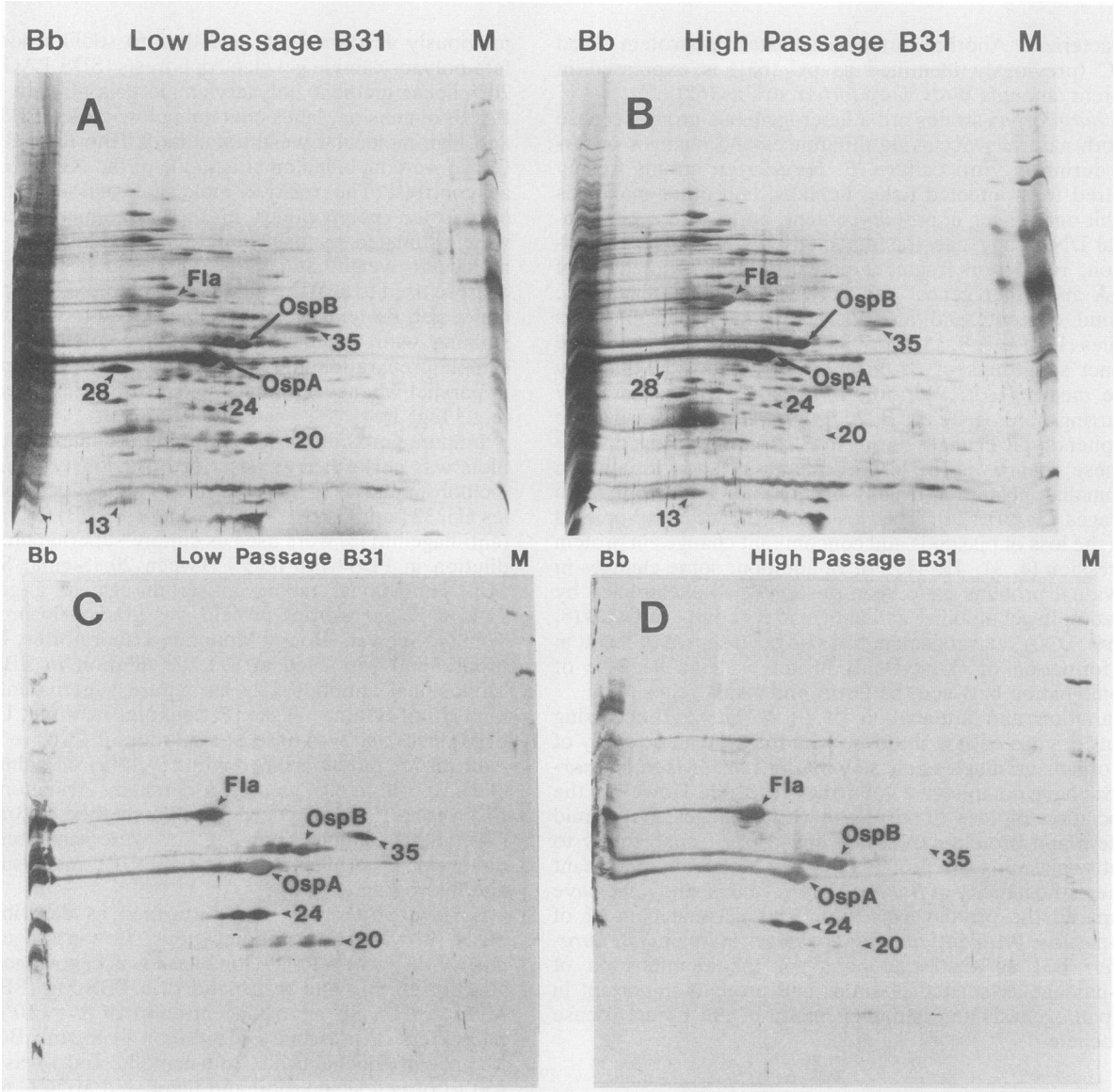

FIG. 1. Comparison of 2DGE patterns of low- and high-passage *B. burgdorferi* (strain B31). (A and B) 2DGE patterns obtained by using NEPHGE in the first dimension, SDS-PAGE in the second dimension, and silver stain for protein visualization. In this and all subsequent figures, the acid end of the NEPHGE separation is oriented to the left, and the 2DGE pattern is flanked by single-dimension lanes containing low-passage B31 (Bb) and Bio-Rad molecular weight standards (M). The locations of polypeptides, including flagellin (Fla), the major surface lipoproteins OspA and OspB, and a minor lipoprotein (M_r = 13,000) are indicated. Low-passage-associated proteins with M_rs of 28,000 (OspD), 35,000, 24,000, and 20,000 are present in low-passage B31 but absent or underexpressed in high-passage B31. (C and D) Immunoblot identification of major *B. burgdorferi* polypeptides. Electroblots of 2DGE patterns identical to those shown in panels A and B were reacted sequentially with the following monoclonal antibodies (MAb) or antiserum: MAb H5322 (anti-OspA), MAb H6831 (anti-OspB), MAb 9724 (anti-flagellin), and rabbit anti-24-kDa protein. MAb H6831 reacted with both OspB and the 20-kDa low-passage-associated protein. The anti-24-kDa antiserum reacted with a 24-kDa spot in both strains and the 35-kDa protein expressed in low-passage *B. burgdorferi* B31.

N-terminal sequences were determined by Richard G. Cook at the Baylor College of Medicine Protein Chemistry Core Facility by using a model 477A sequenator (Applied Biosystems, Foster City, Calif.).

Nucleic acid procedures. Total DNA or total RNA was isolated from borreliae by the methods of Meier et al. (40). Isolation of plasmid DNA from *B. burgdorferi* and from *Escherichia coli* was performed essentially as described previously (12). Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase (Boehringer-Mannheim), and Sequenase (U.S. Biochemical, Cleveland, Ohio) were used as recommended by the manufacturers. DNA was examined by constant-field electrophoresis, pulsed-field electrophoresis, and two-dimensional electrophoresis by the methods of Ferdows and Barbour (23). Field inversion electrophoresis (see Fig. 6) utilized a PPI-100 power inverter (MJ Devices, Waltham, Mass.) at 7 V/cm. The forward pulse time was 0.5 s, and the reverse pulse times consisted of a cycle of 0.25-, 0.15-, and 0.5-s pulses repeated throughout the 14-h electrophoresis period. DNA markers consisted of 8.3- to 48.5-kb size standards (Bethesda Research Laboratories, Gaithersburg, Md.). Southern and Northern (RNA) blots were carried out as described previously (17). Recovery of DNA fragments from gels was performed with an analytical

electroelutor (International Biotechnologies, New Haven, Conn.) or by agarose extraction with GeneClean (Bio101, La Jolla, Calif.). Ligation of insert DNA into pUC18 or pUC19 plasmid vectors and subsequent transformation into competent *E. coli* cells of strains JM109 or SURE (Stratagene, La Jolla, Calif.) were performed by standard techniques (52).

Probes and primers. Custom oligonucleotides were purchased from Midland Certified Reagent Co. (Odessa, Tex.) or were synthesized on an Applied Biosystems DNA synthesizer at the University of Texas Health Science Center at San Antonio. Probes for the 27-kb circular plasmid and the 16-, 29-, and 49-kb linear plasmids of strain B31 were recombinant plasmids pBC27a (31) and pTL16 (33), respectively. Probes in plasmids were labelled with [α-^{32}P]dATP by nick translation; oligonucleotide probes were 5′ end labelled with [γ-^{32}P]ATP and T4 kinase.

DNA sequence analysis. DNA sequences for both strands were determined by using double-stranded recombinant DNA and primer-directed dideoxynucleotide procedures as described previously (5). The transcriptional start site was identified by primer extension analysis of *B. burgdorferi* mRNA (17). The software developed by Harr et al. (30) for VAX computers (Digital Equipment Corporation, Marlborough, Mass.) was used to assemble the DNA sequences. Software from the University of Wisconsin Genetics Computer Group for the VAX was used for subsequent sequence analysis (22). The following nucleic acid and protein data bases were used for sequence comparisons: GenBank (release 70.0), EMBL (29.0), PIR-Protein (30.0), and SwissProt (21.0).

Nucleotide sequence accession number. The sequence shown in Fig. 8 has been assigned the GenBank accession number M97452.

RESULTS

Identification of low-passage-associated proteins. The 2DGE profiles of low-passage and high-passage B31 strains were compared to identify proteins expressed preferentially by low-passage, virulent organisms (Fig. 1A and B). NEPHGE was utilized in the first dimension to permit resolution of highly basic proteins, including OspA and OspB, which elute from the cathodic end of the gel during standard isoelectric focusing. Trailing of proteins from spots toward the acid (left) sides of the gels is due to the nonequilibrium nature of NEPHGE. Immunoblots of low- and high-passage B31 strains were reacted with monoclonal and polyclonal antibodies to verify the identity of some of the major proteins (Fig. 1C and D).

In comparing the low- and high-passage strains, most of the major spots in the 2DGE patterns were similar in mobility and staining intensity, including those associated with OspA, OspB, and flagellin (Fig. 1A and B). However, polypeptides with M_rs of 28,000 and 35,000 were present in low-passage B31 but were not detected in the high-passage strain. In addition, two polypeptides with M_rs of ~24,000 and 20,000 were expressed in lower quantities in the high-passage strain relative to that in its low-passage counterpart. Because this pattern of expression was observed consistently in numerous gels, these four polypeptides were selected for further study.

Most prominent of these low-passage-associated proteins was a slightly acidic polypeptide with an M_r of 28,000 (OspD). OspD was a major constituent in low-passage B31. It migrated just below OspA and thus is not easily identified in standard SDS-polyacrylamide gels; however, it was clearly resolved by 2DGE because of the charge differences of these two proteins.

Other low-passage-associated proteins included those with M_rs of 35,000, 24,000, and 20,000. The 24-kDa polypeptide was present in reduced quantities in high-passage B31 compared with that in the low-passage strain (Fig. 1A and B). It reacted strongly with a monospecific rabbit antiserum prepared against the gel-purified 24-kDa protein of *B. burgdorferi* Sh-2-82 by Tom G. Schwan (Fig. 1C and D). Interestingly, this antiserum also reacted with a basic 35-kDa polypeptide that was present in low-passage B31 (Fig. 1C) but apparently absent in the high-passage strain (Fig. 1D); T. G. Schwan had previously observed this reaction with single-dimension SDS-PAGE immunoblots of low-passage *B. burgdorferi* strains (53). Therefore, these proteins appear to have shared epitopes. The anti-OspC monoclonal antibody L22 (graciously provided by Bettina Wilske) reacted specifically with the 24-kDa polypeptide in two-dimensional immunoblots, with no apparent reaction with the 35-kDa polypeptide (data not shown). On this basis, the 24-kDa polypeptide is apparently equivalent to the membrane-bound lipoprotein OspC (62, 63).

The 20-kDa polypeptide is expressed in much smaller quantities in the high-passage B31 strain relative to that in the low-passage strain. This protein appears to be antigenically related to OspB and may be a truncated form of this protein (15). It migrates at the same position as OspB in the NEPHGE dimension (indicating a similar charge) and reacts strongly with the anti-OspB monoclonal antibody H6831.

Low-passage-associated proteins are lipoproteins. To determine whether OspD and other low-passage-associated proteins were lipoproteins, low- and high-passage B31 strain *B. burgdorferi* were radiolabelled with [^{3}H]palmitate and subjected to 2DGE and autofluorography (Fig. 2). As demonstrated in a previous study by Brandt et al. (14), the major membrane lipoproteins OspA and OspB were heavily labelled, whereas little or no detectable radioactivity was associated with nonlipidated proteins such as the flagellin protein. OspD was labelled at an intensity similar to that of OspA and OspB, indicating that it is also lipidated. The 35-, 24-, and 20-kDa polypeptides identified as low-passage-associated proteins were also labelled with [^{3}H]palmitate. Two additional proteins expressed in both low- and high-passage B31 were radiolabelled: an intensely labelled 13-kDa polypeptide and an acidic protein with an M_r of ~20,000 (Fig. 2). Trailing of the 24- and 13-kDa spots toward the acidic side of the gel was apparently due to the high level of radiolabel incorporated and was less evident with shorter autoradiograph exposures (not shown).

To verify that all of the radiolabelled spots identified by 2DGE were proteins, [^{3}H]palmitate-labelled *B. burgdorferi* was solubilized and treated with proteinase K prior to electrophoresis. No radioactive spots were detected in the resulting two-dimensional gel patterns, indicating that all of the spots detected by this means represented lipidated proteins rather that lipopolysaccharides or lipooligosaccharides (data not shown).

Surface proteolysis. Treatment of intact, low-passage *B. burgdorferi* B31 with proteinase K was used as a measure of surface exposure of the low-passage-associated proteins (Fig. 3). OspD was degraded after as little as 10 min of exposure to proteinase K, even though the organisms remained structurally intact and motile as determined by dark-field microscopy. Spots corresponding to OspB and the 35-, 24-, and 20-kDa low-passage-associated proteins were also decreased in size; OspA was resistant to degradation

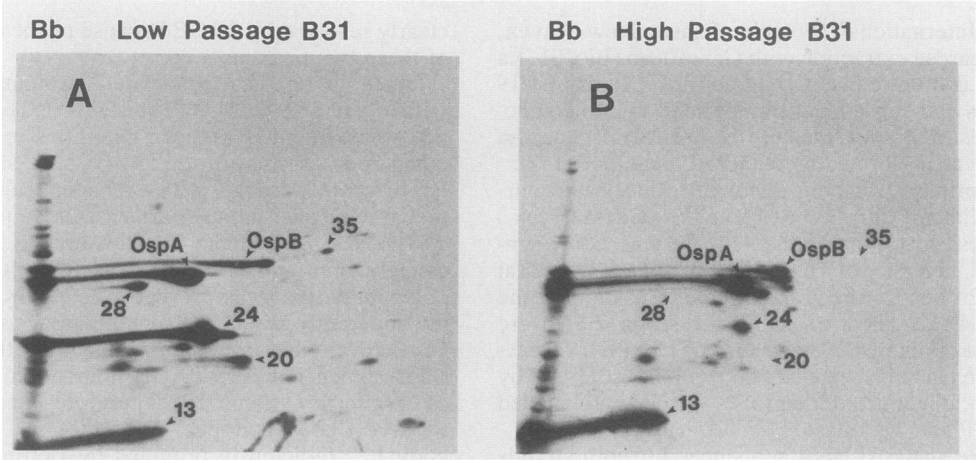

FIG. 2. Lipoproteins of *B. burgdorferi* B31 (low and high passage), as identified by intrinsic radiolabelling with [^{3}H]palmitate. Labeled spots correspond to those visualized by silver staining and immunoperoxidase reactions in Fig. 1. (A) Low-passage B31; (B) high-passage B31.

under these conditions, as noted in previous studies (9). Internally localized proteins such as flagellin (Fla) were not affected by the proteinase treatment (Fig. 3), whereas the flagellin was degraded when partially disrupted organisms were treated in the same manner (results not shown).

Amino acid sequence analysis of OspD. N-terminal amino acid sequence could not be obtained from intact OspD purified by 2DGE, consistent with blockage of the N terminus by acylation. To circumvent this problem, cyanogen bromide-generated cleavage fragments of 2DGE-purified OspD were prepared, separated by SDS-PAGE, transferred to polyvinyl difluoride membranes, and subjected to N-terminal sequencing by Edman degradation. Sequence analysis of two of these fragments yielded overlapping amino acid sequences: XXLAQMAEIDLEKI(H)N and AEIDLEKI (K)N (indeterminate residues are indicated by an X, and tentative identifications are shown in parentheses). This result indicated that the two fragments were generated by cleavage at methionine residues separated by only 5 amino acid residues and that the first peptide represented a partial cleavage product. Analysis of a third peptide yielded a sequence of XXLYKEQQKISE.

Molecular cloning of the *ospD* gene. On the basis of the partial sequence analysis of the protein, the following oligonucleotide was synthesized: 5′-AAT TTT TTC TAA ATC AAT TTC TGC CAT TTG TGC-3′. This probe corresponded to the complementary (antisense) strand of a reverse translation of the amino acids AQMAEIDLEKI, by using the most frequent codon usage found for other *Borrelia* spp. genes (12, 17, 61). Because the A + T content of the *B. burgdorferi* genome is high (70 to 72%), codons containing these bases are highly favored. The oligonucleotide was end labeled and used to probe a Northern blot of RNA from high- and low-passage B31 (Fig. 4). The probe bound to a single transcript in low-passage B31 but did not bind to RNA of high-passage B31 or of *B. hermsii*.

Having demonstrated the reactivity of the probe with a sequence expressed in low-passage B31, we next hybridized the oligonucleotide with a Southern blot of restriction enzyme-digested DNA. The probe bound to a *Hin*dIII fragment of 1.2 kb in low-passage B31 DNA. *Hin*dIII fragments of between 1 and 2 kb were electroeluted and cloned into the plasmid pUC18, and a recombinant plasmid clone containing the *ospD* sequence was identified by Southern blot analysis

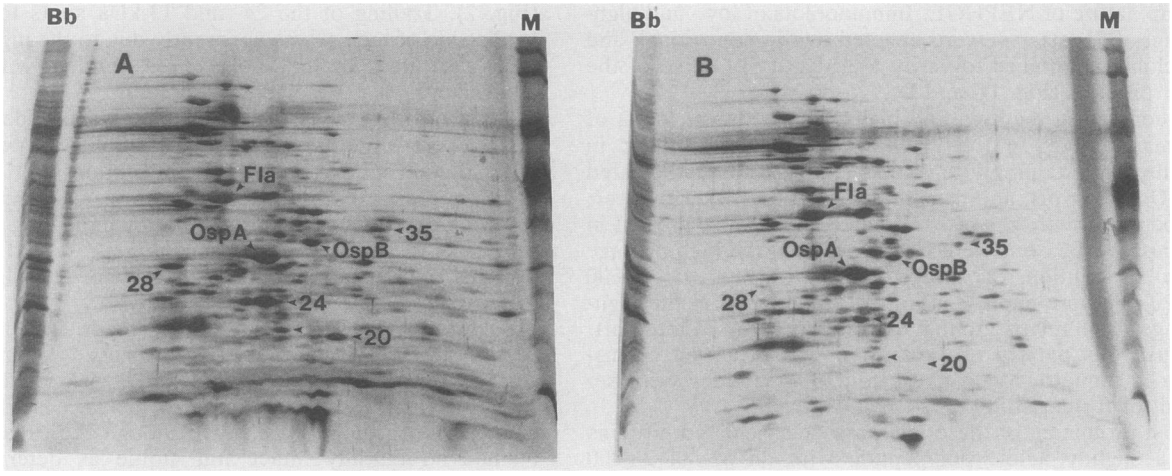

FIG. 3. Surface proteolysis of low-passage *B. burgdorferi* B31, indicating degradation of OspD and other low-passage-associated proteins. Freshly cultured borreliae were washed and exposed to proteinase K (0.5 mg/ml) for 40 min at 24°C. Proteins were then separated by 2DGE. (A) Control without proteinase K treatment; (B) with proteinase K treatment.

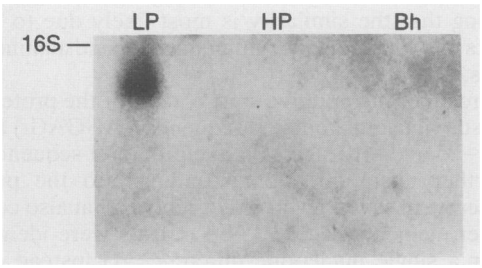

FIG. 4. Expression of RNA containing OspD-encoding sequences by low-passage *B. burgdorferi* B31. A Northern blot was prepared by using RNA purified from low-passage (LP) and high-passage (HP) *B. burgdorferi* B31 and from *B. hermsii* (Bh). The blot was incubated with a radiolabelled oligonucleotide corresponding to an amino acid sequence obtained with purified OspD (see text). The location of 16S rRNA (as determined by acridine orange staining) is indicated.

with the oligonucleotide probe. A *Pst*I-*Hin*dIII fragment of this clone was used subsequently as a hybridization probe to identify a transformant containing an overlapping 2.5-kb *Pst*I fragment. A restriction map of DNA represented by these two clones is shown in Fig. 5.

The *ospD* gene is located on a linear plasmid. The apparent loss of the *ospD* gene during in vitro cultivation suggested to us that the gene was located on a plasmid. It has been demonstrated previously that linear as well as circular plasmids of *B. burgdorferi* are lost during cultivation in the laboratory (4, 34, 54). For these experiments, we used total DNA from low- and high-passage B31 cells. In the first study we separated plasmid and chromosomal DNA by pulsed-field gel electrophoresis and probed the bands in the Southern blot with the oligonucleotide described above. The probe bound to a plasmid with an apparent size of 38 kb that was present in low-passage cells but not in the high-passage cells (Fig. 6).

To determine whether the hybridizing plasmid was a linear or a supercoiled, circular molecule, we subjected the plasmids separated by pulsed-field gel electrophoresis to high-voltage, constant-field electrophoresis at a right angle (Fig. 7). A prior study had shown that linear DNA plasmids migrated in both dimensions but that the 27-kb circular plasmid of *B. burgdorferi* did not migrate appreciably under high-voltage, constant-field conditions (23). In the present study, the 16- and 49-kb plasmids were identified in the blot of the two-dimensional gel with a probe containing sequence that is identical at the left ends of these plasmids (31, 32); this probe also reacts with the 24-kb linear plasmid. The 27-kb circular plasmid was identified in the blot with a probe specific for that replicon (31). The oligonucleotide 5′-GCA ACA AAA AAT ATC AG-3′ (nucleotides 511 to 527 in Fig. 8) was used as a specific probe for the *ospD* gene in these studies. The 38-kb plasmid containing the *ospD* gene mi-

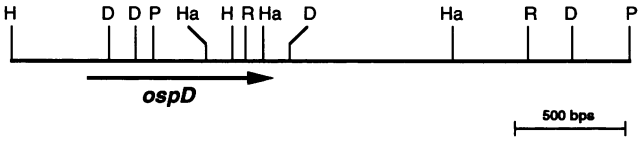

FIG. 5. Restriction endonuclease map of the DNA region containing the *ospD* gene of *B. burgdorferi* (B31 strain). The restriction sites for *Hin*dIII (H), *Dra*I (D), *Pst*I (P), *Hae*III (Ha), and *Rsa*I (R) are shown.

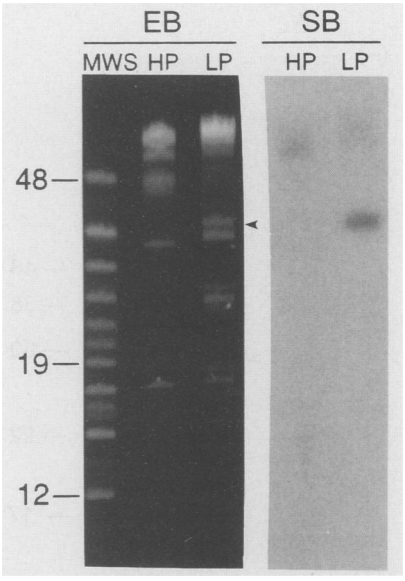

FIG. 6. The *ospD* gene is located on a 38-kb plasmid present in low-passage *B. burgdorferi* B31. Plasmid preparations from low-passage (LP) and high-passage (HP) B31 strains were separated by pulsed-field electrophoresis and visualized by ethidium bromide staining (EB). A Southern blot (SB) of the same gel was hybridized with an oligonucleotide probe based on the OspD amino acid sequence (see text). MWS, DNA size standards, with sizes indicated in kilobases (left side).

grated in the second dimension, similar to the 49- and 16-kb linear plasmids (Fig. 7). The 27-kb supercoiled circular plasmid did not migrate in the second dimension. The second spot that hybridized to the 27-kb probe and migrated more slowly in the first dimension may represent a nicked circle or a concateramized form of the 27-kb plasmid (Fig. 7). On the basis of the results of this experiment, we concluded that the 38-kb plasmid containing the *ospD* gene is linear.

Presence of OspD-encoding plasmids in other *B. burgdorferi* strains. To provide a preliminary indication of the prevalence of OspD-encoding sequences among other strains, the oligonucleotide probe used for isolation of the *ospD* gene was hybridized to Southern blots of plasmid DNA from several different low- and high-passage *B. burgdorferi* isolates (data not shown). The probe hybridized to plasmids in the 35- to 40-kb range in low-passage strains from the United States (HB19, N40, and Veery), Germany (PKa1 and PBi), and Sweden (G25). However, no plasmid in that size range was detected in United States low-passage strains Sh-2-82 and DN127, and OspD expression was not evident in these strains. Conversely, the plasmid was absent from high-passage strains 297 (United States) and ACAII (Sweden), but both the plasmid and OspD expression were identified in a nonvirulent, high-passage clone of HB19. No hybridization was detected with plasmids of *B. hermsii* HS1 (serotype C).

Sequence analysis of *ospD* gene. The sequence of the *ospD* gene and its 5′ and 3′ flanking sequences was determined by primer-directed dideoxynucleotide sequencing of the cloned DNA. The results of this analysis are shown in Fig. 8. A 771-bp open reading frame started in the *Hin*dIII fragment and continued past the internal *Hin*dIII site into the *Pst*I fragment. The deduced amino acid sequence contained the partial amino acid sequences determined previously (underlined in Fig. 8), confirming that the region corresponded to the *ospD* gene.

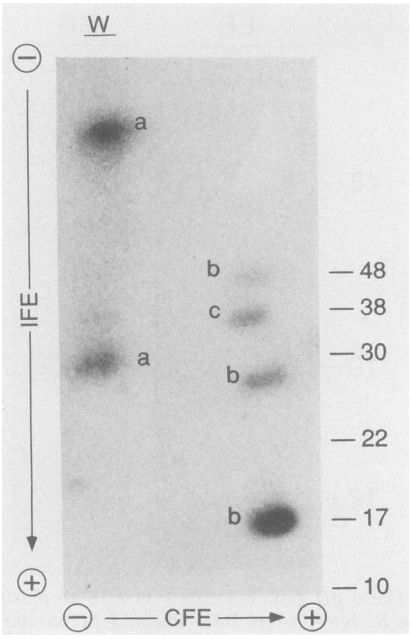

FIG. 7. Linear nature of the 38-kb plasmid containing the *ospD* gene, as demonstrated by two-dimensional agarose gel electrophoresis. A plasmid preparation from low-passage *B. burgdorferi* B31 was subjected to inverted-field electrophoresis (IFE) and then to high-voltage constant-field electrophoresis (CFE). W, location of the well in which the DNA was placed. A Southern blot of the resulting pattern was hybridized sequentially with probes specific for the 27-kb circular plasmid (a), the 16-, 24-, and 49-kb linear plasmids (b), and the 38-kb plasmid containing the *ospD* gene (c). The locations of DNA size standards (in kilobases) after constant-field electrophoresis are indicated on the right side.

The open reading frame encodes a polypeptide of 257 amino acids with a predicted molecular mass of 28,436 Da. The N terminus of the deduced sequence closely resembles the signal peptides of other bacterial lipoproteins. This motif includes three lysine residues near the N terminus, a hydrophobic region, and a sequence (L-S-I-S-C) similar to the consensus signal peptidase II cleavage sequence L-X-Y-C (64). Cleavage at the cysteine residue by signal peptidase II would yield a polypeptide with a predicted mass of 26,267 Da. Beyond the leader sequence, the deduced sequence is largely hydrophilic and has a high content of lysine, glutamic acid, asparagine, leucine, and alanine residues (15.1, 12.8, 10.4, 9.5, and 7.8%, respectively). Only two glycines and one cysteine (in the signal peptidase II site) are present. Secondary structure analyses predicted that OspD is predominantly alpha-helical.

OspD was found to have little or no sequence identity with other *Borrelia* proteins, including OspA, OspB, and the variable major proteins of *B. hermsii*. Homology comparisons were also conducted between the deduced OspD amino acid sequence and protein sequences in the SwissProt, PIR-Protein, and EMBL data bases. The closest match was 27.9% sequence identity between a 140-amino-acid segment (amino acids 51 to 190) of OspD and amino acids 353 to 486 of the *Streptococcus pyogenes* M protein precursor, serotype 12 (48); other M proteins also exhibited some sequence identity. Inclusion of conservative amino acid differences increased the sequence similarity in this region to 68.6%. However, randomized sequences of the M protein region also yielded high degrees of identity to the OspD sequence, indicating that the similarity is most likely due to the high contents of lysine, leucine, glutamic acid, and alanine in both proteins.

Upstream of the putative start codon for the protein was a consensus ribosomal binding sequence (-AGGAG-) and consensus −35 and −10 sigma-70-like promoter sequences (Fig. 8). Further upstream and continuing into the presumed promoter were seven 17-bp direct repeats that also contained promoter elements. These 17-bp repeats were identical except for a single nucleotide difference (G instead of A) at position −34. The sequence contained seven "pairs" of −35 and −10 sequences separated by 22 bp. To determine which of these several possible promoters were active in initiating transcription, primer extension analysis was performed (Fig. 9). This study showed that the A at position +1 was the start of transcription. Consequently, the closest promoter element is the most likely site for RNA polymerase binding.

The 3′ end of the open reading frame is marked by three termination codons followed by an inverted repeat consistent with a transcription termination signal (Fig. 8).

DISCUSSION

Changes in virulence, protein expression, and other phenotypic characteristics occurring during in vitro culture of *B. burgdorferi* could result from three possible mechanisms. The first, and most probable, mechanism is the spontaneous loss of plasmids encoding gene products required for infectivity and virulence. This possibility is supported by the strong association between passage number, plasmid content, and virulence (4, 34, 54). A corollary to this possible mechanism is that *trans*-acting elements encoded by plasmids may either stimulate or repress expression of chromosomal factors. The second possible mechanism is the mutation of genes required for infectivity or pathogenesis. Indeed, Rosa et al. (49) have shown that recombination between homologous regions in the OspA and OspB genes can occur during in vitro culture, resulting in a chimeric product. The third possible mechanism is alteration in gene expression resulting in phenotypic changes in the absence of genotypic change. This prospect seems least likely, because multiple passages (and many generations) are required for the loss of virulence, whereas phenotypic changes should occur shortly after introduction of the bacteria into the in vitro environment. Also, loss of virulence appears to be irreversible.

On the basis of available information, the most likely explanation is as follows: (i) plasmid-encoded proteins are required for infectivity and virulence in mammalian hosts, (ii) plasmids are lost spontaneously during in vitro culture, and (iii) clones lacking these plasmids gradually overgrow the plasmid-containing clones, resulting in the eventual elimination of *B. burgdorferi* cells containing the necessary virulence factors. The latter statement is supported by the finding that virulent isolates of *B. burgdorferi* can be rescued from moderate passage cultures by inoculation and recovery of organisms from mammalian hosts. It is likely that more than one gene product (and encoding plasmid) is required; it is also possible that more than one of the three genetic mechanisms listed above are operative.

In this study, low-passage B31 strain was found to contain several proteins that were absent or expressed in smaller quantities in B31 strain that had been passaged continuously in vitro. Neither OspD nor the 35-kDa protein were detected in the high-passage strain; therefore, it is likely that the 35-kDa protein, like OspD, is encoded by a plasmid lost

```
   -220                -200                -180
******* ** *****
TTTTTTTGCTTGTTTTTAGCATCATTAAACATCCTTTCAATACTCACTATTGTTTTCTTA

   -160                -140                -120
                                    (      .I        )(
GCCTTAAGCTAGCCAAGCTAAATAGAAATTAGTAGGCAATTGATATTAAAATATAATTGA

   -100                -80                 -60
    II.      )(  .     III   .   )(    .  IV     .)(       .V
TATTAAAATATAATTGATATTAAAATATAATTGATATTAAAATATAATTGATATTAAAAT

    -40                -20                 +1
    )(.      VI  .     )(    .  VII    .  )          .       . RBS
ATAATTGATATTGAAATATAATTGATATTAAAATATAATTTAAGACATTATATTTAAGGA

    20                  40                  60
GTATAAATATGAAAAAATTAATAAAAATACTACTGTTAAGTTTATTTTTATTGCTCTCAA
       M  K  K  L  I  K  I  L  L  L  S  S  L  F  L  L  L  S  I
       1                          10
    80                  100                 120
TATCTTGTGTTCATGATAAACAAGAATTATCATCAAAATCTAATTTAAATAATCAAAAAG
  S  C  V  H  D  K  Q  E  L  S  S  K  S  N  L  N  N  Q  K  G
  20                          30
 140                 160                 180
GATATTTAGATAATGAAGGCGCAAATTCAAATTACGAATCAAAAAAACAGAGCATATTAA
  Y  L  D  N  E  G  A  N  S  N  Y  E  S  K  K  Q  S  I  L  S
  40                          50
 200                 220                 240
GTGAGTTAAATCAGTTATTAAAGCAAACTACAAATTCACTAAAAGAAGCCAAAAATACAA
  E  L  N  Q  L  L  K  Q  T  T  N  S  L  K  E  A  K  N  T  T
  60                          70
 260                 280                 300
CAGATAATTTAAATGCATCAAATGAGGCAAATAAAGTTGTAGAAGCGGTTATAAATGCAG
  D  N  L  N  A  S  N  E  A  N  K  V  V  E  A  V  I  N  A  V
  80                          90
 320                 340                 360
TTAATTTAATTTCATCTGCTGCAGATCAAGTAAAAAGTGCAACAAAAAATATGCATGATT
  N  L  I  S  S  A  A  D  Q  V  K  S  A  T  K  N  M  H  D  L
 100                         110
 380                 400                 420
TAGCTCAAATGGCAGAAATAGATTTAGAAAAAATAAAGAACTCTAGTGATAAAGCAATAT
  A  Q  M  A  E  I  D  L  E  K  I  K  N  S  S  D  K  A  I  F
 120                         130
 440                 460                 480
TTGCATCTAATCTTGCAAAAGAAGCATATAGCCTTACTAAAGCAGCAGAACAAAACATGC
  A  S  N  L  A  K  E  A  Y  S  L  T  K  A  A  E  Q  N  M  Q
 140                         150
 500                 520                 540
AAAAACTGTATAAAGAGCAACAAAAAATATCAGAATCAGAATCAGAATCTGACTATTCTG
  K  L  Y  K  E  Q  Q  K  I  S  E  S  E  S  E  S  D  Y  S  D
 160                         170
 560                 580                 600
ATTCTGCTGAAATAAAACAAGCTAAAGAGGCCGTAGAAATAGCTTGGAAAGCTACAGTAG
  S  A  E  I  K  Q  A  K  E  A  V  E  I  A  W  K  A  T  V  E
 180                         190
 620                 640                 660
AAGCAAAAGATAAGTTAATTGATGTAGAAAATACAGTCAAAGAGACATTGGATAAAATAA
  A  K  D  K  L  I  D  V  E  N  T  V  K  E  T  L  D  K  I  K
 200                         210
 680                 700                 720
AGACAGAAACTACGAACAATACAAAGCTTGCAGATATAAAAGAAGCAGCAGAGTTAGTAT
  T  E  T  T  N  N  T  K  L  A  D  I  K  E  A  A  E  L  V  L
 220                         230
 740                 760                 780
TACAAATAGCTAAGAATGCAAAGGAAATAGTACAAGAAGTTGTGGCCTTGTTAAATACTT
  Q  I  A  K  N  A  K  E  I  V  Q  E  V  V  A  L  L  N  T  *
 240                         250
 800                 820                 840
               >>>>>                               <<<<<
AAATAAATAAAAGATGGAAGTAAGGAAGGAAGAATAATTTTGTGCTGGCTTATCCTTCC
   *   *
```

FIG. 8. DNA sequence and deduced amino acid sequence of the *ospD* gene of *B. burgdorferi* (B31 strain). Nucleotides are numbered relative to the transcription initiation site (+1). Significant features of the DNA sequence include the following: a T-rich region ~200 bp upstream of the transcription start site (asterisks); seven identical, direct repeats of a 17-nucleotide sequence containing −35 and −10 sigma-70-like promoter sequences (in brackets, numbered I through VII); the putative active −35 and −10 promoter sequences (underlines); a consensus ribosome binding site (RBS); three termination codons at the 3′ end of the open reading frame (asterisks); and complementary regions consistent with hairpin loop formation (>>>>> and <<<<<). The open reading frame encodes a 257-amino-acid polypeptide with a hydrophobic leader sequence and a consensus signal peptidase II cleavage site (amino acids 1 to 20). The underlined amino acids correspond precisely to the sequences obtained from CNBr cleavage fragments of the OspD protein.

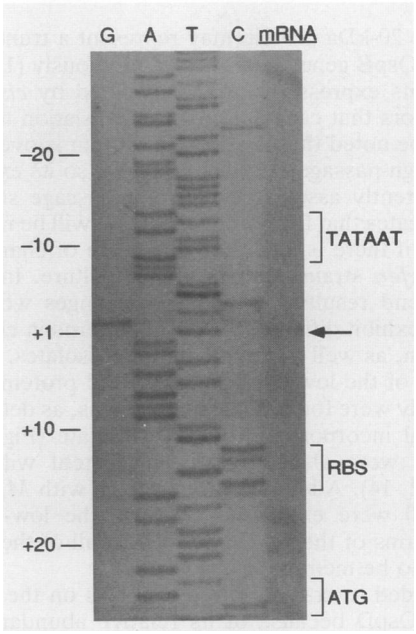

FIG. 9. Primer extension analysis of the 5′ terminus of the *ospD* gene of *B. burgdorferi* B31. The oligonucleotide used as a primer was 5′-TGC GCC TTC ATT ATC-3′, which is the reverse complement of nucleotides 143 to 157 in Fig. 8. The primer was annealed to mRNA, and the DNA was extended with reverse transcriptase. The resulting primer extension product is in the rightmost lane and is indicated by an arrow. A dideoxynucleotide sequencing reaction using the same primer and the *ospD* gene as template was carried out in parallel, yielding a sequence complementary to the coding strand. The locations of the nearest upstream −10 (TATAAT) promoter element, a consensus ribosomal binding sequence (RBS), and the putative start codon are indicated.

during in vitro culture. The 35-kDa protein is expressed in relatively small quantities in the low-passage strain. It also comigrates with another spot in the 2DGE pattern, but its pattern of expression and nonexpression in the low- and high-passage isolates was confirmed by immunoperoxidase staining due to its fortuitous cross-reactivity with antiserum against a 24-kDa protein (Fig. 1C) and by [^{3}H]palmitate labelling. The 24- and 20-kDa low-passage-associated proteins identified in Fig. 1A were present but expressed in smaller quantities in high-passage B31. Thus, their encoding genes were not eliminated during in vitro passage, and the decreased expression of these proteins represents a qualitative difference.

The 24-kDa protein reacted strongly with anti-OspC monoclonal antibody L22 (62), providing evidence that it is the same as the membrane lipoprotein OspC (previously described as the 22-kDa protein pC) (62, 63). Wilske et al. (63) noted that OspC is expressed in different quantities by Lyme disease isolates, with many European strains expressing high levels. In their study, many erythema migrans and lymphocytic meningitis patients had serum antibodies reactive with OspC (63), so OspC may be important immunolog-

ically. The 20-kDa protein may represent a truncated product of the OspB gene, as discussed previously (15). If this is the case, its expression may be affected by *cis*- or *trans*-acting factors that cause premature translation termination. It should be noted that the 20-kDa protein is overexpressed in some high-passage HB19 clones (15), so its expression is not consistently associated with low-passage strains. This result indicates that further investigation will be necessary to determine if there is a consistent pattern of changes among *B. burgdorferi* strains during in vitro culture. Indeed, plasmid loss and resulting phenotypic changes would be expected to exhibit different temporal patterns in clones of the same strain, as well as among different isolates.

All four of the low-passage-associated proteins identified in this study were found to be lipoproteins, as determined by preferential incorporation of [^{3}H]palmitate (Fig. 2). OspA and OspB were also lipidated, consistent with previous reports (12, 14). Additional lipoproteins with M_rs of 13,000 and 20,000 were expressed by both the low- and high-passage forms of the B31 strain. Thus, all of these proteins are likely to be membrane associated.

We decided to concentrate our efforts on the characterization of OspD because of its relative abundance in low-passage B31 cells and its absence in the high-passage B31 strain. The *ospD* gene was found to be associated with a 38-kb linear plasmid present in the low-passage form but missing in the high-passage strain (4, 31); thus, its pattern of expression follows the first mechanism of change (loss of encoding plasmid) listed above. The open reading frame (Fig. 5) is consistent with that of a typical bacterial lipoprotein possessing a hydrophobic leader sequence and a potential signal peptidase II cleavage site. The remainder of the deduced OspD sequence is largely hydrophilic, indicating that the protein is tethered to the membrane surface by a lipidated N terminus.

Beyond the signal peptide sequence, the deduced amino acid sequence of OspD did not show similarity to other known borrelia proteins, so it is unlikely that OspD is related to the membrane lipoproteins characterized thus far (OspA, OspB, and the variable membrane proteins). There was some similarity to the M protein of *S. pyogenes*, which is a surface-exposed protein important in adherence and resistance to phagocytosis. However, the sequence similarities to M proteins seem to reflect factors such as amino acid content and predominance of α-helical structure rather than evolutionary relatedness.

A notable feature of the DNA region upstream from the *ospD* open reading frame was the presence of seven direct repeats of a 17-bp sequence (5′-TTGATATTAAAATAT AAT-3′) containing consensus −35 and −10 sigma-70 recognition sequences (underlined). Because these repeats were in a contiguous array, they form seven pairs of −35 and −10 elements with a spacing appropriate for transcription initiation. However, only one transcription initiation site was identified by primer extension analysis (Fig. 9), and the location was consistent with the last pair of −35 and −10 sequences being active. One could speculate that this region may bind multiple sigma-70 RNA polymerase complexes and serve as a "park and ride lot" for transcription initiation. This site may also undergo recombination, resulting in duplication or deletion of one or more of the direct repeats. Future studies will determine if the multiple repeats have any special properties with regard to transcriptional regulation of the *ospD* gene.

Surface proteolysis of intact low-passage *B. burgdorferi* B31 resulted in nearly complete degradation of OspD and quantitative reductions in the 35-, 24-, and 20-kDa proteins under conditions where internal proteins are not degraded (Fig. 3). This result provides compelling, but not definitive, evidence that OspD and the other low-passage-associated proteins are surface exposed. Further analysis will be necessary to confirm the surface expression of these proteins.

A preliminary examination of the presence of OspD in other *B. burgdorferi* isolates revealed that the protein is expressed by other low-passage strains but that its presence is not the sine qua non for infectivity. Seven of the nine low-passage isolates examined contained a 35- to 38-kb plasmid with sequences homologous to the *ospD* gene; similar plasmids have been identified in other strains previously (4, 54). However, the low-passage strain Sh-2-82, which is virulent in animal models, does not contain a homologous plasmid or express OspD. The OspD-encoding plasmid was also absent from the low-passage California tick isolate DN127, but the virulence of this strain has not been reported. Most of the high-passage, nonvirulent strains tested lacked the plasmid. However, the high-passage, nonvirulent HB19 strain of *B. burgdorferi* was found to contain the plasmid. OspD expression does not appear to be required for infectivity in mammals, although this finding does not rule out the involvement of this protein in some aspect of pathogenesis.

At present, we do not know if the other low-passage-associated proteins identified in this investigation represent *B. burgdorferi* virulence factors. Association between infectivity and the presence of certain plasmids or genes could be established by correlation of infectivity and plasmid or gene content in *B. burgdorferi* clones. Definitive identification of virulence factors would require genetic studies, such as the recovery of infectivity following transfer of the genes encoding the putative factors or mutation and reversion of putative virulence factor genes. Development of these genetic procedures for borreliae would permit a more detailed analysis of the *B. burgdorferi* factors important in the pathogenesis of Lyme disease.

The identification of low-passage-associated proteins may be of practical importance in terms of the diagnosis and control of Lyme disease. Most immunodiagnostic tests utilize high-passage strains of *B. burgdorferi* which may lack these proteins. In future studies, we will determine the immunoreactivity of OspD and other low-passage-associated proteins and the potential usefulness of these proteins in immunodiagnosis and in immunoprotection against Lyme disease.

ACKNOWLEDGMENTS

We thank Tom Schwan and Patricia Rosa of the Rocky Mountain Laboratory and Bettina Wilske of the Pettenkofer Institute, University of Munich, for sharing information and antibodies with us prior to publication.

This work was supported by grant AR41507 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

REFERENCES

1. **Adam, T., G. S. Gassmann, C. Rasiah, and U. B. Göbel.** 1991. Phenotypic and genotypic analysis of *Borrelia burgdorferi* isolates from various sources. Infect. Immun. **59**:2579–2585.
2. **Anderson, J. F., L. A. Magnarelli, R. B. Lefebvre, T. G. Andreadis, J. B. McAninch, G.-C. Perng, and R. C. Johnson.** 1989. Antigenically variable *Borrelia burgdorferi* isolated from cottontail rabbits and *Ixodes dammini* from rural and urban areas. J. Clin. Microbiol. **27**:13–20.
3. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:521–525.

4. **Barbour, A. G.** 1988. Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. J. Clin. Microbiol. **26**:475–478.
5. **Barbour, A. G., C. J. Carter, N. Burman, C. S. Freitag, C. F. Garon, and S. Bergström.** 1991. Tandem insertion sequence-like elements define the expression site for variable antigen genes of *Borrelia hermsii*. Infect. Immun. **59**:390–397.
6. **Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier.** 1986. A *Borrelia* genus-specific monoclonal antibody binds to a flagellar epitope. Infect. Immun. **52**:549–554.
7. **Barbour, A. G., R. A. Heiland, and T. R. Howe.** 1985. Heterogeneity of major proteins in Lyme disease Borreliae: a molecular analysis of North American and European isolates. J. Infect. Dis. **152**:478–484.
8. **Barbour, A. G., S. L. Tessier, and S. F. Haye.** 1984. Variation in a major protein of Lyme disease spirochetes. Infect. Immun. **45**:94–100.
9. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41**:795–804.
10. **Baril, C., C. Richaud, G. Baranton, and I. Saint Girons.** 1989. Linear chromosome of *Borrelia burgdorferi*. Res. Microbiol. **140**:507–516.
11. **Barthold, S. W., K. D. Moody, and D. S. Beck.** 1990. Susceptibility of laboratory rats to isolates of *Borrelia burgdorferi* from different geographic areas. Am. J. Trop. Med. Hyg. **42**:596–600.
12. **Bergström, S., V. G. Bundoc, and A. G. Barbour.** 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochaete *Borrelia burgdorferi*. Mol. Microbiol. **3**:479–486.
13. **Boerlin, P., O. Peter, A.-G. Bretz, D. Postic, G. Baranton, and J.-C. Piffaretti.** 1992. Population genetic analysis of *Borrelia burgdorferi* isolates by multilocus enzyme electrophoresis. Infect. Immun. **60**:1677–1683.
14. **Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect. Immun. **59**:983–991.
15. **Bundoc, V. G., and A. G. Barbour.** 1989. Clonal polymorphisms of outer membrane protein OspB of *Borrelia burgdorferi*. Infect. Immun. **57**:2733–2741.
16. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick borne spirochetosis? Science **216**:1317–1319.
17. **Burman, N., S. Bergström, B. J. Restrepo, and A. G. Barbour.** 1990. The variable antigens Vmp7 and Vmp21 of the relapsing fever bacterium *Borrelia hermsii* are structurally analogous to the VSG proteins of the African trypanosome. Mol. Microbiol. **4**:1715–1726.
18. **Cluss, R. G., and J. T. Boothby.** 1990. Thermoregulation of protein synthesis in *Borrelia burgdorferi*. Infect. Immun. **58**: 1038–1042.
19. **Comstock, L. E., and D. D. Thomas.** 1989. Penetration of endothelial cell monolayers by *Borrelia burgdorferi*. Infect. Immun. **57**:1626–1628.
20. **Comstock, L. E., and D. D. Thomas.** 1991. Characterization of *Borrelia burgdorferi* invasion of cultured endothelial cells. Microb. Pathog. **10**:137–148.
21. **Davidson, B. S., J. MacDougall, and I. Saint Girons.** 1992. Physical map of the linear chromosome of *Borrelia burgdorferi* 212, the causative agent of Lyme disease, and localization of rRNA genes. J. Bacteriol. **174**:3766–3774.
22. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
23. **Ferdows, M. S., and A. G. Barbour.** 1989. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. Proc. Natl. Acad. Sci. USA **86**:5969–5973.
24. **Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell.** 1990. Protection of mice against Lyme disease agent by immunizing with recombinant OspA. Science **250**:553–556.
25. **Fikrig, E., S. W. Barthold, N. Marcantonio, K. DePonte, F. S. Kantor, and R. A. Flavell.** 1992. Roles of OspA, OspB, and flagellin in protective immunity to Lyme borreliosis in laboratory mice. Infect. Immun. **60**:657–661.
26. **Fikrig, E., S. W. Barthold, D. H. Persing, X. Sun, F. S. Kantor, and R. A. Flavell.** 1992. *Borrelia burgdorferi* strain 25015: characterization of outer surface protein A and vaccination against infection. J. Immunol. **148**:2256–2260.
27. **Georgilis, K., A. C. Steere, and M. S. Klemper.** 1991. Infectivity of *Borrelia burgdorferi* correlates with resistance to elimination by phagocytic cells. J. Infect. Dis. **163**:150–155.
28. **Guevera, J., Jr., D. A. Johnston, L. S. Ramagli, L. S. Martin, B. S. Capetillo, and L. V. Rodriguez.** 1982. Quantitative aspects of silver deposition in proteins resolved in complex polyacrylamide gels. Electrophoresis **3**:197–205.
29. **Habicht, G. S., G. Beck, J. L. Benach, J. L. Coleman, and K. D. Leichtling.** 1985. Lyme disease spirochetes induce human and murine interleukin 1 production. J. Immunol. **134**:3147–3154.
30. **Harr, R., P. Fällman, M. Häggström, L. Wahlström, and P. Gustafsson.** 1986. GENEUS, a computer system for DNA and protein sequence analysis containing an information retrieval system for the EMBL data library. Nucleic Acids Res. **14**:273–284.
31. **Hinnebusch, J., and A. G. Barbour.** 1992. Linear- and circular-plasmid copy number in *Borrelia burgdorferi*. J. Bacteriol. **174**:5251–5257.
32. **Hinnebusch, J., S. Bergström, and A. G. Barbour.** 1990. Cloning and sequence analysis of linear plasmid telomeres of the bacterium *Borrelia burgdorferi*. Mol. Microbiol. **4**:811–820.
33. **Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood.** 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. **91**:227–236.
34. **Johnson, R. C., N. Marek, and C. Kodner.** 1984. Infection of Syrian hamsters with Lyme disease spirochetes. J. Clin. Microbiol. **20**:1099–1101.
35. **Kimsey, R. B., and A. Spielman.** 1990. Motility of Lyme disease spirochetes in fluids as viscous as the extracellular matrix. J. Infect. Dis. **162**:1205–1208.
36. **Luft, B. J., P. D. Gorevic, W. Jiang, P. Munoz, and R. J. Dattwyler.** 1991. Immunologic and structural characterization of the dominant 66- and 73kDa antigens of *Borrelia burgdorferi*. J. Immunol. **146**:2776–2782.
37. **Ma, Y., A. Sturrock, and J. J. Weis.** 1991. Intracellular localization of *Borrelia burgdorferi* within human endothelial cells. Infect. Immun. **59**:671–678.
38. **Matsudaira, P.** 1990. Limited N-terminal sequence analysis. Methods Enzymol. **182**:602–613.
39. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**:10035–10038.
40. **Meier, J. T., M. I. Simon, and A. G. Barbour.** 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever *Borrelia* sp. Cell **41**:403–409.
41. **Moody, K. D., S. Barthold, and G. A. Tergwilliger.** 1990. Lyme borreliosis in laboratory animals: effect of host species and in vitro passage of *Borrelia burgdorferi*. Am. J. Trop. Med. Hyg. **43**:87-92.
42. **Norris, S. J.** 1992. Unpublished data.
43. **Norris, S. J., J. F. Alderete, N. H. Axelsen, M. J. Bailey, S. A. Baker-Zander, J. B. Baseman, P. J. Bassford, R. E. Baughn, A. Cockayne, P. A. Hanff, P. Hindersson, S. A. Larsen, M. A. Lovett, S. A. Lukehart, J. N. Miller, M. A. Moskophidis, F. Müller, M. V. Norgard, C. W. Penn, L. V. Stamm, J. D. van Embden, and K. Wicher.** 1987. Identity of *Treponema pallidum* subsp. *pallidum* polypeptides: correlation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis results from different laboratories. Electrophoresis **8**:77–92.
44. **Norris, S. J., N. W. Charon, R. G. Cook, M. D. Fuentes, and R. J. Limberger.** 1988. Antigenic relatedness and N-terminal sequence homology define two classes of periplasmic flagellar proteins of *Treponema pallidum* subsp. *pallidum* and *Treponema phagedenis*. J. Bacteriol. **170**:4072–4082.
45. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High-resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12**:1133–1142.

46. **Postic, D., C. Edlinger, C. Richaud, F. Grimont, Y. Dufresne, P. Perolat, G. Baranton, and P. A. D. Grimont.** 1990. Two genomic species in *Borrelia burgdorferi*. Res. Microbiol. **141:**465–475.
47. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. J. Immunol. **147:**1968–1974.
48. **Robbins, J. C., J. G. Spanier, S. J. Jones, W. J. Simpson, and P. P. Cleary.** 1987. *Streptococcus pyogenes* type 12 M protein gene regulation by upstream sequences. J. Bacteriol. **169:**5633–5640.
49. **Rosa, P. A. (Rocky Mountain Laboratories).** 1992. Personal communication.
50. **Rosa, P. A., D. Hogan, and T. G. Schwan.** 1991. Polymerase chain reaction analyses identify two distinct classes of *Borrelia burgdorferi*. J. Clin. Microbiol. **29:**524–532.
51. **Sadziene, A., D. D. Thomas, V. G. Bundoc, S. C. Holt, and A. G. Barbour.** 1991. A flagella-less mutant of *Borrelia burgdorferi*: structural, molecular, and in vitro functional characterization. J. Clin. Invest. **88:**82–92.
52. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
53. **Schwan, T. G.** 1992. Personal communication.
54. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect. Immun. **56:**1831–1836.
55. **Schwan, T. G., and W. J. Simpson.** 1991. Factors influencing the antigenic reactivity of *Borrelia burgdorferi*, the Lyme disease spirochete. Scand. J. Infect. Dis. **77**(Suppl.)**:**94–101.
56. **Shanafelt, M.-C., P. Hindersson, C. Soderberg, N. Mensi, C. W. Turck, D. Webb, H. Yssel, and G. Peltz.** 1991. T cell and antibody reactivity with the *Borrelia burgdorferi* 60-kDa heat shock protein in Lyme arthritis. J. Immunol. **146:**3985–3992.
57. **Simpson, W. J., C. F. Garon, and T. G. Schwan.** 1990. Analysis of supercoiled circular plasmids in infectious and non-infectious *Borrelia burgdorferi*. Microb. Pathog. **8:**109–118.
58. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
59. **Szczepanski, A., and J. L. Benach.** 1991. Lyme borreliosis: host responses to *Borrelia burgdorferi*. Microbiol. Rev. **55:**21–34.
60. **Thomas, D. D., and L. E. Comstock.** 1989. Interaction of Lyme disease spirochetes with cultured eucaryotic cells. Infect. Immun. **57:**1324–1326.
61. **Wallich, R., S. E. Moter, M. M. Simon, K. Ebnet, A. Heiberger, and M. D. Kramer.** 1990. The *Borrelia burgdorferi* flagellum-associated 41-kilodalton antigen (flagellin): molecular cloning, expression, and amplification of the gene. Infect. Immun. **58:**1711–1719.
62. **Wilske, B. (Pettenkofer Institute, University of Munich).** 1992. Personal communication.
63. **Wilske, B., V. Preac-Mursik, G. Schierz, and K. V. Busche.** 1986. Immunochemical and immunological analysis of European *Borrelia burgdorferi* strains. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **263:**92–102.
64. **Wu, H. C., and M. Tokunaga.** 1986. Biogenesis of lipoproteins in bacteria. Curr. Top. Microbiol. Immunol. **125:**127–157.