Patient Isolates of *Borrelia burgdorferi* Sensu Lato with Genotypic and Phenotypic Similarities to Strain 25015

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Strain 25015 is an atypical tick isolate that belongs to a distinct genomic group (DN127) within the general taxon *Borrelia burgdorferi* sensu lato. Similarities between this strain and a white-footed mouse isolate from Illinois, strain CT39, have been reported. In the course of isolating *B. burgdorferi* sensu lato in culture from Slovenian patients, 9 isolates were identified with the same genetic profiles as strains 25015 and CT39, as evidenced by restriction enzyme *MluI* digestion patterns of genomic DNA. The aim of the present study was to molecularly characterize all 11 isolates to examine the extent of their genotypic and phenotypic similarity. The results of molecular studies suggest a close relationship between the patient isolates and strains 25015 and CT39. However, CT39 and several patient isolates possessed unique characteristics that reflect their discrete ontogeny.

Lyme borreliosis is a multisystem infection caused by tick-transmitted spirochetes of the genus *Borrelia* [1]. In Europe, at least 3 species are associated with the disease: *B. burgdorferi* sensu stricto, *B. garinii,* and *B. afzelii* [2, 3]. In the United States, only *B. burgdorferi* sensu stricto has thus far been detected among patient isolates, although spirochetes belonging to other genomic groups (DN127, *B. andersonii*) have been isolated from ticks [4–6]. The term *B. burgdorferi* sensu lato is used to describe Lyme borreliosis patient and ixodid tick isolates of *Borrelia* that have not been identified to the species level [7].

Strain 25015 was cultured from an *Ixodes scapularis* tick from the northeastern United States and was originally considered an atypical isolate of *B. burgdorferi* sensu stricto [8]. However, it was recently shown to share a 68% level of sequence identity with the type-strain of the latter species and was found to be a member of genomic group DN127, a group that thus far contains only tick isolates [5]. We recently described a Midwestern mouse *(Peromyscus leucopus)* isolate (CT39) with many phenotypic and genotypic similarities to strain 25015 [9]. During culture isolation of *B. burgdorferi* sensu lato for the diagnosis of Lyme borreliosis in Slovenia, we encountered 9 patient isolates with the same genetic profile as strains 25015 and CT39, as evidenced by pulsed-field gel electrophoresis (PFGE) of their chromosomal restriction enzyme fragments. In this study, we did extensive molecular characterization of all 11 strains for comparison of their phenotypic and genotypic properties.

Methods

Patient isolates and reference strains. Processing of skin biopsy and cerebrospinal fluid (CSF) specimens and the culture isolation of spirochetes have been described [10]. Culture tubes containing the original patient sample were used to prepare passage 0 (P_0) frozen stocks. Three patient isolates were derived from extant erythema migrans (EM) lesions (SL-70, -73, -76), 2 from normal-appearing skin at the site of resolved EM lesions (SL-74, -75), 1 from lymphocytoma (SL-78), and 3 from CSF (SL-79, -80, -90). Strain 25015 was isolated from an *I. scapularis* larva obtained from the white-footed mouse, *P. leucopus,* in Millbrook, New York, in 1987 [8]. Strain CT39 was obtained from an ear-punch biopsy of a white-footed mouse captured in northeast Illinois in 1990 [9]. Reference strains of *B. burgdorferi* sensu stricto, *B. garinii,* and *B. afzelii* included for comparison in molecular characterization studies have been described [11].

Molecular characterization of strains. PFGE separation of restriction enzyme *MluI*–digested genomic DNA and determination of large restriction fragment patterns (LRFPs) were done as described previously [9, 10, 12, 13]. Nomenclature follows the system devised by Belfaiza et al. [14]. Since group DN127 strains lack a species designation, we refer to the LRFP associated with strains 25015 and CT39 as MLx [9].

16S rRNA-specific polymerase chain reaction (PCR), plasmid profiling by PFGE, and protein profiling by SDS-PAGE were done as described [9, 10, 12, 13]. rDNA gene restriction patterns for 5S, 16S, and 23S rRNA genes were determined as described by Liveris et al. [15] and by Baranton et al. [2]. PCR amplification of the *rrf* (5S)-*rrl* (23S) intergenic spacer region was done as described by Postic et al. [5].

Growth rate determinations. P_0 frozen stocks of patient isolates and frozen stocks of strains 25015 (number of passages unknown) and CT39 (number of passages <5) were used to inoculate 5-mL cultures of BSK II medium. These were incubated at 33°C

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for 2 days, and the spirochetal cell density of each culture was determined by darkfield microscopy using a Petroff-Hauser counting chamber. Using these counts, fresh 5-mL cultures were started with an initial spirochete concentration of 1.5×10^5 cells/mL. All cultures used the same batch of BSK II medium, the same type of culture tube, and the same total volume of medium. Cultures were incubated at 33°C, and cell numbers were counted at 3-day intervals in a Petroff-Hauser counting chamber.

Results

Genospecies typing and LRFP analysis. In the course of screening Slovenian patient isolates of *B. burgdorferi* sensu lato, 9 isolates were encountered that typed as *B. burgdorferi* sensu stricto by 16S rRNA-specific PCR but possessed an atypical LRFP. The same properties were previously found to characterize North American isolates 25015 and CT39, and the term MLx was devised to refer to their distinctive LRFP [9]. After *Mlu*I digestion of total genomic DNA and separation by PFGE, all 11 strains had three fragments of 440, 300, and 100 kb (data not shown). However, strain SL-80 possessed an extra fragment in its LRFP, intermediate in size between the 440- and 300-kb fragments. Repeated digestion of agarose blocks from this strain with increasing concentrations of *Mlu*I failed to eliminate this band, indicating that it was not a partial digest product. Thus, strain SL-80 has a unique LRFP that appears to be a variant of the MLx type.

Plasmid profiling by PFGE. The complete genomic complement (chromosome and plasmids) of strains 25015 and CT39, and the 9 patient isolates were separated by PFGE (data not shown). All 11 possessed a large (presumably linear) plasmid band that comigrated with the 49-kb linear plasmid of strain B31. With regard to smaller plasmids, strains 25015 and CT39 possessed different plasmid patterns. With the exception of SL-79, all of the patient isolates possessed a complement of four smaller plasmids that comigrated with those found in strain 25015 [9]. Strain SL-79, however, lacked the largest plasmid of this four-plasmid group.

Protein profiling by SDS-PAGE. Although the level of expression of some proteins varied among the isolates, all 11 possessed closely similar protein profiles (data not shown). All had proteins of 41, 35.5, 32.5, and 22 kDa. Strain 25015 was previously shown to possess higher molecular mass outer surface proteins A and B (35 and 32 kDa) [8]. However, the identity of proteins was not determined using monoclonal antibodies. All 9 patient isolates expressed prominent 22-kDa proteins. Strains SL-70, -74, and -79 had lower levels of 35.5- and 32.5-kDa protein expression.

Growth rate of strains. Figure 1 shows growth rates for strains 25015 and CT39 and the 9 patient isolates. All patient isolates grew more slowly and, with the exception of SL-74, to a lower final cell density than strains 25015 or CT39. The patient isolates were divisible into three groups on the basis of their growth rates: fast-growing (SL-70, -74, and -90), intermediate growing (SL-73, -75, -76, -78, and -79), and 1 extremely slow-growing strain (SL-80).

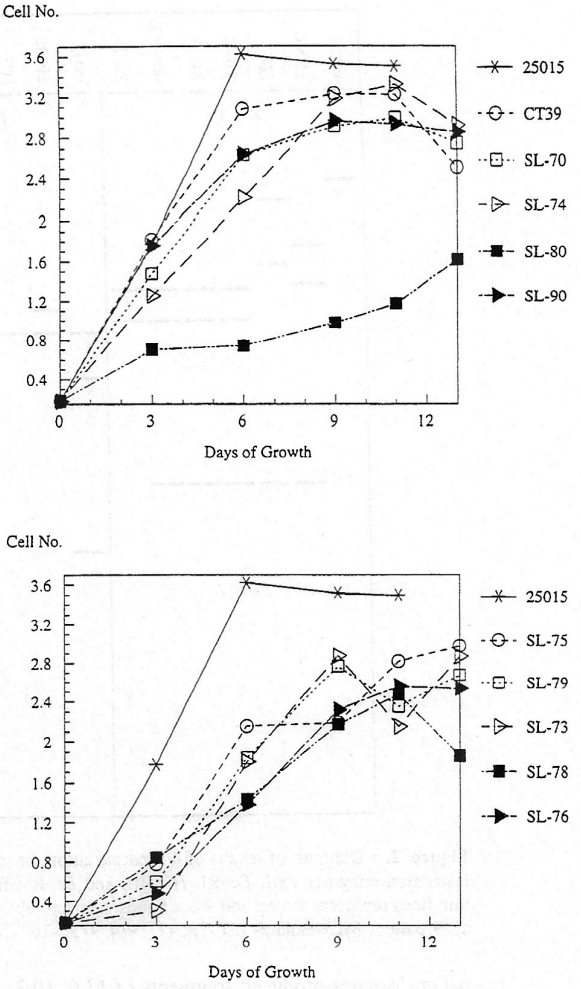

Figure 1. Comparison of growth rates of *B. burgdorferi* 25015 and CT39 and 9 Slovenian (SL) patient MLx isolates.

Ribotyping. rRNA gene restriction patterns resulting from digestion with enzymes *Pst*I, *Eco*RI, *Hin*dIII, and *Eco*RV and probing with 16+23S rRNA probes are shown in figure 2. Strains 25015 and CT39 and the 9 patient isolates could not be distinguished from *B. burgdorferi* sensu stricto B31 on the basis of digestion with *Eco*RV or, with the exception of SL-78, with *Eco*RI. After *Eco*RV digestion, all 11 MLx isolates and strain B31 produced fragments of 6.7 and 3.2 kb. After digestion with *Eco*RI, strains B31, 25015, and CT39, and 8 of the 9 patient isolates produced a single 9.5-kb fragment. SL-78 was unique in that it produced additional fragments of 6.8 and 6.0 kb when digested with *Eco*RI (figure 2).

SL-78 also showed differences when digested with *Pst*I. After *Pst*I digestion, strains 25015 and CT39 and 8 of the 9

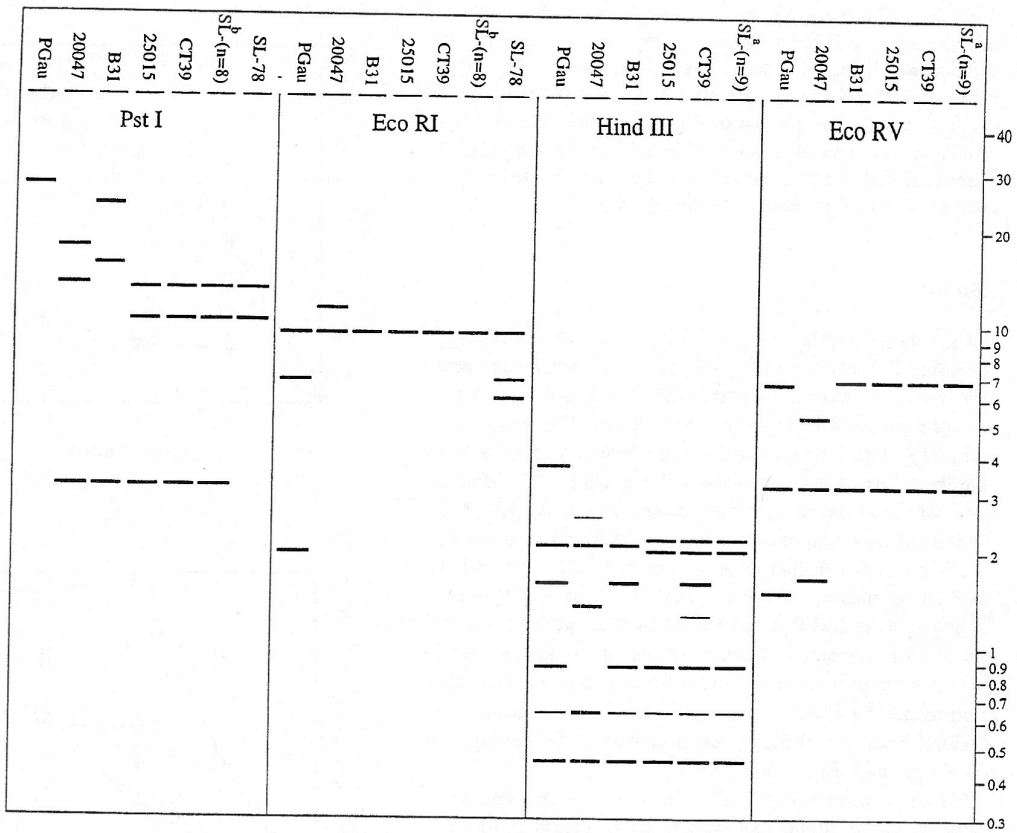

Figure 2. Diagram of results of 4 separate autoradiographs shows migration patterns of rRNA gene restriction fragments after digestion with restriction enzymes *Pst*I, *Eco*RI, *Hin*dIII, and *Eco*RV followed by hybridization with ^{32}P-labeled *Escherichia coli* 16+23S RNA. Thick and thin lines represent strong and weak bands, respectively, as seen on original autoradiographs. Strain designations are above lanes. SL^a comprises all 9 patient ML*x* isolates (SL-70, -73, -74, -75, -76, -78, -79, -80, and -90); SL^b comprises all ML*x* isolates except SL-78.

patient isolates produced fragments of 13.0, 10.3, and 3.2 kb. SL-78, however, produced only two fragments of 13.0 and 10.3 kb. Digestion with *Hin*dIII also revealed differences between the ML*x* isolates and strain B31. All 11 ML*x* strains possessed, in common with B31, fragments of 0.46, 0.64, and 0.87 kb. However, they differed from B31 in that they possessed two fragments of 2.2 and 2.0 kb instead of fragments of 2.1 and 1.6 kb. Strain CT39 also differed from strain 25015 and the 9 patient isolates in possessing a 1.6-kb fragment (in common with B31) and a weak 1.25-kb fragment.

After digestion with *Hpa*I and probing with a 5S rRNA-specific oligonucleotide probe, the band patterns of strains B31, 20047, PGau, and 25015 closely resembled those reported previously [15]. B31 produced 3.0- and 1.9-kb fragments, 20047 had 3.0- and 1.6-kb fragments, and PGau had 3.6- and 3.0-kb fragments. However, in our experiments, strains 25015 and CT39 also produced a weak 1.9-kb band. Of the Slovenian patient isolates, 8 produced 3.0- and 2.6-kb fragments, while 1 (SL-78) produced a single band of 2.6 kb. The weak 1.9-kb band found in strains 25015 and CT39 was not seen in the patient isolates (data not shown).

Amplification of the rrf (5S)-rrl (23S) intergenic spacer region. An amplified fragment of ~250 bp was obtained from all isolates except strain SL-78 (data not shown). The SL-78 genomic DNA preparation was tested using other primer pairs and amplified successfully, indicating that PCR inhibitors were not present.

Discussion

The molecular analyses demonstrated that 9 Slovenian patient isolates had many genotypic and phenotypic similarities to each other and to strains 25015 and CT39. However, among the group of 11 strains, a number of unique features differentiated individual isolates. These included a variant ML*x* LRFP (SL-80), different plasmid patterns (CT39 and SL-79), differences in levels of expression of certain proteins (SL-70, -74, and -79), and different ribotype fragment patterns using combined 16S+23S rRNA probes (strains CT39 and SL-78). Differences among the 9 patient isolates and strains 25015 and CT39 were also detectable by 5S rRNA ribotyping, and the 11

isolates differed (sometimes profoundly, e.g., SL-80) in their growth rates.

SL-78 had several differences from the other patient isolates that point to a major rearrangement of the ribosomal RNA genes in this strain. Thus, SL-78 produced a different pattern of fragments with the restriction enzymes *Eco*RI (yielding two extra fragments) and *Pst*I (lacking one fragment; figure 2). In addition, this strain also possessed a unique 5S rRNA pattern (one fragment only) and failed to produce an amplification product with primers designed to amplify the *rrf* (5S)-*rrl* (23S) intergenic spacer region. These latter findings also support the notion of a major rRNA gene rearrangement.

Strain 25015 and another unusual North American tick isolate, strain DN127, were recently shown to share 83% DNA relatedness and therefore to belong to the same genomic group (DN127) [5]. However, we do not believe that CT39 has been investigated by the same means. DN127 possesses a unique LRFP, quite unlike that of strain 25015 or of the other isolates described [9]. Thus, the DN127 group appears to comprise more than one LRFP, as with *B. burgdorferi* sensu stricto and *B. garinii*. *B. afzelii* is thus far the only species found to possess a single, highly conserved LRFP. Strains 25015 and DN127 also differ in the sizes of fragments obtained after *Mse*I digestion of their PCR-amplified *rrf* (5S)-*rrl* (23S) intergenic spacer regions [5]. The LRFP results are therefore concordant with the previous findings of genomic heterogeneity within the DN127 group.

These studies illustrate the value of PFGE as a classification tool. Currently, LRFP analysis is the easiest and most discriminating method of recognizing MLx isolates. It has also provided additional evidence for genetic heterogeneity within the DN127 group as reported by Postic et al. [5] as well as demonstrating their worldwide distribution and pathogenicity for humans.

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