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RESEARCH BRIEF

# *Trypanosoma avium:* Large Minicircles in the Kinetoplast DNA[1]

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Recently, it was shown that in members of the suborder Bodonina, kinetoplast DNA (kDNA) is organized in a form of noncatenated circular molecules (Maslov and Simpson 1994; Yasuhira and Simpson 1996; Lukeš *et al.* 1998), thus fundamentally differing from the kDNA network structure of trypanosomatids (Shapiro and Englund 1995). In the phylogenetic trees based on the small and large subunit ribosomal RNA gene sequences, *Trypanosoma avium* appears at the base of the monophyletic *Trypanosoma* clade, which represents the earliest branch within the suborder Trypanosomatina (Maslov *et al.* 1996; Lukeš *et al.* 1997). Its phylogenetic position makes *T. avium* an interesting object for the kDNA studies.

*T. avium* parasitizes hundreds of bird species virtually worldwide (Baker 1976), is transmitted by a wide variety of insects and acarids that feed on bird blood, and can cause fatal infections in the bird (Baker 1976: Mungomba *et al.* 1989). Despite their importance as serious pathogens, with few exceptions, only morphological information is available about this group of flagellates.

*T. avium* strains A1412 and A493 were isolated from the bone marrow of a raven, *Corvus frugilegus* (1979, Prague, Czech Republic) and a blackbird, *Turdus merula* (1977, Lindava, Czech Republic), respectively. Both strains were cultivated in the SNB-9 medium (Diamond and Herman 1954) at 24°C. The kDNA was isolated as described elsewhere (Jirku *et al.* 1995).

The restriction analysis revealed that the kDNA of the *T. avium* strains A1412 and A493 contains unusually large minicircles. Undigested kDNA remained in the slot due to the catenated network structure (Fig. 1, lane 2). After the digestion with selected restriction endonucleases, bands of the minicircles appeared in the gel. On the basis of the restriction patterns produced by the digestion with tetranucleotide-target endonucleases (Fig. 1, lanes 4 and 7), which generated a high number of apparently nonstoichiometric bands, the minicircle population appeared heterogeneous in sequence. Hexanucleotide-target restriction enzymes generated single bands of minicircles with the apparent sizes 10 and 6 kb (Fig. 1, lanes 3 and 6).

In order to determine the size of the minicircles and their catenation into a network, we analyzed DNA of cells embedded in the low-melting agarose blocks (final concentration of $5 \times 10^7$ cells/ml) according to Rovai *et al.* (1992). Prior to the restriction digestion, the blocks were incubated overnight at room temperature in an appropriate restriction buffer containing bovine serum albumin at the final concentration 300 mg/ml, then melted for 5 min at 65°C, and cooled to 37°C, and the restriction was performed with 30 U of *Eco*RI and *Pvu*II for 24 h at 37°C. Untreated blocks and blocks in which the DNA was cut by the restriction endonucleases were analyzed by a pulse-field gel electrophoresis in a FIGE Mapper (Bio-Rad) in 1% agarose gel and 0.5× TBE buffer at forward and reverse voltages of 180 and 120 V, respectively, at a linear switch time ramp (0.1–0.8 s) at 10°C for 11 h. In untreated samples the kDNA remained in the slot (Fig. 2, lanes 1 and 3) while after digestion the linearized minicircles entered the gel and, as in the regular agarose gels, migrated at 6 and 10 kb (Fig. 2, lanes 2 and 4). No minor bands were observed.

Since anomalous electrophoretic mobility of kDNA minicircles is

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0014-4894/99 $30.00

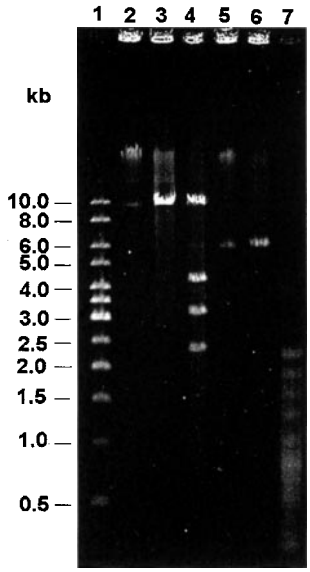



**FIG. 1.** Restriction analysis of *T. avium* kDNAs. 1-kb ladder (lane 1); undigested A1412 kDNA (lane 2); A1412 kDNA cut with *Sac*I and *Pvu*II, respectively (lanes 3 and 4); undigested A493 kDNA (lane 5); A493 kDNA cut with *Sac*I and *Msp*I, respectively (lanes 6 and 7). DNA was detected by UV after ethidium bromide staining of 0.75% agarose gel.

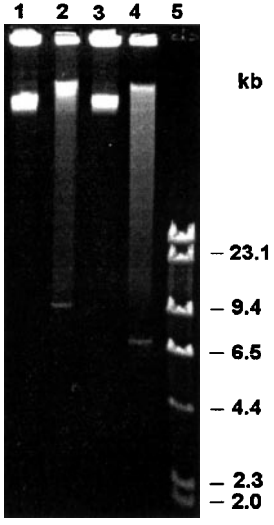



**FIG. 2.** Pulse-field gel electrophoresis of *T. avium* cells embedded in low-melting agarose blocks. Undigested and *Eco*RI-digested blocks with A1412 cells (lanes 1 and 2). Undigested and *Pvu*II-digested blocks with A493 cells (lanes 3 and 4). λ/*Hind*III marker (lane 5).

frequently encountered (Shapiro and Englund 1995), we have also analyzed the size of minicircles by transmission electron microscopy. The kDNA was prepared by the cytochrome *c* method (Fergusson and Davis 1978) and examined with a Philips CM12/STEM electron microscope at 80 kV. The contour lengths of free minicircles found on the grids along with the networks were measured from prints using the HIPAD digitizing tablet, and the precise magnification was determined by replica grating (Balzers).

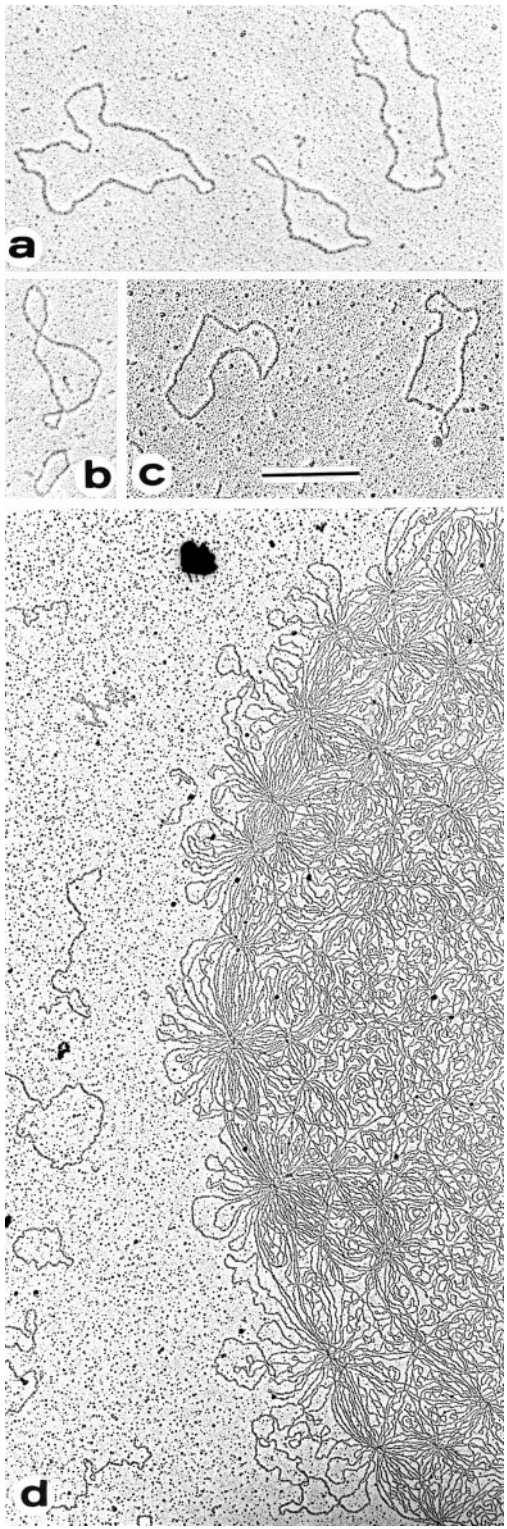
The *T. avium* kDNA networks seem to have structure similar to the *Crithidia fasciculata* networks, with minicircles catenated via a single interlock with their neighbors (Fig. 3d and data not shown). For more accurate measurement, only minicircles free from networks have been measured (Figs. 3a–3c). The majority of minicircles of strain A1412 ($n = 73$) exhibited a mean size of 9.48 kb (SD 0.24 kb) and only approximately 10% minicircles were significantly smaller, ranging in size from 2 to 6 kb. The analysis of the A613 strain showed a major component of 5.90 kb ($n = 112$; SD 0.30 kb), and a few small components (Fig. 4). This analysis confirmed the existence of unusually large kDNA minicircles in both bird trypanosomes.

The size of the kDNA minicircles ranges between 0.6 and 5.2 kb in different trypanosomatid species (Jirku *et al.* 1995) and minicircles of bodonids *Cryptobia helicis* and *Bodo saltans* also fall in this size range (Lukes *et al.* 1998; Blom *et al.* 1998). With the minicircle-like sequences concatenated into 180-kb large circular molecules, *Trypanoplasma borreli* may be the only exception (Yasuhira and Simpson 1996). The 6- and 10-kb minicircles of *T. avium* significantly extend this size range.

Instead of sequencing the entire minicircle, we attempted to analyze its structure by PCR using primers PT1 (TTTAAGCTTAGGGGTTGGTGTAAT) and PT2 (TTGGATCCTATCGAAGCACCAC) that fully and partially, respectively, overlap with the conserved minicircle dodecamer CSB-3 (Ray 1989), contain *Hind*III and *BamH*I restriction sites, and are outward oriented. PCR was performed using 10 ng of the kDNA with initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, 59°C for 1 min, and 72°C for 1.5 min. PCR products were purified in 1% agarose gels and isolated by electroelution. Cloning was performed using the pBS SK(⁻) and *E. coli* XL-1 Blue competent cells (Stratagene) and both strands were sequenced using the Sequenase version 2.0 system (Amersham).

With both *T. avium* strains, two products differing in size and abundance were amplified (data not shown) and three amplified molecules of the A1412 strain were sequenced. The sequences of 835- and 837-bp clones obtained from the large dominant band were 97% identical. The region between the CSB-3, which was part of the PT1 primer, and the CSB-1 like region (GGGCGG) showed a weak similarity with the conserved regions (CRs) of *Trypanosoma cruzi* and *T. carassii* minicircles. In the obtained sequences, we looked for the guide RNA (gRNA) genes by searching for regions that showed complementarity with the edited mRNAs available from different trypanosome species. By this approach we found a 36-bp region (130 bp downstream from

**FIG. 3.** Electron microscopy of *T. avium* kDNA network and free minicircles. (a) Minicircles of the strain A1412; (b,c) minicircles of the strain A493. In (a) and (b) representatives of the major (big circles) and minor (small circles) populations of minicircles are shown. (d) A periphery of the kDNA network. The bar represents 0.5 μm (approximately 1.6 kb) in (a,b,c), and 1 μm (approximately 3.2 kb) in (d).



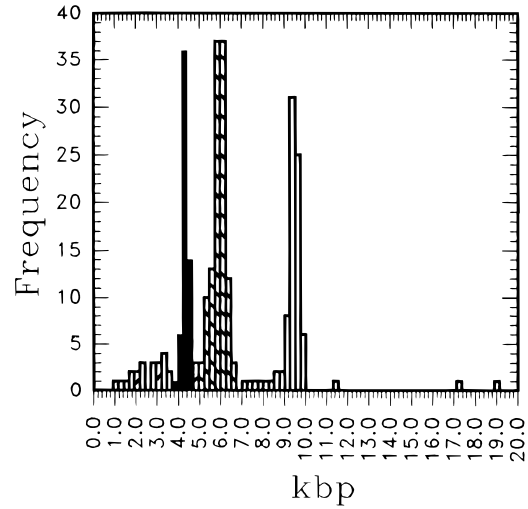




**FIG. 4.** Size distribution of *T. avium* kDNA minicircles in the strains A493 (shaded) and A1412 (open) determined by measuring their contour length. pBR322 plasmid (black) was used as a control.

the CSB-3) that could serve as a template for gRNA involved in the editing of a segment of the cytochrome oxidase subunit III (coIII) mRNA. When the G-U base pairing is allowed, this potential gRNA gene has high complementarity with part of the *T. brucei* coIII edited mRNA (GenBank Accession No. M20379).

Although the size of minicircles is different in the strains A1412 and A493, PCR products of the same size were obtained from both analyzed kDNAs. We assume that the products represent regions between two adjacent CRs. The size uniformity of the sequences from the major amplified band suggest that the CRs are equidistant in the studied minicircles. Moreover, there may be up to 12 CRs in one minicircle, as judged from the size of minicircles and PCR products.

Biological significance of different, but species-specific, sizes of minicircles is unclear. Minicircles bear most of the gRNA genes and this, at present, appears to be their major function. However, the coding capacity for gRNA genes is obviously not influenced by the minicircle size, because only one gRNA gene is encoded by the 2.5-kb *C. fasciculata* minicircles (Yasuhira and Simpson 1995), while smaller minicircles of *T. cruzi* code for up to four gRNA genes (Avila and Simpson 1995). Although the phylogenetic position of *T. avium* has not been unambiguously resolved by the available ribosomal RNA sequence data (Maslov *et al.* 1996; Lukeš *et al.* 1997), it is obvious that any correlation between a minicircle size on one side and a phylogenetic position, final or intermediate hosts, or pathogenicity of a given flagellate on the other side is absent.

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