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DNA Characterization of the Spirochete That Causes Lyme Disease

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Lyme disease, a tick-borne disease long recognized in Europe but only recently recognized in the United States, was shown in 1982-1983 to be caused by a spirochete, the Lyme disease spirochete. Whether one or more species of the spirochete exists is unknown, as is its taxonomic status. To answer these questions, we determined (i) the DNA base (guanine-plus-cytosine) content for five strains; (ii) the DNA relatedness of 10 strains from Europe or the United States (isolated from ticks, humans, and a mouse) by DNA hybridization (hydroxyapatite assay at 50 and 65°C); and (iii) the DNA relatedness to other pathogenic spirochetes. The guanine-plus-cytosine content of the Lyme disease spirochete strains was 27.5 to 29.0 mol%, most similar to those of *Borrelia hermsii* (30.6 mol%) and *Treponema hyodysenteriae* (25.6 mol%) among the other spirochetes tested. DNA hyridization studies with ^{32}P-labeled DNA from Lyme disease spirochete strain TLO-005, a human blood isolate, revealed divergence (unpaired bases) within related nucleotide sequences of only 0.0 to 1.0% for all nine Lyme disease spirochete strains tested for relatedness to TLO-005. Relatedness values of seven strains to TLO-005 were 58 to 98% (mean, 71%) in 50°C reactions and 50 to 93% (mean, 69%) in 65°C reactions. Two other strains, from which very low yields of DNA were obtained, showed less relatedness (36 to 50% at 50°C, 38 to 47% at 65°C). These were nonetheless considered to belong to the same species because of the low amount of divergence in the sequences related to TLO-005 and the absence of decreased relatedness in reactions done at 65°C compared with those done at 50°C. DNA from strain TLO-005 showed relatedness of 1% to DNAs of two leptospires and 16% relatedness to DNA from *T. hyodysenteriae*. *B. hermsii* DNA was 30 to 40% related to three Lyme disease spirochete strains in 50°C reactions. Divergence in these reactions was 16.5 to 18.5%, and relatedness in 65°C reactions was 8 to 10%. On the bases of phenotypic similarity, guanine-plus-cytosine content, and DNA relatedness to *B. hermsii*, we conclude the Lyme disease spirochete is a single previously undescribed species which belongs in the genus *Borrelia*.

Lyme disease is a systemic tick-borne illness characterized by a distinctive skin lesion, erythema chronicum migrans (ECM), and, in many cases, subsequent development of neurological, cardiac, and arthritic complications (19). Although ECM, occasionally with attendant neurological complications, has been recognized in Europe since the early part of this century, the first reported case acquired in the United States occurred in 1969 (17). It was not until 1975 when researchers investigating an unusual cluster of cases of arthritis in East Haddam, Lyme, and Old Lyme, Conn., recognized the entire clinical spectrum of what is now known as Lyme disease (20).

Despite the demonstration in human volunteers in 1955 that ECM could be passed from human to human (5), strongly suggesting that an infectious agent was responsible for the development of ECM, researchers were unable to identify the etiological agent of Lyme disease. In 1982, however, a spirochete was isolated from *Ixodes dammini* ticks (the principal recognized vector of Lyme disease in the United States), which reacted with high titers when tested by indirect immunofluorescence with the convalescent sera from patients with Lyme disease (9). Further, rabbits fed upon by spirochete-infected ticks developed skin lesions similar to ECM (9). Subsequently, this spirochete has been isolated from ECM lesions (4, 18), cerebrospinal fluid (18), and blood (3, 18) of persons acutely ill with Lyme disease. The spirochete has also been isolated from the white-footed mouse (6), a preferred host of immature *I. dammini*, and from *Ixodes ricinus* ticks, the recognized vector of ECM in Europe (1).

The taxonomic status of the spirochete (Lyme disease spirochete) has been unclear (9, 18). Of the five genera of *Spirochaetaceae*, three are pathogenic for humans (*Leptospira*, *Borrelia*, and *Treponema*), and the Lyme disease spirochete resembles most closely by morphology and growth characteristics the treponemes and borreliae (9, 18). To better taxonomically characterize the Lyme disease spirochete and to determine whether it represents one or multiple bacterial species, we determined its guanine-plus-cytosine (G+C) content, DNA relatedness among 10 strains, and relatedness to selected other pathogenic spirochetes.

MATERIALS AND METHODS

Source and cultivation of bacterial strains. The strains used in this study, along with their sources, who they were obtained from, and, if tested, their G+C contents, are listed in Table 1.

Strains of the Lyme disease spirochete and *B. hermsii* were grown in BSK medium (1), in either 1,000-ml Erlenmeyer flasks or 250-ml tissue culture flasks containing ca. 1,000 or 230 ml of medium, respectively. The flasks were incubated at 33°C for 7 to 14 days until growth was about 10^7 to 10^8 cells per ml. Cells were sedimented by centrifugation at 8,000 × *g* for 30 min. Leptospires were grown in Ellinghausen medium at 27°C (22) and harvested by centrifugation at 5,000 × *g* for 25 min after 7 to 10 days of growth. *Treponema hyodysenteriae* was grown in Trypticase soy broth containing 0.25% glucose and 10% bovine fetal serum

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TABLE 1. Source and DNA base content of spirochete strains

Strain[a]	G+C mol%	Source and location	Sender
Lyme disease spirochete B31 (ATCC 35210)	29.0	Tick (*I. dammini*), Shelter Island, N.Y.	A. Barbour[b]
Lyme disease spirochete TLO-004	28.2	Tick (*I. dammini*), Great Island, Mass.	A. Steere[c]
Lyme disease spirochete TLO-005	28.1	Human blood, Connecticut	A. Steere
Lyme disease spirochete TLO-008 (IRS) (ATCC 35211)	28.1	Tick (*I. ricinus*), Switzerland	A. Barbour
Lyme disease spirochete TLO-024	27.5	Tick (*I. dammini*), Monmouth County, N.J.	CDC[d]
Lyme disease spirochete TLO-029	NT[e]	Human skin, New York	B. Berger[f]
Lyme disease spirochete TLO-030	NT	Human spinal fluid, Connecticut	A. Steere
Lyme disease spirochete TLO-031	NT	Human skin, Connecticut	A. Steere
Lyme disease spirochete TLO-032 (50-2)	NT	White-footed mouse (*Peromyscus leucopus*), New York	J. Benach[g]
Lyme disease spirochete TLO-033 (HB4)	NT	Human blood, New York	J. Benach
L. interrogans serotype *mankarso*	36.7		K. Sulzer[d]
L. interrogans serotype *celledoni*	40.2		K. Sulzer
L. biflexa serotype *patoc*	39.0		K. Sulzer
L. biflexa serotype *illini*	47.6		K. Sulzer
T. hyodysenteriae ATCC 27164 (type strain)	25.6		ATCC[h]
B. hermsii strain HS1 (ATCC 35209)	30.6		A. Barbour

[a] The designation number of the sender is shown in parentheses, if appropriate.
[b] Rocky Mountain Laboratory, Hamilton, Mont.
[c] Yale University Medical School, New Haven, Conn.
[d] Centers for Disease Control, Atlanta, Ga.
[e] NT, Not tested.
[f] Southhampton, N.Y.
[g] University of New York at Stony Brook, N.Y.
[h] American Type Culture Collection, Rockville, Md.

under an atmosphere of 90% N_2 and 10% CO_2 at 37°C (14); cells were harvested as above after 3 days of incubation.

G+C content of DNA. The G+C content of DNA was determined by denaturing DNA in a spectrophotometer, determining the thermal midpoint of denaturation, and calculating the moles percent G+C by the method of De Ley (10).

DNA hybridization. The preparation of unlabeled and labeled DNA and the procedure used for DNA hybridization have been described previously (8, 12). Sedimented cells were suspended in a solution containing EDTA, Tris buffer, NaCl, and pronase and lysed by the addition of sodium dodecyl sulfate. DNA was purified by phenol and chloroform extractions, precipitation with ethanol, RNase followed by pronase treatments, additional phenol and chloroform extractions, and finally precipitation with ethoxyethanol. The purified DNA was suspended in 0.01 M NaCl. Lyme disease spirochete strain TLO-005 was labeled in vitro with ^{32}P by nick translation according to the instructions furnished with a commercial nick translation reagent kit (catalog no. 8160; Bethesda Research Laboratories, Inc., Rockville, Md.). Both labeled and unlabeled DNAs were sheared to a size of ca. 2×10^5 daltons by sonication and denatured by boiling before use in DNA hybridization experiments. Approximately 2,000 cpm of labeled DNA (ca. 0.01 μg) was added to 150 μg of unlabeled DNA in 1 ml of 0.28 M phosphate buffer and incubated at 50 or 65°C for 16 h to ensure almost complete reassociation of labeled and unlabeled DNA. The unlabeled DNA concentrations from three Lyme disease spirochete strains (TLO-030, TLO-031, and TLO-032) were only from 55 to 110 μg/ml. These DNAs were incubated for longer periods of time to reach the same time × DNA concentration-incubation exposure. After incubation, the DNA mixtures were diluted to a 0.14 M phosphate buffer concentration and passed through hydroxyapatite. At this concentration, double-, but not single-, stranded DNA binds to hydroxyapatite. Double-stranded DNA was eluted from hydroxyapatite either by the addition of 0.4 M phosphate buffer (at which concentration the DNA is eluted in the double-stranded form) or, to determine divergence in related DNA sequences, by a series of elutions with 0.14 M phosphate buffer at increasing temperature (when the temperature is high enough to denature the double-stranded DNA, it is eluted as single-stranded DNA).

RESULTS

All of the Lyme disease spirochete isolates grew well in BSK medium and had a similar morphology when viewed by dark-field microscopy (about 20 μm long, 0.2 μm in diameter, with rotating motility along the long axis).

The G+C content of five Lyme disease spirochete strains was 27.5 to 29.0 mol%, similar to those of *Borrelia hermsii* (30.6 mol%) and *T. hyodysenteriae* (25.6 mol%) and quite different from that of *Leptospira* spp. (36.7 to 47.6 mol%) (Table 1).

Labeled DNA from Lyme disease spirochete strain TLO-005 was reacted with unlabeled DNAs from nine other strains isolated from ticks, humans with Lyme disease, or from a mouse, as well as with DNAs from *T. hyodysenteriae* and leptospires (Table 2). Due to the low G+C content of Lyme disease spirochete DNAs, 50°C was chosen as the incubation temperature for optimal DNA reassociation, and 65°C was chosen as the incubation temperature for DNA reassociation at a stringent criterion where only very closely related DNA sequences (less than 5% divergence) can hybridize. Strain TLO-005 DNA was 64 to 98% related to DNAs from six Lyme disease strains and 36, 50, and 58%

related to three other Lyme disease strains in 50°C reactions. The divergence in related sequences was quite low, 0.0 to 1.0%. In 65°C reactions, TLO-005 DNA was 61 to 93% related to six Lyme disease spirochete strains and 38, 47, and 50% related to the other three strains. For only one strain (TLO-024) was there a significant decrease in relatedness at 50°C compared with that at 65°C (67 to 50%). Strain TLO-005 DNA was 1% related to DNAs from two leptospires and 16% related to DNA from *T. hyodysenteriae* in 50°C reactions. *B. hermsii* HS1 DNA was 30 to 40% related to unlabeled DNAs from Lyme disease spirochete strains TL-004, TL-005, and TL-033 in 50°C reactions (Table 2). Divergence in related nucleotide sequences was 16.5 to 18.5%. In 65°C reactions, relatedness of *B. hermsii* to these Lyme disease strains decreased to 8 to 10%.

DISCUSSION

On the basis of experience in DNA hybridization with a large number of organisms, Brenner (7) defined a species as "a group of strains with 70% or more relatedness at optimal conditions, with 55% or more relatedness at less than optimal conditions, and with a thermal stability of related sequences within 6°C of reassociated homologous DNAs." There are some exceptions, usually having less than 70% relatedness at optimal reassociation criteria, but fulfilling the two other parts of the species definition. Both Brenner and others (P. A. D. Grimont and M. Popoff, personal communication) believe that the most important part of the definition may be the relative lack of divergence in sequences related among strains of a species. By the criterion of divergence, all strains of the Lyme disease spirochete represent a single species (0.0 to 1.0% divergence in related sequences). At the stringent, 65°C incubation temperature, six strains belong to the same species as the labeled strain (TLO-005). Six strains also are 64% or more related to the labeled strain at the optimal, 50°C incubation temperature (including TLO-024, which was 50% related to TLO-005 in 65°C reactions). Another reason for believing that all strains belong to a single species is the lack of any decrease in relatedness at 65°C compared with that obtained at 50°C. Only strain TLO-024 (67 to 50%) showed a significant decrease in relatedness at the higher incubation temperature.

One possible explanation for low relatedness between strains of the same species is a difference in genome size. For example, if strain A had a genome size of 3×10 daltons and strain B had a genome size of 2×10 daltons, 67% of strain A would be related to 100% of strain B. In the case of the Lyme disease spirochete, we believe that technical difficulties rather than large genome size differences are responsible for the low binding values. The DNA concentrations obtained were from 55 to 390 μg/ml. Strains TLO-030, TLO-031, and TLO-032 were reacted with labeled DNA at final unlabeled DNA concentrations of 55, 105, and 110 μg/ml, respectively. Two of these strains showed low relatedness, possibly due to degradation of DNA during purification or during the longer incubation necessary to reach an incubation constant of 16 h with the 150 μg of DNA routinely used in our experiments (for example, the 55-μg/ml reaction mixture was incubated for almost 48 h). Strain TLO-030, an isolate from human spinal fluid, was also tested by Hyde and Johnson (13), who found it to be 76% related to labeled DNA from another Lyme disease spirochete strain. Their data on this strain provide strong support for the argument that all strains of the Lyme disease spirochete belong to one species.

Additional evidence consistent with a single species for all Lyme disease spirochete strains is the similar G+C content in the five strains tested (Table 1), similar morphology, good growth in the same medium, and reactivity with a monoclonal antibody made against a cell surface antigen from Lyme disease spirochete strain B31 (2). This antibody has been shown not to react with four *Borrelia* species, *Treponema pallidum*, *Treponema phagedenis*, or *Leptospira interrogans*.

Despite some morphological similarity to treponemes, the Lyme disease spirochete was only slightly related (16%) to *T. hyodysenteriae*. *T. hyodysenteriae* DNA had a G+C

TABLE 2. DNA relatedness of Lyme disease spirochete strains[a]

Source of unlabeled DNA	DNA relatedness to labeled DNA from: Lyme disease spirochete strain TLO-005			*B. hermsii* HS1		
	Relative binding ratio (50°C)[b]	Divergence[c]	Relative binding ratio (65°C)	Relative binding ratio (50°C)	Divergence	Relative binding ratio (65°C)
Lyme disease spirochete TLO-005	100	0.0	100	37	16.5	8
Lyme disease spirochete TLO-008	98	1.0	93			
Lyme disease spirochete TLO-032	80	1.0	71			
Lyme disease spirochete B31	67	0.5	71			
Lyme disease spirochete TLO-024	67	0.0	50			
Lyme disease spirochete TLO-004	65	0.0	70	40	18.0	10
Lyme disease spirochete TLO-029	64	1.0	68			
Lyme disease spirochete TLO-033	58	1.0	61	30	18.5	8
Lyme disease spirochete TLO-031	50	0.5	47			
Lyme disease spirochete TLO-030	36	0.0	38			
B. hermsii				100	0.0	100
T. hyodysenteriae	16			9		2
L. interrogans serotype *mankarso*	1					
L. biflexa serotype *illini*	1					

[a] The values given for each reaction are averages of two to four experiments. In control reactions in which labeled DNA was incubated in the absence of unlabeled DNA, 1.0 to 2.5% of labeled DNA bound to hydroxyapatite. These control values were subtracted before the relative binding ratios were calculated. Binding to hydroxyapatite in homologous TLO-005 reactions was 51% at 50°C and 55% at 65°C, and in homologous *B. hermsii* HS1 reactions was 53% at both 50°C and 65°C.

[b] Relative binding ratio = [(heterologous DNA bound to hydroxyapatite)/(homologous DNA bound to hydroxyapatite)] × 100.

[c] Calculated to the nearest 0.5% on the assumption that a 1°C decrease in thermal stability of a heterologous DNA duplex compared with that of the homologous DNA duplex is caused by each 1% of the bases that are unpaired.

content of 25.6 mol%, similar to the value of 25.8 mol% reported by Miao et al. (16). Other treponemes were not tested because their G+C contents range from 38.0 mol% for *T. phagedenis* biotype Kazan 5 to 53.7 mol% for *T. pallidum* (15). The Lyme disease spirochete is not similar to *L. interrogans* or to *Leptospira biflexa* morphologically, in G+C content (Table 1), or in DNA relatedness (Table 2).

Although the Lyme disease spirochete differs from *Borrelia* spp. morphologically and in staining with a monoclonal antibody directed against the Lyme disease spirochete, other observations suggest relatedness to borreliae. First, its G+C content (27.5 to 29.0 mol%) is quite similar to that for *B. hermsii* in our work and to other borreliae in the work of others (13). Second, monoclonal antibodies to borrelial axial filaments cross-react with Lyme disease spirochete axial filaments (A. G. Barbour, personal communication). Third, transmission by ticks is a common feature of the Lyme disease spirochete and of borreliae. Consistent with these similarities between the Lyme disease spirochete and borreliae, we found that DNA from *B. hermsii* was significantly related to the Lyme disease spirochete, sufficient enough to place the organism in the genus *Borrelia*. It is possible, although unlikely, that the Lyme disease spirochete is an existing *Borrelia* species, despite differences in organism morphology and the clinical illnesses produced by *Borrelia* spp. (relapsing fever) and the Lyme disease spirochete. Most likely, the spirochete is a previously undescribed *Borrelia* species; however, since type strains have not been designated for any of the 19 recognized *Borrelia* species, this point cannot be tested directly.

Lyme disease has long been known to occur in Europe, but has only recently been diagnosed in the United States and in Australia (21). Because of the finding that all Lyme disease spirochete strains, including a Swiss strain, are the same species, it is tempting to hypothesize that the organism was transported from Europe to other areas. Nevertheless, some differences may exist between strains. Monoclonal antibodies have been found which do not stain all Lyme disease spirochete strains tested (A. G. Barbour and S. L. Tessier, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 239, 1983). Similarly, differences among the clinical illnesses occurring in Europe and the United States appear to exist. For instance, only recently has arthritis been recognized in patients in Europe (11); this may be due to a simple lack of recognition or actual differences in virulence between strains found in Europe and the United States. These observations indicate that continued study of strain factors complementary to DNA characterization will be necessary to understand the epidemiology and virulence of the Lyme disease spirochete.

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