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Short communication

Rapid identification of *Clavibacter michiganensis* subspecies *sepedonicus* using two primers random amplified polymorphic DNA (TP-RAPD) fingerprints

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Abstract

Twenty strains of *Clavibacter michiganensis* subsp. *sepedonicus* from different geographic origins and other reference strains of the same and different species, including other potato pathogens, were analysed with a new procedure named TP-RAPD that originates fingerprints of bacterial species. This procedure uses two primers to amplify the 16S rDNA gene. At 45 °C of annealing, the PCR product electrophoresed in agarose gels produced a band pattern that was different in all bacterial species studied as well as in the subspecies of *C. michiganensis*. All strains of *C. michiganensis* subsp. *sepedonicus* displayed the same TP-RAPD number of pattern. Unlike Gram negative bacteria, Gram positives of high G+C content, such as *Clavibacter*, produced low bands in TP-RAPD. By using a different set of two primers also based in the 16S rDNA sequence from *Escherichia coli* a more adequate amplification of Gram positives of high G + C including a greater number of bands was obtained. TP-RAPD patterns using the new set of primers described in this work is a reliable and fast method to identify *C. michiganensis* subsp. *sepedonicus*.

Abbreviations: TP-RAPD – Two primers-random amplified polymorphic DNA.

Introduction

The genus *Clavibacter* contains several plant pathogenic bacteria and is classified within Gram positive bacteria of high G + C content. *Clavibacter michiganensis* includes five subspecies that produce different plant diseases. *C. michiganensis* subsp. *sepedonicus* produces potato ring rot, a significant disease affecting potato crops in North America and Europe. In Europe, the diagnosis must be confirmed by the isolation and identification of *C. michiganensis* subsp. *sepedonicus* using biochemical and physiological tests, followed by infectivity tests in *Solanum melongena* (Council of the European Communities, 1993). Therefore, rapid and reliable techniques for identification of this bacterium are desirable.

Several PCR based techniques have been proposed for identification of *C. michiganensis* subsp. *sepedonicus* (Lee et al., 1997; Li and de Boer, 1995; Lows et al., 1998; Mills et al., 1997; Pastrik and Rainey, 1999; Pastrik, 2000). Some of these procedures produce a single band which differentiate isolates of subspecies of *C. michiganensis* on the basis of genomic repetitive sequences (rep-PCR fingerprints). Although differences among strains from the same species or subspecies can be useful in epidemiological studies, it is important for diagnosis at the species or subspecies level to use techniques that do not offer strain dependent variations.

Recently, a new procedure, named TP-RAPD, was proposed to obtain fingerprints of bacteria using two primers in the PCR (Rivas et al., 2001). These primers are widely used to amplify 16S rDNA in Gram negative and Gram positive bacteria. In the amplification conditions of TP-RAPD, the band from 16S rDNA as well as other bands are obtained from both types of bacteria. TP-RAPD fingerprints have an advantage for diagnostic purposes because that strain dependent variations are minimal (Rivas et al., 2001).

In a previous study, the primers used had a low G+C content (Rivas et al., 2001). Pastrik and Rainey (1999) demonstrated that the best RAPD-PCR fingerprints of *C. michiganensis* are obtained using primers with a high G+C percentage. Thus, in the present work, a different forward primer (879F) also based in 16S rDNA sequence of *Escherichia coli* with a high G + C content (75%) was used to obtain TP-RAPD fingerprints of *Clavibacter* subspecies.

Therefore, the aims of this work were (i) to compare the TP-RAPD fingerprints of *C. michiganensis* subsp. *sepedonicus* strains isolated from several geographical locations with those of other subspecies from *C. michiganensis* and of other potato pathogens (ii) to establish the usefulness of TP-RAPD fingerprinting for rapid identification of *C. michiganensis* subsp. *sepedonicus*.

Strains used in this study are listed in Table 1. Strains were grown for 24 h in TY medium (0.4%, tryptone 0.3% yeast extract and 0.09% Ca_2Cl), amended with 0.5% mannitol to cultivate *Clavibacter* strains. Cells (1.5 ml of each culture) were collected by centrifugation at room temperature in a microspin centrifuge at $5000\times g$ and washed with 200 μl of a solution of 0.1% sarkosyl in water. The DNA was extracted with 100 μl of 0.05 M NaOH (DNA-free) heating at 100 °C for 4 min. Samples were placed in an ice bath and 900 μl of water was added to each microtube and mixed thoroughly. After an additional centrifugation at $4000\times g$ for 3 min, 700 μl of the supernatants were harvested and frozen at −20 °C.

Crude DNA (2 μl) was used as template for PCR amplification of 16S rDNA. PCR was performed using an AmpliTaq reagent kit (Perkin-Elmer Biosystems, California, USA) following the manufacturer's instructions (1.5 mM $MgCl_2$, 200 μM of each dNTP and 2 U of Taq polymerase for 25 μl of final volume of reaction). The PCR primers used for amplification were the forward primer 8F (5′-AGAGTTTGATCCTGGCT CAG-3′, *E. coli* positions 8–27) and the reverse primer 1522R (5′-AAGGAGGTGATCCANCCRCA-3′), *E. coli* positions 1509–1522) at a final concentration of 2 μM. We also used an other set with the forward primer 879F (5′-GCCTGGGGAGTACGGCCGCA-3′ *E. coli* positions 879–899) and the same reverse primer 1522R. PCR conditions were as follows: pre-heating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 45 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The PCR products were stored at 4 °C. An alternative set of two primers also based in 16S rDNA sequence was used to compare the patterns obtained with respect to those obtained using the previously published set.

Eight microlitres of PCR product were electrophoresed in 1.5% agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH: 8.5) at 6 V cm^{-1}, stained in a solution containing 0.5 μl ethidium bromide per millilitre and photographed under UV light. Standard VI (Boehringer-Roche, USA) was used as a size marker. To each sample 3 μl of 6× loading solution (30% glycerol, 0.25% xylene cyanol and 0.25% bromophenol blue) were added.

Figure 1 shows the patterns obtained for reference strains of different bacterial species included in this study. Using the primers pair 8F and 1522R, all species showed a different PCR pattern including Gram positive bacteria, such as *Clavibacter*, and Gram negative bacteria, such as *Pectobacterium* (formerly *Erwinia*) and *Ralstonia*. Subspecies from the same species showed a different TP-RAPD pattern. Reference strains of different subspecies of *C. michiganensis* and *P. carotovorum* displayed different patterns. By contrast, the reference strains from the same subspecies, in *C. michiganensis* subsp. *sepedonicus* as well as in *P. carotovorum* subsp. *carotovorum*, displayed the same TP-RAPD pattern.

Figure 2 shows the patterns obtained for strains of *C. michiganensis* subsp. *sepedonicus* from different geographical origins. All strains showed the same TP-RAPD pattern independently of their geographical origin. These results are in agreement with results obtained in our previous work using several strains of species from Order Rhizobiales. However, the patterns obtained in *Clavibacter* species contained fewer bands than those typically found in Gram negative bacteria (e.g. *Pectobacterium* and *Ralstonia*).

Due to the low number of bands obtained using the first set of primers (8F/1522R) and taking into account the results obtained by other authors (Pastrik and Rainey, 1999), a second set of primers, also designed

Table 1. Strains used in this study

Strain	Host plant	Location	Source
C. michiganensis subsp. *sepedonicus*			
95-457, 96-61	*Solanum tuberosum*	Spain/Palencia	This study
95-C6	*S. tuberosum*	Spain/Zamora	This study
95-AS18, 95-AS48	*S. tuberosum*	Spain/Burgos	This study
95-AS79, 95-AS103, 95-M207, 96-M255	*S. tuberosum*	Spain/Burgos	This study
96-BA5	*S. tuberosum*	Spain/Cáceres	This study
97-GAL1	*S. tuberosum*	Spain/Pontevedra	This study
C-R2, C-R12	*S. tuberosum*	Canada	S.H. de Boer
N-87-5, N-92-5	*S. tuberosum*	Norway	A. Sletten
D-285, D-288	*S. tuberosum*	Denmark	K. Mansfeld
D-294	*S. tuberosum*	Germany	K. Mansfeld
S-318	*S. tuberosum*	Sweden	P. Persson
S-403	*S. tuberosum*	Sweden	P. Persson
2049	*S. tuberosum*	USA	CFBP
1154	*S. tuberosum*	USA	CFBP
1792	*S. tuberosum*	USA	CFBP
ATCC33113T	*S. tuberosum*	Canadá	NCPPB
CFBP1154	*S. tuberosum*		
CFBP2049	*S. tuberosum*		
CFBP1792	*S. tuberosum*		
C. michiganensis subsp. *nebraskensis*			
CECT5040T	*Zea mays*	USA	CECT
C. michiganensis subsp *tessellarius*			
CECT4263	*Triticum aestivum*		CECT
C. michiganensis subsp. *insidiosus*			
CFBP5042	*Medicago sativa*	USA	CFBP
C. michiganensis subsp. *michiganensis*			
121.1	*Lycopersicon lycopersicon*	Spain	IVIA
E. crhysanthemi			
1500	*S. tuberosum*	Spain	IVIA
P. carotovorum subsp. *carotovorum*			
194	*S. tuberosum*	Scotland	IVIA
163	*S. tuberosum*	Spain	IVIA
P. carotovorum subsp. *atrosepticum*			
1001	*S. tuberosum*	UK	SCRI
537	*S. tuberosum*	Spain	IVIA
Ralstonia solanacearum			
SMT 24	*S. tuberosum*	Spain	This study
01/006	*S. tuberosum*	Spain	This study

CFBP: Collection Nationale de Bacté ries Phytopathogé nes. INRA: (France); NCPPB: National Collection of Plant Phytopathogenic Bacteria (UK); CECT: Spanish Type Culture Collection; IVIA: Instituto Valenciano de Investigaciones Agrarias (Spain); SCRI: Scotish Crop Research Institute.

based on 16S rDNA sequences, one of them with high G + C content (879F), was assayed. Primer 879F was combined with primer 1522R as an alternative set to obtain TP-RAPD fingerprints.

Patterns obtained using the second set of primers (Figure 3) contain more bands than those obtained using the first primer pair (8F and 1522R), but the results were comparable because the subspecies within *C. michiganensis*, *Pectobacterium* and *Ralstonia* also showed different patterns.

Figure 4 shows the TP-RAPD patterns of strains from *C. michiganensis* subsp. *sepedonicus*. As seen with primers pair 8F/1522R, primers pair 879F/1522R produced the same fingerprint in all strains of *C. michiganensis* subsp. *sepedonicus*. Therefore, this new primers pair can also be used to identify

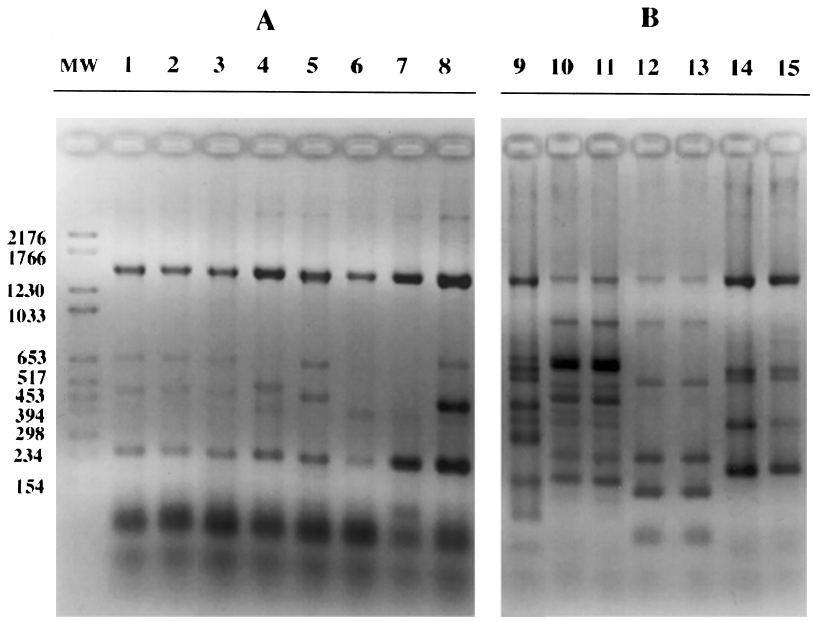

Figure 1. TP-RAPD patterns of reference strains used in this study obtained using 8F and 1522R primers: (A): lane (1) *C. michiganensis* subsp. *sepedonicus* ATCC33113T, lane (2) *C. michiganensis* subsp. *sepedonicus* CFBP 1154, (3) *C. michiganensis* subsp. *sepedonicus* CFPB 2049, lane (4) *C. michiganensis* subsp. *michiganensis* 121.1, lane (5) *C. michiganensis* subsp. *sepedonicus* CFPB 2049, lane (6), *C. michiganensis* subsp. *insidiosus* CECT 5042, lane (7) *C. michiganensis* subsp. *tessellarius* CECT4263, lane (8) *C. michiganensis* subsp. *nebraskensis* CECT 4209. (B): lane (9) *Erwinia chrysanthemi* 1500, lane (10) *Pectobacterium* (*Erwinia*) *carotovorum* subsp. *atrosepticum* 537, lane (11) *Pectobacterium* (*Erwinia*) *carotovorum* subsp. *atrosepticum* 1001, lane (12) *P. carotovorum* subsp. *carotovorum* 194, lane (13) *P. carotovorum* subsp. *carotovorum* 163, lane (14) *R. solanacearum* SMT-24, lane (15) *R. solanacearum* 01/006.

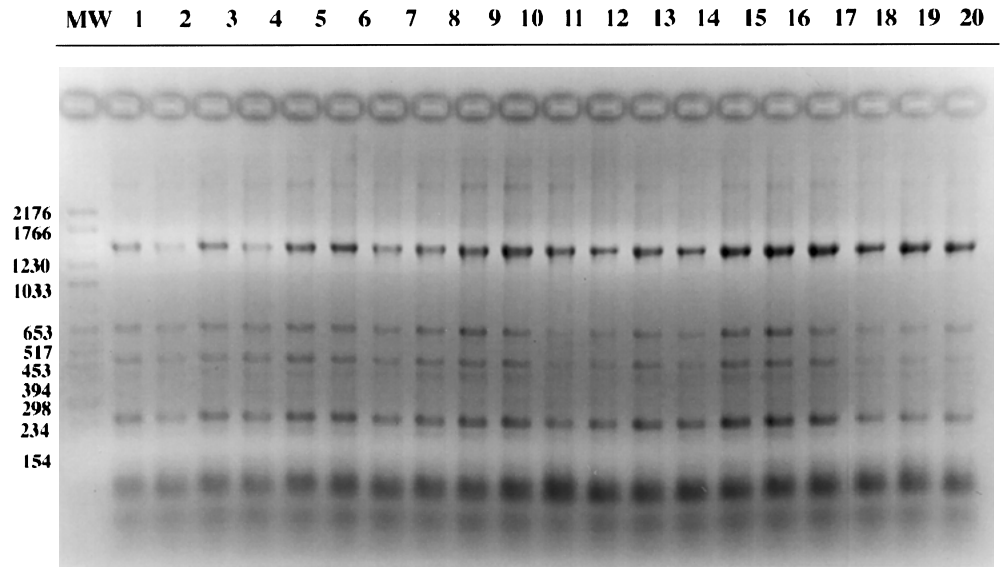

Figure 2. TP-RAPD patterns of strains belong to *C. michiganensis* subsp. *sepedonicus* obtained using 8F and 1522R primers: lane (1) 95-457, lane (2) 96-61, lane (3) 95-C6, lane (4) 95-AS18, lane (5) 95-AS48, lane (6) 95-AS79, lane (7) 95-AS103, lane (8) 95-M207, lane (9) 96-M255, lane (10) 96-BA5, lane (11) 97-GAL1, lane (12) C-R2, lane (13) C-R12, lane (14) N-87-5, lane (15) N-92-5, lane (16) D-285, lane (17) D-288, lane (18) D-294, lane (19) S-318, lane (20) S-403.

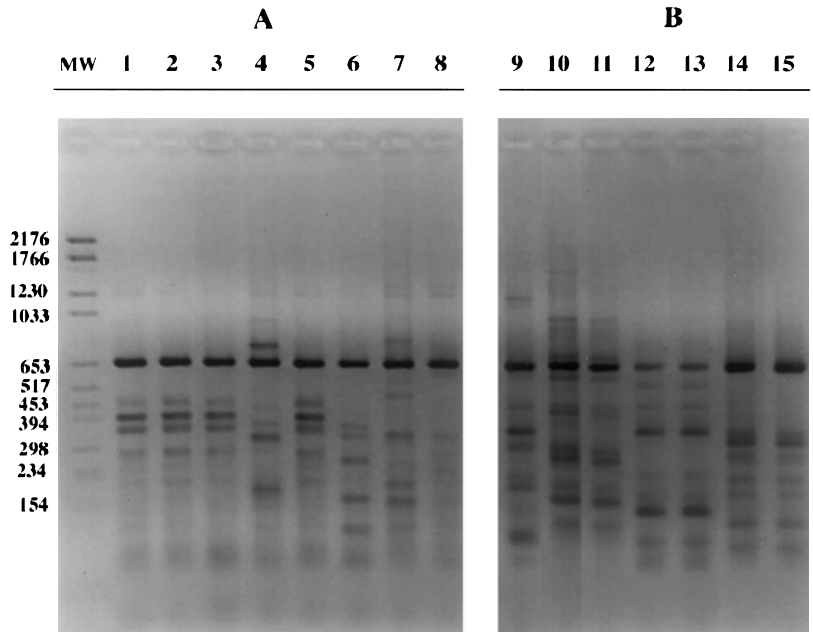

Figure 3. TP-RAPD patterns of reference strains used in this study obtained with primer pair 879F and 1522R: (A): lane (1) *C. michiganensis* subsp. *sepedonicus* ATCC33113T, lane (2) *C. michiganensis* subsp. *sepedonicus* CFBP 1154, (3) *C. michiganensis* subsp. *sepedonicus* CFPB 2049, lane (4) *C. michiganensis* subsp. *michiganensis* 121.1, lane (5) *C. michiganensis* subsp. *sepedonicus* CFPB 2049, lane (6) *C. michiganensis* subsp. *insidiosus* CECT 5042, lane (7) *C. michiganensis* subsp. *tessellarius* CECT4263, lane (8) *C. michiganensis* subsp. *nebraskensis* CECT 4209. (B): lane (9) *Erwinia chrysanthemi* 1500, lane (10) *Pectobacterium* (*Erwinia*) *carotovorum* subsp. *atrosepticum* 537, lane (11) *Pectobacterium* (*Erwinia*) *carotovorum* subsp. *atrosepticum* 1001, lane (12) *P. carotovorum* subsp. *carotovorum* 194, lane (13) *P. carotovorum* ssp. *carotovorum* 163, lane (14) *R. solanacearum* SMT-24, lane (15) *R. solanacearum* 01/006.

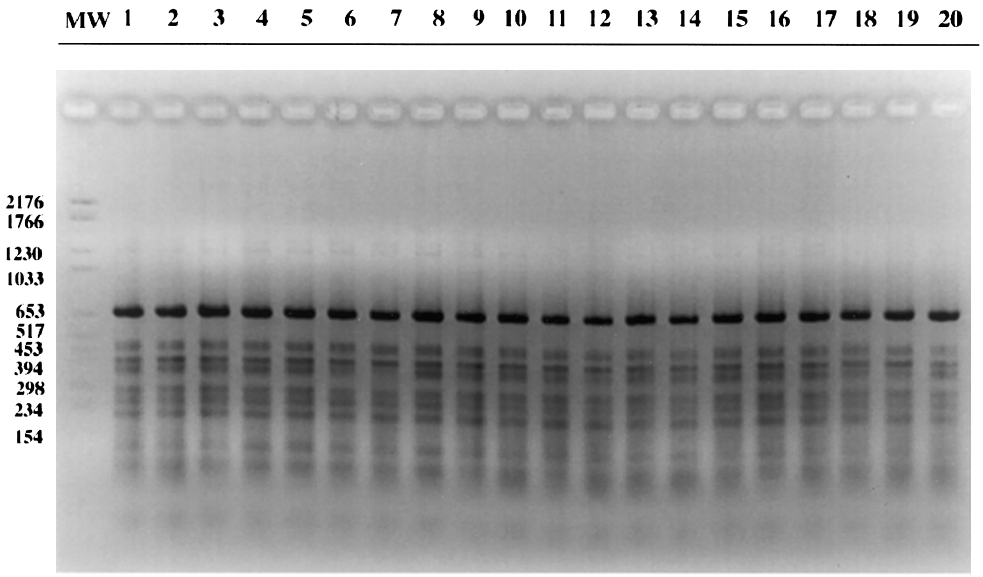

Figure 4. TP-RAPD patterns of strains belong to *C. michiganensis* subsp. *sepedonicus* obtained with primer pair 879F and 1522R primers: lane (1) 95-457, lane (2) 96-61, lane (3) 95-C6, lane (4) 95-AS18, lane (5) 95-AS48, lane (6) 95-AS79, lane (7) 95-AS103, lane (8) 95-M207, lane (9) 96-M255, lane (10) 96-BA5, lane (11) 97-GAL1, lane (12) C-R2, lane (13) C-R12, lane (14) N-87-5, lane (15) N-92-5, lane (16) D-285, lane (17) D-288, lane (18) D-294, lane (19) S-318, lane (20) S-403.

C. michiganensis subsp. *sepedonicus* and it has the advantage of identifying the subspecies from *C. michiganensis* on the basis of a greater number of bands which allow a more reliable differentiation among closely related microorganisms.

In conclusion, we have demonstrated that: (i) the primer pairs 8F/1522R and 879F/1522R differentiate subspecies of *C. michiganensis* (ii) the patterns obtained with primer pair 879F/1522R may be of greater value than those obtained with 8F/1941R to identify Gram positive bacteria of high G + C content, such as *Clavibacter*; (iii) the TP-RAPD patterns are uniform among strains of the same subspecies; and (iv) the TP-RAPD patterns of *C. michiganensis* subsp. *sepedonicus* are a realiable fingerprint of this bacterium.

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