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Phenotypic and Genotypic Analysis of *Borrelia burgdorferi* Isolates from Various Sources

THOMAS ADAM, GABRIELE S. GASSMANN, CHRISTIANE RASIAH, AND ULF B. GÖBEL*

Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Freiburg, Federal Republic of Germany

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A total of 17 *B. burgdorferi* isolates from various sources were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins, restriction enzyme analysis, Southern hybridization with probes complementary to unique regions of evolutionarily conserved genes (16S rRNA and *fla*), and direct sequencing of in vitro polymerase chain reaction-amplified fragments of the 16S rRNA gene. Three groups were distinguished on the basis of phenotypic and genotypic traits, the latter traced to the nucleotide sequence level.

The spirochete *Borrelia burgdorferi* has been isolated from a variety of ticks, animals, and patients suffering from a multistage infection named Lyme disease or Lyme borreliosis, considered now the most prevalent tick-borne disease in Europe, northern America, and other parts of the world with a moderate climate (2, 15, 38, 52, 54, 55). There are several clinical manifestations of Lyme borreliosis, depending upon the stage of the infection. Localized skin lesions, erythema chronicum migrans, develop first around the site of the tick bite. Subsequent dissemination of bacteria from the primary site of infection results in a variety of symptoms, e.g., migratory pain in joints and muscles, headache, polyneuropathy, or atrioventricular nodal block. Late infection is characterized by chronic arthritis (Lyme arthritis), progressive encephalomyelitis, or acrodermatitis chronica atrophicans. Whereas some of the signs, such as acrodermatitis chronica atrophicans, were seen primarily in Europe, others, such as arthritis, were diagnosed more frequently in American patients (54). It has been speculated that genetic and phenotypic differences between European and American isolates may account for the multifaceted clinical appearance of *B. burgdorferi* infections in different geographic regions (4, 7, 8, 32, 34, 35, 53, 58, 60).

This diversity among *B. burgdorferi* isolates was assessed by a variety of methods, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, electron microscopy, plasmid analysis, restriction endonuclease analysis, and DNA hybridization (3, 7–9, 24, 27, 31, 34–36, 53, 58–60). Some of the differences were found to be laboratory artifacts, as plasmids and major proteins have been lost after prolonged cultivation (32, 44–46). Despite the marked variability among isolates from different sources, *B. burgdorferi* is still considered a homogenous species, and no subspecies have been defined so far (31, 43). This inconsistency prompted us to reassess the intraspecies heterogeneity.

The aim of this study was to compare several approaches which in future may lead to a more reliable classification of clinical or vector isolates and laboratory strains, a prerequisite for associating particular genotypes or phenotypes with certain clinical manifestations. Seventeen *B. burgdorferi* strains, 1 American and 16 European isolates from both ticks and diseased patients, were classified by SDS-PAGE, restriction enzyme analysis, genetic fingerprinting with oligonucleotide probes complementary to defined regions of unlinked, phylogenetically conserved genes (16S rRNA and flagellin genes) both located on the chromosome, and direct sequencing of in vitro-amplified 16S rRNA gene fragments (10, 12, 13, 18, 21, 22, 39a, 41). We were able to define three distinct groups, characterized by similar protein patterns, identical genomic fingerprints, and defined nucleotide substitutions within their respective 16S rRNA genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains were kept for more than 25 passages before inclusion in this study. Strain designations and sources are listed in Table 1. The bacteria were cultivated to late logarithmic growth in plastic tissue culture flasks (Costar) at 33°C in 50 ml of a modified Kelly's medium supplemented with 2% (vol/vol) bovine serum albumin (35% solution; Sigma) and 5% (vol/vol) heat-inactivated rabbit serum (GIBCO/BRL) (4). Bacteria were harvested by centrifugation at 5,000 × *g* for 30 min at room temperature.

Protein analysis. Pellets were washed three times in phosphate-buffered saline containing proteinase inhibitors (5 mM EDTA, 5 mM sodium tetrathionate, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine). The final pellet was resuspended in 1 ml of the same buffer, ultrasonicated (Branson sonifier) for 10 min in an ice bath, and mixed with sample buffer. Aliquots (12 μl, containing about 3 μg of protein) were separated by one-dimensional SDS-PAGE (12.6% acrylamide) as described previously (33).

DNA preparation and restriction endonuclease digestion. Bacteria were grown for 3 to 4 days as described above. Cultures (50 ml) were harvested by centrifugation, and the pellets were washed three times with phosphate-buffered saline. Final pellets were resuspended in 240 μl of TES buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA). After addition of 60 μl of lysozyme (5 mg/ml), the mixture was incubated for 15 min at room temperature. Subsequently, 300 μl of 1% (vol/vol) sodium deoxycholate in TES buffer and 10 μl of proteinase K (10 mg/ml) were added and incubated for another 50 min at 37°C. The mixture was then extracted twice with phenol-chloroform and then with two cycles of chloroform. The DNA was precipitated overnight

* Corresponding author.

TABLE 1. *B. burgdorferi* strains

Isolate	Source[a]	Location[b]
B31 (ATCC 35210)	Tick	New York, New York
Z25	Tick	Freiburg, F.R.G.
Z37	Tick	Freiburg, F.R.G.
Z118	Tick	Freiburg, F.R.G.
Z136	Tick	Freiburg, F.R.G.
Z160	Tick	Freiburg, F.R.G.
ZS7	Tick	Freiburg, F.R.G.
ZQ1	Tick	Freiburg, F.R.G.
A	Tick	Cologne, F.R.G.
GeHo	Skin (ECM)	Freiburg, F.R.G.
Bo23	Skin (ECM)	Freiburg, F.R.G.
PKo	Skin (ECM)	Munich, F.R.G.
ACA1	Skin (ACA)	Stockholm, Sweden
387	CSF (EM)	Würzburg, F.R.G.
IP1	CSF (EM)	Paris, France
IP2	CSF (EM)	Paris, France
IP3	CSF (EM)	Paris, Fance

[a] ECM, erythema chronicum migrans; ACA, acrodermatitis chronica atrophicans; CSF, cerebrospinal fluid; EM, encephalomyelitis.
[b] F.R.G., Federal Republic of Germany.

at −20°C by adding 1/10 volume of 3 M sodium acetate and 2 volumes of ice-cold ethanol. The precipitate was dissolved in 400 μl of TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) buffer, and RNA was removed by a 3-h incubation at 37°C after the addition of 20 μl of RNase A (5 mg/ml). The DNA was extracted twice with equal volumes of phenol and chloroform, precipitated with ethanol, and resuspended in 50 μl of TE buffer.

Oligonucleotide labeling. The probes used were BBU30 (5′-AACTTCCTCTATCAGACTCTAGACATATAG-3′), complementary to region V4 (20) of the *B. burgdorferi* 16S rRNA (18), and BBFN27 (5′-AGCTGATGTATTATGATT GATAATCAT-3′), complementary to the extreme 5′ end of the *B. burgdorferi* flagellin structural gene; the probes were 30 and 27 bases in length, respectively. Both oligonucleotides were synthesized on a Cyclone DNA synthesizer (Milligen/Biosearch) by using β-cyanoethyl phosphoramidite chemistry (49). The oligonucleotide probes were labeled at the 5′ end with T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]dATP (Amersham; 5,000 Ci/mmol) and purified by preparative PAGE essentially as described previously (17, 23).

Southern blot hybridization. DNA was digested with *Hin*dIII (GIBCO/BRL) as recommended by the manufacturer. Resulting DNA fragments were separated on 0.8% agarose gels, stained with ethidium bromide, and transferred to nylon membranes (Biodyne A; Pall) by the method of Southern (51). After being baked (2 h at 80°C), the filters were prehybridized, hybridized, washed, and autoradiographed as described earlier (17, 18, 23). BBU30 was hybridized at medium-stringency (60°C) and high-stringency (69°C) temperatures; BBFN27 was used only at 50°C.

PCR and direct sequencing of amplified DNA fragments. Several sets of amplification primers were used. For sequencing, the 16S rRNA genes (domains I and II) from strains IP3, B31, and PKo were amplified by forward primer TPU1 (5′-AGAGTTTGATC[A,C]TGGCTCAG-3′) and reverse primer RTU6 (5′-ATTGTAGCACGTGTGT[A,C]GC CC-3′). The entire 16S rRNA sequences will be described elsewhere. For diagnostic purposes, region 16SV4 was amplified by using the *B. burgdorferi*-specific primers BBUTP1 (5′-TACCACAGCTCAACTGTGGACCTA-3′) and BBUR-T1 (5′-TCGGTACTAACTTTTAGTTAACA-3′). DNA (1 μg), primers (60 pmol), and deoxynucleotides (dATP, dCTP, dGTP, and dTTP; 200 μM each) were brought to 100 μl with polymerase chain reaction (PCR) buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin). DNA was then amplified with 2.5 U of *Taq* polymerase (Cetus) for 30 cycles with an automatic thermocycler (Hybaid). The first cycle involved DNA denaturation at 93°C for 2 min, primer annealing at 40°C, and primer extension at 72°C for 1 min for each step. During cycles 2 to 4, strand separation was done at 92.5°C for 1 min and all other conditions were kept constant. Cycles 5 to 30 were performed at 92.5°C for 1 min, followed by annealing at 50°C and extension at 72°C for 1 min each. TE buffer was then added (2-ml total volume), and amplified DNA was purified by three repeated cycles of ultrafiltration with Centricon-100 devices (Amicon) to remove amplification primers, *Taq* polymerase, and salts. Amplified fragments were sequenced by a modified dideoxy chain termination procedure with the Sequenase kit (USB) according to the manufacturer's recommendations.

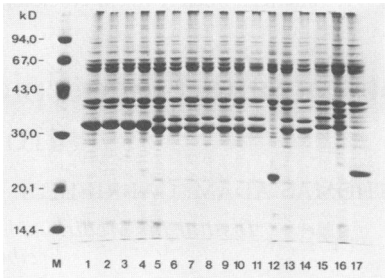

FIG. 1. SDS-PAGE of whole-cell proteins from various European *B. burgdorferi* isolates and the B31 type strain. Polyacrylamide gel (12.6%) stained with Coomassie blue. The sizes of molecular size markers (lane M) are given in kilodaltons. Lanes 1 to 17 represent protein profiles from strains ZQ1, A, 387, IP3, IP2, IP1, GeHo, ZS7, Z25, Z37, Z118, Z136, Z160, B31, Bo23, ACA1, and PKo, respectively.

RESULTS

Protein analysis. All isolates were subjected to phenotype analysis by comparing the protein profiles of *B. burgdorferi* whole-cell lysates. They are characterized by several major peptide bands, the strongest appearing in the range of about 30 to 35 kDa. Despite some minor overall variations, patterns of low-molecular-weight proteins allow a preliminary grouping. Group I strains ZQ1, A, 387, and IP3 (Fig. 1, lanes 1 to 4) show a single prominent band, about 32 kDa in size. A second group (group II; Fig. 1, lanes 5 to 14) comprises strains IP2, IP1, GeHo, ZS7, Z25, Z37, Z118, Z136, Z160, and B31, characterized by two bands of 31 and 34 kDa, representing OspA and OspB proteins, respectively (9, 54). The remaining three isolates, Bo23, ACA1, and PKo (group III; Fig. 1, lanes 15 to 17), are distinguished by two major peptides, about 35 and 32 kDa in size. Two strains, Z136 and PKo (lanes 12 and 17), show only traces of the 31- and 34-kDa or 32- and 35-kDa Osp proteins but have nonetheless been assigned to the respective groups. Both strains exhibit major bands at 21 kDa (Z136) or 22 kDa (PKo), described as cationic surface proteins, named pC (24, 59).

Restriction enzyme analysis and genomic fingerprinting. In

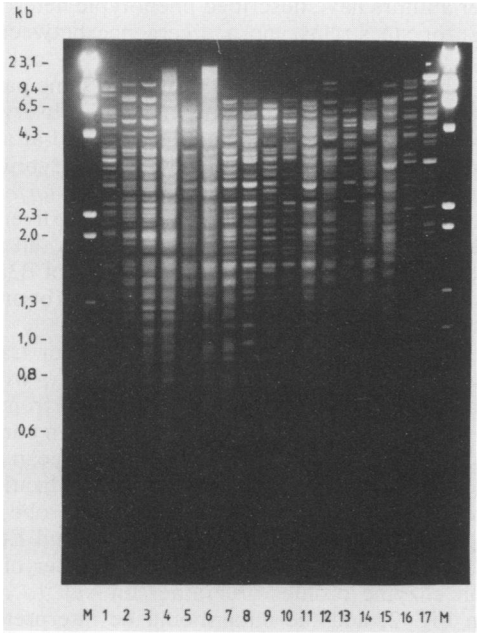

FIG. 2. Restriction enzyme analysis of *Hin*dIII-digested DNA from *B. burgdorferi* isolates (lanes 1 to 17). Strains were applied to the same lanes as in Fig. 1. Ethidium bromide-stained 0.8% agarose gel. The sizes of DNA markers (lanes M) are given in kilobase pairs.

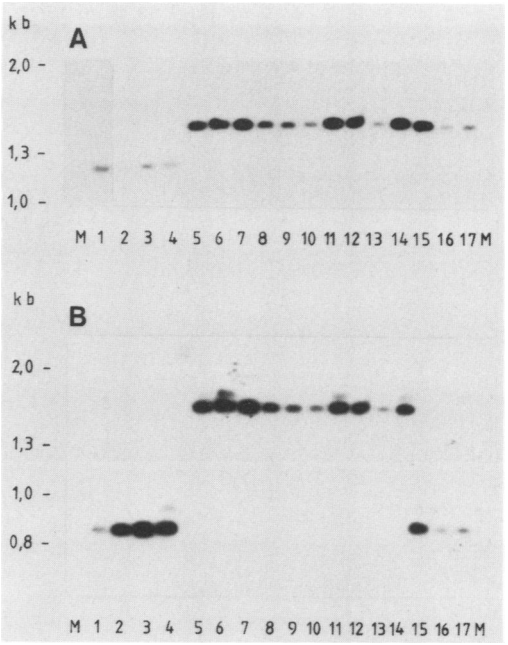

FIG. 3. Genomic fingerprints of *Hin*dIII-digested DNA from the isolates given in the legend to Fig. 1 (lanes 1 to 17) with ^{32}P-labeled oligonucleotide probes BBU30, complementary to region V4 of the 16S rRNA gene (A), and BBFN27, complementary to the 5′ end of the flagellin structural gene (B). The sizes of DNA markers (lanes M) are given in kilobase pairs.

contrast, restriction enzyme patterns of whole *Hin*dIII-digested (Fig. 2) or EcoRI-digested (data not shown) *B. burgdorferi* DNA were difficult to interpret. The many individual bands did not allow identification of particular clusters. We therefore used two well-characterized probes for genomic fingerprinting of the isolates. One probe, BBU30, was complementary to region V4 of the *B. burgdorferi* 16S rRNA (18). The second probe hybridized to the extreme 5′ end of the *fla* gene, encoding the monomeric protein of *B. burgdorferi* endoflagella. We found a distinct restriction fragment length polymorphism (RFLP) for both probes. The 16S rRNA-specific probe identified a small *Hin*dIII fragment of about 1.2 kb in group I strains ZQ1, A, 387, and IP3 (Fig. 3A, lanes 1 to 4). A larger *Hin*dIII fragment, about 1.5 kb in length, was found in DNA digests from the remaining 13 strains (Fig. 3A, lanes 5 to 15). When BBU30 was hybridized under higher stringency (69°C), the small 1.2-kb *Hin*dIII fragments were no longer detectable (data not shown). Similarly, the *fla* gene probe recognized a short 0.9-kb *Hin*dIII fragment in group I isolates (Fig. 3B, lanes 1 to 4) and group III strains Bo23, ACA1, and PKo (Fig. 3B, lanes 15 to 17). A 1.6-kb *Hin*dIII *fla* fragment was found in digested DNA from group II isolates (Fig. 3B, lanes 5 to 14). None of the probes cross-hybridized with DNA from closely related spirochetes, such as *B. hermsii*, *B. parkeri*, and *B. turicatae* (data not shown).

In vitro amplification and direct PCR sequencing. The primary observation that probe BBU30 failed to hybridize under high-stringency conditions to *Hin*dIII-digested DNA from group I strains prompted us to confirm this result by hybridizing the same probe with in vitro-amplified DNA to exclude the possibility that negative hybridization was due to low concentrations of the respective *Hin*dIII fragment in previous Southern hybridization experiments (Fig. 4). Subsequent DNA sequencing of amplified 16S rRNA genes from representative strains of each group, *B. burgdorferi* IP3 (group I), B31 (group II), and PKo (group III), revealed several base substitutions in various regions (data not shown). Signature nucleotides within region V4 which differentiate the three groups are shown in Fig. 5. There was a single base substitution (G to A transition) in 16S V4 from strain IP3 (group I) compared with the sequence of the B31 type strain (group II), situated within the binding region of BBU30. Group III strains are characterized by a point mutation (T to C transition) only a few bases upstream of the BBU30 binding region.

DISCUSSION

Several methods are available to classify *B. burgdorferi* strains, none of which is widely accepted and generally applicable. Phenotypic analyses, such as comparison of protein profiles or antigenic characterization of surface proteins by various monoclonal antibodies, are commonly used. They are prone to error, as major antigenic changes occur during cultivation, partly due to loss of plasmids carrying genes encoding surface proteins, gene rearrangements resulting in altered antigenicity of known peptides (48), or altered gene expression as a consequence of DNA methylation (28), and regulatory processes, such as thermoregulation (19). It was therefore unexpected that all *B. burgdorferi* isolates included in this study could be distinguished by their Osp profiles, representing three distinct clusters. The 49-kb linear plasmid, however, encoding both OspA and OspB proteins was shown to be rather stable during continuous cultivation (5, 44, 46). Heterogeneity of Osp proteins, including appearance and loss of the 21- to 22-kDa pC protein, which may be a member of the Osp family (11, 14, 37), possibly results from insertion of transposable elements or other DNA rearrangements (6, 29, 43).

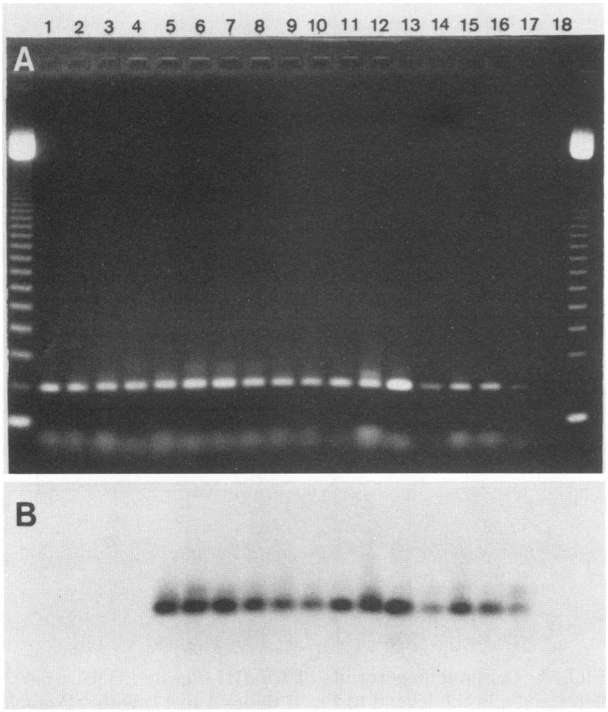

FIG. 4. In vitro (PCR)-amplified V4 of 16S rRNA genes from strains shown in Fig. 1 (lanes 1 to 17). (A) Visualization of the expected 244-bp amplification product on an ethidium bromide-stained 1% agarose gel. (B) Autoradiograph of a nylon filter carrying amplified DNA fragments hybridized to ^{32}P-labeled probe BBU30 under high stringency. Lane 18, negative control. Lanes to the far left and right contain DNA marker molecules (123-bp ladder [Bethesda Research Laboratories]).

The phenotypic stability found in our study may have resulted from the high passage number (>25) of our strains. One might speculate that unstable genetic elements, e.g., the 7.6-kb circular and the 22-kb linear plasmids, carrying genes or regulatory elements possibly involved in phenotypic variation or infectivity were lost during early cultivation (5, 43). Other authors have described phenotypic heterogeneity among European strains and a divergence between European and American isolates (7, 58, 60). In our hands, however, the American isolate B31 had the same pattern as most of the European strains (group II). This observation is supported by data from genetic analyses aimed at avoiding the pitfalls of phenotypic characterization. Hybridization experiments in which cloned genomic *B. burgdorferi* DNA fragments were used to analyze RFLPs among five strains, two American, including B31, and three Swiss, all isolated from ticks, revealed a rather close relationship of B31 to one of the Swiss isolates (53). These data were corroborated by analyses of linear and circular plasmids.

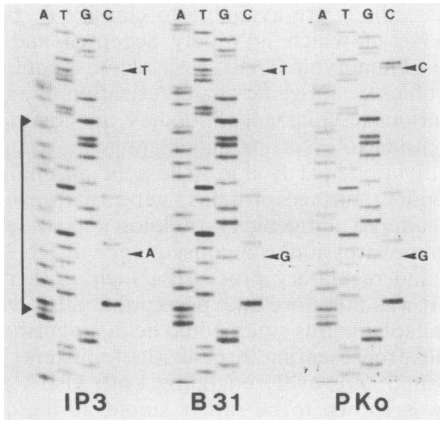

FIG. 5. Direct sequencing of PCR-amplified 16S rRNA gene V4 rDNA fragments from *B. burgdorferi* strains, IP3, B31, and PKo. Autoradiograph of a representative sequencing gel. Signature nucleotides are indicated by arrowheads. The sequence complementary to BBU30 is marked by a vertical bar at the left margin.

Hybridization studies with entire plasmids or fragments thereof as probes revealed genetic relatedness, which appeared to be more pronounced among plasmids from strains clustering within one group (5, 29, 53). This is in contrast to a study comparing North American and European isolates by restriction endonuclease analysis and hybridization with whole genomic DNA (strain B31) as the probe, which showed considerable differences among B31 and European isolates (36). However, due to the large number of bands, restriction enzyme profiles are rather difficult to read, as shown in Fig. 2, and the data should be interpreted with caution. In addition, RFLP and plasmid analyses with whole genomic or uncharacterized, randomly cloned fragments derived from either genomic or plasmid DNA are hampered by a number of problems associated with both methods. Plasmid analysis is impeded by the frequent loss of such extrachromosomal elements during cultivation (5, 43, 45). Estimation of sequence divergence by comparing restriction endonuclease-digested DNA fragments is only valid if DNA rearrangements or repeats are ruled out. Similarly, assessment of genetic distance by RFLP analysis with uncharacterized probes can be misleading, as little is known about genetic recombination in borreliae, with the exception of *B. hermsii*, for which tandem insertion sequence-like elements have been described (6). In addition, recent evidence suggests that localized recombination by horizontal transfer of chromosomal genes between related species seems to occur more frequently than anticipated and may thus play a significant role (50).

Comparison by pulsed-field gel electrophoresis of larger DNA fragments, spanning broader regions of the bacterial chromosome, generated by rare cutting restriction enzymes may be more reliable (57). The same is true for multilocus enzyme electrophoresis, a method assessing the variation in the amino acid sequences of metabolic enzymes by virtue of their different electrophoretic patterns. It has been shown that the allelic variation of enzyme loci correlated significantly with results from DNA-DNA reassociation studies (47).

Alternatively, comparison of rRNA gene restriction patterns (ribotyping) with a variety of probes complementary to an entire rRNA or parts thereof has been shown to be a powerful tool in molecular epidemiology and taxonomy (23, 25, 38, 56). As most bacteria possess several rRNA genes, which vary in number and location on the chromosome, probes recognize multiple restriction fragments. According to Grimont and Grimont, "Different patterns within the same species could be correlated with significant divergence in DNA/DNA hybridization" (25). This technique, together with DNA-DNA hybridization, revealed two genomic species in *B. burgdorferi*, characterized by the strains B31 (*B. burgdorferi* sensu strictu) and 20047 (*Borrelia* group 20047) (40). Hybridization with short oligonucleotide probes com-

TABLE 2. Phenotypic and genotypic characteristics of *B. burgdorferi* strain clusters

Group	Characteristic[a] protein(s) (kDa)	Size (kb) of *Hin*dIII fragment[b] carrying:		Amplification[c]	
		fla	*rrn*	Hybridization	Sequencing
I	32	0.9	1.2	Negative	A, T
II	34, 31	1.6	1.5	Positive	G, T
III	35, 32	0.9	1.5	Positive	G, C

[a] Sizes of characteristic low-molecular-mass proteins.
[b] Sizes of *Hin*dIII fragments carrying parts of the flagellin (*fla*) or 16S rRNA (*rrn*) gene.
[c] Results from high-stringency hybridization with BBU30 as the probe and direct PCR sequencing of 16S rRNA region V4. Signature nucleotides (A, T, C, and G) are indicated.

plementary to distinct regions within the 16S rRNA gene result in rather simple patterns, as divergence depends upon variation within the structural gene or adjacent flanking regions. In bacteria with only one or two rRNA operons, single bands are not uncommon (Fig. 3) (16, 23). Intraspecies rRNA sequence heterogeneity, however, does not occur frequently and was found to be stable within clusters of different isolates of a given species. This has been shown convincingly by comparative sequence analysis of 16S rRNA genes from members of the genus *Mycobacterium*, including BCG strains, which have been passaged for almost a century, where species and even serotypes could be identified by a few stable signature nucleotide differences (12, 13, 41).

Heterogeneity among *B. burgdorferi* 16S rRNA sequences was first identified by high-stringency hybridization with the probe BBU30. Subsequent sequence analysis of PCR-amplified 16S rRNA gene fragments revealed a single base change within the region complementary to BBU30, underlining the specificity of DNA hybridization. This G to A transition results in a highly unstable C-A mismatch disrupting the heteroduplex between BBU30 and its target DNA in group I strains (30). To test whether our grouping of *B. burgdorferi* isolates based on the microheterogeneity of rRNA genes, assessed by either hybridization or comparative PCR sequencing of rRNA signature sequences, was meaningful, we used another oligonucleotide probe (BBFN27), complementary to the structural gene coding for the phylogenetically conserved Fla protein, showing a high degree of functional constancy (21). Definition of similar groups (Table 2) by RFLP analysis of this gene, unrelated to rRNA genes by both function and chromosomal localization (39a), suggests the phylogenetic relevance of our data. In addition, the effectiveness of this approach has been shown for several *Mycoplasma gallisepticum* strains, hybridized in parallel with probes specific for the genes for elongation factor (EF-Tu) and rRNA, where both probes defined identical clusters (61).

An even stronger indication that analysis of unrelated but essential genes yielded similar phylogenetic trees was given by comparative analysis of 16S rRNA nucleotide and ATP-synthase amino acid sequences (1). A recent article, published after submission of our manuscript, identified two distinct classes of *B. burgdorferi* by PCR analysis of a hitherto uncharacterized gene of unknown function (42). Most interestingly, those of our strains which were included in the studies mentioned above belong to the genomic species *B. burgdorferi* sensu strictu (40). Our data suggest that there are further subtypes within this genospecies. Although the selection of strains included in this study is by no means statistically representative, we are confident that the classification of *B. burgdorferi* isolates, based primarily on the molecular analysis of phylogenetically relevant genes, particularly rapid direct sequencing of in vitro-amplified 16S rRNA genes, might contribute to direct classification of new strains isolated from ticks or humans.

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