

# Transmission Frequency of *Clavibacter michiganensis* subsp. *insidiosus* to Alfalfa Seed and Identification of the Bacterium by PCR

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## ABSTRACT

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A sensitive polymerase chain reaction (PCR)-based identification method was developed for *Clavibacter michiganensis* subsp. *insidiosus*, the causal agent of alfalfa bacterial wilt. The method, which targets a high-copy-number insertion element, is rapid and specific for this plant pathogen. The assay was used to determine the frequency of transmission of the pathogen to alfalfa seed. Seed was produced from infected plants grown and pollinated in the greenhouse, from infected plants grown in the field and transplanted to the greenhouse to produce seed, and from diseased 2-year-old field-grown plants. Seed from each infected plant were assayed to identify infected seed lots. Seed were ground to a fine powder and soaked in a liquid medium, after which a portion of the seed slurry was plated on a semi-selective agar medium. The PCR assay was used to identify *C. michiganensis* subsp. *insidiosus* colonies on plates. Approximately 6.3 to 7.7% of diseased plants transmitted *C. michiganensis* subsp. *insidiosus* to seed. In assays in which individual seed were analyzed from infected seed lots, approximately 2.5 to 8.7% of seed contained the bacterium.

Additional keywords: bacterial wilt, *Medicago sativa*, polymerase chain reaction, seed-borne pathogen

Bacterial wilt of alfalfa (*Medicago sativa* L.), caused by the gram-positive coryneform bacterium *Clavibacter michiganensis* subsp. *insidiosus*, is found in most alfalfa-growing areas in North America. This disease was recognized very early as a major limiting factor to alfalfa production (16,18) and was the focus of the first disease-resistance breeding programs in alfalfa. Resistance to bacterial wilt is incorporated into practically all alfalfa varieties

currently grown in North America. Due to the genetic heterogeneity of alfalfa, generally 50 to 75% of the plants in a "resistant variety" will exhibit resistance to the disease. Infection occurs through wounds such as those made by mowing, freezing and thawing, and feeding by insects and nematodes (17,21). The progress of the disease is greatest at moderate temperatures and in soils with abundant moisture (18,19,28). Bacteria multiply in xylem vessels and eventually cause wilting, stunting, and leaf yellowing. Stems of infected plants proliferate, become spindly, and recover slowly after mowing (18). Diseased plants produce relatively few seed (6,18). Roots of infected plants have a pale yellow to brown discoloration in the stele. However, in field-grown plants, symptoms of the disease do not become evident or severe until the second or third year after infection (18,19). Resistance to the disease is associated with vascular morphology (3,28).

The bacterium can persist in dried plant material for 8 to 10 years and can be recovered from seed from diseased plants (6). In histological studies, Cormack and Moffatt (7) observed *C. michiganensis* subsp. *insidiosus* in varying numbers in the vascular system of the floral rachis and pedicels. Bacteria were not found in flowers but were found in the vascular tissue of several seed pods. Bacteria were infrequent in seed and confined to the aleurone layer

of the endosperm. The occurrence of seed transmission resulted in the creation of phytosanitary regulations in a number of countries; however, the frequency of seed transmission in seed production fields is unknown.

In pure culture, *C. michiganensis* subsp. *insidiosus* grows slowly as fluidal colonies with a pale yellow to gray color; older colonies often develop a dark blue pigment. However, fluidity and pigmentation are unstable characteristics in culture, and nonfluidal and non-pigmented pathogenic isolates can be recovered from diseased plants (13,27). A rapid, sensitive, and specific test is therefore required to positively identify *C. michiganensis* subsp. *insidiosus* cultured from plant material. Detection techniques have been developed for the closely related bacteria *C. michiganensis* subsp. *michiganensis* (8,29) and *C. michiganensis* subsp. *sepedonicus* (10,15,22,29,35) based on DNA amplification using the polymerase chain reaction (PCR). The methods developed by one of the present authors (26) targets a multicopy insertion (IS) element, IS1121 (25). A total of approximately 50 copies of this element is found in the genome of the potato ring rot pathogen (39). Of more than 40 species of microbes tested, a related IS element was found only in *C. michiganensis* subsp. *insidiosus*. The genome of the alfalfa wilt pathogen was shown to contain approximately 33 copies of an element termed IS1122. These two IS elements exhibit approximately 88% sequence identity, and each has a size of 1,078 bp (39). Because of the limited distribution of this class of elements, a localized region within the IS elements of greater sequence divergence and relatively low GC content that is easily amplified represents an attractive target for a PCR-based detection method for *C. michiganensis* subsp. *insidiosus*. The objectives of this study were to develop tests for identifying *C. michiganensis* subsp. *insidiosus* isolated from plant material on a semi-selective medium and to determine the frequency of transmission of the pathogen to seed in greenhouse-grown and field-grown plants. A preliminary report of this research has been published (32).

## MATERIALS AND METHODS

**Microbial culture and DNA isolation.** For development of the PCR procedure, *C. michiganensis* spp. were grown in NBY

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broth (38) at 22 to 24°C with gyratory shaking (175 rpm). Other organisms were grown in a similar manner in nutrient-broth yeast extract (NBY) or in media recommended by the American Type Culture Collection. Bacterial DNA was isolated by the method of Comai and Kosuge (5).

**Polymerase chain reaction.** Amplification of target sequences present in purified bacterial DNA or bacterial cells was performed with primers CIRS-1 (5'-TTCAACCGCACCTCGCGAC-3') and CIRS-2 (5'-CGTCAGCCCGTGGCTCGA-GT-3'), purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Most of the other reagents were components of the GeneAmp kit of Applied Biosystems (Foster City, CA). The PCR reaction contained the primers (1 µM each), four dNTPs (200 µM each), 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin, 5% acetamide, 2.5 units *Taq* DNA polymerase, and template DNA in a total volume of 30 µl. Reactions were prepared in a laminar flow hood, using aerosol barrier pipette tips. Chill-out 14 wax (MJ Research, Cambridge, MA) was used to prevent evaporation of water during amplification. PCR was conducted in a thermal cycler (Model 480, Perkin-Elmer Cetus, Foster City, CA) for 30 cycles with the following parameters: denaturation at 94°C, 2 min (first 4 cycles) or 1 min (last 26 cycles); annealing at 58°C, 1 min; and elongation at 75°C, 1 min (29 cycles) or 5 min (last cycle). The method of Rychlik et al. (31) was used for selection of the annealing temperature during thermal cycling.

**Analysis of PCR products.** Amplification products were analyzed by electrophoresis and by hybridization of DNA blots. A portion (20 µl) of the amplified mixture was loaded on a horizontal gel composed of 3% NuSieve 3:1 agarose (FMC, Rockland, ME). *Hae*III restriction fragments of phage φX174 DNA (Life Technologies, Gaithersburg, MD) were used as molecular weight standards. Electrophoresis was carried out for 30 min at 150 V in Tris-borate-EDTA (TBE) buffer (24). The presence of a 132-bp PCR product was detected by visual examination of a photograph of the ethidium bromide-stained gel or by densitometry of the photographic negative. To verify that the observed fragment was an amplification product of *C. michiganensis* subsp. *insidiosus* DNA, standard protocols (33) were used to transfer the DNA to a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA), prepare a 5'-<sup>32</sup>P-labeled oligonucleotide probe, and conduct the hybridization and washing of the membrane. The probe, CIRS-3 (5'-GCCAGAACCGACGCCCT-TGATCCGTGGA-3'), is an *IS1122*-specific sequence. Autoradiography was performed for an interval sufficient to provide a strong signal with positive control samples on the membrane.

**Plant culture and inoculation.** To generate infected seed lots, alfalfa seed was produced from plants grown in three ways: diseased plants grown and pollinated in the greenhouse, diseased plants grown in the field and transplanted to the greenhouse to produce seed, and diseased 2-year-old plants grown and pollinated in the field. For all experiments, alfalfa seed scarified with sandpaper were sown in steam-sterilized sand benches and the resulting plants were grown in the greenhouse for 8 weeks. Plants were lifted from the sand benches and roots were washed and then inoculated by soaking for 30 min in a suspension of ground infected root material (50 g/liter). The inoculum was derived from plants inoculated in a similar manner and displaying symptoms of bacterial wilt.

For production of seed from greenhouse-grown plants, plants of the bacterial wilt-susceptible cultivar Narragansett were inoculated as described above and transplanted into 15-cm pots containing steamed sand:soil (1:1, vol/vol). Plants were grown in the greenhouse for 12 weeks and then upper stem sections (4 stems/plant) were assayed for the presence of *C. michiganensis* subsp. *insidiosus*. Plants positive for the presence of the bacterium in stem sections were cross-pollinated by hand and mature seed collected. From each plant, duplicate samples of approximately 250 seed (0.5 g), representing approximately half of the seed produced from each plant, were ground and plated (see below) to identify infected seed lots. Individual seed (200) were assayed from infected seed lots to determine the frequency of transmission of the pathogen to seed.

For production of seed from field-grown plants transferred to the greenhouse, three germ plasms varying in fall dormancy were used: Turkestan (nondormant), Flemish (moderate dormancy), and Narragansett (dormant). After inoculation, roots and shoots were trimmed and plants were transplanted to the field at the University of Minnesota Experiment Station, Rosemount, Minnesota. After 16 weeks, a subset of the plants were dug from the field and rated for symptoms of bacterial wilt on a 0 to 5 scale, (where 0 = no symptoms; 1 = small dark strands in the stele; 2 = discoloration up to one-third of the stele; 3 = nearly entire stele discolored, cortex white; 4 = discoloration throughout stele and cortex; and 5 = plant dead) by sectioning roots (12). Plants were potted in 15-cm pots containing steamed sand:soil (1:1, vol/vol) and grown in the greenhouse. Resistant plants, rated 0 or 1, were cross-pollinated by hand within each germ plasm, as were susceptible plants rated 2 or 3. After seed pods were removed, 4 seed-bearing stems from each plant were assayed for the presence of *C. michiganensis* subsp. *insidiosus* (see below). The presence of the pathogen in seed was assayed

by grinding all seed harvested from individual racemes in lots of 3 to 20 seed, soaking disrupted seed in 0.5 ml of YEP medium (10 g yeast extract, 5 g NaCl, and 10 g peptone per liter), and spreading 100 µl of liquid from the seed slurry on duplicate plates of TBY medium (23) (10 g tryptone, 5 g NaCl, 5 g yeast extract, and 15 g agar per liter amended after autoclaving with 25 ml 20% glucose) with antibiotics. The antibiotics included to suppress other seed-associated microorganisms were: 200 mg cycloheximide, 0.4 mg tobramycin, 10 mg nalidixic acid, and 12.5 mg polymyxin B sulfate (7600 IU/mg) per liter (J. W. Sheppard, *personal communication*).

For production of seed in the field, plants not removed to the greenhouse, as described above, were allowed to set seed the following summer. Once seed had matured, plants were dug and scored for bacterial wilt symptoms by sectioning roots, and seed was collected from 50 diseased plants. From 26 plants with disease ratings of 2, 3, or 4, duplicate samples of 0.5 g surface-sterilized seed (approximately 250) were ground, and 100 µl of the seed slurry and two serial 10-fold dilutions were plated on duplicate plates of TBY with antibiotics to identify infected seed lots. Individual surface-sterilized seed (200) were assayed from infected seed lots to determine the frequency of transmission of the pathogen to seed. From 24 plants with seed lots of less than 1 g, seed was pooled together, a portion was surface sterilized, and 400 individual seed assayed for the bacterium. For surface sterilization, seed were immersed in 70% ethanol for 2 min and in a 10% bleach solution (0.525% sodium hypochlorite) for 10 min with gentle shaking at room temperature, then rinsed three times with sterile distilled water and dried in a sterile petri dish in a laminar flow hood.

**Isolation of *C. michiganensis* subsp. *insidiosus* from stems and seed.** To assay stems for the presence of *C. michiganensis* subsp. *insidiosus*, a 10-cm-long stem section from each stem tested was stripped of leaves, cut into 1- to 3-mm pieces with a sterile scalpel, and soaked in YEP medium, 1 ml/stem section, for 30 min with gentle shaking at room temperature. Aliquots of 100 µl of the stock material and two serial 10-fold dilutions were plated in duplicate on TBY plates (23) containing antibiotics. Plates were enclosed in clear plastic boxes and incubated at room temperature (22 to 24°C) for 14 days.

The presence of *C. michiganensis* subsp. *insidiosus* in seed was assayed by grinding seed to a fine powder with a sterilized mortar and pestle. The disrupted seed were soaked in YEP, 1 ml/0.1 g seed, for 30 min with gentle shaking at room temperature. Duplicate plates of TBY plus antibiotics were spread with 100 µl of the seed slurry and two serial 10-fold dilutions. Individual

seed were assayed by crushing single seeds between plastic wrap and sterile weighing paper with a large pestle and suspending the crushed seed in 20  $\mu$ l YEP for 30 min. Liquid from each slurry was spotted on plates of TBV with antibiotics. Plates were placed in clear plastic boxes and incubated at room temperature for 14 days.

Colonies arising on plates were tested by the KOH test for Gram-stain differentiation (36). Colonies with a negative reaction (Gram positive) were assayed for PCR amplification of the 132-bp portion of IS1122 using oligonucleotide primers CIRS-1 and CIRS-2. Using a sterile plastic

pipette tip, a very small amount of each colony was removed from the plate and swirled into 10  $\mu$ l sterile water in a PCR tube. For each sample, 20  $\mu$ l of the PCR reaction mix was added to the bacterial cell suspension. The final concentration of components in each reaction was 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 1  $\mu$ M each primer, and 1 unit *Taq* polymerase. Reactions were overlaid with mineral oil and amplified using a Coy TempCycler II programmed with an initial incubation at 80°C for 1 min followed by four cycles of 94°C for 2 min, 58°C for 1 min, and 75°C for 1

min. Twenty-five additional cycles of 94°C for 1 min, 58°C for 1 min, and 75°C for 1 min were performed, after which the reactions were held at 4°C. Positive controls of *C. michiganensis* subsp. *insidiosus* DNA (1 pg/30  $\mu$ l reaction) and bacterial cells were included with each set of reactions. Negative controls included reactions with sterile water alone and with *Escherichia coli* cells. From each reaction, 15  $\mu$ l was analyzed by electrophoresis in a 3% agarose gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0). After electrophoresis the gel was stained with ethidium bromide, bands visualized with UV light, and photographed. Lanes containing a 132-bp band were scored as positive for *C. michiganensis* subsp. *insidiosus*.

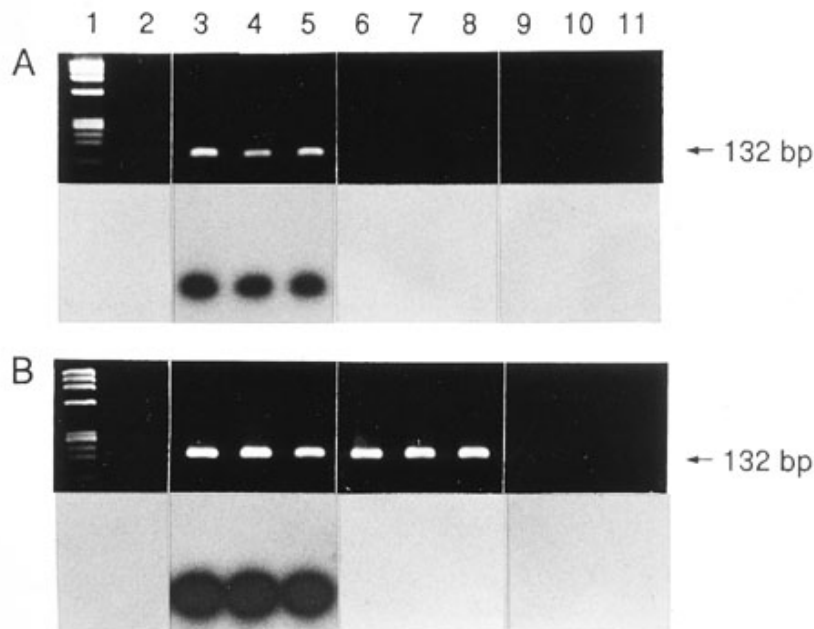
## RESULTS

**PCR detection system for *C. michiganensis* subsp. *insidiosus*.** PCR methods based on amplification of IS elements are well-established approaches to detection of microbial pathogens (1,9), and the high copy number of IS1122 makes this genetic element a desirable target for the detection of *C. michiganensis* subsp. *insidiosus*. However, much of this sequence is shared with IS1121, the related insertion element of *C. michiganensis* subsp. *sepidonicus*. In order to develop a detection system specific for the alfalfa wilt pathogen, the sequences of these two elements, GenBank accession numbers AF079817 and AF079818 (39), were compared to identify regions that have divergent sequence. Because amplification is inefficient with GC-rich target sequences that tend to form stable secondary structures (20), the IS1122 sequence was also examined to detect localized regions of reduced GC content. The sequence selected for amplification is shown in Figure 1. The 132-bp region has a GC content of 61%, compared to a value of 67% for the full sequence of IS1122. Insertion elements IS1122 and IS1121 differ by a total of three bases in the regions selected as PCR primers, and 10 of the 28 bases are different in the amplified region chosen for the hybridization probe.

Specificity of the amplification and hybridization steps of the IS1122-based detection method is demonstrated in Figure 2. Amplification performed with 0.5 pg (approximately 100 cell equivalents) of template DNA from three different strains of *C. michiganensis* subsp. *insidiosus* yielded the expected 132-bp product (Fig. 2A, lanes 3 to 5). A barely detectable amount of an amplification product was observed with this quantity of template from strains of *C. michiganensis* subsp. *sepidonicus* (Fig. 2A, lanes 6 to 8), but a larger quantity of product was formed when the amount of template was increased 2,000-fold (Fig. 2B, lanes 6 to 8). Densitometric analysis indicated that the amount of product from 0.5 pg of *C. michiganensis* subsp. *sepidonicus* DNA

	910	920	930	940	950
IS1121	TTCAACCG	GACCCTCGCG	ACCGAGTGGG	CCTACCGGCA	ACCCTTCACC
(COMMON)	TTCAACCG	*ACCCTCGCG	ACCGAGTGGG	CCTACCGGCA	ACCCTTCACC
IS1122	<u>TTCAACCG</u>	<u>CACCCTCGCG</u>	<u>ACCGAGTGGG</u>	<u>CCTACCGGCA</u>	<u>ACCCTTCACC</u>
	CIRS-1				
	960	970	980	990	1000
IS1121	AGCAACCAAC	ACCGGGCCGA	CGCGCTTGAC	CCCTTCATCG	AGCACTACAA
(COMMON)	AGCAACCAA*	*C*G**CCGA	CG*CTTGA*	CC*T**ATCG	AGCACTACAA
IS1122	<u>AGCAACCAAG</u>	<u>CCAGAACC GA</u>	<u>CGCCCTTGAT</u>	<u>CCGTGGATCG</u>	<u>AGCACTACAA</u>
	CIRS-3				
	1010	1020	1030		
IS1121	CACTGAACGA	ATCCACTCAA	GCCACGGGCT	CACG	
(COMMON)	CACTGAACGA	ATCCACTC*A	GCCACGGGCT	*ACG	
IS1122	CACTGAACGA	<u>ATCCACTCGA</u>	<u>GCCACGGGCT</u>	<u>GACG</u>	
	CIRS-2				

**Fig. 1.** Sequence of the amplified region of IS1122 and its counterpart in IS1121. The common sequence displays nucleotides that are identical and different (\*) in the two insertion elements. The numerals represent positions in the 1078-base pair sequence.



**Fig. 2.** Specificity of the amplification and hybridization steps of the detection method for *Clavibacter michiganensis* subsp. *insidiosus*. Experimental samples contained (A) 0.5 pg of DNA template from the sources indicated, and (B) 1 ng of DNA template. In each panel, the upper portion is a photograph of the ethidium bromide-stained agarose gel containing the polymerase chain reaction product, and the lower portion is the corresponding autoradiogram of the DNA blot probed with <sup>32</sup>P-labeled CIRS-3 oligomer. Lane 1, size standards (*Hae*III restriction fragments of  $\phi$ X174 DNA); lane 2, no-template control; lanes 3-5, DNA from *C. michiganensis* subsp. *insidiosus* strains 239, 10253, and 33114; lanes 6-8, DNA from *C. michiganensis* subsp. *sepidonicus* strains 3R, 33113, and 106; and lanes 9-11, DNA from *C. michiganensis* subsp. *michiganensis* 4450, *C. michiganensis* subsp. *nebraskensis* 3294, and *C. michiganensis* subsp. *tessellarius* 7221, respectively.

was approximately 5% of that obtained from *C. michiganensis* subsp. *insidiosus* DNA. However, these IS1121-derived products from *C. michiganensis* subsp. *sepidonicus* failed to hybridize with <sup>32</sup>P-labeled CIRS-3 (Fig. 2B, lanes 6 to 8). No amplification product was observed for the tested strains of other subspecies of *C. michiganensis*: *michiganensis*, *nebraskensis*, and *tessellarius* (lanes 9 to 11). Further studies with DNA templates from uninoculated alfalfa and 39 other microbes, including another species of *Clavibacter*, other plant- and soil-associated bacteria, and many organisms with GC-rich genomes, indicated that the method is selective for *C. michiganensis* subsp. *insidiosus*. The amplification procedure was performed with 1 ng purified template DNA (approximately 10<sup>5</sup> bacterial cells). Other organisms that tested negative were Alfalfa cv. Regen SY, Alfalfa cv. Saranac, *Agrobacterium tumefaciens*, *Agromyces ramosus*, *Arthrobacter ilicis*, *A. globiformis*, *Aurobacterium testaceum*, *Bacillus cereus*, *B. subtilis*, *Bordetella pertussis*, *Brevibacterium casei*, *Clavibacter xyli cynodontis*, *Corynebacterium glutamicum*, *C. pseudodiphtheriticum*, *Curtobacterium flaccumfaciens* subsp. *betae*, *C. flaccumfaciens* subsp. *flaccumfaciens*, *C. flaccumfaciens* subsp. *oortii*, *C. flaccumfaciens* subsp. *pointsettiae*, *Erwinia carotovora* subsp. *amylovora*, *E. carotovora* subsp. *carotovora*, *E. herbicola*, *E. stewartii*, *Escherichia coli*, *Microbacterium lacticum*, *Micrococcus luteus*, *Mycobacterium phlei*, *Pseudomonas aurantiaca*, *P. solanacearum*, *Rathayibacter iranicus*, *R. rathayi*, *R. toxicus*, *R. tritici*, *Rhizobium phasioli*, *Rhodococcus fascians*, *Salmonella typhimurium*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Streptococcus lactis*, *Streptomyces griseus*, *S. scabies*, and *Thermus aquaticus*.

Results of experiments performed to assess the sensitivity of the method with three different strains of *C. michiganensis* subsp. *insidiosus* are presented in Figure 3. The dose-response curve was similar for all three strains, and the lower limit of detection was approximately 2 to 4 cells.

#### Transmission frequency of *C. michiganensis* subsp. *insidiosus* to alfalfa seed.

Seed was collected from 16 Narragansett plants inoculated, grown, and intercrossed in the greenhouse. Bacteria with the colony morphology of *C. michiganensis* subsp. *insidiosus* were recovered on plates from the seed-producing stems of all plants. PCR amplification from these isolates resulted in production of the 132-bp band diagnostic of IS1122 from *C. michiganensis* subsp. *insidiosus*. PCR-positive isolates from the 16 plants were used to inoculate alfalfa seedlings and all isolates incited symptoms of bacterial wilt. A number of white, fluidal, KOH-negative, rapidly growing bacteria were also isolated from stem tissues on antibiotic-amended TB. These bacteria did not produce the 132-bp

band after PCR amplification and did not incite symptoms of bacterial wilt. Bacterial colonies identified as *C. michiganensis* subsp. *insidiosus*, using the PCR assay, were obtained on plates spread with seed extracts from 1 plant. From this plant, 5 out of 200 individual seeds that were ground and plated contained *C. michiganensis* subsp. *insidiosus*. In this experiment, transmission of the bacterium to seed occurred in the greenhouse in 1 of 16 diseased plants (6.3%), with approximately 2.5% of seed from the one plant containing *C. michiganensis* subsp. *insidiosus*. Based on seed tested from the 16 infected plants (approximately 8,200 seed), transmission of *C. michiganensis* subsp. *insidiosus* to seed occurred in approximately 0.21% of seed.

From inoculated field-grown plants, 108 plants were successfully transplanted to soil in the greenhouse. Seed were produced from resistant (rated 0 or 1) and susceptible plants (rated 2 or 3) within each germ plasm. Stems from all plants, 4 stems/plant, were assayed for the presence of *C. michiganensis* subsp. *insidiosus* after seed were harvested. A number of plants initially rated as resistant contained the bacterium in seed-bearing stems (Table 1). A number of susceptible plants with initial disease ratings of 2, showing discoloration in up to one-third of the stele, did not contain *C. michiganensis* subsp. *insidiosus* in seed-bearing stems. All plants rated as 3, with nearly the entire stele discolored, contained the pathogen in upper stem sec-

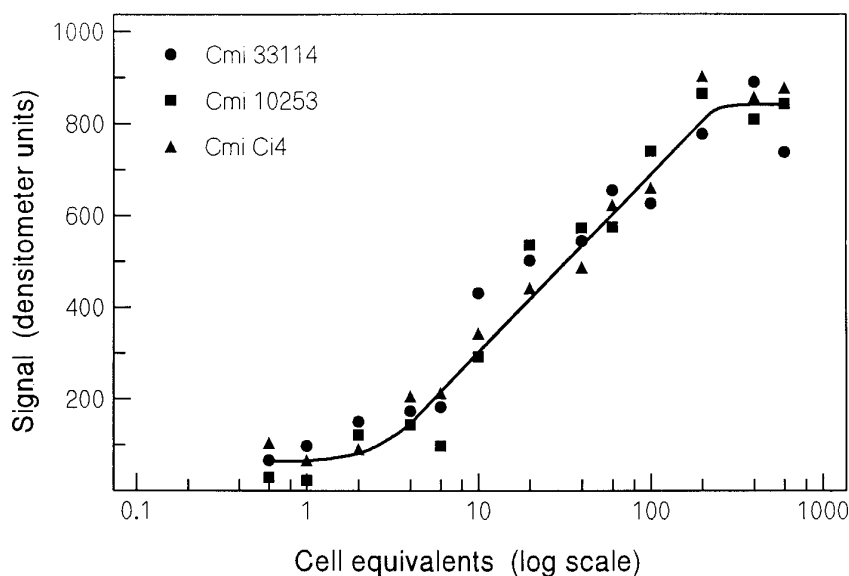


Fig. 3. Sensitivity of detection of three strains of *Clavibacter michiganensis* subsp. *insidiosus* by the polymerase chain reaction (PCR) method. Cells of strains 33114, 10253, and Ci4 were diluted into a solution containing 10 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, and 15% acetamide, and portions of the solutions were subjected to the PCR protocol. Negatives from photographs of the ethidium bromide-stained gels were scanned in a laser densitometer to quantify the amount of 132-base pair amplification product formed at each level of pathogen.

Table 1. Percentage of plants positive for *Clavibacter michiganensis* subsp. *insidiosus* and proportion of healthy and infected plants producing seed

Germplasm	Rating <sup>a</sup>	No. plants	Infected (%)	Proportion with seed	
				Healthy	Infected
Turkestan	0	5	0	3/5	NA <sup>b</sup>
	1	17	24	10/13	3/4
	2	17	41	8/10	5/7
	3	6	100	NA	1/6
Flemish	0	5	60	2/2	2/3
	1	20	25	14/15	4/5
	2	7	71	2/2	4/5
	3	5	100	NA	2/5
Narragansett	0	9	33	5/6	3/3
	1	5	20	4/4	1/1
	2	7	71	1/2	2/5
	3	5	100	NA	2/5

<sup>a</sup> Rating scale: 0 = no symptoms; 1 = small dark strands in the stele; 2 = discoloration up to one-third of the stele; 3 = nearly entire stele discolored, cortex white; 4 = discoloration throughout stele and cortex; and 5 = plant dead.

<sup>b</sup> NA = not applicable.

tions. Bacterial colonies testing positive in the PCR assay were mucoid, pale yellow with some colonies developing blue granules upon long incubation. PCR-positive, non-mucoid, blue-pigmented colonies were isolated from one plant. From colonies positive by PCR, 28 were chosen to inoculate alfalfa plants and all caused symptoms of bacterial wilt.

Seed was produced from 49 plants lacking the bacterium and 29 infected plants. Of plants lacking *C. michiganensis* subsp. *insidiosus*, 83% produced seed, while only 59% of the diseased plants produced seed. Most plants that did not produce seed had the higher disease ratings (Table 1). The average number of seed produced per plant and average seed weight were reduced in infected plants compared to healthy plants (Table 2) but these differences were significant (*t* test) only for seed produced from Turkestan plants.

To determine the distribution of infected seed produced by a diseased plant, seed harvested from individual racemes were assayed in lots of 3 to 20 seeds. From the 29 infected plants producing seed, colonies of *C. michiganensis* subsp. *insidiosus* were recovered from seed of 2 plants, or approximately 6.9% of diseased plants. There was no correlation between transmission of bacteria to seed and fall dormancy rating. No *C. michiganensis* subsp. *insidiosus* colonies were recovered from seed assayed in the same manner from the 49 healthy plants. The distribution of infected seed from the first plant (Naragansett 1-3) was highly localized. The pathogen was recovered from seed harvested in 1 out of 5 racemes on 1 stem. This raceme contained 88 seeds; 15 seeds were assayed on the plate containing colonies of *C. michiganensis* subsp. *insidiosus*. Thus, 1 to 15 of the total 286 seeds from this plant, or 0.35 to 5.2%, were infected. From the second plant (Turkestan 3-1), colonies identified as *C. michiganensis* subsp. *insidiosus* were recovered from seed harvested from 4 out of 19 racemes on 2 stems. Plates positive for *C. michiganensis* subsp. *insidiosus* had been spread with a seed slurry from 3, 14, 15, and 20 seeds from each of the four positive racemes. From a total of 180 seeds produced by this plant, possibly 4 to 52 seeds, or approximately 2.2 to 29%, were infected. Because all seed were assayed in this experiment, it was not possible to ascertain a more precise transmission rate in these two infected plants. Based on all seed produced from infected plants in this experiment (approximately 4,232 seeds), 0.12 to 1.6% of seed from infected plants contained the bacterium. The lower transmission rate would occur if only one seed in each group plated (5 positive groups) was infected, and the higher rate would occur if all seed tested in each group (a total of 67 seeds) were infected.

To compare transmission of the pathogen to seed in greenhouse- and field-grown

plants, seed were harvested from 50 plants with disease ratings of 2, 3, or 4 and foliar symptoms of bacterial wilt that had been inoculated with *C. michiganensis* subsp. *insidiosus* and grown in the field for 2 years at Rosemount, Minnesota. From 26 plants, duplicate seed samples of 0.5 g were assayed by grinding and plating on TBY medium amended with antibiotics. Seed from two plants, or approximately 7.7% of infected plants, contained bacteria identified by the PCR assay as *C. michiganensis* subsp. *insidiosus*. From each of these two plants, 200 individual seeds were ground and plated. The bacterium was recovered from 8.7% of seed from one plant and 6.1% from the other. Based on the seed assayed from diseased plants (approximately 13,400), and the estimated number of infected seed in the two positive seed lots, 44 and 31, respectively, the pathogen was transmitted to approximately 0.55% of seed from diseased plants. The remaining 24 plants had seed lots of less than 1 g, so seed was pooled, and 400 individual seed were assayed. Bacteria identified as subsp. *insidiosus* by the PCR assay were recovered from 3.5% of pooled seed.

In summary, from the three sources of infected plants, greenhouse-grown, field-grown plants transplanted to the greenhouse for seed production, and field-grown plants, *C. michiganensis* subsp. *insidiosus* was recovered from seed of 6.3, 6.9, and 7.7% of infected plants, respectively. In assays in which individual seed were analyzed from diseased plants that transmitted the bacterium to seed, 2.5 to 8.7% of seed contained *C. michiganensis* subsp. *insidiosus*. From all infected greenhouse-grown and field-grown plants, an estimated 0.21 to 0.55% of seed contained the pathogen. There was no correlation between seed size (shriveled or plump) or seed color and the presence of the pathogen.

## DISCUSSION

The PCR-based method described for identifying *C. michiganensis* subsp. *insidiosus* is very rapid, sensitive, and specific. In addition to standard components, the reaction mixture contains 5% acetamide to aid in denaturation of GC-rich DNA and unfolding of regions of denatured DNA with secondary structure (20,26,30). This organic analog of urea also aids in cell disruption, and it is not

necessary to isolate bacterial DNA or pre-treat samples before PCR. The procedure is capable of detecting less than 5 pathogen cells in a reaction mixture. The high sensitivity of the assay reflects the presence of multiple copies of the target *IS1122* sequence in the pathogen. More than 30 copies of this 1.1-kbp sequence exist in the genome of *C. michiganensis* subsp. *insidiosus*. Insertion sequences are potentially mobile genetic elements that can give rise to transpositions of DNA sequences in prokaryotes (14), and presumably *IS1122* may function in this capacity in *C. michiganensis* subsp. *insidiosus*. No evidence exists for a relationship between this repeated sequence and pathogenicity. *IS1121*, the related element in *C. michiganensis* subsp. *sepidonicus*, is present in bacterial strains that vary widely in virulence (26). Although *IS1121* and *IS1122* have an overall sequence homology of 88%, regions of significant diversity exist. It is from these regions that oligomers for the PCR-hybridization-based detection of *C. michiganensis* subsp. *insidiosus* were designed. The primers used in this assay (CIRS-1 and CIRS-2) amplify a 132-bp region of the repeated element. A small amount of an identically-sized product is formed when these primers are used in a reaction with a large quantity of purified DNA from *C. michiganensis* subsp. *sepidonicus*, but the two products can be distinguished by use of CIRS-3, a subspecies-specific oligonucleotide hybridization probe that binds to the amplified region. The selectivity of the PCR phase of the method for *C. michiganensis* subsp. *insidiosus* versus *C. michiganensis* subsp. *sepidonicus* can be increased by raising the annealing temperature, but this alteration in the thermal cycling protocol also causes some loss of sensitivity due to reduced amplification efficiency (A. E. Oleson, unpublished). No PCR product was detected with a wide variety of other plant- or soil-associated bacteria or with alfalfa DNA.

The titers of *C. michiganensis* subsp. *insidiosus* in infected alfalfa foliage and seed are too low for routine detection solely by the PCR method described here. The pathogen could be detected in two of four infected plants if the tissue was suspended in buffer containing acetamide and subjected to vigorous agitation with glass beads in a micro-scale cell disrupter, but this technique did

**Table 2.** Mean number of seed produced per plant and average seed weight for healthy and infected plants from three germplasms

Germplasm	Disease rating	Mean seed number	Mean seed weight (mg)
Turkestan	Infected	155.3 <sup>a*</sup>	1.78 <sup>**</sup>
Turkestan	Healthy	248.1	2.18
Flemish	Infected	128.4	1.87
Flemish	Healthy	193.6	1.98
Naragansett	Infected	203.1	1.59
Naragansett	Healthy	532.8	2.03

<sup>a</sup> \* and <sup>\*\*</sup> indicate significant difference between healthy and infected plants within a germplasm at the 5 and 1% probability levels, respectively.

not allow formation of a PCR product when tested with infected lots of alfalfa seed.

Transmission of *C. michiganensis* subsp. *insidiosus* from infected plants to seed occurred at a low frequency, with a few infected plants producing a limited number of infected seed. Although the pathogen was isolated from stems of infected plants before and after seed production, the bacterium was only rarely recovered from seed. Infected seed were localized to specific racemes rather than dispersed over the plant, suggesting the presence of a barrier to bacterial invasion of ovules. As observed previously (7,18), infected plants tended to produce fewer seed than healthy plants. The inefficient transmission of *C. michiganensis* subsp. *insidiosus* to seed is in contrast with high levels of transmission of other subspecies of *C. michiganensis* to seed of other hosts. In tomato, transmission of *C. michiganensis* subsp. *michiganensis* to seed occurs in 0.25 to 100% of seed (37), and transmission of *C. michiganensis* subsp. *nebraskensis* occurs in 17.1 to 30.7% of seed harvested from infected corn plants (2). Transmission of other seed-borne pathogens to alfalfa seed is also low. Alfalfa mosaic virus is found in 0.2 to 6% of alfalfa seed produced from infected plants (11). *Verticillium albo-atrum*, the causal agent of Verticillium wilt in alfalfa, is found in 0.03% of field-grown seed (34) and 5 to 10% of seed from infected greenhouse-grown plants (4). A number of alfalfa diseases reported to be seed-borne may be due to contamination of seed lots with plant debris rather than actual presence of the pathogen within seed. The potential for dissemination of pathogens associated with debris has been largely eliminated with modern seed cleaning methods. In addition, since the late 1970s most of the U.S. alfalfa seed production is concentrated in western states where drier conditions have reduced the occurrence of seed-borne pathogens once common in seed produced in the Midwest.

Detection systems such as PCR-based methods and serological methods are used extensively to identify potato plants infected with the bacterial ring rot pathogen, *C. michiganensis* subsp. *sepedonicus*. This bacterium can cause latent infections in which plants show no symptoms but produce tubers that are infected. The identification of *C. michiganensis* subsp. *insidiosus* in plants originally rated as resistant with restricted or no symptoms indicates that these plants initially escaped infection or had latent infections that were only identified by subsequent isolation of the pathogen.

The PCR-based identification method will be useful in identifying bacteria isolated from alfalfa seed lots and in distinguishing alfalfa plants resistant to the pathogen without relying on disease symptoms.

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