Southern Hybridization and PCR for Specific Detection of Phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

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Clavibacter michiganensis subsp. *michiganensis* is the causal agent of bacterial wilt and canker in tomato (*Lycopersicon esculentum*). Specific detection of this important bacterial pathogen was possible using Southern hybridization with DNA-probes derived from plasmid-borne genes, *cel*A encoding an endocellulase and *pat*-1 involved in pathogenicity. The *cel*A probe differentiated the *C. m.* subsp. *michiganensis*, *insidiosus*, *nebraskensis*, *tessellarius*, and *sepedonicus*; moreover, the *pat*-1 probe distinguished virulent from avirulent strains of *C. m. michiganensis*. A polymerase chain reaction based on primers derived from the *pat*-1 region was developed. Virulent strains were detectable in homogenates prepared from infected tomato plants, naturally contaminated seeds, and healthy plant homogenates containing as few as 2×10^2 bacteria per milliliter.

Bacterial wilt and canker of tomato (*Lycopersicon esculentum* Mill.) is caused by the coryneform bacterium *Clavibacter michiganensis* subsp. *michiganensis* (8). In this vascular disease, the bacteria spread throughout the plant via the xylem vessels (32). *C. m. michiganensis* is the cause of crop failures in all main cultivation areas (35,40) since neither resistant tomato cultivars nor effective chemical controls of this pathogen are available (42). *C. m. michiganensis* generally is transmitted by contaminated seeds (14, 43) or transplants (18, 19), and therefore, there is great demand for reliable diagnostic methods to detect this important pathogen.

Diagnostic tests useful for a general application have to be highly specific, reliable, and rapid. Although *C. michiganensis* subspecies are very host specific, the presence of saprophytes on plant material and low bacterial titers during early stages of infection impede detection of these bacteria. Chemotaxonomic markers employed for classification of coryneform bacteria-like fatty acids (6,21), menaquinons (5), polar lipids (5), cellular proteins (4), allozyme patterns (31), and enzymatic relatedness (10) are useless for a rapid diagnosis because they require time-consuming enrichment and complicated detection procedures. Phage typing is hampered due to the limited number of phage available (12,36,45). Currently, serological techniques such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assays with polyclonal antisera are in use. However, they are not always reliable due to strong cross-reactions with other subspecies (17,28,30), although the introduction of monoclonal antibodies has improved specificity (9). The best procedure, the isolation of the microorganism on semiselective media (14) followed by a direct test for virulence on the host plant (44) or induction of a hypersensitive reaction on nonhost plants (18), is very time-consuming and unsuitable for large-scale screening.

Nucleic acid hybridization and polymerase chain reaction (PCR) offer alternatives for rapid, highly sensitive, and specific identification of pathogenic bacteria (16,22,34). In recent reports, DNA-probes have been used to identify genes for virulence factors (33), ribosomal RNA (11,27), and cryptic chromosomal fragments (41) unique to pathogens. The extensive chromosomal homology among *C. michiganensis* subspecies has complicated the development of specific DNA-probes (38). However, we recently found that in strain NCPPB382 pathogenicity genes and a gene for an endocellulase are carried on two plasmids, pCM1 and pCM2 (13,25). The discovery of the genes *cel*A, encoding the endocellulase, and *pat*-1, with unknown function, suggested they might be useful targets in specific DNA-based detection methods. We report DNA-probes derived from *cel*A and *pat*-1 that allow specific identification of *C. michiganensis* subspecies. We also describe DNA primers for PCR that distinguish between virulent and avirulent strains of *C. m. michiganensis*. The PCR assay provides a rapid test for identification of virulent *C. m. michiganensis* strains in infected plant tissue.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacteria (Table 1) were obtained either from the National Collection of Plant Pathogenic Bacteria (NCPPB; Hatching Green, Harpenden, England) or the American Type Culture Collection (ATCC; Rockville, MD).

Escherichia coli and *Bacillus subtilis* were grown in TBY medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7.2) at 37 C, whereas *Rhizobium meliloti* was cultured in TBY at 25 C. *Clavibacter* and other coryneform bacterial strains were grown in TBY medium supplemented with 5 g of glucose per liter (C medium) at 24–26 C. A modified semiselective SCM medium was used (2,14) to determine bacterial titers of plant homogenates.

Phage assay. The lytic phage CMP1 has been useful for detecting *C. m. michiganensis* (12). Lysates containing 10^{11} plaque-forming units per milliliter of CMP1 were prepared from confluently lysed agar lawns of *C. m. michiganensis* strain NCPPB382 as described by Shirako et al (36). Serial dilutions of the phage lysates were made in SM buffer (5.8 g of NaCl, 2.4 g of Tris-HCl, 2.5 g of $MgSO_4$, and 0.1 g of gelatin per liter, pH 7.5); 10-μl drops of each dilution were spotted on agar plates seeded with cells of the indicator bacteria as described by Echandi and Sun (12). After three days of incubation at 20 C, the plates were scored for the occurrence of plaques.

Preparation of antisera. Cultures of *C. m. michiganensis* NCPPB382 were grown in TBY, and cells were harvested by centrifugation for 10 min at 3,000 *g*. Pelleted cells were washed twice in PBS

TABLE 1. Bacterial strains used in this study

Species	Source	Origin	Host plant
Clavibacter michiganensis subsp. *michiganensis*[a]	NCPPB382	UK, 1956	*Lycopersicon esculentum*
	NCPPB1064	Italy, 1961	*L. esculentum*
	NCPPB1468	UK, Channel Isl., 1962	*L. esculentum*
	NCPPB1496	C. M. Moskovets, 1963	*L. esculentum*
	NCPPB1572	Hungary, 1963	*L. esculentum*
	NCPPB1573	Hungary, 1963	*L. esculentum*
	NCPPB2979	Hungary, 1957	*L. esculentum*
	NCPPB3120	UK, 1979	*L. esculentum*
	NCPPB3121	UK, 1979	*L. esculentum*
	NCPPB3223	UK, 1981	*L. esculentum*
	NCPPB3224	UK, 1981	*L. esculentum*
	NCPPB3226	UK, Jersey, 1981	*L. esculentum*
	NCPPB3227	UK, Jersey, 1981	*L. esculentum*
	NCPPB3264	UK, 1983	*L. esculentum*
	NCPPB3284	UK, 1983	*L. esculentum*
	NCPPB3454	Hungary, 1985	*L. esculentum*
C. m. michiganensis[b]	NCPPB254	Australia, 1933	*L. esculentum*
	NCPPB399	H. L. Jensen, 1957	*L. esculentum*
	NCPPB515	Italy, 1956	*L. esculentum*
	NCPPB861	USA, 1939	*Cyphomandra betacea*
	NCPPB3123	USA, 1972	*L. esculentum*
	NCPPB3285	UK, 1983	*L. esculentum*
	NCPPB3286	UK, 1983	*L. esculentum*
C. m. insidiosus	NCPPB83	USA, 1934	*Medicago sativa*
	NCPPB1109	USA, 1955	*M. sativa*
	NCPPB1110	USA, 1943	*M. sativa*
	NCPPB1660	UK, 1964	*M. sativa*
	NCPPB1686	Italy, 1962	*M. sativa*
	NCPPB1687	Italy, 1961	*M. sativa*
C. m. nebraskensis	NCPPB2578	USA, 1971	*Zea mays*
	NCPPB2579	USA, 1971	*Z. mays*
	NCPPB2580	USA, 1971	*Z. mays*
	NCPPB2581	USA, 1971	*Z. mays*
	NCPPB2582	USA, 1974	*Z. mays*
	ATCC27794	USA, 1971	*Z. mays*
C. m. sepedonicus	NCPPB299	Canada, 1951	*Solanum tuberosum*
	NCPPB378	Sweden, 1956	*S. tuberosum*
	NCPPB2140	USA, 1942	*S. tuberosum*
	NCPPB2913	Sweden, 1977	*S. tuberosum*
	NCPPB3323	J. van Vaerenburgh, 1983	*S. tuberosum*
	NCPPB3468	Poland, 1985	*S. tuberosum*
C. m. tessellarius	ATCC33566	A. K. Vidaver, USA	*Triticum aestivum*
C. iranicus	NCPPB2253	Iran, 1966	*Triticum aestivum*
C. tritici	NCPPB255	Egypt, 1941	*T. aestivum*
	NCPPB1857	Egypt, 1966	*T. aestivum*
C. toxicus	NCPPB2980	New Zealand, 1969	*Dactylis glomerata*
Curtobacterium flaccumfaciens pv. *oortii*	NCPPB2113	Netherlands, 1967	*Tulipa gesneriana*
Rhodococcus fascians	NCPPB1488	UK, 1963	*Lathyrus odoratus*
Arthrobacter ilicis	NCPPB1228	USA, 1960	*Ilex opaca*
Bacillus subtilis	ATCC6051	H. J. Conn, Marburg strain	
Escherichia coli	EC294	Hanahan, 1983	
Rhizobium meliloti	2011	A. Pühler, Bielefeld	*Medicago sativa*

[a] Virulent on *Lycopersicon esculentum* cv. Moneymaker.
[b] Avirulent on cv. Moneymaker.

buffer (68.5 mM NaCl, 20 mM KH_2PO_4/K_2HPO_4, pH 7.2), and the bacterial titer was adjusted to approximately 1×10^8 cells per milliliter with PBS buffer. The cell suspension (0.5 ml) was injected subcutaneously into rabbits, followed by a second injection after 3–4 wk. Serum was prepared from blood 10 wk after the second injection. The serum was tested at different dilutions with strain NCPPB382 in an ELISA, as described below, and then generally used diluted 1:200. Specificity was tested in comparison to commercial antisera with the strains listed in Figure 1.

ELISA. *C. m. michiganensis* antisera, used for indirect sandwich ELISA, were obtained from several sources. A mouse monoclonal antibody against *C. m. michiganensis* was provided by Dr. Rabenstein (Bundesanstalt für Züchtungsforschung, Aschersleben, Germany); polyclonal rabbit antisera against *C. m. michiganensis* were obtained either from Loewe Biochemica GmbH (kit 07063K, Otterfing, Germany) or were prepared as described above.

ELISA plates (Corning Inc., Corning, NY) were coated with antiserum diluted 1:500 in coating buffer (1.5 g of Na_2CO_3, 2.91 g of $NaHCO_3$, and 0.20 g of NaN_3 per liter, pH 9.6) by incubation for 4 h at 37 C, and then washed five times with washing buffer (8 g of NaCl, 2.9 g of $Na_2HPO_4 \times 12\ H_2O$, 0.2 g of KH_2PO_4, 0.2 g of KCl, 0.2 g of NaN_3, and 0.5 ml of Tween 20 per liter, pH 7.2–7.4). Bacteria were collected from agar plates and resuspended in PBS buffer, inactivated by heating (10 min, 95 C), and washed three times with PBS buffer. The inactivated cells were adjusted to a concentration of 10^8 cells per milliliter in washing buffer containing 20 mg of polyvinylpyrrolidone (K_{10}–K_{40}) per milliliter and 2 mg of bovine serum albumin per milliliter and were transferred to the wells of the ELISA plate (0.2 ml per well). The plates were incubated overnight at 4 C and washed five times with washing buffer. Alkaline-phosphatase–labeled rabbit antibodies (diluted 1:500, 0.2 ml) directed against *C. m. michiganensis* (Loewe Biochemica) or 0.2 ml of alkaline-phosphatase–labeled goat antibodies diluted 1:7,500 directed against the F_c domain of mouse immunoglobulin (Ig) G (Serva, Heidelberg, Germany) were added, and the plates incubated for 4 h at 37 C. Plates were then washed five times with washing buffer followed by the addition of 0.2 ml of substrate solution (1 mg of *p*-nitrophenylphosphate per milliliter of substrate buffer containing 7.3 ml of glycerin, 0.2 g of MgCl, 0.014 g of $ZnCl_2$ per liter adjusted to pH 10.5 with KOH). The OD_{405} was determined 30 min after the addition of the substrate, using an ELISA plate reader. *B. subtilis* strain ATCC6051 served as the negative control in all assays.

Virulence assay. Infection of tomato plants was carried out as described previously (44). The petiole of the first true leaf of 4-wk-old tomato plants (*L. esculentum* cv. Moneymaker) was cut off near the stem with a scalpel that had been dipped into the bacterial suspension (10^9 cfu per milliliter) to be tested. A virulent strain will cause wilting of leaves 6–20 days after infection. Plants were cultivated in a growth chamber with a 16-h photoperiod at 25 C and 80% relative humidity.

Seeds contaminated with *C. m. michiganensis* were obtained from infected tomato plants (cv. Moneymaker). The peduncles of 4-wk-old plants were inoculated with approximately 50 µl of a bacterial suspension containing 10^9 cfu per milliliter of NCPPB382, using a syringe with a 27-gauge needle. Ripe fruits were harvested, and the seeds were removed, washed in tap water, placed onto paper towels, and dried overnight at room temperature.

DNA preparation and Southern hybridization. Preparation of plasmid DNA from *E. coli* or *C. m. michiganensis* was carried out as described previously (26). Plasmids pCM1 and pCM2 were prepared from the two partially cured *C. m. michiganensis* strains, CMM101 with pCM1 and CMM102 with pCM2, respectively (26). Total DNA was prepared from 5 ml of late log-phase cultures as described by Hopwood et al (23).

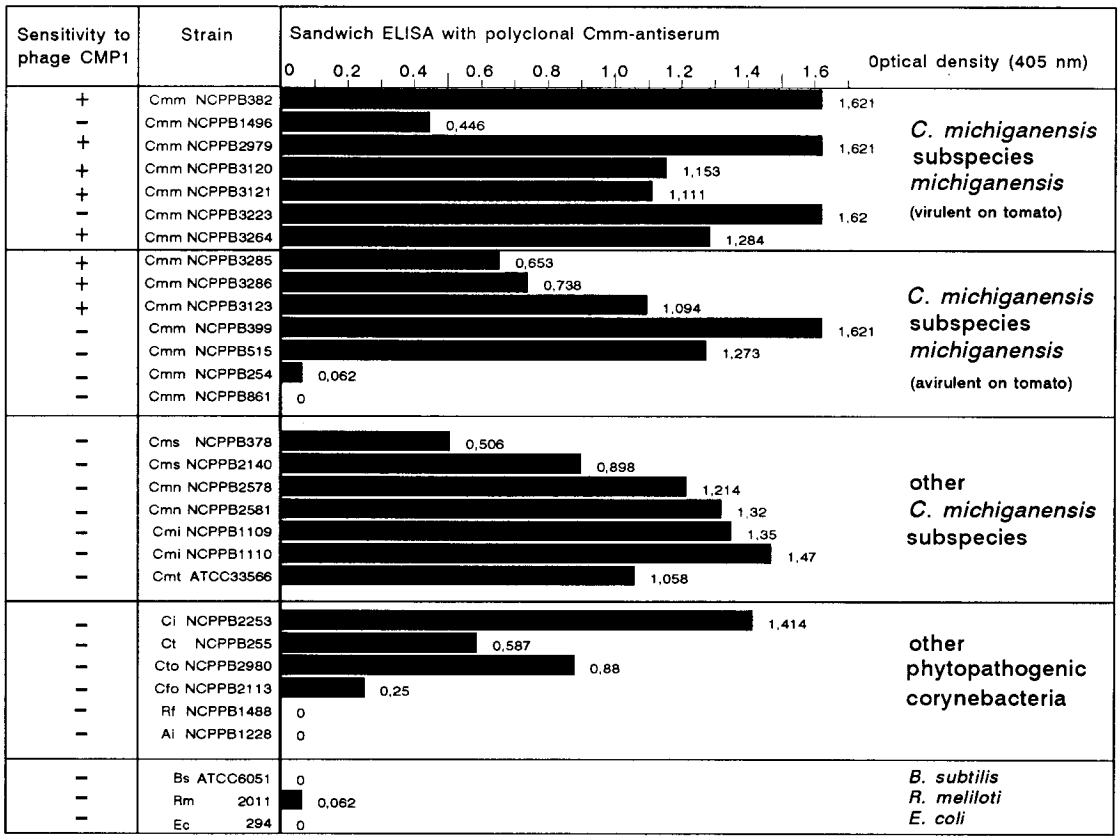

Fig. 1. Detection of *Clavibacter michiganensis* subsp. *michiganensis* by enzyme-linked immunosorbent assay (ELISA) and sensitivity to phage CMP1. ELISA was performed with the polyclonal *C. m. michiganensis* antiserum (Loewe Biochemica GmbH). Phage typing was performed with the *C. m. michiganensis* phage CMP1. Strain abbreviations: Cmm (*C. m. michiganensis*), Cms (*C. m. sepedonicus*), Cmn (*C. m. nebraskensis*), Cmi (*C. m. insidiosus*), Cmt (*C. m. tessellarius*), Ci (*C. iranicus*), Ct (*C. tritici*), Cto (*C. toxicus*), Cfo (*Curtobacterium flaccumfaciens* pv. *oortii*), Rf (*Rhodococcus fascians*), Ai (*Arthrobacter ilicis*), Bs (*Bacillus subtilis*), Rm (*Rhizobium meliloti*), and Ec (*Escherichia coli*).

For extraction of *C. m. michiganensis*, DNA from tomato plants was harvested 3 wk after inoculation by cutting above the root. At this time, plants inoculated with virulent strains had developed disease symptoms. Infected and noninfected control plants were homogenized by grinding in a mortar with the addition of 1 ml of TE buffer A (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) per gram of plant material. Serial dilutions of this homogenate were plated on semiselective SCM-medium (2,14). When scoring the plates after incubation at 24–26 C for 5 days, the yellow-orange colonies formed by *C. m. michiganensis* could be distinguished easily from the few colonies produced by saprophytes. To remove free DNA, 400-μl aliquots of the plant homogenate were centrifuged (10 min at 3,000 *g*), the pellet was resuspended in TE buffer A, and the process was repeated with TE buffer B (100 mM EDTA, 200 mM Tris-HCl, pH 8.0). The final pellet, consisting of plant material and bacteria, was resuspended in 400 μl of extraction buffer (TE buffer B plus 75 μl of NaCl, 25 μl of 10% sodium dodecyl sulfate [SDS], and 60 μl of 10% cetyltri-methylammonium bromide [CTAB]) and incubated at 65 C with shaking at 1,300 rpm for 10 min on a heating stir plate. The mixture was extracted twice with phenol-chloroform, and the DNA in the aqueous phase was precipitated with isopropanol. The resulting pellet was washed with 70% ethanol, and the DNA was dissolved in 100 μl of TE buffer A. Fifty tomato seeds from infected or noninfected plants were homogenized by grinding in a mortar after addition of 0.5 ml of TE buffer A, and DNA was prepared as described above.

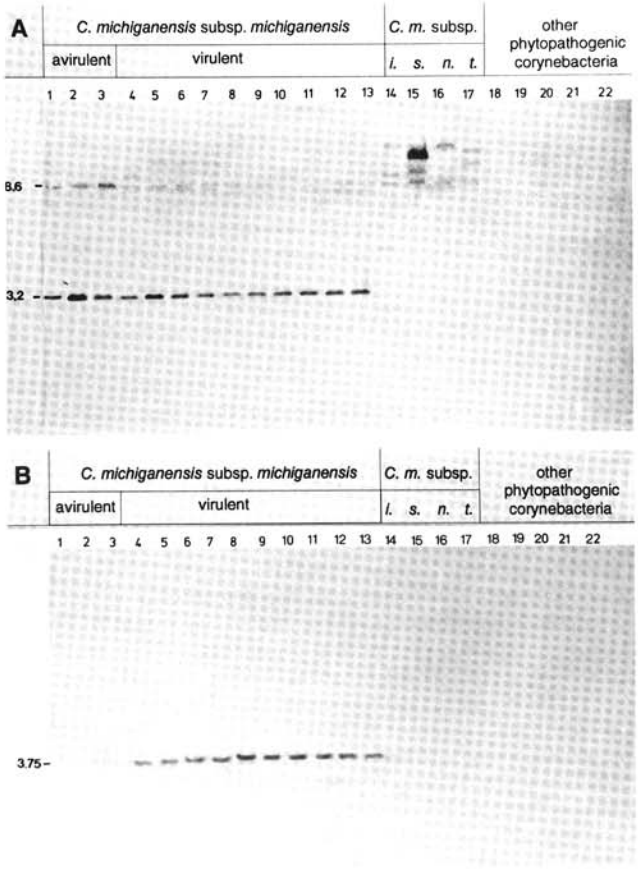

Fig. 2. Southern hybridization of genomic DNA with probes derived from **A,** *cel*A and **B,** the *pat*-1 region. Lanes 1–3: avirulent *Clavibacter michiganensis* subsp. *michiganensis* strains (Cmm) NCPPB399, NCPPB515, and NCPPB3123, respectively. Lanes 4–13: virulent *C. m. michiganensis* strains NCPPB382, NCPPB1064, NCPPB1496, NCPPB1572, NCPPB2979, NCPPB3120, NCPPB3223, NCPPB3264, NCPPB3284, and NCPPB3454, respectively. Lanes 14–17: *C. michiganensis* subspecies: lane 14, *C. m. insidiosus* NCPPB1660 (Cmi); lane 15, *C. m. sepedonicus* NCPPB2140 (Cms); lane 16, *C. m. nebraskensis* NCPPB2578 (Cmn); and lane 17, *C. m. tessellarius* ATCC33566 (Cmt). Lanes 18–22: phytopathogenic corynebacteria: lane 18, *C. iranicus* NCPPB2253; lane 19, *C. toxicus* NCPPB2980, lane 20, *C. tritici* NCPPB255; lane 21, *Curtobacterium flaccumfaciens* pv. *oortii* NCPPB2113; and lane 22, *Rhodococcus fascians* NCPPB1488.

To determine the detection limit of *C. m. michiganensis* in plant tissue, bacteria from a freshly prepared overnight culture (about 5×10^9 cfu per milliliter) were added at various concentrations to homogenate from noninfected plants. After removing a sample for verification of the bacterial titer, DNA was extracted as described above.

The DNA-probes used for Southern hybridization were derived from two plasmid-encoded pathogenicity regions, *cel*A and *pat*-1, of strain NCPPB382 (25). The *cel*A probe was an internal 1.1-kb DNA PCR fragment isolated from pCM1 (D. Meletzus, *unpublished data*). The *pat*-1 probe represents a 0.2-kb fragment of the repetitive sequence motif found on the 3.75-kb *Bgl*II fragment of plasmid pCM2 (13), which was obtained by PCR. Both DNA fragments were cloned with the vector pSVB30 (1) and labeled with digoxigenin-11-dUTP by nick-translation as described by Maniatis et al (24).

For Southern hybridization, DNA fragments were generated using appropriate restriction enzymes, resolved by agarose gel electrophoresis (37), and transferred to Hybond N nylon membranes (Amersham Inc., Arlington Heights, IL) with a LKB 2016 VacuGene blotting apparatus. Prehybridizations were performed at 42 C for 2 h in a solution of 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 50% formamide, 2% blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany), 0.02% SDS, and 0.1% *N*-lauroylsarcosine. The denatured probe was added to the prehybridization solution followed by gentle agitation at 42 C for 16 h. The nylon membranes were washed twice for 10 min at room temperature in a 2× SSC and 0.1% SDS solution and twice for 15 min at 65 C in a solution consisting of 0.1× SSC and 0.1% SDS. The hybridization results were visualized by using the DIG nucleic acid detection kit supplied by Boehringer.

PCR. Polymerase chain reactions were carried out using the GeneAmp DNA amplification kit and a DNA thermal cycler, both from Perkin-Elmer Cetus (Norwalk, CT). In general, approximately 50 ng of template DNA and 20 pmol of each primer DNA were used per reaction. The set of synthetic oligonucleotide primers used had the following nucleotide sequence: 5′-GCGAATAAGCCCATATCAA-3′ (CMM-5) and 5′-CGTCAGGAGGTCGCTAATA-3′ (CMM-6). They were derived from partial nucleotide sequence information of the *pat*-1 region and generated a 614-bp PCR amplification product. Generally, 30 PCR cycles were run with denaturation at 94 C for 1 min, annealing at 55 C for 1.5 min, and extension at 72 C for 1 min.

RESULTS

Virulence assay. The virulence of all the *C. m. michiganensis* strains used in our study was determined by inoculation of a susceptible tomato cultivar. Only those strains inducing typical wilt symptoms were classified as virulent (Table 1). Our tests confirmed the classification by NCPPB, with the exception of strains NCPPB3285 and NCPPB3286, which were described as weakly aggressive but were avirulent under our test conditions.

Serological specificity. To evaluate whether a serological procedure could specifically detect *C. m. michiganensis*, we tested two polyclonal antisera and a monoclonal antibody in an ELISA. All three antibodies gave similar results, and representative ELISA data obtained with the antibody from Loewe Biochemica are shown in Figure 1. All *C. m. michiganensis* strains gave a positive reaction ($OD_{405} > 0.4$) in indirect ELISA, with the exception of two of the avirulent strains, NCPPB254 and NCPPB861. A strong reaction also was observed with all subspecies of *C. michiganensis* and some phytopathogenic corynebacteria.

Sensitivity to phage CMP1. Reliable identification of *C. m. michiganensis* was not possible with the lytic phage CMP1. This bacteriophage is related to two other phage, MiP_1 and MiP_{1h}, which are described as specific for *C. m. michiganensis* (45). Although *C. m. insidiosus, sepedonicus, nebraskensis,* and *tessellarius* were not lysed by CMP1, the phage also did not lyse six strains of *C. m. michiganensis* (Fig. 1). For example, strains

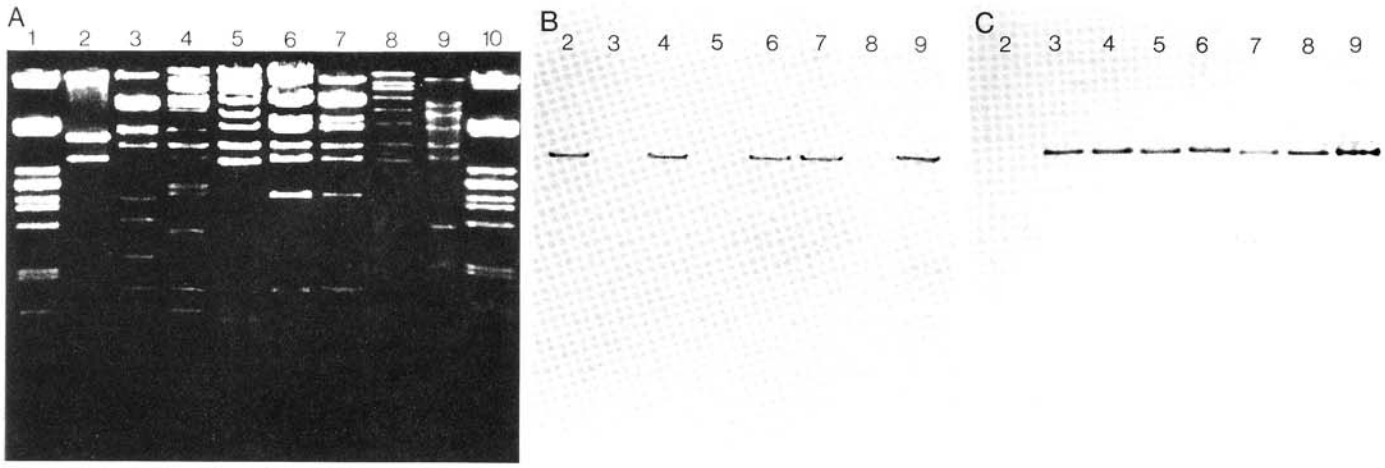

Fig. 3. Gel electrophoresis and Southern analysis of plasmid DNA isolated from virulent strains of *Clavibacter michiganensis* subsp. *michiganensis*. **A,** Plasmid DNA was digested with *Bgl*II, subjected to electrophoresis on a 0.8% agarose gel, and stained with ethidium bromide. DNA was prepared from strains NCPPB382 (lanes 2 and 3, pCM1 and pCM2, respectively), NCPPB1496 (lane 4), NCPPB2979 (lane 5), NCPPB3120 (lane 6), NCPPB3121 (lane 7), NCPPB3223 (lane 8), NCPPB3264 (lane 9). Lanes 1 and 10, molecular marker λ-DNA digested with *Pst*I. **A,** Southern blot of the agarose gel probed with digoxigenin-11-dUTP–labeled probes **B,** *cel*A and **C,** *pat*-1.

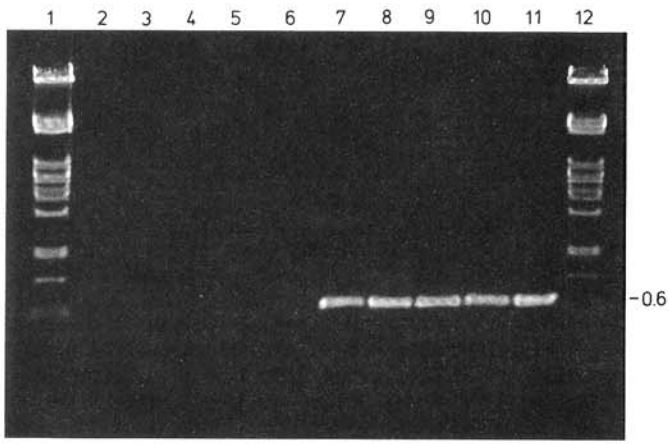

Fig. 4. Electrophoretic analysis of DNA from *Clavibacter michiganensis* subsp. *michiganensis* strains amplified by polymerase chain reaction. The position of the predicted product (0.6 kb) is indicated. Lanes 1 and 12, λ *Pst*I marker; lane 2–6, avirulent strains of *C. m. michiganensis* NCPPB254, NCPPB861, NCBBP399, NCPPB515, and NCPPB3123; lanes 7–11, virulent strains of *C. m. michiganensis*, NCPPB382, NCPPB1496, NCPPB2979, NCPPB3120, and NCPPB3264.

NCPPB1496 and NCPPB3223, which were virulent on tomato plants, were resistant in this phage assay.

Detection of *C. michiganensis* subspecies by Southern hybridization. When total bacterial DNA was cleaved with *Bgl*II and subjected to Southern hybridizations with the *cel*A probe, a distinct restriction fragment length polymorphism (RFLP) was characteristic of *C. m. michiganensis* (Fig. 2A). All *C. m. michiganensis* strains listed in Table 1 gave a strong hybridization signal with a 3.2-kb *Bgl*II DNA fragment from which the 1.1-kb *cel*A subfragment was derived. Additional hybridizing bands, especially an 8.6-kb fragment, indicated other homologies. Other *C. michiganensis* subspecies exhibited different hybridization patterns, which were characteristic for these bacteria, that always lacked the 3.2-kb band. Figure 2A (lanes 14–17) shows the characteristic RFLP common to all strains of *C. michiganensis* subspecies tested (Table 1). Strains NCPPB254 and NCPPB861 did not have the 3.2-kb hybridizing band (data not shown). The probe for the *pat*-1 region hybridized with a 3.75-kb *Bgl*II DNA fragment found only in virulent strains of *C. m. michiganensis*, whereas no signal was obtained with avirulent strains or other phytopathogenic bacteria (Fig. 2B).

Both DNA-probes used were derived from plasmid-encoded pathogenicity genes of strain NCPPB382. Since most *Clavibacter* strains harbor cryptic plasmids (15,20), we examined the possibility that these genes are carried on the plasmids present in these *Clavibacter* strains. Southern blots probed with the *cel*A region (Fig. 3B) revealed that this gene was not always located on plasmids; for example, in strains NCPPB2979 and NCPPB3223 it was located on the chromosome because hybridization signals were only obtained with total DNA preparations (Fig. 2A). In contrast, the *pat*-1 region was always plasmid-borne (Fig. 3C). In summary, the results from Southern hybridization with the two DNA-probes demonstrated that the *cel*A probe could be used for the detection of *C. m. michiganensis*, whereas the *pat*-1 probe allowed further differentiation between virulent and avirulent *C. m. michiganensis* strains.

Detection of *C. m. michiganensis* by PCR. The specificity of PCR with the CMM-5 and CMM-6 primers was tested by screening for an amplification product with the predicted size (614 bp) using total DNA isolated from 23 *C. m. michiganensis* strains (Table 1). The proper amplification product was observed only with DNA extracted from virulent *C. m. michiganensis* strains, whereas no amplification products were observed with DNA from avirulent strains (Fig. 4); a result that agrees with the hybridization data. Also, DNA prepared from other bacterial species and related phytopathogenic corynebacteria listed in Table 1 did not yield a PCR amplification product (data not shown).

The PCR assay was evaluated for detection of *C. m. michiganensis* in infected plant tissue and seeds as would be required for routine diagnosis. Amplification of target DNA with intact, untreated cells of *C. m. michiganensis* using the method described for *C. m. sepedonicus* (16) was unsuccessful. However, the CTAB protocol for extraction of *C. m. michiganensis* DNA from plant homogenate gave satisfactory results. A specific amplification of target DNA was observed with extracts prepared from plants infected with strain NCPPB382, which induced typical wilt symptoms and colonized the plant effectively ($1–3 \times 10^9$ cfu per gram of plant material) (Fig. 5). Amplification products were not observed when extracts from healthy, noninfected control plants were assayed nor when extracts from plants infected with the avirulent strain NCPPB3123 were tested ($1–2 \times 10^4$ cfu per milliliter of plant homogenate). PCR also was tested on seeds harvested from tomato plants infected with *C. m. michiganensis* strain NCPPB382. It was possible to detect the pathogen in DNA extracts from 50 seeds containing 1×10^3 bacteria (Fig. 6).

Sensitivity of the PCR assay. To estimate the detection limit of PCR assay, DNA preparations from plant homogenates containing 2×10^7 to 2×10^1 cfu per milliliter of strain NCPPB382 were tested in three independent experiments. It was possible to detect the 614-bp amplification product by Southern hybridization against a 3.75-kb *Bgl*II *pat*-1 probe down to a concentration

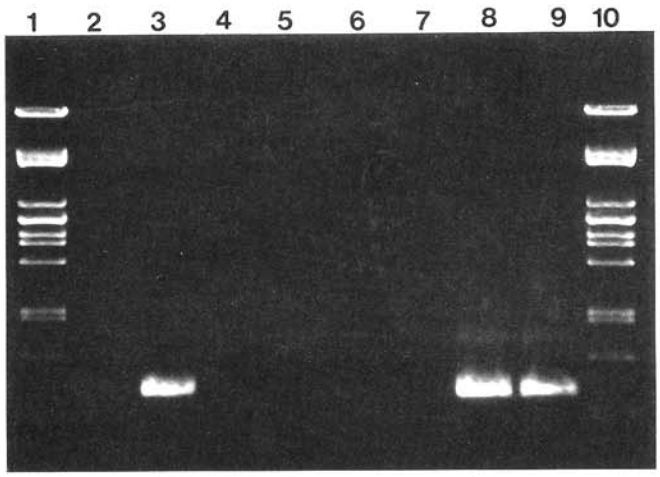

Fig. 5. Detection of *Clavibacter michiganensis* subsp. *michiganensis* strain NCPPB382 after cetyltri-methylammonium bromide extraction of infected tomato plants by polymerase chain reaction assay. Lanes 1 and 10, λ *Pst*I marker; lane 2, negative control (bidest H_2O); lane 3, positive control (NCPPB382 DNA); lanes 4–9: DNA extracted from tomato plants: lanes 4 and 5: noninfected plants; lanes 6 and 7, NCPPB3123-infected plants; and lanes 8 and 9, NCPPB382-infected plants.

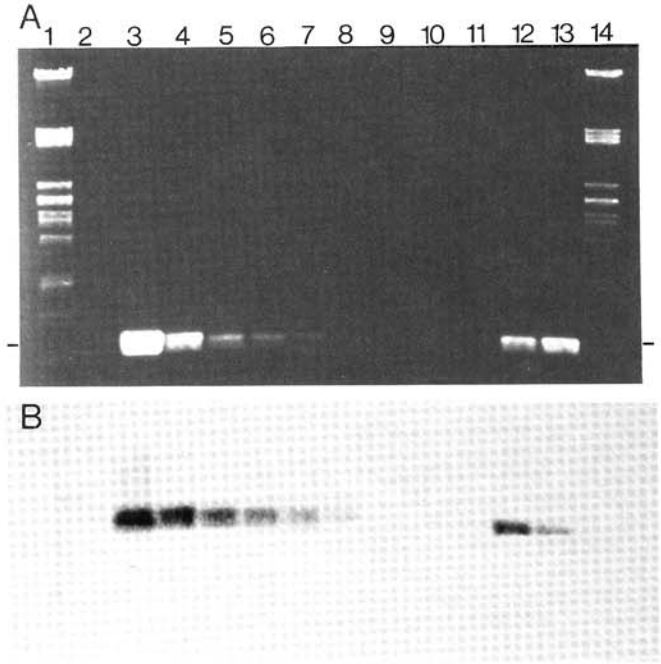

Fig. 6. Sensitivity of polymerase chain reaction (PCR) detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato homogenates and seeds. Homogenates were inoculated with various cell densities of strain NCPPB382. Seeds were obtained from tomato plants infected with strain NCPPB382. **A,** After gel electrophoretic separation of the amplification products, the gel was blotted on nylon membrane and **B,** hybridized against the *pat*-1 probe (3.75 kb) labeled with digoxigenin-11-dUTP. Lanes 1 and 14, λ *Pst*I marker; lane 2, negative control (homogenate from noninfected plants); lanes 3–9, 10-fold dilutions (2×10^7 to 2×10^1 cfu per milliliter) of NCPPB382; lanes 10 and 11, homogenate from noninfected tomato seeds; and lanes 12 and 13, homogenate from 50 seeds collected from tomato plants infected with NCPPB382 containing 1×10^3 cfu.

of 2×10^2 cells of *C. m. michiganensis* per milliliter of plant homogenate (Fig. 6).

DISCUSSION

Since the main source for transmission of *C. m. michiganensis* is contaminated seeds and transplants, the control of this plant disease requires reliable and sensitive methods of detecting the pathogen in these tissues. Immunofluorescence and ELISA are hampered by the fact that available antibodies lack specificity to allow differentiation at the subspecies level (17). Strong cross-reactions observed in our work indicate that similar antigenic epitopes are common to all subspecies of *C. michiganensis*. A prominent antigen is the exopolysaccharide with an identical carbohydrate composition in *C. m. michiganensis* and the alfalfa pathogen *C. m. insidiosus* (3). The detection limit for ELISA with pure cultures was approximately 10^4 cfu per milliliter (39)—a sensitivity sufficient to detect the pathogen in symptomless plants (19). Phage typing is not practicable for rapid diagnosis, because bacterial cultures are required, and thus, phage typing is more useful for taxonomic classification, provided a number of phage with different host specificity are available.

Recently a DNA-probe directed against the 16S ribosomal RNA genes of *C. m. sepedonicus* was described (27). However, due to high conservation of 16S rRNA genes among *C. michiganensis* subspecies, detection of *C. m. michiganensis* depends on an appropriate hybridization stringency. Thompson et al (41) described DNA-probes derived from a chromosomal library of *C. m. michiganensis* to detect *C. m. michiganensis* by dot blot that suggested sufficient specificity; however, very few strains were tested.

As reported here, plasmid-borne genes (25) proved to be useful for identification of *C. m. michiganensis*. Plasmids frequently play a role in virulence of phytopathogenic bacteria (7), and this was the case in *C. m. michiganensis* strain NCPPB382 (25,26). The probing of plasmids from various strains for the presence of the pathogenicity gene *pat*-1 (Fig. 3) suggests that in *C. m. michiganensis* virulence genes generally seem to be located on plasmids. This contradicts earlier reports by Gross et al (20) who saw no correlation between plasmids and virulence in *C. m. michiganensis*. Therefore, as discussed by Finnen et al (15), it would be interesting to study the molecular properties of plasmids from *C. m. michiganensis* isolates to gain more information on their relationship and to obtain data on the epidemiology of this important pathogen.

Southern hybridization with the *cel*A probe allowed differentiation between *C. michiganensis* subspecies and other phytopathogenic coryneform bacteria. In addition, an analysis of RFLP and the development of specific PCR primers for the *cel*A region may be helpful for identification of other subspecies.

The avirulent *C. m. michiganensis* strains NCPPB254 and NCPPB861 were negative in Southern hybridizations with the *cel*A probe (data not shown), and since these strains also gave no positive signal in ELISA (Fig. 1), they seem to be incorrectly classified. In fact, strain NCPPB861 was isolated from *Cyphomandra betacea* and, thus, may represent a different bacterial species.

An important finding of this work is the specificity of the *pat*-1 probe and its correlation with virulence. This region can be amplified by PCR and provides a sensitive detection assay for virulent strains of *C. m. michiganensis*. Our results indicate that PCR-based analysis without prior isolation and enrichment of the pathogen from plant tissue has a detection limit of 2×10^2 cells per milliliter of plant homogenate. This sensitivity should even be sufficient for the detection of the pathogen in latently infected plants, provided that the titer is in the range of the detection limit. Disease symptoms are observed only when bacteria reach a titer of 10^8–10^9 cfu per gram of plant tissue (25). We demonstrated that this technique also is appropriate for rapid screening of heavily contaminated seeds. The application of "booster" PCR may further enhance the sensitivity, as has been shown recently for *Agrobacterium tumefaciens* (29). As is the case for serological assays, DNA methods do not provide information about the viability of the pathogen but rapidly identify potentially contaminated plant and seed samples for successive confirmation by microbiological tests. Therefore, PCR assay should be a useful addition to standard detection methods because it combines speed, sensitivity, and specificity, which are critical parameters for any detection assay.

LITERATURE CITED

1. Arnold, W., and Pühler, A. 1988. A family of high-copy-number plasmid vectors with single end-label sites for rapid nucleotide sequencing. Gene 70:171-179.
2. Beimen, A., Bermpohl, A., Meletzus, D., Eichenlaub, R., and Barz, W. 1992. Accumulation of phenolic compounds in leaves of tomato plants after infection with *Clavibacter michiganense* subsp. *michiganense* strains differing in virulence. Naturforsch. 47:898-909.
3. Bermpohl, A. 1994. Untersuchung der pathogenen Wechselwirkung zwischen *Clavibacter michiganensis* subsp. *michiganensis* und der Tomate: Bedeutung der bakteriellen Exopolysaccharid- und Auxin-Biosynthese. Ph.D. thesis. University of Bielefeld, Bielefeld, Germany.
4. Carlson, R. R., and Vidaver, A. K. 1982. Taxonomy of *Corynebacterium* plant pathogens, including a new pathogen of wheat, based on polyacrylamide gel electrophoresis of cellular proteins. Int. J. Syst. Bacteriol. 32:315-326.
5. Collins, M. D., Goodfellow, M., and Minnikin, D. E. 1980. Fatty acid, isoprenoid quinone and polar lipid composition in the classification of *Curtobacterium* and related taxa. J. Gen. Microbiol. 118:29-37.
6. Collins, M. D., and Jones, D. 1980. Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2,4 diaminobutyric acid. J. Appl. Bacteriol. 48:459-470.
7. Coplin, D. L. 1989. Plasmids and their role in the evolution of plant pathogenic bacteria. Annu. Rev. Phytopathol. 27:187-212.
8. Davis, M. J., Gillaspie, A. G., Vidaver, A. K., and Harris, R. W. 1984. *Clavibacter*: A new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermudagrass stunting disease. Int. J. Syst. Bacteriol. 34:107-117.
9. De Boer, S. H., and Wieczorek, A. 1984. Production of monoclonal antibodies to *Corynebacterium sepedonicum*. Phytopathology 74:1431-1434.
10. De Bruyne, E., Swings, J., and Kersters, K. 1992. Enzymatic relatedness amongst phytopathogenic coryneform bacteria and its potential use for their identification system. Appl. Microbiol. 15:393-401.
11. DeParasis, J., and Roth, D. A. 1990. Nucleic acid probes for identification of phytobacteria: Identification of genus-specific 16S rRNA sequences. Phytopathology 80:618-621.
12. Echandi, E., and Sun, M. 1973. Isolation and characterization of a bacteriophage for the identification of *Corynebacterium michiganense*. Phytopathology 63:1398-1401.
13. Eichenlaub, R., Meletzus, D., Bermpohl, A., Dreier, J., and Bahro, R. 1992. Molecular analysis of the pathogenic interaction of the bacterium *Clavibacter michiganense* with the host plant. Pages 17-25 in: Plant Tissue Culture and Gene Manipulation for Breeding and Formation of Phytochemicals. K. Ono, K. Oouo, T. Hirabayashi, S. Kikuchi, H. Handa, and K. Kajiwara, eds. National Institute of Agrobiological Resources, Tsukuba, Japan.
14. Fatmi, M., and Schaad, N. W. 1988. Semiselective agar medium for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology 78:121-126.
15. Finnen, R. L., Dhanvantari, B. N., and Trevors, J. T. 1990. Plasmid profiles and restriction endonuclease analysis of genomic DNA of *Clavibacter michiganensis* ssp. *michiganensis*. J. Microbiol. Methods 12:57-64.
16. Firrao, G., and Locci, R. 1994. Identification of *Clavibacter michiganensis* subsp. *sepedonicus* using polymerase chain reaction. Can. J. Microbiol. 40:148-151.
17. Franken, A. A. J. M., Kamminga, G. C., Snijders, W., van der Zouwen, P. S., and Birnbaum, Y. E. 1993. Detection of *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds by immunofluorescence microscopy and dilution plating. Neth. J. Plant Pathol. 99:125-137.
18. Gitaitis, R. D. 1990. Induction of a hypersensitivelike reaction in four-o'clock by *Clavibacter michiganensis* subsp. *michiganensis*. Plant Dis. 74:58-60.
19. Gitaitis, R. D., Beaver, R. W., and Voloudakis, A. E. 1991. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomless tomato transplants. Plant Dis. 75:834-838.
20. Gross, D. C., Vidaver, A. K., and Keralis, M. B. 1979. Indigenous plasmids from phytophathogenic *Corynebacterium* species. J. Gen. Microbiol. 115:479-489.
21. Henningson, P. J., and Gudmestad, N. C. 1991. Fatty acid analysis of phytopathogenic coryneform bacteria. J. Gen. Microbiol. 137:427-440.
22. Henson, J. M., and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol. 31:81-109.
23. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Schrempf, H. 1985. Genetic Manipulation of Streptomyces: A Laboratory Manual. John Innes Foundation, Norwich, England.
24. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular cloning: A laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.
25. Meletzus, D., Bermpohl, A., Dreier, J., and Eichenlaub, R. 1993. Evidence for plasmid-encoded virulence factors in the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. J. Bacteriol. 175:2131-2136.
26. Meletzus, D., and Eichenlaub, R. 1991. Transformation of the phytopathogenic bacterium *Clavibacter michiganense* subsp. *michiganense* by electroporation and development of a cloning vector. J. Bacteriol. 173:184-190.
27. Mirza, M. S., Rademaker, J. L. W., Janse, J. D., and Akkermans, A. D. L. 1993. Specific 16S ribosomal targeted oligonucleotide probe against *Clavibacter michiganensis* subsp. *sepedonicus*. Can. J. Microbiol. 39:1029-1034.
28. Nemeth, J., Laszlo, E., and Emody, L. 1991. *Clavibacter michiganensis* ssp. *insidiosus* in lucerne seeds. Bull. OEPP 21:713-718.
29. Picard, C., Ponsonnet, C., Paget, E., Nesme, X., and Simonet, P. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. 58:2717-2722.
30. Riley, I. T. 1987. Serological relationships between strains of coryneform bacteria responsible for annual ryegrass toxicity and other plant pathogenic corynebacteria. Int. J. Syst. Bacteriol. 37:153-159.
31. Riley, I. T., Reardon, T. B., and McKay, A. C. 1988. Genetic analysis of plant pathogenic bacteria in the genus *Clavibacter* using allozyme electrophoresis. J. Gen. Microbiol. 134:3025-3030.
32. Rothwell, A. 1968. Bacterial canker of tomato caused by *Corynebacterium michiganense* (Smith) Jensen. Rhod. Agric. J. 1871:75-78.
33. Schaad, N. W., Azad, H., Peet, R. C., and Panopoulos, N. J. 1989. Identification of *Pseudomonas syringae* pv. *phaseolicola* by a DNA hybridization probe. Phytopathology 79:903-907.
34. Schneider, B. J., Zhao, J., and Orser, C. S. 1993. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by DNA amplification. FEMS Microbiol. Let. 109:207-212.
35. Shirakawa, T., Sasaki, T., and Ozaki, K. 1991. Ecology and control of tomato bacterial canker and detection methods of this pathogen. JARQ 25:27-32.
36. Shirako, Y., Vidaver, A. K., and Ackermann, H.-W. 1986. Partial characterization of bacteriophages for *Clavibacter michiganense* subsp. *nebraskense*. Ann. Phytopathol. Soc. Jpn. 52:793-800.
37. Southern, E. 1979. Gel electrophoresis of restriction fragments. Methods Enzymol. 68:153-176.
38. Starr, M. P., Mandel, M., and Murata, N. 1975. The phytopathogenic coryneform bacteria in the light of DNA base composition and DNA-DNA segmental homology. J. Gen. Appl. Microbiol. 21:13-26.
39. Stephens, C. T., Stebbins, T., and Fulbright, D. W. 1988. Detection of *Cmm* using four selective media and Pathoscreen ELISA assay (Agdia). Pages 6-14 in: The Proceedings of the 4th Bacterial Canker Workshop.
40. Strider, D. L. 1969. Bacterial canker of tomato caused by *Corynebacterium michiganense*. A literature review and bibliography. N.C. Agric. Exp. Stn., Tech. Bull. 193.
41. Thompson, E. T. 1986. The toxicity of a number of different bactericides to *Clavibacter michiganense* subsp. *michiganense* (Smith 1910) Jensen 1934 comb. nov. basonym *Corynebacterium michiganense* pv. *michiganense* (AL) and to the tomato plant, *Lycopersicon esculentum*. J. Appl. Bacteriol. 61:427-436.
42. Thompson, E., Leary, J. V., and Chun, W. W. C. 1989. Specific detection of *Clavibacter michiganense* subsp. *michiganense* by a homologous DNA probe. Phytopathology 79:311-314.
43. Tsiantos, J. 1987. Transmission of bacterium *Corynebacterium michiganense* pv. *michiganense* by seeds. J. Phytopathol. 119:142-146.
44. Van Steekelenburg, N. A. M. 1985. Resistance to *Corynebacterium michiganense* in tomato genotypes. Euphytica 34:245-250.
45. Wakimoto, S., Uematsu, T., and Mizukami, T. 1969. Bacterial canker disease of tomato in Japan. 2. Properties of bacteriophages specific for *Corynebacterium michiganense* (Smith) Jensen. Ann. Phytopathol. Soc. Jpn. 35:168-173.