Taxonomic classification of 29 *Borrelia burgdorferi* strains isolated from patients with Lyme borreliosis: a comparison of five different phenotypic and genotypic typing schemes

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Abstract. Twenty-nine European and North American *Borrelia burgdorferi* strains isolated from patients with Lyme borreliosis, were investigated by restriction fragment length polymorphism (RFLP) of two phylogenetically highly conserved chromosomal genes encoding flagellin (*fla*) and the p60 common antigen (*CA*), as well as of the plasmid-borne outer surface protein A (*ospA*) gene. RFLP of the *ospA*, *fla* and *CA* gene revealed five, two and four distinct subspecies-specific patterns, respectively. RFLP classification of the *B. burgdorferi* strains was compared with four different classification schemes proposed by others: (i) molecular mass profile of OspA and OspB (Adam et al. [1]); (ii) OspA serotyping (Wilske et al. [34]); (iii) genomic fingerprinting on the central region of the *B. burgdorferi fla* gene (Picken [24]) and (iv) 16S rRNA signature nucleotide analysis (Marconi and Garon [19]). Results obtained with the different methods correlated highly. All strains classified as *B. burgdorferi* sensu stricto and *B. afzelii* could be unequivocally identified as one distinct group by all five typing methods. *B. garinii* isolates, however, were more heterogeneous and according to RFLP of the *CA* and *ospA* gene fell into either two or three subgroups. The agreement of the different approaches supports the recent concept that *B. burgdorferi* sensu lato strains should be delineated to three genomic groups and that *B. burgdorferi* sensu lato is clonal. All 12 US strains were *B. burgdorferi* sensu stricto, whereas the 17 European isolates belonged to any of three genospecies. Among European *B. burgdorferi* isolates there was an association between *B. burgdorferi* genospecies and the clinical manifestation of Lyme borreliosis. *B. afzelii* strains were found to predominate in 11 skin isolates (75%), whereas all 6 cerebrospinal fluid isolates from patients with neuroborreliosis were *B. garinii*. These findings support the concept of a strain-dependent organotropism of *B. burgdorferi*.

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Introduction

Lyme borreliosis is a tick-borne spirochetosis caused by *Borrelia burgdorferi*. The infection is increasingly recognized and is now the most common vector-transmitted human infectious disease in Europe and North America.

When *Borrelia burgdorferi* in 1984 was described as a new species of the genus *Borrelia* [14], the definition was based on the characterization of only few strains including only one European isolate. Meanwhile, numerous strains have been isolated from patients with Lyme borreliosis and from a variety of reservoir hosts and ticks in Europe, North America and Asia.

In recent years several attempts have been made to classify *B. burgdorferi* isolates on the basis of phenotypic and of genotypic traits. The phenotypic approaches were: electrophoretic profiles of *B. burgdorferi* proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [1, 4, 6, 7, 32] and antigenic diversity of outer surface protein A (OspA) [7, 33, 34]. The genetic diversity has been studied by restriction endonuclease analysis [17], plasmid analysis [5], DNA relatedness [4, 25], multilocus enzyme electrophoresis [9], arbitrarily primed polymerase chain reaction (PCR) [31], 16S rRNA sequencing [1, 19, 20], restriction fragment length polymorphism (RFLP) of the 16S rRNA gene [4, 25], the ospA gene [6, 30] and the flagellin gene (*fla*) [30] and comparison of protein-encoding gene sequences [1, 10, 34]. All these studies revealed a significant heterogeneity of *B. burgdorferi*, resulting in the denomination of two to seven different groups. On the basis of DNA relatedness and RFLP of the 16S rRNA gene Baranton et al. [4] proposed, that *B. burgdorferi* strains should be divided into three genospecies: *B. burgdorferi* sensu stricto (genospecies I), *B. garinii* sp. nov. (genospecies II) and *B. burgdorferi* group VS461 (genospecies III). Recently, *B. burgdorferi* group VS461 was named *B. afzelii* sp. nov. [21]. The term *B. burgdorferi* sensu lato was introduced as a common designation of all three *B. burgdorferi* species.

The heterogeneity among *B. burgdorferi* strains has important implications for epidemiological tracing, selection of suitable antigens for diagnostic assays and in order for an understanding of the possible role of strain-dependent organotropism. Subspecies differences might explain why approximately 90% of patients recover spontaneously after an erythema migrans (EM), whereas only 5–10% progress to a disseminated infection [2], or why some patients exclusively develop neurological disease or arthritis. In accordance with this concept Wilske et al. [33] found a predominance of OspA serotype 2 among European skin isolates. Subspecies differences might also explain the different clinical presentation of Lyme borreliosis in Europe and the US [27].

The aims of this study were (i) to study the genetic diversity of *B. burgdorferi* strains isolated from patients with Lyme borreliosis by RFLP; for this purpose we studied two unlinked, phylogenetically highly conserved chromosomal genes – the *fla* gene and the common antigen gene (*CA*) – and additionally the plasmid-encoded *B. burgdorferi*-specific *ospA* gene; (ii) to compare the classification of *B. burgdorferi* strains obtained by RFLP with serological and genetic classification schemes proposed by others: (a) molecular mass profile of OspA and OspB [1], (b) OspA serotyping [34], (c) genomic fingerprinting based on the nucleic acid sequence of the central region of the *fla* gene [24], and (d) 16S rRNA signature nucleotide analysis [19]; and (iii) to investigate a possible taxonomic correlation with geographic origin and with the type of clinical manifestation of Lyme borreliosis.

Materials and methods

Spirochetal strains

A panel of 29 B. burgdorferi strains was used as summarized in Table 1. Except for the US type strain B31, which is a tick isolate, all other strains were isolated from patients with Lyme borreliosis and, thus, known to be pathogenic for man; 17 strains were from Europe and 12 from North America. To control the specificity of our investigation we also included B. hermsii the etiological agent of relapsing fever.

Purification of spirochetal DNA

B. burgdorferi and B. hermsii were grown in BSK medium and whole cell DNA extracted as previously described [12, 16]. DNA concentrations were determined spectrophotometrically at a wave length of 260 nm (A_{260}) [18].

RFLP of the B. burgdorferi ospA, fla and CA genes

Approximately 0.5 µg of whole-cell DNA from all of the Borrelia strains listed in Table 1 was completely digested with the restriction endonuclease HindIII (Bethesda Research Laboratories, INC., Gaithersburg, Md.). DNA digests were then separated by agarose gel electrophoresis (0.8%). Agarose gels were placed in 0.37% (vol/vol) HCl for 10 min and subsequently Southern blotting was performed as previously described [16]. DNA probes complementary to the ospA, the fla and the CA genes were generated by PCR amplification. The sequences and the locations of synthetic oligonucleotide primers used are specified in Table 2. They were based on previously published nucleotide sequences [8, 11, 26]. As template DNA for the fla and the CA gene probe served 0.2 µg of purified DNA from B. burgdorferi DK1 and for the ospA gene probe 1 µg of the plasmid pTHR-44 carrying the ospA gene [13]. PCR was performed using a standard PCR mixture. After amplification, products were applied to agarose gel electrophoresis. Under UV illumination the slice of agarose containing the specific DNA fragment was cut out and the DNA was recovered from the gel segment by repeated freezing/thawing and elution in TRIS-EDTA buffer (pH 7.0). After centrifugation approximately 1 µg DNA in the supernatant was labelled as probe by random primed ^{32}P-labelling according to a standard protocol [22]. The membranes were hybridized and washed at 60 °C and subsequently autoradiographed for 24 h as described previously [16]. The size of DNA fragments was determined using a [^{35}S]-labelled molecular weight standard containing 14 DNA fragments from 0.41 to 22.01 kbp as a reference (no. SJ 5000, Amersham International, Buckinghamshire, UK).

OspA/B molecular mass determination by SDS-PAGE

SDS-PAGE for molecular mass analysis of OspA/OspB was performed according to a standard protocol [12] using approximately 10^7 spirochetes per lane. The evaluation of the OspA/B molecular mass profile was performed according to Adam et al. [1] and the 29 B. burgdorferi strains grouped into four different groups according to the molecular mass of the OspA and OspB. Group 1 was defined as having a molecular mass of 31 and 34 kDa of the OspA and OspB, respectively, and group 2 by expressing the OspA and OspB at 32 and 35 kDa. Group 3 had an OspA of 32 kDa and OspB was not expressed. Group 4 was characterized by OspA and OspB of 32 and 34 kDa.

OspA serotyping

All strains listed in Table 1 were subjected to OspA serotyping according to Wilske et al. [34]. Based on the Western blot immunoreactivity of OspA with eight different monoclonal antibod-

Table 1. *Borrelia burgdorferi* sensu lato strains studied for classification

Strain	Geographic source	Biological source	RFLP			Mol. mass profile OspA/B	OspA serotyping	Flagellin genotyping	16s rRNA genotyping	Supplier[a]
			ospA	Fla	CA					
DK1	DK	Skin, EM	II	II	I	2	2	P/Sto	Ba	SSI
DK2	DK	Skin, ACA	II	II	I	2	2	P/Sto	Ba	SSI
DK3	DK	Skin, ACA	II	II	I	2	2	P/Sto	Ba	SSI
DK4	DK	Skin, EM	II	II	I	2	2	P/Sto	Ba	SSI
DK5	DK	Skin, ACA	V	II	I	2	2	P/Sto	Ba	SSI
DK6	DK	CSF, LMR	I	I	II	4	4	B31	Bg[f]	SSI
DK7	DK	Skin, ACA	I	I	II	1	1	P/Bi	SS[g]	SSI
DK27	DK	Skin, EM	IV	II	Ii	3	X	P/Bi	Bg	SSI
DK29	DK	Skin, EM	III	II	III	3	6	P/Bi	Bg	SSI
P/Ko	G	Skin, EM	II	II	I	2	2	P/Sto	Ba	V. Preac-Mursic
P/Bi	G	CSF, LMR	V	II	II	4	4	P/Bi	Bg	V. Preac-Mursic
P/Tr	G	Skin, ACA	II	II	III	n.d.	2	P/Sto	Ba	V. Preac-Mursic
SL10	SW	CSF, LMR	III	II	III	3	6	P/Bi	Bg	M. Karlsson
SL14	SW	CSF, LMR	III	II	III	3	6	P/Bi	Bg	M. Karlsson
SL20	SW	CSF, LMR	III	II	III	3	6	P/Bi	Bg	M. Karlsson
SL42	SW	CSF, LMR	III	II	III	3	6	P/Bi	Bg	M. Karlsson
ACA1	SW	Skin, ACA	II	II	I	2	2	P/Sto	Ba	E. Åsbrink
297	US	CSF	I	I	IV	1	1	B31	SS	A. C. Steere
272	US	Skin	I	I	IV	1	1	B31	SS	A. C. Steere
245	US	Blood	I	I	IV	1	1	B31	SS	A. C. Steere
TXGW	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
MIJ	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
ROB	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
MUL	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
BUR	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
KIPP	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
DUNKIRK	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
LIPITZ	US	Skin	I	I	IV	1	1	B31	SS	R. C. Johnsson
B31	US	Tick	I	I	IV	1	1	B31	SS	A. G. Barbour

RFLP, Restriction fragment length polymorphism; EM, erythema migrans; ACA, acrodermatitis chronica atrophicans; CSF, cerebrospinal fluid; LMR, lymphocytic menigoradiculitis; n.d., not done; DK, Denmark; G, Germany; SW, Sweden; Ba, *B. afzelii*; Bg, *B. garinii*; BB, *B. burgdorferi* sensu stricto
[a] SSI, Statens Seruminstitut, Copenhagen; M. Karlsson, Danderyd Sjukhus, Stockholm, Sweden [15]; E. Åsbrink, Södersjukhuset, Stockholm, Sweden [3]; V. Preac-Mursic, Max von Pettenkofer Institute, Munich, Germany; A. C. Steere, Tufts New England Medical Center, Boston, Mass. [28]; A. G. Barbour, Health Science Center, San Antonio, Tex.

Table 2. RFLP: Nucleotide sequences and positions of primers used for DNA probe generation

Gene encoding the *B. burgdorferi*	Primer	Sequence	Coding strand	Location (nucleotide)[a]
OspA	OA_1	CAA GCT TGA GCT TAA AGG	+	177–194
Strain B31	OA_2	GCA GCT TGG AAT TCA GGC	–	640–657
Flagellin protein	F_1	ATT AAC GCT AAT CTT AGT	+	52–72
Strain B31	F_3	GTA CTA TTC TTT ATA GAT TC	–	823–842
Common antigen Strain ACA-1	CA_1	TAA AAT TTT TAT GGC TAA AGA CAT ATA T	+	–10–18
	CA_2	CCG GCA AAA ACA CAA GCT CAC CGG T	–	1649–1674

[a] Base positions are numbered according to the published sequence of the *B. burgdorferi* outer surface protein A (*ospA*) gene [8], flagellin (*fla*) gene [11] and common antigen (*CA*) gene [26]

ies (mAbs) seven distinct OspA serotypes were defined. Isolates having mAb patterns different from those of types 1 to 7 were considered to belong to a heterogeneous group designated type X, and strains that could not be analyzed for the OspA serotype due to a lack of OspA expression were designated serotype 0.

Genomic fingerprinting of the B. burgdorferi fla gene

The central region of the *B. burgdorferi fla* gene (bp 517 to 742) is variable. Using DNA probes Fl8, Fl15 and Fl16 complementary to this central region derived from three different *B. burgdorferi* strains (B31, P/Bi, P/Sto) Picken [24] divided *B. burgdorferi* isolates into three different groups. Briefly, a 275-bp fragment of the central region of the *B. burgdorferi fla* gene was PCR amplified and subsequently hybridized at 59 °C with the radioactively labeled oligonulceotide probes. The filters were washed at 67°, 64° or 69 °C using the probe Fl8, Fl15 or Fl16, respectively. Using this method we typed all isolates listed in Table 1.

16S rRNA signature nucleotide analysis

Based on partial 16S rRNA gene sequences from *B. burgdorferi*, Marconi and Garon [19] identified signature nucleotides unique to each genospecies. Consequently three PCR primer sets were designed to differentiate between *B. burgdorferi* sensu stricto (BB primer set), *B. garinii* (BG primer set), and *B. afzelli* (VS461 primer set). A further primer set designed to amplify all *B. burgdorferi* sensu lato strains was designated LD primers. We performed the 16S rRNA signature nucleotide analysis of *B. burgdorferi* isolates listed in Table 1 according to the protocol of Marconi and Garon [19].

Results

RFLP of B. burgdorferi ospA, fla and CA genes

The position and size of the *ospA, fla* and *CA* gene probes used to visualize the restriction patterns of *B. burgdorferi* are schematically shown in relation to the complete genes in Fig. 1 A–C. The *Hind*III sites in each gene are indicated and, thus, the expected size and number of restriction fragments to be obtained. Figure 2 and
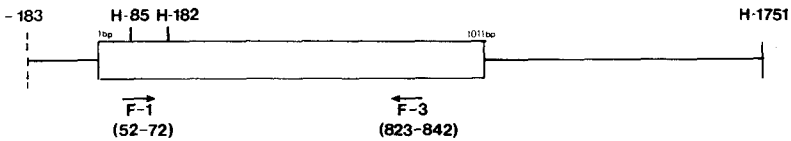
A

Osp A GENE
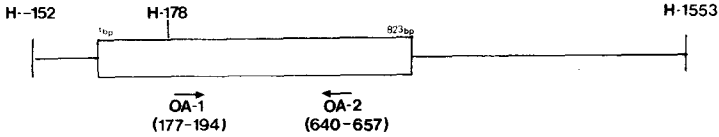
Expected restriction fragment profile
(B.burgdorferi strain B31):
- 1,375 bp
- 330 bp

B

FLAGELLIN GENE

Expected restriction fragment profile
(B.burgdorferi strain B31):
- 1,569 bp
- > 268 bp
- 97 bp

C

COMMON ANTIGEN GENE

Expected restriction fragment profile
(B.burgdorferi strain ACA-1):
- 1,045 bp
- > 1,042 bp
- 834 bp
- 21 bp

Fig. 1. Schematic maps of (**A**) the *B. burgdorferi* outer surface protein A (*ospA*) gene [8] the flagellin (*fla*) gene [11] and (**C**) the common antigen (*CA*) gene [26]. The *boxes* indicate the open reading frame and the *full lines* the upstream and downstream sequences. The *dotted horizontal lines* (**B**, **C**) illustrate, that the upstream sequences are only determined until the base positions indicated. The base positions of expected *Hind*III restriction sites (H) and the expected restriction fragment profile according to sequence data are indicated. In parentheses are shown the base positions of the oligonucleotide primers used in the PCRs for generation of the hybridization probes. The orientations of primers are shown with *arrows* pointing in the 5' to 3' direction

Table 3 show representative RFLP patterns of *Hind*III-digested genomic DNA of the 29 *B. burgdorferi* strains following hybridization with radioactively labelled *ospA*, *fla*, and *CA* geneprobes.

RFLP analysis for *ospA* using *Hind*III revealed five distinct hybridization patterns among the 29 *B. burgdorferi* sensu lato strains tested. The gene probe iden-

Table 3. Characteristic patterns of restriction fragments of *B. burgdorferi* subgroups

| Representative strain according to Fig. 2 A–C	*ospA* geneprobe		*fla* geneprobe		*CA* geneprobe	
	Type	Fragment sizes (kbp)	Type	Fragment sizes (kbp)	Type	Fragment sizes (kbp)
DK7	I	1.3; 0.33	I	1.6	IV	1.8
DK1	II	1.7	II	1.2; 0.32	I	1.7; 1.0; 0.8
DK29	III	1.0; 0.6	II	1.2; 0.32	III	1.7; 1.5; 1.0; 0.8
DK27	IV	1.9	II	1.2; 0.32	III	1.7; 1.5; 1.0; 0.8
DK6	V	2.4; (1.7)	II	1.2; 0.32	II	2.3; (1.0); 0.8

tified two *Hind*III fragments of about 1.3 and 0.3 kbp in group I^{OspA} strains (Fig. 2A, lane DK7), as expected from the sequence data from strain B31. A larger *Hind*III fragment, about 1.7 kbp, was found in group II^{OspA} strains (Fig. 2A, lane DK1), whereas group III^{OspA} isolates were characterized by two fragments of approximately 1.0 and 0.6 kbp (Fig. 2A, lane DK29) and group IV^{OspA} isolates exhibited a profile of only one *Hind*III-band of about 1.9 kbp (Fig. 2A, lane DK27). A larger *Hind*III fragment of approximately 2.4 kbp was found in group V^{OspA} (Fig. 2A, lane DK6). Additonally a weak *Hind*III fragment of 1.7 kbp was often seen. No cross hybridization with DNA from the closely related spirochete *B. hermsii* was seen using the *ospA* probe (Fig. 2A, lane *B. hermsii*).

As shown in Fig. 2B two different restriction fragment patterns could be identified by using the *fla* gene probe. A 1.5-kbp *Hind*III fragment was recognized in group I^{fla} isolates (Fig. 2B, lane DK7), whereas group II^{fla} strains were characterized by two fragments of approximately 1.2 and 0.32 kbp (Fig. 2B, lane DK1). *Hind*III digests of DNA from *B. hermsii* showed banding pattern clearly distinguishable from all *B. burgdorferi* strains. With the *CA* gene probe four hybridization groups could be distinguished. The expected restriction fragment profile according to the *CA* sequence of strain ACA-1 were three major *Hind*III fragment (1.7, 1.0 and 0.8 kbp)-characterized strains belonging to group I^{CA} (Fig. 2C, lane DK1); two *Hind*III fragments of about 2.3 and 0.8 kbp were found in DNA digests of group II^{CA} strains (Fig. 2C, lane DK6). Group III^{CA} strains exhibited a profile of four bands at approximately 1.7, 1.5, 1.0 and 0.8 kbp (Fig. 2C, lane DK29), whereas a 1.8-kbp *Hind*III *CA* fragment was found in digested DNA from group IV^{CA} isolates (Fig. 2C, lane DK7). *Hind*III digests of DNA from *B. hermsii* showed banding patterns clearly distinguishable from all *B. burgdorferi* strains. The classification based on RFLP of all 29 *B. burgdorferi* isolates is summarized in Table 1.
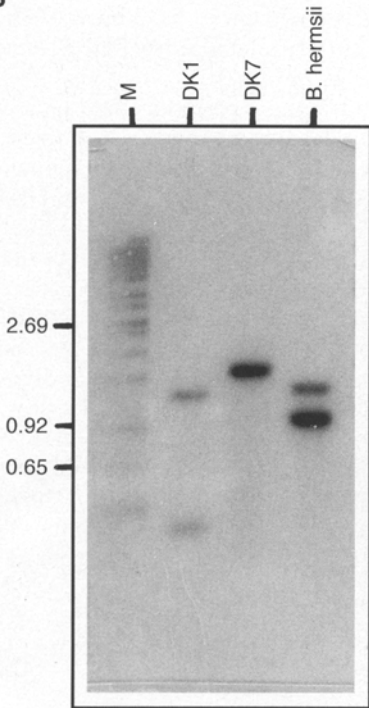
Molecular mass analysis of OspA/B

According to Adam et al. [1], four different groups of *B. burgdorferi* can be identified based on the electrophoretic mobility of the OspA and OspB. All 29 strains were referable to one of these four groups and the molecular mass OspA/B phe-
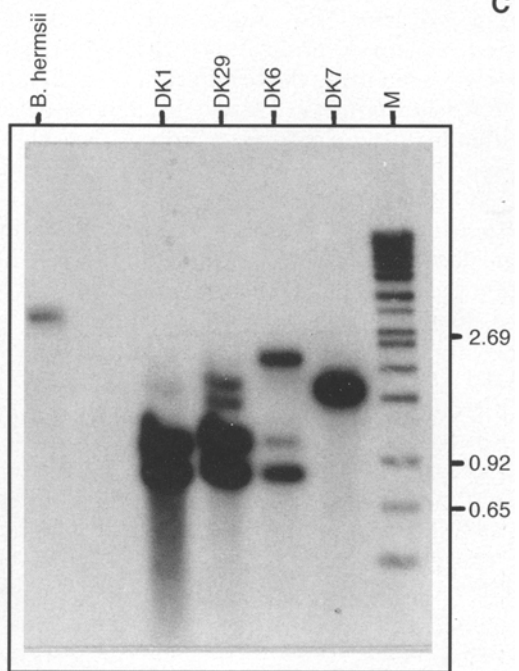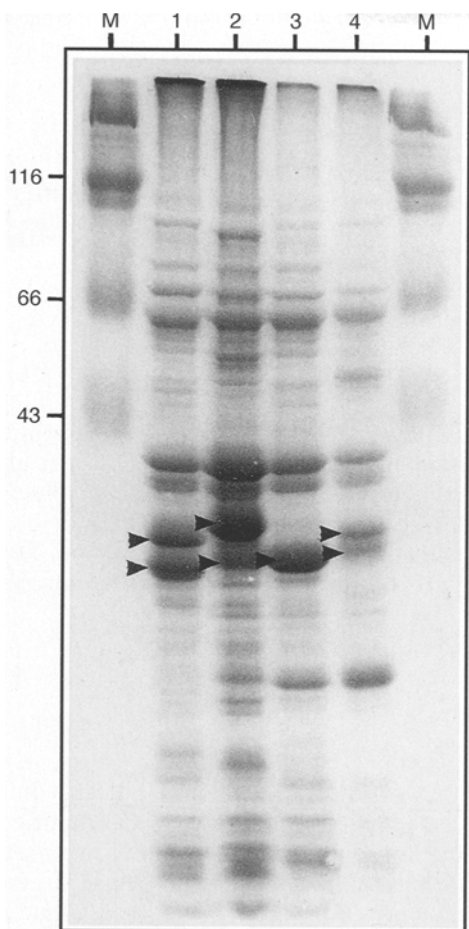
A
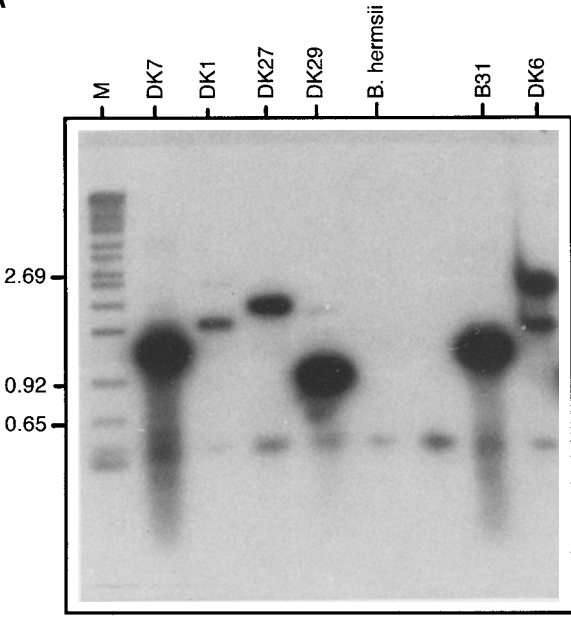
B

C

Fig. 3. SDS-PAGE of whole cell protein from four different *B. burgdorferi* sensu lato isolates. Polyacrylamide gel (12%) stained with Coomasie blue. *Lane 1, B. burgdorferi* sensu stricto (strain B31); *lane 2, B. afzelii* (strain ACA-1); *lane 3, B. garinii* (strain SL10); and *lane 4, B. garinii* (strain DK6). *Lane M*, molecular size marker in kDA (cat. No. 161-0303, Bio-Rad Laboratories, Richmond, Calif.)

Fig. 2A–C. Restriction fragment length polymorphism (RFLP) analysis of *Borrelia* isolates. Whole cell DNA derived from the indicated *Borrelia* isolates (designated above the lanes) was digested with *Hind*III and analyzed by Southern blot. The blots were probed with either (**A**) the *ospA* geneprobe, (**B**) fla geneprobe or (**C**) the *CA* geneprobe. *Lane M*, DNA size markers in kbp (^{35}S-labelled DNA size markers). The band of approximately 0.3 kbp was regarded as an artifact as it was present in all lanes including the ones where no DNA was added. Furthermore the 0.3-kbp band was absent in a repeat of the RFLP experiment. Two different restriction fragment patterns could be identified using the *fla* gene probe: group I[fla] strains (**B**, *lane* DK7) gave rise to a single hybridization band of about 1.5 kbp as predicted from the sequence data from strain B31. Group II[fla] strains were characterized by two fragments of approximately 1.2 and 0.32 kbp (**B**, lane DK1); a restriction fragment profile in agreement with the appearance of an extra *Hind*III site at base position 503. Only a G to T shift at the base postition 507 will provide the construction of the recognition sequence for the enzyme *Hind*III. The remaining RFLPs can be explained likewise

notype classification of all *B. burgdorferi* strains is listed in Table 1. Figure 3 shows representative results.

OspA serotyping

The panel of 29 *B. burgdorferi* strains comprised strains belonging to five different OspA serotypes (serogroup 1, 2, 4, 6 and X) as shown in detail in Tables 1 and 4.

Genomic fingerprinting based on differences in the fla gene sequences

As expected this technique divided the 29 *B. burgdorferi* isolates in three genomic groups. The Fl8 probe defining the B31 group hybridized exclusively DK7 and all 12 US strains. The Fl15 or P/Bi probe hybridized to eight strains, two skin isolates and all European cerebrospinal fluid (CSF) isolates. The third probe, Fl16, hybridized to eight European skin isolates referring these strains to the P/Sto group. The results are summarized in details in Table 1. Southern blots with representative patterns are shown in Fig. 4 A–C.

16S rRNA signature nucleotide analysis

The BB-primer set amplified exlusively DNA from DK7 and the 12 US strains thus comprising the *B. burgdorferi* sensu stricto group. The BG-primer set amplified DNA from eight strains thus comprising the *B. garinii* group. The third primer set, VS461, amplified DNA from eight European skin isolates referring these strains to the *B. afzelii* group. The results are summarized in Table 1 and Table 4 and a representative genospecific 16S rRNA gene amplification is shown in Fig. 5.

Comparison of the ospA, fla and CA gene RFLP classification with four other taxonomic approaches

Table 4 compares 16S rRNA signature nucleotide analysis with RFLP grouping, *fla* sequence fingerprinting, OspA/B molecular mass analysis and OspA serotyping. An almost complete agreement with respect to the obtained classification of *B. burgdorferi* subspecies was found. All strains classified as *B. burgdorferi* sensu stricto could unequivocally be associated with RFLP genogroup I^{OspA}, I^{fla}, IV^{CA}; the *fla* genotype B31; the OspA/B molecular mass pheno-group 1 and OspA serotype 1. Regarding the genospecies *B. afzelii* complete accordance was seen to RFLP genogroup II^{OspA}, I^{CA}; the *fla* genotype P/Sto; OspA/B molecular mass phenogroup 2 and OspA serotype 2. RFLP typing of the *fla* gene did not differentiate between *B. afzelii* and *B. garinii*. Isolates belonging to *B. garinii* were considerably more heterogeneous. Based on RFLP of *CA* and *ospA*, OspA/B molecular mass analysis and OspA serotyping *B. garinii* fell into two or three groups.

Table 4. Comparison of five typing schemes to classify *B. burgdorferi* isolates

Typing schemes

16S rRNA genotyping	Flagellin genotyping	RFLP ospA	fla	CA	Mol. mass profile OspA/B	OspA serotyping
Bb sensu stricto US strains DK7	B31 US strains DK7	I US strains DK7	I US strains DK7	IV US strains DK7	1 US strains DK7	1 US strains DK7
B. afzelii DK1–DK5 P/Ko, P/Tr ACA-1	P/Sto DK1–DK5 P/Ko, P/Tr ACA-1	II DK1–DK5 P/Ko, P/Tr ACA-1	II DK1–DK5 P/Ko, P/Tr ACA-1	I DK1–DK5 P/Ko, P/Tr ACA-1	2 DK1–DK5 P/Ko ACA-1	2 DK1–DK5 P/Ko, P/Tr ACA-1
B. garinii SL10, SL14 SL20, SL42 DK29	P/Bi SL10, SL14 SL20, SL42 DK29	III SL10, SL14 SL20, SL42 DK29	SL10, SL14 SL20, SL42 DK29	II SL10, SL14 SL20, SL42 DK29	3 SL10, SL14 SL20, SL42 DK29	6 SL10, SL14 SL20, SL42 DK29
DK27	DK27	IV DK27	DK27	DK27	DK27	X DK27
DK6, P/Bi	DK6, P/Bi	V DK6, P/Bi	DK6, P/Bi	III DK6, P/Bi	4 DK6, P/Bi	4 DK6, P/Bi

US strains are 297, 272, 245, TXGW, MIJ, ROB, MUL, BUR, KIPP, DUNKIRK, LIPITZ, and B31

Bb, *B. burgdorferi*
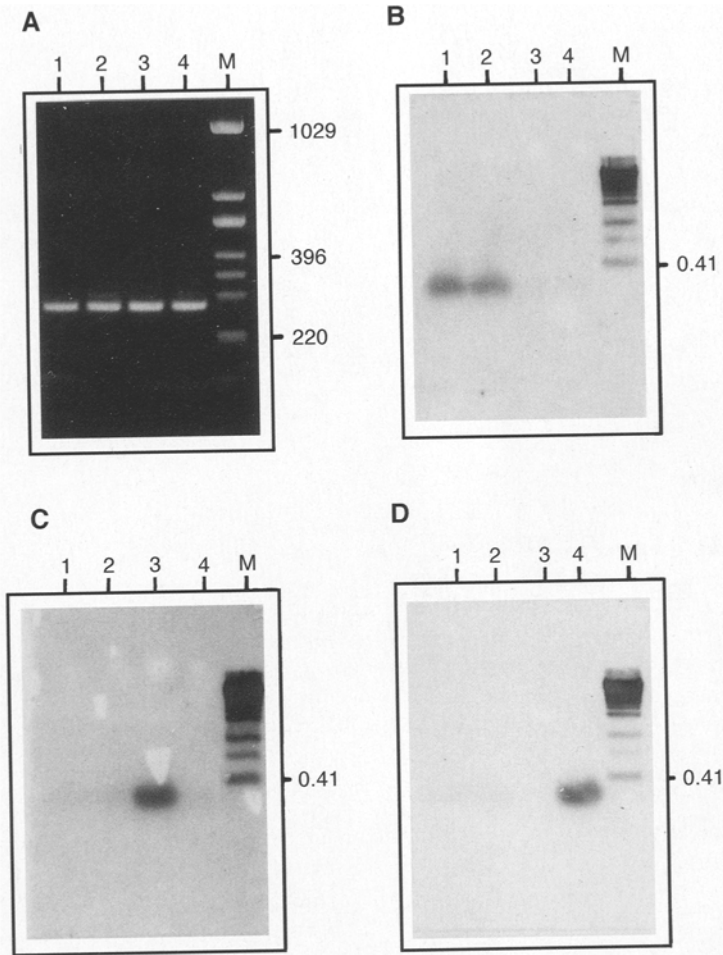
Fig. 4. A PCR amplification with 1 ng of template DNA from *B. burgdorferi* sensu stricto (strain B31; *lane 1*); *B. burgdorferi* sensu stricto (strain DK7; *lane 2*); *B. garinii* (strain DK6; *lane 3*); and *B. afzelii* (strain DK1; *lane 1*) demonstrated by agarose gel electrophoresis after ethidium bromide staining. *Lane M*, DNA size marker in bp (pBR322 digested with *Hind*III-*Hinf*I). **B** Southern blot of the gel shown in **A** hybridized with the oligonucleotide probe Fl8 whose sequence was derived from *B. burgdorferi* strain B31. **C** Southern blot of the gel shown in **A** hybridized with the oligonulceotide probe Fl15 whose sequence was derived from *B. burgdorferi* strain P/Bi. **D** Southern blot of the gel shown in **A** hybridized with the oligonulceotide probe Fl16 whose sequence was derived from *B. burgdorferi* strain P/Sto. *Lane M*; DNA size marker in kbp (^{35}S-labelled DNA size marker, Amersham)

Discussion

We studied RFLP for taxonomic classification of *B. burgdorferi* using hybridization probes complementary to the *ospA* gene and to two chromosomal genes encoding the *fla* and *CA*, respectively. The *fla* and *CA* genes were chosen because they are phylogenetically highly conserved and, thus, should be suitable to uncover
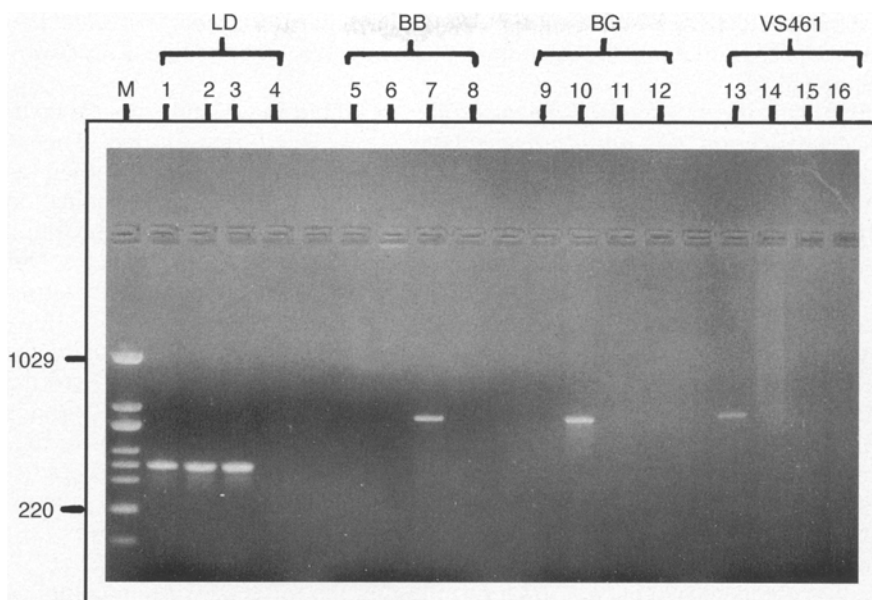
Fig. 5. 16S rRNA nucleotide analysis. PCR amplification with 10 ng of purified DNA from *B. afzelii* (strain DK1; *lanes 1, 5, 9, 13*); *B. garinii* (strain DK6; *lanes 2, 6, 10, 14*) and *B. burgdorferi* sensu stricto (strain DK7; *lanes 3, 7, 11, 15*) using primer set LD (*lanes 1–4*); primer set BB (*lanes 5–8*); primer set BG (*lanes 9–12*); primer set VS461 (*lanes 13–16*). Negative control (*lanes 4, 8, 12, 16*). *Lane M*, DNA size marker in bp (pBR322 digested with *Hind*III-*Hinf*I)

a limited but significant heterogeneity within the species of *B. burgdorferi*. While this study was performed, several other attempts have been made to classify *B. burgdorferi* isolates on the basis of phenotypic characteristics [1, 4, 23, 33, 34] and of genotypic traits [1, 4, 9, 19, 20, 25, 31, 35]. All these studies confirmed a significant heterogeneity within the species *B. burgdorferi*, resulting in the denomination of two to seven different groups. Our study was, thus, extended to compare five different proposals of taxonomic classification using a large panel of human *B. burgdorferi* isolates. Regarding RFLP of the *ospA*, *fla* and *CA* genes all 13 strains classified as *B. burgdorferi* sensu stricto could without exception be associated with one single group regardless of what typing scheme was used. RFLP results on *B. afzelii* showed an almost complete correlation with the different methods; only RFLP typing with the *fla* gene probe was unable to differentiate between *B. afzelii* and *B. garinii*. *B. garinii* isolates, however, revealed a greater extent of heterogeneity, as RFLP of the *ospA* and *CA* gene resulted in two to three subgroups. A similar heterogeneity was obtained using phenotypic markers. Since the composition of the *B. garinii* subgroups was rather uniform regardless of the method used, this might indicate that the subspecies *B. garinii* may need to be further subdivided. In accordance with this finding is: (i) that Baranton et al. [4] observed a more pronounced heterogeneity in rRNA gene RFLP of *B. garinii*; and (ii) that Wilske et al. [34] found *B. garinii* to comprise OspA serotypes 3–7. Our data corroborate the concept of Baranton et al. [4] – since different approaches using different genes and even phenotypic characteristics highly correlated yielding an iden-

tical subclassification. Besides supporting the idea that *B. burgdorferi* sensu lato strains consists of three distinct genomic groups, it also supports that *B. burgdorferi* is clonal.

RFLP using the *ospA* or the *CA* gene probe is a simple and reliable tool for taxonomic classification of *B. burgdorferi* isolates. Based on our findings RFLP needs only to be performed on one gene, e.g., the *CA* gene. In addition, this method (as OspA serotyping) provides data on the subspecies of *B. garinii*. Further indication for differences due to strain selection was provided by Wilske et al. [34], who found seven strains with significantly different *ospA* sequences among 136 isolates. Our data corroborate recent results of Wallich et al. [30], who also evaluated restriction fragment patterns of *Hind*III-digested genomic *B. burgdorferi* DNA following hybridization with the *fla*, the *CA* and the *ospA* gene. They found: (i) using the *fla* gene, two genogroups A and B consistent with our group I^{fla} and II^{fla}; (ii) using the *CA* gene, three restriction fragment profiles identical to our genogroups I^{CA}, III^{CA} and IV^{CA} and (iii) using the *ospA* gene, six different genogroups, four of which exhibited patterns identical to our genogroups. The minor differences between our and the results of Wallich et al. [30] could be due to a different composition of the *B. burgdorferi* strain panel tested. They included 36 tick/reservoir host isolates and 17 clinical isolates.

Results obtained by genomic fingerprinting on the *fla* gene were in complete agreement with ribotyping. Compared to RFLP, genomic fingerprinting on the *fla* gene is less laborious, but the stringency of the washing of the Southern blot's has to be very carefully optimized to avoid cross hybridization between the three genogroups. Furthermore, genomic fingerprinting on the *fla* gene did not allow subdivision of *B. garinii* strains.

We expected that nucleotide sequence analysis on the 16S rRNA gene by PCR performed according to Marconi and Garon [19] to be the easiest and most suitable typing method. However, the technique required very precise optimization of the annealing temperatures of the different primers. To avoid cross amplification the annealing temperatures had to be very close to the Tm of the primers, which often revealed in complete failure of amplification.

In agreement with previous reports [4, 25, 30, 31, 34, 35] all US *B. burgdorferi* isolates belonged to *B. burgdorferi* sensu stricto. European *B. burgdorferi* isolates, however, comprised all three genospecies. In agreement with Wilske et al. [34] the number of the European human *B. burgdorferi* strains classified as sensu stricto was low, only 1 out of 17. It is, furthermore, remarkable that the only European sensu stricto strain *B. burgdorferi* DK7 was isolated from a patient with acrodermatitis chronica atrophicans (ACA) – a skin manifestation which is very rare in the US. The question why US isolates so far only comprises *B. burgdorferi* sensu stricto remains unsolved. An explanation proposed was that *B. burgdorferi* could have been introduced into the American continent relatively late, thus leaving a limited time for genetic diversity to develop. Another explanation could be that *B. afzelii* and *B. garinii* cannot efficiently use the predominate US tick *Ixodes scapularis* as a vector or that the US mammalian reservoir hosts do not support the propagation of *B. garinii* and *B. afzelii* suffficiently [31].

It has been considered whether there is a strain-dependent difference in organotropism of *B. burgdorferi*. Among the European clinical *B. burgdorferi* isolates in this study, there was a correlation between the *B. burgdorferi* genotype and the type of clinical manifestation, either dermatoborreliosis or neuroborreliosis, present in the patient who gave rise to the strain. *B. afzelli* was predominant among

isolates from patients with dermatoborreliosis (73%; 8 out of 11). The remaining skin isolates belonged either to *B. garinii* ($n = 2$, both EM) or to sensu stricto ($n = 1$, ACA). This observation is in accordance with two recent studies [29, 34] finding that 79% of 62 and 98% of 58 *B. burgdorferi* isolates from EM and ACA lesions, respectively, belonged to *B. afzelii*. All 6 European CSF isolates tested in our study belonged to *B. garinii*. In accordance with this observation, van Dam et al. [29] found that all seven strains from either CSF or skin from patients with neuroborreliosis were *B. garinii*. It is, therefore, tempting to suggest that primarily *B. garinii* strains have the pathogenic potential to cause neuroborreliosis. If so, this could explain why only some EM patients (5–10%), if untreated, develop neuroborreliosis, while the remaining recover spontaneously. However, Wilske et al. [34] reported that only 9 out of 16 European CSF isolates were *B. garinii*, 2 CSF isolates were *B. afzelii* and the remaining ones *B. burgdorferi* sensu stricto.

To elucidate whether *B. burgdorferi* subspecies show a specific organotropism further studies should concentrate on human *B. burgdorferi* isolates, because only such strains have proven to be pathogenic. This recommendation is further emphasized as the genotype composition among tick isolates is significantly different to that seen in strains isolated from patients with Lyme borreliosis [30, 34]. Wilske et al. [34] reported *B. afzelii* to comprise only 6% of tick isolates versus 79% of human skin isolates. To solve the question about strain-dependent organotropism larger panels of human *B. burgdorferi* isolates especially from CSF need to be typed. The demonstration of a specific organotropism of *B. burgdorferi* subspecies will have important implications for the understanding of the pathogenesis of Lyme borreliosis.

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