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FEMS Microbiology Letters 205 (2001) 65–69

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Kinetoplast DNA minicircles of phloem-restricted *Phytomonas* associated with wilt diseases of coconut and oil palms have a two-domain structure

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Received 1 August 2001; accepted 15 September 2001

First published online 26 October 2001

Abstract

We report the cloning and sequencing of the first minicircle from a phloem-restricted, pathogenic *Phytomonas* sp. (Hart 1) isolated from a coconut palm with hartrot disease. The minicircle possessed a two-domain structure of two conserved regions, each containing three conserved sequence blocks (CSB). Based on the sequence around CSB 3 from Hart 1, PCR primers were designed to allow specific amplification of *Phytomonas* minicircles. This primer pair demonstrated specificity for at least six groups of plant trypanosomatids and did not amplify from insect trypanosomatids. The PCR results were consistent with a two-domain structure for other plant trypanosomatids.

Keywords: Conserved sequence block; Hartrot; Marchitez sorpressiva; Polymerase chain reaction; Plant trypanosomatid

1. Introduction

Some phloem-restricted trypanosomatids are specifically associated with sudden wilts of coconut (hartrot) and oil palm (marchitez sorpressiva) endemic to Latin America [1]. These trypanosomatids are transmitted through pentatomid bugs from the genus *Lincus* [2]; however, the natural plant reservoir(s) of the trypanosomatids that kill these two introduced plants is unknown.

The taxonomy of trypanosomatids isolated from plants is complicated by the observation that several monoxenous genera of nebulous membership known as 'insect trypanosomatids', namely *Leptomonas*, *Herpetomonas*, and *Crithidia*, can multiply in fruit [3]. All kinetoplastids found in plants, including fruit, have typically been placed together under the designation *Phytomonas*. Thus there is a need to distinguish between trypanosomatids from plants and insects.

Several markers have been used to explore the relative classification within the phytomonads. Isoenzyme, RAPD and 5S rDNA analyses have defined a minimum of eight groups, currently designated A–H [4–6]. Studies of the SL-RNA gene have defined differences within phloem-restricted parasites [7].

Mitochondrial minicircles contain the genes for guide RNAs involved in the editing of mitochondrial RNA [8]. They have been useful molecular markers for taxonomic, phylogenetic, and diagnostic studies of trypanosomatids [9]. Minicircle templates are small and abundant, and have been exploited to create sensitive diagnostic and molecular epidemiological tools using PCR [10]. The kDNA minicircles of plant trypanosomatids show a range of different sizes [11–13]; however, sequences of minicircles of plant trypanosomatids have been obtained only for fruit, specifically tomato, isolates [14,15]. In this paper, we report the first minicircle sequence from a pathogenic, phloem-restricted *Phytomonas* sp. and explore the use of the minicircles from *Phytomonas* spp. as informative molecular tools.

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0378-1097/01/$20.00
PII: S0378-1097(01)00448-7

2. Materials and methods

2.1. Trypanosomatids

The isolate Hart 1 [11] was obtained from a coconut tree affected with hartrot in Saut Sabbat, French Guiana. The previously described *Phytomonas* spp. isolates used for PCR amplification are: Ps1G [16]; Lima.Pe, Berg.Br, E.hi.Sur, Mand.sc.Br, Tom.Sp, E.M.1, E.hi.Ind, E.hi.Sen, Mani.Br, E.hi.Vz, E.hy.Gu, Hart 1, Mar 1 and Ascl.cu.Vz [6]; Aca [11]; Mar 6 [7]; Blepharodon, Rhabdadenia, Trifolium and Amaranthus [5]. Previously undescribed Venezuelan isolates from *Euphorbia heterophila* (E.het.Vz) and *Euphorbia prostata* (E.pro.Vz) were collected by M.D. from San Augustin, Estado Sucre in 1991 and Puerto Chama, Estado Zulia in 1992, respectively.

The following insect trypanosomatid DNAs were used as controls: Tangerine (Saõ Paulo TCC 237), *Leptomonas mirabilis* (ATCC 30263), *Leptomonas seymouri* (ATCC 30220), *Crithidia oncopelti* and *Crithidia luciliae* (kindly provided by Theo Baltz, University of Bordeaux II), *Crithidia deanei* (ATCC 30255), *Crithidia acanthocephali* (ATCC 30251), *Herpetomonas muscarum* (Liverpool School of Tropical Medicine), *Herpetomonas megaseliae* (ATCC 30209) and *Herpetomonas samuelpessoai* (kindly provided by G. Riou, Institute Gustave Roussy).

2.2. kDNA extraction, cloning, sequencing and analysis

kDNA for cloning was obtained through fractionation in a CsCl-propidium iodide gradient as described previously [11]. Minicircles were cut with *Mbo*I (one cut), and cloned into the *Bam*HI site of the plasmid pBluescript SK+. Candidate clones were purified, analyzed by digestion with *Mbo*I, and screened for the presence of inserts corresponding to the size of Hart 1 minicircles [11,12]. A 0.8-kb *Pst*I fragment was subcloned to allow for complete sequencing of the minicircle clone. Template plasmids were purified using the QIAquick PCR Purification Kit (Qiagen) and sent to Davis Sequencing for dideoxy sequencing. Sequence information was compiled and analyzed using the UW GCG package.

2.3. PCR amplification

Synthetic oligonucleotides used for *Phytomonas* minicircle-specific amplifications from the CSB-3 motif were: mcCSB3a, 5′-GGTTT TTTAG GGGTT GGTAT AAT; mcCSB3b, 5′-TACCA ACCCC TAAAA AACCC C; mcCSB3aa, 5′-AAGGT TTTTT AGGGG TTGGT ATA; mcCSB3bb, 5′-GGTAC CAACC CCTAA AAAAC CC. PCR reactions were carried out using reagents from the Qiagen PCR Core Kit. Amplification was obtained routinely from 10 ng of total cell DNA or directly from 1/1000 dilutions of guanidine isothiocyanate cell lysates (4×10^8 cells ml^{-1}) into H_2O. The cycle profile used was 94°C for 2 min; 30 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 1 min; 72°C for 9 min. Reaction products were separated on 1.0% SeaKem LE agarose gels in 1×TBE buffer.

3. Results and discussion

Sequence characterization of a cloned minicircle from the Hart 1 isolate of *Phytomonas* sp. revealed a molecule of 1604 bp (GenBank accession No. AF397906), whose features are summarized in Fig. 1A. Analysis of the sequence identified two conserved regions (CR) of approximately 220 bp, containing variants on the conserved sequence block (CSB) 1, 2 and 3 motifs found in all Trypanosomatidae [17]. The conserved regions extended upstream from CSB 3 and were nearly identical, with minor variability in the area 27–56 bp upstream from CSB 1 (data not shown). The spacing among the CSB elements in Hart 1 was shorter than described for tomato fruit isolates [14,15], and more closely approached the spacing found in *Trypanosoma brucei*.

The Hart 1 minicircle structure is similar to that described in *Phytomonas serpens* [15], with one variable region showing a strong bias in G and T residues and the other containing a potential guide RNA gene in the vicinity of the *Mbo*I site. Unlike *P. serpens*, the sequence between CSB 1 and CSB 3 is identical for both CRs in the Hart 1 minicircle.

Based on the alignment of the Hart 1 minicircle CSBs

Table 1
Plant trypanosomatid minicircle and PCR product sizes

Group [4–6]	Isolate	Size (kb)	PCR (kb)
A	1G	1.44 [15]	0.76
	Berg	ND[a]	0.7
	Lima	1.45 [11]	0.76
B	Aca	1.66 [11]	0.8
	E.hi.Sur	ND	0.86
	Blephar.	ND	0.74
	Rhabda.	ND	0.85
C	Mand.sc.Br	1.62 [11]	0.86
	Tom.Sp	1.64 [13]	0.9
	Trifolium	ND	0.85
	Amaran.	ND	0.84
D	E.hi.Ind	2.30 [11]	–[b]
	E.hi.Sen	2.20 [11]	–
	E.M.1	2.8 [11]	1.5
F	Mani.Br	ND	0.74
G	E.hy.Gu	1.30 [11]	0.68
	E.hi.Vz	ND	0.68
H-I	Hart 1	1.6 [11]	0.82
H-IIa	Mar 1	1.8 [13]	0.875
H-IIb	Mar 6	1.8 [13]	0.875
I	Ascl.cu.Vz	0.85 [11]	–
ND	E.pro.Vz	ND	0.64
	E.het.Vz	ND	0.85

[a]ND, not determined.
[b]–, not amplified.

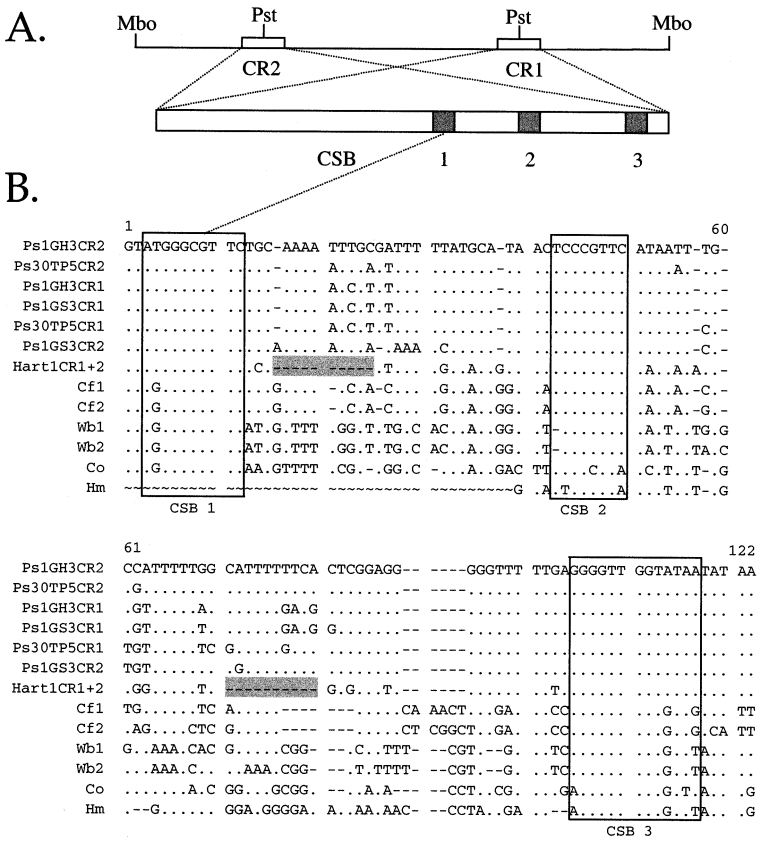

Fig. 1. The Hart 1 minicircle contains two conserved regions. A: Schematic diagram of the Hart 1 minicircle showing conserved regions (CRs; open box). The location of conserved sequence blocks (CSB, shaded boxes) 1, 2 and 3 within the CR is shown below. B: Multiple sequence alignment of the portion of the CRs containing the CSBs with tomato-isolated fruit phytomonads and insect trypanosomatids. The PILEUP program was used to generate this alignment, with a gap weight of 0.5 and a gap length weight of 0.1. Conserved nucleotides are represented by dots (.), gaps by hyphens (-), and missing sequence by squiggles (∼). The shaded boxes represent deletions in the Hart 1 sequence relative to the sequence from plant trypanosomatids isolated from tomato. The sequence abbreviations are as follows: Ps1GH3CR1, *P. serpens* 1G *Hin*dIII CR1 [15]; Ps1GH3CR2, *P. serpens* 1G *Hin*dIII CR2 [15]; Ps1GS3CR1, *P. serpens* 1G *Sau*3A CR1 [15]; Ps1GS3CR2, *P. serpens* 1G *Sau*3A CR2 [15]; Ps30TP5CR1, *P. serpens* 30T CR1 [14]; Ps30TP5CR2, *P. serpens* 30T CR2 [14]; Hart1CR1+CR2, *Phytomonas* sp. Hart 1 CR1 and CR2 (AF397906); Cf1 and Cf2, *Crithidia fasciculata* (M19266); Wb1 and Wb2, *Wallaceina brevicula* (Z32854); Co, *C. oncopelti* (X17109); Hm, *H. megaseliae* (X76660).

with those of other plant and insect trypanosomatids (Fig. 1B), oligonucleotide primers were designed to promote the specific amplification of *Phytomonas* spp. minicircle fragments from the CSB-3 region (mcCSB3a and mcCSB3b). Application of these primers to amplification of templates Hart 1 and Ps1G resulted in the expected products that were approximately half the size of the minicircles (lanes 1 and 12, Fig. 2A). Half-sized products were obtained for a further eight isolates (Lima, Aca, Mand.sc.Br, Tom.Sp, E.M.1, E.hy.Gu, Mar 1 and Mar 6; Fig. 2A and Table 1). The primers were successful with DNAs from most plant trypanosomatid isolates attempted. The exceptions were Ascl.cu.Vz, E.hi.Ind and E.hi.Sen, which did not yield products either at a lowered annealing temperature (45°C) or with variation of $MgCl_2$ concentration. A second set of primers was designed (mcCSB3aa and mcCSB3bb) to accommodate variance at the 3′ end of the original primers, but this set also failed to amplify from the refractory templates (data not shown).

The PCR fragments sizes supported the existing sequence data suggesting that each minicircle contains two conserved regions. Product sizes were not strictly conserved within the 5S rRNA groups. The phloem-restricted isolates in group H [4–6] showed PCR product size differences that were consistent with full-length minicircle size [13] and SL RNA gene subgrouping [7]. The existence of different minicircle PCR product sizes within 5S rRNA groups A and C suggests possible subdivisions within these groups as well.

Phytomonas spp. DNAs of unknown minicircle size were subjected to CSB-3 amplification (Fig. 2). These isolates were selected to represent a spectrum of phytomonads, as characterized by nuclear markers [4–6]. The amplification products obtained ranged in size from between 650 bp and 900 bp, with the notable exception of the *Amaranthus* isolate, which yielded a product of approximately 1.4 kb.

To test the specificity of the CSB-3 primers for the phytomonads, DNAs from insect trypanosomatids were used as templates for amplification. These templates did not

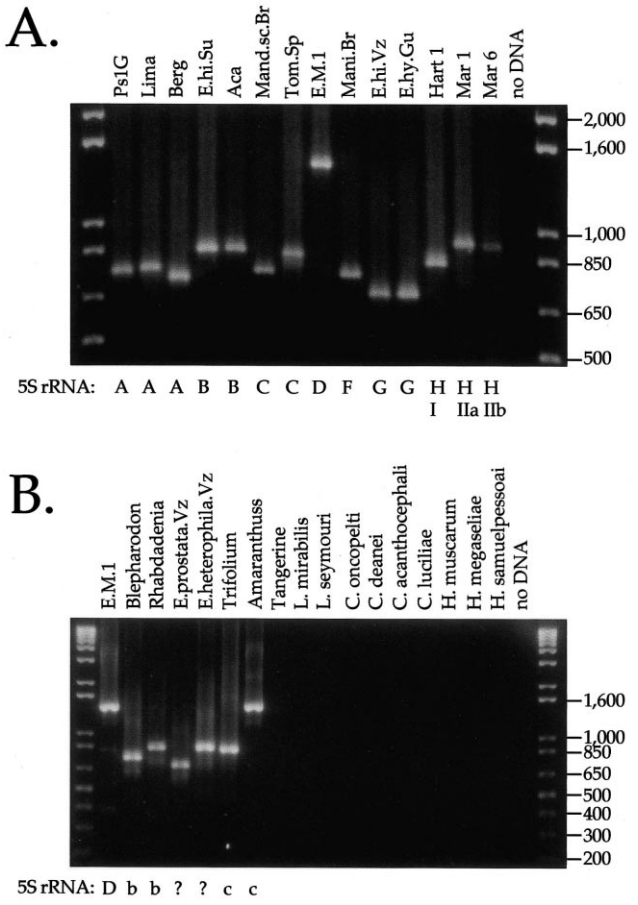

Fig. 2. Amplification of *Phytomonas* spp. minicircle domains. A: Strains of known minicircle size. B: Strains of unknown minicircle size and of insect trypanosomatids. Primers mcCSB3a and mcCSB3b were used for the reactions, and the products separated through a 1% agarose gel containing ethidium bromide. E.M.1 was included as a positive control. For the phytomonads, the 5S group (uppercase letters, with SL RNA subgroups [7]) and RAPD (lower case letters) designations are shown underneath. Lanes flanking each gel are molecular size markers shown in base pairs (1 kb Plus DNA Ladder, Gibco BRL).

yield products (Fig. 2B), as expected for some based on their minicircle sequence (Fig. 1B). Thus, the primer set mcCSB3a and mcCSB3b does not amplify minicircles from insect trypanosomatids, but yields products for most plant trypanosomatids. We will pursue the direct cloning of minicircles from the three refractory isolates to examine their sequences.

The Hart 1 minicircle sequence represents the first minicircle data from a pathogenic, phloem-restricted member of the *Phytomonas* genus. Our analyses of the nucleotides in the conserved region, and specifically in the region of CSB 3, have allowed the design of primer sets for amplification from many, but not all, of the phytomonad groups. Our future goals include the use of minicircles to distinguish between true plant trypanosomatids and opportunistic insect trypanosomatids, as well as among three types of plant trypanosomatids found in fruit, latex, or phloem. Further studies of minicircle amplification products will be useful for determining the relationships between close isolates using the sequence information contained in the CRs [18].

Acknowledgements

D.A.C. was a grantee of CIRAD. We thank Theo Baltz, Erney Camargo, Peter Kastelein, Guy Riou and Manuel Sanchez-Moreno for providing cell lines.

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