Borrelia turcica sp. nov., isolated from the hard tick *Hyalomma aegyptium* in Turkey

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Previously, a novel, fast-growing spirochaete was isolated from the hard tick *Hyalomma aegyptium*, which infests tortoises (*Testudo graeca*), by using Barbour–Stoenner–Kelly (BSK) II medium; the tick samples were taken from the Istanbul area in northwestern Turkey [Güner *et al.* (2003). *Microbiology* **149**, 2539–2544]. Here is presented a detailed characterization of the spirochaete. Electron microscopy revealed that strain IST7^T is morphologically similar to other spirochaetes of the genus *Borrelia* and possesses 15 to 16 flagellae that emerge from both polar regions. PFGE analysis revealed the genome to comprise a linear chromosome of approximately 1 Mb; two large linear plasmids of approximately 145 and 140 kb, and several small plasmids ranging from 50 to 20 kb in size were also found. The 16S rRNA gene sequence of this *Borrelia* isolate exhibited 99.4 to 99.8% identity with other strains isolated from *H. aegyptium* and less than 99% similarity with those of other *Borrelia* species. A phylogenetic tree, generated from 16S rRNA gene sequences, demonstrated that the spirochaete isolates from *H. aegyptium* clustered together and branched off from both Lyme-disease-related and relapsing-fever-associated *Borrelia* species. A single copy of the *rrs* gene was detected in the genome of strain IST7^T by Southern hybridization. DNA–DNA hybridization results showed that strain IST7^T was distinct from Lyme-disease-related *Borrelia*, *Borrelia burgdorferi* and the relapsing-fever-associated species *Borrelia hermsii*. The G + C content of strain IST7^T is 30.0 mol%. From these genetic features, a novel *Borrelia* species, *Borrelia turcica* sp. nov., is proposed; the type strain is IST7^T (=JCM 11958^T = DSM 16138^T).

Lyme borreliosis and relapsing fever are caused by *Borrelia* species (Barbour & Hayes, 1986). Lyme-disease-related *Borrelia* have been classified into at least 11 species (Masuzawa *et al.*, 2001; Miyamoto & Masuzawa, 2002) and are transmitted by hard ticks of the family Ixodes.

Abbreviations: BSK, Barbour–Stoenner–Kelly; CFE, constant-field electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences reported in this article are AB111849–AB111854.

Transmission electron micrographs, a PFGE profile, a two-dimensional gel electrophoresis profile, determination of the *rrs* copy number and a physical map of the DNA fragment containing the *rrs* gene of strain IST7^T are available as supplementary material in IJSEM Online.

Relapsing-fever-associated *Borrelia* species, including *Borrelia coriaceae*, are transmitted by soft ticks of the genus *Ornithodoros*. An avian borreliosis agent, *Borrelia anserina*, is also carried by soft ticks, *Argas persicus*. Other relapsing-fever-associated *Borrelia* species are *Borrelia recurrentis*, transmitted by the human body louse (*Pediculus humanus*), and a bovine borreliosis agent, *Borrelia theileri* (Armstrong *et al.*, 1996), transmitted by *Rhipicephalus evertsi* and *Boophilus* spp. (Barbour & Hayes, 1986). Furthermore, *Borrelia miyamotoi* (Fukunaga *et al.*, 1995), ‘*Borrelia lonestari*’ (Barbour *et al.*, 1996) and *B. miyamotoi*-like *Borrelia* (Fraenkel *et al.*, 2002) carried by hard ticks, *Ixodes persulcatus* in Japan, *Amblyomma americanum* in the United States and *Ixodes ricinus* in

Sweden, respectively, are classified within the relapsing-fever-associated *Borrelia*. Previously, we have reported the isolation of six pure cultures of a novel, fast-growing spirochaete from the hard tick *Hyalomma aegyptium*: tick samples were taken from a summer resort in the Istanbul area of Turkey in the summer of 2000 and the novel spirochaete was isolated by using Barbour–Stoenner–Kelly (BSK) II medium (Güner *et al.*, 2003). The optimal growth temperature of the spirochaete in BSK II medium was between 34 and 37 °C, and doubling times at 34 and 37 °C were 5·3 and 5·1 h, respectively. The six strains were morphologically similar to other *Borrelia* species and contained a 41 kDa flagellin protein. Analysis of *flaB* sequences suggested that the unique spirochaete belonged to the genus *Borrelia*, but differed from previously described species (Güner *et al.*, 2003). The aim of the present study was to characterize the novel, fast-growing spirochaete by using 16S rRNA gene sequence data, DNA–DNA relatedness, G + C content and chromosome and plasmid organization. Based on the results of these analyses, we propose a novel species of the genus *Borrelia*, *Borrelia turcica* sp. nov.

Strain IST7^T was purified from a colony formed on BSK II agar medium (Barbour, 1984) at 34 °C under 5 % carbon dioxide. To determine the number of flagellae, cross-sections of strain IST7^T were prepared as described previously (Masuzawa *et al.*, 2001). Photomicrographs were taken with a Hitachi H-7000 electron microscope at an acceleration voltage of 75 kV. Chromosome and plasmid organization was examined by PFGE as described by Cutler *et al.* (1997). The DNA contained in an agarose plug was run at 150 V with the pulse time ramped from 5 to 125 s for a total of 36 h, at 150 V with the pulse time ramped from 0·5 to 20 s for a total of 36 h or at 200 V with a pulse time of 2 s for 18 h. Furthermore, to investigate the structure of the chromosome and plasmids, two-dimensional gel electrophoresis was carried out as described by Ferdows *et al.* (1996). The DNA contained in an agarose plug was electrophoresed in the first dimension by PFGE at 200 V with the pulse time ramped from 0·5 to 15 s for 24 h in 0·5 × TBE. After the PFGE, the gel was placed in a horizontal unit and set perpendicular to the position of the first-dimension gel. Constant-field electrophoresis (CFE) was performed for 3·5 h at 80 V in 0·5 × TBE. Template DNAs for 16S rRNA gene sequencing were prepared as described previously (Masuzawa *et al.*, 1996). Aliquots (0·5 ml) of cultures were washed. The cells were resuspended in 100 µl of water and boiled at 100 °C for 10 min. After centrifugation at 10 000 g for 5 min, the supernatant was collected as template DNA. The 16S rRNA gene sequences were amplified with the primers 16SF1, corresponding to the 5' end of the 16S rRNA gene (5'-ATA ACG AAG AGT TTG ATC CTG GC-3'), and 16SR, corresponding to the 3' end of the 16S rRNA gene (5'-CAG CCG CAC TTT CCA GTA CG-3'), by PCR (Masuzawa *et al.*, 2001). The amplified 16S rRNA gene fragments (about 1350 bp) were purified by using a DNA purification column (Microcon-PCR; Millipore). For DNA sequencing, the same

sequence primers, labelled with Texas-red (Proligo), were used. The DNA cycle sequencing reaction was performed using the Thermo Sequenase pre-mixed cycle sequencing kit (Amersham Biosciences) according to the manufacturer's recommendation. DNA sequences were determined using a SQ5500EL DNA-sequencer (Hitachi). CLUSTAL_X software (Thompson *et al.*, 1997) was used to align the sequences, and the phylogenetic distance was calculated by using the neighbour-joining method (Saitou & Nei, 1987) and UPGMA method with Genetyx-Mac version 10. To determine DNA–DNA homology values, DNAs purified from strain IST7^T and representative *Borrelia* species, *Borrelia hermsii* strain HS1 and *Borrelia burgdorferi* strain B31^T as described previously (Kawabata *et al.*, 1993) were subjected to microplate hybridization using photobiotin-labelled DNA (Ezaki *et al.*, 1988; Masuzawa *et al.*, 2001). Briefly, DNA coated onto microplates was pre-treated with pre-hybridization solution [2 × SSC (1 × SSC is 0·15 M NaCl plus 0·015 M sodium citrate), 5 × Denhardt's solution and 50 % formamide] containing 200 µg denatured salmon sperm DNA ml⁻¹. Photobiotin-labelled DNA prepared as reported previously (Ezaki *et al.*, 1989) was hybridized in the presence 50 % formamide at 26 °C (Blüthmann *et al.*, 1973). The G + C content of DNA was determined by HPLC (Noguchi *et al.*, 1988), using a DNA-GC kit (Seikagaku-kogyo). The copy number of the 16S rRNA gene was determined by Southern blot analysis. To prepare the DNA probes, primers 8UA (5'-AGAGTTTGA-TCTGGCTCAG-3'; Komatsu *et al.*, 1996), 16SMR (5'-CCTCCCTTACGGGTTAGAG-3'; GenBank accession no. M88329, position 1452–1435) and 350R (5'-CTGCTGCCTCCCGTAG-3'; Sawada *et al.*, 1993) were used for amplification of part of the *rrs* gene (Fig. IV, available as supplementary material in IJSEM Online). The products amplified by using 8UA/16SMR or 8UA/350R were used as probe A and probe B, respectively. The purified genomic DNA was digested with restriction enzymes and separated on a 0·8 % agarose gel and transferred to a Hybond-N⁺ nylon membrane (Amersham Biosciences). The hybridization was performed with the probes labelled by an ECL Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences).

Spirochaetes isolated from *H. aegyptium* were slender and helically shaped (Fig. I, available as supplementary material in IJSEM Online). The length of the cells varied from 10 to 25 µm, and the diameter was 0·2 µm to 0·28 µm (Güner *et al.*, 2003). Eight to 23 flagellae were observed at both ends. The average number of flagellae observed was 15 to 16.

Strain IST7^T had chromosomal DNA that migrated as a 1 Mb band similar to that of other borrelia, *B. hermsii*, *Borrelia parkeri*, *Borrelia turicatae* and *B. burgdorferi* on the PFGE analysis (Fig. IIa, available as supplementary material in IJSEM Online). Furthermore, with a pulse time of 2 s, or ramped from 0·5 to 20 s, a relatively small plasmid was separated. Strain IST7^T had plasmids of about 145 and 140 kb in size, and at least seven plasmids smaller than 50 kb

(Fig. IIB, c, available as supplementary material in IJSEM Online). The genomic organization of spirochaetes in the genus *Borrelia* is unique in that the chromosome and most plasmids are linear (Barbour & Hayes, 1986). Two-dimensional gel electrophoresis was performed to investigate the genomic structure of strain IST7^T. All DNA bands of strain IST7^T with chromosomal DNA migrated in the second-dimension gel (Fig. III, available as supplementary material in IJSEM Online). An open circular DNA may enter the gel in PFGE of the first dimension, but it will be arrested in CFE of the second dimension (Ferdows & Barbour, 1989; Serwer & Allen, 1984). In this experiment, the 1 Mb chromosome and 140 and 145 kb plasmids of strain IST7^T behaved in a similar manner to the linear DNA molecule of *Saccharomyces cerevisiae* and of *B. hermsii* in the second-dimension gel. Therefore, the results indicated that these were linear DNA molecules.

It was reported that there was a single copy of the *rrs* gene in spirochaetes of the genus *Borrelia* (Schwartz *et al.*, 1992). We determined whether the *rrs* gene of the *Hyalomma*-originating isolate was present as a single copy by Southern blot hybridization with two probes that were complementary to a specific sequence in the gene. Restriction enzymes that cleave inside the gene (strain IST7^T; GenBank accession no. AB111849, this study) were selected for the analysis. The results using *Hind*III, *Kpn*I and *Xba*I and the restriction map containing the *rrs* gene are shown in Fig. IV (available as supplementary material in IJSEM Online). DNA fragments of 5.7 and 3.7 kb were hybridized with probe A in the *Xba*I digestion, but the 3.7 kb band was not observed with probe B. DNA fragments of 9.4 and 7.8 kb were hybridized with probe A in the *Kpn*I digestion, but the 7.8 kb band was absent when probe B was used. DNA fragments of 1.4 and 1.9 kb were hybridized with probe A in the *Hind*III digestion, whereas the 1.9 kb band was absent when probe B was used. A single band ranging from 4.5 to 20 kb in length was observed with probe A when restriction enzymes *Bam*HI, *Bgl*II, *Nco*I, *Nhe*I, *Spe*I, *Sph*I and *Xho*I, which did not cleave inside of the *rrs* gene, were used (data not shown). These data, together with the results of *rrs* gene sequencing, indicate that a single copy of *rrs* is present in the genome.

A phylogenetic tree, based on 16S rRNA gene sequences, was constructed by using the neighbour-joining method (Fig. 1). The phylogenetic relationship among relapsing-fever-associated *Borrelia* species and Lyme-disease-related *Borrelia* species was reported previously by Ras *et al.* (1996). The 16S rRNA gene sequences of six strains, ISTF1, IST2, ISTF2, IST4, IST6 and IST7^T, isolated from *H. aegyptium* formed a monophyletic cluster and showed similarity values ranging from 99.4 to 99.8%. Interestingly, spirochaetes isolated from *H. aegyptium* formed a separate branching root from both Lyme-disease-related *Borrelia* species and relapsing-fever-associated *Borrelia* species (similarity values ranging from 93.9 to 98.2%). A similar tree on the topology of branches was constructed by using the UPGMA

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence data. Sequences of strains ISTF1, ISTF2, IST2, IST4, IST6 and IST7^T were determined in this study. Accession numbers are shown in parentheses. Bar, 1.0% sequence divergence. *Spirochaeta littoralis* (GenBank accession no. M88723) was used as an outgroup (not shown).

method (data not shown). Our findings suggested that the *Hyalomma*-originating isolates were unique and phylogenetically divergent from both Lyme-disease-related and relapsing-fever-associated *Borrelia* species. Furthermore, the level of DNA relatedness in strain IST7^T was less than 20% when compared with the Lyme-disease agent, *B. burgdorferi*, or the relapsing-fever agent, *B. hermsii*. These results indicated that strain IST7^T was different from *B. burgdorferi* and *B. hermsii*. The G + C content of the DNA of strain IST7^T was 30.0 mol%. The G + C content of the DNA of the other borrelia species, *B. hermsii*, *B. parkeri*, *B. turicatae* and *B. burgdorferi*, examined in this study was 30.6, 30.6, 29.4 and 29.0 mol%, respectively. These values were similar to those reported previously (Barbour & Hayes, 1986; Hyde & Johnson, 1984; Johnson *et al.*, 1984; Kawabata *et al.*, 1993; Schmid *et al.*, 1984). Based on these findings, and the results of *flaB* and phenotypic analyses reported previously (Güner *et al.*, 2003), a novel *Borrelia* species, *Borrelia turcica* sp. nov., is proposed.

Description of *Borrelia turcica* sp. nov.

Borrelia turcica (tur.ci'ca. N.L. fem. adj. *turcica* referring to Turkey, the country in which the species was isolated).

Isolated from the hard tick *Hyalomma aegyptium* from samples collected in Istanbul, Turkey. Morphology is as

described previously for the genus (Barbour & Hayes, 1986; Güner *et al.*, 2003). Length of the cells is variable, about 10–25 µm, and the diameter is 0.2–0.28 µm, with eight to 23 flagellae. The average number of flagellae is 15 to 16. The optimal growth temperature in BSK II medium is between 34 and 37 °C; cells can grow at 39 °C. Doubling times at 34 and 37 °C are 5.3 and 5.1 h, respectively. Contains a 41 kDa protein that is reactive with mAb H9724 (Barbour *et al.*, 1986), which is specific to the flagellin of *Borrelia* species.

The type strain is IST7^T (=JCM 11958^T = DSM 16138^T). Its G + C content is 30.0 mol%.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research B (No. 13576014) from the Japan Society for the Promotion of Science (JSPS).

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