JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1995, p. 2304–2315 Vol. 33, No. 9
0095-1137/95/$04.00+0

Genotypic and Phenotypic Characterization of *Borrelia burgdorferi* Isolated from Ticks and Small Animals in Illinois

ROGER N. PICKEN,[1]* YU CHENG,[1] DONGMEI HAN,[1] JEFFREY A. NELSON,[2] A. GIRIDHER REDDY,[3] MARY K. HAYDEN,[2] MARIA M. PICKEN,[4] FRANC STRLE,[5] JOHN K. BOUSEMAN,[6] AND GORDON M. TRENHOLME[2]

Department of Immunology/Microbiology[1] and Section of Infectious Disease,[2] Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612; Divisional Protein-Peptide Core Facility, University of Chicago, Chicago, Illinois 60637[3]; Department of Pathology, Loyola University Medical Center, Maywood, Illinois 60153[4]; Department of Infectious Diseases, University Medical Centre, 61000 Ljubljana, Slovenia[5]; and Illinois Natural History Survey, Champaign, Illinois 61820[6]

Received 9 May 1995/Accepted 9 June 1995

We have characterized 33 isolates of *Borrelia burgdorferi* from northern Illinois (32 isolates) and Wisconsin (1 isolate) representing the largest series of midwestern isolates investigated to date. The techniques used for molecular analysis of strains included (i) genospecies typing with species-specific PCR primers, (ii) plasmid profiling by pulsed-field gel electrophoresis of total genomic DNA, (iii) large-restriction-fragment pattern (LRFP) analysis by pulsed-field gel electrophoresis of *Mlu*I-digested genomic DNA (J. Belfaiza, D. Postic, E. Bellenger, G. Baranton, and I. Saint Girons, J. Clin. Microbiol. 31:2873–2877, 1993), (iv) sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total proteins, (v) microsequencing of high-performance liquid chromatography-purified peptides derived from proteins showing high levels of expression, (vi) amino acid composition analysis of proteins, and (vii) immunological analysis of proteins with a polyclonal antiserum of human origin. Five reference strains as well as two atypical tick isolates from California (DN127) and New York (25015) were included for comparison. All of the Illinois and Wisconsin isolates were typed as *B. burgdorferi* sensu stricto with genospecies-specific PCR primers. The isolates were found to be heterogeneous with regard to their plasmid and protein profiles. One isolate from Illinois possessed two large-molecular-size plasmids instead of the usual 49-kb plasmid. Fragment patterns resulting from *Mlu*I digestion of genomic DNA from the 33 isolates and strains DN127 and 25015 were separable into six distinct LRFPs, five of which have not previously been described. Strain 25015 and an isolate from Illinois (CT39) shared an unusual LRFP that is not typical of other *B. burgdorferi* sensu stricto strains, suggesting that they may represent a fifth species of *B. burgdorferi* sensu lato. Five of the 33 isolates and strains DN127 and 25015 showed high-level expression of proteins with molecular masses of approximately 22 kDa. Investigation of these proteins by microsequencing of individual peptides and total amino acid composition analysis indicated that the 22-kDa proteins expressed by the seven strains were polymorphic OspC proteins. By using a polyclonal serum of human origin, expression of OspC could be detected in all 33 Illinois and Wisconsin isolates.

Lyme borreliosis is a multisystem disorder caused by spirochetes of the genus *Borrelia* and transmitted by ixodid ticks. The disease has a worldwide prevalence in temperate zones that harbor the tick vector and is currently the leading tick-borne infection in North America, Europe, and Asia. Initially it was believed that Lyme borreliosis was caused by a single *Borrelia* species, *Borrelia burgdorferi* (16, 25), although this is now known not to be the case. In the United States, the three main foci of Lyme borreliosis are the northeast (Massachusetts through Pennsylvania), the northern Midwest (Minnesota and Wisconsin), and the Pacific coast (California, Oregon, and Washington) (47). Despite its proximity to the expanding midwestern endemic focus of northwestern Wisconsin (19), Illinois has not experienced an extensive history of Lyme borreliosis and is not a region where the disease continually prevails. Thus, it would not generally be considered an area of endemicity. However, the presence of *Ixodes scapularis* (formerly *Ixodes dammini*) ticks in certain well-defined regions of the state has been documented (14, 15), and it has further been demonstrated that a proportion of the ticks in these areas are infected with *Borrelia* spp. (18, 26, 38). Thus, Illinois may be considered an area of focal endemicity for both the tick vector and the Lyme borreliosis spirochete.

Early molecular investigations of North American and European isolates of *Borrelia* spirochetes from both patients and ixodid ticks focused upon their major protein antigens. These investigations indicated that North American strains were more homogeneous than their European counterparts (1, 6–9, 17, 49, 57, 58). Analysis of North American isolates by DNA-DNA reassociation studies confirmed the high level of relatedness between strains (22, 23). However, when these techniques were later applied to a collection of European isolates, three genomic groups within the species *B. burgdorferi* could be delineated. Two of these groups have now been accorded separate species status, and the three genospecies are referred to as *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (4, 37). The genetic investigations therefore supported the phenotypic heterogeneity seen in the total protein profiles of European strains. While *B. burgdorferi* sensu stricto has been found on the North American, European, and Asian conti-

* Corresponding author. Mailing address: Section of Infectious Disease, Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL 60612. Phone: (312) 942-3223. Fax: (708) 240-0467.

nents, *B. garinii* and *B. afzelii* have thus far been isolated only in Europe and Asia. Unclassified isolates of *Borrelia* spp. from ixodid ticks or Lyme borreliosis patients have been termed *B. burgdorferi* sensu lato (4).

Since northern Illinois has thus far been shown to be an area of only focal endemicity, the following question arises: do *Borrelia* spp. isolated from *I. scapularis* ticks in this region differ in some important regard from isolates of *B. burgdorferi* sensu lato derived from true regions of endemicity? We therefore considered it of interest to investigate the extent of the genetic and phenotypic diversity prevalent among spirochetes from Illinois and to compare our unclassified midwestern isolates with reference strains of *B. burgdorferi* sensu lato derived from patients and ticks in the United States and Europe. Also, although large numbers of isolates from the northeastern and Pacific foci have been investigated in detail, an extensive series of isolates from the Midwest has not previously been characterized.

One of the earliest and most important observations concerning the differences between North American and European isolates was the perception that nearly 50% of European strains expressed a major 22-kDa protein, termed pC (later OspC), whereas proteins of this size were only rarely detected in North American isolates (58, 59). During the course of our molecular characterization of the midwestern isolates we noticed that several strains possessed prominent proteins of approximately 22 kDa, as determined by examination of their total protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recently, the deduced amino acid sequences of OspC (21, 24, 50, 56) and partial amino acid sequences of several other 22-kDa proteins (30, 31, 33, 53) from *B. burgdorferi* sensu lato isolates have been published. We therefore considered it worthwhile to characterize the prominent 22-kDa proteins from these strains and to compare their amino acid sequences with published sequences. We also included in these investigations two other North American isolates with prominent 22-kDa proteins. These strains were from California (DN127) and New York (25015). Since it has been shown that *ospC* is present as a cryptic gene in almost all strains of *B. burgdorferi* sensu lato, even when it is not expressed (36), we chose to investigate our midwestern isolates by amino acid sequencing and composition analysis rather than by cloning and determination of nucleic acid sequences.

MATERIALS AND METHODS

Collection of ticks, trapping of rodents, and isolation of spirochetes. Adult and nymph *I. scapularis* ticks were collected from vegetation with cloth drags (15) and preserved in collection tubes containing moist filter paper. For the isolation of *Borrelia* spp., ticks were immersed in 70% ethanol for 2 to 5 min to reduce surface bacterial contamination and placed on a clean glass slide and the midguts were removed with two sterile 18-gauge needles. The midguts were then placed in BSK II medium (modified as described below) containing rifampin (40 μg/ml) and ciprofloxacin (0.4 μg/ml) (11). *Peromyscus leucopus* mice were collected in baited traps (H. B. Sherman Traps, Inc., Tallahassee, Fla.), and 2-mm ear-punch tissue specimens were removed and placed in the same medium. Cultures were incubated at 33°C and checked periodically for the growth of spirochetes.

Bacterial strains and growth conditions. The *B. burgdorferi* sensu lato isolates used in this study are listed in Table 1. All strains were grown in BSK II medium (5) modified by the addition of L-cysteine (1 mM) and dithiothreitol (1 mM) (2). All cultures were incubated at 33°C. Isolates from Illinois and Wisconsin were all low-passage strains (fewer than five passages in artificial growth medium). The seven reference strains have undergone an unknown number of passages.

Genospecies typing. Previously described, species-specific PCR primers were used to test tick and mouse isolates. These primers were designed to differentiate *B. burgdorferi* sensu lato into the three separate genospecies *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (27).

PFGE and digestion of DNA in agarose. Preparation of large-molecular-size genomic DNA and pulsed-field gel electrophoresis (PFGE) were performed as described previously (20). A contour-clamped homogeneous electric field pulsed-field apparatus (CHEF-DRII; Bio-Rad Laboratories, Richmond, Calif.) was used for all separations. For the separation of undigested genomic DNA, pulse times were ramped from 1 to 6 s for 24 h at 200 V, and for the separation of *Mlu*I-digested DNA, pulse times were ramped from 5 to 35 s for the same time period. Agarose (1%) gels were used for both types of separation.

Restriction endonuclease *Mlu*I was purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's instructions. Genomic DNA, in low-melting-temperature agarose plugs (0.8%), was digested with 40 U of *Mlu*I in a total volume of 200 μl of digestion buffer for 24 h at 37°C. The λ concatamers used as size markers for Fig. 1 and 2 were purchased from FMC Bioproducts Corp. The monomer size of these markers is 48.5 kb, resulting in bands of the following approximate sizes: 49, 97, 146, 194, 243, 291, 340, 388, 437, and 485 kb. The large-molecular-size markers used for Fig. 1 and 2 were purchased from Gibco-BRL Life Technologies, Inc., Gaithersburg, Md., and produce bands of the following sizes: 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb.

Large restriction fragment patterns (LRFPs). In describing unique *Mlu*I-PFGE profiles, we have followed the definition and nomenclature previously devised by Belfaiza et al. (10) for bands greater than 70 kb in size.

SDS-PAGE and preparation of total cellular proteins. *B. burgdorferi* sensu lato strains were grown in modified BSK II medium as described above, harvested by centrifugation, and washed three times in phosphate-buffered saline. Approximately 10^8 cells were boiled for 5 min in a sample buffer containing 2% SDS, 0.125 M Tris (pH 6.8), 10% glycerol, 1% 2-mercaptoethanol, and 0.02% pyronin Y tracking dye and were applied to the gel in various volumes (up to 20 μl). Separation of *B. burgdorferi* sensu lato polypeptides was performed with a Bio-Rad mini-Protean II apparatus with a Laemmli discontinuous buffer system (29), a 12.5% separating gel (pH 8.8), and a 4.5% stacking gel (pH 6.8). Low-range molecular mass markers (containing polypeptides of 14.4, 21.5, 31, 45, 66.2, and 97.4 kDa) and prestained molecular mass markers for immunoblots (containing polypeptides of 20.7, 28.6, 33.3, 47.8, 86.8 and 139.9 kDa) were obtained from Bio-Rad Laboratories, Inc.

Immunoblot analysis. Proteins separated by SDS-PAGE were transferred to nitrocellulose and incubated with patient serum by a modification of the method described by Towbin et al. (51). Prestained molecular mass markers were used (Bio-Rad Laboratories, Inc.). Transfer of proteins to nitrocellulose was performed with a Bio-Rad semidry apparatus and CAPS buffer, pH 10.5 (10 mM cyclohexylaminopropane sulfonic acid, 0.05% dithiothreitol, 15% methanol). Transfer was carried out for 30 min at 15 V. Nonspecific binding to membranes was blocked by incubation overnight at 25°C in TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dried milk (BLOTTO). Blots were washed three times with TBST and then incubated for 1 h at 25°C with patient sera (1:100 in TBST–5% BLOTTO). The blots were then washed four times with TBST and incubated with rabbit anti-human μ-chain antibody conjugated to alkaline phosphatase (Dako Corporation, Carpinteria, Calif.). Finally, the blots were washed four times in TBST before the addition of substrate (nitroblue tetrazolium-BCIP [5-bromo-4-chloro-3-indolylphosphate toluidinium salt]). The patient serum used in these experiments possessed a high-level immunoglobulin M (IgM) antibody titer that appeared to be directed almost exclusively against a 22-kDa protein of the patient's autologous *B. afzelii* isolate. The serum also reacted strongly with the prominent 22-kDa proteins from seven isolates investigated by microsequencing that were shown to be homologs of OspC.

Transfer of protein bands to nitrocellulose membranes and digestion with trypsin. After SDS-PAGE, samples were transferred to nitrocellulose membranes with a Bio-Rad semidry apparatus and CAPS buffer, pH 10.5. Transfer was carried out for 30 min at 15 V. After transfer, membranes were stained with 0.1% Ponceau S in 1% aqueous acetic acid for 1 min with gentle agitation; staining was followed by incubation in 1% acetic acid for 1 min. Protein bands of interest were excised with a new, unused razor blade, and the fragments of membranes containing the protein were destained in 0.2 mM sodium hydroxide for 1 min with vortexing. Membrane fragments were then blocked with 0.5% polyvinylpyrrolidone 40 in 0.1 M acetic acid for 30 min at room temperature with gentle agitation. The membrane fragments were washed five times in distilled water and cut into 1- to 2-mm pieces. These smaller membrane fragments were then incubated with 10% trypsin in 0.1 M Tris-HCl [pH 8.0]–1 mM calcium chloride–10% (vol/vol) acetonitrile at room temperature for 16 h. After digestion, the membrane fragments were pelleted by centrifugation and the supernatant was collected for separation of peptides by high-performance liquid chromatography (HPLC).

HPLC separation of peptides. HPLC separation was carried out with a Perkin-Elmer (Norwalk, Conn.) series 410 HPLC system fitted with a Rainin Instruments (Woburn, Mass.) reversed-phase C_{18} column (1 by 30 cm). Buffer A was 5% acetonitrile–0.1% trifluoroacetic acid in distilled water. Buffer B was 70% acetonitrile–0.085% trifluoroacetic acid in distilled water. The column was equilibrated with 95% buffer A–5% buffer B. For the separation of peptides, a flow rate of 2 ml/min was used. The column gradients used were 5% buffer B to 50% buffer B within 40 min, 50% buffer B to 100% buffer B within 5 min, 100% buffer B held for 15 min, and finally to 95% buffer B within 2 min. Elution at 215 nm was detected with a Perkin-Elmer model LC90 UV spectrophotometric detector and flatbed chart recorder. Peptides were collected and concentrated in a Speed-vac concentrator (Savant Instruments, Farmingdale, N.Y.) for microsequencing.

TABLE 1. *Borrelia* species and strains

Species	Strain designation	LRFP	Biological origin	Geographic location
B. burgdorferi	B31 (ATCC 35210)	MLb1	Tick (*I. scapularis*)	New York
	297 (ATCC 53899)	MLb2	CSF	United States
B. garinii	20047	MLg1	Tick (*I. ricinus*)	France
	PBi	MLg2	CSF	Germany
B. afzelii	PGau	MLa1	Skin (ACA[a])	Germany
	SL-77	MLa1	Skin (EM[b])	Slovenia
B. burgdorferi	DN127	MLb14	Tick (*I. pacificus*)	Northern California
	25015	ML*x*	Tick (*I. scapularis*)	New York
	CT39	ML*x*	Mouse (*P. leucopus*)	NE[c] Illinois
	Og3	MLb2	Tick (*I. scapularis*)	NW[d] Illinois
	Og7	MLb12	Tick (*I. scapularis*)	NW Illinois
	Og16	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og19	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og21	MLb13	Tick (*I. scapularis*)	NW Illinois
	Og22	MLb11	Tick (*I. scapularis*)	NW Illinois
	Og25	MLb13	Tick (*I. scapularis*)	NW Illinois
	Og27	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og28	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og30	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og31	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og32	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og34	MLb11	Tick (*I. scapularis*)	NW Illinois
	Og36	MLb2	Tick (*I. scapularis*)	NW Illinois
	Og38	MLb2	Tick (*I. scapularis*)	NW Illinois
	OgJA	MLb2	Tick (*I. scapularis*)	NW Illinois
	CR7	MLb2	Tick (*I. scapularis*)	NW Illinois
	CR17	MLb2	Mouse (*P. leucopus*)	NW Illinois
	CR19	MLb13	Mouse (*P. leucopus*)	NW Illinois
	CR23	MLb2	Mouse (*P. leucopus*)	NW Illinois
	CR24	MLb2	Mouse (*P. leucopus*)	NW Illinois
	CR25	MLb2	Mouse (*P. leucopus*)	NW Illinois
	CR35	MLb2	Mouse (*P. leucopus*)	NW Illinois
	CR49	MLb2	Mouse (*P. leucopus*)	NW Illinois
	CR57	MLb2	Mouse (*P. leucopus*)	NW Illinois
	LT1	MLb2	Tick (*I. scapularis*)	NW Illinois
	LT4	MLb12	Tick (*I. scapularis*)	NW Illinois
	LT6	MLb13	Tick (*I. scapularis*)	NW Illinois
	65468	MLb2	Tick (*I. scapularis*)	NW Illinois
	114236	MLb2	Tick (*I. scapularis*)	NW Illinois
	IS17	MLb12	Mouse (*P. leucopus*)	Wisconsin
	LT13	MLb2	Tick (*I. scapularis*)	NW Illinois

[a] ACA, acrodermatitis chronica atrophicans.
[b] EM, erythema migrans.
[c] NE, northeast.
[d] NW, northwest.

Microsequencing of isolated peptides. Peptide sequence analysis was performed with a model 473A pulsed-liquid protein sequencer (Applied Biosystems, Inc., Foster City, Calif.). All tryptic fragments were applied to trifluoroacetic acid-treated glass fiber filters which had been pretreated with 3 mg of Biobrene Plus (Polybrene) and subjected to three "filcon" (filter conditioning) cycles on the sequencer. This procedure was performed to enhance sample attachment to the filter and to prevent sample washout during the initial Edman degradation sequencing cycles.

Transfer of proteins to PVDF membranes and determination of amino acid composition. Samples were transferred to polyvinylidene difluoride (PVDF) membranes with the apparatus and buffers described above for transfer to nitrocellulose. PVDF membranes were wetted in 100% methanol and then soaked in transfer buffer before being placed on the PAGE gel. After transfer, the membranes were stained with 0.25% Coomassie brilliant blue in 25% isopropanol–10% glacial acetic acid for 5 min with gentle agitation; staining was followed by destaining (three times for 5 min each time) in 40% methanol–10% acetic acid. Protein bands of interest were excised with an unused razor blade, and the fragments of membranes containing the protein were used for composition analysis.

Amino acid analysis was performed on a Beckman (Palo Alto, Calif.) model 121M amino acid analyzer by using postcolumn ninhydrin-dimethyl sulfoxide detection at both 440- and 570-nm wavelengths with a 690-nm reference wavelength. The 570-nm wavelength was used to identify and quantify all primary amino acids, and the 440-nm wavelength was used to identify and quantify secondary amino acids. Before analysis, samples were subjected to a 24-h acid hydrolysis at 120°C with 6 N hydrochloric acid (double distilled constant boiling with 0.001 M phenol and mercaptoethanol protection). Samples were then dried in a Speedvac concentrator (Savant Instruments) and taken up in 0.15 M lithium citrate buffer (pH 2.2) before being loaded onto the amino acid analyzer. The resulting chromatogram was calculated to generate both moles percent and number-of-residues compositional data based on the molecular masses of the samples. No alkaline hydrolysis was performed to determine the tryptophane content of the sample.

RESULTS

The techniques that we chose to employ for the molecular characterization of strains included (i) genospecies typing with PCR primers designed to differentiate *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*; (ii) PFGE of total genomic DNA to determine plasmid profiles; and (iii) SDS-PAGE to determine the protein profiles of strains. We also found that digestion of total genomic DNA with the restriction enzyme *Mlu*I and separation of the resulting fragments by PFGE could be used to differentiate isolates (and reference strains) into

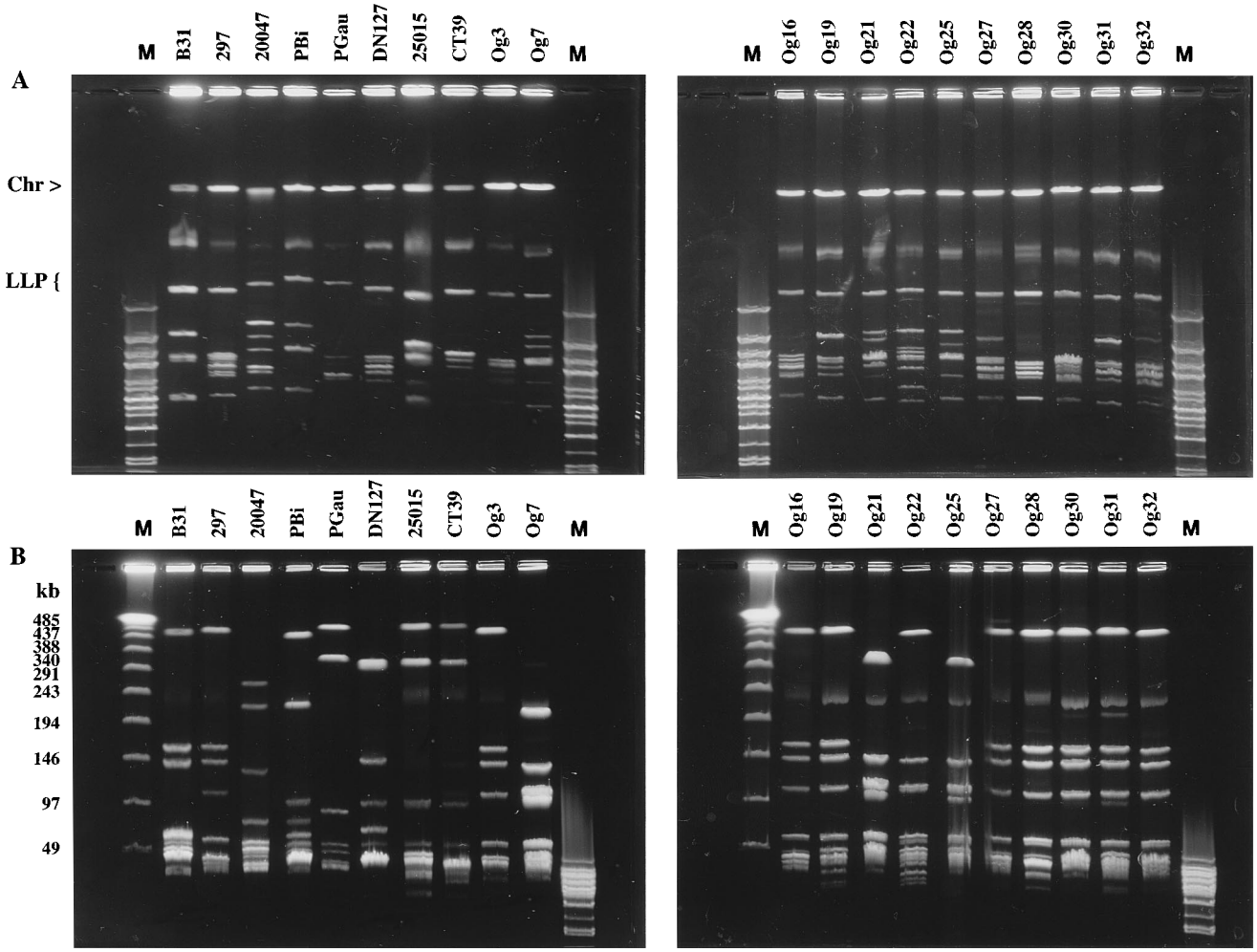

FIG. 1. Genetic characterization of *B. burgdorferi* isolates from ticks and small animals collected in Illinois in comparison to some reference strains. Strain designations are shown above their respective lanes. (A) PFGE separation of the total genomic complement of the *B. burgdorferi* isolates. Lanes M, DNA molecular size markers of 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb. Chr>, position of the 950-kb chromosome. LLP, position of the largest linear plasmid. The diffuse band of DNA between the chromosome and the largest linear plasmid represents randomly sheared, chromosomal breakdown products. (B) PFGE separation of restriction enzyme *Mlu*I digests of total genomic DNA from the *B. burgdorferi* isolates. Lanes M contain DNA molecular size markers. The small-molecular-size markers are as listed in panel A; the larger-molecular-size markers are concatamers of bacteriophage λ resulting in bands of the approximate sizes shown. The diffuse band of DNA corresponding in size to ~243 kb represents randomly sheared, chromosomal breakdown products.

distinct classes. In the course of this work, similar findings were published by Belfaiza et al. (10). We have therefore retained their definitions and nomenclature for the classification of *Mlu*I digestion patterns (LRFPs).

We used these techniques to investigate 32 unclassified isolates of *Borrelia* spp. from Illinois derived from either ticks (*I. scapularis*) or white-footed mice (*Peromyscus leucopus*) and one isolate from a white-footed mouse captured in Wisconsin. For comparison, we included seven well-characterized strains of *B. burgdorferi* sensu lato. These were B31 (*B. burgdorferi* sensu stricto, type strain [ATCC 35210]), 297 (*B. burgdorferi* sensu stricto, North American cerebrospinal fluid [CSF] isolate [ATCC 53899]), 20047 (*B. garinii* type strain), PBi (*B. garinii*), PGau (*B. afzelii*), and two tick isolates from California (DN127) and New York (25015) that had previously been observed to possess atypical protein profiles (3, 12). In the course of this molecular characterization, it was noticed that four of the Illinois isolates, the Wisconsin isolate, and strains DN127 and 25015 (as reported previously [3, 12]) possessed prominent protein bands with molecular masses of approximately 22 kDa. We therefore decided to investigate these proteins in greater detail using (i) microsequencing of HPLC-purified peptides derived from the proteins, (ii) amino acid composition analysis of the proteins, and (iii) Western blot (immunoblot) analysis with a polyclonal antiserum against OspC derived from a European patient with culture-confirmed Lyme borreliosis.

Genospecies typing and plasmid profiles. Investigation of the 33 isolates with genospecies-specific PCR primers (27) indicated that all of the strains belonged to the species *B. burgdorferi* sensu stricto. PFGE analysis of the total genomic complement of the isolates showed that they were heterogeneous with regard to their plasmid profiles (Figures 1A and 2A). Among the 33 strains, we could identify eight distinct DNA bands with various intensities representing plasmids of different molecular sizes. Open circular forms of two of the plasmids were apparent in some gels, indicating that at least two of the plasmids were supercoiled and not linear. We noted that the largest of the linear plasmids from the European isolates of *B. garinii* (20047 and PBi) and *B. afzelii* (PGau)

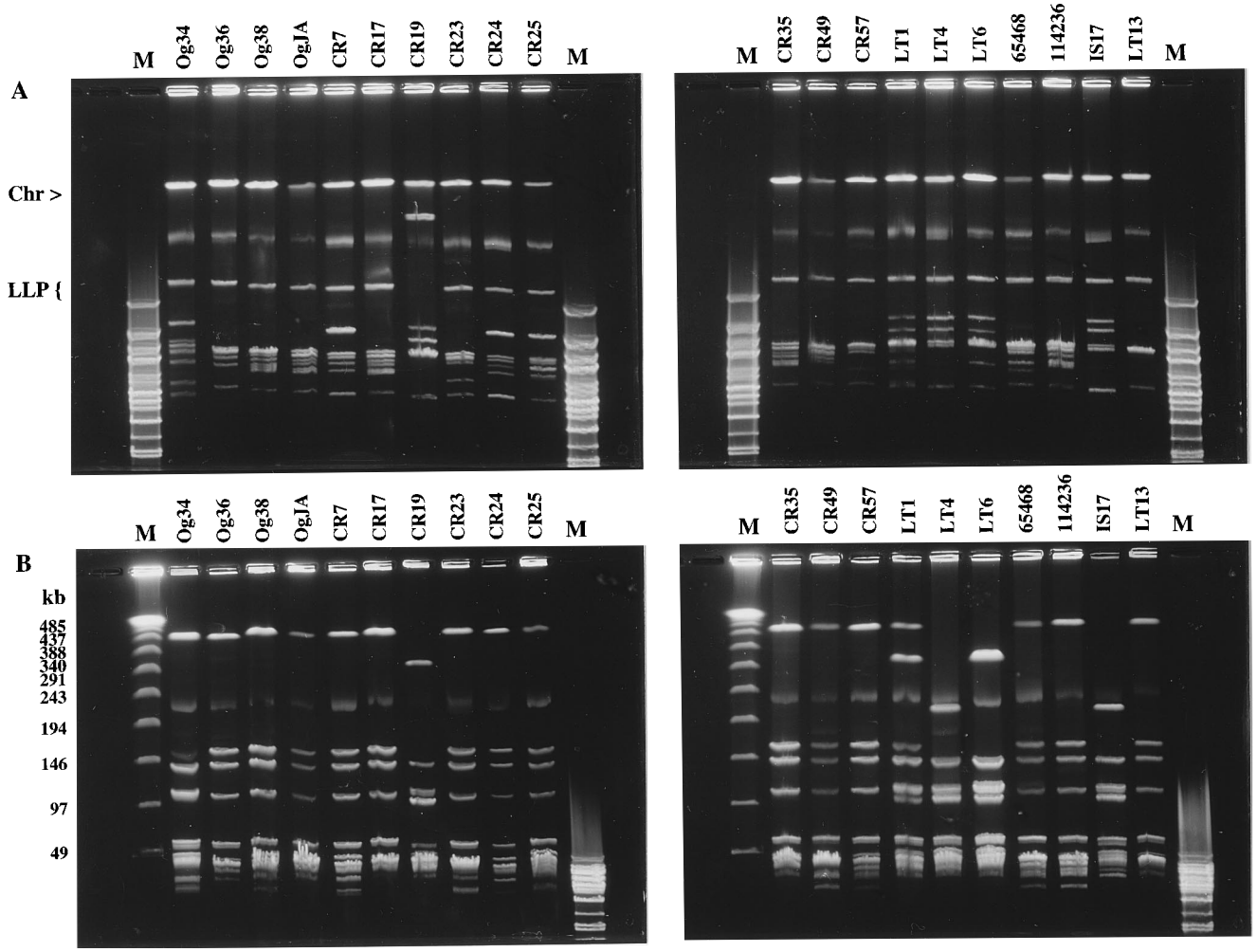

FIG. 2. Genetic characterization of 20 additional *B. burgdorferi* isolates from ticks and small animals collected in Illinois and Wisconsin (strain IS17). Strain designations are shown above their respective lanes. (A) PFGE separation of the total genomic complements of the *B. burgdorferi* isolates. Lanes M, DNA molecular size markers of 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb. Chr>, position of the 950-kb chromosome. LLP, position of the largest linear plasmid. The diffuse band of DNA between the chromosome and the largest linear plasmid represents randomly sheared, chromosomal breakdown products. (B) PFGE separation of restriction enzyme *Mlu*I digests of total genomic DNA from the *B. burgdorferi* isolates. Lanes M contain DNA molecular size markers. The small-molecular-size markers are as listed in panel A; the larger-molecular-size markers are concatamers of bacteriophage λ resulting in bands of the sizes shown. The diffuse band of DNA corresponding in size to ~243 kb represents randomly sheared, chromosomal breakdown products.

appeared to have slightly larger molecular sizes than the largest, linear plasmid (49 kb) of strains B31 and 297 and the Illinois and Wisconsin isolates (Fig. 1A). This fact has been commented upon previously (45). One Illinois isolate (CR19) possessed, instead of the typical 49-kb linear plasmid, two very much larger plasmids with undetermined molecular sizes (>150 kb). These appear as a doublet in the pulsed-field gel of Fig. 2A, immediately below the chromosome.

Identification of distinct LRFPs and determination of fragment sizes. Investigation of the 33 Illinois and Wisconsin isolates and 7 reference strains by *Mlu*I digestion of genomic DNA and separation of the fragments by PFGE revealed a number of distinct patterns, or LRFPs, some of which have been described previously (10). The sizes of fragments obtained were determined by comparison to λ standards. The results from measuring the bands of the Illinois and Wisconsin strains and of the 7 reference strains are shown in Table 2.

PFGE separation of *Mlu*I digestion fragments showed that 22 of the 33 Illinois and Wisconsin isolates had LRFPs that were identical to that of strain 297, a North American CSF isolate. This LRFP was designated MLb2 by Belfaiza et al. (10). The 11 other Illinois and Wisconsin strains did not have *Mlu*I digestion profiles that matched any of the nine other LRFPs previously described by these authors (MLb1 and MLb3 to MLb10). The 11 isolates were divisible into three groups, each with a new LRFP. We therefore propose the designations MLb11 (strains Og22 and Og34), MLb12 (strains Og7, LT4, and IS17 [Wisconsin]), and MLb13 (strains Og21, Og25, CR19, and LT6) for the new LRFPs found among these strains. One isolate, LT1, was found to have an LRFP that was a composite of MLb2 and MLb13. Presumably the *I. scapularis* tick from which this isolate was derived must have been infected with two different strains of *B. burgdorferi*. We also found that strain DN127 possessed a hitherto-undescribed LRFP; we propose the designation MLb14 for this. Its LRFP was characterized by the 140-kb fragment that has thus far been found in the LRFPs of all *B. burgdorferi* sensu stricto isolates examined to date (reference 10 and this study).

One Illinois isolate, CT39 (described elsewhere as Illinois 1 [34–36]), shared a new and unusual LRFP with strain 25015.

TABLE 2. Sizes of bands obtained by *Mlu*I digestion of DNA from Illinois *B. burgdorferi* isolates

LRFP	Isolate(s)	Presence of band with the following size (kb):														
		440	410	390	320	300	290	260	220	210	160	140	110	100	90	80	70
MLb1	B31		+								+	+					
MLb2	297 (ATCC 53899), Ill. group 1 (22 isolates)		+								+	+	+				
MLb11	Ill. group 2 isolates Og22 and Og34		+									+	+				
MLb12	Ill. group 3 isolates Og7, LT4, and IS17									+		+	+	+			
MLb13	Ill. group 4 isolates Og21, Og25, CR19, and LT6						+					+	+	+			
MLb14	DN127					+	+					+		+			+
ML*x*	25015 and CT39	+				+								+			
MLg1	20047							+	+			+				+	
MLg2	PBi			+					+					+		+	
MLa1	PGau	+			+										+		

Their LRFPs were very similar to the MLa1 LRFP of *B. afzelii* PGau. Thus, both LRFPs were characterized by three fragments. However, for strain PGau we measured these to be 440, 320, and 90 kb, and for strains CT39 and 25015 we measured these to be 440, 300, and 100 kb. Both CT39 and 25015 lacked the 140-kb fragment mentioned above in connection with strain DN127.

SDS-PAGE of total proteins. All 33 isolates were found to be very similar with regard to the size and level of expression of their higher-molecular-mass proteins (>41 kDa) (Fig. 3A and 4A). With the exception of strains CT39 and Og38, all of the isolates also possessed proteins of 41, 39, 34, and 31 kDa. Polypeptides of this size are consistent with the flagellin (52), P-39 (48), OspB (4, 55), and OspA (4, 55) proteins, respectively; OspB and OspA proteins of this size are also typical for *B. burgdorferi* sensu stricto. Strain CT39 (like strain 25015) possessed higher-molecular-mass OspA and OspB proteins that are similar in size to those of *B. afzelii* PGau. The levels of expression of the 41-, 39-, and 31-kDa proteins were very similar for all of the Illinois and Wisconsin isolates (Fig. 3A and 4A). There was apparent variability, however, in the level of expression of the 34-kDa protein: strain Og38 appeared to lack a 34-kDa protein entirely, whereas strains Og36, CR7, CR23, CR24, CR25, CR35, CR57, LT1, LT4, LT6, 65468, and 114236 possessed prominent bands of this size. As noted previously for these isolates (3, 12), strain 25015 possessed higher-molecular-mass OspA and OspB proteins of 32.5 and 35.5 kDa and DN127 appeared to lack OspA and OspB proteins entirely.

The 33 isolates were heterogeneous with regard to the size and level of expression of lower-molecular-mass proteins. Five of the Illinois and Wisconsin isolates possessed a prominent protein of approximately 22 kDa, as did strains 25015 and DN127. However, the levels of expression of these proteins varied. Strains DN127, 25015, CT39, and CR17 showed high-level expression (Fig. 3A and 4A), whereas strains LT4, Og19, and IS17 showed lower levels of expression. We considered it worthwhile to investigate these proteins in more detail to see if they were related to the OspC protein (21, 24, 50, 56) or other 22-kDa proteins of *B. burgdorferi* sensu lato that have recently been described (30, 31, 33, 53). Protein bands, separated by SDS-PAGE, were therefore transferred to nitrocellulose and PVDF membranes and investigated further by microsequencing and amino acid composition analysis.

HPLC separation and microsequencing of peptides derived from the 22-kDa proteins. As reported previously for similar attempts to microsequence the OspC protein of *B. afzelii* PKo (21) and the p23 protein of *B. burgdorferi* 2591 (40), we found the 22-kDa proteins from the seven strains to be N-terminally blocked. We therefore transferred the protein bands to nitrocellulose membranes, excised the proteins of interest, and digested them with trypsin to release individual peptide fragments as described in Materials and Methods. Peptide fragments were then separated by HPLC, and individual, well-separated peaks were microsequenced as described in Materials and Methods. Seventeen of 20 HPLC peaks sequenced produced readable amino acid sequences.

The peptide sequences obtained from microsequencing experiments are shown in Fig. 5 in relation to the published, deduced amino acid sequences of OspC from the European strain PKo and the North American strain 2591 (21, 40). Digestion with trypsin breaks the amino acid chain at lysine and arginine residues. It may be seen from Fig. 5 that the majority of the peptide sequences obtained begin and end at lysine residues. For some peptides, the yield from microsequencing of the last few residues was insufficient for an unambiguous determination of their identities to be made. Since samples were not subjected to modification reactions designed to produce pyridyl ethyl cysteine residues, the microsequencing procedure was not able to distinguish cysteine. These residues therefore appear as blanks in the sequences obtained. Thus, the first residue of the DN127 peptide ?SDDFTK, shown in Fig. 5, is most probably a cysteine, as in the sequences of strains PKo and 2591. All of the peptide sequences obtained possessed considerable overall sequence identity with OspC but also contained amino acid substitutions in certain positions (Fig. 5).

Amino acid composition analysis of the 22-kDa proteins. Sequence analysis of peptides from the seven isolates investigated showed that these strains expressed polymorphic OspC proteins. However, since only a few peptide sequences from each strain were obtained, we performed amino acid composition analysis of intact 22-kDa proteins from the isolates in order to gain a better understanding of their overall level of sequence identity. The results of these analyses are presented in Table 3 in comparison to the deduced amino acid composition of strain PKo (21).

Immunoblot analysis. SDS-PAGE of the 40 strains investigated in this study identified 7 isolates that showed high-level expression of 22-kDa proteins. Microsequencing experiments demonstrated that these proteins were homologs of OspC. Close scrutiny of the SDS-PAGE gels shown in Fig. 3A and 4A revealed that faint bands with approximately the same molecular masses were visible in the protein profiles of 38 of the 40

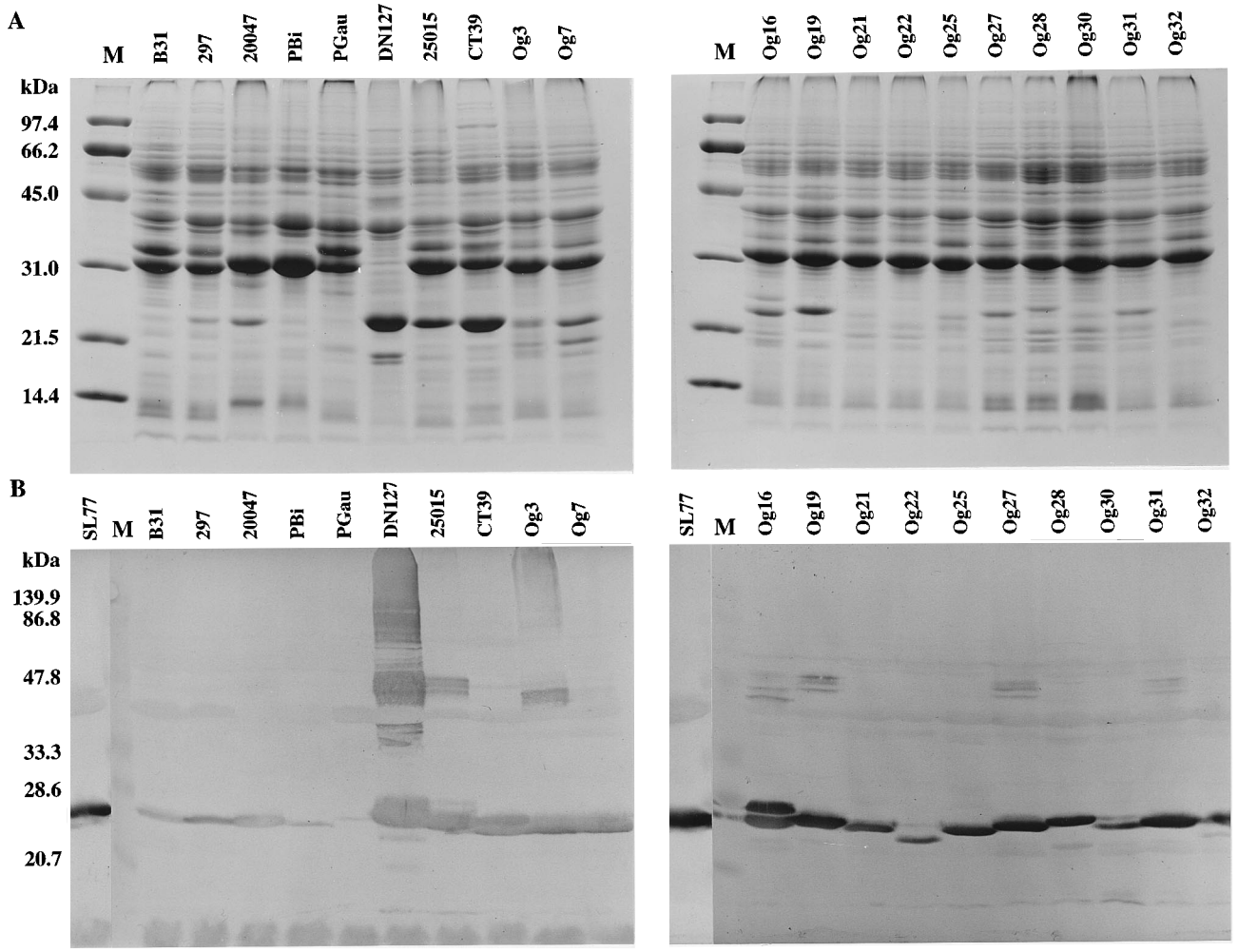

FIG. 3. Protein profiles and antigenic characterization of the *B. burgdorferi* isolates and reference strains in Fig. 1. Strain designations are shown above their respective lanes. (A) Denaturing PAGE of total proteins from the *B. burgdorferi* sensu lato strains. Lanes M contain molecular mass markers of the sizes shown. (B) Immunoblot analysis of the strains in panel A with sera from a European Lyme borreliosis patient with a high-level antibody titer against an abundant 22-kDa protein of the patient's autologous *B. afzelii* isolate (strain SL-77). The first lane in each gel contains total proteins from strain SL-77 as a control and shows the antibody response to the patient's autologous isolate. This lane has been moved from the right side to the left side of the gel in order to align the strains in the SDS-PAGE gel and immunoblot. Lanes M contain prestained molecular mass markers of the sizes shown.

isolates. We therefore investigated all 40 strains using more-sensitive immunoblotting techniques that would be able to demonstrate low-level expression of OspC. For this we used a polyclonal serum of human origin that showed a strong, almost-exclusive IgM reactivity with the 22-kDa proteins from the seven isolates investigated by microsequencing and shown to be homologs of OspC.

The results of this Western blot evaluation are shown in Fig. 3B and 4B. The patient's autologous isolate (strain SL-77) has also been included on these blots as a control. It can be seen from this that the serum reacted almost exclusively with a 22-kDa protein of strain SL-77 and had less reactivity with a 41-kDa protein. It can also be seen from Fig. 3B and 4B that the serum reacted strongly with the OspC proteins of strains DN127, 25015, CT39, Og19, CR17, LT4, and IS17. All 33 Illinois and Wisconsin isolates investigated showed some measure of reactivity with the SL-77 serum. Some of the reference strains showed very weak or no reactivity. Thus, all of the midwestern isolates expressed OspC, although the levels of expression and molecular masses of the proteins appeared to be variable. Two Illinois strains, Og16 and Og34, showed the presence of two bands with very similar molecular masses that reacted with the serum. Strain Og38, which appeared to lack a 34-kDa protein entirely, also showed a very weak reactivity with the SL-77 serum. Strains Og21, Og22, and Og25 appeared to possess OspC proteins with lower molecular masses.

DISCUSSION

We have carried out a molecular analysis of 33 unclassified *Borrelia* spp. isolated from *I. scapularis* ticks and white-footed mice collected within Illinois (32 isolates) and Wisconsin (1 isolate) during 1990 and 1992. To our knowledge, this represents the largest collection of midwestern strains that have been characterized to date. Typing with genospecies-specific PCR primers indicated that all of the strains belonged to the species *B. burgdorferi* sensu stricto. However, although the isolates were obtained from a relatively small geographic region, they were found to be genetically and phenotypically diverse. The isolates were heterogeneous with regard to their protein profiles and plasmid profiles and to the physical maps of their linear chromosomes as evidenced by *Mlu*I LRFPs.

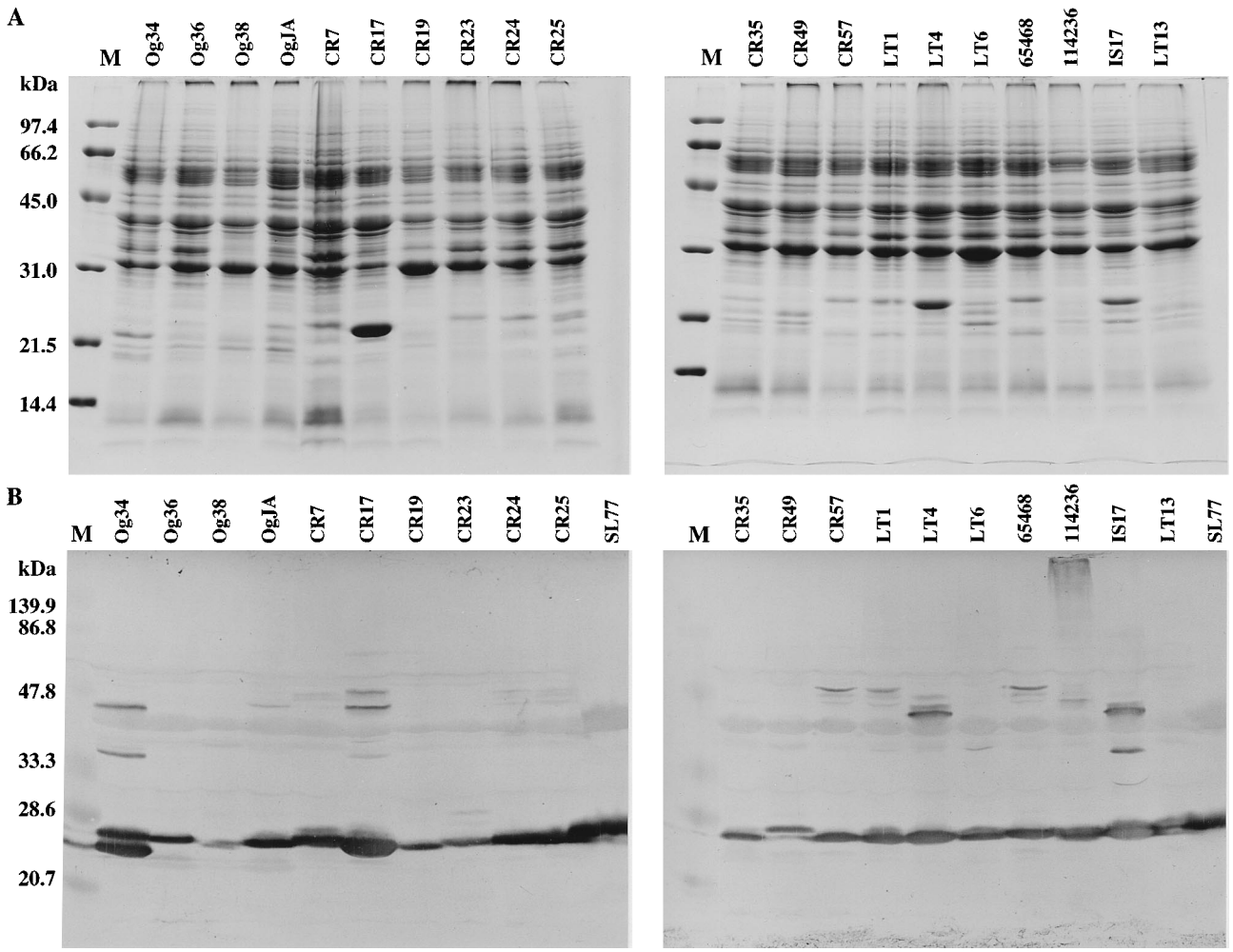

FIG. 4. Protein profiles and antigenic characterization of the *B. burgdorferi* isolates shown in Fig. 2. Strain designations are as shown above their respective lanes. (A) Denaturing PAGE of total proteins from the *B. burgdorferi* isolates. Lanes M contain molecular mass markers of the sizes shown. (B) Immunoblot analysis of the strains in panel A with sera from a European Lyme borreliosis patient with a high-level antibody titer against an abundant 22-kDa protein of the patient's autologous *B. afzelii* isolate (SL-77). Lanes M contain prestained molecular mass markers of the sizes shown. The last lane in each gel contains total proteins from strain SL-77 as a control and shows the antibody response to the patient's autologous isolate.

With the exception of the largest linear plasmid, which approximated in size that of strain B31 in all but one of the Illinois and Wisconsin isolates, the plasmid profiles of these low-passage strains were widely variable. In aggregate, we were able to identify at least eight plasmids with different molecular sizes among the 33 isolates, of which at least two were supercoiled. All of the strains contained a variable subset of this plasmid array. These variations were not related to the biological origin of the strains (i.e., isolation from *P. leucopus* versus *I. scapularis* ticks) and showed no correlation with other molecular characteristics such as protein profiles or LRFPs. We therefore concluded that plasmid profiles were not a meaningful basis on which to classify strains. One isolate from Illinois (CR19) was very unusual in that it possessed, instead of the typical largest, linear plasmid of 49 kb, two plasmids with very similar, but very much larger, molecular sizes. At this time, it is not known whether these plasmids are linear or supercoiled. In pulsed-field gels they migrate with a relative mobility less than that of the 146-kb λ molecular size standard (unpublished data). We therefore conclude that they are at least 146 kb or greater in size. However, the molecular sizes of neither supercoiled nor linear plasmids can be assessed by comparison with linear molecular size standards (39). Thus, regardless of their conformation, we were not able to obtain an accurate assessment of the sizes of the plasmids from the gel shown in Fig. 2A.

In carrying out LRFP analysis of the isolates, we found certain differences between our results and those of Belfaiza et al. (10). Most of these were minor differences arising from the difficulty in making precise measurements from pulsed-field gels. For example, the latter authors described a fragment of 135 kb that was present in the LRFPs of all *B. burgdorferi* sensu stricto strains (MLb1 to MLb10) and which they therefore inferred to be diagnostic of the species. We determined the size of this fragment to be 140 kb and refer to it as such. They also described a fragment of 145 kb that was present in all MLb LRFPs except MLb6, MLb7, and MLb9. Although the overall patterns of the fragments for LRFPs MLb1 and MLb2 were the same in our gels as in the study by Belfaiza et al. (10), we measured this particular fragment as 160 kb. The PFGE-*Mlu*I profiles of strains B31 (MLb1) and 297 (MLb2) in Fig. 1B demonstrate that the fragment referred to migrates above the third λ marker of 146 kb. Therefore, we conclude that the size of this fragment more closely approximates 160 than 145 kb.

LRFP analysis of the 33 strains revealed that all except one

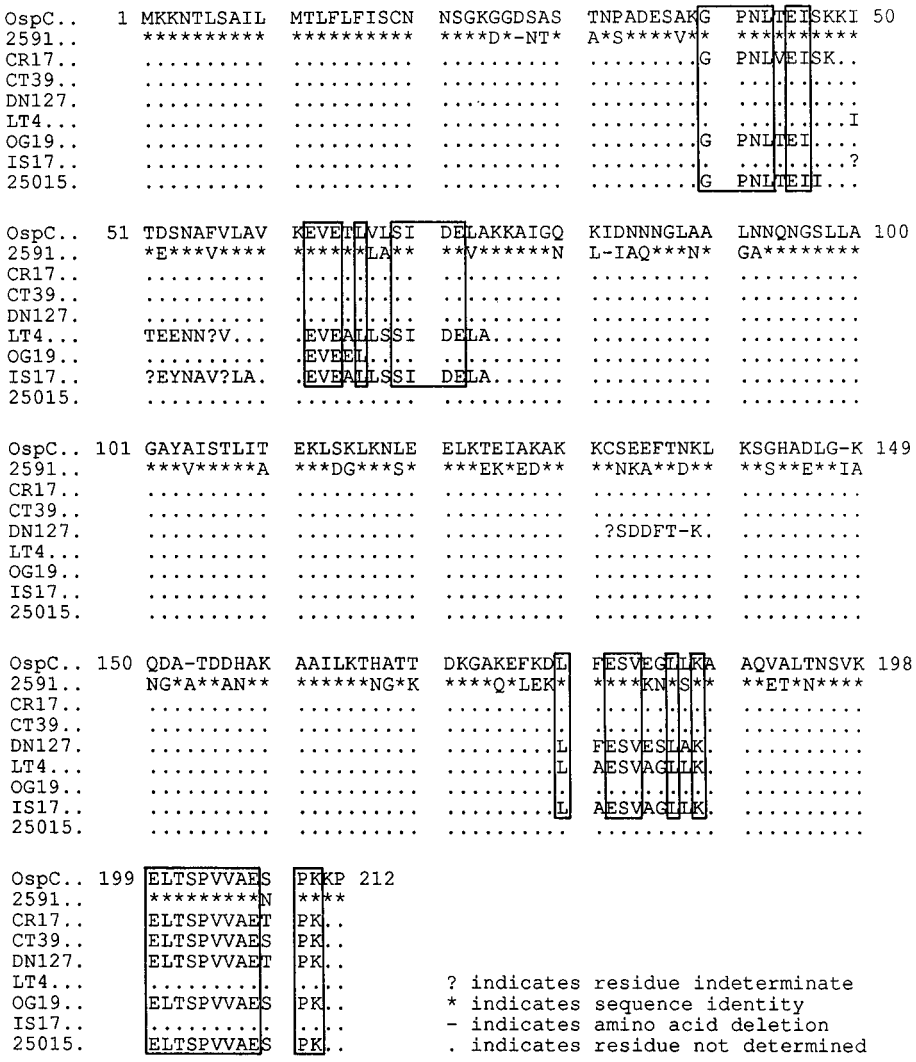

FIG. 5. Data from microsequencing of individual peptides derived from the prominent 22-kDa proteins of strains DN127 and 25015; the Illinois isolates CT39, Og19, CR17, and LT4; and the Wisconsin isolate IS17. Amino acid sequences are shown in comparison to the published OspC sequences of strains PKo (from Germany) and 2591 (from the United States).

of the Illinois and Wisconsin isolates (strain CT39) were found to have four distinct LRFPs, of which three have not previously been described. This latter finding was surprising considering the small geographic area from which the ticks and mice were collected and the fact that Belfaiza et al. (10) have already examined a sizable collection of *B. burgdorferi* sensu stricto isolates from both North America and Europe. It is noteworthy, however, that all three of the new midwestern LRFPs possessed the 140-kb fragment, which the latter authors considered typical of *B. burgdorferi* sensu stricto. Twenty-two of the 33 isolates had the same LRFP as strain 297, a North American CSF isolate. This finding indicates that the 22 Illinois isolates are genetically related to strain 297 with regard to the physical maps of their chromosomes. It remains to be seen if determination of genetic relatedness through LRFP typing will serve to identify *B. burgdorferi* sensu stricto isolates with equivalent pathogenic potential. If LRFPs can be taken as an accurate predictor of pathogenicity, these findings presumably indicate that the majority of the isolates obtained from ticks and mice in northern Illinois are potentially infectious for humans, although the incidence of overt Lyme borreliosis in

TABLE 3. Composition analysis of OspC proteins from Illinois isolates

Amino acid	No. of residues/strain:							
	PKo[a]	DN127	25015	CT39	Og19	CR17	LT4	IS17
Ala	26	35	16	19	25	29	33	32
Arg	0	0	1	0	1	1	0	1
Asx	26	8	10	10	4	5	6	5
Cys	2	0	0	0	0	0	0	0
Glx	20	39	30	43	29	29	33	32
Gly	12	19	18	11	14	17	18	15
His	3	2	3	3	7	2	2	2
Ile	11	14	19	22	25	18	15	16
Leu	26	33	29	38	44	37	33	34
Lys	29	18	19	17	15	20	17	19
Met	2	0	2	1	1	1	0	1
Phe	6	2	10	10	11	4	4	6
Pro	5	11	14	9	3	6	8	8
Ser	18	5	5	0	2	9	11	8
Thr	16	11	9	4	6	12	10	8
Tyr	1	1	6	7	6	2	2	3
Val	9	16	19	17	20	19	21	22

[a] Data taken from Fuchs et al. (21).

this region is very low. In 1992, we studied the infection rate among ticks at a site in Ogle County in northwestern Illinois using both culture and PCR for detection of spirochetes. The infection rates that we found were 37.5% by culture and 39.5% by PCR (unpublished data). These figures were derived from two independent determinations, using 40 to 48 ticks per study, and agree very closely. Given these findings, it seems surprising that more cases of Lyme borreliosis from the surrounding area are not reported. However, factors other than the genetic structure of isolates could also play a role in determining the incidence of Lyme borreliosis in the region.

Strain CT39 was unique among the 33 midwestern isolates investigated. In its genotypic and phenotypic characteristics, it was unlike any of the other midwestern or reference strains but closely resembled strain 25015, which was from Millbrook, N.Y. Thus, in addition to having the same unique LRFP, both strains possessed OspA and OspB proteins with higher molecular masses than are typical for North American isolates (3). These isolates have been the subject of other molecular characterizations (34–36) that have shown them to be very similar to each other but different from typical *B. burgdorferi* sensu stricto strains. Thus, they were shown to resemble each other in the conformation of their *ospAB* operons (35) and *ospC* genes (36) and in the identities of the nucleotides present at two positions in their 16S rRNA sequences (34). Computer analysis of the latter findings led the authors to conclude that these strains possessed a peripheral relationship to the species *B. burgdorferi* sensu stricto.

With genospecies-specific PCR primers derived from the above-mentioned study (27), strains CT39 and 25015 were typed as *B. burgdorferi* sensu stricto: the primers do not contain sequences that discriminate between these isolates and typical *B. burgdorferi* sensu stricto strains. LRFP analysis of the isolates showed they lacked the 140-kb fragment considered typical for *B. burgdorferi* sensu stricto (10). Thus far, this fragment has been found in the LRFPs of all isolates of this species except CT39 and 25015 (reference 10 and this study). The results of LRFP analysis, together with the findings of the other molecular analyses mentioned above, suggest that CT39 and 25015 could possibly represent a fifth genospecies of *B. burgdorferi* sensu lato. Recently, findings that support this view have been published (32, 42). In view of the highly unusual and atypical nature of the LRFP from these two strains, we propose that it be designated ML*x* until the exact taxonomic status of these isolates has been decided.

Strain DN127 is an unusual *I. pacificus* tick isolate from California (13) that has previously been shown to differ from typical North American *B. burgdorferi* sensu stricto isolates in both phenotypic and genotypic characteristics (12, 41, 44, 54). LRFP analysis revealed that this strain possesses a new and unique pattern. The fragments that characterized it were a large-molecular-size doublet of 300 and 290 kb and additional fragments of 140, 100, and 70 kb. The 140-kb fragment is that which typifies *B. burgdorferi* sensu stricto. The small-molecular-size fragment of 70 kb is, thus far, unique to this strain. Therefore, by LRFP analysis, DN127 shows both similarities to and differences from *B. burgdorferi* sensu stricto. In the study referred to above, Liveris et al. (32) investigated both DN127 and strain 25015 by PCR-restriction fragment length polymorphism analysis. They found that the two isolates differed from *B. burgdorferi* sensu stricto in the size of the fragment amplified by PCR, but they also found that the strains could be distinguished from each other by *Hin*fI restriction fragment length polymorphism analysis. Postic et al. (42) examined these strains using DNA-DNA reassociation and found that they had only a 68% level of DNA relatedness with *B. burgdorferi* sensu stricto; the two strains had 83% DNA relatedness with each other. The above-described findings, and those of the present study, indicate to us that DN127 and 25015 are different from typical *B. burgdorferi* sensu stricto isolates and also from each other.

Investigation of the prominent 22-kDa proteins from five Illinois and Wisconsin isolates, DN127, and 25015 by microsequencing demonstrated that they contained a number of peptides with sequence identity to the OspC proteins of *B. afzelii* PKo and *B. burgdorferi* sensu stricto 2591. However, heterogeneity was also apparent within these amino acid sequences, indicating that the OspC proteins of the seven North American isolates are polymorphic. This study therefore confirms the findings of Schwan et al. (46), based on immunologic studies, that the 25-kDa protein of strain DN127 (12, 28) is a homolog of OspC. It also demonstrates for the first time that the prominent 23.5-kDa protein of strain 25015 (3) is a homolog of OspC. The results of amino acid composition analysis (Table 3) also indicate that certain isolates possess related OspC proteins. Thus, strains CT39 and 25015 show many similarities, as do strains CR17, LT4, and IS17. However, even in these cases there are some important differences. For example, the OspC protein of strain 25015 was found to contain five serine residues, while that of strain CT39 contained none. This suggests that, despite the genotypic and other similarities between the two strains (references 34 to 36 and this study), their OspC proteins are not identical. As determined by amino acid composition analysis, the OspC proteins of strains DN127 and Og19 are considerably different from the five other North American isolates and from each other. Overall, however, the OspC proteins of the North American strains are more similar to each other than to the OspC protein of the European *B. afzelii* strain PKo. The composition analysis of intact proteins thus reflects the geographic separation in the origins of these isolates and the fact that they belong to separate genospecies.

Molecular heterogeneity of OspC proteins has previously been demonstrated for European isolates of *B. burgdorferi* sensu lato (24, 50, 56). One of these studies also identified four hypervariable regions (around amino acids 30, 90, 150, and 180) and three conserved regions (amino acids 1 to 19, 35 to 55, and 195 to 212) within the OspC amino acid sequences examined. In the present study, we obtained microsequencing data for two of the three conserved regions, at amino acid positions 35 to 55 and 195 to 212. In agreement with the findings of Jauris-Heipke et al. (24), certain sequences from these regions appeared to be well conserved. However, peptide sequence data from one of the proposed hypervariable regions (amino acid 180) showed that the peptide LAESVAGLLK (amino acids 178 to 187) was reasonably well conserved among the three North American strains that we studied and strain PKo (8 of 10 amino acids were identical). Thus, some of our peptide sequence data appear to fit the hypothesis of Jauris-Heipke et al. (24) concerning the existence of well-conserved and hypervariable regions, while data for other peptides appear to be in disagreement. Theisen et al. (50) concluded from their studies that the level of sequence identity correlated with the genospecies of strains. However, they also found that the overall level of sequence identity between isolates ranged as low as 60.5% and that differences were scattered throughout the amino acid sequences. The above-described findings could also be interpreted as supporting this view. More OspC protein sequences (particularly from North American isolates) need to be analyzed and compared in order to provide a better insight into the existence or lack thereof of conserved and hypervariable regions.

It has previously been shown that monoclonal antibodies

directed against OspC do not react with the 22-kDa proteins of all strains (46), presumably because of the polymorphic nature of their proteins (24, 50, 56). For this reason, we decided to investigate the 33 Illinois and Wisconsin isolates using a polyclonal serum. In the course of performing a Western blot evaluation of European patient sera, using the patients' autologous isolates of *B. burgdorferi* sensu lato, we encountered a serum sample (SL-77) with a strong, almost-exclusive IgM reactivity to the 22-kDa protein of the patient's *B. afzelii* isolate. In Europe, OspC is expressed in culture as a major protein by about 45% of *B. burgdorferi* sensu lato isolates (58, 59) and IgM antibodies to OspC are detected in a higher proportion of patients with early disease manifestations than any other protein (60). This suggested that the above-mentioned patient serum most probably contained polyclonal IgM antibodies directed against OspC. In support of this notion, the serum reacted strongly in Western blots with the 22-kDa proteins of strains DN127, 25015, CT39, CR17, LT4, Og19, and IS17, which were shown by microsequencing to be homologs of OspC. Theisen et al. (50) also encountered one patient serum sample that recognized all of the strains used in their study, irrespective of genospecies.

In our experiments, the only strains that did not react with this serum were the isolates PBi and PGau; for these strains no appropriately sized protein was detectable in polyacrylamide gels stained with Coomassie brilliant blue. In agreement with the findings of Theisen et al. (50), we did detect low-level expression of OspC in the American type strain B31. The most interesting finding to emerge from our immunoblotting studies was the discovery of isolates (Og16 and Og34) that express two proteins with very similar molecular masses, both of which reacted with the human polyclonal SL-77 antiserum. It is therefore possible that these isolates possess genes for two related OspC proteins that contain different numbers of amino acids. The presence of OspC has recently been detected in other isolates of *B. burgdorferi* sensu stricto from North America by immunological methods (46). Those authors also noticed extensive variation in the molecular mass and amount of protein expressed. Interestingly, only 14 of 32 isolates bound a monoclonal antibody (86DN-1) for OspC, supporting the contention that the OspC proteins of North American isolates are polymorphic and may possess unique, non-cross-reactive epitopes.

It has recently been noted that, in addition to OspA, the OspC protein could be a viable candidate for the development of a *Borrelia* vaccine (43) and that this would be especially useful in Europe, where patient isolates occasionally lack OspA but where a strong immunologic response is usually mounted against OspC (58, 60). The data presented here, documenting the expression of OspC in midwestern *Borrelia* isolates, as well as the above-mentioned investigations of Californian strains that express OspC but lack OspA, indicate that an OspC-based vaccine could be as applicable to North American Lyme borreliosis as to European borreliosis. For this reason, the heterogeneous nature of the OspC proteins of North American isolates needs to be investigated further.

In conclusion, we find that LRFP analysis provides a meaningful basis for the classification of *B. burgdorferi* sensu lato isolates. We consider it the method of choice since it is more discriminatory than typing by genospecies-specific PCR. It permits the recognition of established genospecies and provides insight into the existence of additional genomic groups. Thus, at this time, it is by far the most convenient means of discriminating isolates of the type represented by strains 25015 and CT39. In contrast, protein profiling of isolates, although it provides additional evidence as to their genospecies, is mostly pertinent to the antigenic characterization of strains, while plasmid profiling appears to provide very little information useful for their classification. We also conclude that the expression of OspC among North American isolates may be more widespread than has hitherto been considered. It appears to be a consistent feature of the midwestern strains isolated to date as well as strains of the ML*x* group.

ACKNOWLEDGMENTS

This work was supported by grant AR 41517 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (to R.N.P.) and by grants from the Microscan Division of Baxter Diagnostics, Inc., (to R.N.P.), and the Schweppe Foundation (to M.M.P.).

We also thank Steven M. Callister, John F. Anderson, and Uriel Kitron, who participated in the isolation of some strains.

REFERENCES

1. **Anderson, J. F., P. H. Duray, and L. A. Magnarelli.** 1987. Prevalence of *Borrelia burgdorferi* in white-footed mice and *Ixodes dammini* at Fort McCoy, Wis. J. Clin. Microbiol. **25:**1495–1497.
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 dentatus* in rural and urban areas. J. Clin. Microbiol. **27:**13–20.
3. **Anderson, J. F., L. A. Magnarelli, and J. B. McAninch.** 1988. New *Borrelia burgdorferi* antigenic variant isolated from *Ixodes dammini* from upstate New York. J. Clin. Microbiol. **26:**2209–2212.
4. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J.-C. Piffaretti, M. Assous, and P. A. D. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. **42:**378–383.
5. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
6. **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.
7. **Barbour, A. G., and M. E. Schrumpf.** 1986. Polymorphisms of major surface proteins of *Borrelia burgdorferi*. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **263:** 83–91.
8. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface 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. **Belfaiza, J., D. Postic, E. Bellenger, G. Baranton, and I. Saint Girons.** 1993. Genomic fingerprinting of *Borrelia burgdorferi* sensu lato by pulsed-field gel electrophoresis. J. Clin. Microbiol. **31:**2873–2877.
11. **Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman.** 1992. Cultivation of *Borrelia burgdorferi* from erythema migrans lesions and perilesional skin. J. Clin. Microbiol. **30:**359–361.
12. **Bissett, M. L., and W. Hill.** 1987. Characterization of *Borrelia burgdorferi* strains isolated from *Ixodes pacificus* ticks in California. J. Clin. Microbiol. **25:**2296–2301.
13. **Bisset, M., W. Hill, W. Probert, and S. Kurashige.** 1988. An unusual isolate of *Borrelia burgdorferi* from a tick in California. Ann. N. Y. Acad. Sci. **539:**369–371.
14. **Bouseman, J. K., and C. E. Kirkpatrick.** 1988. New tick in Illinois. Ill. Nat. Hist. Surv. Rep. **277:**2–3.
15. **Bouseman, J. K., U. Kitron, C. E. Kirkpatrick, J. Siegel, and K. S. Todd.** 1990. The status of *Ixodes dammini* (Acari:Ixodidae) and Lyme disease in Illinois. J. Med. Entomol. **27:**556–560.
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. **Burgdorfer, W., R. S. Lane, A. G. Barbour, R. A. Gresbrink, and J. R. Anderson.** 1985. The western black-legged tick, *Ixodes pacificus*: a vector of *Borrelia burgdorferi*. Am. J. Trop. Med. Hyg. **34:**925–930.
18. **Callister, S. M., J. A. Nelson, R. F. Schell, D. A. Jobe, R. Bautz, W. A. Agger, and J. Coggins.** 1991. Survey for *Ixodes* spp. and *Borrelia burgdorferi* in southeastern Wisconsin and northeastern Illinois. J. Clin. Microbiol. **29:**403–406.
19. **Davis, J. P., W. L. Schell, T. E. Amundsen, M. J. Godsey, A. Spielman, W. Burgdorfer, A. G. Barbour, M. LaVenture, and R. A. Kaslow.** 1984. Lyme disease in Wisconsin: epidemiologic, clinical, serologic, and entomologic findings. Yale J. Biol. Med. **57:**685–696.
20. **Ferdows, M. S., and A. G. Barbour.** 1989. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*. Proc. Natl. Acad. Sci. USA **86:**5969–5973.
21. **Fuchs, R., S. Jauris, F. Lottspeich, V. Preac-Mursic, B. Wilske, and E. Soutschek.** 1992. Molecular analysis and expression of a *Borrelia burgdorferi*

gene encoding a 22 kDa protein (pC) in *Escherichia coli*. Mol. Microbiol. **6:**503–509.
22. **Hyde, F. W., and R. C. Johnson.** 1984. Genetic relationship of Lyme disease spirochetes to *Borrelia*, *Treponema*, and *Leptospira* spp. J. Clin. Microbiol. **20:**151–154.
23. **Hyde, F. W., and R. C. Johnson.** 1986. Genetic analysis of *Borrelia*. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **263:**119–122.
24. **Jauris-Heipke, S., R. Fuchs, M. Motz, V. Preac-Mursic, E. Schwab, E. Soutschek, G. Will, and B. Wilske.** 1993. Genetic heterogeneity of the genes coding for the outer surface protein C (OspC) and the flagellin of *Borrelia burgdorferi*. Med. Microbiol. Immunol. **182:**37–50.
25. **Johnson, R. C., G. P. Schmid, F. W. Hyde, A. G. Steigerwalt, and D. J. Brenner.** 1984. *Borrelia burgdorferi* sp. nov.: etiological agent of Lyme disease. Int. J. Syst. Bacteriol. **34:**496–497.
26. **Kitron, U., C. J. Jones, and J. K. Bouseman.** 1991. Spatial and temporal dispersion of immature *Ixodes dammini* on *Peromyscus leucopus* in northwestern Illinois. J. Parasitol. **77:**945–949.
27. **Kuiper, H., A. P. van Dam, L. Spanjaard, B. M. de Jongh, A. Widjojokusumo, T. C. P. Ramselaar, I. Cairo, K. Vos, and J. Dankert.** 1994. Isolation of *Borrelia burgdorferi* from biopsy specimens taken from healthy-looking skin of patients with Lyme borreliosis. J. Clin. Microbiol. **32:**715–720.
28. **Kurashige, S., M. Bisset, and L. Oshiro.** 1990. Characterization of a tick isolate of *Borrelia burgdorferi* that possesses a major low-molecular-weight surface protein. J. Clin. Microbiol. **28:**1362–1366.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **Lam, T. T., T.-P. K. Nguyen, E. Fikrig, and R. A. Flavell.** 1994. A chromosomal *Borrelia burgdorferi* gene encodes a 22-kilodalton lipoprotein, P22, that is serologically recognized in Lyme disease. J. Clin. Microbiol. **32:**876–883.
31. **LeFebvre, R. B., W. S. Probert, and G.-C. Perng.** 1993. Characterization of a chromosomal gene and the antigen it expresses from the Lyme disease agent, *Borrelia burgdorferi*. J. Clin. Microbiol. **31:**2146–2151.
32. **Liveris, D., A. Gazumyan, and I. Schwartz.** 1995. Molecular typing of *Borrelia burgdorferi* sensu lato by PCR-restriction fragment length polymorphism analysis. J. Clin. Microbiol. **33:**589–595.
33. **Luft, B. J., W. Jiang, P. Munoz, R. J. Dattwyler, and P. D. Gorevic.** 1989. Biochemical and immunological characterization of the surface proteins of *Borrelia burgdorferi*. Infect. Immun. **57:**3637–3645.
34. **Marconi, R. T., and C. F. Garon.** 1992. Development of polymerase chain reaction primer sets for diagnosis of Lyme disease and for species-specific identification of Lyme disease isolates by 16S rRNA signature nucleotide analysis. J. Clin. Microbiol. **30:**2830–2834.
35. **Marconi, R. T., M. E. Konkel, and C. F. Garon.** 1993. Variability of *osp* genes and gene products among species of Lyme disease spirochetes. Infect. Immun. **61:**2611–2617.
36. **Marconi, R. T., D. S. Samuels, and C. F. Garon.** 1993. Transcriptional analyses and mapping of the *ospC* gene in Lyme disease spirochetes. J. Bacteriol. **175:**926–932.
37. **Marin Canica, M., F. Nato, L. du Merle, J. C. Mazie, G. Baranton, and D. Postic.** 1993. Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand. J. Infect. Dis. **25:**441–448.
38. **Nelson, J. A., J. K. Bouseman, U. Kitron, S. M. Callister, B. Harrison, M. J. Bankowski, M. E. Peeples, B. J. Newton, and J. F. Anderson.** 1991. Isolation and characterization of *Borrelia burgdorferi* from Illinois *Ixodes dammini*. J. Clin. Microbiol. **29:**1732–1734.
39. **Norton Hughes, C. A., C. B. Kodner, and R. C. Johnson.** 1992. DNA analysis of *Borrelia burgdorferi* NCH-1, the first northcentral U.S. human Lyme disease isolate. J. Clin. Microbiol. **30:**698–703.
40. **Padula, S. J., A. Sampieri, F. Dias, A. Szczepanski, and R. W. Ryan.** 1993. Molecular characterization and expression of p23 (OspC) from a North American strain of *Borrelia burgdorferi*. Infect. Immun. **61:**5097–5105.
41. **Picken, R. N.** 1992. Polymerase chain reaction primers and probes derived from flagellin gene sequences for specific detection of the agents of Lyme disease and North American relapsing fever. J. Clin. Microbiol. **30:**99–114.
42. **Postic, D., M. V. Assous, P. A. D. Grimont, and G. Baranton.** 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rrf* (5S)-*rrl* (23S) intergenic spacer amplicons. Int. J. Syst. Bacteriol. **44:**743–752.
43. **Preac-Mursic, V., B. Wilske, E. Patsouris, S. Jauris, G. Will, E. Soutschek, S. Reinhardt, G. Lehnert, U. Klockmann, and P. Mehraein.** 1992. Active immunization with pC protein of *Borrelia burgdorferi* protects gerbils against *Borrelia burgdorferi* infection. Infection **20:**342–349.
44. **Ralph, D., D. Postic, G. Baranton, C. Pretzman, and M. McClelland.** 1993. Species of *Borrelia* distinguished by restriction site polymorphisms in 16S rRNA genes. FEMS Microbiol. Lett. **111:**239–244.
45. **Samuels, D. S., R. T. Marconi, and C. F. Garon.** 1993. Variation in the size of the *ospA*-containing linear plasmid, but not the linear chromosome, among the three *Borrelia* species associated with Lyme disease. J. Gen. Microbiol. **139:**2445–2449.
46. **Schwan, T. G., M. E. Schrumpf, R. H. Karstens, J. R. Clover, J. Wong, M. Daugherty, M. Struthers, and P. A. Rosa.** 1993. Distribution and molecular analysis of Lyme disease spirochetes, *Borrelia burgdorferi*, isolated from ticks throughout California. J. Clin. Microbiol. **31:**3096–3108.
47. **Sigal, L. H., and A. S. Curran.** 1991. Lyme disease: a multifocal worldwide epidemic. Annu. Rev. Public Health **12:**85–109.
48. **Simpson, W. J., M. E. Schrumpf, and T. G. Schwan.** 1990. Reactivity of human Lyme borreliosis sera with a 39-kilodalton antigen specific to *Borrelia burgdorferi*. J. Clin. Microbiol. **28:**1329–1337.
49. **Stanek, G., G. Wewalka, V. Groh, R. Newmann, and W. Kristoperitsch.** 1985. Differences between Lyme disease and European arthropod-borne *Borrelia* infections. Lancet **i:**401.
50. **Theisen, M., B. Frederiksen, A.-M. Lebech, J. Vuust, and K. Hansen.** 1993. Polymorphism in *ospC* gene of *Borrelia burgdorferi* and immunoreactivity of OspC protein: implications for taxonomy and for use of OspC protein as a diagnostic antigen. J. Clin. Microbiol. **31:**2570–2576.
51. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
52. **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.
53. **Wallich, R., M. M. Simon, H. Hofmann, S. E. Moter, U. E. Schaible, and M. D. Kramer.** 1993. Molecular and immunological characterization of a novel polymorphic lipoprotein of *Borrelia burgdorferi*. Infect. Immun. **61:**4158–4166.
54. **Welsh, J., C. Pretzman, D. Postic, I. Saint Girons, G. Baranton, and M. McClelland.** 1992. Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. Int. J. Syst. Bacteriol. **42:**370–377.
55. **Wilske, B., V. Preac-Mursic, U. B. Göbel, B. Graf, S. Jauris, E. Soutschek, E. Schwab, and G. Zumstein.** 1993. An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. J. Clin. Microbiol. **31:**340–350.
56. **Wilske, B., V. Preac-Mursic, S. Jauris, A. Hofmann, I. Pradel, E. Soutschek, E. Schwab, G. Will, and G. Wanner.** 1993. Immunological and molecular polymorphisms of OspC, an immunodominant major outer surface protein of *Borrelia burgdorferi*. Infect. Immun. **61:**2182–2191.
57. **Wilske, B., V. Preac-Mursic, and G. Schierz.** 1985. Antigenic heterogeneity of European *Borrelia burgdorferi* strains isolated from patients and ticks. Lancet **i:**1099.
58. **Wilske, B., V. Preac-Mursic, G. Schierz, and K. V. Busch.** 1986. Immunochemical and immunological analysis of European *Borrelia burgdorferi* strains. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **263:**92–102.
59. **Wilske, B., V. Preac-Mursic, G. Schierz, R. Kuhbeck, A. G. Barbour, and M. Kramer.** 1988. Antigenic variability of *Borrelia burgdorferi*. Ann. N. Y. Acad. Sci. **539:**124–143.
60. **Wilske, B., V. Preac-Mursic, G. Schierz, G. Liegl, and W. Gueye.** 1989. Detection of IgM and IgG antibodies to *Borrelia burgdorferi* using different strains as antigen. Zentralbl. Bakteriol. Mikrobiol. Hyg. Suppl. **18:**299–309.