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# Polymerase Chain Reaction Primers and Probes Derived from Flagellin Gene Sequences for Specific Detection of the Agents of Lyme Disease and North American Relapsing Fever

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**By cloning and sequencing the flagellin gene of *Borrelia hermsii* and comparing this sequence with that of the corresponding gene from *B. burgdorferi*, I identified a central region within the two genes which showed a reduced level of sequence similarity. Oligonucleotide sequences selected from this region produced species-specific amplimers when used in polymerase chain reaction experiments. Thus, primers derived from the *B. burgdorferi* sequence amplified a 276-bp fragment from 22 strains of *B. burgdorferi* of diverse geographic origin but not from 5 strains of *B. hermsii*, 5 other *Borrelia* species, 16 *Treponema*, *Leptospira*, and *Spirochaeta* species, or representatives of 10 other bacterial genera. However, when the amplified fragments were tested for hybridization with an oligonucleotide probe derived from the nonhomologous region, seven strains from either Germany or Switzerland did not hybridize. Cloning and sequencing of the amplified fragments from these strains revealed that the 22 strains of *B. burgdorferi* tested could be divided into three groups based on the nucleic acid sequence of the central region of the flagellin gene. With this information, oligonucleotide probes that hybridized to the amplified fragments and were able to differentiate the three groups of *B. burgdorferi* were designed. The corresponding primers, derived from the *B. hermsii* gene sequence, were tested for their ability to amplify DNA from this collection of strains. Although no amplification was obtained with representatives of the three groups of *B. burgdorferi* or various *Treponema*, *Leptospira*, and *Spirochaeta* species, amplification was obtained with the five other *Borrelia* species (*B. parkeri*, *B. turicatae*, *B. crocidurae*, *B. anserina*, and *B. coriaceae*) in addition to the five strains of *B. hermsii*. Sequencing of the amplified fragments from one strain of *B. hermsii* as well as *B. parkeri* and *B. turicatae* allowed the design of oligonucleotide probes that were able to differentiate the three species of North American relapsing fever spirochetes into two separate groups. These studies suggest that there is sufficient diversity within the flagellin gene sequences of closely related *Borrelia* species to differentiate them into groups and to pursue taxonomic studies both within and between species.**

Pathogenic spirochetes of the genus *Borrelia* have been associated worldwide with a number of epidemic and endemic tick-borne diseases, including North American and Mediterranean relapsing fevers (*Borrelia hermsii*, *B. parkeri*, *B. turicatae*, and *B. crocidurae*), avian borreliosis (*B. anserina*), and epidemic bovine abortion (*B. coriaceae*) (7). However, since 1975, these diseases and the *Borrelia* spp. that cause them have been overshadowed by the most recently discovered member of the genus, *Borrelia burgdorferi*, the causative agent of Lyme disease (12, 25, 50, 51).

Current methods used for the diagnosis of these spirochetal infections depend upon serologic testing (14, 21, 30, 50) and have shown themselves to be both insufficiently sensitive for the detection of early disease (13, 47) and insufficiently specific in the later stages (6, 31). Particularly in the case of Lyme disease, detection of the organism by microscopic examination (16) or culturing of specimens (49) is impractical, since spirochetes are rare and difficult to cultivate. The best solution to these problems would therefore appear to be direct detection of *Borrelia* spp. by a sensitive and specific assay. An example of such an assay, which has the potential for combining extreme sensitivity with extreme specificity, is a DNA probe test designed to detect fragments of DNA amplified from the *Borrelia* genome by an amplification procedure, such as the polymerase chain reaction (PCR) (33). A further important advantage of such an assay is that it could be used on any specimen that contains or once contained live spirochetes, without the need for isolation of the organism in pure culture (37).

Ideally, such a PCR-DNA probe system should embody two important attributes: it must be able to exclude other related species but at the same time retain the ability to recognize all available strains or serotypes of the species of interest. Thus, a DNA probe test for Lyme disease should not cross-react with relapsing fever spirochetes and vice versa. Investigation of the protein profiles of geographically diverse isolates of *B. burgdorferi* over a number of years (4, 8, 9, 55) as well as DNA homology studies (23, 24, 46) have demonstrated that the species is heterogeneous and genetically diverse. At the same time, these DNA homology studies have indicated that *B. burgdorferi* shares a considerable level of homology with North American relapsing fever spirochetes (*B. hermsii*, *B. parkeri*, and *B. turicatae*) (23). Therefore, it could be anticipated that this genomic diversity and close relationship to related *Borrelia* species would make the isolation of species-specific but all-inclusive PCR primers and DNA probes problematic.

Recently, a number of PCR-DNA probe systems were described for the detection and diagnosis of Lyme disease. Some of these have been unable to fully satisfy both requirements: systems that have been shown to be specific often have failed to detect certain isolates of *B. burgdorferi* (38, 42), while others that have relied upon the use of sequences

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TABLE 1. Nucleotide sequences of the PCR primers and probes

| Species | Oligonucleotide | Sequence |
|---|---|---|
| *B. burgdorferi* | FL6 | 5′ TTC AGG GTC TCA AGC GTC TTG GAC T 3′ |
| | FL7 | 5′ GCA TTT TCA ATT TTA GCA AGT GAT G 3′ |
| | FL8 | 5′ CTC TGG TGA GGG AGC TCA AAC TGC TCA GGC TGC ACC GGT TCA AGA GGG T 3′ |
| | FL15 | 5′ CTC TGG TGA AGG AGC TCA GGC TGC TCA GAC TGC ACC TGT TCA AGA AGG 3′ |
| | FL16 | 5′ TGC TGG TGA GGG AGC TCA AGC TGC TCA GGC TGC ACC TGT TCA AGA GGG TGC T 3′ |
| *B. hermsii* | FL11 | 5′ AGC TGG ATC ACA AGC TTC ATG GAC A 3′ |
| | FL12 | 5′ CCC TCT ATC TTT GCA AGT GAC A 3′ |
| | FL13 | 5′ TGC AGG TGA AGG CGC TCA GGC TGC TCC AGT GCA AGA GAT A 3′ (T[a]; A[a]) |
| | FL17 | 5′ TGC AGG TGA AGG CGC TCA GGC TGC TCC AGT GCA AGA GAT AGG A 3′ |
| | FL18 | 5′ TGC AGG TGA AGG TGC GCA GGT TTC TCC AGC TCA GGA AGG TGC A 3′ |

[a] Redundancy in the sequence of FL17.

from highly conserved genes have cross-reacted with *B. hermsii* (18, 29). For one such system, these difficulties were circumvented by the use of multiple primer sets (41) but, to date, no single set of PCR primers has been able to detect all known isolates of *B. burgdorferi* without cross-reacting with other species. Thus far, scant attention has been paid to the development of PCR-DNA probe systems for other *Borrelia* species. At the present time, only one such system has been described for the detection of relapsing fever spirochetes (41). This system consists of a set of PCR primers which amplify *B. hermsii*, *B. parkeri*, and *B. turicatae* as one group.

The present investigation describes an attempt to devise species-specific but all-inclusive PCR-DNA probe systems for the detection and diagnosis of Lyme disease and North American relapsing fever by exploiting fine differences between highly conserved genes. The gene that was chosen for study was the *Borrelia* flagellin gene. To circumvent the problems of cross-reactivity, I did an analysis of the degree of sequence identity between the flagellin gene of *B. burgdorferi* and that of the most closely related species, *B. hermsii* (23). This analysis led to the identification of specific sequences that can be amplified from either *B. burgdorferi* or the relapsing fever spirochetes. Further sequence analysis of amplified fragments allowed the design of internal probe oligonucleotides that are capable of subdividing individual *Borrelia* species into several subgroups.

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## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Borrelia* cultures were maintained in BSKII medium (5) at 33°C and passaged once per week by making two 50-fold dilutions into fresh medium. *B. hermsii* HS1 (ATCC 35209), *B. burgdorferi* B31 (ATCC 35210) and IRS (ATCC 35211), and *B. coriaceae* Co53 (ATCC 43381) were obtained from the American Type Culture Collection (ATCC) (Rockville, Md.). *B. burgdorferi* MMI (W. Schmid), MMTI (R. Bey), IPS (*Ixodes pacificus* spirochete) (R. Lane), P/Bi (V. Preac-Mursic) (55), and P/Sto (V. Preac-Mursic) (55) as well as *B. parkeri* and *B. turicatae* were obtained from Russell C. Johnson, University of Minnesota, Minneapolis. *B. burgdorferi* N40 (2), Son 328 (10), Minnesota Mouse (38), Lake 339 (10), 20001 (3), CD16 (38), 25015 (2), P/Gau (V. Preac-Mursic) (55), and GTI (German tick isolate) (38) were obtained from Stephen W. Barthold, Yale University School of Medicine, New Haven, Conn. Lyophilized spirochetes from these strains were also obtained from David H. Persing, Mayo Clinic, Rochester, Minn. *B. burgdorferi* VS215, NE56, VS3, and VS185 (A. Aeschliman) (32) were obtained from Jürg Meyer, University of Basel, Basel, Switzerland. *B. burgdorferi* DN127 (10) and *B. hermsii* YOR-1, CON-1, and MAN-1 (26) were obtained from J. Michael Janda, California Department of Health Services, Berkeley. *B. burgdorferi* G2 (22) as well as purified DNAs from *B. hermsii* Frogner, *B. parkeri*, *B. turicatae*, *B. crocidurae*, *B. anserina*, and *B. coriaceae* were obtained from Thomas G. Schwann, Rocky Mountain Laboratories, Hamilton, Mont. *Treponema pallidum* and *T. phagedenis* spirochetes were obtained from Thomas J. Fitzgerald, University of Minnesota, Duluth. The names in parentheses represent the persons who isolated the strains (when known).

Other *Treponema*, *Leptospira*, and *Spirochaeta* strains and other bacterial genera were obtained from the ATCC and propagated in the appropriate ATCC medium.

**DNA purification.** Total genomic *Borrelia* DNA was prepared essentially as described previously (42).

**PCR amplifications.** All amplifications were carried out with a Perkin-Elmer-Cetus thermal cycler. Routinely, 100-μl reaction mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each deoxynucleoside triphosphate, 100 pmol each of the 5′ and 3′ primers, and 2.5 U of *Taq*I DNA polymerase (Amplitaq; Perkin Elmer Cetus). Unless otherwise specified, 1 ng of template DNA was amplified for 30 cycles with the following cycle parameters: 94°C for 1 min (denaturation), 37°C for 2 min (annealing), 72°C for 3 min (extension), 7 min at 72°C (final extension). For the specific amplification of *B. burgdorferi* flagellin gene sequences, the optimum annealing temperature (54°C) was calculated with the computer program Oligo (43). For the specific amplification of flagellin gene sequences from other *Borrelia* species, an annealing temperature of 49°C was used. The oligonucleotides used in this study as PCR primers and probes are listed in Table 1.

For analysis of the amplification products, a 15-μl aliquot of the reaction mixture was subjected to electrophoresis on a 2% SeaKem LE–1× Tris-Borate-EDTA (TBE) agarose gel. Amplified bands were visualized by ethidium bromide staining, and the gels were photographed. The gels were blotted by the Southern method (44) and probed with radioactively labeled oligonucleotide probes as described below.

**Construction and screening of genomic libraries.** Chromosomal DNA from *B. hermsii* was partially digested with *Sau*3A, and the digestion was monitored by agarose gel electrophoresis. For prevention of the cloning of multiple inserts, suitable aliquots containing fragments in the size range of ~9 to 23 kb were subjected to a partial filling-in reaction with the large fragment of DNA polymerase I (Klenow fragment) in the presence of dGTP and dATP. This material was cloned into λ replacement vector λ GEM 11 (Promega Corp., Madison, Wis.) which had been digested with *Xho*I and partially filled in with dTTP and dCTP. Ligations were packaged with Gigapack packaging extracts (Stratagene, Inc., La Jolla, Calif.) and plated on *Escherichia coli* LE392. The same procedures were used to construct a *B. burgdorferi* genomic partial digest library.

Recombinant λ phage containing *B. hermsii* flagellin gene sequences were isolated by the plaque lift technique as described previously (39). Plaque lift filters were probed with a 300-bp fragment probe which had been amplified by PCR from *B. hermsii* chromosomal DNA with *B. burgdorferi* primers. The amplified fragment was isolated from agarose gels with powdered glass (53) and $^{32}P$ labeled, either by nick translation or by further cycles of PCR with one labeled deoxynucleoside triphosphate. Prehybridization and hybridization of filters were carried out as described previously (39). Positive plaques were picked directly from the primary isolation plates into SM buffer (44) and subjected to two further rounds of single-plaque isolation to ensure their purity before large-scale λ DNA preparations were made from them (44).

For cloning of the corresponding genomic segment from *B. burgdorferi*, a λ library of DNA fragments from this organism was probed with oligonucleotide probes derived from the *B. burgdorferi* flagellin gene sequence (20). These probes were 5′ end labeled with T4 polynucleotide kinase and [γ-$^{32}P$]ATP as described previously (39). After hybridization, filters were washed twice for 30 min each time at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% sodium dodecyl sulfate and autoradiographed.

**RE mapping of genomic clones.** Purified DNA from candidate clones was digested with the following enzymes, which do not cut the λ GEM 11 genome: *Sfi*I, *Sac*I, *Bam*HI, *Eco*RI, and *Xba*I. Resulting fragment sizes were determined by agarose gel electrophoresis against standards. Gels were also blotted by the Southern method (44) and probed either with oligonucleotides (*B. burgdorferi*-derived clone) or with the amplified fragment of *B. hermsii* (see above) (*B. hermsii*-derived clones) to determine which fragments carried the flagellin genes. This determination allowed the construction of preliminary physical and restriction enzyme (RE) maps of the clones. For the construction of complete maps, the largest *Bam*HI-*Sac*I fragments from clones 35209-1, 35209-3, and 35209-4 and the *Sac*I fragments from clone 35210-8 were subcloned into plasmid vector pBluescript II (Stratagene) and mapped in more detail by further RE digestion or by partial digestion of 3′-end-labeled fragments (48).

**Southern blot hybridization.** Southern blot hybridization was performed by alkaline dry transfer. After being stained and photographed, gels were soaked for 20 min in 0.4 N NaOH–0.6 M NaCl, placed on a sheet of Saran Wrap, and overlaid sequentially with one dry sheet of Pall Biodyne B membrane (0.45-μm pore size) and with two sheets of Schleicher & Schuell gel blot paper GB002 wetted with the alkaline transfer solution. After the addition of a stack of dry gel blot paper GB004 and a suitable weight, transfer was allowed to proceed overnight. Before prehybridization, the nylon membrane was washed three times for 5 min each time in 2× SSC to remove the alkaline transfer solution and dried under a heat lamp for 10 min. Prehybridization, hybridization, washing, and autoradiography of filters were done as described previously (39).

**Cloning of PCR-amplified fragments.** PCR reactions (100 μl) were extracted with chloroform to remove mineral oil, and the amplified DNA fragments were precipitated. After resuspension in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA buffer, they were treated with the large fragment of DNA polymerase I (Klenow fragment) in the presence of a mixture of four deoxynucleotide triphosphates to ensure that the ends were blunt and double stranded. The PCR products were applied to a 2% low-melting-temperature agarose gel and separated by electrophoresis, and the fragments were excised and purified free of agarose with powdered glass (53). After ethanol precipitation, the fragments were resuspended in distilled water and treated with T4 polynucleotide kinase in the presence of ATP to phosphorylate the 5′ ends. The phosphorylated fragments were ethanol precipitated to remove the kinase buffer and used directly in ligation reactions with M13 vector molecules.

M13mp18 or M13mp19 vector DNA was digested with RE *Sma*I and dephosphorylated with calf intestinal alkaline phosphatase (44). Vector molecules were ligated to amplified fragments at 4°C overnight and transformed into *E. coli* JM109 (44). Recombinant plaques containing amplified fragments in both orientations were selected by the plaque lift technique and by probing with 5′ $^{32}P$-labeled primer oligonucleotides as previously described (39).

**DNA sequencing.** The complete nucleotide sequence of the *B. hermsii* flagellin gene was determined by the M13 dideoxy chain termination technique (45) with a commercially available $^{35}S$ sequencing kit (Sequenase; U.S. Biochemicals, Cleveland, Ohio). Sequencing was performed initially by using the M13 forward sequencing primer and then by using oligonucleotide primers whose sequences had been derived from previous sequencing experiments (52). In all cases, both strands were sequenced to ensure the accuracy of sequence data. PCR-amplified fragments which had been cloned into M13 were sequenced by using the M13 forward sequencing primer, and in each case four independent clones (two in each orientation) were sequenced. All sequence data were manipulated and analyzed with the University of Wisconsin Genetics Computer Group sequence analysis package (17).

**Nucleotide sequence accession numbers.** The nucleotide sequences reported here have been assigned the following GenBank accession numbers: *B. hermsii* flagellin gene sequence (M33839); *B. burgdorferi* 0.68-kb *Xba*I fragment (M34710); PCR-amplified fragments from *B. burgdorferi* P/Bi (M67456), VS185 (M67457), P/Sto (M67458), and 25015 (M67459); *B. hermsii* YOR-1 (M67460); *B. parkeri* (M67461); and *B. turicatae* (M67462).

## RESULTS

**Cloning and sequencing of the *B. hermsii* flagellin gene.** Primer sequences, chosen at random from the published sequence of the *B. burgdorferi* flagellin gene, were tested in PCR amplification experiments with genomic DNA from *B. hermsii* HS1, *B. coriaceae* Co53, and two *B. burgdorferi* strains (B31 and IRS). Analysis of the amplification products by agarose gel electrophoresis showed that bands of the appropriate sizes (based on the sequence of the *B. burgdor-*

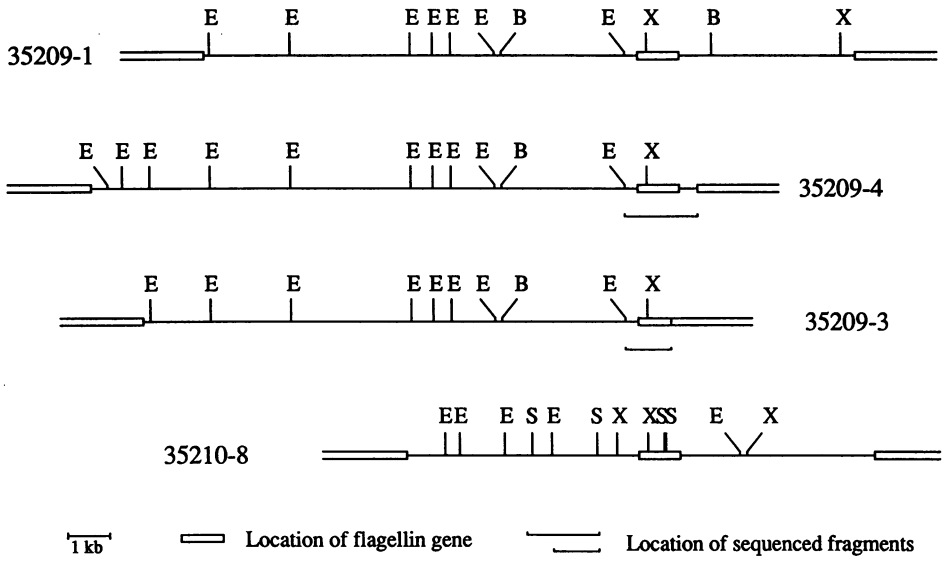


FIG. 1. RE maps of three *B. hermsii* λ clones (35209-1, 35209-3, and 35209-4) and one *B. burgdorferi* λ clone (35210-8) showing the positions within these clones of the flagellin genes (closed boxes). The four RE maps are drawn to scale and have been aligned relative to the locations of the flagellin genes of the two organisms. The open boxes at the ends of the clones represent bacteriophage λ vector DNA. Abbreviations: E, *Eco*RI; B, *Bam*HI; S, *Sac*I, X, *Xba*I. The sequences of the multiple *Eco*RI sites in the clones were determined by 3′ end label mapping (48).

*feri* flagellin gene) could be amplified from the two *B. burgdorferi* strains. The primers also amplified a faint band of DNA (of the correct size) from *B. hermsii*, indicating considerable levels of identity between the flagellin gene sequences from the two organisms. Similar results have been described recently (29).

For cloning and sequencing of the flagellin gene from *B. hermsii* HS1, the faint band of amplified material produced in PCR with *B. burgdorferi* flagellin gene primers was excised from agarose gels and purified. This DNA fragment, of approximately 300 bp, was assumed to possess a sequence structure such that the first and last 25 bp were identical to the primer sequences used for amplification but that there was also an intervening region of approximately 250 bp with perfect sequence identity to the *B. hermsii* flagellin gene. This purified DNA fragment was radioactively labeled and used as a probe to screen a *B. hermsii* genomic library of *Sau*3A partial digest fragments in phage λ. A number of reactive clones were obtained; three of them were mapped in detail by RE analysis. For selection of clones which contained the *B. burgdorferi* flagellin gene, a similar *Sau*3A partial digest library of *B. burgdorferi* fragments was constructed and screened with radioactively labeled oligonucleotide probes derived from the *B. burgdorferi* flagellin gene sequence (20). Figure 1 depicts the RE maps of three clones derived from *B. hermsii* (35209-1, 35209-3, and 35209-4) and one clone derived from *B. burgdorferi* (35210-8). The order of the RE sites within the three 35209 clones shows that they contain the same segment of the *B. hermsii* genome but with variable flanking regions, as expected for clones derived from a *Sau*3A partial digest library. Thus, the RE map shown in Fig. 1 may be taken to represent a discrete segment of the *B. hermsii* genome. The RE map of the corresponding segment of the *B. burgdorferi* genome is shown for comparison.

With the radioactively labeled fragment probe described above, the various subfragments of the three *B. hermsii* clones shown in Fig. 1 were investigated by Southern blot analysis. By this means, subfragments which hybridized to the probe and therefore presumably contained the *B. hermsii* flagellin gene were identified. The locations of these subfragments are also shown in Fig. 1. Two such subfragments (1.74-kb *Eco*RI-*Sac*I fragment from clone 35209-4 and 1.07-kb *Eco*RI-*Sac*I fragment from clone 35209-3) were subcloned into a bacteriophage M13 vector, and their nucleic acid sequences were determined. (Clone 35209-3 was eventually found to contain a truncated version of the flagellin gene.) The complete nucleotide sequence of the 1.74-kb subfragment from clone 35209-4 is shown in Fig. 2. Computer analysis of this sequence identified within it a single open reading frame (ORF) of 1,005 bp (6 bp shorter than the *B. burgdorferi* flagellin gene sequence). The deduced amino acid sequence of this ORF consists of 334 amino acids, 2 fewer than the *B. burgdorferi* flagellin protein sequence. Figure 3 shows a comparison of the amino acid sequences of the flagellin genes of the two organisms. The first 16 residues of the *B. hermsii* ORF are identical to the N-terminal sequence of the *B. burgdorferi* flagellin gene, as determined by protein sequencing (19).

**Sequence comparison of the flagellin genes of *B. hermsii* and *B. burgdorferi*.** DNA sequence analysis of the *B. hermsii* ORF and the *B. burgdorferi* flagellin gene showed that they possess an overall sequence similarity of 85%, with regions at the N and C termini showing 90 and 88% similarities, respectively (Fig. 4A). When the sequences were translated and their amino acid sequences were compared, these levels rose to 95, 96, and 95%, respectively (Fig. 4A). These findings support the conclusion that the ORF located within the nucleotide sequence of Fig. 2 represents the flagellin gene of *B. hermsii*. Interestingly, computer analysis of the upstream and downstream flanking sequences of the two flagellin genes showed that these high levels of sequence identity were not maintained outside of the coding region (data not shown). (The noncoding sequences 5′ to the flagellin gene of *B. burgdorferi* were derived by sequencing a 0.68-kb *Xba*I fragment from clone 35210-8 [Fig. 1] which


FIG. 2. Complete nucleotide sequence of the 1,736-bp *Eco*RI-*Sau*3A fragment from clone 35209-4. The sequence was determined on both strands. The deduced amino acid sequence of a 1,005-bp ORF found within this fragment is shown below the nucleotide sequence.

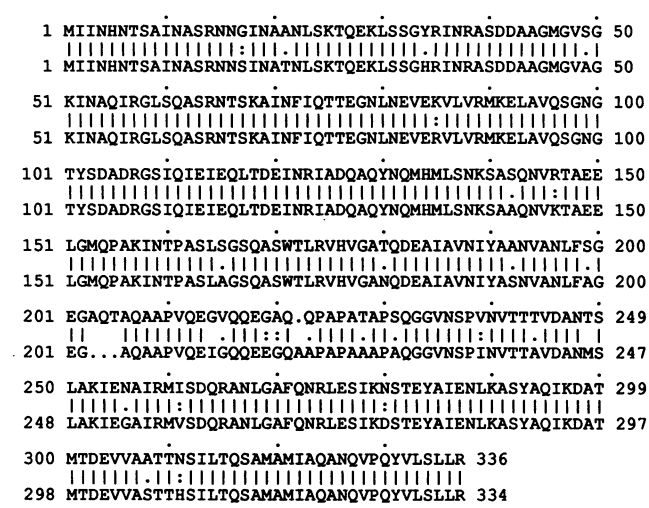


FIG. 3. Deduced amino acid sequences of the flagellin genes of *B. burgdorferi* (top) and *B. hermsii* (bottom), showing the extent of sequence similarity.

contains the first 190 bp of the flagellin gene.) The flagellin gene of *B. hermsii* showed considerably less sequence identity with the *flaB2* endoflagellum subunit of *T. pallidum* (36). The calculated degrees of sequence similarity were 62% at the amino acid level and 57% at the nucleotide level (data not shown).

From Fig. 4A it is apparent that although the two genes possess a high overall degree of similarity, there is a central region (from bp 491 to 764 on the *B. burgdorferi* sequence) of reduced sequence identity (73%). Figure 4B shows a detailed comparison of the two sequences in this central region. It is evident that there are numerous mismatches and at least one deletion (or duplication) within this segment. It therefore seemed possible that oligonucleotide sequences derived from this region might serve as primers and probes for the specific amplification and detection of *B. burgdorferi*. On the basis of these results, primer sequences (bp 492 to 516 [FL6] and bp 743 to 767 [FL7]) and a probe sequence (bp 594 to 642 [FL8]) were chosen from the *B. burgdorferi* flagellin gene and tested for their ability to specifically amplify and detect *B. burgdorferi* genomic DNA. For this initial experiment, equal amounts (1 ng) of chromosomal DNAs from four *Borrelia* strains were used (*B. hermsii* HS1, *B. burgdorferi* B31, *B. burgdorferi* IRS, and *B. coriaceae* Co53). Analysis of PCR products by agarose gel electrophoresis revealed that the primers generated an amplified fragment of DNA from the two strains of *B. burgdorferi* but not from *B. hermsii* or *B. coriaceae*. When these fragments were transferred to a hybridization membrane by the Southern method (44) and the membrane was probed with oligonucleotide probe sequence FL8, a signal was detectable only from the two *B. burgdorferi* strains. Thus, the primer-probe system specifically amplified and detected the two strains of *B. burgdorferi* but did not cross-react with *B. hermsii* or *B. coriaceae* (data not shown).

**Testing of the *B. burgdorferi*-specific PCR primer-probe system.** The primer-probe system described above was tested more exhaustively in PCR experiments by including a wide variety of *Borrelia* strains and other spirochetes obtained from diverse geographic locations. The primer pair FL6 and FL7 amplified the appropriate segment of the flagellin gene from all strains of *B. burgdorferi* tested (22 strains) but not from 5 strains of *B. hermsii* or 5 other *Borrelia* species (Table 2). Other spirochetes from the genera *Treponema*, *Leptospira*, and *Spirochaeta* as well as members of 10 unrelated genera of bacteria were all negative, both for amplification of fragments and for hybridization of the oligonucleotide probe. The bacterial strains tested were as follows: *T. pallidum*, *T. pectinovorum*, *T. phagedenis*, *T. denticola* (ATCC 33520), *T. socranskii* subsp. *buccale* (ATCC 35534), *T. socranskii* subsp. *paredis* (ATCC 35535), *T. socranskii* subsp. *socranskii* (ATCC 35536), *T. vincentii* (ATCC 35580), *Leptospira interrogans* serovar autumnalis (ATCC 23476), *L. interrogans* serovar budapest (ATCC 23581), *L. interrogans* serovar icterohaemorrhagiae (ATCC 43642), *L. weilii* serovar celledoni (ATCC 43285), *L. inadai* serovar lyme (ATCC 43289), *Spirochaeta* sp. (ATCC 43810), *Spirochaeta* sp. (ATCC 43811), *Spirochaeta aurantia* (ATCC 25082), *E. coli*, *Salmonella typhi* (ATCC 19430), *Shigella* sp., *Proteus* sp., *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 12600), *Klebsiella pneumoniae* (ATCC 13883), *Serratia marcescens* (ATCC 13880), *Enterobacter aerogenes* (ATCC 13048), and *Streptococcus pyogenes* group A (ATCC 12344).

However, amplified fragments from certain *Borrelia* strains did not hybridize to the oligonucleotide sequence (FL8) selected as a confirmatory probe (Table 2). All of these strains were isolated in Germany or Switzerland, and one of them (P/Bi) was a member of a group of strains which has recently been shown to share only 50% DNA homology with the type strain of *B. burgdorferi*, B31 (ATCC 35210) (40). These results therefore seemed to be in agreement with the premise that *B. burgdorferi*, as presently designated, actually comprises more than one subspecies or group.

**Differentiation of *B. burgdorferi* strains into three groups based on flagellin gene sequences.** The above-described findings were investigated further by cloning and sequencing of the amplified flagellin gene fragments from all of the aberrant European strains (seven strains) and one unusual North American strain (25015). The sequence data (Fig. 5) revealed that the unusual North American strain, which has recently been shown to be nonpathogenic (2), differed only slightly from the *B. burgdorferi* type strain, B31, and therefore may be considered to belong to this group or subtype. However, the aberrant European strains were found to fall into two groups—the P/Bi group (five strains) and the P/Sto group (two strains)—which differed significantly from each other and from the B31 group. Within these groups, the sequences of all members were found to be identical, with the exception of that of VS185, which may be considered a minor variant of the P/Bi group. Translation of the nucleotide sequences obtained further reinforced the notion that the strains belong to three different groups of organisms, in that significant differences in amino acid sequences are apparent among the groups (Fig. 6).

The sequence data presented in Fig. 5 suggested the possibility of designing oligonucleotide probes capable of distinguishing the three groups of *B. burgdorferi*. The original probe, FL8 (bp 594 to 642 of the B31 sequence; Fig. 5), would serve to distinguish the B31 group of strains. In addition, an oligonucleotide derived from the P/Bi sequence, FL15 (bp 594 to 641; Fig. 5), was selected as a putative specific probe for the P/Bi group of strains, and an oligonucleotide derived from the P/Sto sequence, FL16 (bp 594 to 644; Fig. 5), was selected for the group comprising strains P/Sto and P/Gau. For testing of these probe sequences, PCR amplifications were carried out with a standard amount (1 ng) of purified genomic DNA from the 22 strains of *B.*

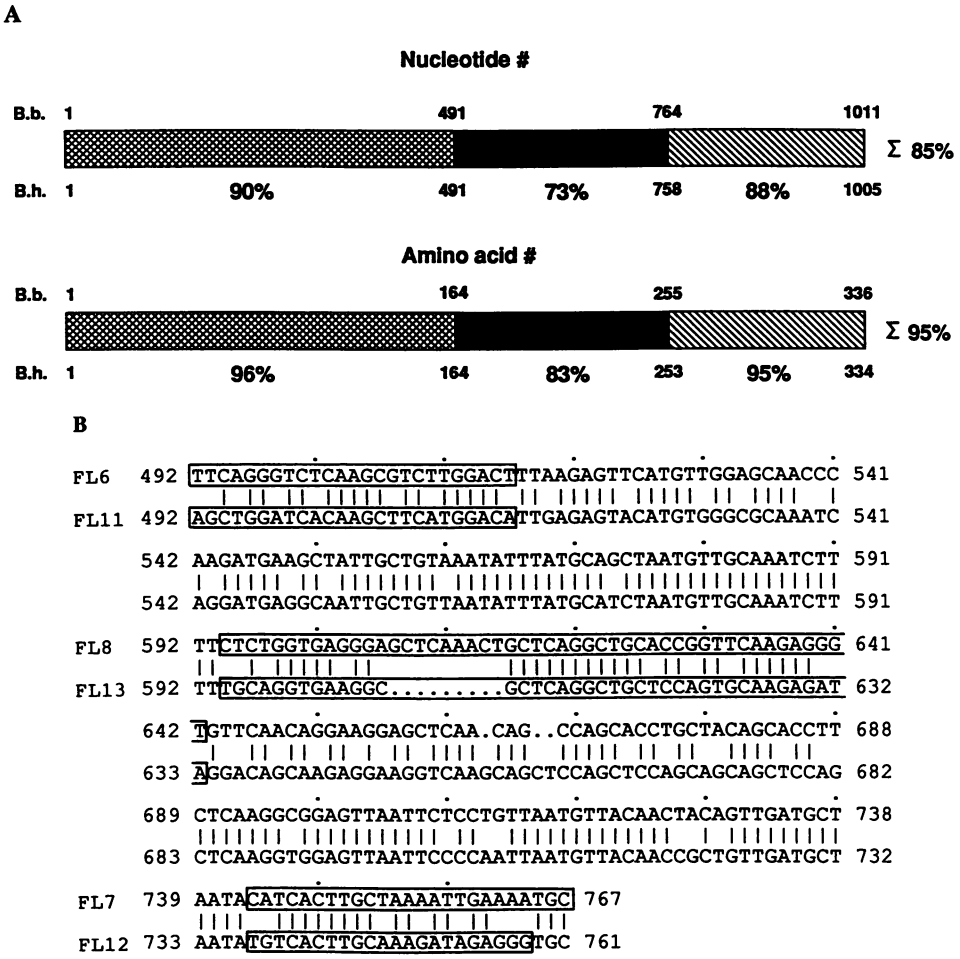


FIG. 4. (A) Different degrees of sequence similarity existing between corresponding segments of the flagellin genes of *B. burgdorferi* (B.b.) and *B. hermsii* (B.h.) at both the nucleotide and the amino acid levels. Symbols: ▩, N terminus; ▨, C terminus; Σ, overall homology. (B) Detailed comparison of the central segments of the two genes. The *B. burgdorferi* sequence is positioned above the *B. hermsii* sequence. The locations of *B. burgdorferi* primer sequences FL6 (bp 492 to 516) and FL7 (bp 743 to 767) and probe sequence FL8 (bp 594 to 642) and of *B. hermsii* primer sequences FL11 (bp 492 to 516) and FL12 (bp 737 to 758) and probe sequence FL13 (bp 594 to 633) are boxed.

*burgdorferi*, 5 strains of *B. hermsii*, 1 strain each of *B. parkeri*, *B. turicatae*, *B. crocidurae*, *B. anserina*, and *B. coriaceae*, and 5 strains from the related genera *Treponema* and *Leptospira* (see the legend to Fig. 7). Aliquots from these PCR amplifications were subjected to electrophoretic separation on three identical agarose gels (Fig. 7A), and the amplified fragments were transferred to hybridization membranes by the Southern method (44). The primer sequences yielded specific amplification from *B. burgdorferi* strains only (Fig. 7A). The three identical Southern blots were probed with the oligonucleotide probes described above (FL8, FL16, and FL15), and autoradiography revealed the patterns of reactivity shown in Fig. 7B to D. The B31 group probe (Fig. 7B) hybridized to the first 15 strains only, comprising the B31 group. The P/Sto group probe hybridized only to strains P/Sto and P/Gau (Fig. 7C) but cross-reacted slightly with two unusual isolates, DN127 and 25015. The P/Bi group probe hybridized exclusively to the five strains from this group, P/Bi, GTI, G2, VS3, and VS185 (Fig. 7D).

**PCR primer-probe system for the detection and differentiation of other *Borrelia* species.** On the basis of the determined nucleotide sequence of the *B. hermsii* flagellin gene (Fig. 4B), the corresponding oligonucleotide sequences were tested as a primer-probe system for the specific amplification and detection of this organism. The primer sequences used contained bp 492 to 516 (FL11) and bp 737 to 758 (FL12) (Fig. 4B). The confirmatory probe sequence used contained bp 594 to 633 (FL13) (Fig. 4B). The primer-probe system was tested against different *Borrelia* species, including four other strains of *B. hermsii* as well as *B. parkeri*, *B. turicatae*, *B. crocidurae*, *B. anserina*, and *B. coriaceae*. Interestingly, the primers amplified a fragment of the appropriate size from all *Borrelia* species other than *B. burgdorferi* (Fig. 8A). However, oligonucleotide probe FL13 hybridized only to the five strains of *B. hermsii* and *B. anserina* (Fig. 8B). In addition, one strain of *B. hermsii* (YOR-1) reacted somewhat poorly with the probe. Since *B. hermsii*, *B. parkeri*, and *B. turicatae* have been reported to share from 77 to 95% total DNA homology (23), this result was unexpected. It was therefore considered of interest to clone the amplified fragments from the North American relapsing fever spirochetes and determine their nucleotide sequences. The sequence data for *B. hermsii* HS1 (type strain), *B. parkeri*, *B. turicatae*, and the one strain of *B. hermsii* (YOR-1) which hybridized poorly to the probe are shown in Fig. 9.

The aberrant strain of *B. hermsii* (YOR-1) differs only

TABLE 2. *Borrelia* species and strains tested

| Species | Strain | Biological origin[a] | Geographic location | PCR primer[b] reactivity | Probe[c] reactivity |
|---|---|---|---|---|---|
| *B. burgdorferi* | B31 (ATCC 35210) | Tick (*Ixodes dammini*) | New York | + | + |
| | IRS (ATCC 35211) | Tick (*Ixodes ricinus*) | Switzerland | + | + |
| | MMI | Mouse (*Peromyscus* sp.) | Minnesota | + | + |
| | MMTI | Mouse tick (*Ixodes* sp.) | Minnesota | + | + |
| | IPS | Tick (*Ixodes pacificus*) | California | + | + |
| | N40 | Tick (*I. dammini*) | New York | + | + |
| | Son 328 | Tick (*I. pacificus*) | California | + | + |
| | Minnesota Mouse | | Minnesota | + | + |
| | Lake 339 | Tick (*I. pacificus*) | California | + | + |
| | 20001 | Tick (*I. ricinus*) | France | + | + |
| | CD16 | | Minnesota | + | + |
| | VS215 | Tick (*I. ricinus*) | Switzerland | + | + |
| | NE56 | Tick (*I. ricinus*) | Switzerland | + | + |
| | DN127 | Tick (*I. pacificus*) | California | + | ± |
| | 25015 | Tick (*I. dammini*) | New York | + | ± |
| | P/Sto | Skin (ACA) | Germany | + | − |
| | P/Gau | Skin (ACA) | Germany | + | − |
| | P/Bi | Cerebrospinal fluid | Germany | + | − |
| | G2 | Cerebrospinal fluid | Germany | + | − |
| | GTI | Tick | Germany | + | − |
| | VS3 | Tick (*I. ricinus*) | Switzerland | + | − |
| | VS185 | Tick (*I. ricinus*) | Switzerland | + | − |
| *B. hermsii* | HS1 (ATCC 35209) | Tick (*Ornithodorus hermsii*) | Washington | − | − |
| | Frogner | Human blood | Washington | − | − |
| | YOR-1 | Human blood | California | − | − |
| | CON-1 | Human blood | California | − | − |
| | MAN-1 | Human blood | California | − | − |
| *B. parkeri* | | | | − | − |
| *B. turicatae* | | | | − | − |
| *B. crocidurae* | | | | − | − |
| *B. anserina* | | | | − | − |
| *B. coriaceae* | Co53 (ATCC 43381) | Tick (*Ornithodorus coriaceus*) | California | − | − |

[a] When known. ACA, acrodermatitis chronica atrophicans.
[b] Primers FL6 and FL7 (Table 1).
[c] Probe FL8 (Table 1).

slightly from the type strain in its nucleotide sequence and may therefore be considered a true member of the species *B. hermsii*. However, the sequences of *B. parkeri* and *B. turicatae* are appreciably different from that of *B. hermsii* and very similar to each other (Fig. 9). This result is even more apparent when the deduced amino acid sequences are compared (Fig. 10). These findings seem to indicate that *B. parkeri* and *B. turicatae* are more closely related to each other than to *B. hermsii* and suggest the possibility of designing a PCR-DNA probe test for relapsing fever *Borrelia* species that would be capable of distinguishing the causative organism as being of either the *B. hermsii* group or the *B. parkeri-B. turicatae* group. Thus, on the basis of the sequence data in Fig. 9, two further oligonucleotide probes were designed. The first probe (FL17), which was designed to detect all five strains of *B. hermsii* with equal intensity, has the same sequence as FL13 (bp 594 to 633; Fig. 9) but contains a C/T redundancy at base 606 and an A/T redundancy at base 623. The second probe (FL18), derived from the *B. parkeri* sequence (bp 594 to 636; Fig. 9), was designed to detect *B. parkeri* and *B. turicatae* only.

The putative *B. hermsii*-specific and *B. parkeri-B. turicatae*-specific probes were tested against the same set of strains as that used previously by probing Southern blots of agarose gels identical to that in Fig. 8A. The results of these experiments are also shown in Fig. 8, in which it can be seen that probe FL17 detected all five strains of *B. hermsii* with equal intensity (Fig. 8C) but still cross-reacted with *B. anserina*. Probe FL18 detected *B. parkeri* and *B. turicatae* exclusively (Fig. 8D).

## DISCUSSION

The present study represents an attempt to devise species-specific but all-inclusive PCR-DNA probe systems for the direct detection of *B. burgdorferi* and the North American relapsing fever spirochetes. For guaranteeing the all-inclusive aspect of the requirements, the gene that was targeted for the development of these systems was the *Borrelia* flagellin gene. Previous DNA homology studies on various *Borrelia* species had demonstrated that the species with the greatest homology to *B. burgdorferi* is *B. hermsii* (~58% overall homology) (24). It was therefore anticipated that a similar or even greater degree of sequence similarity would

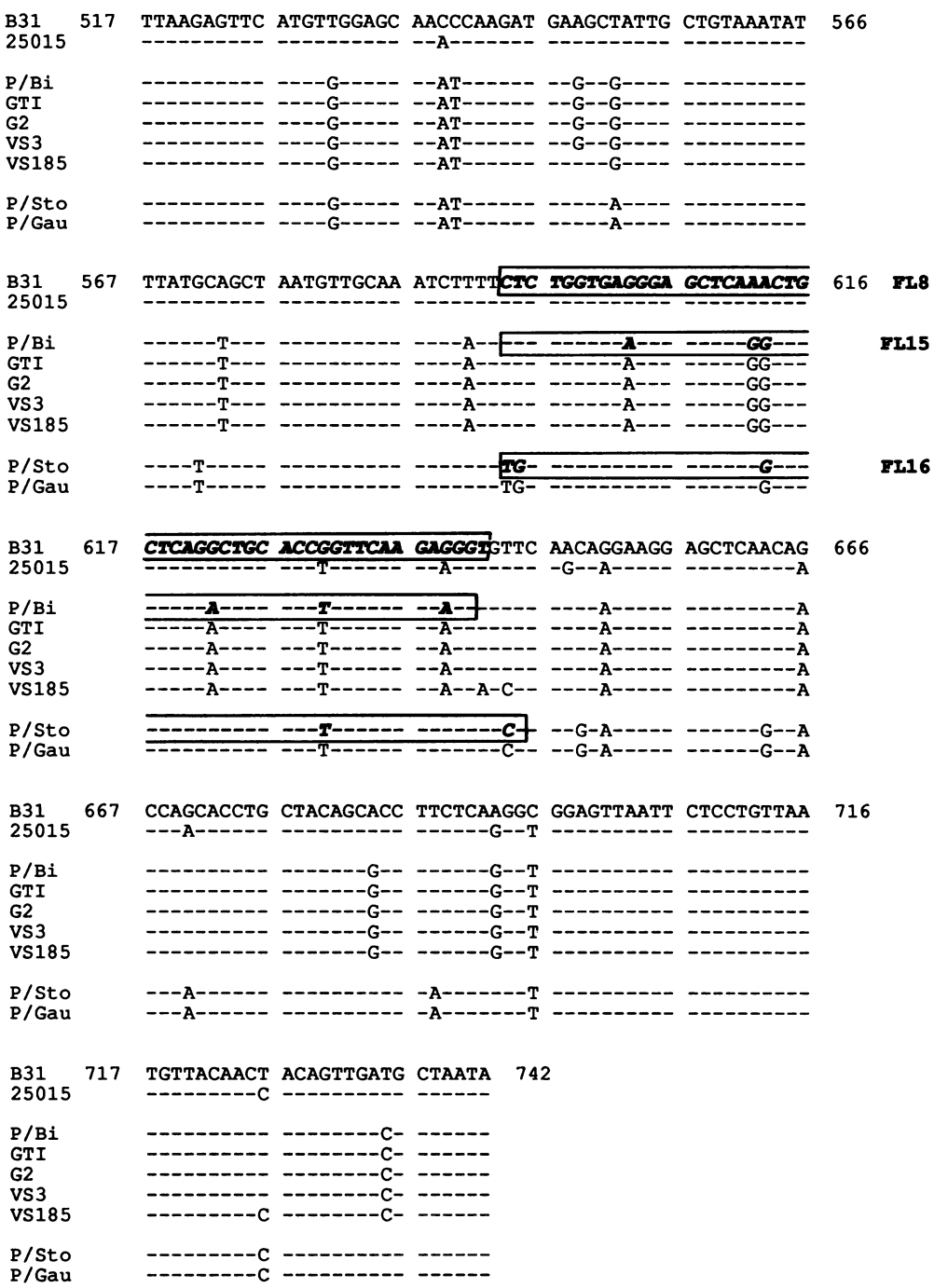


FIG. 5. Nucleotide sequences of the amplified fragments (not including primer sequences) from the seven aberrant strains of *B. burgdorferi* (P/Bi, GTI, G2, VS3, VS185, P/Sto, and P/Gau), one aberrant North American strain (25015), and the type strain, B31, illustrating their division into three basic groups. A dash in the sequence indicates identity with the B31 sequence. The locations of oligonucleotide sequences used as probes to distinguish the three groups (FL8, FL15, and FL16) are shown in boldface italic type and boxed.

be encountered within the flagellin genes of the two organisms, given the highly conserved nature of these genes throughout the bacterial kingdom. For this reason, the chosen strategy was to clone and sequence the flagellin gene from *B. hermsii*, compare the sequence with the *B. burgdorferi* sequence, and determine whether species-specific sequences could be identified. As expected, the two genes shared a very high degree of overall sequence identity (85%). A surprising aspect of this study, however, was the finding that even within the flagellin genes of two such closely related species, the same central variable region as that identified previously from a comparison of the *B. burgdorferi* gene with those of relatively unrelated bacterial genera existed (54). Within this central region, there appears to have

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B31 | 173 | *Leu* | *Arg* | *Val* | *His* | *Val* | *Gly* | *Ala* | *Thr* | *Gln* | *Asp* | *Glu* | *Ala* | *Ile* | *Ala* | *Val* | *Asn* 188 |
| 25015 | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| P/Bi | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| GTI | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| G2 | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| VS3 | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| VS185 | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| P/Sto | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| P/Gau | | - | - | - | - | - | - | - | *Asn* | - | - | - | - | - | - | - | - |
| B31 | 189 | *Ile* | *Tyr* | *Ala* | *Ala* | *Asn* | *Val* | *Ala* | *Asn* | *Leu* | *Phe* | **Ser** | **Gly** | **Glu** | **Gly** | **Ala** | **Gln** 204 |
| 25015 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| P/Bi | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| GTI | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| G2 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| VS3 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| VS185 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| P/Sto | | - | - | *Ser* | - | - | - | - | - | - | - | **Ala** | - | - | - | - | - |
| P/Gau | | - | - | *Ser* | - | - | - | - | - | - | - | *Ala* | - | - | - | - | - |
| B31 | 205 | **Thr** | **Ala** | **Gln** | **Ala** | **Ala** | **Pro** | **Val** | **Gln** | **Glu** | **Gly** | *Val* | *Gln* | *Gln* | *Glu* | *Gly* | *Ala* 220 |
| 25015 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| P/Bi | | **Ala** | - | - | **Thr** | - | - | - | - | - | - | - | - | - | - | - | - |
| GTI | | *Ala* | - | - | *Thr* | - | - | - | - | - | - | - | - | - | - | - | - |
| G2 | | *Ala* | - | - | *Thr* | - | - | - | - | - | - | - | - | - | - | - | - |
| VS3 | | *Ala* | - | - | *Thr* | - | - | - | - | - | - | - | - | - | - | - | - |
| VS185 | | *Ala* | - | - | *Thr* | - | - | - | - | - | - | *Ala* | - | - | - | - | - |
| P/Sto | | **Ala** | - | - | - | - | - | - | - | - | - | **Ala** | - | *Glu* | - | - | - |
| P/Gau | | *Ala* | - | - | - | - | - | - | - | - | - | *Ala* | - | *Glu* | - | - | - |
| B31 | 221 | *Gln* | *Gln* | *Pro* | *Ala* | *Pro* | *Ala* | *Thr* | *Ala* | *Pro* | *Ser* | *Gln* | *Gly* | *Gly* | *Val* | *Asn* | *Ser* 236 |
| 25015 | | - | - | - | *Thr* | - | - | - | - | - | - | - | - | - | - | - | - |
| P/Bi | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| GTI | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| G2 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| VS3 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| VS185 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| P/Sto | | - | - | - | *Thr* | - | - | - | - | - | *Thr* | - | - | - | - | - | - |
| P/Gau | | - | - | - | *Thr* | - | - | - | - | - | *Thr* | - | - | - | - | - | - |
| B31 | 237 | *Pro* | *Val* | *Asn* | *Val* | *Thr* | *Thr* | *Thr* | *Val* | *Asp* | *Ala* | *Asn* | *Thr* 248 | | | | |
| 25015 | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |
| P/Bi | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |
| GTI | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |
| G2 | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |
| VS3 | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |
| VS185 | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |
| P/Sto | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |
| P/Gau | | - | - | - | - | - | - | - | - | - | - | - | - | | | | |

FIG. 6. Deduced amino acid sequences of the amplified fragments from the nine strains of *B. burgdorferi* whose nucleotide sequences are shown in Fig. 5. A dash in the sequence indicates identity with the type strain (B31) sequence. The portions of the sequences from which oligonucleotide probes were derived are shown in boldface type.

been either a short 9-bp duplication of the *B. burgdorferi* sequence (bp 607 to 615 and bp 616 to 624; Fig. 4B), with the concomitant loss of 3 bp at positions 655, 659, and 660, or a 9-bp deletion of the *B. hermsii* sequence, with the same loss of 3 bp from the *B. burgdorferi* gene. The former hypothesis also assumes the mutation of 2 bp of the duplicated 9-mer (GCTAAACT to GCTAGGCT). In total, however, the two genes were found to differ by 6 bp, equivalent to 2 amino acids.

These unexpected findings allowed the identification of primer sequences which, as the results presented here show, are capable of amplifying specifically from *B. burgdorferi*. This single set of primers is all inclusive in that the primers amplify from all North American and European isolates of *B. burgdorferi* tested to date, without cross-reacting with other *Borrelia* or *Treponema* species. Furthermore, the exploitation of fine differences within the sequence of the variable region allowed the design of oligonucleotide probes capable

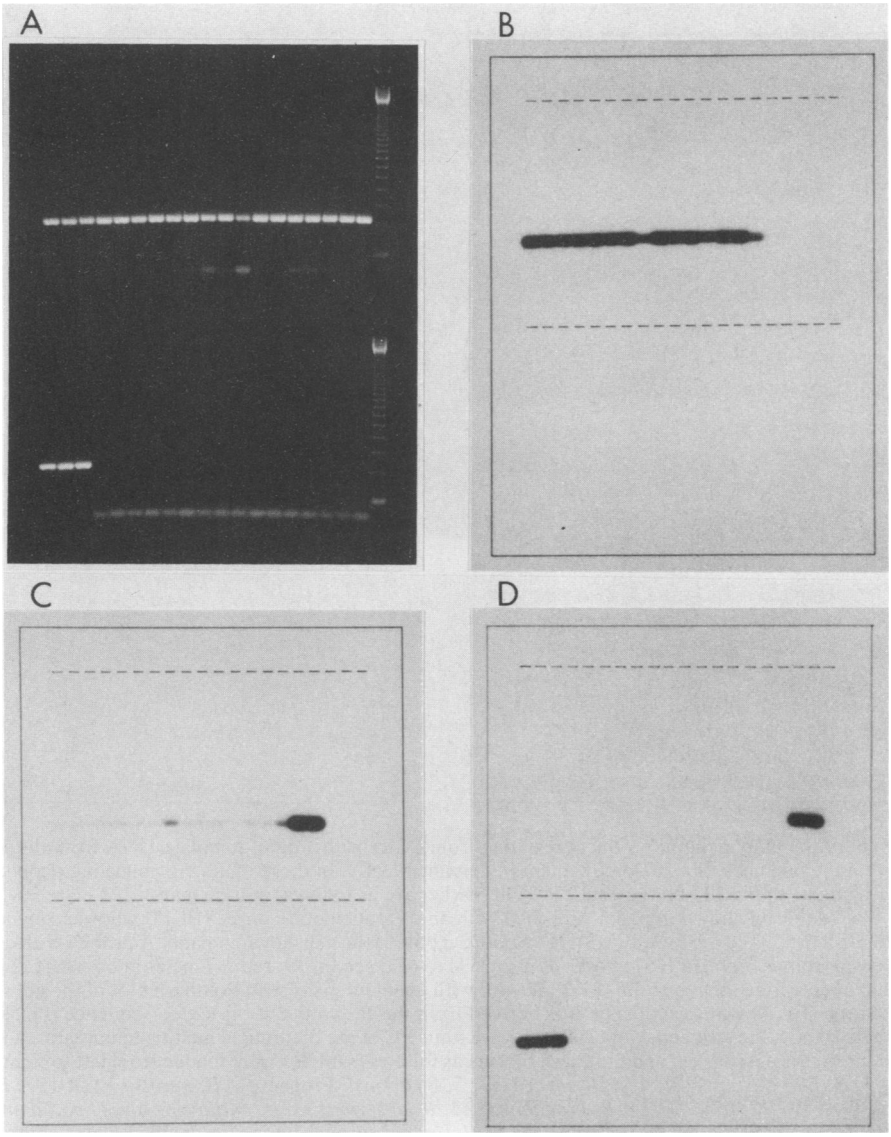


FIG. 7. Agarose gel of the amplification products resulting from PCRs with 1 ng of template DNA from 37 different strains listed below. Lanes 1 to 19 and 21 to 23 reveal the presence of a 276-bp fragment of amplified DNA. In these PCRs, an annealing temperature of 54°C was used. The increasing sizes of fragments in the 123-bp molecular weight marker are as follows (in base pairs): 123, 246, 369, 492, 615, 738, 861, 984, and 1107. (B) Southern blot of the gel shown in panel A probed with an oligonucleotide probe (FL8) whose sequence was derived from the type strain of *B. burgdorferi*, B31 (bp 594 to 642; Fig. 5). (C) Southern blot of the gel shown in panel A probed with an oligonucleotide probe (FL16) whose sequence was derived from *B. burgdorferi* P/Sto (bp 594 to 644; Fig. 5). (D) Southern blot of the gel shown in panel A probed with an oligonucleotide probe (FL15) whose sequence was derived from *B. burgdorferi* P/Bi (bp 594 to 641; Fig. 5). In the Southern blots in panels B to D, the stringency of washing was within 1°C of the theoretical melting temperature (39) of the oligonucleotide probes. The order of strains on the agarose gel and Southern blots is as follows (samples 1 to 20 were loaded from left to right on the upper half of the gel, and samples 21 to 40 were loaded from left to right on the lower half of the gel): 1, B31 (ATCC 35210); 2, IRS (ATCC 35211); 3, MMI; 4, MMTI; 5, IPS; 6, N40; 7, Son 328; 8, DN127; 9, Minnesota Mouse; 10, Lake 339; 11, 20001; 12, CD16; 13, VS215; 14, NE56; 15, 25015; 16, P/Sto; 17, P/Gau; 18, P/Bi; 19, GTI; 20, 123-bp molecular weight marker; 21, G2; 22, VS3; 23, VS185; 24, HS1 (ATCC 35209); 25, Frogner; 26, YOR-1; 27, CON-1; 28, MAN-1; 29, *B. parkeri*; 30, *B. turicatae*; 31, *B. crocidurae*; 32, *B. anserina*; 33, *B. coriaceae*; 34, *T. pallidum*; 35, *T. pectinovorum*; 36, *T. phagedenis*; 37, *L. interrogans* serovar icterohaemorrhagiae; 38, *L. inadai* serovar lyme; 39, distilled water control; 40, 123-bp molecular weight marker. Samples 1 to 19 and 21 to 23 were *B. burgdorferi*. Samples 24 to 28 were *B. hermsii*.

of differentiating *B. burgdorferi* strains into three distinct groups. Whether these groups are definitive or whether additional groups also exist remains to be discovered. Similarly, the significance of the three groups defined by the flagellin gene sequences presented here and their possible relationship to the two groups of *B. burgdorferi* found by others (32, 40, 41) are still unclear. It is tempting to speculate that there might be a correlation between these groups and the perceived differences in the clinical presentation of Lyme borreliosis in North America and Europe, most notably, the lower incidence of arthritis in northern Europe. In this regard, one important advantage of a PCR-DNA probe

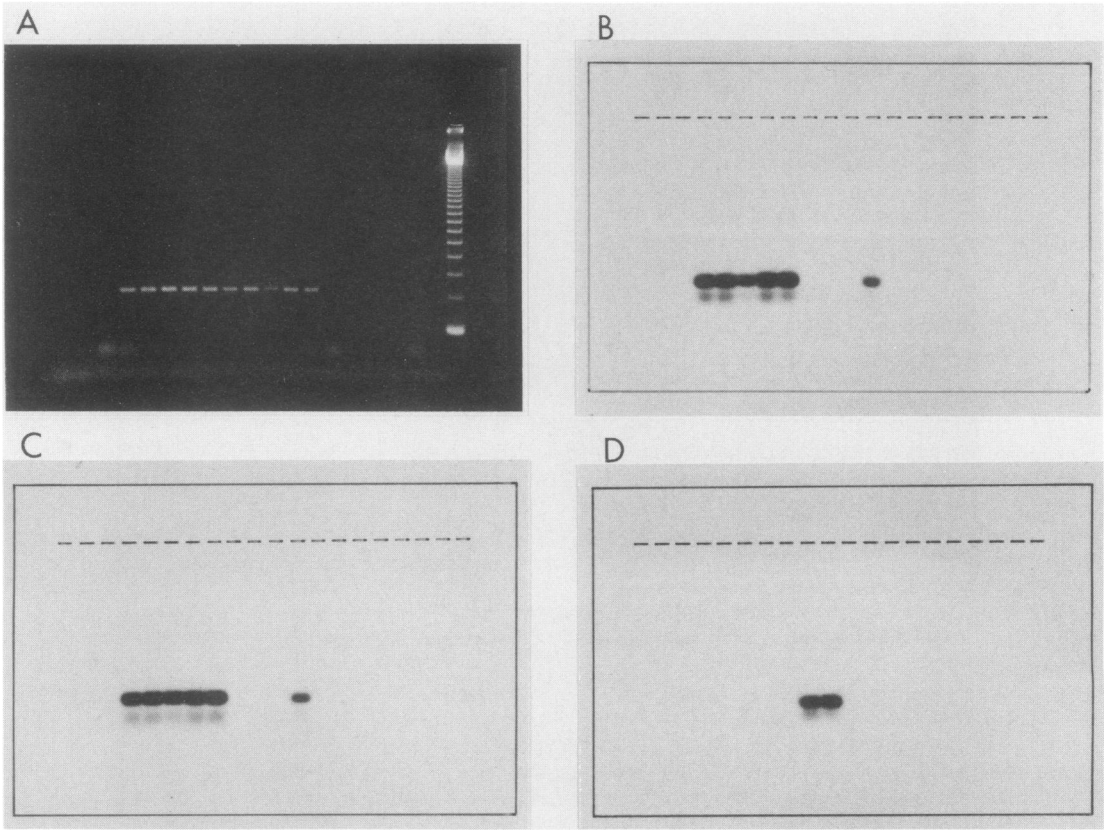


FIG. 8. (A) Agarose gel of the amplification products resulting from PCRs with 1 ng of template DNA from the 18 different strains listed below. Lanes 4 to 13 reveal the presence of a 267-bp fragment of amplified DNA. In these PCRs, an annealing temperature of 49°C was used. The increasing sizes of fragments in the 123-bp molecular weight marker are as follows (in base pairs): 123, 246, 369, 492, 615, 738, 861, 984, and 1107. (B) Southern blot of the gel shown in panel A probed with an oligonucleotide probe (FL13) whose sequence was derived from the type strain of *B. hermsii*, HS1 (bp 594 to 633; see Fig. 9). (C) Southern blot of the gel shown in panel A probed with an adjusted probe (FL17) whose sequence was derived from *B. hermsii* HS1 and YOR-1 (bp 594 to 633; see Fig. 9; redundancies at bp 606 [C/T] and bp 623 [A/T]). This probe is therefore capable of detecting all five strains of *B. hermsii* with equal intensity. (D) Southern blot of the gel shown in panel A probed with an oligonucleotide probe (FL18) whose sequence was derived from the *B. parkeri-B. turicatae* sequence (bp 594 to 636; see Fig. 9). In the Southern blots in panels B to D, the stringency of washing was within 3°C of the theoretical melting temperature (39) of the oligonucleotide probes. The order of strains on the agarose gel and Southern blots is as follows (samples were loaded from left to right): 1, *B. burgdorferi* B31; 2, *B. burgdorferi* P/Sto; 3, *B. burgdorferi* P/Bi; 4, *B. hermsii* HS1; 5, *B. hermsii* Frogner; 6, *B. hermsii* YOR-1; 7, *B. hermsii* CON-1; 8, *B. hermsii* MAN-1; 9, *B. parkeri*; 10, *B. turicatae*; 11, *B. crocidurae*; 12, *B. anserina*; 13, *B. coriaceae*; 14, *T. pallidum*; 15, *T. phagedenis*; 16, *T. denticola*; 17, *L. interrogans* serovar icterohaemorrhagiae; 18, *L. inadai* serovar lyme; 19, distilled water control; 20, 123-bp molecular weight marker.

test is its capacity to provide for specific and sensitive detection concomitantly with the provision of epidemiologically significant data (e.g., subgroup typing of strains).

The North American strains investigated in this study appeared to be more homogeneous in their genetic makeup than the European strains. With the exception of two unusual strains (Millbrook 25015 and DN127), all the North American strains tested fell into one group (the B31 group). This group was also represented among the European strains. In contrast, the European strains contained representatives of two additional groups with no North American counterparts. These two groups (P/Bi and P/Sto) differed significantly from each other and from the B31 group in the nucleotide sequence of the variable region in the flagellin gene. Translation of these sequences revealed the presence of amino acid substitutions, which further reinforced the notion of two separate groups. Two members of the P/Bi group (P/Bi and G2) have been investigated by others; P/Bi was found by Postic et al. (40) to fall into their *Borrelia* group 20047, and G2 was classified into a separate European group by Rosa et al. (41). This latter group also contained one other strain investigated by Postic et al. (G25) and found to belong to *Borrelia* group 20047. However, Rosa et al., using their PCR system, found no distinction between G2 and strain P/Gau, which in the present study was found to belong to a second distinct group of European strains (with P/Sto). On the basis of these findings, the tempting prospect of establishing a correlation between the division of strains into subgroups and their biological origin or the original disease state from which they were isolated arises. Thus, of the five members of the P/Bi group, the two human strains (P/Bi and G2) were both cultured from cerebrospinal fluid, while both members of the P/Sto group (P/Sto and P/Gau) were derived from acrodermatitis chronica atrophicans lesions. However, more clinically characterized strains need to be tested to determine whether such correlations are meaningful.

The PCR-DNA probe assay system, as presently configured, thus consists of a single set of PCR primers which are

```
HS1   517  TTGAGAGTAC ATGTGGGCGC AAATCAGGAT GAGGCAATTG CTGTTAATAT  566
YOR-1      ---------- -------A-- ---------- ---------- ----------

park       --A------- -------T-- ---------- --A------- ----------
turi       --A------- -------T-- ---------- --A------- ----------


HS1   567  TTATGCATCT AATGTTGCAA ATCTTTTTGC AGGTGAAGGC GCTCAGGCTG  616
YOR-1      ---------- ---------- -------[--- ---------T ----------]  FL17

park       ---------- ---------- -C-----[--- ---------T --G----T-T]  FL18
turi       -------G-- ---------- -C-------- ---------T --G----T-T


HS1   617  CTCCAGTGCA AGAGATAGGA CAGCAAGAGG AAGGTCAAGC AGCTCCAGCT  666
YOR-1      ------A--- ----------] ---------- ---------- ----------

park       ------CT-- G--AGGT-C-] --A------- G--T------ T--------A
turi       ------CT-- G--AGGT-C- --A------- G--T------ T--------A


HS1   667  CCAGCAGCAG CTCCAGCTCA AGGTGGAGTT AATTCCCCAA TTAATGTTAC  716
YOR-1      ---------- ---------- ------G--- -----T---- ----------

park       ---------- ---------- ---------- -----A---G ----------
turi       ---------- ---------- ---------- -----T---G ----------


HS1   717  AACCGCTGTT GATGCTAATA  736
YOR-1      ---------- ----------

park       ---TA----- ----------
turi       ---TA--A-- ----------
```

FIG. 9. Nucleotide sequences of the amplified fragments (not including primer sequences) from *B. hermsii* HS1 and YOR-1 as well as *B. parkeri* (park) and *B. turicatae* (turi), illustrating their division into two basic groups. A dash in the sequence indicates identity with the HS1 sequence. The locations of oligonucleotide sequences used as probes to distinguish the two groups (FL17 and FL18) are shown in boldface italic type and boxed.

capable of amplifying specifically from all Lyme disease-associated strains of *B. burgdorferi* tested to date and three oligonucleotide probes which are able to differentiate these strains into three (possibly epidemiologically significant) groups. This configuration is particularly suited to the development of automated PCR-DNA probe assay systems. Thus, the first step in the investigation of clinical specimens suspected of containing *B. burgdorferi* would be an amplification step with a single set of universal primers. Amplified nucleic acids would then be detected by hybridization of the three oligonucleotide probes, each labeled with a different reporter molecule, e.g., different fluorescent labels. Moreover, the hybridization step could take place in solution with the three probes added simultaneously as, for instance, in fluorescence polarization assays (34), in which hybridization of the labeled probe is sufficient to generate a detectable signal. In such a case, the wavelength of the emitted signal would indicate which of the three probes had hybridized to the amplified target sequence and thus allow two simultaneous results: first, determination of whether *B. burgdorferi* was present in the sample, and second, identification of the *B. burgdorferi* subgroup. This approach, using fluorescence measurements, would obviate the need for nested sets of primers to reduce nonspecific amplification of eukaryotic sequences.

Determination of the nucleotide sequence of the flagellin gene of *B. hermsii* allowed the design and testing of primer and probe sequences for the specific amplification and detection of this organism. In this case, it was a surprising observation that the primers chosen were able to amplify from six different *Borrelia* species other than *B. burgdorferi*. More surprising was the result obtained in hybridization experiments. The first probe tested, which was based on the *B. hermsii* flagellin gene sequence, hybridized only to strains of *B. hermsii* and *B. anserina*. This observation was unexpected, since prior DNA homology studies (23) had indicated that *B. hermsii*, *B. parkeri*, and *B. turicatae* shared from 77 to 95% total genomic homology and should perhaps more properly be considered a single species. Consistent with this view, the PCR primer system devised by Rosa et al. amplified from all three species with equal facility (41). However, nucleotide sequence determinations of the amplified fragments from *B. parkeri* and *B. turicatae* revealed that, in the variable regions of their flagellin genes, the sequences of the two species were almost identical but were markedly different from that of *B. hermsii*. Furthermore, an oligonucleotide probe based on these sequences detected only *B. parkeri* and *B. turicatae*. There was no cross-reactivity with *B. hermsii* or other *Borrelia* species. These results raise the possibility of designing a PCR-DNA probe system for the relapsing fever borrelias which would be able to distinguish the causative agent as being of either the *B. hermsii* or the *B. parkeri-B. turicatae* group. Since no cross-reactivity of either the *B. hermsii* or the *B. parkeri-B. turicatae* probes was observed with *B. crocidurae* or *B. coriaceae*, it is possible that cloning and sequencing of the amplified fragments from these two species would allow the design of *B. crocidurae* and *B. coriaceae* probes. One note of caution which should be added is that, at the present time, very few strains of these organisms are available for study, and the results described here are based on only one strain of each species. Furthermore, single-colony cloning of stock and reference cultures of these organisms has not routinely been carried out. However, on acrylamide gels, the protein

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HS1 | 173 | *Leu* | *Arg* | *Val* | *His* | *Val* | *Gly* | *Ala* | *Asn* | *Gln* | *Asp* | *Glu* | *Ala* | *Ile* | *Ala* | *Val* | *Asn* 188 |
| YOR-1 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| park | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| turi | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| HS1 | 189 | *Ile* | *Tyr* | *Ala* | *Ser* | *Asn* | *Val* | *Ala* | *Asn* | *Leu* | *Phe* | **Ala** | **Gly** | **Glu** | **Gly** | **Ala** | **Gln** 204 |
| YOR-1 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| park | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| turi | | - | - | - | *Ala* | - | - | - | - | - | - | - | - | - | - | - | |
| HS1 | 205 | **Ala** | **Ala** | **Pro** | **Val** | **Gln** | **Glu** | **Ile** | *Gly* | *Gln* | *Gln* | *Glu* | *Glu* | *Gly* | *Gln* | *Ala* | *Ala* 220 |
| YOR-1 | | - | - | - | *Glu* | - | - | - | - | - | - | - | - | - | - | - | |
| park | | **Val** | **Ser** | - | **Ala** | - | - | **Gly** | **Ala** | - | - | - | *Gly* | *Val* | - | - | - |
| turi | | *Val* | *Ser* | - | *Ala* | - | - | *Gly* | *Ala* | - | - | - | *Gly* | *Val* | - | - | - |
| HS1 | 221 | *Pro* | *Ala* | *Pro* | *Ala* | *Ala* | *Ala* | *Pro* | *Ala* | *Gln* | *Gly* | *Gly* | *Val* | *Asn* | *Ser* | *Pro* | *Ile* 236 |
| YOR-1 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| park | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | *Val* |
| turi | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | *Val* |
| HS1 | 237 | *Asn* | *Val* | *Thr* | *Thr* | *Ala* | *Val* | *Asp* | *Ala* | *Asn* 245 | | | | | | | |
| YOR-1 | | - | - | - | - | - | - | - | - | - | | | | | | | |
| park | | - | - | - | - | *Thr* | - | - | - | - | | | | | | | |
| turi | | - | - | - | - | *Thr* | *Ile* | - | - | - | | | | | | | |

FIG. 10. Deduced amino acid sequences of the amplified fragments from the two strains of *B. hermsii* (HS1 and YOR-1), *B. parkeri* (park), and *B. turicatae* (turi) whose nucleotide sequences are shown in Fig. 9. A dash in the sequence indicates identity with the type strain (HS1). The portions of the sequences from which oligonucleotide probes were derived are shown in boldface type.

profiles of the strains of *B. parkeri* and *B. turicatae* used in this study were markedly different (data not shown).

The protean clinical manifestations of chronic Lyme borreliosis have been commented upon extensively (1, 15, 35). Recently, Lavoie et al. (28) described several cases of chronic disease in which the symptoms were similar to those of chronic Lyme borreliosis, there was a history of exposure to *Ornithodorus hermsii*, and serum antibody titers to *B. hermsii* were in excess of those to *B. burgdorferi*. A similar instance was also described by Lange et al. (27) and prompted the authors to hypothesize that in certain situations or in selected individuals, relapsing fever can produce a chronic clinical picture analogous to Lyme disease. These findings suggest that in certain parts of North America, such as the northwestern United States, supposed cases of Lyme disease could be caused by other *Borrelia* species. In addition, it has been pointed out (11) that, at the present time, for most cases of tick-borne relapsing fever, taxonomic identification of the etiologic agent is presumptive and is usually based on the history of exposure to a particular vector. Also, because of the sporadic occurrence of tick-borne relapsing fever, extremely little is known about the worldwide incidence of this disease. A PCR-DNA probe system that is capable of detecting and differentiating relapsing fever and other borreliae could be of significant value in the investigation of field specimens of ticks and in worldwide epidemiological studies of relapsing fever. Furthermore although the precise role of PCR-DNA probe tests in the diagnosis of chronic and acute disease is still unclear, such tests might aid in the investigation of clinical specimens and specific incidents of chronic disease mimicking Lyme borreliosis. These considerations enhance the relevance of the question of cross-reactivity of PCR primers and probes with *B. hermsii*. In view of the recent findings described above, it would seem desirable to attempt to be as specific as possible in developing tests for these organisms and particularly to investigate chronic disease with primers and probes that do not exhibit interspecies cross-reactivity.

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