Determination of members of a *Borrelia afzelii*-related group isolated from *Ixodes nipponensis* in Korea as *Borrelia valaisiana*

Toshiyuki Masuzawa,¹ Takako Fukui,¹ Masaki Miyake,¹ Hee-Bok Oh,² Min-Kee Cho,³ Woo-Hyun Chang,³ Yasuyuki Imai¹ and Yasutake Yanagihara^{1†}

Author for correspondence: Toshiyuki Masuzawa. Tel: +81 54 264 5710. Fax: +81 54 264 5715.
e-mail: masuzawa@u-shizuoka-ken.ac.jp

¹ Department of Microbiology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan

² Department of Microbiology, National Institute of Health, Seoul, Korea

³ Department of Microbiology, College of Medicine, Hallym University, Chunchon, Korea

The 16S rRNA sequences of the Korean *Borrelia* strains 5MT and 9MT, isolated from *Ixodes nipponensis*, showed identities of 99.0–99.1% to that of *B. afzelii*. The strains were tentatively classified as belonging to the *B. afzelii*-related group. In this study, Korean isolates, including these strains, were characterized further and compared with recently described new species. These strains generated a RFLP pattern that has not been found previously in RFLP analysis of the 5S–23S rRNA intergenic spacer and the flagellin gene. When phylogenetic trees were constructed, based on the 5S–23S rRNA intergenic spacer, flagellin gene and 16S rRNA sequences, these Korean isolates formed a cluster with the *Borrelia* strain Am501 isolated from *Ixodes columnae* in Japan and *Borrelia valaisiana* strains VS116^T and UK isolated from *Ixodes ricinus* in Europe and were distinguishable from the other species. However, these three groups of strains were divergent from each other in the molecular masses of the putative outer surface protein A (OspA) and in the sequences of the *ospA* gene. These findings suggest that these Korean isolates and one Japanese isolate are members of *B. valaisiana* and that OspA of this species is divergent, as is that of *Borrelia garinii*. This led to the speculation that *B. valaisiana* strains are adapted to the vector ticks found in each locality.

Keywords: *Borrelia valaisiana*, Lyme disease, *Ixodes nipponensis*, 16S rRNA, Korea

INTRODUCTION

Lyme borreliosis is the most prevalent tick-borne zoonotic disease (Steere, 1989) in Europe, North America and Far Eastern countries such as Japan, Russia and China (Anderson, 1989). The causative agent, *Borrelia burgdorferi sensu lato*, is classified into 10 species: *B. burgdorferi sensu stricto* (Burgdorfer *et al.*, 1982; Johnson *et al.*, 1984), isolated in North America and Europe; *Borrelia garinii* (Baranton *et al.*, 1992) and *Borrelia afzelii* (Canica *et al.*, 1993), isolated in Europe and east Asian countries; *Borrelia japonica*,

Borrelia tanukii and *Borrelia turdi*, isolated from *Ixodes ovatus*, *Ixodes tanuki* and *Ixodes turdus*, respectively, in Japan (Fukunaga *et al.*, 1996b; Kawabata *et al.*, 1993); *Borrelia andersonii*, isolated from *Ixodes dentatus* in North America (Marconi *et al.*, 1995); *Borrelia valaisiana* (Wang *et al.*, 1997), formerly genomic group VS116, and *Borrelia lusitaniae* (Le Flèche *et al.*, 1997), formerly genomic group PotiB2, isolated in Europe; and '*Borrelia bissettii*' (Postic *et al.*, 1998), formerly genomic group DN127, isolated in North America.

In Korea, *B. burgdorferi sensu lato* has been isolated from species of tick (*Ixodes granulatus*, *Ixodes nipponensis* and *Ixodes persulcatus*) and wild rodent (*Apodemus agrarius*). However, no cases of Lyme disease have been documented in Korea. These isolates were identified as *B. garinii* or *B. afzelii* by the ribotyping method, 16S rRNA-PCR (Park *et al.*, 1993), reactivity with specific mAbs and 16S rRNA sequencing (Kee

[†] Present address: Department of Medical Microbiology, College of Public Health, University of the Philippines Manila, 625 Pedro Gil Street, Ermita, Manila 1000, The Philippines.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA, 5S–23S intergenic spacer, *fla* and *ospA* sequences reported in this paper are AB013913–AB013917, AB014677–AB014679, AB016974–AB016979 and AB017479.

et al., 1994, 1996). Interestingly, some isolates from *I. nipponensis* were relatively similar, but not identical, to *B. afzelii* and were tentatively classified as belonging to the *B. afzelii*-related group (Kee *et al.*, 1996). On the other hand, no isolates have been found from *I. nipponensis* in Japan.

In this study, Korean isolates tentatively classified as belonging to the *B. afzelii*-related genomic group were further characterized on the basis of sequencing of 16S rRNA, the 5S–23S rRNA intergenic spacer, the flagellin gene and the outer surface protein A (*OspA*) gene and compared with recently described new species.

METHODS

Borrelia isolates. Eight Korean isolates, 5MT, 9MT and 10MT isolated from *I. nipponensis*, 5Y, 17Y and 18Y from *I. granulatus*, 935T from *I. persulcatus* and 934U from *A. agrarius* collected in Korea (Kee *et al.*, 1994, 1996), were cultivated at 30 °C in Barbour–Stoenner–Kelly II medium (Barbour, 1984). The standard strains listed in Table 1 were used for comparison of genetic characteristics.

PCR and RFLP analysis of the 5S–23S rRNA intergenic spacer and the flagellin gene. Primers corresponding to the 3' end of the 5S rRNA (*rrf*) (5' CTGCGAGTTCGCGGGAGA 3'), the 5' end of the 23S rRNA (*rrl*) (5' TCCTAGGCA-TTCACCATA 3'), the 5' end of the flagellin gene (5' GCAGTTCAATCAGGTAACGG 3') and 3' end of the flagellin gene (5' AGGTTTTCAATAGCATACTC 3'), described previously (Fukunaga *et al.*, 1996c; Masuzawa *et al.*, 1996a; Postic *et al.*, 1994), were synthesized by a custom

oligonucleotide synthesis service (Bex Co., Tokyo, Japan). Aliquots (2 ml) of cultures were washed and the cells were resuspended in 100 µl water. The resultant cell suspensions were boiled at 100 °C for 10 min. PCR was performed by the method described previously (Fukunaga *et al.*, 1996c; Postic *et al.*, 1994). The amplification products of the 5S–23S rRNA PCR were digested with *MseI* and *DraI* and the digested DNA was electrophoresed through 16% polyacrylamide gels. The amplified DNA from the flagellin gene was digested with *Bpu1102I*, *HhaI*, *HincII* or *DdeI* and subsequently electrophoresed in 12% polyacrylamide gels. DNA fragments of pBR322 digested with *MspI*, ranging from 26 to 622 bp, were electrophoresed as molecular size markers. DNA bands were visualized by ethidium bromide staining.

Sequencing of 16S rRNA, the 5S–23S rRNA intergenic spacer and the flagellin and *ospA* genes. The *ospA* gene was amplified with primers A8 (5' CAATTTTCTATTTGTTA-TTTGTTAATC 3'), corresponding to the 5' end of *ospA*, and BR1 (5' TGAATCAGCACCTTTTTGTGC 3'), corresponding to the 5' end of *ospB*, or BR2 (5' AAATCC-TAGTAAATATTGTTT 3'), corresponding to the 5' end of *ospB*. The 16S rRNA sequences and flagellin (*fla*) gene were amplified with primer pairs 16SF1 (5' ATAACGAAGAG-TTTGATCCTGGC 3'), corresponding to the 5' end of 16S rRNA, and 16SR (5' CAGCCGCACTTTCCAGTACG 3'), corresponding to the 3' end of 16S rRNA, and *flaF* (5' GATTATAAATCATAATAGGTCAGC 3'), corresponding to the 5' end of *fla*, and *flaR2* (5' CTCCTCAATAA-GCCTGCATTATGC 3'), corresponding to the 3' end of *fla*. The amplicons of the *ospA* gene (about 980 bp), flagellin gene (about 970 bp), 16S rRNA (about 1350 bp) and the intergenic spacer sequence (about 250 bp) were cloned into the pGEM-T vector and the recombinant plasmids were transformed into *Escherichia coli* JM109 using the pGEM-T

Table 1. RFLP analysis of the 5S–23S rRNA intergenic spacer and flagellin gene

Taxa are listed as reported previously or as identified in this study. Exact restriction fragment sizes were determined from sequences. NA, Sequence not available; NS, no site; NT, not tested.

Taxon	Strain	Isolated from	5S–23S rRNA intergenic spacer				Flagellin gene			
			Amplicon (bp)	Restriction fragments (bp)		Amplicon (bp)	Restriction fragments (bp)			
				<i>DraI</i>	<i>MseI</i>		<i>Bpu1102I</i>	<i>DdeI</i>	<i>HhaI</i>	<i>HincII</i>
<i>B. garinii</i>	935T	<i>I. persulcatus</i>	237	185, 52	107, 79, 51	584	NS	329, 153, 72, 21, 9	405, 179	453, 131
<i>B. afzelii</i>	934U	<i>A. agrarius</i>	246	174, 52, 20	107, 68, 51, 20	NT	NT	NT	NT	NT
<i>B. afzelii</i>	17Y	<i>I. granulatus</i>	246	174, 52, 20	107, 68, 51, 20	NT	NT	NT	NT	NT
<i>B. afzelii</i>	5Y	<i>I. granulatus</i>	~ 250	Similar to 17Y	Similar to 17Y	NT	NT	NT	NT	NT
<i>B. afzelii</i>	18Y	<i>I. granulatus</i>	~ 250	Similar to 17Y	Similar to 17Y	NT	NT	NT	NT	NT
<i>B. valaisiana</i>	5MT	<i>I. nipponensis</i>	254	173, 81	107, 59, 43, 24, 22	585	NS	252, 188, 90, 33, 22	NS	NS
<i>B. valaisiana</i>	9MT	<i>I. nipponensis</i>	~ 250	Similar to 5MT	Similar to 5MT	~ 590	NS	Similar to 5MT	NS	NS
<i>B. valaisiana</i>	10MT	<i>I. nipponensis</i>	254	173, 81	150, 58, 24, 22	585	NS	221, 135, 118, 90, 21	NS	NS
<i>B. burgdorferi</i>	B31 ^T	<i>I. scapularis</i>	254	144, 53, 29, 28	107, 52, 38, 29, 28	584	NS	338, 225, 21	NS	NS
<i>B. garinii</i>	20047 ^T	<i>I. ricinus</i>	253	201, 52	107, 95, 51	584	NS	329, 153, 72, 21, 9	405, 179	453, 131
<i>B. garinii</i>	NT29	<i>I. persulcatus</i>	253	144, 57, 52	107, 57, 51, 38	NA	NA	NA	NA	NA
<i>B. garinii</i>	ChY13p	<i>I. persulcatus</i>	237	185, 52	107, 79, 51	NA	NA	NA	NA	NA
<i>B. afzelii</i>	VS461 ^T	<i>I. ricinus</i>	246	174, 52, 20	107, 68, 51, 20	584	NS	305, 183, 42, 33, 21	NS	NS
<i>B. japonica</i>	HO14 ^T	<i>I. ovatus</i>	236	NS	107, 78, 51	584	NS	305, 225, 33, 21	348, 236	NS
<i>B. valaisiana</i>	VS116 ^T	<i>I. ricinus</i>	255	203, 52	174, 51, 23, 7	584	NS	188, 135, 117, 90, 33, 21	NS	NS
<i>B. valaisiana</i>	Am501	<i>I. columbae</i>	248	NS	168, 51, 23, 6	584	NS	188, 135, 117, 90, 33, 21	NS	NS
<i>B. lusitanae</i>	PotB2 ^T	<i>I. ricinus</i>	257	145, 83, 29	107, 82, 39, 29	584	NS	338, 225, 21	393, 179, 12	453, 131
<i>B. andersonii</i>	21123	<i>I. dentatus</i>	266	NS	119, 67, 52, 28	584	NS	238, 225, 100, 21	NS	NS
<i>B. tanukii</i>	Hk512	<i>I. tanukii</i>	245	173, 52, 20	174, 51, 20	584	NS	338, 225, 21	328, 256	NS
<i>B. turdi</i>	Ya501 ^T	<i>I. turdus</i>	248	144, 81, 23	107, 51, 38, 21, 16, 8, 7	584	NS	225, 221, 117, 21	405, 179	453, 131
' <i>B. bissestii</i> '	DN127 ^T	<i>I. neotomae</i>	257	144, 53, 33, 27	107, 52, 38, 33, 27	584	NS	221, 153, 117, 72, 21	405, 179	NS

vector system (Promega) according to the manufacturer's instructions. The recombinant plasmids were extracted from *E. coli* cultures using a PERFECT prep plasmid DNA kit (5 prime-3 prime) and sequenced by the dideoxy chain-termination method using a *Taq* Dye-Deoxy Terminator cycle sequencing kit with primers M13 (–29) and M13 reverse and a model 373A DNA sequencer (Applied Biosystems) or Sequi Therm EXEL II Long-Read DNA Sequencing Kit LC (Epicentre Technology) and an LI-COR 4000 DNA sequencer. At least three clones were sequenced for determination of each strain.

Nucleotide sequence accession numbers. The sequences of the 5S–23S rRNA intergenic spacer, flagellin gene, 16S rRNA gene and *ospA* gene determined in this study have been submitted to the DDBJ, EMBL and GenBank databases under the accession numbers listed in the legends to Figs 1–3 and in Table 3.

Phylogenetic analysis. Sequences were aligned and a similarity matrix and neighbour-joining phylogenetic tree were constructed using the DNASTAR program.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were carried out according to a previously described method (Masuzawa *et al.*, 1991). Ten micrograms protein was applied to each lane of a 12.5% polyacrylamide gel. After electrophoresis, the gels were stained with Coomassie brilliant blue. Antigens were transferred to PVDF membranes (Bio-Rad) and specific antigen bands were subsequently detected by immunostaining with mAbs diluted 1:50 to 1:100. The mAbs used were: H9724 and O1441b to flagellin; P31a, P31b, P31c and P31d to *OspA* (Masuzawa *et al.*, 1996c); G7 to *OspC* (Masuzawa *et al.*, 1996b); and O62a to heat-shock protein 60 (Hsp60, Masuzawa *et al.*, 1996c).

RESULTS AND DISCUSSION

Phylogenetic analysis of the *B. afzelii*-related group on the basis of 16S rRNA gene sequences

A phylogenetic tree based on 16S rRNA gene sequences was constructed on the basis of a sequence-similarity matrix of the 16S rRNA gene (1367 bp) (Fig. 1). The 16S rRNA sequence of strain 10MT was determined in this study, while the other sequences were obtained from previous reports as described in the figure legend. The sequences of the three strains

Fig. 1. Phylogenetic tree based on 16S rRNA sequences. The 16S rRNA sequence of strain 10MT (accession no. AB016974) was determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: 5MT (U44938), 9MT (L39080), VS116^T (X98232), UK (X98233), Am501 (D67021), B31^T (B59293), DK21 (X85191), 20047^T (D67018) and PBi (X85199). Bar, sequence divergence of 1.0%.

Fig. 2. Phylogenetic tree based on 5S–23S rRNA intergenic spacer sequences. Sequences of strains 17Y (accession no. AB013913), 5MT (AB013914), 10MT (AB013915), 934U (AB013917) and 935T (AB013916) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: VS116^T (L30134), UK (L30133), Am501 (D84402), B31^T (L30127), 20047^T (L30119), NP81 (D84406), NT29 (L30130), ASF (D84403), ChY13p (AB003785), VS461^T (L30135), NT28 (D84405), HO14^T (L30128), 19952 (L30118), 21123 (L30120), CA2 (L30123), Hk501^T (D84404), Ya501^T (D84407), PotiB2^T (L30131), PotiB3 (L30132), DN127^T (L30126), CA55 (L30124) and 25015 (L30122). Bar, sequence divergence of 5%.

5MT, 9MT and 10MT clustered with those of strains VS116^T and UK of the recently described new species *B. valaisiana* and *Borrelia* sp. strain Am501, isolated from *Ixodes columnae* in Japan, and showed the highest identities, 99.2–99.9%, to these strains. Kee *et al.* (1996) tentatively classified these strains as belonging to the *B. afzelii*-related genomic group on the basis of 16S rRNA gene sequences. However, the strains showed greater sequence identity to *B. valaisiana* than to *B. afzelii*. These observations strongly suggested that the Korean isolates from *I. nipponensis*, the European isolates from *Ixodes ricinus* and the Japanese isolates from *I. columnae* are closely related.

RFLP analysis of the 5S–23S rRNA intergenic spacer and flagellin gene

The results of RFLP analysis of the 5S–23S rRNA intergenic spacer sequence and flagellin gene are summarized in Table 1. Strains 5Y, 17Y, 18Y and 934U generated RFLP patterns for the 5S–23S rRNA intergenic spacer identical to that of *B. afzelii* strain VS461^T. Strain 935T generated a pattern identical to that of *B. garinii* strain ChY13p, isolated from *I. persulcatus* in northern China (Li *et al.*, 1998), while strains 5MT, 9MT and 10MT, isolated from *I. nipponensis*, showed unique RFLP patterns that have not been reported previously. These three strains also generated unique patterns on RFLP analysis of the flagellin gene.

Fig. 3. Phylogenetic tree based on flagellin gene sequences. Sequences of strains 5MT (accession no. AB014677), 10MT (AB014678) and 935T (AB017479) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: VS116^T (D82854), Am501 (D82855), B31^T (X15661), GeHo (X56334), 20047^T (D82846), HT17 (D63363), HT19 (D63371), JEM4 (D63364), JEM5 (D63370), HT37 (D63369), VS461^T (D63365), HT61 (D63366), HO14^T (D82852), 21123 (D83764), Hk501^T (D82847), Ya501^T (D82849), PotiB2^T (D85856) and DN127^T (D82857). Bar, sequence divergence of 2%.

Phylogenetic analysis of the 5S–23S rRNA intergenic spacer and flagellin gene

Figs 2 and 3 show the phylogenetic trees constructed on the basis of the 5S–23S rRNA intergenic spacer and flagellin gene sequences. 5S–23S rRNA intergenic spacer sequences of strains 17Y and 935T showed greatest identities to *B. afzelii* strain VS461^T (99.6%) and *B. garinii* strain ChY13p (95.4%), respectively. Furthermore, the sequences from strains 5MT and 10MT, which generated unique RFLP patterns, showed the greatest identity (94.9–97.3%) to *B. valaisiana* strains VS116^T and UK, isolated from *I. ricinus* in Europe, and formed a cluster with these strains. Similar results were obtained from sequence analysis of the flagellin gene (Fig. 3). Sequences of strains 5MT and 10MT showed the greatest identity (about 97%) to strain VS116^T but were also similar to strain Am501, isolated from *I. columnae* in Japan. The Korean strains formed a cluster with the *Borrelia* sp. strain Am501 and *B. valaisiana* strain VS116^T and were distinct from other strains. These facts indicate that the Korean strains and Am501 should be classified as *B. valaisiana*. The Korean isolates identified as *B. valaisiana* showed specific RFLP patterns for the 5S–23S rRNA intergenic spacer and flagellin gene sequences. These methods are useful for the identification of *Borrelia* species on the basis of DNA extracted from vector ticks and/or small amounts of culture material. From the results obtained, the methods could be applicable to an epidemiological survey in this area.

Protein profiles and characterization of the *ospA* gene

Strains 5MT and 10MT each contained a 32 kDa protein that reacted with the anti-OspA mAb P31c (Fig. 4 and Table 2). Strain 9MT had a protein profile similar to those of strains 5MT and 10MT with respect to the molecular mass of OspA and other major

Fig. 4. SDS-PAGE profiles of *Borrelia* strains used in this study. Ten micrograms protein was applied to each lane of a 12.5% polyacrylamide gel. Arrowheads indicate the position of OspA reactive to mAb P31c. M, molecular mass markers.

proteins (data not shown), as previously reported (Kee *et al.*, 1994). However, strains VS116^T and Am501 contained OspA proteins of different sizes and these did not react with mAb P31c. European strains classified as *B. valaisiana* express proteins that appear to be OspA, in the size range 32–35 kDa (Nohlmans *et al.*, 1995; Péter & Bretz, 1992; Wang *et al.*, 1997). Specific mAbs are useful for the detection of specific *Borrelia* species in the tick midgut and identify them by immunological reactivity. However, in this study, we could not identify a mAb that reacted specifically with *B. valaisiana* among a battery of mAbs tested. Previously, Wang *et al.* (1997) also reported that the mAbs tested in their study were not reactive to *B.*

Table 2. Reactivity of the isolates with various mAbs

The targets of the various mAbs tested are indicated in parentheses.

Taxon	Strain	062a (Hsp60)	H9724 (flagellin)	01441b (flagellin)	P31a (OspA)	P31b (OspA)	P31c (OspA)	P31d (OspA)	G7 (OspC)
<i>B. burgdorferi</i>	297	+	+	—	—	—	—	—	+
<i>B. garinii</i>	HP1	+	+	—	—	+	+	—	+
<i>B. afzelii</i>	PGau	+	+	—	—	—	+	+	+
<i>B. valaisiana</i>	5MT	+	+	—	—	—	+	—	+
<i>B. valaisiana</i>	10MT	+	+	—	—	—	+	—	+
<i>B. valaisiana</i>	VS116 ^T	+	+	—	—	—	—	—	+
<i>B. valaisiana</i>	Am501	+	+	—	—	—	—	—	+
<i>B. japonica</i>	HO14 ^T	+	+	+	—	—	+	—	+

Table 3. Sequence identity matrix of partial sequences (819 bp) of *ospA* genes

The sequences shown were all determined in this study.

Taxon	Strain	5MT	10MT	VS116 ^T	Am501	Ya501 ^T	Accession no.
<i>B. valaisiana</i>	5MT	100	99.6	87.4	93.3	98.4	AB016977
<i>B. valaisiana</i>	10MT		100	87.0	92.9	98.0	AB016978
<i>B. valaisiana</i>	VS116 ^T			100	90.8	86.5	AB016979
<i>B. valaisiana</i>	Am501				100	92.4	AB016976
<i>B. turdi</i>	Ya501 ^T					100	AB016975

valaisiana. Further study is necessary to identify a mAb that reacts specifically with this species.

In order to estimate the similarity among strains 5MT, 10MT, Am501 and VS116^T, *ospA* sequences were determined and compared with each other (Table 3). The greatest identity (99.6%) was observed between strains 5MT and 10MT. Interestingly, relatively greater identity (98.0–98.4%) was found to the *ospA* sequence of *B. turdi* strain Ya501^T, but sequences from strains Am501 and VS116^T were about 93 and 87% identical to those of strains 5MT and 10MT, respectively. These results clearly indicate that the *ospA* sequences of strains 5MT and 10MT were quite similar to one another and relatively divergent from those of strains Am501 and VS116^T. Other strains belonging to *B. valaisiana* could not be examined in this study as *ospA* sequences are not yet available for these strains. Based on the currently available information, *ospA* sequences from European and Korean strains and Japanese strain Am501, which are classified as *B. valaisiana*, are heterogeneous, like that of *B. garinii*.

The three Korean strains were isolated from *I. nipponensis* collected in Haenam, located at the southern tip of the Korean peninsula (Kee *et al.*, 1994, 1996). We performed a survey in Choongju, located in the central part of Korea, in 1997 and isolated typical *B. garinii* and *B. afzelii* strains from *I. nipponensis* collected around this area, but did not obtain strains similar to *B. valaisiana* (unpublished results).

The geographical distribution of *B. valaisiana* is still unclear. Strains have been isolated and/or specific DNA has been detected from *I. ricinus* collected in the Netherlands (Rijpkema *et al.*, 1995), Switzerland (Filipuzzi-Jenny *et al.*, 1993; Péter & Bretz, 1992), the United Kingdom (Livesley *et al.*, 1995), Italy (Cinco *et al.*, 1998), Croatia (Golubic *et al.*, 1998; Rijpkema *et al.*, 1996) and Ireland (Kirstein *et al.*, 1997). A species of *Borrelia* was also isolated from *I. persulcatus* in the central Urals (Lesnki *et al.*, 1997). These reports suggest that *B. valaisiana* is distributed in various European countries and central Eurasia. Also, strain Am501, isolated from *I. columnae* in Japan, has been identified as *B. valaisiana* on the basis of flagellin (Fukunaga & Koreki, 1996; Fukunaga *et al.*, 1996c) and 16S rRNA gene sequences (Fukunaga *et al.*, 1996b; Le Flèche *et al.*, 1997; Wang *et al.*, 1997). Recent taxonomic characterization of *Borrelia* has indicated that the ticks *I. ovatus*, *I. turdus* and *I. tanuki* transmit *B. japonica*, *B. turdi* and *B. tanukii*, respectively (Fukunaga *et al.*, 1996a; Kawabata *et al.*, 1993; Nakao & Miyamoto, 1993). In contrast, *I. ricinus* ticks can transmit five species of *Borrelia*, *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae* (Baranton *et al.*, 1992; Canica *et al.*, 1993; Le Flèche *et al.*, 1997; Postic *et al.*, 1994; Wang *et al.*, 1997). Also, *I. persulcatus* carries *B. garinii*, *B. afzelii* and possibly *B. valaisiana* but not *B. burgdorferi sensu stricto* (Li *et al.*, 1998; Masuzawa *et al.*, 1997; Postic *et al.*, 1997; Sato *et al.*, 1996). Marti Ras *et al.* (1997)

proposed that European *B. burgdorferi sensu stricto* strains may have been imported recently from North America and have begun to evolve and diverge in Europe. The American ancestor of *B. burgdorferi* has probably evolved to *B. burgdorferi sensu stricto*, *B. andersonii* and '*B. bissettii*', each adapted to their own vector tick species in North America. These observations lead us to speculate that the Korean *Borrelia* strains (5MT, 9MT and 10MT) and the Japanese isolate strain Am501, as members of *B. valaisiana*, are adapted to the ticks, *I. nipponensis* and *I. columnae*, that are found in their respective localities and are differentiated into these borrelia groups by *ospA* gene sequences and their products.

ACKNOWLEDGEMENTS

We thank Dr G. Baranton, Pasteur Institute, Paris, and Dr M. Nakao, Asahikawa Medical College, Japan, for providing *Borrelia* strains. This study was supported in part by Grants-in-Aid for International Cooperative Research (nos 08044310, 08041181 and 10041204) and Grants-in-Aid for Scientific Research (nos 07670320, 08670312 and 09670294) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- Anderson, J. F. (1989). Epizootiology of *Borrelia* in *Ixodes* tick vectors and reservoir hosts. *Rev Infect Dis* **11** (Suppl. 6), S1451–S1459.
- Baranton, G., Postic, D., Saint Girons, I., Boerlin, P., Piffaretti, J.-C., Assous, M. & Grimont, P. A. D. (1992). Delineation of *Borrelia burgdorferi sensu stricto*, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. *Int J Syst Bacteriol* **42**, 378–383.
- Barbour, A. G. (1984). Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med* **57**, 521–525.
- Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E. & Davis, J. P. (1982). Lyme disease – a tick-borne spirochetosis? *Science* **216**, 1317–1319.
- Canica, M. M., Nato, F., du Merle, L., Mazie, J. C., Baranton, G. & Postic, D. (1993). Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. *Scand J Infect Dis* **25**, 441–448.
- Cinco, M., Padovan, D., Murgia, R., Poldini, L., Frusteri, L., van de Pol, I., Verbeek-De Kruif, N., Rijpkema, S. & Maroli, M. (1998). Rate of infection of *Ixodes ricinus* ticks with *Borrelia burgdorferi sensu stricto*, *Borrelia garinii*, *Borrelia afzelii* and group VS116 in an endemic focus of Lyme disease in Italy. *Eur J Clin Microbiol Infect Dis* **17**, 90–94.
- Filipuzzi-Jenny, E., Blot, M., Schmid-Berger, N., Meister-Turner, J. & Meyer, J. (1993). Genetic diversity among *Borrelia burgdorferi* isolates: more than three genospecies? *Res Microbiol* **144**, 295–304.
- Fukunaga, M. & Koreki, Y. (1996). A phylogenetic analysis of *Borrelia burgdorferi sensu lato* isolates associated with Lyme disease in Japan by flagellin gene sequence determination. *Int J Syst Bacteriol* **46**, 416–421.
- Fukunaga, M., Hamase, A., Okada, K., Inoue, H., Tsuruta, Y., Miyamoto, K. & Nakao, M. (1996a). Characterization of spirochetes isolated from ticks (*Ixodes tanuki*, *Ixodes turdus*, and *Ixodes columnae*) and comparison of the sequences with those of *Borrelia burgdorferi sensu lato* strains. *Appl Environ Microbiol* **62**, 2338–2344.
- Fukunaga, M., Hamase, A., Okada, K. & Nakao, M. (1996b). *Borrelia tanukii* sp. nov. and *Borrelia turdae* sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis. *Microbiol Immunol* **40**, 877–881.
- Fukunaga, M., Okada, K., Nakao, M., Konishi, T. & Sato, Y. (1996c). Phylogenetic analysis of *Borrelia* species based on flagellin gene sequences and its application for molecular typing of Lyme disease borreliae. *Int J Syst Bacteriol* **46**, 898–905.
- Golubic, D., Rijpkema, S., Tkalec-Makovec, N. & Ruzic, E. (1998). Epidemiologic, ecologic and clinical characteristics of Lyme borreliosis in northwest Croatia. *Acta Med Croatica* **52**, 7–13.
- Johnson, R. C., Schmid, G. P., Hyde, F. W., Steigerwalt, A. G. & Brenner, D. J. (1984). *Borrelia burgdorferi* sp. nov.: etiologic agent of Lyme disease. *Int J Syst Bacteriol* **34**, 496–497.
- Kawabata, H., Masuzawa, T. & Yanagihara, Y. (1993). Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. *Microbiol Immunol* **37**, 843–848.
- Kee, S. H., Hwang, K. J., Oh, H. B. & Park, K. S. (1994). Identification of *Borrelia burgdorferi* isolated in Korea using outer surface protein A (OspA) serotyping system. *Microbiol Immunol* **38**, 989–993.
- Kee, S. H., Yoon, J. H., Oh, H. B., Park, Y. H., Kim, Y. W., Cho, M. K., Park, K. S. & Chang, W. H. (1996). Genetic analysis of *Borrelia burgdorferi sensu lato* in Korea using genomic hybridization and 16S rRNA gene sequence determination. *Microbiol Immunol* **40**, 599–605.
- Kirstein, F., Rijpkema, S., Molkenboer, M. & Gray, J. S. (1997). The distribution and prevalence of *B. burgdorferi* genospecies in *Ixodes ricinus* ticks in Ireland. *Eur J Epidemiol* **13**, 67–72.
- Le Flèche, A., Postic, D., Girardet, K., Péter, O. & Baranton, G. (1997). Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. *Int J Syst Bacteriol* **47**, 921–925.
- Lesnki, O. M., Istomina, O. I., Rijpkema, S., Bruininck, H. & Beliaeva, M. L. (1997). The clinical manifestations of Lyme borreliosis in the Middle Urals and their association with *Borrelia burgdorferi* genospecies. *Ter Arkh* **69**, 9–12.
- Li, M., Masuzawa, T., Takada, N. & 7 other authors (1998). Lyme disease *Borrelia* species in northeastern China resemble those isolated from far eastern Russia and Japan. *Appl Environ Microbiol* **64**, 2705–2709.
- Livesley, M. A., Thompson, I. P., Rainey, P. B. & Nuttall, P. A. (1995). Comparison of *Borrelia* isolated from UK foci of Lyme disease. *FEMS Microbiol Lett* **130**, 151–157.
- Marconi, R. T., Liveris, D. & Schwartz, I. (1995). Identification of novel insertion elements, restriction fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme disease spirochetes: phylogenetic analysis of rRNA genes and their intergenic spacers in *Borrelia japonica* sp. nov. and genomic group 21038 (*Borrelia andersonii* sp. nov.) isolates. *J Clin Microbiol* **33**, 2427–2434.
- Marti Ras, N., Postic, D., Foretz, M. & Baranton, G. (1997). *Borrelia burgdorferi sensu stricto*, a bacterial species 'Made in the U. S. A.'? *Int J Syst Bacteriol* **47**, 1112–1117.
- Masuzawa, T., Okada, Y., Yanagihara, Y. & Sato, N. (1991). Antigenic properties of *Borrelia burgdorferi* isolated from *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan. *J Clin Microbiol* **29**, 1568–1573.
- Masuzawa, T., Komikado, T., Iwaki, A., Suzuki, H., Kaneda, K. &

- Yanagihara, Y. (1996a).** Characterization of *Borrelia* sp. isolated from *Ixodes tanuki*, *I. turdus*, and *I. columnae* in Japan by restriction fragment length polymorphism of *rrf* (5S)-*rrl* (23S) intergenic spacer amplicons. *FEMS Microbiol Lett* **142**, 77–83.
- Masuzawa, T., Suzuki, H., Kawabata, H., Ishiguro, F., Takada, N. & Yanagihara, Y. (1996b).** Characterization of *Borrelia* spp. isolated from the tick, *Ixodes tanuki* and small rodents in Japan. *J Wildl Dis* **32**, 565–571.
- Masuzawa, T., Wilske, B., Komikado, T. & 9 other authors (1996c).** Comparison of OspA serotypes for *Borrelia burgdorferi* sensu lato from Japan, Europe and North America. *Microbiol Immunol* **40**, 539–545.
- Masuzawa, T., Iwaki, A., Sato, Y., Miyamoto, K., Korenberg, E. I. & Yanagihara, Y. (1997).** Genetic diversity of *Borrelia burgdorferi* sensu lato isolated in far eastern Russia. *Microbiol Immunol* **41**, 595–600.
- Nakao, M. & Miyamoto, K. (1993).** Isolation of spirochetes from Japanese ixodid ticks, *Ixodes tanuki*, *Ixodes turdus*, and *Ixodes columnae*. *Jpn J Sanit Zool* **44**, 49–52.
- Nohlmans, L. M. K. E., de Boer, R., van den Bogaard, A. E. J. M. & van Boven, C. P. A. (1995).** Genotypic and phenotypic analysis of *Borrelia burgdorferi* isolates from The Netherlands. *J Clin Microbiol* **33**, 119–125.
- Park, K. H., Chang, W. H. & Schwan, T. G. (1993).** Identification and characterization of Lyme disease spirochetes, *Borrelia burgdorferi* sensu lato, isolated in Korea. *J Clin Microbiol* **31**, 1831–1837.
- Péter, O. & Bretz, A. G. (1992).** Polymorphism of outer surface proteins of *Borrelia burgdorferi* as a tool for classification. *Int J Med Microbiol Virol Parasitol Infect Dis* **277**, 28–33.
- Postic, D., Assous, M. V., Grimont, P. A. D. & Baranton, G. (1994).** Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rrf* (5S)-*rrl* (23S) intergenic spacer amplicons. *Int J Syst Bacteriol* **44**, 743–752.
- Postic, D., Korenberg, E., Gorelova, N., Kovalevski, Y. V., Bellenger, E. & Baranton, G. (1997).** *Borrelia burgdorferi* sensu lato in Russia and neighbouring countries: high incidence of mixed isolates. *Res Microbiol* **148**, 691–702.
- Postic, D., Marti Ras, N., Lane, R. S., Henderson, M. & Baranton, G. (1998).** Expanded diversity among Californian borrelia isolates and description of *Borrelia bissettii* sp. nov. (formerly *Borrelia* group DN127). *J Clin Microbiol* **36**, 3497–3504.
- Rijpkema, S. G. T., Molkenboer, M. J. C. H., Schouls, L. M., Jongejan, F. & Schellekens, J. F. P. (1995).** Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. *J Clin Microbiol* **33**, 3091–3095.
- Rijpkema, S., Golubic, D., Molkenboer, M., Verbeek-De Kruif, N. & Schellekens, J. (1996).** Identification of four genomic groups of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected in a Lyme borreliosis endemic region of northern Croatia. *Exp Appl Acarol* **20**, 23–30.
- Sato, Y., Miyamoto, K., Iwaki, A. & 7 other authors (1996).** Prevalence of the Lyme disease spirochetes in *Ixodes persulcatus* and wild rodents in far eastern Russia. *Appl Environ Microbiol* **62**, 3887–3889.
- Steere, A. C. (1989).** Lyme disease. *New Engl J Med* **321**, 586–596.
- Wang, G., van Dam, A. P., Le Flèche, A., Postic, D., Péter, O., Baranton, G., de Boer, R., Spanjaard, L. & Dankert, J. (1997).** Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* genomic groups VS116 and M19). *Int J Syst Bacteriol* **47**, 926–932.