

Pflanzenschutzamt Hannover, Hannover, Germany and Department of Biological Sciences Louisiana State University Baton Rouge, LA, USA

Identification and Differentiation of *Clavibacter michiganensis* Subspecies by Polymerase Chain Reaction-based Techniques

K.-H. PASTRIK¹ and F. A. RAINEY²

Authors' addresses: Pflanzenschutzamt Hannover, Wunstorfer Landstr. 9, 30453 Hannover, Germany; ²Department of Biological Sciences Louisiana State University, Baton Rouge, LA, USA (correspondence to K.-H. Pastrik; E-mail: pastrik@Lawikhan-de)

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Abstract

For the identification and differentiation of the five subspecies of *Clavibacter michiganensis*, two different polymerase chain reaction (PCR)-based techniques were employed: amplification with subspecies-specific primers and amplification with random primers (RAPD). Based on the sequence data of the intergenic spacer region between 16S and 23S rRNA genes, primers were designed for the identification of each subspecies. Using the designed primer pairs it was possible to identify each subspecies of *C. michiganensis* according to the amplification of a specific DNA fragment. No amplification products were obtained, when bacteria belonging to other genera were submitted to PCR under the same conditions. RAPD-PCR conditions suitable for the differentiation of *C. michiganensis* subspecies were developed. RAPD typing was capable of distinguishing subspecies of *C. michiganensis* as well as strains within subspecies. Both genomic variation between subspecies and genetic polymorphisms between bacterial strains were identified as differences in the size and numbers of DNA fragments obtained.

Zusammenfassung

Title ???

Für die Identifizierung und Differenzierung der fünf Subspecies von *Clavibacter michiganensis* wurden zwei PCR-Techniken angewendet, die Amplifikation mit Subspecies-spezifischen Primern und die Amplifikation mit Zufalls-Primern (RAPD-PCR). Es wurden spezifische Primer entwickelt, die auf den Sequenzdaten der Intergenic Spacer Region zwischen den 16S und 23S rRNA-Genen basierten und eine Identifikation der jeweiligen Subspecies ermöglichten. Mit diesen entwickelten Primerpaaren, war es möglich, die jeweilige Subspecies

von *C. michiganensis* anhand der Amplifikation von spezifischen DNA-Fragmenten zu identifizieren. Außerdem wurden RAPD-PCR Bedingungen entwickelt, die eine Differenzierung der fünf Subspecies von *C. michiganensis* und eine Identifizierung von Bakterien-Stämmen innerhalb einer Subspecies ermöglichten. Sowohl die genomische Variation der Subspecies, als auch der genetische Polymorphismus von Bakterien-Stämmen konnte identifiziert werden als Unterschied von Größe und Anzahl amplifizierter DNA Fragmente.

Introduction

The genus *Clavibacter* contains plant-pathogenic bacteria characterized by the presence of 2,4-diaminobutyric acid as a cell wall component (Davis et al., 1984; Riley, 1987). Within the gram-positive species of *Clavibacter michiganensis*, there are the five subspecies *C.m. sepedonicus* (Cms), *C.m. michiganensis* (Cmm), *C.m. insidiosus* (Cmi), *C.m. tessellarius* (Cmt) and *C.m. nebraskensis* (Cmn), which are causal agents of various diseases in agriculture (Davis, 1986).

Many attempts have been made to identify and differentiate the subspecies of *Clavibacter michiganensis* according to standard biochemical and physiological tests (Vidaver, 1980; Henningson and Gudmestad, 1991), pathogenicity tests, cultural characteristics, pigmentation and growth rate (Moffett et al., 1983). In addition, chemotaxonomic markers used to classify these coryneform bacteria include polar lipids (Collins et al., 1980), cellular proteins (Carlson and Vidaver, 1982), allozyme patterns (Riley et al., 1988), fatty acids (Henningson and Gudmestad, 1991) and DNA homology (Starr et al., 1975). For rapid diagnosis, these techniques are unsuitable because they require time-consuming enrichment and complicated detection procedures. Currently, serological

techniques such as enzyme-linked immunosorbent assays and immunofluorescence assays are in use (DeBoer et al., 1988; Nemeth et al., 1991; Franken et al., 1993). However, they are not always reliable due to cross-reactions with other bacteria (Calzolari et al., 1982; DeBoer, 1982; Mills et al., 1997).

Nucleic acid hybridization and polymerase chain reaction (PCR) offer alternatives for rapid, highly sensitive and specific identification of pathogenic bacteria. For some, but not for all, subspecies of *Clavibacter michiganensis*, probes or PCR-systems have been described. These methods identify specific DNA sequences on plasmids (Schneider et al., 1993; Firrao and Locci, 1994), chromosomal DNA (Mills et al., 1997), 16S rDNA (Mirza et al., 1993; Lee et al., 1997a) and the intergenic spacer (Li and DeBoer, 1995). The extensive chromosomal homology among the *Clavibacter michiganensis* subspecies has complicated the development of specific DNA-assays (Starr et al., 1975).

The objective of this work was the development of easy and rapid PCR-based methods for the identification and differentiation of *Clavibacter michiganensis* subspecies. Two different PCR-based techniques were employed; amplification with subspecies-specific primers based on sequence data of the intergenic spacer region between 16S and 23S rRNA genes, and amplification with random primers (RAPD).

The intergenic spacer in the rRNA operon between 16S and 23S loci has been used to compare both closely and distantly related organisms (Jenson et al., 1993). This region of the genome is not subject to the same selective pressure as the rRNA structural genes and has been demonstrated to exhibit considerable variability for specific identification of bacteria (Barry et al., 1991; East and Collins, 1993; Wunschel et al., 1994; Li and DeBoer, 1995).

In the RAPD-PCR technique, DNA fragments are amplified using single random primers of arbitrary sequence (Welsh and McClelland, 1990; Williams et al., 1990). Over the last few years, RAPD-PCR has evolved as a powerful tool for genetic analysis (Williams et al., 1993) and diagnosis of plant pathogens (Henson and French, 1993), including nematodes (Braasch et al., 1995; Pastrik et al., 1995), fungi (Guthrie et al., 1992; Strongman and MacKay, 1993) and bacteria (Mäki-Valkama and Karjalainen, 1994; Makino et al., 1995). Reaction conditions for RAPD-PCR must be very carefully optimized to obtain informative and reproducible results. Once standardized, the technique is amenable to routine testing due to its speed and simplicity. In the present work, RAPD-PCR conditions suitable for the differentiation of *Clavibacter michiganensis* subspecies were developed.

Materials and Methods

Bacterial strains and culture conditions

Bacteria (Table 1) were obtained either from the Göttingen Collection of Phytopathogenic Bacteria (GSPB, Göttingen, Germany) or from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braun-

Table 1
Bacterial strains used in this study

Strains	Source and culture code
<i>Clavibacter michiganensis</i> ssp. <i>sepedonicus</i>	DSM 46300
	GSPB 1522
	GSPB 2238
	GSPB 2249
	GSPB 2237
	GSPB 2242
	GSPB 2250
<i>Clavibacter michiganensis</i> ssp. <i>michiganensis</i>	DSM 20134
	DSM 46364
	GSPB 382
	GSPB 390
	GSPB 392
	GSPB 2315
<i>Clavibacter michiganensis</i> ssp. <i>insidiosus</i>	DSM 20157
	GSPB 2225
<i>Clavibacter michiganensis</i> ssp. <i>tesselarius</i>	DSM 20741
<i>Clavibacter michiganensis</i> ssp. <i>nebraskensis</i>	DSM 20400
	DSM 20401
	DSM 7483
<i>Ralstonia solanacearum</i>	GSPB 2223
	DSM 9544
	GSPB 1958
	GSPB 1960
	GSPB 2124
<i>Erwinia carotovora</i> ssp. <i>atroseptica</i>	GSPB 2126
	DSM 60424
	GSPB 401
	GSPB 410
<i>Erwinia carotovora</i> ssp. <i>carotovora</i>	GSPB 1255
	DSM 30168
	GSPB 133
<i>Erwinia chrysanthemi</i>	GSPB 425
	GSPB 445
<i>Erwinia rhapontici</i>	GSPB 421
	DSM 30177
<i>Erwinia rhapontici</i>	GSPB 454
	GSPB 455
	GSPB 1440
<i>Pseudomonas syringae</i> pv. <i>atrofaciens</i>	GSPB1392
	DSM 50302
<i>Pseudomonas syringae</i> pv. <i>morsprunorum</i>	
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	GSPB 1495
	GSPB 567
	GSPB 2203
	GSPB 2204

GSPB, Göttingen Collection of Phytopathogenic Bacteria, Göttingen, Germany; DSMZ, German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany.

schweig, Germany). All *Clavibacter* spp. were grown on yeast extract glucose mineral salts agar (YGM-Agar) at 23°C (Anonymous, 1993). Other bacteria were cultured on YPN agar (Rhodes, 1959) at ambient temperature.

Isolation of nucleic acids

For the isolation of bacterial genomic DNA, a loopful of a bacterial culture was suspended in 1 ml PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; pH 7.4) and centrifuged for 2 min at 13 000 × g and 4°C. The pellet was resuspended in 320 µl lysis-buffer (100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0), placed on a heating block at 95°C for 10 min and cooled on ice for 5 min. Then 80 µl lysozyme stock

solution (50 mg/ml lysozyme in 10 mM Tris-HCl, pH 8.0) was added, and the sample was incubated for 30 min at 37°C. The DNA was purified using the Easy-DNA-Extraction-kit (Invitrogen, De Schelp, Netherlands). Solution A (220 µl) was added and the mixture was incubated for 30 min at 65°C. After addition of 100 µl solution B and mixing, 500 µl chloroform was added and the mixture was centrifuged for 20 min at 20 000 × g. The aqueous phase was transferred to a new tube and DNA was precipitated by addition of ethanol to 70%, and the resulting pellet was washed with 80% ethanol. After the final centrifugation the DNA was resuspended in 100 µl of sterile water (bidest.). Nucleic acid concentration was estimated from the intensity of ethidium bromide fluorescence (Sambrook et al., 1989) by using 2D-Densitometry-Software (Cybertech, Berlin, Germany) and DNA molecular weight marker I (Boehringer Mannheim, Germany) as a standard DNA.

Sequence analysis of the intergenic spacer region between 16S and 23S rRNA genes

For the amplification of the intergenic spacer region between 16S and 23S rRNA genes the eubacterial universal primers 1500 forward (16S rDNA; 5'-GTGGATCACCTCCTTC-3') and 241 reverse (23S rDNA; 5'-TTGCCTCGCCCTAC-3') were used. The reaction mixture contained 1 × reaction-buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl; pH 8.4), 1 mM of each dNTP, 0.5 µg of each primer and 1 ng DNA in 100 µl reaction volume overlaid with one drop of mineral oil. After denaturation for 3 min at 94°C, 2 U of Taq DNA polymerase (Boehringer Mannheim) was added and the reaction mixture was submitted to 25 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 60 s with a final extension of 72°C for 10 min. The PCR products were purified by Prep-a-Gene (Biorad, USA). The purified PCR products were used as template for sequencing reactions. Sequencing reactions were carried out with a Taq DyeDeoxy[®] Terminator Cycle Sequencing Kit (Applied Biosystems, USA), according to the manufacturer's instructions, using a Perkin Elmer 9600 thermocycler. The sequence reactions were electrophoresed using the Applied Biosystems 373 A DNA Sequencer.

Amplification with subspecies-specific primers

The primers used for the specific amplification were purified using high-performance liquid chromatography (HPLC) and purchased from Roth (Karlsruhe, Germany). The PCR was performed in a MJ Research thermocycler (PTC 200). The 25 µl PCR reaction mixture contained 1 × reaction buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 1.5 mM MgCl₂, 100 µM of each dNTP (Boehringer Mannheim), 0.2 µM of each primer (forward and reverse), 1 U Taq DNA polymerase (Life Technologies, Germany) and 1 ng of DNA. The following PCR conditions were used: 94°C for 2.30 min, followed by 30 reaction cycles of 94°C for 30 s, 60–65°C for 20 s and 72°C for 45 s. The rate of heating from 60–65°C to 72°C was regulated to 0.5°C/sec. After the final reaction cycle the mixture was held at 72°C for 5 min and stored

at 4°C. After the PCR, 12 µl aliquots of the reaction mixture were resolved by electrophoresis in a 2% agarose gel and DNA fragments were visualized by staining in 0.5 µg/ml ethidium bromide.

RAPD-PCR

Random primers (10 base oligonucleotides with 60–80% G + C content and random sequence) were purchased from Genosys Biotechnologies (Cambridge, UK) and Roth. RAPD-PCR was carried out in a MJ Research thermocycler (PTC 200) using 'Gold Star' Taq DNA polymerase (Eurogentec, Belgium). The reaction mixture (25 µl) contained 50–100 ng template DNA, 1 U 'Gold Star' Taq DNA polymerase, 100 µM of each dNTP (Boehringer Mannheim), 0.4 µM of a single primer and 2 mM MgCl₂ in 1 × reaction buffer (75 mM Tris-HCl, pH 9.0; 20 mM (NH₄)₂SO₄; 0.01% Tween (20)). The reaction mixture was heated for 2.5 min to 94°C and submitted to 40 reaction cycles of 94°C for 20 s, 38°C for 15 s and 72°C for 60 s. The rate of heating from 38°C to 72°C was regulated to 0.3°C/sec. After the final reaction cycle, the mixture was held at 72°C for 10 min and stored at 4°C. A negative control without DNA was included in each RAPD run. After the PCR, 12 µl aliquots of the reaction mixture were resolved by electrophoresis in a 2% agarose gel and DNA fragments were visualized by staining in 0.5 µg/ml ethidium bromide. Please note, that the 'Gold Star' Taq DNA polymerase commercially available now is not absolutely identical to the 'Gold Star' Taq DNA polymerase used in this study. After completion of this work Taq DNA polymerases from Life Technologies (Germany) and Quiagen (Hilden, Germany) have also been tested. Slightly varying RAPD patterns were obtained with different brands of this enzyme. With all Taq DNA polymerases tested the subspecies of *Clavibacter michiganensis* and strains within subspecies could be differentiated on the basis of specific features in the RAPD patterns.

Results

Selection of subspecies-specific primers and amplification with specific primers

The eubacterial universal primers 1500 forward and 241 reverse amplified a DNA-fragment of approximately 700 bp in all five subspecies of *Clavibacter michiganensis*. The complete spacer regions between the 16S and 23S rRNA genes of the five subspecies were sequenced and manually aligned. Specific PCR primers were designed for each subspecies on the basis of the sequence data of the spacer region. Each primer pair consisted of a subspecies-specific forward primer and a reverse primer which was universal for all subspecies (Table 2). The primer pairs amplified a specific DNA fragment, respectively, only from DNA of the subspecies they were designed for and not from other related subspecies (Fig. 1). To test the specificity of the designed primer pairs, amplification was carried out with genomic DNA of all bacterial strains listed in Table 1. Amplification products were not obtained from DNA of bacteria of other subspecies or genera (data not shown).

Table 2
Subspecies-specific primers with primer sequence, annealing temperature and size of the amplified DNA-fragment

Primer	Bacteria	Primer sequence 5'-3'	Annealing temperature	Size of amplified DNA-fragment
Forward:				
PSA-1	<i>C.m. sepedonicus</i>	CTC CTT GTG GGG TGG GAA AA	62°C	502 bp
PSA-4	<i>C.m. michiganensis</i>	TCA TTG GTC AAT TCT GTC TCC C	63°C	270 bp
PSA-5	<i>C.m. insidiosus</i>	CCC TTT CCG TCG TCC CGG A	64°C	393 bp
PSA-2	<i>C.m. tessellarius</i>	CAC GCG TCA GGC GTT CGT C	65°C	587 bp
PSA-7	<i>C.m. nebraskensis</i>	CCC TTT CCG TCG TCC TTT CG	60°C	393 bp
Reverse:				
PSA-R		TAC TGA GAT GTT TCA CTT CCC C		

C.m., *Clavibacter michiganensis*.

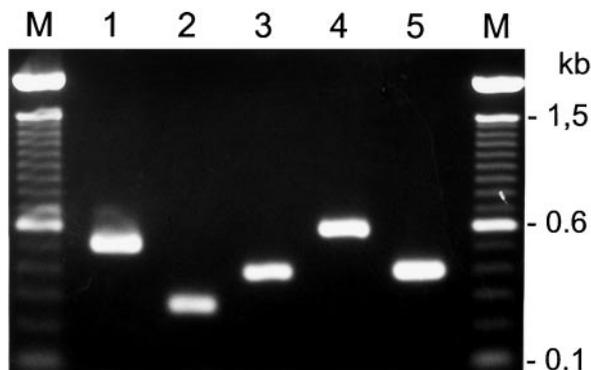


Fig. 1 Agarose gel electrophoresis of DNA fragments amplified with subspecies-specific primer pairs. Lane 1: *Clavibacter michiganensis sepedonicus* (DSM 46300) amplified with PSA-1/PSA-R; lane 2: *C.m. michiganensis* (DSM 20134) amplified with PSA-4/PSA-R; lane 3: *C.m. insidiosus* (DSM 20157) amplified with PSA-5/PSA-R; lane 4: *C.m. tessellarius* (DSM 20741) amplified with PSA-2/PSA-R; lane 5: *C.m. nebraskensis* (DSM 20400) amplified with PSA-7/PSA-R. DNA size markers (100 bp ladder; Life Technologies) were applied in lanes M

RAPD-PCR

Sixty different 10mer random primers from the primer kits of Genosys (GEN 1-RE and GEN 3-RE) and Roth (180, 280, 380 and 480) were tested for their ability to differentiate the five *Clavibacter michiganensis* subspecies. In these experiments, only random primers with a GC-content of 80% were observed to generate reproducible informative patterns of amplified DNA fragments, due to the high GC-content (72–75 mol percentage) of the *Clavibacter michiganensis* genome (Carlson and Vidaver, 1982). For reliable identification of the *Clavibacter michiganensis* subspecies certain amplified DNA fragments were selected to be obviously distinctive and stable among all strains of a given subspecies. This condition was met for more tested primers than the three illustrated below (data not shown). The reproducibility of the RAPD patterns was examined using different template DNA concentrations in the PCR. The patterns were not significantly affected by changes in template DNA levels between 25 ng and 150 ng per 25 μ l reaction (Fig. 2). As a control for the reproducibility of the RAPD patterns two different template concentrations (50 ng and 100 ng) from each strain were always tested per RAPD run. The repro-

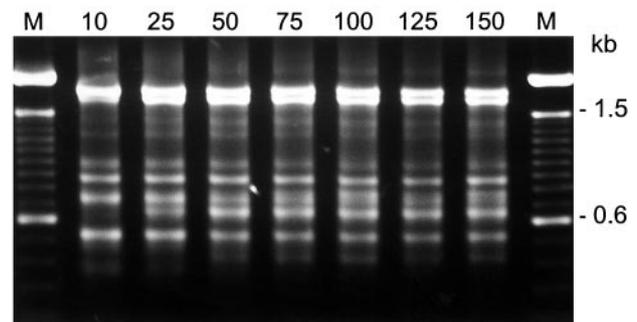


Fig. 2 Effect of template DNA concentration on the reproducibility of RAPD patterns. Different template DNA concentrations (10 ng – 150 ng) of *Clavibacter michiganensis sepedonicus*-DNA (GSPB 2238) were amplified by primer RE-01 (5'-CGGGATCCGC-3') from Genosys. DNA size markers (100 bp ladder; Life Technologies) were applied on lanes M

ducibility of the RAPD assay was further tested by repeating each experiment at least twice. Repeated RAPD's with a given primer always exhibited identical RAPD patterns with minor variations in intensity of some bands, even if different DNA preparations of the same strain were used (data not shown).

Several primers were selected, which generated informative reproducible banding patterns of 1–15 distinct DNA fragments. The amplified bands ranged from about 100 bp to 3000 bp. Representative RAPD patterns obtained with three primers (180–4, RE-01, 180–8) are shown in Fig. 3(a–c). The RAPD profiles amplified with primer 180–4 (Fig. 3a) revealed subspecies-specific DNA fragments permitting the identification and differentiation of the *Clavibacter michiganensis* subspecies (850 bp for Cms, 1060 bp for Cmm, 700 bp and 1600 bp for Cmi, 1000 bp for Cmt and 2070 bp for Cmn). Furthermore, it was possible to identify and discriminate strains within the subspecies Cms, Cmm and Cmi when this primer was used. The primer RE-01 amplified a common DNA fragment of 1800 bp from DNA of all strains of Cms, Cmm and Cmi (Fig. 3b). In addition the Cmm, Cmn and Cmt subspecies could be clearly distinguished on the basis of their specific DNA fragments. RAPD patterns obtained with primer 180–8 contained subspecies-specific bands for Cms, Cmi and Cmn (Fig. 3c).

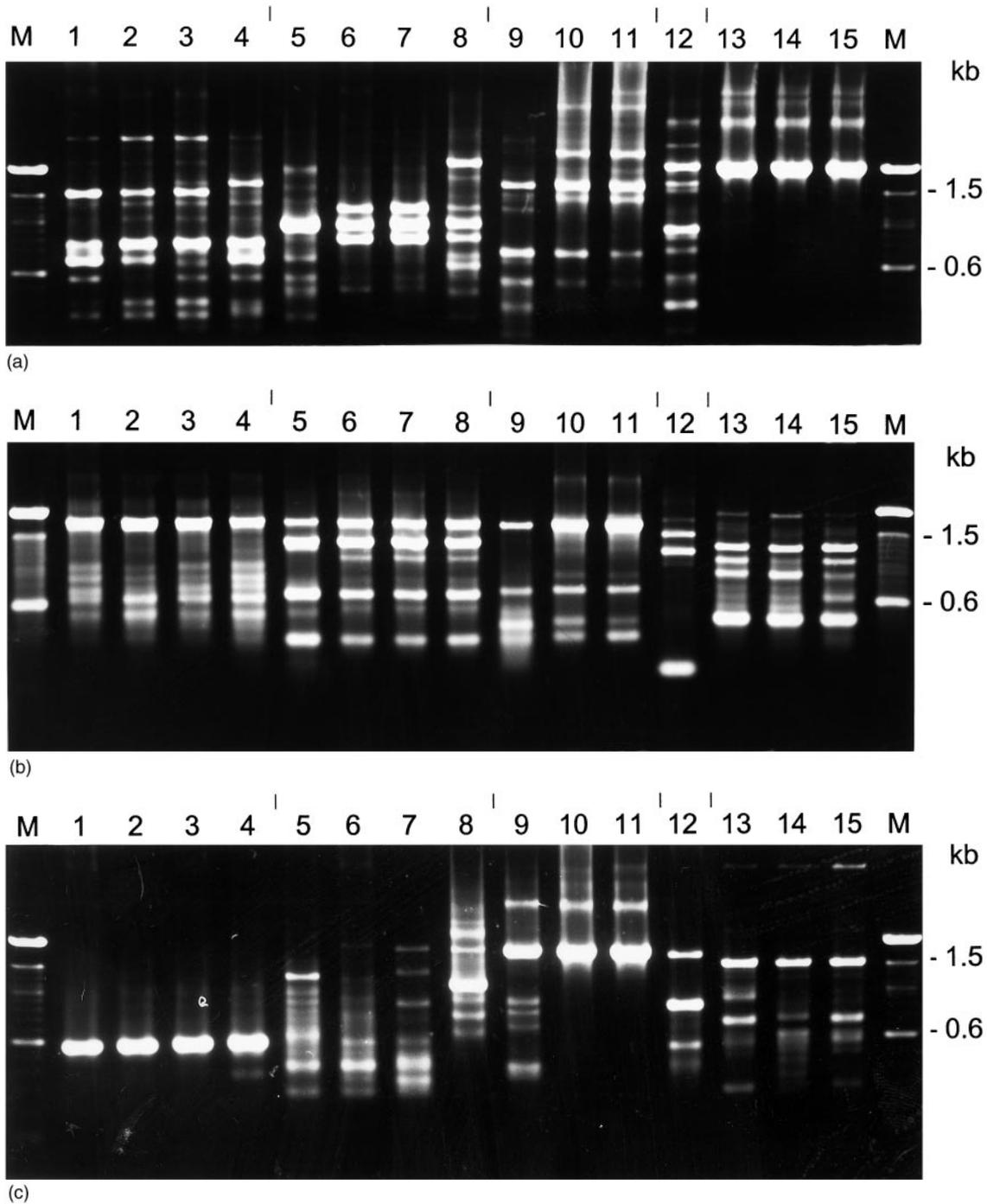


Fig. 3 RAPD profiles of *Clavibacter michiganensis sepedonicus*-(lane 1-4), *C.m. michiganensis*-(lane 5-8), *C.m. insidiosus*-(lane 9-11), *C.m. tessellarius*-(lane 12) and *C.m. nebraskensis*-strains (lane 13-15) amplified with primer 180-4 (5'-CGCCCGATCC-3') from Roth (3a), RE-01 (5'-CGGGATCCGC-3') from Genosys (3b) and 180-8 (5'-CGCCCTCAGC-3') from Roth (3c). 1 = GSPB 1522, 2 = GSPB 2238, 3 = GSPB 2249, 4 = DSM 46300, 5 = GSPB 382, 6 = GSPB 390, 7 = GSPB 392, 8 = DSM 20134, 9 = GSPB 2225, 10 = DSM 20157, 11 = GSPB 29, 12 = DSM 20741, 13 = DSM 20400, 14 = DSM 20401, 15 = DSM 7483. DNA size markers (100 bp ladder; Life Technologies) were applied on lanes M

All Cms strains showed a uniform profile displaying little genetic divergence and shared a major common specific band of 550 bp allowing an easy classification of this subspecies. In contrast, this primer was very suitable for the differentiation of Cmm strains. Using this primer the high discriminatory power of RAPD-PCR was indicated by the patterns obtained from the Cmm strain DSM 20134 (lane 8). In conclusion, depending on the primer

used, sufficient information was generated to distinguish the *Clavibacter michiganensis* subspecies and to identify strains within a subspecies on the basis of differences between the genomic fingerprints generated.

Discussion

For the identification of the five subspecies of *Clavibacter michiganensis*, two different PCR-based techniques were

employed; amplification with specific primers and RAPD-PCR. Based on the sequence data of the intergenic spacer between 16S and 23S rRNA genes, primers were designed for the identification of each subspecies. For the potential use in a multiplex PCR (Chamberlain et al., 1988; Way et al., 1993) a primer pair consists of a subspecies-specific forward primer and a universal reverse primer. In an independent study by Li and DeBoer (1995) the intergenic spacer region was also used for the detection of Cms in a PCR assay with a resulting amplification product of 215 bp. In this work, a slightly longer forward primer and a different reverse primer were used, resulting in a PCR product of 502 bp. Using the designed primer pairs it was possible to identify each subspecies of *Clavibacter michiganensis* according to the amplification of a specific DNA fragment. No amplification products were obtained, when bacteria belonging to other genera were submitted to PCR under the same conditions. These results demonstrate the potential of the described primer pairs for the identification of the *Clavibacter michiganensis* subspecies.

The sequencing results of the intergenic spacer between 16S and 23S rRNA genes and of the 16S rRNA gene (Lee et al., 1997b) provided data indicating relatively little genetic divergence between the five subspecies of *Clavibacter michiganensis*. The conservative nature of the 16S rRNA gene and the intergenic spacer limits the discriminatory power of these regions for distinguishing closely related strains. In comparison with the sequencing data, the high discriminatory potential of the RAPD-PCR was apparent. RAPD typing was capable of distinguishing subspecies of *Clavibacter michiganensis* as well as strains within subspecies. Both genomic variation between subspecies and genetic polymorphisms between bacterial strains were identified as differences in the size and numbers of DNA fragments obtained.

Recently, the subspecies of *Clavibacter michiganensis* were classified by restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified 16S rDNA sequences (Lee et al., 1997b). Using different restriction enzymes the authors were able to differentiate the subspecies, but were unable to identify strains within subspecies.

Sensitivity of RAPD patterns to changes in primer, template DNA concentrations and Taq polymerase were described (Schierwater and Ender, 1993; He et al., 1994; Niederhauser et al., 1994; Rawadi et al., 1995). For this reason, identical conditions were used for the generation of all RAPD profiles, resulting in highly reproducible patterns of amplified DNA fragments in replicate experiments. This implies, that differences in RAPD patterns obtained from different strains of *Clavibacter michiganensis* subspecies reflect DNA sequence diversity among their genomes.

The RAPD-PCR is a rapid and simple technique which requires no previous knowledge of nucleotide sequence, requires a minimum amount of template DNA and because it potentially analyses the whole genome, it is highly discriminatory. This technique has been recently exploited for the characterization and identification of a

number of different pathogens (Farber and Addison, 1994; Mäki-Valkama and Karjalainen, 1994; Rasmussen et al., 1994; Sandery et al., 1994). Depending on the primer used in RAPD analysis, information of variability at the strain level can be obtained, providing useful methods for epidemiological studies of the *Clavibacter michiganensis* subspecies (Marquet-Van Der Mee et al., 1995). Moreover, the RAPD-PCR will be a useful tool for the development of PCR assays based on cloned subspecies-specific RAPD fragments, without the need of previous knowledge of the microorganisms genome (Mäki-Valkama and Karjalainen, 1994; Martinez-Murcia and Rodriguez-Valera, 1994).

In conclusion, the PCR-based techniques described here can permit rapid and reliable means for the identification and differentiation of the *Clavibacter michiganensis* subspecies and should provide effective alternative methods to the conventional tests used.

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