

rep-PCR–Mediated Genomic Fingerprinting: A Rapid and Effective Method to Identify *Clavibacter michiganensis*

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ABSTRACT

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The genomic DNA fingerprinting technique known as repetitive-sequence–based polymerase chain reaction (rep-PCR) was evaluated as a tool to differentiate subspecies of *Clavibacter michiganensis*, with special emphasis on *C. michiganensis* subsp. *michiganensis*, the pathogen responsible for bacterial canker of tomato. DNA primers (REP, ERIC, and BOX), corresponding to conserved repetitive element motifs in the genomes of diverse bacterial species, were used to generate genomic fingerprints of *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *nebraskensis*, *C. michiganensis* subsp. *tessellarius*, and *C. michiganensis* subsp. *insidiosum*. The rep-PCR–generated patterns of DNA fragments observed after agarose gel electrophoresis support the current division of *C. michiganensis* into five subspecies. In addition, the rep-PCR fingerprints identified at least four types (A, B, C, and D) within *C. michiganensis* subsp. *michiganensis* based on limited DNA polymorphisms; the ability to differentiate individual strains may be of potential use in studies on the epidemiology and host-pathogen interactions of this organism. In addition, we have recovered from diseased tomato plants a relatively large number of naturally occurring avirulent *C. michiganensis* subsp. *michiganensis* strains with rep-PCR fingerprints identical to those of virulent *C. michiganensis* subsp. *michiganensis* strains.

Additional keywords: bacterial canker of tomato, bacterial mosaic of wheat, ring rot of potato, taxonomy, wilt and leaf blight of corn, wilt of alfalfa.

A taxonomic reevaluation of phytopathogenic, xylem-inhabiting, gram-positive coryneform bacteria has led to a reorganization of the taxonomy of these bacteria and the establishment of the species *Clavibacter michiganensis* (8). This species has been further subdivided into subspecies based on differences in phenotypic traits such as pigmentation, growth on differential media, total protein profiles, and serological reactions, as well as distinct differences in host range (4). Each subspecies of *C. michiganensis* causes disease on an important crop plant including ring rot of potato (*C. michiganensis* subsp. *sepedonicus*), wilt of alfalfa (*C. michiganensis* subsp. *insidiosum*), wilt and leaf blight of corn (*C. michiganensis* subsp. *nebraskensis*), bacterial mosaic of wheat (*C. michiganensis* subsp. *tessellarius*), and bacterial canker of tomato (*C. michiganensis* subsp. *michiganensis*). The diagnosis and identification of these gram-positive pathogens are based on a limited number of biochemical and physiological characteristics, as well as time-consuming pathogenicity assays (13,40). Immunologically based diagnostic kits are available for some subspecies, but these kits have experimental limitations because of serological cross-reactivity (7,9). DNA-based detection protocols including Southern blot hybridization and the analysis of polymerase chain reaction (PCR)–amplified DNA products, have also been reported (11,15,16,18,24,28,29,35).

Recently, a technique for subspecific differentiation of some pathovars of *Pseudomonas syringae* and *Xanthomonas* species has been reported (20,21,26,41). This technique, termed repetitive-sequence–based (rep)-PCR genomic fingerprinting, is based on PCR-mediated amplification of DNA sequences located between specific interspersed repeated sequences in prokaryotic genomes. These repeated sequences are termed BOX, REP, and ERIC elements. Amplification of the DNA sequences between primers based on these repeated elements generates an array of differently sized DNA fragments from the genomes of individual strains. The separation of these fragments on agarose gels yields highly specific DNA fingerprints (10,22,27,29,39).

This technique has proven useful in the rapid identification of bacteria associated with plant diseases (20,26,30). Because several primers are useful for many different genera, rep-PCR is a promising technique suitable for rapid identification of bacteria. The rep-PCR genomic fingerprinting protocol can be performed on whole cells (individual colonies), bypassing DNA extraction, and can also be directly applied to cell suspensions generated from symptomatic plant tissues (20).

The rep-PCR–generated genomic fingerprint patterns can be useful in differentiating isolates of the same pathovar. For example, during a recent rep-PCR genomic fingerprinting study of *X. campestris* pv. *vesicatoria* strains (21), it could be determined rapidly that the isolates fell into at least two distinct groups, independently corroborating polyphasic taxonomic studies that showed that *X. campestris* pv. *vesicatoria* is composed of two distinct lineages (3,34,37). Similarly, rep-PCR effectively corroborated the detection of distinct genomic lineages in natural populations of *X. oryzae* pv. *oryzae* by restriction fragment length polymorphism (RFLP) analysis (38).

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The objectives of this study were to determine whether rep-PCR genomic fingerprinting could be used to differentiate the currently recognized subspecies of *C. michiganensis* and to determine whether rep-PCR genomic fingerprints could distinguish specific strains of the tomato pathogen *C. michiganensis* subsp. *michiganensis*. If so, the technique might be useful in tracking specific isolates in epidemiological experiments and in studies pertaining to tomato cultivar infection.

MATERIALS AND METHODS

Bacterial strains. The 183 *C. michiganensis* strains obtained from culture collections and used in this study are summarized in Tables 1 and 2. All bacteria were stored in glycerol (15% final concentration) at -20 or -70°C . These strains were grown on nutrient broth yeast extract (NBY) agar (with or without the addition of glucose) (32) at 27°C for 3 to 7 days depending on the subspecies.

Pathogenicity and hypersensitive response (HR) assays. Broth cultures of *C. michiganensis* subsp. *michiganensis* were grown in liquid NBY medium without glucose for 24 to 48 h with shaking at room temperature. Cell suspensions were prepared according to Gitaitis (13). Tomato seedlings (*Lycopersicon esculentum* Mill. cv. Bonny Best) and four o'clocks (*Mirabilis jalapa*) were grown in clay pots in a commercial potting mixture, either in growth chambers maintained at 25°C with 80 to 90% humidity, and a 12-h photoperiod or in the greenhouse maintained at 25 to 35°C with relative humidity ranging from 55 to 73%. Tomato seedlings were inoculated according to Thyr (36). Three seedlings were inoculated with each strain, and symptoms were monitored over several weeks. Sterile water and cells of known virulent strains (strains Cmm 68, Cmm 290, or Cmm 300) were used as negative and positive controls, respectively.

The hypersensitive response was determined as described by Gitaitis (13), except that a needleless hypodermic syringe was used to infiltrate the leaves. Sterile water and known hypersensitive-positive strains (same as for pathogenicity tests) were used as negative and positive controls, respectively. All tests were done in duplicate.

Cultures of *C. michiganensis* subsp. *tessellarius* (2 days old), grown by shaking at room temperature, were inoculated into 14-day-old winter wheat seedlings grown in a greenhouse. Approximately 1×10^8 CFU/ml were infiltrated into the leaves by placing the beveled edge of a 22-gauge needle mounted on a 3-ml syringe against the abaxial side of the youngest fully developed leaf. The plants were placed in plastic bags for 24 h, and symptoms were scored 7 days after inoculation (4). Pathogenicity of *C. michiganensis* subsp. *sepedonicus* strains was determined by inoculating eggplant seedlings as described by Hu et al. (15).

Immunological assays for *C. michiganensis* subsp. *michiganensis* and subsp. *sepedonicus*. Selected *C. michiganensis* subsp. *michiganensis* strains were tested for their enzyme-linked immunosorbent assay (ELISA) reaction following the protocol provided by the manufacturer (Agdia, Inc., Elkhart, IN). All *C. michiganensis* subsp. *sepedonicus* strains included in this study were tested for their reaction with a monoclonal antibody as described by Hu et al. (15).

DNA isolation. Total genomic DNA from Cmm 936 and other strains was prepared using a modification of the procedure of Ausubel et al. (1). Cultures were grown in 40 ml of liquid Luria-Bertani medium (31) for 48 h at 27°C and 200 rpm. Cells were centrifuged at $4,000 \times g$ for 10 min at 4°C and washed twice in 5 ml of 0.85% NaCl and once in 0.1 M NaPO_4 buffer (pH 6.8). Cells were lysed using mutanolysin (10 units per ml at 37°C for 30 min; Sigma Chemical Co., St. Louis). Lysis was facilitated with the addition of 1 ml of 10% sarcosyl, and the resulting lysate was treated with 5 mg of pronase per ml at 37°C for 2 h. NaCl was added to a final concentration of 1 M, and the DNA was purified using a solution of 10% hexadecyltrimethyl ammonium bromide (CTAB)

in 1 M NaCl at 65°C for 10 min, followed by phenol-chloroform and chloroform extractions. DNA was recovered by isopropanol precipitation, redissolved in Tris-EDTA (TE; 10 mM Tris, 1 mM EDTA, pH 8.0), and quantified spectrophotometrically at 260 nm.

Whole-cell PCR amplification protocols and electrophoresis. rep-PCR protocols were carried out as described by Versalovic et al. (39) with the following modifications. Autoclaved, membrane-filtered ($0.22 \mu\text{m}$), double-distilled H_2O was used instead of high-performance liquid chromatography (HPLC)-grade H_2O , and the 25- μl PCR reaction mixture contained 1 μl of Tween 20 (Sigma Chemical Co.). For rep-PCR reactions with genomic DNA as template, 1 μl of a 50-ng/ml solution of purified DNA isolated from Cmm 936 was added to the reaction mixture. Otherwise, the rep-PCR reactions contained whole cells from single colonies grown on NBY agar medium (without glucose) and harvested with a 1- μl , sterile, disposable inoculation loop (LPS, Rochester, NY), as described by Schneider and de Bruijn (33).

BOX, REP, and ERIC primers (39) were synthesized by the Macromolecular Structure, Sequence and Synthesis Facility at Michigan State University as previously described (20,21). DNA fragments in 6 μl of the amplified PCR product were separated by gel electrophoresis at 4°C in 1.5% agarose gels in $0.5 \times$ Tris-acetate-EDTA (TAE; 0.4 M Tris-acetate and 1 mM EDTA, pH 8.0) buffer with electrophoretic and photographic conditions similar to those reported by Louws et al. (20). Each strain included in Table 1 was included in at least two BOX-PCR reactions carried out on separate days. Each strain included in Figure 1 was included in at least two BOX-PCR, ERIC-PCR, and REP-PCR reactions carried out on separate days.

Analysis of DNA fingerprints. Computer-image analysis of photographs of the ethidium bromide-stained agarose gels was performed using the gel analysis system (AMBIS, San Diego, CA) following the recommendations of the manufacturer. Photographs were captured with the optical imaging system, and the images were stored. The DNA fragments in each lane were standardized to the 1 kilobase pair (kbp) marker lanes in each gel, and the stored images were scanned in the 2.0- to 0.2-kbp range. The lanes were normalized, and a similarity matrix was generated using the simple matching similarity coefficient and unweighted pair group with arithmetic averages (UPGMA) clustering, as previously described (39). In addition, cluster analysis was performed on the combined REP, ERIC, and BOX rep-PCR banding patterns in Figure 1 using the similarity coefficient of Nei and Miller (25) and UPGMA clustering (SPSS 6.1; SPSS Inc., Chicago).

RESULTS

Identification and pathogenicity characteristics of the *C. michiganensis* isolates used. A collection of 163 gram-positive, yellow-pigmented strains (Table 1) thought to be *C. michiganensis* subsp. *michiganensis* were tested for virulence on tomato and HR on four o'clock plants. Of these strains, 109 were fully virulent (causing cankers and wilt in tomato), and 54 showed reduced virulence including 31 strains that were characterized as avirulent (Table 1). Strains of intermediate virulence reactions produced only cankers at the site of inoculation, and avirulent strains produced neither cankers nor wilt. All 109 strains showing virulence on tomato produced a strong HR on four o'clock plants; 29 of 31 avirulent strains failed to produce HR. Twenty-one of twenty-three strains showing intermediate virulence produced HR. The remaining two avirulent strains and two strains of intermediate virulence were inconsistent in HR response. Strong positive reactions were obtained when selected strains (Table 1), representing virulent, intermediate, and avirulent phenotypes, were tested immunologically by ELISA. All strains of *C. michiganensis* subsp. *sepedonicus* used in this study caused disease symptoms on eggplant and tested positive as subsp. *sepedonicus* in immunological assays (data not shown) (Table 2). Both strains of *C. michi-*

TABLE 1. Strains of *Clavibacter michiganensis* subsp. *michiganensis* used in this study

Strain(s) ^a	PCR ^b type	Virulence ^c	HR ^d	Year	State - site ^e	Source ^f
253	A	V	+	1987	OH - 5	DF
672, 676	A	V	+	1991	MI - 19	FL
936*, 937, 938	A	V	+	1993	MI - 21	FL
T1, T4, T5, T8, T12, T13, T35, T39, T41, T42, T43, T44, T45, T57, T63, T64, T66, T67, T69, T70, T73, T74, T80	A	V	+	1993	MI - 22	FL
T17, T18, T19, T20, T23, T24, T27, T56	A	V	+	1993	MI - 23	FL
127*, 128*, 130*, 139*, 210*, 211*, 229*, 297*	A	V	+	1994	MI - 25	DF
281, 291, 292, 349	A	V	+	1994	MI - 26	DF
294, 295, 296	A	V	+	1994	MI - 27	DF
283, 299	A	V	+	1994	MI - 28	DF
300*, 301*	A	V	+	1994	MI - 29	DF
352, 353	A	V	+	1994	MI - 30	DF
671, 677	A	I	-	1991	MI - 19	FL
T33*	A	I	+	1993	MI - 22	FL
129*	A	I	+/-	1994	MI - 25	DF
282	A	I	+	1994	MI - 28	DF
287	A	I	+	1994	MI - 34	DF
29*, 32*	A	AV	-	1987	OH - 6	DF
25*	A	AV	-	1987	MI - 11	DF
67*	A	AV	+/-	1987	MI - 11	DF
123*	A	AV	-	1988	OH - 14	DF
9*	A	AV	-	1988	OH - 15	DF
673, 674, 675	A	AV	-	1991	MI - 19	FL
T2*	A	AV	+/-	1993	MI - 22	FL
T9*	A	AV	-	1993	MI - 22	FL
98*, 131*, 132*, 136*, 138*, 209*	A	AV	-	1994	MI - 25	DF
208*, 298*	A	AV	-	1994	MI - 27	DF
126*, 133*, 134*, 135*, 207*	A	AV	-	1994	MI - 29	DF
303*, 304*, 305*, 306*, 350*, 351*	A	AV	-	1994	MI - 30	DF
30, 35	B	V	+	1986	MI - 3	DF
68, 69	B	V	+	1987	OH - 7	DF
71	B	V	+	1987	OH - 8	DF
75*, 76, 77	B	V	+	1987	OH - 9	DF
12	B	V	+	1987	MI - 13	DF
226	B	V	+	1988	OH - 16	DF
R28	B	V	+	1990	ON - nk ^g	MR
38	C	V	+	1981	MI - 1	DF
21, 54	C	V	+	1984	NC - 2	DE-EE
R4	C	V	+	1984	NC - nk	MR-EE
3	C	V	+	1986	NC - 4	DE-EE
4, 8, 10, 18, 44, 53	C	V	+	1987	MI - 11	DF
237	C	V	+	1987	MI - 12	DF
117, 122, 230, 234, 268	C	V	+	1988	OH - 15	DF
27	C	V	+	1988	OH - 16	DF
13	C	V	+	1988	MI - 17	DF
115, 116, 222, 231, 232, 236	C	V	+	1988	MI - 18	DF
R19	C	V	+	1990	OH - nk	MR
R24	C	V	+	1990	ON - nk	MR
954, 955, 956, 957, 958, 961, 962, 963	C	V	+	1992	MI - 20	FL-SM
81	C	V	+	1993	MI - 24	FL
280, 290	C	V	+	1994	MI - 31	DF
CIC 14*	C	V	+	nk	NE - nk	CI
26*	C	I	+	1987	OH - 10	DF
15, 16, 23, 31, 33, 56	C	I	+	1987	MI - 11	DF
24	C	I	+/-	1987	MI - 11	DF
65*	C	I	+	1987	MI - 13	DF
216, 218	C	I	+	1988	MI - 18	DF
CIC 23*	C	I	+	nk	NE - nk	CI
CDA 4* ^h	C	AV	-	nk	nk - nk	DO
285*	D	V	+	1994	MI - 32	DF
284*, 286*	D	I	+	1994	MI - 32	DF
288*, 289*, 293*	D	I	+	1994	MI - 33	DF

^a Strains marked with * were positive when tested by enzyme-linked immunosorbent assay.

^b PCR = polymerase chain reaction.

^c Virulent = producing both canker and wilt; I = intermediate, producing canker only; and AV = avirulent, producing no symptoms.

^d HR = hypersensitive reaction; + = positive; - = negative; and +/- = inconsistent between trials.

^e Strains from each site are independent isolates obtained from the same greenhouse or field in the same year.

^f CI = C. Ishimaru, Colorado State University; DE = D. Ematty, Heinz, Inc.; DF = D. Fulbright, Michigan State University; DO = D. Opeganorth, California Department of Food and Agriculture; EE = E. Echandi, North Carolina State University; FL = F. Louws, Michigan State University; MR = M. Riker, Heinz, Inc.; and SM = S. Miller, Ohio State University.

^g nk = not known.

^h Originally obtained as ATCC 4450 by DO from the American Type Culture Collection, Rockville, MD 20852.

ganensis subsp. *tessellarius* used in this study were pathogenic on wheat in greenhouse assays. The five strains of *C. michiganensis* subsp. *nebraskensis* and one strain of *C. michiganensis* subsp. *insidiosum* used in this study were not tested by our laboratories for pathogenicity and serological reaction.

rep-PCR genomic fingerprinting clearly distinguishes different subspecies. Three rep-PCR primers (BOX, ERIC, and REP) were used for genomic fingerprinting; this analysis generated distinct sets of patterns for each *C. michiganensis* subspecies tested (Fig. 1A, B, and C). No differences were noted in the “quality” of the fingerprints obtained from purified DNA and from whole cells (Fig. 1, lanes 1 and 2). DNA fingerprint patterns of *C. michiganensis* subsp. *sepedonicus* and of *C. michiganensis* subsp. *nebraskensis* strains listed in Table 2 but not shown in Figure 1 had identical fingerprint patterns to those strains shown in Figure 1. Thus, whole-cell rep-PCR was used to type all of the strains listed in Tables 1 and 2. The rep-PCR-generated DNA fingerprint, in the 2,000- to 200-bp range, was examined by AMBIS analysis, and a distinct pattern was recognizable for each subspecies. The fingerprint patterns were also easily distinguished by eye and only one or two strongly amplified bands in each subspecies were observed to comigrate with bands in another subspecies (e.g., the 750-bp and

500-bp DNA fragments of *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus* generated by the BOX-PCR reaction) (Fig. 1A). Avirulent strains of *C. michiganensis* subsp. *michiganensis* showed identical fingerprint patterns to those of virulent *C. michiganensis* subsp. *michiganensis* strains (Fig. 2).

Further subdivision of *C. michiganensis* subsp. *michiganensis*. rep-PCR genomic fingerprints generated with the BOX primer from the 109 virulent *C. michiganensis* subsp. *michiganensis* isolates and strains in this study enabled us to further subdivide this subspecies into four distinct groups arbitrarily designated types A, B, C, and D (Fig. 1A, lanes 2 to 13). Most of the DNA fragments generated by BOX PCR were similar; however, distinct DNA polymorphisms in the region around 1,000 bp could be identified for

TABLE 2. Reference strains of *Clavibacter michiganensis* subsp. *sepedonicus*, *nebraskensis*, *tessellarius*, and *insidiosum* used in this study

Subspecies	Strains
<i>sepedonicus</i> ^a	CIC4, NCPPB2139 ^b , CIC5 ^b , CIC7 ^b , CIC29 ^{b,c} , CIC194 ^{b,c} , CDA12, ATCC9850 ^{d,e} , CDA16St, R9 ^{d,f} , CDA18, R10 ^{d,f} , CDA20, R14 ^{d,f} , CDA30 ^d , CDA32 ^d , SS#43 ^g
<i>nebraskensis</i> ^h	CIC13 ^{b,i} , CIC15 ^{b,i} , CIC16 ^{b,i} , CIC17 ^{b,i} , CIC53 ^b
<i>tessellarius</i> ^j	CIC21 ^{b,i} , CIC22 ^{b,i}
<i>insidiosum</i> ^h	CDA2, ATCC3314 ^{d,e}

^a All strains of *C. michiganensis* subsp. *sepedonicus* virulent on eggplant.

^b Strains received from C. Ishimaru.

^c Original source of strain, C. Orser.

^d Strain received from D. Opgenorth.

^e Original source of strain, American Type Culture Collection in 1993.

^f Original source of strain was S. DeBoer in 1993.

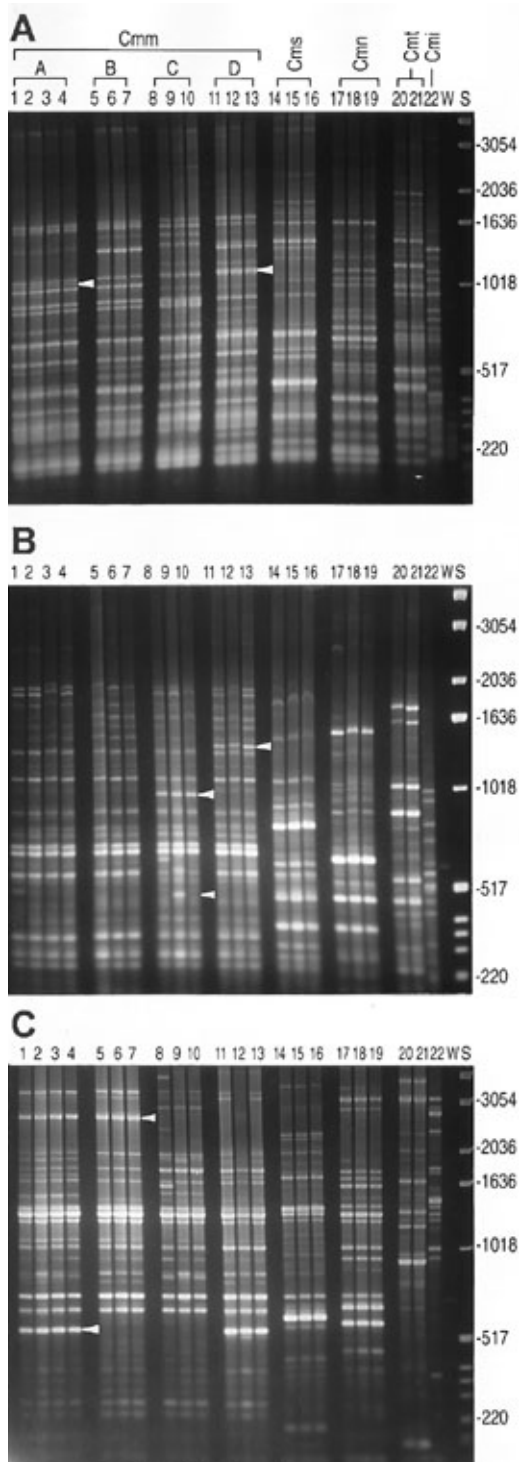
^g Strain received from M. Lacy, Michigan State University; original source of strain was W. Fry, Cornell University.

^h Strains of subspecies not tested for virulence.

ⁱ Originating from A. Vidaver isolations from Nebraska.

^j Strains of subspecies pathogenic on wheat.

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) fingerprint patterns obtained from strains representing five subspecies of *Clavibacter michiganensis* using primers corresponding to repetitive extragenic palindromic sequences (rep-PCR). **A**, BOX-PCR pattern, **B**, ERIC-PCR pattern, and **C**, REP-PCR pattern. A 6- μ l portion of each of the rep-PCR mixtures was loaded onto a 1.5% agarose gel. Lanes 1 to 13, Electrophoretic patterns for *C. michiganensis* subsp. *michiganensis* including representatives of designated type A strains Cmm 936 (lanes 1 and 2), Cmm 300 (lane 3), and Cmm 301 (lane 4); designated type B strains Cmm 35 (lane 5), Cmm 68 (lane 6), and Cmm 77 (lane 7); designated C type strains Cmm CDA 4 (lane 8), Cmm 4 (lane 9), and Cmm 3 (lane 10); and designated D type strains Cmm 285 (lane 11), Cmm 288 (lane 12), and Cmm 289 (lane 13). Electrophoretic fingerprints of other *C. michiganensis* subspecies including subsp. *sepedonicus* strains CIC 4 (lane 14), CDA 12 (lane 15), and SS#43 (lane 16); subsp. *nebraskensis* strains CIC 16 (lane 17), CIC 17 (lane 18), and CIC 53 (lane 19); subsp. *tessellarius* strains CIC 21 (lane 20) and CIC 22 (lane 21); and subsp. *insidiosum* strain CDA 2 (lane 22) are also represented. Lane 1, rep-PCR reaction products of purified genomic DNA-extracted Cmm 936; lane 2, rep-PCR reaction products of a whole cell preparation of Cmm 936. Lane W, the same rep-PCR mixture but lacking template DNA (control); lane S, DNA molecular size markers (1-kbp ladder; Gibco BRL, Gaithersburg, MD); the sizes are indicated in base pairs. Arrowheads indicate polymorphisms among the A, B, C, and D type *C. michiganensis* subsp. *michiganensis* strains identified by rep-PCR as indicated in the text. Cmm = *C. michiganensis* subsp. *michiganensis* strains, Cms = *C. michiganensis* subsp. *sepedonicus* strains, Cmn = *C. michiganensis* subsp. *nebraskensis* strains, Cmt = *C. michiganensis* subsp. *tessellarius* strains, and Cmi = *C. michiganensis* subsp. *insidiosum* strain.



each type (Fig. 1A, lanes 2 to 13). Type A strains displayed three bands in this region, while type B strains had only two (Fig. 1A, lanes 1 to 4 and 5 to 7, arrow). Type C and type D strains both displayed a single band in the same 1,000-bp region. The single type D band was found to migrate slightly slower than the type C single DNA band and was always brighter (Fig. 1A, lanes 8 to 10 and 11 to 13, arrow). The single band in type C strains, although slightly faster in mobility than the band in type D strains, appeared similar in mobility to the top band of the triplet observed in strains with type A and B patterns.

The fingerprint types defined on the basis of the 1,000-bp region of the BOX-PCR fingerprint pattern can also be distinguished by fragment pattern differences obtained with REP and ERIC primers (Fig. 1B and C). For example, strains having BOX-pattern types A and B did not have distinguishable fingerprint patterns when amplified with ERIC primers, but strains having BOX-pattern type C were distinguishable from the three other types by a fragment of approximately 1,000 bp in the ERIC fingerprint (Fig. 1B, arrow). Also, the ERIC fingerprint pattern of type D strains possessed an approximately 1,500-bp fragment not shared by BOX-PCR types A, B, and C. Similarly, the REP-PCR fingerprint patterns of BOX-PCR types A and B possessed an approximately 3,000-bp fragment (outside the defining 2,000- to 200-bp region used in the AMBIS analysis) not shared by BOX types C and D, and BOX types A and D possessed unique bands slightly larger than 500 bp in size (Fig. 1C).

Most of the strains classified by BOX PCR as types A, B, C, and D did not show DNA polymorphisms within the fingerprint types. However, a few strains did show unique bands in DNA patterns generated by BOX PCR. For example, three strains, Cmm 65, Cmm 13, and Cmm 81, were grouped into type C based on the

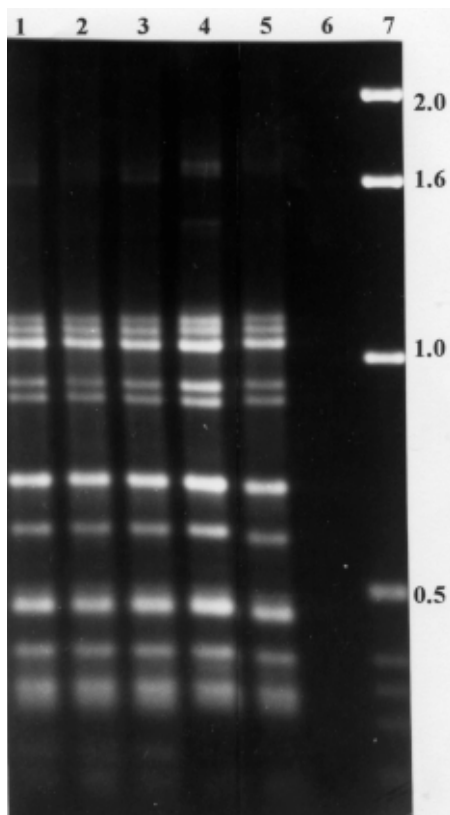


Fig. 2. Agarose gel electrophoresis of polymerase chain reaction (PCR) fingerprint patterns obtained from BOX-PCR reaction products representing virulent *Clavibacter michiganensis* subsp. *michiganensis* type A strains Cmm 287 (lane 1), Cmm 353 (lane 2), and Cmm 297 (lane 3); and avirulent *C. michiganensis* subsp. *michiganensis* type A strains Cmm 135 (lane 4) and Cmm 132 (lane 5). Lane 6, H₂O control reaction; and lane 7, molecular size markers. Approximate molecular sizes of the bands in lane 7 are given on the right in kilobase pairs.

DNA fragment patterns found in the 1,000-bp region of the BOX-PCR fingerprint; however, unique polymorphisms in the 700- to 900-bp region could be used to distinguish these type C isolates from each other (Fig. 3, lanes 3 to 5, arrows). Furthermore, strains that give identical banding patterns with one primer may not show identical patterns with another primer. For example, the three type C strains in Figure 1 have identical BOX-PCR fingerprints (Fig. 1A), but one strain (Cmm 4) displays a polymorphism in its ERIC-PCR fingerprint (Fig. 1B, lane 9, arrow).

Definite patterns linking fingerprint types to geographical areas or year of isolation have not been evident; however, we have observed a trend in which type A strains have been more frequently associated with processing varieties of tomato; type B and C strains have been associated with both fresh market and processing tomato varieties; and type D strains have, so far, only been associated with fresh market varieties (data not shown).

Cluster analysis. Cluster analysis of the combined data sets of BOX-, ERIC-, and REP-PCR experiments revealed that the subspecies of *C. michiganensis* could be separated into five distinct groups. Because the banding patterns of all strains of each subspecies, except *C. michiganensis* subsp. *michiganensis*, were identical and because the banding patterns of each subgroup of *C. michiganensis* subsp. *michiganensis*, except type C, were also identical (Fig. 1), only the consensus pattern for each subspecies or subgroup of *C. michiganensis* subsp. *michiganensis* was used in the

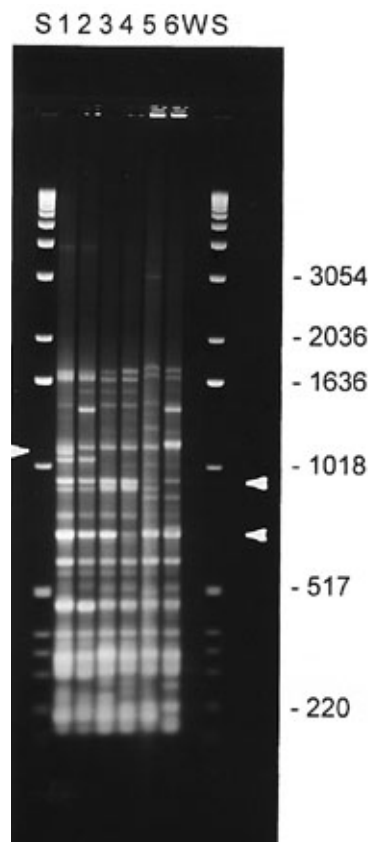


Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) fingerprint patterns obtained from BOX-PCR reaction products representing *Clavibacter michiganensis* subsp. *michiganensis* strains Cmm 936 (type A; lane 1), Cmm 3 (type B; lane 2), Cmm 65 (type C; lane 3), Cmm 13 (type C; lane 4), Cmm 81 (type C; lane 5), and Cmm 285 (type D; lane 6). Based on the DNA banding pattern at approximately 1,100 bp (arrow in left margin), the strains were placed into their designated types. The C type strains shown are examples of how unique and consistent polymorphic DNA fragments (arrows in right margin at approximately 700 and 900 bp) can be used to distinguish these C type strains from each other. Lane W, The same rep-PCR mixture but lacking template DNA (control); lane S, DNA molecular size marker (1-kbp ladder; Gibco BRL, Gaithersburg, MD); the sizes are indicated in base pairs.

analysis. The polymorphisms present within *C. michiganensis* subsp. *michiganensis* type C were excluded. Similarities in rep-PCR genomic fingerprint patterns between the five subspecies were less than 40%; therefore, the rep-PCR technique apparently distinguishes the subspecies unambiguously. Patterns within subspecies *C. michiganensis* subsp. *michiganensis* were very similar, with most strains clustering at greater than 80% similarity to one another.

DISCUSSION

Bacterial canker of tomato is a severe and constant threat to the tomato-growing industry, and epidemics are unpredictable. Recent epidemiological studies have given us a better understanding of the spread of the pathogen in the field under various cultivation schemes (5,6,14). Although the isolation and identification of *C. michiganensis* subsp. *michiganensis* has been improved by the use of semiselective media (12), selection of presumptive colonies is usually based on pigmentation, gram stain, and fluidal colony morphology. Differentiation of *C. michiganensis* subsp. *michiganensis* colonies from those of similar-appearing saprophytes remains problematic, because the correct identification of *Clavibacter* spp. is based on relatively few obvious phenotypic and physiological characteristics (4,32,40).

Biochemical and physiological characteristics described in previous studies have revealed that subspecies of *C. michiganensis* are closely related (4,8). Cluster analysis of the data on cell wall and metabolic characteristics of *C. michiganensis* subspecies reported by Davis et al. (8) was performed in our laboratory using the simple matching coefficient (including negative matches) and UPGMA (data not shown). The five subspecies were more than 80% similar. In addition, the total protein profiles obtained by Carlson and Vidaver (4) were nearly identical. Furthermore, studies employing conserved 16S rDNA and 16-23S intergenic transcribed spacer sequences (17,19) have demonstrated close phylogenetic relationships among *C. michiganensis* subspecies.

Our initial concern, therefore, was whether there would be enough diversity in rep-PCR-generated DNA fragment patterns among the subspecies to distinguish them. This concern, however, was not borne out. Our results show that each subspecies of *C. michiganensis* does have a distinct genomic rep-PCR fingerprint and that the strains comprising each subspecies have identical or nearly identical rep-PCR DNA fingerprint patterns as the other strains of that subspecies (except *C. michiganensis* subsp. *insidiosum*, in which only one strain was examined). The marked differences among the fingerprint patterns (<40% similarity) also provide confidence in the ability of this technique to identify *C. michiganensis* strains to the subspecies level.

The strains used in the analysis of *C. michiganensis* subsp. *sepedonicus* were similar in morphology to the type species and did not represent a diversity of types, such as nonmucoid strains (2). Studies comparing variants using the rep-PCR technique could offer insights into the relatedness of strains. Obviously, not many conclusions based on rep-PCR-generated DNA fingerprint patterns regarding *C. michiganensis* subsp. *nebraskensis*, subsp. *tessellarius*, or subsp. *insidiosum* can be made, because few strains were used in this study.

These data, when considered in conjunction with the findings of Carlson and Vidaver (4), Davis et al. (8), and Lee et al. (17), support the idea that the *C. michiganensis* subspecies represent genetically well-defined evolutionary lineages isolated by specialization on different plant hosts. Such specialization may be reflected in the fact that unique DNA sequences can be isolated from *C. michiganensis* subsp. *sepedonicus* by subtractive hybridization with DNA from other *C. michiganensis* subspecies (23). The pathogens differ in host range and also display differences in their genome organization. This last presumption, in turn, is based on the hypothesis that rep-PCR genomic fingerprint differences reflect distinct differences in genome organization (27). However, there

is always the possibility that other lineages of a given subspecies could be so deeply divided as to generate distinct DNA fingerprint patterns that would lead to misidentification.

The only subspecies in which we examined sufficient numbers of strains to detect genetic variation below the subspecies level was *C. michiganensis* subsp. *michiganensis*, and this subspecies appears to have a limited number of polymorphisms (>80% similarity in rep-PCR fingerprint patterns). This situation is in contrast to the one that exists for *Xanthomonas campestris* pv. *vesicatoria*, in which strains that infect a single host can vary widely in their rep-PCR genomic fingerprints (21). Since we appear to be able to identify specific strains of *C. michiganensis* subsp. *michiganensis* by virtue of their unique DNA polymorphisms, it should be possible to utilize these in epidemiological studies where it may be necessary to track a particular strain for which only antibiotic resistance markers have been available in the past (14). This is the first report in which *C. michiganensis* subsp. *michiganensis* has been subdivided below the subspecies level. It was important to include as many strains as possible in these studies to reveal the phenotypic and genotypic diversity that exists. At the current time, the biological significance of our type designations is not entirely clear. A substantial number (30%) of type A strains of *C. michiganensis* subsp. *michiganensis* were found to be nonpathogenic when tested in stem-inoculation assays; they were also incapable of causing a HR on four o'clock plants. A few of these avirulent strains had been stored in culture collections for years, but the majority were isolated from tomato and assayed for virulence soon after isolation. Finally, the fact that avirulent and virulent strains can be found at the same site in the same year (e.g., site 25 in Table 1) raises basic questions about the genetics and ecology of virulence and avirulence in this plant pathogen.

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