***Borrelia sinica* sp. nov., a Lyme disease-related *Borrelia* species isolated in China**

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A survey was performed for Lyme disease borrelia in the southern part of China, in Zhejiang, Sichuan and Anhui provinces, along the Yangtze River valley, in May of 1997 and 1998. Twenty isolates from *Ixodes granulatus*, *Ixodes ovatus*, *Apodemus agrarius* and *Niviventer confucianus* were obtained. These isolates were characterized by RFLP of the 5S–23S rDNA intergenic spacer, sequence analysis of the intergenic spacer, 16S rDNA and flagellin gene, DNA–DNA hybridization analysis, SDS-PAGE and Western blotting with mAbs. Six isolates from *A. agrarius*, five from *I. granulatus* collected in Zhejiang province and one from *N. confucianus* in Sichuan province were highly similar to strains 10MT and 5MT isolated in Korea and classified as *Borrelia valaisiana*. Four isolates from *A. agrarius* and *I. granulatus* collected in Zhejiang province generated unique RFLP patterns and phylogenetic analysis of the 16S rDNA and flagellin gene sequences suggested that the isolates should be classified as *B. valaisiana*. Furthermore, three isolates (CMN1a, CNM2, CMN3^T) from *N. confucianus* captured in Sichuan province and one (CWO1) from *I. ovatus* in Anhui province showed lower 16S rDNA sequence similarity (less than 99.0%) to sequences of previously described Lyme disease-related *Borrelia* species. DNA–DNA hybridization results revealed that strains CMN3^T and CMN1a were clearly distinct from all other known Lyme disease *Borrelia* species. Electron microscope observation showed the spirochaetes to be morphologically similar to those of *Borrelia*, but the cells contained only four periplasmic flagella inserted at each end of the spirochaetes. Based on these results, a new *Borrelia* species, *Borrelia sinica* sp. nov., is proposed. Strain CMN3^T is the type strain of this new species.

Keywords: *Borrelia sinica*, *Borrelia valaisiana*, China, *Ixodes ovatus*, Lyme disease

INTRODUCTION

Lyme borreliosis is the most prevalent tick-borne zoonotic disease in Europe, North America and Far-Eastern countries such as Japan, Russia and China (Steere, 1989). The causative agent, *Borrelia burgdorferi* (Johnson *et al.*, 1984), was isolated from the tick *Ixodes dammini* (Burgdorfer *et al.*, 1982). *B.*

burgdorferi sensu lato is classified into 10 species: *B. burgdorferi sensu stricto*, 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*, respectively isolated from *Ixodes ovatus*, *Ixodes tanuki* and *Ixodes turdi* in Japan (Fukunaga *et al.*, 1996; Kawabata *et al.*, 1993); ‘*Borrelia andersonii*’, isolated from *Ixodes dentatus* in North America (Marconi *et al.*, 1995); *Borrelia valaisiana* (Wang *et al.*, 1997) and *Borrelia lusitaniae* (Le Fleche *et al.*, 1997), isolated from *Ixodes ricinus* in Europe; and ‘*Borrelia bissettii*’ (Postic *et al.*, 1998), isolated in North America. Furthermore, *B. valaisiana*

Abbreviations: Hsp, heat-shock protein; Osp, outer surface protein.

The DDBJ accession numbers for the 16S rDNA, flagellin gene and 5S–23S rDNA intergenic spacer sequences reported in this paper are AB022101 and AB022124–AB022146.

has been found in Korea, Taiwan and Japan (Kee *et al.*, 1996; Masuzawa *et al.*, 1999, 2000; Wang *et al.*, 1997).

We previously performed a survey in the north-eastern part of China and isolates from *Ixodes persulcatus* and from the rodent *Apodemus peninsulae* were identified as *B. afzelii* and *B. garinii*, but no isolates were determined as *B. burgdorferi sensu stricto* (Li *et al.*, 1998). In order to examine the prevalence of Lyme disease *Borrelia* and to discover new *Borrelia* species, we performed a survey in the southern part of China in 1997 and 1998. Consequently, 20 isolates were obtained from *Ixodes granulatus*, *I. ovatus*, *Apodemus agrarius* and *Niviventer confucianus*. The aim of this study was to characterize these isolates based on their genetic and phenotypic properties. On the basis of the results of the present study, we propose that isolates from *N. confucianus* captured in Sichuan province and *I. ovatus* collected in Anhui province should be described as *Borrelia sinica* sp. nov.

METHODS

Survey sites and isolation of *Borrelia*. The *Borrelia* isolates from China are listed in Table 1. Ticks and wild rodents were collected in the southern part of China, from Zhejiang, Sichuan and Anhui provinces, in May of 1997 and 1998. Ixodid ticks were collected by dragging vegetation at woodland areas. At the same time, wild rodents were also

captured in Sherman live traps. Spirochaetes were isolated from earlobe tissues of rodents (*A. agrarius* and *N. confucianus*) and the midgut of two species of tick (*I. granulatus* and *I. ovatus*) by the method described previously (Takada *et al.*, 1998) and were cultivated at 30 °C in Barbour–Stoenner–Kelly II medium (Barbour, 1984).

PCR and RFLP analysis of 5S–23S rDNA intergenic spacer. Primers corresponding to the 3' end of the 5S rDNA (*rrf*) (5'-CTGCGAGTTCGCGGGAGA-3') and the 5' end of the 23S rDNA (*rrl*) (5'-TCCTAGGCATTACCAT-3') (Masuzawa *et al.*, 1996a; Postic *et al.*, 1994) were synthesized. PCR was performed by the method described previously (Postic *et al.*, 1994). The amplification products of 5S–23S rDNA amplicons were digested with *MseI* and *DraI* and the digested DNA was electrophoresed through 16% polyacrylamide gels. DNA bands were visualized by ethidium bromide staining.

Sequencing of 5S–23S rDNA intergenic spacer, the flagellin gene and 16S rDNA. 16S rDNA sequences and flagellin genes were amplified with primer pairs 16SF1 (5'-ATAACGAA-GAGTTTGATCCTGGC-3') and 16SR (5'-CAGCCGCA-CTTCCAGTACG-3'), corresponding to the 5' and 3' ends of the 16S rDNA, and flaF (5'-GATTATAAATCATAA-TAGGTCAGC-3') and flaR2 (5'-CTCCTCAATAAGCC-TGCATTATGC-3'), corresponding to the 5' and 3' ends of *fla*. The amplicons from 16S rDNA (about 1350 bp), the flagellin gene (about 970 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 vector system (Promega) according to the manufacturer's instructions. The

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

Taxa are listed as reported previously or as identified in this study.

Strain	Isolated from:	Location	Size (bp)		
			Amplicon	<i>DraI</i> fragments	<i>MseI</i> fragments
<i>B. valaisiana</i>					
CKA2a*, CKA3b*, CKA5b	<i>A. agrarius</i>	Hangzhou, Zhejiang	254	173, 81	150, 58, 24, 22
CKG1, CKG2, CKG3, CKG5, CKG6	<i>I. granulatus</i>	Hangzhou, Zhejiang	~ 250	CKA2a type	CKA2a type
CMN1b	<i>N. confucianus</i>	Nanchuan, Sichuan	~ 250	CKA2a type	CKA2a type
CKA2b*, CKA4a, CKA4b	<i>A. agrarius</i>	Hangzhou, Zhejiang	254	173, 81	107, 58, 43, 24, 22
CKA1*, CKA5a	<i>A. agrarius</i>	Hangzhou, Zhejiang	253	144, 52, 29, 28	145, 51, 29, 21, 7
CKA3a*	<i>A. agrarius</i>	Hangzhou, Zhejiang	246	144, 102	145, 59, 28, 14
CKG4	<i>I. granulatus</i>	Hangzhou, Zhejiang	~ 250	CKA1 type	CKA1 type
<i>Borrelia sinica</i> sp. nov.					
CMN3 ^T *	<i>N. confucianus</i>	Nanchuan, Sichuan	235	157, 49, 29	107, 48, 38, 29, 13
CMN1a*, CMN2	<i>N. confucianus</i>	Nanchuan, Sichuan	236	144, 50, 42	107, 49, 38, 29, 13
CWO1*	<i>I. ovatus</i>	Mt Huangshan, Anhui	236	144, 50, 42	107, 49, 38, 29, 13
Reference strains					
<i>B. valaisiana</i> VS116 ^T *	<i>I. ricinus</i>	Switzerland	255	203, 52	174, 51, 23, 7
<i>B. valaisiana</i> 10MT*	<i>I. nipponensis</i>	Korea	254	173, 81	150, 58, 24, 22
<i>B. valaisiana</i> 5MT*	<i>I. nipponensis</i>	Korea	254	173, 81	107, 58, 43, 24, 22

* Exact fragment sizes were determined from the sequences. Sequences of strains CKA2a (accession no. AB022124), CKA3b (AB022125), CKA2b (AB022126), CKA1 (AB022127), CKA3a (AB022128), CMN1a (AB022129), CWO1 (AB022130) and CMN3^T (AB022131) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: VS116^T (L30134), 5MT (AB013914) and 10MT (AB013915).

Fig. 1. Phylogenetic tree based on flagellin gene sequences. Sequences of strains CKA1 (accession no. AB022132), CKA2a (AB022133), CMN1b (AB022134), CKA3a (AB022135), CKA4a (AB022136), CMN1a (AB022137), CMN3^T (AB022138) and CKA3b (AB022139) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: VS116^T (D82854), 10MT (AB014678), 5MT (AB014677), Am501 (D82855), B31^T (X15661), 20047^T (D82846), HT17 (D63363), HT19 (D63371), HT37 (D63369), VS461^T (D63365), HT61 (D63366), HO14^T (D82852), 21123 (D83764), Hk501^T (D82847), Ya501^T (D82849), PotiB2^T (D85856), DN127 (D82857) and the relapsing fever borrelia, *Borrelia hermsii* strain HS1 (M86838). Bar, sequence divergence of 1.0%. Chinese isolates are indicated in bold type.

recombinant plasmids were extracted from *E. coli* cultures using a Plasmid Miniprep kit (Bio-Rad) 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 Them EXEL II Long-Read DNA sequencing kit LC (Epicentre Technology) and LICOR 4000 DNA sequencer. At least three clones were sequenced.

Nucleotide sequence accession numbers. The intergenic spacer, flagellin gene and 16S rDNA sequences were submitted to DDBJ, EMBL and GenBank with the accession numbers listed in the legends to Figs 1 and 2 and in Table 1.

Phylogenetic analysis. The CLUSTAL X software, originally described by Thompson *et al.* (1997), was used to align the sequences and phylogenetic distances were calculated by the neighbour-joining method (Saitou & Nei, 1987). The phylogenetic tree was drawn using NJplot software. Bootstrap values were obtained with 1000 resamplings.

Determination of DNA–DNA hybridization. DNAs were extracted and purified from strains CMN3^T and CMN1a and 10 strains of *B. burgdorferi sensu lato* species by the method described previously (Kawabata *et al.*, 1993). Purified reference DNAs were used to determine DNA–

DNA hybridization by the microplate hybridization method (Ezaki *et al.*, 1988; Kawabata *et al.*, 1993). Aliquots containing 1 µg heat-denatured DNA solution were incubated for 12 h at 37 °C in microdilution plates (Dynatech Laboratories). The DNA solution was discarded and then dried overnight at 60 °C. Two-hundred microlitres of pre-hybridization solution [2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 × Denhardt's solution, 50 % formamide] containing 200 µg denatured salmon sperm DNA ml^{–1} was added to microdilution wells that had been coated with reference DNAs. The microdilution plates were incubated at 37 °C for 1 h and then washed. Photobiotin-labelled DNA was prepared as described previously (Ezaki *et al.*, 1989) and 50 ng labelled DNA was then added. Hybridization was performed with hybridization solution (2 × SSC) containing 50 % formamide at 26 °C (Blüthmann *et al.*, 1973). After hybridization, the microdilution plates were washed with 2 × SSC buffer. Streptavidin-β-D-galactosidase (Gibco) was added to the wells and incubated at 37 °C for 30 min. After incubation, the wells were washed and 4-methylumbelliferyl β-D-galactopyranoside (Wako) was added as substrate. After incubation, the fluorescence intensity was measured with a MicroFluor reader (Dynatech Laboratories) at a wavelength of 360 nm for excitation and 450 nm for emission. The experiment was performed in triplicate.

Fig. 2. Phylogenetic tree based on 16S rDNA sequences. Sequences of strains CNM3^T (AB022101), CKA3a (AB022140), CKA1 (AB022141), CKA2a (AB022142), CKA4a (AB022143), CMN1a (AB022144), CMN2 (AB022145) and CWO1 (AB022146) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: 5MT (U44938), 9MT (L39080), 10MT (AB016974), VS116^T (X98232), UK (X98233), Am501 (D67021), B31^T (B59293), DK21 (X85191), 20047^T (D67018), PBi (X85199) and *B. hermsii* strain H51 (U42292). Bar, sequence divergence of 0.2%. Chinese isolates are indicated in bold type.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were carried out according to methods described previously (Masuzawa *et al.*, 1991). After electrophoresis, the gels were stained with Coomassie brilliant blue. Antigens were transferred onto PVDF membranes (Bio-Rad) and specific antigen bands were subsequently detected by immunostaining with mAbs. The mAbs used were: H9724 and O1441b to flagellin; LA2, H5332, P31b, P31c and P31d to outer surface protein A (OspA) (Masuzawa *et al.*, 1996c); G7 to OspC (Masuzawa *et al.*, 1996b); O62a to heat-shock protein 60 (Hsp60) (Masuzawa *et al.*, 1996c); and D6 to 12 kDa antigen (Baranton *et al.*, 1992).

Electron microscopy. To prepare negatively stained specimens, a drop of the cell suspension concentrated by centrifugation was allowed to adsorb onto carbon-coated Formvar film supported on copper grids. The cells were stained with 2% sodium phosphotungstate for 30 s. The specimens were examined with a Hitachi H-7000 electron microscope at an acceleration voltage of 100 kV. To determine the numbers of flagella, cross-sections of *Borrelia* cells were prepared. Aliquots of 5 ml borrelial culture (strains CMN1a and CMN3^T) in BSK II medium were fixed by addition of an equal volume of 4% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 6 h at 4 °C. The cells

were harvested by centrifugation, washed gently in buffer and post-fixed with cold 1% osmium tetroxide for 3.5 h. The pellet was mixed with 0.5 ml 2% agarose. The agarose blocks were dehydrated in graded acetone and embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead acetate before observation with a Hitachi H-600 electron microscope at an acceleration voltage of 75 kV.

G+C contents of DNAs. DNA base compositions were determined by HPLC (Noguchi *et al.*, 1988) using a DNA-GC kit (Yamasa Shoyu).

RESULTS AND DISCUSSION

RFLP analysis and sequencing of the 5S–23S rDNA intergenic spacer

The results of RFLP analysis of the 5S–23S rDNA intergenic spacer sequence are summarized in Table 1. Exact fragment sizes generated after digestion with restriction enzymes *Dra*I and *Mse*I were determined by sequencing analysis of the amplicon. Eight strains (CKA2a, CKA3b, CKG1, CKG2, CKG3, CKG5,

Table 2. DNA–DNA relatedness of *B. sinica* sp. nov. and other species of *B. burgdorferi sensu lato*

Strain	Level of DNA relatedness with labelled DNA from:					
	1	2	3	4	5	6
1. CMN3 ^T	100.0	75.8	36.5	41.7	59.7	54.3
2. CMN1a	84.9	100.0	57.8	59.8	58.2	54.8
3. <i>B. burgdorferi</i> B31 ^T	58.5	34.0	100.0	35.2	38.7	32.6
4. <i>B. garinii</i> 20047 ^T	52.5	66.5	48.7	100.0	53.8	49.3
5. <i>B. tanukii</i> Fi81t	50.8	59.6	52.3	53.8	100.0	61.2
6. <i>B. japonica</i> HO14 ^T	52.1	59.8	49.5	57.0	67.9	100.0
7. <i>B. afzelii</i> VS461 ^T	43.1	54.4	45.9	57.0	57.4	45.7
8. <i>B. turdi</i> Ya501 ^T	46.4	61.7	48.5	60.3	57.4	51.0
9. ' <i>B. andersonii</i> ' 21123	38.8	49.5	69.9	55.7	52.4	47.0
10. <i>B. valaisiana</i> VS116 ^T	30.4	49.3	61.5	59.7	62.5	59.6
11. <i>B. lusitaniae</i> PotiB2 ^T	47.4	64.8	47.3	65.4	51.4	46.3
12. ' <i>B. bissettii</i> ' DN127	47.0	46.8	72.8	45.2	49.9	47.1

Fig. 3. SDS-PAGE profiles of Chinese *Borrelia* strains. Lanes: M, molecular mass markers; 1, CKA1; 2, CKA2a; 3, CKA3a; 4, CKA3b; 5, CMN1a; 6, CMN1b; 7, CMN2; 8, CMN3^T; 9, CWO1; 10, B31^T. Ten micrograms of protein was applied to each lane of the 12.5% polyacrylamide gel. Arrowheads indicate the position of OspA reactive to mAb P31c.

CKG6 and CKA5b) isolated from *A. agrarius* and *I. granulatus* collected in Hangzhou, Zhejiang province, and one isolate (CMN1b) from *N. confucianus* in Nanchuan, Sichuan province, generated RFLP patterns identical to that of strain 10MT, isolated from *Ixodes nipponensis* collected in Korea. Three isolates (CKA2b, CKA4a, CKA4b) from *A. agrarius* collected in Zhejiang province showed patterns identical to that of strain 5MT, also isolated from *I. nipponensis* collected in Korea. Kee *et al.* (1996) tentatively classified Korean strains 10MT and 5MT as belonging to the *B. afzelii*-related genomic group on the basis of 16S rDNA gene sequences. The strains showed higher similarity to *B. valaisiana* than to *B. afzelii* in their 16S rDNA sequences and were distinguishable from *B. afzelii* by RFLP analysis of the intergenic spacer sequence (Masuzawa *et al.*, 1999). Consequently, we classified these Chinese isolates as *B. valaisiana*. The

other eight strains listed in Table 1 generated unique RFLP patterns that could be differentiated from those of species reported previously (Masuzawa *et al.*, 1999).

Phylogenetic analysis based on flagellin gene sequences

A phylogenetic tree based on flagellin gene sequences (Fig. 1) was constructed on the basis of a sequence-similarity matrix of the flagellin gene. The sequences of the six strains CKA2a, CMN1b, CKA3b, CKA4a, CKA1 and CKA3a showed higher similarity values, ranging from 97.1 to 99.7%, to strains 5MT, 10MT, Am501 and VS116^T and were clustered with these strains. Strains CMN1a and CMN3^T, isolated from *N. confucianus* captured in Sichuan, diverged from *B. valaisiana* and other previously described species.

Phylogenetic analysis of 16S rDNA sequences

A phylogenetic tree based on 16S rDNA sequences (Fig. 2) was constructed on the basis of a sequence-similarity matrix (1367 bp). The sequences of the four strains CKA1, CKA2a, CKA3a and CKA4a clustered with *B. valaisiana* strains VS116^T, UK, Am501, 10MT and 5MT and showed the highest similarity to strains of this group, ranging from 98.8 to 99.9%.

Strains CMN1a, CMN2, CWO1 and CMN3^T showed relatively lower similarity to previously described species, whereas these strains showed high levels of similarity to each other, ranging from 99.8 to 100%. These observations suggested that these isolates were different from previously described species.

DNA–DNA hybridization

Chromosomal DNA–DNA hybridization values among strains of 10 *B. burgdorferi sensu lato* species are shown in Table 2. The levels of DNA relatedness of

Table 3. Reactivity of the isolates with various mAbs

The targets of the various mAbs tested are indicated in parentheses. All strains tested reacted with mAbs O62a (Hsp60) and H9724 (flagellin). NT, Not tested.

Strain	Isolated in:	O1441b (flagellin)	P31b (OspA)	P31c (OspA)	P31d (OspA)	H5332 (OspA)	LA2 (OspA)	G7 (OspC)	D6 (12 kDa antigen)
<i>B. sinica</i> sp. nov. CMN3 ^T , CMN1a, CMN2, CWO1	China	—	—	—	—	—	—	+	—
<i>B. valaisiana</i> CKA1, CKA2a	China	—	—	+	—	—	—	+	—
CKA3a, CKA3b	China	—	—	+	—	+	—	+	—
CMN1b	China	—	—	+	—	—	—	—	—
5MT	Korea	—	—	+	—	NT	NT	+	NT
10MT	Korea	—	—	+	—	NT	NT	+	NT
VS116 ^T	Switzerland	—	—	—	—	NT	NT	+	NT
Am501	Japan	—	—	—	—	NT	NT	+	NT
<i>B. burgdorferi</i> B31 ^T	USA	—	—	—	—	+	+	+	—
<i>B. garinii</i> HP1	Japan	—	+	+	—	—	—	+	+
<i>B. afzelii</i> PGau	Germany	—	—	+	+	—	—	+	—
<i>B. japonica</i> HO14 ^T	Japan	+	—	+	—	—	—	+	—

strains CMN3^T and CMN1a were less than 70 % when compared with the type strains of 10 species described previously. This result indicated that strains CMN3^T and CMN1a were not members of previously known species.

Protein profiles and reactivity with mAbs

Strains classified as *B. valaisiana* showed variable molecular masses of OspA and OspB. Likewise, four strains of the novel taxon had variable sizes of proteins that seemed to be OspA and OspB, ranging from 31 to 35 kDa (Fig. 3). All Chinese strains tested reacted with mAbs H9724 to flagellin and O62a to Hsp60, which are specific to *Borrelia* species or *B. burgdorferi sensu lato*, but did not react with O1441b, specific for flagellin of *B. japonica*, LA2, specific for OspA of *B. burgdorferi sensu stricto*, D6, specific for *B. garinii*, or P31d, specific for OspA of *B. afzelii* (Table 3). Chinese isolates classified as *B. valaisiana* reacted with mAb P31c, specific for OspA, as did Korean strains 5MT and 10MT, members of *B. valaisiana*. No reactivity of strains CMN3^T, CMN1a, CMN2 or CWO1 was observed with mAbs reactive to OspA in this study. This result suggested that the four strains differed from known species. However, the group of *Borrelia* could not be identified and distinguished by SDS-PAGE protein profiles alone.

Morphology

On electron microscope observation of negatively stained specimens, the cells showed the characteristic borrelial shape, i.e. a helical shape with tapered ends.

The length of the cells was variable, about 10–25 µm, and the diameter was 0.25–0.3 µm (Fig. 4a). The maximum number of flagella observed at the tapered end of the cells was four. An insertion point was observed in the preparation (Fig. 4b). In order to determine the number of flagella, cross-sections of *Borrelia* cells were prepared; two to four flagella were observed within the ridge (Fig. 4c–f). These results suggested that cells of the novel taxon have two to four flagella at each end. Cells of *B. burgdorferi sensu lato* have approximately seven to fourteen flagella at each end (Burgdorfer *et al.*, 1982; Hovind-Hougen *et al.*, 1986; Oliver *et al.*, 1993; Yano *et al.*, 1997; Zung *et al.*, 1989). Barbour & Hayes (1986) clearly demonstrated seven periplasmic flagellar insertion points in the terminus portion of *B. burgdorferi*. The number of flagella in these novel isolates was thus very low in comparison with other borrelial species. Since the flagella are generally responsible for motility, the number of flagella may also affect their movement. In fact, these isolates cannot move actively in medium and readily developed into cell clusters in medium (data not shown); therefore, the exact doubling time could not be determined.

G+C content of DNA

The G+C content of the DNA of strain CMN3^T, isolated from *N. confucianus*, was 29.6 mol%. This value is in agreement with those reported previously for *B. burgdorferi sensu lato* (Barbour & Hayes, 1986; Johnson *et al.*, 1984; Kawabata *et al.*, 1993). From these findings, a new *Borrelia* species, *Borrelia sinica* sp. nov., was designated.

Fig. 4. Electron micrographs of *B. sinica* sp. nov. (a) Morphology of a negatively stained cell of *B. sinica* strain CMN3^T. Bar, 5.0 µm. (b) Negatively stained cell of *B. sinica* strain CMN3^T showing insertion points of flagella marked by arrowheads. Bar, 0.5 µm. (c)–(d) Cross-sections of cells of *B. sinica* strain CMN3^T bearing two (c) or four (d) flagella, indicated by arrowheads. Bars, 0.2 µm. (e)–(f) Cross-sections of cells of *B. sinica* strain CMN1a bearing two (e) or four (f) flagella, indicated by arrowheads. Bars, 0.2 µm.

Description of *Borrelia sinica* sp. nov.

Borrelia sinica (si'ni.ca. L. fem. adj. *sinica* of China, the country from which the organisms were isolated).

Morphology was as described previously for the genus (Burgdorfer & Hayes, 1986). However, counting of flagella showed a maximum of four at each end. Cultivated in BSK II medium at 30–34 °C, similar to *B. burgdorferi* (Johnson *et al.*, 1984). Differentiated from other *B. burgdorferi sensu lato* species by analysis of *Dra*I and *Mse*I restriction polymorphism of the 5S–23S rDNA (*rrf-rrl*) spacer. The G+C content of the DNA is 29.6 mol % for the type strain.

The type strain, CMN3^T, has been deposited in the Japan Collection of Microorganisms (RIKEN) as strain JCM 10505^T. This strain was isolated from *N. confucianus* captured in Sichuan province, China.

ACKNOWLEDGEMENTS

We thank Drs Ma (Zhejiang Institute of Microbiology), H. Fujita (Ohara Hospital) and H. Ohtake (Miyagi Prefectural University of Agriculture) for help with the survey in China. We also thank Dr K. Tsuchiya (Miyazaki Medical University) for identification of rodents. We are grateful to Drs A. G. Barbour (University of California), E. Isogai (Health Science University of Hokkaido), D. Postic (Institut Pasteur) and O. Péter (Institut Central des Hopitaux Valaisans) for providing mAbs. 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, 09670294 and 11670267) from the Ministry of Education, Science and Culture of Japan.

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