Specific Detection of *Clavibacter michiganense* subsp. *michiganense* by a Homologous DNA Probe

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ABSTRACT

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A chromosomal DNA library for strain 23 of *Clavibacter michiganense* subsp. *michiganense* was constructed in the cosmid vector pLAFR5 and transduced into strain HB101 of *Escherichia coli*. Random clones were selected and screened against several strains of *C. m. michiganense*: *C. michiganense* subsp. *insidiosum, C. rathayi, Curtobacterium flaccumfaciens* pv. *poinsettiae, Rhodococcus fascians, Pseudomonas corrugata, P. syringae* pv. *tomato*, and a variety of other bacteria, including saprophytes isolated from field-grown tomato plants and surrounding weeds. Several clones were identified that hybridized with *C. m. michiganense*, *C. m. insidiosum*, and *C. f. poinsettiae* but not to other bacteria tested. One cosmid clone (pTLC1-44), after digestion with the restriction endonucleases *Eco*RI and *Hind*III, yielded fragments of approximately 13, 11, and 5 kilobases. When the 5-kb fragment was used as a probe, it specifically distinguished *C. m. michiganense* from an avirulent strain and all other bacteria tested. The 5-kb fragment was subcloned into pDSK519 and demonstrated the same specificity.

Additional keywords: bacterial canker, DNA probes.

Clavibacter michiganense subsp. *michiganense*, a seedborne pathogen, is the causal agent of bacterial canker of tomato. This disease can be very destructive if not identified early and controlled. Outbreaks of the disease continue to cause serious problems wherever tomatoes are grown in the United States. A major difficulty in controlling bacterial canker of tomatoes is that the disease is not easy to diagnose. The symptoms are variable and often bear a confusing similarity to spray damage in the early stages of pathogenesis (16). There is often a long latency period before any development of symptoms (3), thus allowing undetected spread of the pathogen in seed beds.

Seed transmission of *C. m. michiganense* is known to occur at levels of less than 1% (7), making detection of the pathogen difficult because of its low frequency of occurrence and the overgrowth of saprophytic seed bacteria during enrichment procedures (20). Methods of diagnosis include serology, cell wall composition, phospholipid composition, metaquinone analyses, phage and bacteriocin sensitivity, and DNA:DNA hybridization (22). A rapid, reliable, and practical identification method has not yet been developed. Techniques such as serology have been either too specific, as with monoclonal antibodies, or found to produce too many cross reactions, as with immunofluorescence tests employing polyclonal antibodies, to be effective in diagnosis (19). Techniques involving biochemical analyses, although highly specific, are too laborious for screening a large number of samples. Phage and bacteriocin assays are sensitive and specific but impractical at this time. Thus, there is "no easy or certain means of identification of these bacteria below the genus level—apart from their ability to cause particular plant diseases" (22). Development of a simple, specific, and reliable diagnostic test would therefore have significant value in disease control.

DNA probes for homologous sequences are quite useful in the identification of animal and human pathogens (8,13). A recent report by Manicom et al (15) described the potential use of random DNA probes in the taxonomy of phytopathogenic fusaria. In the case of bacterial pathogens, differentiation of a particular phytopathogenic bacterium by homologous DNA probes requires DNA specific to that phytopathogenic pathovar, subspecies, etc. Closely related phytopathogens, such as the subspecies of *C. michiganense*, exhibit a large degree of chromosomal DNA/DNA homology (21,22). Plasmids are often associated with particular phenotypes in limited numbers of strains such as drug resistance (12) and heavy metal resistance (1). Therefore, such unique plasmid traits are specific, as frequently are the plasmids coding for those phenotypes. It is also easier to develop specific probes with plasmid DNA, simply because there are fewer random fragments. However, if most or all of the bacteria of interest contain a diverse number of plasmids and there is no particular plasmid(s) common among strains, or if there is no known association of a plasmid with a common phenotype such as pathogenicity or virulence, then chromosomal DNA must be used.

Very little is known about the genetics of the plant pathogenic *Clavibacter* spp. It is thought that several genes mediate pathogenicity (21), but these genes have not been identified. The presence of plasmids in various *Clavibacter* spp. has been reported (8). However, the plasmid content varies among strains and there has been no demonstration of a correlation between the presence of plasmids and phenotype.

This report describes the construction of DNA probes from the chromosomal DNA of *C. m. michiganense* and their use in identifying homologous sequences in pathogenic *C. m. michiganense*.

MATERIALS AND METHODS

Bacterial cultures. The strains of *C. m. michiganense* and the other plant pathogenic bacterial species used in this study are listed in Table 1. All strains were repeatedly tested for their pathogenicity to tomato plants by the cotyledon assay (3,10,19). Forty bacterial saprophytes were isolated from tomato plants and various weeds found in a tomato field in San Diego County, CA on nutrient-dextrose agar (Difco nutrient agar supplemented with D-glucose at 5 g/L). The majority of the saprophytes were identified as coryneform bacteria by their colony morphology, the positive Gram-strain, the production of yellow pigment, and growth characteristics. The single avirulent strain of *C. m. michiganense* utilized was characterized as above and by biochemical tests. It was considered avirulent because of the failure to produce symptoms in the cotyledon assay. Cultures were stored at 4 C in sterile water.

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Single colonies grown on Luria-Bertani (LB) agar (10 g of tryptone, 10 g of sodium chloride, 5 g of yeast extract, and 15 g of Bacto agar per liter of water, pH 7.5) were used to start 25-ml LB broth cultures, which were grown for 18 hr on an orbital shaker at 200 rpm at 25 C.

Plasmid extraction and visualization. The mini-preparation of de Bruijn and Lupski's alkali lysis method (2) was used to isolate plasmids. The DNA was separated by electrophoresis on 0.8% agarose in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (0.04 M Tris-HCl, 0.02 mM acetic acid, 0.001 M EDTA, pH 8) for 1 hr at 10 volts/cm, stained in ethidium bromide (1 μg/ml), and visualized with a Fotodyne 3-300 transilluminator.

Extraction of chromosomal DNA. Single bacterial colonies were inoculated into 500 ml of LB broth and the DNA extracted by sodium dodecyl sulfate (SDS) lysis (18). The DNA was purified on cesium chloride-ethidium bromide density gradients as described by Maniatis et al (14).

Construction of a chromosomal DNA library. All enzyme reactions were carried out according to the manufacturer's specifications. The cosmid vector pLAFR5 (11) was digested with *Bam*HI and *Sca*I. The purified chromosomal DNA from isolate 23 of *C. m. michiganense* (an isolate producing typical bacterial canker symptoms in tomatoes) was partially digested with *Sau*3A, treated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis), and ligated to the digested vector in the presence of T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD). The ligation products were packaged into lambda phage heads (Boehringer Mannheim) and transduced into *Escherichia coli* HB101 cells. The transformed colonies of *E. coli* were selected on LB medium containing tetracycline at 50 μg/ml.

TABLE 1. List of bacterial isolates and their sources

Organism	Isolate no.	Source
Clavibacter m.	1,2,7–43	S. California (W. Chun)
subsp. *michiganense*	3	Nebraska (A. K. Vidaver)
	5,6	Hawaii (W. Chun)
Avirulent *C. m. michiganense*	44	S. Calif. (D. A. Cooksey)
C. m. insidiosum	45	ATCC 10253
Clavibacter rathayi	46	ATCC 13659
	54	ATCC 1082-25
Pseudomonas syringae pv. *tomato*	47	S. Calif. (D. A. Cooksey)
Pseudomonas corrugata	48	ATCC 29736
Xanthomonas campestris	49	Mexico (D. A. Cooksey)
pv. *vesicatoria*	59	S. Calif. (D. A. Cooksey)
Bacillus subtilis	52	S. Calif. (D. A. Cooksey)
Curtobacterium flaccumfaciens subsp. *poinsettiae*	53	ATCC 9682
Rhodococcus fascians	56	ATCC 12975
	57	ATCC 12974
	58	ATCC 13000

Selected colonies were analyzed for the presence of pLAFR5 plus inserts by digesting plasmids isolated by the above miniprep method with *Eco*RI and *Hin*dIII.

Colony hybridizations. Colonies of *E. coli* from the genomic library for *C. michiganense* were either spotted by hand or replica plated onto 0.2 μ Biotrans nylon membranes (ICN Biomedicals, Inc.). The colonies were lysed and the DNA denatured and immobilized onto the filters according to the procedure of Maniatis et al (14). Purified DNA isolated from *C. m. michiganense* and other bacteria was spotted over a vacuum, in 500-ng quantities, onto nylon filters dampened with 6× saline sodium citrate (SSC). The DNA was immobilized in the same way as the DNA derived from the colony spots. Probe DNA was labeled with ^{32}P-dCTP by nick translation (17). Filters were prehybridized as described by Maniatis et al (14), for a minimum of 4 hr. The nick translated probe was boiled for 2 min, added to the DNA hybridization fluid (6× SSC, 0.5% SDS, 10 mM EDTA, 5× Denhardt's solution [14], denatured salmon sperm DNA at 0.1 mg/ml) and agitated overnight at 220 rpm at 65 C. After hybridization, the filters were washed twice in 2× SSC and 0.1% SDS for 15 min at 35 C and 30 C, respectively. The third wash was a high-stringency wash containing 0.1× SSC and 0.1% SDS at 65 C for 10 min. The filters were then exposed at −80 C to X-ray film (Kodak XAR-5) by using intensifier screens.

Colony hybridizations with restriction fragments of clone pTLC1-44. Clone pTLC1-44 DNA was digested with *Eco*RI and *Hin*dIII. The doubly digested DNA was visualized as described previously except that 0.8% low melting point agarose in Crouse salts (6) was used for electrophoresis. The single 5-kb fragment was excised separately, and the 13- and 11-kb fragments were excised together, extracted from the agarose by soaking the agarose slices in water for 2 hr, removing the water, adding a 6× volume of TE buffer, melting at 65 C for 5 min, and then extracting with phenol as described by Maniatis et al (14). The phenol-purified DNA was passed through a Sephadex spun column (14) and precipitated with ethanol. The fragments were nick translated, and colony hybridizations performed as described above.

Dot blot hybridization with plant sap. Two procedures were used to obtain sap from diseased tomato stems for hybridization with radio isotope-labeled pTLC1-44 DNA and the *Hin*dIII/*Eco*RI fragments from pTLC1-44. In the first, 2-cm sections of stem tissue were squeezed until sap was exuded. The sap was collected and 20 μl spotted directly onto nylon filters. In the second, similar sections of stem tissue each were cut longitudinally into eight pieces, which were placed in microfuge tubes and centrifuged for 20 min. The pellet was resuspended in 200 μl of TE buffer, centrifuged for 5 min, resuspended in 100 μl of TE buffer, and 20 μl per spot was applied to the nylon filter.

Bacteria obtained from each treatment were lysed and the DNA fixed to the filter as previously described. In both procedures, 20 μl

TABLE 2. Hybridization of radio isotope-labeled cosmid clones randomly selected from a chromosomal library of *C. m. michiganense* with various bacterial colonies[a]

	Bacterial colonies									
Clones	Cm (42)	Ci (1)	Av (1)	Cfp (1)	Rf (3)	Cr (2)	Pc (1)	Pt (1)	Xv (3)	Bs (1)
pTLC1-44	+	+	+	+	−	−	−	−	−	−
pTLC2-14	+	+	+	+	−	−	−	−	−	−
pTLC3-28	+	+	+	+	−	−	−	−	−	−
pTLC7-7	+	+	+	+	+	−	−	−	−	−
pTLC10-4	+	+	+	+	−	+	+	+	−	−
pTLC10-5	+	+	+	+	−	+	−	−	−	−
pTLC10-23	+	+	+	+	−	+	−	−	−	−
pTLC10-32	+	+	+	+	−	−	−	−	−	−
pTLC1-44 top	+	+	+	+	−	−	−	−	−	−
pTLC1-44 bot	+	−	−	−	−	−	−	−	−	−

[a] Cm = *C. m. michiganense*; Ci = *C. m. insidiosum*; Av = avirulent *C. m. michiganense*; Pc = *Pseudomonas corrugata*; Xv = *Xanthomonas campestris* pv. *vesicatoria*; Cfp = *Curtobacterium flaccumfaciens* subsp. *poinsettiae*; Rf = *Rhodococcus fascians*; Cr = *Clavibacter rathayi*; Pt = *Pseudomonas syringae* pv. *tomato*; Bs = *Bacillus subtilis*. The numbers in parentheses represent the number of strains tested. pTLC1-44 top = the 13- and 11-kb *Eco*RI/*Hin*dIII fragments of clone pTLC1-44; pTLC1-44 bot = the 5-kb *Eco*RI/*Hin*dIII fragment of clone pTLC1-44.

TABLE 3. Hybridization of cosmid clones randomly selected from a chromosomal library of *C. m. michiganense* with colonies of various saprophytic bacteria from tomatoes and weeds[a]

Clones	Saprophytes							
	T5	T6	1.5	2.3	4.5	4.6	4.7	Others
pTLC1-44	+	+	+	+	+	+	+	4
pTLC2-14	−	+	+	−	−	+	−	1
pTLC3-28	+	+	−	+	+	+	+	0
pTLC7-7	−	+	+	+	−	+	−	1
pTLC10-4	−	+	+	+	−	−	+	0
pTLC10-5	−	+	+	+	−	+	+	1
pTLC10-23	+	+	−	+	+	+	+	4
pTLC10-32	−	−	+	+	+	+	+	0
pTLC1-44 top	+	+	+	+	+	+	+	18
pTLC1-44 bot	−	−	−	−	−	−	−	0

[a]The saprophytes most frequently found to share homology with random DNA cosmid clones generated from chromosomal DNA of *C. m. michiganense*. T5 and T6 are bacteria isolated from tomatoes, and 1.5, 2.3, 4.5, 4.6, and 4.7 were isolated from weeds growing adjacent to tomato plants. pTLC1-44 top = the 13- and 11-kb *Eco*RI/*Hin*dIII fragments of clone pTLC1-44; pTLC1-44 bot = the 5-kb *Eco*RI/*Hin*dIII fragment of clone pTLC1-44.

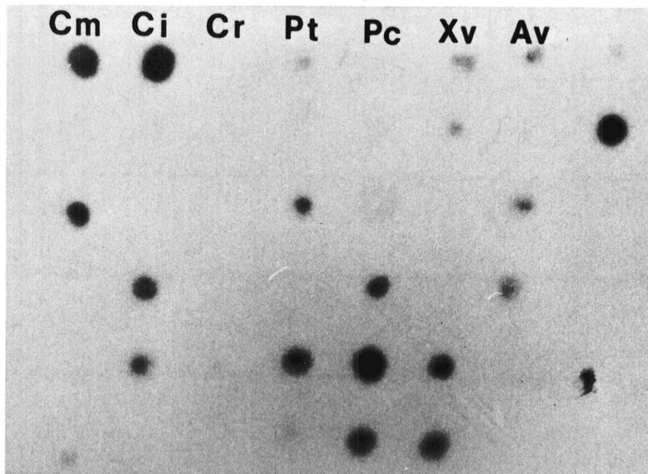

Fig. 1. Autoradiogram of colony blot hybridization with probe pTLC1-44. The headings refer to the spots immediately below them as follows: Cm = pathogenic *C. m. michiganense*; Cr = *C. rathayi*; Pt = *Pseudomonas syringae* pv. *tomato*; Xv = *Xanthomonas campestris* pv. *vesicatoria*; Av = an avirulent strain of *C. m. michiganense*. The remaining blots are 40 saprophytic coryneform isolates from tomato plants and various solanaceous weeds.

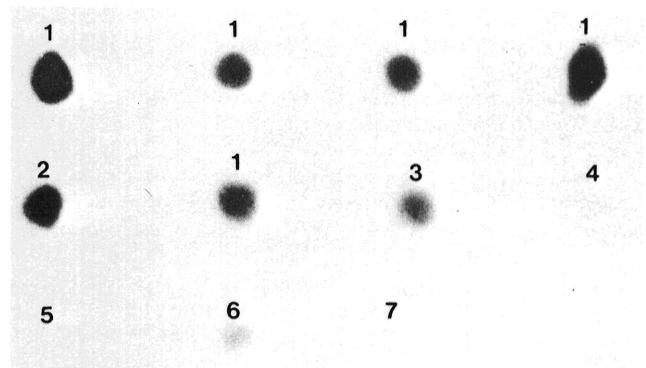

Fig. 2. Autoradiogram of Southern blot hybridization of probe pTLC1-44 with purified DNA of pathogenic *C. m. michiganense* (1), an avirulent *C. m. michiganense* (2), *C. m. insidiosum* (3), *Pseudomonas corrugata* (4), *Pseudomonas putida* (5), *Pseudomonas alcaligenes* (6), and *Xanthomonas campestris* pv. *vesicatoria* (7).

of a dilution series were plated to determine the number of *C. m. michiganense* present in each 20 μl sample.

RESULTS

Detection of plasmid DNA. Plasmids were detected in all strains of *C. m. michiganense* examined. Plasmids detected in *C. m. michiganense* varied in size from 35 to 115 kb. There was no correlation between the presence or absence of common plasmids and the pathogenicity of the various strains.

Screening of the chromosomal library for *C. m. michiganense*. A genomic library was constructed and more than 1,200 tetracycline-resistant colonies were picked. Restriction endonuclease analysis of a random sample of five library clones demonstrated unique restriction fragments, with an average insert size of 24–28 kb.

Colony and Southern blot hybridizations. Eight randomly selected library clones hybridized with colonies of *C. m. michiganense* (Table 2) and purified DNA (data not given). All of the probes hybridized to some degree with the closely related pathogen of alfalfa, *C. m. insidiosum*, as well as to a nonpathogenic strain of *C. m. michiganense* designated Av. Only one clone, pTLC10-4, hybridized with colonies of *P. corrugata* or *P. tomato* (Table 2). None of the clones showed any homology with the purified DNA of tomato pathogens *P. corrugata* and *Xanthomonas vesicatoria* or to colonies of *Bacillus subtilis* (Table 2). All of the clones that were tested hybridized to a closely related pathogen, *Curtobacterium flaccumfaciens* subsp. *poinsettiae* (Table 2), and to at least four out of the 40 tomato and weed saprophytes tested (Table 3). Based on these hybridization studies, clone pTLC1-44 was selected for development as a specific probe of *C. m. michiganense*, because it exhibited the greatest homology to both the colonies (Fig. 1) and the isolated DNA of the pathogen (Fig. 2).

The derivation of the probe pTLC1-44 from chromosomal DNA was verified by Southern blot hybridizations with purified plasmid and chromosomal DNA from strain 23 of *C. m. michiganense*. The probe hybridized with the chromosomal DNA but not with plasmid DNA (data not shown).

Colony hybridizations with restriction fragments of clone pTLC1-44. The 13- and 11-kb fragments of *Eco*RI- and *Hin*dIII-digested clone pTLC1-44 hybridized with colonies and purified DNA from virulent and avirulent *C. m. michiganense* and to DNA from *C. poinsettiae* but not to DNA from any of the other tomato pathogens (Table 2). Hybridization was observed with 25 out of the 40 saprophytic isolates (Table 3). The 5-kb fragment of clone pTLC1-44 was extremely specific and showed homology only to the isolates of *C. m. michiganense* and not to any of the other bacterial pathogens or saprophytes (Tables 2 and 3, Fig. 3).

Dot blot hybridization with plant sap. Each of the procedures used to obtain plant sap containing *C. m. michiganense* yielded approximately 10^6 colony-forming units per 20 μl of sap.

The pTLC1-44 probe and the 13- and 11-kb restriction fragments hybridized with bacterial DNA in infected plant sap and not with sap from uninfected controls. The 5-kb pTLC1-44 fragment produced less intense spots (Table 4) probably because there was approximately 70% less probe DNA than in the other two probes. The effect of the reduced amount of probe DNA can be seen from the data for the amount of DNA each probe was able to detect (Table 4).

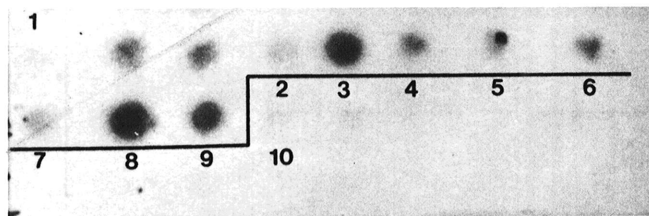

Fig. 3. Autoradiogram of colony blot hybridization of the 5-kb *Eco*RI/*Hin*dIII fragment of probe pTLC1-44 with pathogenic *C. m. michiganense* (1), an avirulent *C. m. michiganense* (2), *C. m. insidiosum* (3), *C. rathayi* (4), *Pseudomonas syringae* pv. *tomato* (5), *Pseudomonas corrugata* (6), *Xanthomonas campestris* pv. *vesicatoria* (7), *Bacillus subtilis* (8), *Curtobacterium flaccumfaciens* subsp. *poinsettiae* (9), and *Rhodococcus fascians* (10).

TABLE 4. Dot blot hybridization of pTLC1-44 and the *Eco*RI/*Hin*dIII fragments as probes against varying concentrations of DNA of *C. m. michiganense* and sap from diseased plants[a]

Probe	DNA concentration detected (ng)	Hybridization with *C. michiganense* in plant sap
pTLC1-44	<100	++
pTLC1-44 top	<100	++
pTLC1-44 bot	100–250	+

[a] pTLC1-44 top = the 13- and 11-kb *Eco*RI/*Hin*dIII fragments of clone pTLC1-44; pTLC1-44 bot = the 5-kb *Eco*RI/*Hin*dIII fragment of clone pTLC1-44. + = weak signal but clearly above background and negative controls; ++ = strong signal.

DISCUSSION

All isolates possessed at least one plasmid, but the yields were very low, suggesting very low copy numbers. These results are similar to the findings of Gross et al (9), who determined that there are only one to two copies of a single plasmid present per cell in isolate 156-2 of *C. m. michiganense*.

The ideal diagnostic probe would share homology with all strains of the bacterium of interest and it should be a fragment of DNA that has a stable association with all strains of the pathogen. This does not appear to be the case with plasmids of *C. m. michiganense*. Unlike other plant pathogenic bacteria investigated, such as *Erwinia stewartii* (5) and various pathovars of *Pseudomonas syringae* (4), there did not appear to be any one plasmid in common among the strains. Thus, chromosomal DNA was used in the construction of the library for *C. m. michiganense*.

Although *C. m. insidiosum* hybridized to virtually all of the probes tested, this was not considered a problem for diagnosis of bacterial canker of tomato because the habitats of the two pathogens do not overlap. Under natural conditions, all of the pathogenic coryneforms except *C. fascians* are host specific (21). More serious was the lack of differentiation between tomato saprophytes, closely associated weed saprophytes, and *C. m. michiganense* (Table 3). The combined 13- and 11-kb fragments (*Eco*RI/*Hin*dIII) from clone pTLC1-44 were extremely specific to *C. m. michiganense* when compared with other pathogens. However, this probe also showed limited homology with some saprophytes. The 5-kb fragment did not hybridize with any of the coryneform saprophytes tested, with the other bacterial genera that are tomato pathogens, with *C. m. insidiosum*, or with the avirulent *C. m. michiganense*, which is nonpathogenic. Therefore, it is extremely specific for DNA from *C. m. michiganense*, which is pathogenic to tomato.

Avirulent strains of *C. m. michiganense* appear to be extremely rare. Chaldecott (3) screened a large number of strains isolated worldwide and never found an avirulent strain. However, he did find differences in the level of virulence. This raises the question of whether the "avirulent" strain we examined is truly *C. m. michiganense* or some other closely related bacterium. The important point, however, is that the probe described here is specific for *C. m. michiganense* and differentiates between *C. m. michiganense* and saprophytes associated with tomato plants. It should therefore be possible to develop a rapid diagnostic method by using this DNA probe for the identification of *C. michiganense*. The 5-kb fragment of pTLC1-44 has been subcloned into pDSK519 (11) and is currently being tested with infected tomato seed and plant tissue.

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