JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2004, p. 1270–1273 Vol. 42, No. 3
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.3.1270–1273.2004

Evaluation of *groEL* Gene Analysis for Identification of *Borrelia burgdorferi* Sensu Lato

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Received 22 July 2003/Returned for modification 13 November 2003/Accepted 21 November 2003

The nucleotide sequences of the *groEL* genes, the flagellin genes, and the 16S rRNA genes from 22 reference strains of *Borrelia* were compared. *groEL* sequence analysis is useful not only in interspecies differentiation but also in intraspecies differentiation of *Borrelia afzelii* and *Borrelia garinii* isolates.

Borrelia burgdorferi sensu lato, the causative spirochete of Lyme disease, is transmitted to humans and animals through *Ixodes* ticks (1, 3). Lyme disease is one of the most prevalent tick-borne infectious diseases in Europe and North America and now occurs all over the world (1, 12). Various DNA-based techniques have recently been developed for the species identification of *B. burgdorferi* because characterization and identification by conventional methods are time-consuming and expensive (2, 5, 6, 11). A previous study proposed that *groEL* gene analysis is useful for the differentiation of *B. burgdorferi* sensu lato (9). In the present study, *groEL* gene analysis was compared with the sequence analyses of the 16S rRNA and the flagellin gene to determine the role of the *groEL* gene in defining evolutionary relationships among strains of *B. burgdorferi* sensu lato.

Twelve *Borrelia* strains were recently isolated from *Ixodes granulatus*, *Ixodes nipponensis* (tick vectors for Lyme spirochetes in rare cases), and *Ixodes persulcatus*, and 11 strains were isolated from *Apodemus agrarius* (8, 10). In previous studies, they were characterized as *Borrelia afzelii*, *Borrelia garinii*, and unclassified Haenam strains (8, 10). In the present study, a comparative sequence analysis of the *groEL* gene from Korean isolates was performed to determine their relationships with the known species of the genus *Borrelia*.

Twenty-two reference strains (Table 1) and 23 Korean isolates of the genus *Borrelia* were used in this study. The strains were cultivated at 32°C in Barbour-Stoenner-Kelly II (BSKII) medium. DNA was extracted by a modified version of a previously described method (4). The *groEL* genes of 22 reference strains and 23 Korean isolates and the flagellin genes and the 16S rRNA genes of 22 reference strains were amplified as presented in Table 2. The nucleotide sequences of the recombinant DNA were determined using the CEQ L DNA Analysis System and the CEQ 2000 Dye Terminator Cycle Sequencing kit (Beckman Coulter Inc., Fullerton, Calif.) with forward and reverse sequencing primers (M13) and sequencing primers (Table 2). The multiple-alignment algorithm in the MegAlign software package (Windows version 3.12e; DNASTAR, Madison, Wis.) was used to align the sequences. All positions with alignment gaps were excluded from the pairwise sequence comparison. Phylogenetic trees were constructed by the unweighted pair group method with arithmetic averages using the MEGA program (7). A bootstrap analysis (100 replicates) was performed to evaluate the topology of the phylogenetic tree.

In this study, interspecies differences in the *groEL* genes (positions 552 to 861 in *B. burgdorferi* $B31^T$ numbering; 310 bp) of *B. burgdorferi* strains sensu lato were compared with those in the flagellin genes (positions 280 to 789 in the *B. burgdorferi* $B31^T$ numbering; 510 bp) and 16S rRNA genes (positions 44 to 849 in *B. burgdorferi* $B31^T$ numbering; 806 bp). Moreover, intraspecies differences in the *groEL* genes from *B. burgdorferi*, *B. afzelii*, and *B. garinii* were compared with those in the flagellin and 16S rRNA genes. However, intraspecies differences in the *groEL* genes of other *Borrelia* species could not be compared with those in the flagellin and 16S rRNA genes, because the *groEL* gene sequence of just one strain per species was available (9).

groEL gene analysis has several characteristics different from those of analyses of other genes. Compared with the 16S rRNA genes, *groEL* sequences have higher divergence for strains of *B. burgdorferi* sensu lato. More than 91.6% similarity of the *groEL* gene sequences was observed among strains of *B. burgdorferi* sensu lato. On the other hand, more than 95.4% similarity of the 16S rRNA gene sequences was observed among strains of *B. burgdorferi* sensu lato (data not shown). The *groEL* gene sequence similarities in *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains were 99.7 to 100%, 99.0 to 99.4%, and 96.8 to 100%, respectively (Table 3). On the other hand, the 16S rRNA gene sequence similarities in *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains were 99.6 to 100%, 99.6 to 100%, and 99.1 to 100%, respectively (Table 3). These results showed that the *groEL* gene is more heterogeneous than the 16S rRNA gene and is useful in intraspecies differentiation. Compared with the flagellin gene analysis, more than 92.0% similarity of the flagellin gene sequences was observed in strains of *B. burgdorferi* sensu lato (data not shown). The *groEL* gene sequence similarities in *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains were

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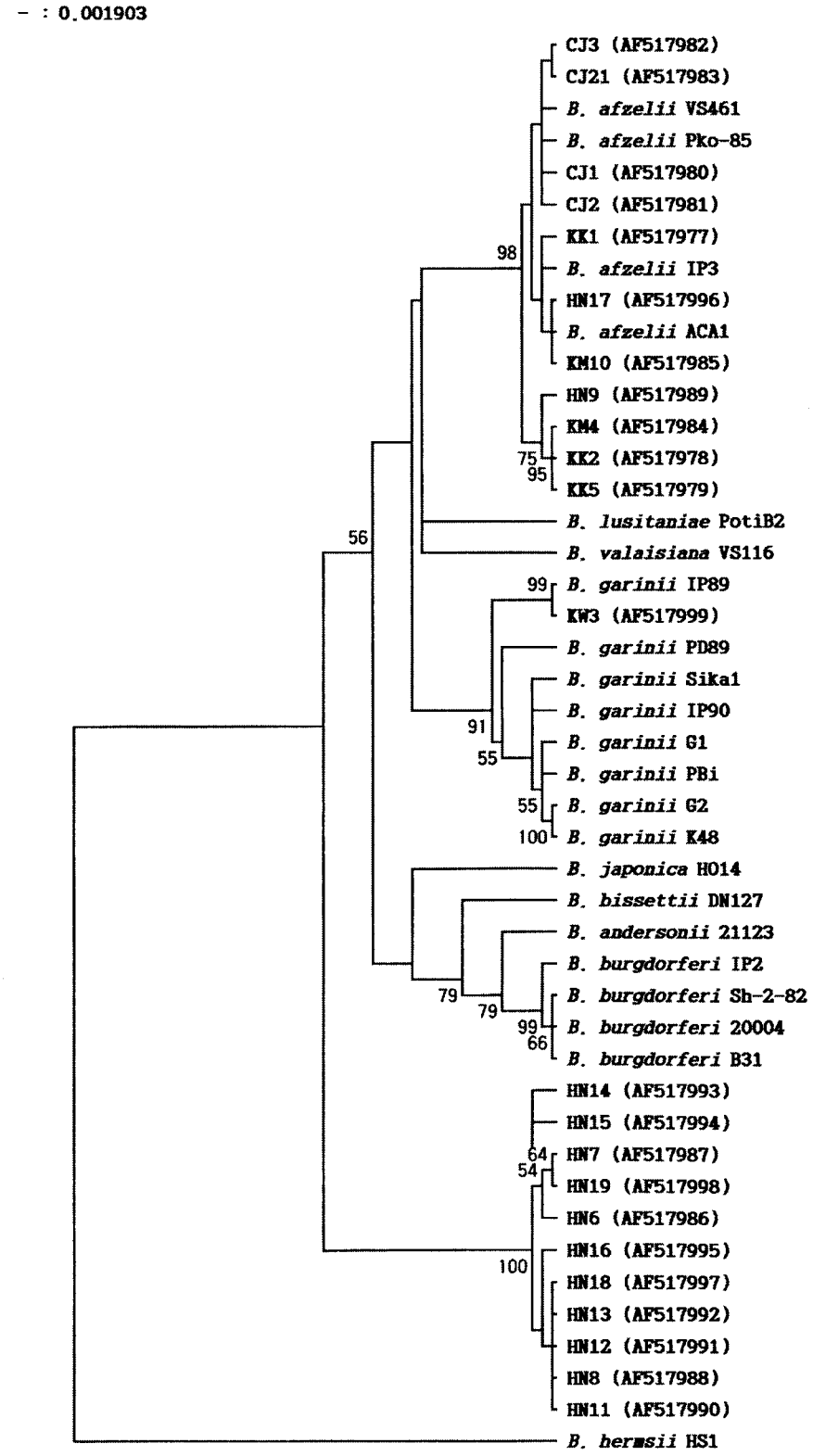

FIG. 1. Phylogenetic tree based on *groEL* gene sequences of *Borrelia* strains. The phylogenetic tree was constructed by the unweighted pair group method with arithmetic averages using MEGA software. Bootstrap analysis was performed with 100 replicates. The GenBank accession numbers are shown in parentheses.

TABLE 1. *Borrelia* reference strains used in this study

Borrelia species	Strain	Source	Geographic location	GenBank accession no.		
				groEL	Flagellin gene	16S rRNA
B. burgdorferi	B31^T	*Ixodes scapularis*	US[b]	AE001166	X15661	U03396
	Sh-2-82	*Ixodes dammini*	US	AF517948	AY342019	M60969
	20004	*Ixodes ricinus*	France	AF517951	AY342018	M64310
	IP2	Human (CSF[a])	France	AF517952	AB057452	AY342028
B. afzelii	Iper3	*Ixodes ricinus*	Russia	AF517953	AY342020	M84815
	VS461^T	*Ixodes ricinus*	Switzerland	AF517954	D63365	AY342034
	ACA1	Human (skin)	Sweden	X54059	AB035613	AB035404
	Pko-85	Skin	Germany	AF517956	AY342021	AY342030
B. garinii	PBi	Human (CSF)	Germany	AF517957	AB035595	X85199
	PD89	Human (blood)	China	AF517958	AY342022	AY342031
	IP90	*Ixodes persulcatus*	Russia	AF517959	L42885	M89937
	G1	Human (CSF)	Germany	AF517960	AY342023	M64311
	G2	Human (CSF)	Germany	AF517961	AY342024	M60967
	Sika1	*Ixodes ovatus*	Japan	AF517963	AY342025	AY342029
	K48	*Ixodes ricinus*	Slovakia	AF517968	AY342026	AY342032
	IP89	*Ixodes persulcatus*	Russia	AF517969	AY342027	AY342033
B. japonica	HO14^T	*Ixodes ovatus*	Japan	AF517970	D82852	L40597
B. valaisiana	VS116^T	*Ixodes ricinus*	Switzerland	AF517976	D82854	X98232
B. lusitaniae	PotiB2^T	*Ixodes ricinus*	Portugal	AF517971	D82856	X98228
B. bissettii	DN127^T	*Ixodes pacificus*	US	AF517974	D82857	L40596
B. andersonii	21123	*Ixodes dentatus*	US	AF517975	D83764	ND[c]
B. andersonii	21038	*Ixodes dentatus*	US	ND	ND	L46701
B. hermsii	HS1^T	*Ornithodoros coriaceus*	US	AF518000	M86838	U42292

[a] CSF, cerebrospinal fluid.
[b] US, United States.
[c] ND, not done.

99.7 to 100%, 99.0 to 99.4%, and 96.8 to 100%, respectively, whereas the flagellin gene sequence similarities in *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains were 99.0 to 99.8%, 99.6 to 100%, and 98.0 to 100%, respectively (Table 3). These results showed that the *groEL* gene is more heterogeneous than the flagellin gene in *B. afzelii* and *B. garinii*, whereas the flagellin gene is more heterogeneous than the *groEL* gene in *B. burgdorferi*.

The *B. garinii* IP89 strain showed the lowest similarity (96.8 to 98.4%) to other *B. garinii* strains (data not shown). This strain was previously classified as a different group with *B. garinii* using multilocus enzyme electrophoresis (2). This strain also showed a different restriction fragment length polymorphism (RFLP) pattern of 5S-23S intergenic spacer amplicons from one of the *B. garinii* strains (11). These results showed that *groEL* sequence analysis is useful not only in interspecies differentiation but also in intraspecies differentiation of *B. garinii* strains. *groEL* gene sequence analysis may be useful for

TABLE 2. Sequences of primers and PCR conditions

Gene (DNA size)	Primer	Sequence	Positions[c]	PCR conditions	
				No. of cycles	Cycle steps[d]
groEL (310 bp)	GF[a]	5′-TACGATTTCTTATGTTGAGGG-3′	552–572	30	94°C for 30 s, 59°C for 45 s, 72°C for 45 s
	GR[a]	5′-CATTGCTTTTCGTCTATCACC-3′	861–841		
Flagellin gene (584 bp)	F1[a]	5′-GCAGTTCAATCAGGTAACGG-3′	280–299	30	94°C for 30 s, 56°C for 45 s, 72°C for 45 s
	F2[a]	5′-AGGTTTTCAATAGCATACTC-3′	863–844		
16S RNA (1,427 bp)	B1[a,b]	5′-CAGTGCGTCTTAAGCATGC-3′	40–58	30	94°C for 30 s, 59°C for 45 s, 72°C for 45 s
	B2[b]	5′-CGACCTTCTTCATTCACGC-3′	416–398		
	B3[b]	5′-GCAGCTAAGAATCTTCCGCAATGG-3′	340–373		
	B4[b]	5′-AAGTTCGCCTTCGCCTCCGGTA-3′	735–714		
	B5[b]	5′-TGTAAGGGTGGAATCTGTTG-3′	681–700		
	B6[b]	5′-CAACCATGCAGCACCTGTATAT-3′	1053–1032		
	B7[b]	5′-TATACAGGTGCTGCATGG-3′	1033–1040		
	B8[a,b]	5′-CCTTAAATACCTTCCTCCC-3′	1466–1448		

[a] Oligonucleotide primers used for PCR amplication.
[b] Oligonucleotide primers used for sequencing.
[c] Position numbers were determined from *B. burgdorferi* B31^T.
[d] Steps in one cycle of PCR.

TABLE 3. Intraspecies variation in the *groEL*, flagellin, and 16S rRNA gene sequences of *Borrelia* strains

Species	No. of strains	% Identity		
		groEL gene	Flagellin gene	16S rRNA gene
B. burgdorferi	4	99.7–100.0	99.0–99.8	99.6–100.0
B. afzelii	4	99.0–99.4	99.6–100.0	99.6–100.0
B. garinii	8	96.8–100.0	98.0–100.0	99.1–100.0

intraspecies differentiation of *B. afzelii* and *B. garinii* strains, whereas flagellin gene sequence analysis may be useful for intraspecies differentiation of *B. burgdorferi* strains.

Twenty-three Korean isolates were characterized by phylogenetic analysis based on *groEL* gene sequences. Eleven strains (KK1, KK2, KK5, KM4, KM10, CJ1, CJ2, CJ3, CJ21, HN9, and HN17), identified as *B. afzelii* through PCR-RFLP analyses of the *ospC* gene and the *rrf-rrl* intergenic spacer in a previous study (10), were also identified as *B. afzelii* by *groEL* gene analysis (Fig. 1). The nucleotide sequence of strain KW3 was identical to that of *B. garinii* IP89 (Fig. 1), and KW3 also showed the same restriction pattern as *B. garinii* IP89 in RFLP analysis of the 5S-23S intergenic spacer amplicons (data not shown). Eleven Haenam strains formed a distinctive cluster, separated from other strains of *B. burgdorferi* sensu lato in the phylogenetic tree (Fig. 1). The sequence similarities among 11 Haenam strains (HN6, HN7, HN8, HN11, HN12, HN13, HN14, HN15, HN16, HN18, and HN19) were 98.7 to 100%. In general, they showed 89.7 to 94.8% similarity with other strains of *B. burgdorferi* sensu lato. The *Mse*I and *Dra*I restriction patterns of the 5S-23S intergenic spacer amplicons of Haenam strains differed from those of other strains of *B. burgdorferi* sensu lato. Furthermore, in the phylogenetic tree based on 16S ribosomal DNA sequences, Haenam strains also formed a distinctive cluster (8).

In conclusion, the *groEL* gene is useful for the identification and characterization of *B. burgdorferi* sensu lato despite the fact that it has a shorter nucleotide sequence (310 bp) than the flagellin gene (510 bp) and the 16S rRNA gene (806 bp).

This work was supported in part by the Ministry of Science and Technology through the Bio-Food and Drug Research Center at Konkuk University, Chungju, Korea.

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