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Molecular and Pathogenic Characterization of *Borrelia burgdorferi* Sensu Lato Isolates from Spain

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Fifteen *Borrelia burgdorferi* sensu lato isolates from questing ticks and skin biopsy specimens from erythema migrans patients in three different areas of Spain were characterized. Four different genospecies were found (nine *Borrelia garinii*, including the two human isolates, three *B. burgdorferi* sensu stricto, two *B. valaisiana*, and one *B. lusitaniae*), showing a diverse spectrum of *B. burgdorferi* sensu lato species. *B. garinii* isolates were highly variable in terms of pulsed-field gel electrophoresis pattern and OspA serotype, with four of the seven serotypes described. One of the human isolates was OspA serotype 5, the same found in four of seven tick isolates. The second human isolate was OspA serotype 3, which was not present in ticks from the same area. Seven *B. garinii* isolates were able to disseminate through the skin of C3H/HeN mice and to cause severe inflammation of joints. One of the two *B. valaisiana* isolates also caused disease in mice. Only one *B. burgdorferi* sensu stricto isolate was recovered from the urinary bladder. One isolate each of *B. valaisiana* and *B. lusitaniae* were not able to disseminate through the skin of mice or to infect internal organs. In summary, there is substantial diversity in the species and in the pathogenicity of *B. burgdorferi* sensu lato in areas in northern Spain where Lyme disease is endemic.

Lyme borreliosis (LB) is considered the most prevalent tick-borne disease worldwide. In Europe, the causative agent, *Borrelia burgdorferi* sensu lato, is diverse and has been divided into several species or genomic groups, three of which (*B. burgdorferi* sensu stricto [29] [*B. burgdorferi* in this paper], *B. garinii* [7], and *B. afzelii* [13]) are pathogenic for humans. The pathogenicity of *B. lusitaniae* (35), also present in Europe, remains to be elucitated, since it has been isolated only from *Ixodes ricinus*. *B. valaisiana* (61), isolated for the first time in Switzerland (45), has been detected by PCR in skin lesions of erythema migrans (EM) patients (52), and there is some evidence of pathogenic potential in humans (53). There have also been descriptions of genotypic and phenotypic similarities of human European isolates to strain 25015 of *B. bissettii* (47, 58), but this strain has been isolated only from ticks and small mammals (50). In addition, Wang et al. (62) have suggested that apart from the established genospecies, there is another *Borrelia* genomic group with culture-confirmed pathogenic potential for causing human LB.

Investigations into the geographical distribution of *B. burgdorferi* sensu lato in Europe have revealed that *B. garinii* is the most frequently cultured species, followed by *B. afzelii*, *B. burgdorferi*, and *B. valaisiana* in that order (25, 54). *B. valaisiana* and *B. lusitaniae* have been isolated from or detected in *I. ricinus* in a few countries (25). A genospecies specificity has been proposed in Eurasia, with rodents as the main host for *B. afzelii* (19, 24, 26, 27, 38, 39), and birds as the main host for *B. garinii* (33, 41), where a migration restlessness-associated transient spirochetemia occurs (23). However, there are some descriptions of the existence of such cycles for *B. garinii* and *B. valaisiana* in different Eurasian countries (19, 26, 33, 38). Other authors argue against this, describing an even distribution of *Borrelia* species in local ticks and rodents (51), proposing a one-vector–one-reservoir system. In addition, *B. garinii* has been detected in small rodents in other studies (28, 32), and all three genospecies were detected in larval ticks feeding on birds (42).

In Spain, the first isolation of *B. burgdorferi* (strain Esp1) from *I. ricinus* was reported in 1992 (17). Previously, Oteo Revuelta et al. (44) had described spirochetes in the midgut of *I. ricinus* in a different area from the one where the strain Esp1 was isolated. Although LB has been reported in Spain since 1977 (60) and several series of cases have been studied (2, 20, 55), it was not until 1998 that the first isolation of *B. garinii* from an EM lesion was described (43), confirming the role of this strain as a human pathogen in Spain.

Since information about the prevalence of *Borrelia* spirochetes in tick populations and about the different genospecies is essential for our understanding of the epidemiology, diagnosis, and prevention of LB, we have conducted the first study involving the molecular and pathogenic characterization of *B. burgdorferi* sensu lato isolates from ticks from different areas of Spain known to harbor populations of *I. ricinus* (8, 16), as well as from skin biopsy specimens from patients with LB.

MATERIALS AND METHODS

Isolation of the spirochetes. Questing *I. ricinus* ticks were collected by flagging at three regions in the northern half of Spain (Basque Country, La Rioja, and Castilla-León), in areas known to harbor dense populations of *I. ricinus* (8, 16). The ticks were disinfected by serial passages of 2 min in 2-propanol and 70% ethanol, serially washed in phosphate-buffered saline and Barbour-Stoenner-Kelly medium II (BSKII), and placed in fresh BSKII, where the specimens were broken up with two needles. The suspension was either filtered through a syringe filter (μStar 0.45-μm-pore-size filter; Corning Inc., Corning, N.Y.) and added to a 5-ml culture tube containing 4.5 ml of BSK supplemented with 6% rabbit

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TABLE 1. *B. burgdorferi* sensu lato strains used in this study and their characteristics

Strain	Source[a]	Country of origin	PFGE pattern	OspA serotype	Genospecies	Reference
B31^T	*I. dammini*	United States	MLb1	1	*B. burgdorferi*	65
272	Skin	United States	NA[b]	1	*B. burgdorferi*	65
297	Human CSF	United States	MLb2	1	*B. burgdorferi*	65
Esp1	*I. ricinus*	Spain	MLb2	1	*B. burgdorferi*	17
PBi	Human CSF	Germany	MLg2	4	*B. garinii*	65
DK27	Skin of EM patient	Denmark	NA	NA	*B. garinii*	59
R-IP90	*I. persulcatus*	Russia	NA	NA	*B. garinii*	36
DK6	Human CSF	Denmark	NA	4	*B. garinii*	65
WABSou	Skin	Austria	NA	5	*B. garinii*	65
PHei	CSF	Germany	NA	5	*B. garinii*	65
TN	*I. ricinus*	Germany	NA	6	*B. garinii*	65
PWudII	Skin of EM patient	Germany	MLg2	6	*B. garinii*	65
TIs I	*I. ricinus*	Germany	NA	6	*B. garinii*	65
DK29	Skin of EM patient	Denmark	NA	6	*B. garinii*	65
Gö2	*I. ricinus*	Germany	NA	6	*B. garinii*	65
T25	*I. ricinus*	Germany	MLg3	7	*B. garinii*	65
PBr	CSF	Germany	MLg2	3	*B. garinii*	65
PLa	CSF	Germany	NA	8	*B. garinii*	65
VS461^T	*I. ricinus*	Switzerland	MLa1	2	*B. afzelii*	10
DK1	Skin of EM patient	Denmark	NA	2	*B. afzelii*	65
DK2	Skin of ACA patient	Denmark	NA	2	*B. afzelii*	65
M49	*I. ricinus*	The Netherlands	NA	NA	*B. valaisiana*	62
VS116^T	*I. ricinus*	Switzerland	NA	NA	*B. valaisana*	48
POTIB1	*I. ricinus*	Portugal	NA	NA	*B. lusitaniae*	48
POTIB2^T	*I. ricinus*	Portugal	NA	NA	*B. lusitaniae*	48
H014^T	*I. ovatus*	Japan	NA	NA	*B. japonica*	48
IKA2	*I. ovatus*	Japan	NA	NA	*B. japonica*	31
19857	Rabbit	United States	NA	NA	*B. andersonii*	48
21038^T	*I. dentatus*	United States	NA	NA	*B. andersonii*	37
DN127^T	*I. pacificus*	United States	MLb14	NA	*B. bissettii*	48

[a] CSF, cerebrospinal fluid; ACA, acrodermatitis chronica atrophicans.
[b] NA, not available.

serum (BSK-RS) (14) or directly added without previous filtration to a BSK-RS tube supplemented with 0.4 μg of ciprofloxacin per ml and 40 μg of rifampin per ml (BSK-CR) (11). The second type of medium used, to which unfiltered tick suspension was added, was composed of BSK-RS supplemented with 8 μg of kanamycin per ml and 230 μg of 5-fluorouracil per ml (BSK-K5) (30). Blind passages were done always at 24 to 48 h of inoculation to avoid possible toxicity of tick debris and to prevent any adverse effects of the antibiotics on the growth of the spirochetes (6). Cultures were examined by dark-field microscopy weekly for the first month and twice a month for the second and third months after inoculation. Spirochetes from positive cultures were frozen at −70°C in BSK supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich Química S.A., Alcobendas, Madrid, Spain). When possible, only isolates from the first blind passage in antibiotic-free medium were used throughout all the study. Skin biopsy specimens from patients with EM were shipped to the laboratory in complete BSK medium. They were processed as described previously (43).

In addition to the spirochetes isolated in this study, a total of 30 *B. burgdorferi* sensu lato strains were used for comparison as shown in Table 1.

Sequencing of the 16S rRNA gene and phylogenetic analysis. Partial sequencing of the 16S rRNA gene was done by PCR with primers constructed as described previously (3). The primers used were based on the published sequences of the bacterial 16S rRNA (4, 18, 36, 56). For this study we used primer 16-1 (5′-CGAAGAGTTTGATCCTGGCTTAG-3′) as the forward primer and primer 16-3 (5′-GCGGCTGCTGGCACGTAATTAGC-3′) as the reverse primer. The amplified fragment was 519 bp long. Products were purified using the Qiaquick PCR purification columns (Qiagen Inc., Chatsworth, Calif.) as specified by the manufacturer and sequenced using the ABI PRISM Dye Terminator cycle-sequencing ready reaction kit (Perkin-Elmer Co.) on an ABI 377 DNA sequencer.

The DNASTAR package (DNASTAR, Inc., Madison, Wis.) and the Clustal method were used for sequence alignment and construction of the phylogenetic tree. 16S rRNA sequences from other *B. burgdorferi* strains were used in the analysis to construct the phylogenetic tree. These included *B. burgdorferi* 272 (GenBank accession number X85189), 297 (X85204), and Esp1 (U28501); *B. garinii* DK27 (X85193), R-IP9 (M89937), and Rio1 (U28500); *B. afzelii* DK1 (X85190) and DK2 (X85188); *B. valaisiana* M49 (U78155) and VS116^T (X98232); *B. lusitaniae* POTIB1 (X98226) and POTIB2^T (X98228); *B. japonica* H014^T (L40597) and IKA2 (L40598); *B. andersonii* 19857 (L46688) and 21038^T (L46701); and *B. bissettii* DN127^T (L40596). The 16S rRNA sequence from *Treponema pallidum* (M88726) was used as well.

PFGE. Pulsed-field gel electrophoresis (PFGE) was done as described previously (10, 46). Two restriction endonucleases were used: *Mlu*I and *Sma*I (MBI Fermentas, Amherst, N.Y.). 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, we used a pulse time ramped from 1 to 6 s for 24 h at 200 V; for the separation of digested DNA, pulse times were ramped from 3 to 40 s for 20 h. Lambda concatamers with a monomer size of 48.5 kbp (Boehringer, Mannheim, Germany) and a high-molecular-weight marker (Gibco-BRL Life Technologies, Inc., Gaithersburg, Md.) were used as standards. In describing *Mlu*I- and *Sma*I PFGE profiles, we followed the definition and nomenclature previously devised by Belfaiza et al. (10) and Picken et al (46), with the inclusion of the pulsotypes MLv1 and SMv1 for the pattern observed in the *B. valaisiana* isolates tested.

SDS-PAGE. For protein analysis, whole-cell sonicates of cultured spirochetes were prepared from *Borrelia* isolates from ticks and EM patient biopsy specimens, as well as from *B. burgdorferi* (strain B31^T and strain Esp1), *B. garinii* (strain PBi^T), and *B. afzelii* (strain VS461^T). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Laemmli's discontinuous buffer system and 10% polyacrylamide gels (34). The gels were stained with Coomassie brilliant blue R-250 (Merk AG, Darmstadt, Germany). Protein molecular weight standards (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.) were used to determine the relative molecular mass of major proteins by comparison. Monoclonal antibody 84C (15) was used to assess the expression of OspB.

Western blotting. Sera from the mice inoculated with the different isolates were tested for reactivity to the respective homologous strain by Western blotting, following the previously described protocol (2) with the minor modification of using NuPAGE Bis-Tris System (Novex, San Diego, Calif.).

Partial sequencing of *ospA* genes. Nested PCR was carried out as described elsewhere (22). Each PCR amplification product was purified using the Qiaquick PCR purification columns (Qiagen Inc.) as specified by the manufacturer and sequenced as above. The DNASTAR package and the Clustal method were used for sequence alignment and construction of the phylogenetic tree. For comparison of the sequences, a series of other strains were included in this study: Phei (GenBank accession number X80251), TN (X80252), PWudII (X80253), T25 (X80254), PBr (X80256), WABSou (X85441), TIs I (X85440), DK29 (X63412), Gö2 (X60300), DK6 (L38657), PBi (S48323), and PLa (X95355) (genospecies for these strains are indicated in Table 1).

TABLE 2. Pathogenicity scores in C3H mice

Pathogenicity	TTJ inflammation	EPB culture	No. of organs colonized
Nonpathogenic	−	−	0
Low pathogenicity[a]	+/−	+/−	0 or 1[b]
Pathogenic	+	+	1–6

[a] At least one of the three must be positive.
[b] Bladder.

Animal studies. The C3H/He Lyme disease mouse model (9) was used to assess the pathogenicity of the strains from this study. A total of 20 mice were injected intradermally in the lower back with 10^4 spirochetes of each isolate. The percentage of mice that developed arthritis after injection was determined for each isolate by monitoring signs of inflammation of the tibiotarsal joints (TTJ) daily during the first week after injection, every other day during the second week, and twice a week until the end of the fourth week. The level of spirochete dissemination through the skin was determined on day 15 by culturing in BSK-RS 3-mm-diameter ear punch biopsy specimens (EPB) from two mice in each group that had shown signs of inflammation (in the groups where no mice showed signs of inflammation, the two mice were selected on the basis of the level of antibodies to the homologous strain). On day 30, the selected mice were euthanized in CO_2 chambers and necropsy material from the liver, kidneys, heart, brain, spleen, and bladder was collected and cultured in BSK-RS. Citrated blood samples were also cultured for each mouse to ensure that the isolates were tissue and not blood associated.

A score for pathogenicity was constructed as explained in Table 2.

Level of IL-6 in serum. Quantification of interleukin-6 (IL-6) in the sera of the same mice selected for culture was done using the InterTest-6X mouse IL-6 enzyme-linked immunosorbent assay ELISA kit (Genzyme Diagnostics, Cambridge, Mass.) as specified by the manufacturer.

Nucleotide sequence accession numbers. Partial sequences of the *ospA* gene generated in this study were deposited in GenBank under the following accession numbers: AF227323 (Rio1), AF227319 (Rio2), AF227320 (Rio3), AF227321 (Rio4), AF227322 (Rio5), AF227316 (PV4), AF227317 (PV5), AF227318 (PV6), and AF227315 (CL1). Partial sequences of the 16S RNA generated in this study were deposited in GenBank under the following accession numbers: AF245110 (Rio1), AF245111 (Rio2), AF245097 (Rio3), AF245102 (Rio4), AF245108 (Rio5), AF245109 (Rio6), AF245103 (PV1), AF245105 (PV2), AF245106 (PV3), AF245098 (PV4), AF245107 (PV5), AF245100 (PV6), AF245099 (PV7), AF245101 (PV8), and AF245104 (CL1).

RESULTS

Four *B. burgdorferi* genospecies are present in Spain. A total of 13 isolates were obtained from pooled *I. ricinus*. Eight isolates (PV1 to PV8) were derived from ticks collected in the Basque Country, one isolate (CL1) was derived from Castilla-León, and four isolates (Rio3 to Rio6) were derived from La Rioja. We also obtained one more isolate from a skin biopsy specimen of an EM patient in La Rioja (Rio2). The human strain Rio1, previously isolated in our laboratory (43), was also used in this study. For isolate PV8, only PCR-related tests were possible, due to its slow growth.

Sequencing of a fragment at the 3′ end of the 16S rRNA (519 bp long) and subsequent phylogenetic analysis grouped our isolates as follows (Fig. 1): Rio1, Rio2, Rio3, Rio4, Rio5, PV4, PV5, PV6, and CL1 grouped with other *B. garinii* strains; PV1, PV2, and PV3 grouped with the *B. burgdorferi* cluster; PV7 and Rio6 formed a branch group with the recently named *B. valaisiana* species; and PV8 grouped with the *B. lusitaniae* strains.

There is genotypic and phenotypic variability in the isolated strains. Figure 2A and Table 3 show the results of the restriction fragment length polymorphism patterns of *Mlu*I-digested total genomic DNA by PFGE. According to nomenclature previously described (10, 46), 3 of the 14 isolated strains were *B. burgdorferi* (PV1 is MLb13, and PV2 and PV3 are MLb2). All of them had the 135-kbp characteristic band. There were nine *B. garinii* strains (PV4, PV5, PV6, Rio3, Rio4, Rio5, and CL1 from ticks and Rio1 and Rio2 from skin biopsy specimens). These had the two *B. garinii* characteristic bands of 220 and 80 kbp, and all corresponded to pattern MLg2. PV7 and Rio6, which grouped with *B. valaisiana* in the phylogenetic analysis, shared an atypical pattern, with three fragments of 380, 320, and 90 kbp. This pattern was named MLv1 in this study. There was a total correlation of these results with the ones obtained by analyzing the 16S rRNA gene. Both analyses yielded the same result with respect to the genospecies of each isolate.

A higher variability was found when *Sma*I was used, since some isolates that had the same *Mlu*I pulsotype had different *Sma*I patterns. PV2 and PV3 (MLb2) showed different restriction bands (named types SMb2 and SMb1, respectively). PV1, which was MLb13, had the same pulsotype as PV3 (SMb1). For the nine *B. garinii* isolates, there were four different patterns (named SMg1 to SMg4). The two strains that grouped with *B. valaisiana* in the phylogenetic analysis had the same *Sma*I pattern (named SMv1). Figure 2B and Table 3 show the results of the LRFP patterns of the *Sma*I-digested total genome.

When the total genome (chromosome and plasmids) of the isolates was separated by PFGE, there was a variable number of plasmids, ranging between two and seven, per strain (Fig. 3, Table 3). All isolates contained a large plasmid in the 45- to 50-kbp range, which was identified as the linear *ospAB*-containing plasmid (12). The size of this plasmid varied, but the variation did not correlate with the different genospecies. The diffuse band of DNA immediately below the chromosome in same strains could correspond to a multimeric form of a small circular plasmid. The plasmid content of each strain, expressed as the number of bands seen in four size ranges (60 to 40, 39 to 30, 29 to 20, and <20 kb), is shown in Table 3.

The phenotypic variability of the isolates was demonstrated by SDS-PAGE (Fig. 4). The protein profiles were compared with the profile of the reference strains, demonstrating a correlation with the results obtained in the genotypic analysis. All the isolates had a protein profile consistent with that for each *B. burgdorferi* sensu lato species. All had protein bands of

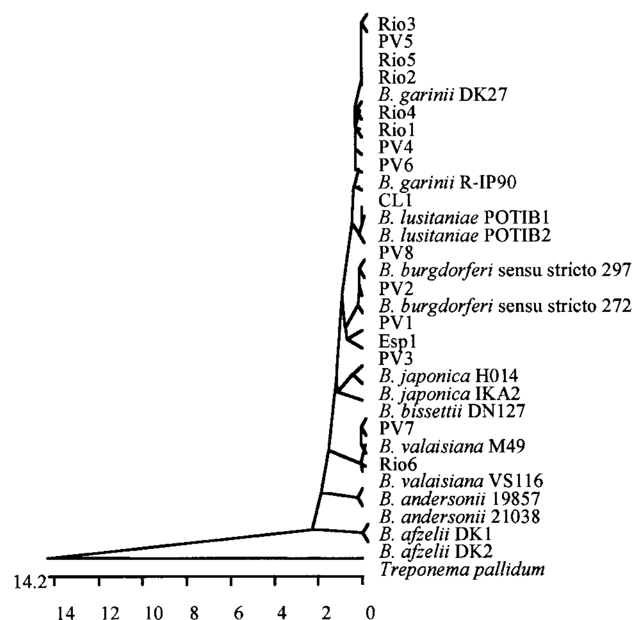

FIG. 1. Phylogenetic tree of *B. burgdorferi* strains based on the sequence of the 16S rRNA gene as described in the text. The scale under the tree measures the distance between sequences.

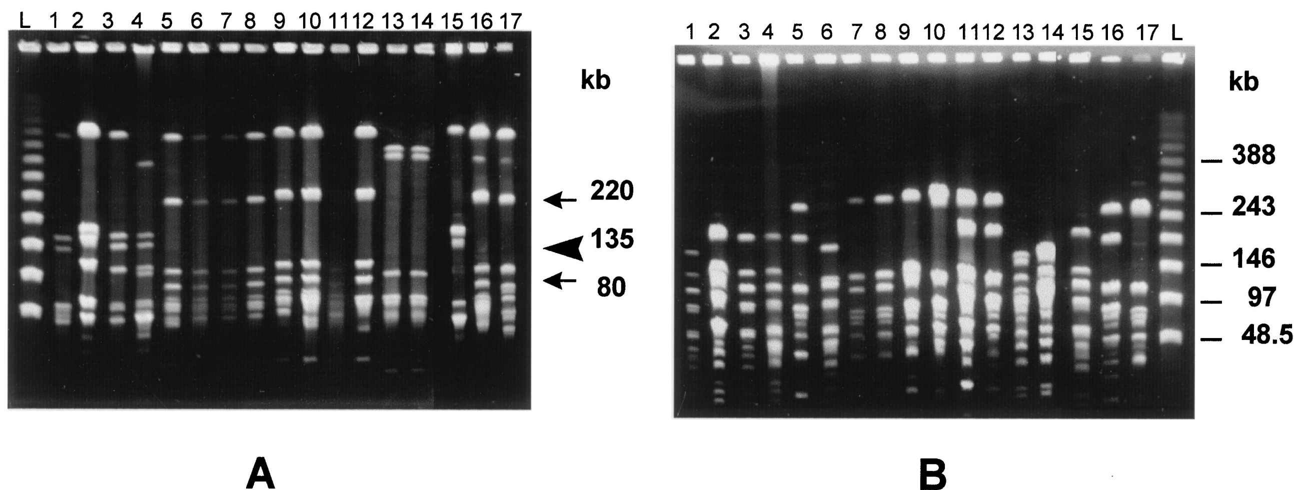

FIG. 2. PFGE separation of *Mlu*I-digested (A) and *Sma*I-digested (B) genomic DNA. Lanes: L, DNA lambda concatemers (48.5 to 485 kb); 1, strain TI-1; 2, Esp1; 3, PV2; 4, PV1; 5, PBi; 6, Rio4; 7, PV6; 8, PV4; 9, Rio1; 10, Rio2; 11, Rio3; 12, Rio5; 13, PV7; 14, Rio6; 15, PV3; 16, PV5; 17, CL1. The arrowhead indicates the 135-kbp band characteristic of *B. burgdorferi* sensu stricto, and the arrows indicate the 220- and 80-kbp bands characteristic of *B. garinii*.

various sizes ranging from 13 to 97 kDa. They were homogeneous with regard to the size and level of expression of their higher-molecular-mass proteins (>41 kDa) but heterogeneous in their lower-molecular-mass proteins (<41 kDa). The sizes of OspA and OspB of the *B. burgdorferi* isolates correlated with those previously described by Baranton et al. (7). Seven of the *B. garinii* isolates and the two *B. valaisiana* isolates expressed a protein with a molecular mass higher than that of the OspA protein, which was confirmed to be OspB (by reactivity with monoclonal antibody 84C [data not shown]). The level of expression of a protein in the appropriate size range for OspC (22 to 25 kDa) (64) varied highly among the genospecies (Fig. 4).

Partial sequencing of the *ospA* gene was performed, and a phylogenetic tree was constructed using representative strains of each serotype described for *B. garinii* (63, 64) (Fig. 5). Among the *B. garinii* isolates, PV4, PV5, PV6, Rio1, and Rio3 are serotype 5, Rio4 and Rio5 are serotype 6, Rio2 is serotype 3, and CL1 is serotype 8.

***B. burgdorferi* isolates have low pathogenicity in C3H mice.** Of the three *B. burgdorferi* isolates tested, PV2 was nonpathogenic (Table 3) for mice, PV1 did not induce inflammation of the TTJ but was recovered from urinary bladder, and PV3 induced inflammation of the TTJ in only 5 of 20 mice and was considered to be of low pathogenicity (Table 3). The sera of the mice inoculated with PV1 and PV3 showed a reactivity in Western blots (with the respective homologous strain) to the 41-kDa protein, OspA, and OspB (Fig. 6). The isolate that was recovered from urinary bladder also induced secretion of IL-6 at a low level.

***B. garinii* isolates are pathogenic for mice.** Eight of nine *B. garinii* isolates disseminated through the skin and induced inflammation of the TTJ (in 25% of mice for two strains, PV5 and Rio1). The rate of recovery from organs varied from iso-

TABLE 3. Summary of results

Isolate	Species[a]	PFGE pattern		No. of plasmids				OspA serotype	% Arthritogenicity[b]	Recovery from:		IL-6 secretion	Pathogenicity[d]
		*Mlu*I	*Sma*I	60–40 kb	39–30 kb	29–20 kb	<20 kb			EPB	Organs[c]		
PV1	S	MLb13	SMb1	2	2	1	0	1	0	−	B	+	LP
PV2	S	MLb2	SMb2	2	2	1	0	1	0	−	−	−	NP
PV3	S	MLb2	SMb1	3	0	1	0	1	25	−	−	−	LP
PV4	G	MLg2	SMg1	2	1	2	0	5	100	+	B, S, H, Br	++	P
PV5	G	MLg2	SMg2	4	0	2	0	5	25	−	B	−	LP
PV6	G	MLg2	SMg1	3	2	2	0	5	100	+	B, S, H, Br, K, L	+++	P
PV7	V	MLv1	SMv1	2	0	1	0	ND[e]	0	−	−	−	NP
PV8	L	ND	ND	ND	ND	ND	ND	ND	0	−	−	−	NP
CL1	G	MLg2	SMg3	3	0	2	0	8	100	+	B	++	P
Rio1	G	MLg2	SMg1	ND	ND	ND	ND	5	25	+	B, S	−	P
Rio2	G	MLg2	SMg3	2	1	2	0	3	100	+	−	+	LP
Rio3	G	MLg2	SMg2	3	0	2	0	5	100	+	S, H	++	P
Rio4	G	MLg2	SMg4	2	1	1	1	6	100	+	B, H, K	+	P
Rio5	G	MLg2	SMg2	2	2	1	0	6	100	+	B, K	+++	P
Rio6	V	MLv1	SMv1	1	0	1	0	ND	25	+	B, K	−	P

[a] S, *B. burgdorferi* sensu stricto; G, *B. garinii*; V, *B. valaisiana*; L, *B. lusitaniae*.
[b] Percentage based on 20 mice.
[c] B, urinary bladder; S, spleen; H, heart; Br, brain; K, kidney; L, liver.
[d] NP, nonpathogenic; LP, low pathogenicity; P, pathogenic.
[e] ND, not determined.

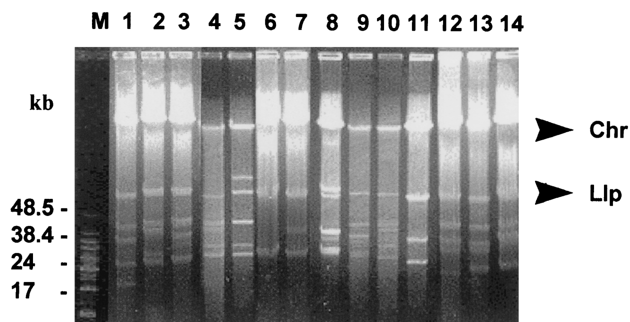

FIG. 3. PFGE separation of the total undigested genome of the *B. burgdorferi* sensu lato isolates and reference strains. Lanes: M, DNA molecular size markers of 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb; 1, Rio4; 2, Rio3; 3, Rio5; 4, Rio2; 5, CL1; 6, Rio6; 7, PV7; 8, PV6; 9, PV4; 10, PV5; 11, Esp1; 12, PV2; 13, PV1; 14, PV3.

late to isolate. One isolate of human origin (Rio2) was recovered from EPB and induced inflammation of the TTJ in 100% of mice and secretion of IL-6 but was not recovered from internal organs, suggesting low disseminating capabilities in this model; considering this discrepancy, it was classified as having low pathogenicity. PV5 did not migrate through the skin and induced TTJ inflammation in only 25% of mice, was recovered only from the urinary bladder, and did not induce IL-6 secretion; it was also classified as having low pathogenicity. A third isolate, CL1, induced TTJ inflammation in 100% of mice and secretion of IL-6 and was recovered from EPB but was recovered only from the urinary bladder; it was therefore classified as pathogenic. For the rest of the strains, a positive EPB, inflammation of the TTJ in 100% of mice, secretion of IL-6, and recovery from at least two organs were seen; they were also classified as pathogenic. All the *B. garinii* strains showed a strong antibody reactivity to the homologous isolate by Western blotting (data not shown).

***B. valaisiana* is pathogenic for mice.** Of the two *B. valaisiana* isolates analyzed, one (PV7) did not show any sign of pathogenicity and the other (Rio6) was recovered from EPB, induced TTJ inflammation in 25% of mice, and was recovered from the urinary bladder and kidneys; it was classified as pathogenic. Both isolates showed a high reactivity with the 41-kDa protein and OspA by Western blotting to the homologous strain, and Rio6 was also reactive with a band in the range of the 22-kDa protein (Fig. 6).

In summary, the pathogenicity for mice was higher among the *B. garinii* isolates, one isolate of *B. valaisiana* was considered pathogenic, and all the *B. burgdorferi* isolates showed milder signs of pathogenicity. The isolates that were recovered from EPB were *B. garinii* and *B. valaisiana* strains. Also, recovery from the urinary bladder was considered of low significance since, even with no arthritogenicity or recovery from EPB, some isolates were found in this organ, suggesting that it was preferentially infected, with low pathogenic significance. The secretion of IL-6 at low level (1+) did not always correlate with inflammation of TTJ in our system, but secretion at 2+ to 3+ level was always associated with positive EPB.

DISCUSSION

Fifteen *B. burgdorferi* sensu lato isolates were recovered from Spanish *I. ricinus* ticks and biopsy specimens from EM patients. Of these, three were *B. burgdorferi* sensu stricto, nine were *B. garinii*, two were *B. valaisiana*, and one was *B. lusitaniae*. These findings indicate greater genospecies diversity of *B. burgdorferi* sensu lato in Spain than in other parts of Europe. The only genospecies not present was *B. afzelii*, even though this species is the second most frequently isolated throughout Europe (25, 54). Based on these results, *B. afzelii* may be absent at the southwestern margin of the continent. Accordingly, *B. afzelii* has not been detected in patients in Spain (1), and there has been an absence of descriptions of *B. afzelii*-related cutaneous manifestations in clinical series (2, 5, 20, 21). In contrast, *B. lusitaniae* is present in southern Europe and North Africa (40, 65) but it is not frequent in eastern Europe (49). Overlapping geographic areas in the Iberian peninsula with highly diverse *B. burgdorferi* populations as well as relapsing-fever borrelia (4) could create the necessary conditions for genetic exchanges and for the origin of new genospecies.

The high intraspecies variability detected on the basis of all the parameters studied is reflected in the behavior of the iso-

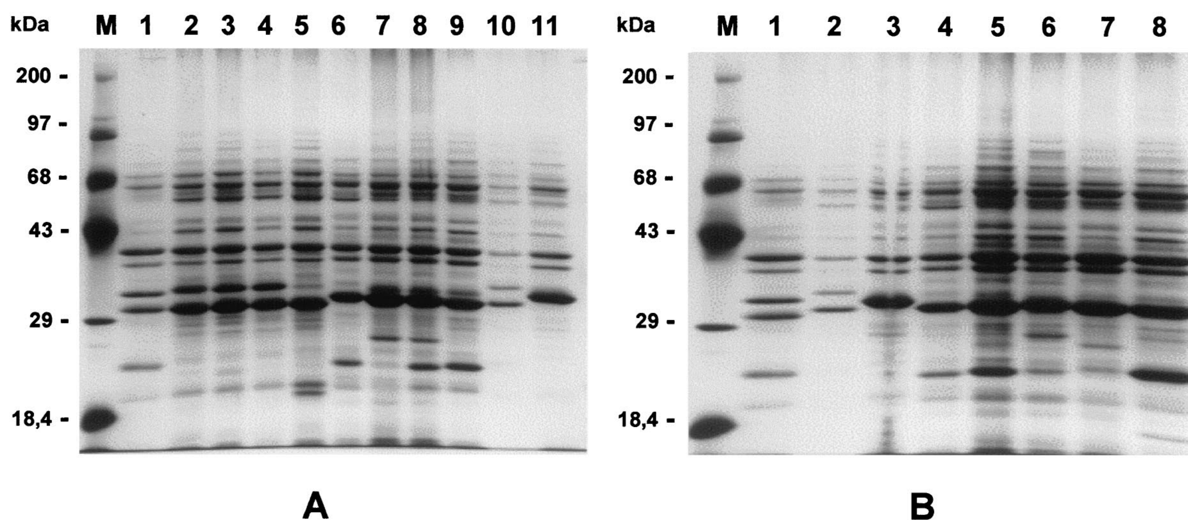

FIG. 4. SDS-PAGE and Coomassie blue staining of *B. burgdorferi* sensu lato isolates. In each panel, the protein profiles of B31^{T} (*B. burgdorferi* sensu stricto), PBi (*B. garinii*), and VS461^{T} (*B. afzelii*) are also given. Lanes M contain molecular mass markers of the sizes shown. (A) Isolates from Basque Country. Lanes: 1, B31; 2, Esp1; 3, PV2; 4, PV1; 5, PV3; 6, PBi; 7, PV6; 8, PV4; 9, PV5; 10, VS461; 11, PV7. (B) Isolates from La Rioja. Lanes: 1, B31; 2, VS461; 3, Rio6; 4, PBi; 5, Rio4; 6, Rio2; 7, Rio3; 8, Rio5.

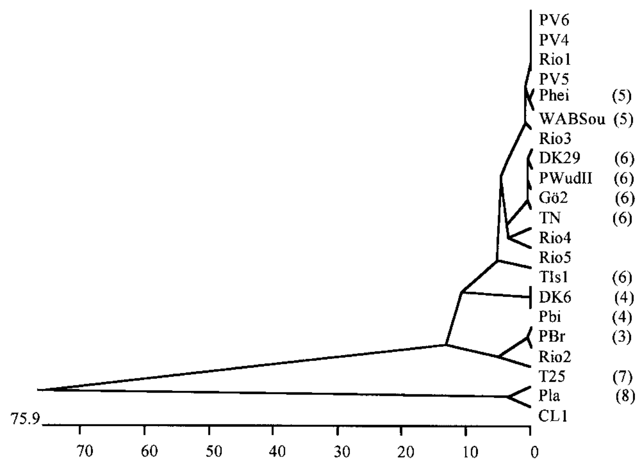

FIG. 5. Phylogenetic tree of *B. burgdorferi* strains based on the sequence of the *ospA* gene as described in the text. The scale under the tree measures the distance between sequences. OspA serotypes of each strain are given in parentheses.

lates in C3H mice (Table 3). Seven of the nine *B. garinii* isolates (CL1, Rio1, Rio3, Rio4, Rio5, PV4, and PV6) disseminated through the skin, induced severe TTJ inflammation in 20 of 20 mice, and caused disseminated infection in C3H mice. Organisms were recovered from a battery of internal organs (Table 3). The two remaining *B. garinii* strains (Rio2 and PV5) showed low pathogenicity, even though one of them was an isolate of human origin, which was the only one that disseminated through the skin of C3H mice. None of the three *B. burgdorferi* isolates were virulent to C3H mice (only strain PV1 was recovered from urinary bladder, and strain PV3 induced TTJ inflammation in 25% of mice). None of them were recovered from EPB. Interestingly, one of the two *B. valaisiana* isolates (Rio6) was able to disseminate through the skin and to induce severe TTJ inflammation in 25% of mice and was recovered from the kidneys and urinary bladder, suggesting that a tick-mouse cycle could maintain this isolate in nature.

Several authors have suggested that *B. afzelii* is preferentially or exclusively maintained in cycles involving small rodents and ticks (19, 24, 26, 27, 38). *B. burgdorferi* has been largely associated with small rodents (57). Associations for *B. garinii* appear to be more heterogeneous: a cycle involving sea birds has been well characterized for this species (41), and a mechanism of transient spirochetemia associated with migrating birds has been described (23). Several other studies have pointed out the existence of such cycles for *B. garinii* and *B. valaisiana* in different Eurasian countries (19, 23, 26, 33, 38). However, other descriptions have found *B. garinii* associated with small rodents (28, 51). Whether a bird-tick or rodent-tick cycle or perhaps both maintain local variants of *B. garinii* and *B. valaisiana* in nature remains to be elucidated, but, given the high frequency of *B. garinii* isolates in the areas studied and the data from the animal model, we have shown that at least some variants of *B. garinii* can infect mice and disseminate through the skin from day 7 after infection until at least day 90 (data not shown). Since *B. garinii* is a very heterogeneous species in terms of pulsotype (Fig. 2, Table 3), plasmid content (Fig. 3, Table 3) and OspA serotype (Fig. 5, Table 3), some of these differences could account for a distinct susceptibility of different hosts. Data about the transmission of the human isolate Rio1 from syringe-infected C3H mice to xenodiagnostic larval *I. ricinus* and the subsequent transmission of the organisms to mice via a tick bite from the derived nimphal ticks (M. M. Vitutia, unpublished data) support this hypothesis. We can assume that other *B. garinii* isolates that exhibited a full spectrum of pathogenicity could at least be equally and efficiently maintained in a tick-mouse cycle.

We did not find an association between OspA serotype and pathogenicity to mice. In fact, the only *B. garinii* strain that was not recovered from EPB was OspA serotype 5 (PV5), in common with four additional isolates that were cultured with this method (PV4, PV6, Rio1, and Rio3). Consequently, different degrees of pathogenicity to mice are found among serotype 5 *B. garinii* isolates. In summary, in this study, isolates belonging to OspA serotypes 5, 6, and 8 were pathogenic to mice and a serotype 3 isolate had low pathogenicity, suggesting that other factors seem to influence the behavior of *B. garinii* in mice.

These differences in pathogenicity to C3H mice found in this work for each isolate could be used, given the constraints of extrapolation to humans, to hypothesize about the risk for humans of contracting Lyme disease in a certain area. Given that the isolates represent a highly variable population, they could form the basis for a variable clinical spectrum in humans.

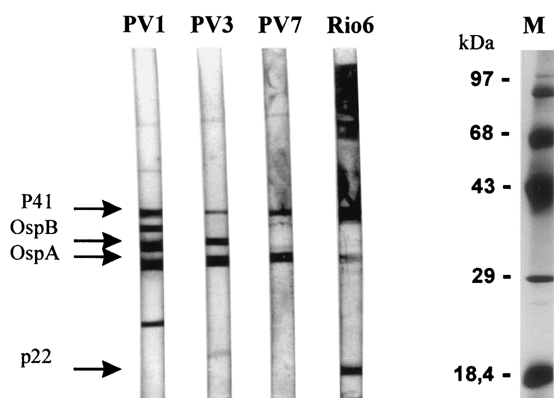

FIG. 6. Reactivity of sera from the *B. burgdorferi* PV1, PV3, PV7, and Rio6 isolates by Western blotting to the respective homologous strain. M, molecular mass markers of the sizes shown.

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