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Characterization of *Phytomonas* sp. kinetoplast DNA

A plant pathogenic trypanosomal species

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Phytomonas sp. which belongs to the Trypanosomatidae family, is a pathogen of plants. Its kinetoplast DNA consists of a huge network of about 7000 catenated minicircles of 2880 ± 30 base pairs each. It represents 30–33% of the total cellular DNA. An AT composition of 65% has been evaluated from the buoyant density $\varrho = 1.694$ g/ml. The restriction endonuclease *Hpa*II was the only one able to cleave the minicircles into a single fragment. The other enzymes so far tested (*Eco*RI, *Hind*III, *Hae*III, *Bam*HI) do not cleave these minicircles significantly. Analysis of the kDNA by Southern blot hybridization shows that minicircles of *Phytomonas* sp. have little sequence homology with minicircles of other species of trypanosomes. Maxicircles, present in low proportions, have been identified. Our data indicate that the kinetoplast DNA of *Phytomonas* sp. presents biochemical characteristics which could be used to identify the *Phytomonas* genus.

kDNA; Restriction endonuclease; Topoisomerase II; Southern blotting; (Plant trypanosome)

1. INTRODUCTION

It has been known since 1909 that plants can harbor trypanosomes and, in 1910, the genus *Phytomonas* was termed on the basis of no strict criterion [1]. Since it was difficult to obtain in vitro cultures of *Phytomonas*, little information became available on the biochemical properties of this new genus, especially since the first in vitro grown '*Phytomonas*' [2] showed many biochemical properties similar to those of the *Herpetomonas* genus (Camargo, E.P., personal communication). The characterization of *Phytomonas* by serological techniques, using polyclonal or monoclonal antibodies, revealed numerous cross-reactions between *Phytomonas* from different origins and other members of the Trypanosomatidae family (*Herpetomonas, Trypanosoma* or *Crithidia*) [3].

Phytomonas can parasitize different kinds of plant tissues, some being found in laticiferous tubes, while others are phloem-restricted [1]. Some species are specifically associated with certain pathological syndromes, like the Hartrot of the coconut, the Marchitez sorpresiva of the oil palm, or the phloem necrosis of the coffee tree [1]. All these diseases are economically important in Latin America [1].

The variation in nutritional requirements observed during primoculture experiments points to the existence of different species of *Phytomonas* in plants [4]. We have tried to characterize *Phytomonas* by studying their kinetoplast DNA (kDNA).

The kDNA of Trypanosomatidae is composed of a majority of minicircles, whose function remains unknown [5–7], and of maxicircles which

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contain the mitochondrial, ribosomal, and structural genes [6,7]. The minicircles of the various trypanosome species have different biochemical properties and their characterization is, therefore, a valuable means of identifying the species. It should be noted that kDNA restriction enzyme patterns have successfully been used for species identification of insect trypanosomatids [8,9].

2. MATERIALS AND METHODS

2.1. *Cells and cultures*

We have used *Phytomonas* sp. strain EM1, clone C''1C[5] isolated from *Euphorbia pinea* [4]. Trypanosomes were cultured at 26°C in 250 ml of the medium described by Yunker et al. [10]. Cells were collected by centrifugation after about 7 days when their concentration had reached about 10^7 cells/ml. The cells were washed 3 times in 50 ml of 0.15 M NaCl, 0.015 M Na citrate. *Trypanosoma cruzi* and *Herpetomonas samuelpessoai* were grown in vitro as in [11]. *T. equiperdum* was obtained from infested rat blood [12].

2.2. *kDNA preparations*

Total cell DNA was prepared and the kDNA fractionated by sedimentation in 3 M CsCl, as described [13]. The kDNA was then purified in a CsCl-ethidium bromide gradient [11].

2.3. *Preparation of free minicircles*

Free minicircles were obtained by decatenation of the kDNA network with calf thymus or trypanosome topoisomerase II prepared in this laboratory [14]. After incubation of the kDNA with enzyme under the conditions in [15], the reaction mixture was electrophoresed in a 2% agarose gel. Minicircles were eluted, purified in a CsCl gradient and used for electron microscope study or as DNA probes for Southern blot hybridization.

2.4. *Digestion of kDNA with restriction enzymes and electrophoretic analysis*

The kDNA was incubated with restriction enzymes (*Hpa*II, *Eco*RI, *Hae*III, *Bam*HI, *Hin*dIII) under the conditions recommended by the supplier (Boehringer, Mannheim). The reaction mixtures were electrophoresed in 2% agarose gel, as in [11]. The gels were stained with ethidium bromide (5 mg/l for 10 min) and photographed under UV light with a Polaroid camera (665 film).

2.5. *Southern blot hybridization*

kDNAs from *T. cruzi, T. equiperdum, H. samuelpessoai* and *Phytomonas* sp. were incubated with topoisomerase II, electrophoresed in a 1.5% agarose slab gel and analyzed by the Southern blot hybridization technique [16] using minicircle probes. Free minicircles from *Phytomonas* sp. and/or *H. samuelpessoai* were labeled with [^{32}P]dCTP to a specific activity of about $5–7 \times 10^7$ cpm/μg by nick-translation [16]. Hybridization was performed under stringent conditions ($T_m = -20$°C). Blots were autoradiographed on Kodak XAR5 films for 4 h.

3. RESULTS

3.1. *The DNA of* Phytomonas *sp.*

The DNA content of *Phytomonas* sp. in the exponential growth phase was evaluated by spectrofluorimetry, using Hoechst dye 33258 [17] and found to be $0.7 \pm 0.15 \times 10^{-13}$ g per cell. Total cell DNA was prepared and analyzed by analytical ultracentrifugation at equilibrium in a CsCl gradient. The densitometer tracing of fig.1 reveals the

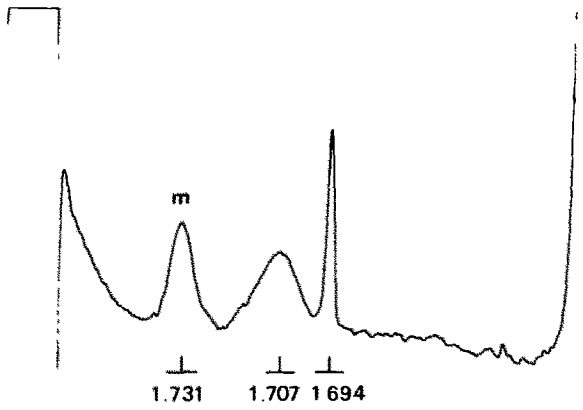

Fig.1. Analytical ultracentrifugation at equilibrium in a neutral CsCl gradient of the DNA extracted from *Phytomonas* sp. kDNA, ϱ = 1.694 g/ml; nuclear DNA, ϱ = 1.707 g/ml; m, *Micrococcus luteus* DNA used as density marker, ϱ = 1.731 g/ml. The assay was carried out in an L5 65 Beckman ultracentrifuge, equipped with a UV scanner system (Rotor AN-E', at 40000 rpm, for 36 h, at 25°C).

presence of two DNA peaks: the peak of buoyant density ϱ = 1.707 g/ml corresponds to nuclear DNA, while that of density ϱ = 1.694 g/ml corresponds to kDNA. The sharp profile of the latter is characteristic of high-M_r trypanosomal kDNA [18,19]. After fractionation and purification of the kDNA in a CsCl gradient, only the peak of ϱ = 1.694 g/ml was observed. The kDNA accounts for about 30–33% of the total DNA as evaluated by measuring the areas under the peaks of the photoelectric scanner tracing. From measurement of the buoyant densities [20], a proportion of 65% of AT base pairs can be estimated for the kDNA and 52% for the nuclear DNA.

3.2. *Electron microscope study of the kDNA*

Purified *Phytomonas* kDNA was spread by the method of Dubochet et al. [21] and observed under the electron microscope. Like the kDNA of other Trypanosomatidae species [12,22], the *Phytomonas* kDNA is a huge network composed of thousands of interlocked minicircles. Larger molecules, stretching out of the edge of the kDNA network, are also observed (not shown). The presence of maxicircles catenated to the network has, however, rarely been observed. The minicircles must be associated by catenation since the kDNA network is resolved into free minicircles by type II topoisomerase [14,15] (fig.2, lane 5). Minicircles (n = 55 molecules) were measured in the presence of circular DNA molecules from phage ϕX174 RFII. The mean perimeter was 0.98 ± 0.01 μm, corresponding to 2880 ± 30 base pairs. The number of minicircles can be estimated at about 7000 per kDNA network. Fig.2 shows the result of the electrophoresis of kDNA after incubation with a type II topoisomerase. The intact kDNA network (lane 4) was resolved into minicircles (lane 5). The main band consisted of relaxed minicircles, while the other fast migrating bands were minicircle topoisomers [15]. Together with the free minicircles, some long and intricate molecules, expected to be maxicircles, were also observed under the electron microscope. Because of the high degree of complexity after spreading, these molecules were difficult to measure. However, their length was estimated to be between 9.8 and 12 μm. These free large molecules, as well as those extruding from the intact network, were rarely observed, suggesting that maxicircles are

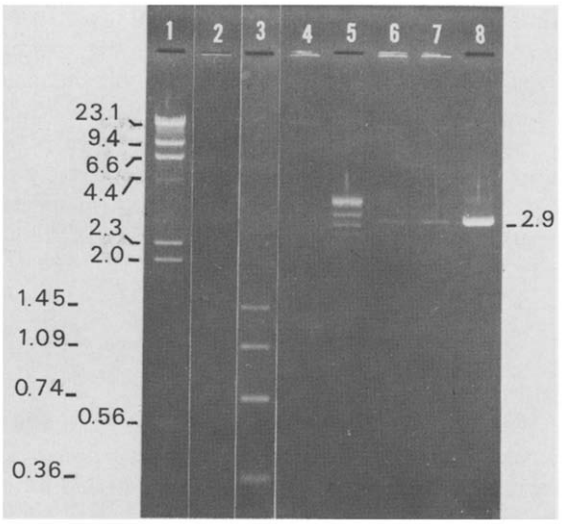

Fig.2. Electrophoretic analysis in 2% agarose slab gels of kDNA networks from *Phytomonas* sp. incubated with calf thymus topoisomerase II and restriction endonucleases. (1) Phage DNA + *Hin*dIII; (2) *T. cruzi* kDNA; (3) + *Hae*III, the four DNA fragments of the minicircle usually observed are present [11]; (4) *Phytomonas* sp. kDNA; (5) + topoisomerase II, the minicircles are liberated and several topoisomers are observed [15]; (6) + *Eco*RI; (7) + *Hae*III; (8) + *Hpa*II, the minicircles are cleaved in a single linear DNA fragment. The lengths of DNA fragments are given in kilobase pairs.

present in a low proportion in the *Phytomonas* kDNA network.

3.3. *Analysis of the* Phytomonas *kDNA by restriction enzymes*

The kDNA was incubated with the following restriction endonucleases: *Hpa*II, *Eco*RI, *Hae*III, *Bam*HI and *Hin*dIII. When electrophoresed in agarose gels, the intact kDNA cannot penetrate the gel (fig.2, lanes 2,4), while after cleavage smaller DNA molecules are formed. Among the 5 restriction enzymes, which usually cleave the kDNA of other trypanosomal species (fig.2, lane 3) [11,12,22], only *Hpa*II was able to cleave the kDNA minicircles of *Phytomonas* sp., the generated fragments having the length of a minicircle (fig.2, lane 8). The other enzymes were ineffective, even when they were incubated with *Phytomonas* free minicircles obtained by decatenation of the kDNA. Furthermore, free minicircles

Fig.3. Electrophoresis in 1.5% agarose gel of kDNAs incubated with calf thymus topoisomerase II (A) and Southern blot hybridization analysis (B,C). A: (1) *T. cruzi* kDNA; (2) + topoisomerase II; (3) *H. samuelpessoai* kDNA; (4) + topoisomerase II; (5) *Phytomonas* sp. kDNA; (6) + topoisomerase II; (7) *T. equiperdum* kDNA; (8) + topoisomerase II. Gel was stained with ethidium bromide. B: The gel in (A) was transferred to a GeneScreenPlus membrane according to the Southern blot technique and blot hybridized with ^{32}P-labeled minicircles from *Herpetomonas* and exposed to Kodak XAR5 film for 4 h. Hybridization bands are only obtained with *Herpetomonas* kDNA. C: The blot in (B) was dehybridized and then probed with ^{32}P-labeled minicircle from *Phytomonas* and exposed to Kodak XAR5 film for 4 h. Hybridization bands are only obtained with *Phytomonas* kDNA. kDNAs in B (lane 3) and C (lane 5) are not labeled as they are not transferred.

incubated with *Hpa*II and *Hae*III provided the DNA fragments generated by *Hpa*II alone (not shown).

3.4. *Analysis of kDNAs by Southern blot hybridization*

kDNAs from *T. cruzi, H. samuelpessoai, Phytomonas* sp. and *T. equiperdum* were incubated with topoisomerase II and electrophoresed in a 1.5% agarose gel (fig.3A). Fig.3B,C presents autoradiograms after Southern blot and hybridization with ^{32}P-labeled minicircles from *Herpetomonas* (B) and *Phytomonas* (C). The results show that minicircles of *Phytomonas* sp. have little sequence homology with those of the other species of trypanosome.

4. DISCUSSION

We have purified the kDNA of *Phytomonas* sp., a trypanosome species isolated from plants and cultured in vitro. It represents more than 30% of the total cell DNA, which is the highest proportion of kDNA ever found in a cultured form of trypanosome [7]. An AT composition of 65% was deduced from the buoyant density value (ϱ = 1.694 g/ml). We have also found that the *Phytomonas* kDNA is composed of catenated minicircles, since each network was resolved by type II topoisomerases into about 7000 minicircles of 2880 ± 30 base pairs each. These minicircles are the largest ever found [7]. They also present the remarkable characteristic of being cleaved only by *Hpa*II restriction enzyme. The 4 other enzymes used (*Eco*RI, *Hae*III, *Bam*HI, *Hin*dIII), which cleave the kDNA of other trypanosomal species [7], are ineffective on the *Phytomonas* minicircles. Analysis of the kDNA by Southern blot hybridization shows that minicircles of *Phytomonas* sp. have little sequence homology with those of the other species of trypanosomes.

If confirmed in other strains, these properties could permit the distinction of the *Phytomonas* species from other species of Trypanosomatidae and, more particularly, from the *Herpetomonas* species with which it is often confused. Our data are different from those obtained by Cheng and Simpson [23] for the kDNA of *Phytomonas davidi*, a *Phytomonas* species described in 1909 [24] and recently cultured in vitro [2]. The

characterization of the *Phytomonas* maxicircles is still incomplete. In conclusion, our data show that, because of its original properties, the kDNA of *Phytomonas* sp. will allow the identification of the parasite in infested plants.

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