Semiselective Agar Medium for Isolation of *Clavibacter michiganense* subsp. *michiganense* from Tomato Seed

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ABSTRACT

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A semiselective agar medium was developed for isolating *Clavibacter michiganense* subsp. *michiganense*, the causal agent of bacterial canker of tomato, from tomato seed. The new medium, designated SCM (semiselective medium for *C. michiganense*), contained sucrose, mono- and dipotassium phosphate, magnesium sulfate, yeast extract, nicotinic acid, boric acid, nalidixic acid, potassium tellurite, and cycloheximide. Recovery of 34 strains of *C. michiganense* on SCM in comparison to nutrient broth yeast extract (NBY) medium ranged from 85 to 132%, and more than 98% of the recoverable tomato seed saprophytic bacteria growing on NBY did not grow on SCM. SCM was quantitatively and qualitatively superior to other selective media for *C. michiganense*. Extracting seed by blending for 10–15 min in a commercial laboratory blender resulted in an increase of 100% or more over washing seeds in a flask at 4 C for 72 hr. A single contaminated seed containing as few as 50 colony-forming units of *C. michiganense* was detectable in samples of 10,000 seeds.

Additional key word: bacteria.

Clavibacter michiganense subsp. *michiganense* (7) causes bacterial canker of tomato (26), one of the world's major diseases of tomato (*Lycopersicum esculentum*) (29). Several other plants, including some common weeds, are known hosts of *C. michiganense* (29,31,32). In addition, pepper and eggplant are susceptible under laboratory conditions (16). Pepper has also been found to be naturally infected in the field (18). Although *C. michiganense* may survive in soil (2,8,20,28), tomato seed is considered the most important source of inoculum of this organism (5,29,30,36). The percentage of seed transmission is reported to range from 0.25 to 100% (3,5,13,14,21,30).

Seed treatment cannot always eradicate *C. michiganense* (25,33). Therefore, detection and elimination of seed lots contaminated with *C. michiganense* is an effective method of controlling this serious seedborne pathogen. Several detection techniques are available, including: culturing from seed onto a semisolid (5) or differential medium (28), grow-out tests (1,14), inoculating tomato plants with ground seed filtrate (30), phage-plaque counting tests (9), immunofluorescence (IF) (11,34), and a combination of IF and plant inoculation (37). Direct plating assays have several advantages over the other methods (23). Plating assays are easier to do, are less expensive, and result in the recovery of a viable culture (23) that can be used to determine pathogenicity.

The purpose of this study was to develop a semiselective agar medium for isolating *C. michiganense* from tomato seed. We determined the nutritional requirement of *C. michiganense* and then the susceptibility of *C. michiganense* and saprophytic microorganisms associated with tomato seeds to different inhibitors and antibiotics. Finally, we determined the optimal conditions for the extraction of *C. michiganense* from tomato seeds. A preliminary report has been published (12).

MATERIALS AND METHODS

Source of strains. Strains of *C. michiganense* were isolated freshly or obtained from culture collections (Table 1).

Development of semiselective agar medium. Four strains of *C. michiganense* (H112, H122, H124, H126) were used for comparing growth on D2 (selective medium for *C. michiganense*) (17), CNS (selective medium for *C. nebraskense*), and YDS (yeast extract dextrose selective medium) (15), using the dilution plating method (24,38). For routine growth of *C. michiganense*, nutrient broth yeast extract (NBY) medium (15) was used. The dilution plating method was used to determine a suitable source of nitrogen and carbohydrate. Inorganic nitrogen compounds, KNO_3, NH_4Cl, $(NH_4)_2 \cdot HPO_4$, $(NH_4)_2 \cdot SO_4$, and NH_4NO_3, were tested at 1 g/L, whereas the amino acids, biotine, thiamine, pyridoxine, and nicotinic acid, were tested at 2 mg/L. The basal medium used for determination of nitrogen source contained 2 g/L of K_2HPO_4, 0.5 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4 \cdot 7H_2O$, 5 g/L of sucrose, and 15 g/L of purified agar. Sucrose was chosen as a carbohydrate from results of previous work (11,27). After determining an appropriate nitrogen source, the following carbohydrates (Sigma Chemical Co., St. Louis, MO) were tested at 5 g/L: adonitol,

dextrin, dulcitol, glycerol, lactose, levulose, maltose, melibiose, melizitose, raffinose, rhamnose, sorbitol, starch, sucrose, trehalose, and D-xylose. Triplicate plates were seeded with approximately 100 colony-forming units (cfu) of *C. michiganense*. After 4 days of incubation at 26 C, growth was recorded. To determine the selectivity of the carbohydrates on growth of saprophytic bacteria associated with tomato seeds, 3 g of a mixture of five seed lots were washed in 20 ml of saline in a 250-ml Erlenmeyer flask for 6 hr at room temperature. The washing solution was atomized using a chromatographic sprayer onto triplicate plates of the basal medium containing different carbohydrates and 200 mg/L of cycloheximide. NBY with and without cycloheximide was used as controls for growth of *C. michiganense*. The basal medium without any carbohydrate source was also used as a control.

Susceptibility of *C. michiganense* to inhibitors. Ampicillin, bacitracin, carbenicillin, chloramphenicol, cephalexin, cephalothin, erythromycin, gentamycin, kanamycin, kasugamycin, neomycin, nitrofuratoin, novobiocin, penicillin, polymyxin B sulfate, pyridomycin, rifampicin, streptomycin, tobramycin, tyrothricin, nalidixic acid, boric acid, potassium tellurite, LiCl, $LiNO_3$, Li_2SO_4, $LiSeO_3$, $C_2H_3LiO_2$, $CaCl_2$, and sodium dichromate were initially screened at several concentrations. The impregnated paper disk method was used (22). The compounds were tested against *C. michiganense* strains H112, H113, and H122 and bacterial flora of tomato seed. For bacterial flora, 3 g of tomato seed from a mixture of five different seed lots were washed in 20 ml of saline in a 250-ml Erlenmeyer flask for 6 hr at room temperature. The seed washing was then sprayed, as described above, onto triplicate plates of NBY containing the chemically impregnated paper disk. For the control, sterile distilled water was used. The diameter of the clear zone (no bacterial growth) around the disks was recorded after 3 days of incubation at 26 C. Positive inhibitors were tested in the final agar medium to determine maximum inhibition of seed-associated saprophytic bacteria without reducing the recovery of strains H112, H113, and H122.

TABLE 1. Source and recovery of strains of *Clavibacter michiganense* subsp. *michiganense* on a semiselective medium

Laboratory strain no.	Received as strain	Source[a]	Mean recovery (%)[b]
H 160	440	1	132
H 165	445	1	128
H 172	HMB293	2	127
H 175	1465	3	126
H 113	Cm 152-2	4	124
H 166	446	1	120
H 127		5	118
H 161	441	1	118
H 112	Cm 15-2	4	117
H 114	Cm 156-1	4	114
H 159	Original	6	113
H 170	HMB67	7	112
H 123	Original	8	111
H 176	0974	9	111
H 174	1463	3	110
H 177	1080	9	110
H 157	Original	10	109
H 126		5	108
H 120	Original	8	106
H 115	Cm 3 D21	11	103
H 122	Original	8	103
H 167	447	1	102
H 125	Original	8	100
H 158	Original	10	99
H 141	030-5	12	97
H 124	Original	8	97
H 168	448	1	97
H 173	1461	3	96
H 134	Cm 18	13	92
H 143	123-1	12	92
H 171	Cm 5	14	92
H 111	Cm 14-4	4	90
H 164	444	1	90
H 144	158-6	12	85

[a] Source names and location: 1, J. Colin, Agadir, Morocco; 2, H. Maraite, University Catholique De Louvain, Laboratoire de Phytopathologie, Place Croix du Sud, 3, SC1. 15D, B-1348, Louvain-La-Neuve, Belgium, originally from Turkey; 3, L. Gardan, INRA Station de Pathologie Vegetale, Phytobacteriologie, Angers, France; 4, Anne Vidaver, University of Nebraska, Lincoln, originally from E. Echandi, North Carolina State University, Raleigh; 5, seeds, J. A. Dick, Canadian Canners, Ontario, Canada; 6, seeds, M. D. Ricker, Campbell Institute, Napoleon, OH; 7, H. Maraite (see above), originally from South Africa; 8, plant debris, E. Braun, University of Iowa, Ames; 9, M. L. Moffett, Plant Pathology Branch, Agricultural Research Laboratories, Meiers Road, Indooroopilly QLd 406, Australia; 10, seeds, J. A. Dick, Canadian Canners, Ontario, Canada; 11, Anne Vidaver, University of Nebraska, Lincoln, originally from C. I. Kado, University of California, Davis; 12, Maria Milagros Lopez, Spain; 13, M. D. Ricker, Campbell Institute, Napoleon, OH; and 14, H. Maraite (see above).

[b] Percentage of recovery based on comparison to nutrient broth yeast extract medium.

Colony differentiation. Nineteen dyes—Alcian blue, basic fuchsine, brilliant blue R, brilliant cresyl blue, bromcresol purple, bromothymol blue, congo red, crystal violet, fast green FCF, gentian blue, malachite green, matrius yellow, methyl green, methyl violet 2B, night blue, neutral red, phenol red, tetrazoluim chlorid, and trypan blue—and potassium tellurite (Difco) (6) were tested at 1 mg/L and 1×10^{-2}%, 5×10^{-3}%, and 1×10^{-3}%, respectively, in the basal medium for dye uptake. Thiamine-HCl (4) was also tested in the basal medium for increase of yellow pigmentation at 2, 20, 100, and 200 mg/L.

Evaluation of the semiselective SCM medium. Recovery of 34 strains of *C. michiganense* in comparison to NBY medium was determined on the final semiselective medium (SCM medium) using the dilution plate technique (24,38). The efficiency of the SCM medium in controlling the saprophytic bacteria was also determined using tomato seed lots of different origins.

Survival in liquid media. The following liquid media were tested for survival of *C. michiganense*: 0.85% NaCl (saline); distilled water; 0.05 M phosphate buffer, pH 7.45 (PB-2); PB-2 plus 0.1% Ca CO_3; pH 7.45 and 0.005 M phosphate-buffered saline (PBS-3). Erlenmeyer flasks (250 ml) containing 100 ml of sterile medium or 100 ml of sterile medium plus 4 g of tomato seeds and 0.02% Tween 20 were seeded with approximately 1×10^4 viable cells of *C. michiganense*. Three flasks (replicates) were used per treatment. The flasks were shaken on a New Brunswick G-24 rotatory shaker (100 rpm) in a cold room at 4 C. After zero time, 5 min, and 2, 15, and 24 hr, the number of cfu of *C. michiganense* was determined by dilution plating. Triplicate 0.1-ml samples were pipetted onto petri plates of NBY medium containing 200 mg/L of cycloheximide. All plates were incubated at 26 C and the number of colonies was counted after 3 days (colonies were approximately 2 mm in diameter).

Extraction of *C. michiganense*. To determine the effect of the volume of liquid on extraction of *C. michiganense*, 10,000 tomato seeds (24 g) from a naturally contaminated seed lot were added to each of three 500-ml Erlenmeyer flasks containing 125, 150, and 200 ml of PB-2 plus 0.02% Tween 20 (PBT-2). The flasks were shaken as above. After 1 and 15 hr of shaking, triplicate 0.1-ml samples were assayed on SCM medium. After 7 days of incubation at 26 C, the number of cfu per plate was determined. A comparison was made between washing seeds in a flask and blending them in a commercial laboratory blender (Stomacher, model no. STO-400, Tekmar Company, Cincinnati, OH) for extraction of *C. michiganense*. For washing, three 500-ml Erlenmeyer flasks containing 150 ml of PBT-2 at 2–4 C were seeded with 10,000 tomato seeds (500 seeds from a contaminated seed lot plus 9,500 seeds from a healthy seed lot) and shaken on a Lab Line orbital shaker (Melrose Park, IL) at 4 C. After 5 min and 2, 6, 15, 24, 48, 72, and 96 hr, 1-ml samples were removed and assayed by dilution plating on SCM. For the laboratory blender, three samples of 10,000 seeds, as above, and 150 ml of PBT-2 were placed in sterile, heavy-duty polyethylene plastic bags (18 cm × 30 cm, Tekmar Company). Each bag was placed in the blender at room

temperature. After 30 sec and 3, 5, 8, 10, 12, 15, and 20 min, samples were removed and assayed as above.

Recovery of *C. michiganense* from naturally contaminated seeds. Naturally contaminated seeds were used to evaluate the efficiency of SCM in recovering *C. michiganense* from tomato seeds. Contaminated tomato seeds were obtained by inoculating plants of cultivar Glamour, as described (11). Briefly, flowers of plants at the first flower-stage of growth were sprayed with a suspension of sterile distilled water containing 10^6 cfu of *C. michiganense* per milliliter using a chromatographic atomizer. The inoculation was repeated twice until fruit set. Later, when the green fruits reached a diameter of 3–4 cm, peduncules were inoculated with a similar suspension using a 26-gauge needle and syringe. The mature fruits were harvested and the tomato seeds were removed, washed in tap water, placed onto paper towels, and dried overnight at room temperature in a Laminair flowhood (Bellco Glass, Inc., Vineland, NJ). To determine the number of contaminated seeds in the sample of harvested seed, two replicates of 100 seeds each were placed onto SCM medium and incubated at 26 C for 8–10 days. In addition, 20 seeds from the same dried sample were chosen randomly. Ten of the seeds were assayed separately to determine the range of cfu of *C. michiganense* per seed. Each seed was ground separately in 3 ml of PBT-2 using a sterile pestal and mortar. Serial 10-fold dilutions were made, and 0.1 ml from the nondiluted, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were pipetted onto triplicate plates of SCM medium. The plates were incubated at 26 C for 7 days and the number of cfu of *C. michiganense* was counted. One seed from the remaining 10 seeds was then added to each of 10 samples of 10,000 healthy seeds in sterile polyethylene plastic bags containing 150 ml of PBT-2 at 4 C and the seeds were blended for 15 min, as described previously.

Identification of colonies of *C. michiganense*. Suspected colonies of *C. michiganense* with typical morphology were purified by streaking onto NBY medium (15) and tested for Gram staining, as described (10). Pathogenicity was tested by inoculating stems of tomato plants at the second or third true leaf stage (cultivar Glamour), as described (19).

RESULTS

Development of a semiselective agar medium. None of the strains tested grew on D2 medium. The mean percentage of recovery of *C. michiganense* on CNS and YDS media, respectively, ranged from 11 to 62.4%, with a mean of 47.8%, and from 6.7 to 45.1%, with a mean of 21.7%, compared to growth on NBY medium (Table 2). Besides the poor growth on these media, colonies of *C. michiganense* were difficult to differentiate from the many yellow-pigmented saprophytic flora associated with tomato seeds. Compared to the control basal salt medium, only nicotinic acid increased growth of *C. michiganense*. Growth of *C. michiganense* was not different on the basal salt nicotinic acid medium or basal salt nicotinic acid medium plus other sources of nitrogen. The final defined basal medium contained the following ingredients per liter: 2 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of nicotinic acid (free acid), and 15 g of agar. Of the 16 carbohydrates studied, only sucrose, melizitose, rhamnose, raffinose, glycerol, trehalose, and D-sorbitol were used by the three strains of *C. michiganense* tested (Table 3). Of the 16 carbohydrates tested, sucrose gave the highest reduction of tomato seed saprophytic bacteria while allowing all strains of *C. michiganense* to grow (Table 3).

Susceptibility to inhibitors. Nalidixic acid, gentamycin, kanamycin, chloramphenicol, polymyxin B sulfate, streptomycin, tobramycin, LiCl, boric acid, sodium dichromate, and potassium tellurite suppressed the growth of saprophytic bacteria associated with tomato seed in the sucrose basal agar medium. However, growth of *C. michiganense* was also affected. The maximum concentration of chemical resulting in no inhibition of growth of *C. michiganense*, as compared to growth on basal medium without chemicals, was as follows: 30 μg/ml for nalidixic acid and sodium dichromate, 20 μg/ml for polymyxin B sulfate, 10 μg/ml for potassium tellurite, < 10 μg/ml for streptomycin, chloramphenicol, and tobramycin, < 3 μg/ml for gentamycin and kanamycin, 5 g/L for LiCl, and 1.5 g/L for boric acid. Nalidixic acid at 30 mg/L, boric acid at 1.5 g/L, and potassium tellurite at 10 mg/L resulted in relatively high inhibition in growth of seed saprophytic bacteria and low inhibition in growth of *C. michiganense*.

Colony differentiation. The dyes tested either inhibited growth of *C. michiganense* or prevented development of pigmentation. None of the amino acids or thiamine-HCl induced yellow pigmentation when added to the basal medium. However, potassium tellurite was partially taken up, resulting in formation of gray-to-black speckled colonies (Fig. 1). The SCM medium contained the following (per liter of distilled water): 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KH_2PO_4, 2 g of K_2HPO_4, 10 g of sucrose, 0.1 g of yeast extract, 1.5 g of boric acid, 15 g of agar, 200 mg of cycloheximide (2 ml of 100 mg/ml stock solution dissolved in 75% methanol), 30 mg of nalidixic acid (salt)(3 ml of a 10 mg/ml stock solution dissolved in 2% 0.1 N NaOH), 10 mg of potassium tellurite (1 ml of 1% Chapman tellurite solution from Difco), and 100 mg of nicotinic acid (free acid) (10 ml of 10 mg/ml stock solution dissolved in distilled water). Stock solutions of nalidixic acid, nicotinic acid, and cycloheximide were filter-sterilized and added

TABLE 2. Recovery of *Clavibacter michiganense* subsp. *michiganense* on *Corynebacterium nebraskense* (CNS), yeast extract dextrose (YDS), and *Corynebacterium* spp. (D2) media[a]

Media	Average recovery[b] (%) of *C. michiganense* strain			
	H 112	H 122	H 124	H 126
CNS	62.4 ± 12.3	11.0 ± 3.3	61.1 ± 8.1	56.7 ± 8.4
YDS	45.1 ± 3.3	6.7 ± 1.8	24.3 ± 8.1	10.9 ± 7.0
D2	0.0	0.0	0.0	0.0

[a] The percentage of recovery was based on nutrient broth yeast extract medium (15). The media tested were CNS, a selective medium for *Corynebacterium nebraskense* (15), a yeast extract dextrose selective medium (15), and D2, a selective medium for *Corynebacterium* spp. (17).
[b] Average percentage of recovery per plate from four plates and standard deviation.

TABLE 3. Growth of *Clavibacter michiganense* subsp. *michiganense* on different carbon sources

Carbon source[a]	*C. michiganense* strain[b,c]			Reduction of saprophytes (%)[c]
	H 112	H 113	H 122	
Lactose	–[b]	–	–	94
Maltose	–	–	+	77
Melobiose	+	+	–	65
Sucrose	+	+	+	38
Melizitose	+	+	+	35
Rhamnose	+	+	+	33
Raffinose	+	+	+	29
Dextrin	+	–	+	27
Glycerol	+	+	+	21
Trehalose	+	+	+	20
D-Sorbitol	+	+	+	6
Levulose	–	–	–	ND
D-Xylose	–	–	–	ND
Dulcitol	–	+	+	ND
Adonitol	–	–	–	ND
Starch	+	–	–	ND

[a] Carbon source (5 g/L) was added to basal medium consisting of 2 g/L of K_2HPO_4, 0.5 g/L of KH_2PO_4, 0.25 g/L $MgSO_4$, 7 H_2O, 0.1 g/L of nicotinic acid, and 15 g/L of purified agar.
[b] + = Growth, visible single colonies. – = No growth, no visible colonies after 8 days of incubation at 26 C.
[c] The percentage of tomato seed saprophytic bacteria was based on growth on nutrient broth yeast extract medium. ND = Not determined.

after the medium was autoclaved and cooled to 45–50 C. Colonies of *C. michiganense* on SCM medium were visible after 4–5 days' incubation at 26 C. After 6–7 days, colonies were 1–2 mm in diameter, circular, speckled gray-to-black, and mucoid.

Evaluation of SCM medium. Plating efficiencies on SCM medium in comparison to NBY medium for the 34 strains of *C. michiganense* ranged from 85 to 132%, with a mean of 107%. Reduction in number of saprophytic bacteria on SCM medium in comparison to NBY medium for the 11 tomato seed lots ranged from 92.6 to 99.8%, with a mean of 98%.

Survival of *C. michiganense* in liquid media. There was no significant difference in the survival of *C. michiganense* in various liquid media alone, and no reduction in the cfu of *C. michiganense* occurred after 24 hr of incubation. However, when seeds were added to the media, a significant difference in the survival of *C. michiganense* occurred (Table 4). In distilled water, 25 and 100% of the cells died after 5 min and 2 hr, respectively. Similarly, in saline, 45 and 95% of the cells died after 5 min and 2 hr, respectively. In PBS-3, the figures were 33 and 54%, respectively. On the other hand, no significant decrease occurred in the population of *C. michiganense* after 24 hr in PB-2, whereas in PB-2 plus $CaCO_3$, only 8% of the cells died. PB-2 was the only solution tested that maintained the pH level of the different seed lots throughout the extraction period (Table 5). These experiments were repeated several times with similar results. The final extraction solution consisted of PBT-2.

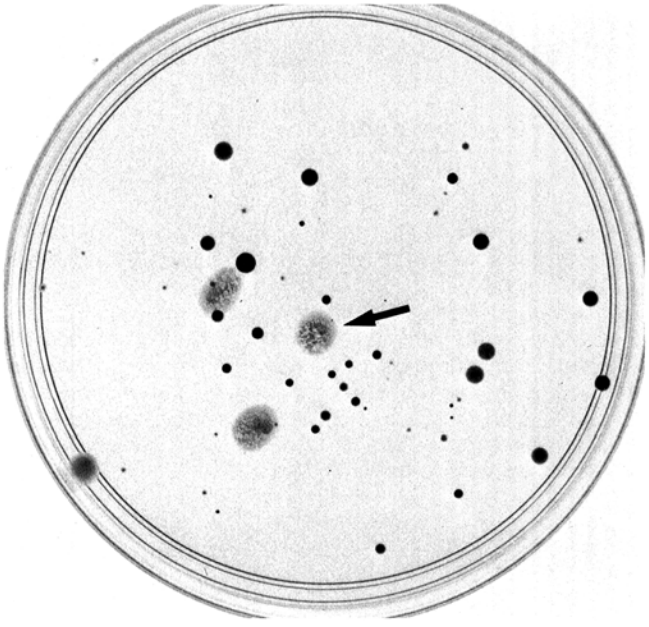

Fig. 1. Effectiveness of SCM medium in suppressing growth of seed-associated bacteria. Arrow points to a typical colony of *Clavibacter michiganense* subsp. *michiganense* after 9 days at 26 C.

TABLE 4. Survival and growth of *Clavibacter michiganense* subsp. *michiganense* in different liquid media with and without tomato seeds

Media	Recovery on nutrient broth yeast extract medium agar (%)				
	0 min	5 min	2 hr	15 hr	24 hr[a]
Without seed					
Distilled water	100	98	101	92	108 a
Saline[b]	100	104	97	91	87 b
PBS-3[c]	100	93	103	99	95 a
PB-2[d]	100	107	103	90	97 a
PB-2 + $CaCO_3$[e]	100	115	104	93	96 a
With seed					
Distilled water	100	74	0	0	0 D
Saline	100	54	5	0	0 D
PBS-3[c]	100	67	46	27	1 C
PB-2[d]	100	107	114	85	100 A
PB-2 + $CaCO_3$[e]	100	107	78	70	92 B

[a] Means (recorded at 24 hr) followed by the same letter are not significantly different from one another ($P = 0.05$) by Duncan's multiple range test.
[b] Saline = 0.85% NaCl.
[c] PBS-3 = 0.005 M phosphate buffer, 0.85% NaCl.
[d] PB-2 = 0.05 M phosphate buffer.
[e] PB-2 + $CaCO_3$ = PB-2 + 1 g/L of $CaCO_3$.

Extraction of *C. michiganense*. Significantly more cells of *C. michiganense* were extracted from 10,000 seeds in 150 ml than in either 125 or 200 ml. Washing 10,000 seeds in flasks for 5 min or 2, 6, 15, 24, 48, 72, and 96 hr resulted in recoveries of 5, 52, 146, 444, 167, 449, 1,933, and 99 cfu of *C. michiganense*, respectively. Blending seeds for 10–15 min resulted in an increase of 100% or more over washing for 72 hr (Table 6). Increasing the time in a laboratory blender from 0.5 to 15 min generally increased recovery of *C. michiganense* (Table 6). Similar results were obtained with repeated experiments.

Recovery of *C. michiganense* from naturally contaminated seeds. Each of the 10 naturally contaminated seeds assayed separately was positive. The number of cfu of *C. michiganense* per each of the 10 naturally contaminated seeds was as follows: 2.9×10^4, 9.3×10^3, 6.8×10^3, 2.5×10^3, 2.4×10^3, 2.3×10^3, 7.2×10^2, 5.8×10^2, 3.0×10^2, and 3.0×10^2. The number of cfu of *C. michiganense* per each of the 10 samples of 10,000 healthy seeds to which was added one naturally contaminated seed was as follows: 9.5×10^3, 6.0×10^3, 5.5×10^3, 4.0×10^3, 3.3×10^3, 2.5×10^3, 2.0×10^3, 5.0×10^2, 5.0×10^2, and 5.0×10^1.

Recovery of *C. michiganense* from commercial seed lots. *C. michiganense* was detected in 4 of 17 commercial seed lots tested on SCM medium (Table 7).

Recommended procedure for assaying tomato seed for *C. michiganense*. Place 10,000 seeds in a sterile, heavy-duty plastic

TABLE 5. Effect of the extraction solution on the pH of different tomato seed lots[a]

Extraction solution[b]	Initial pH	Tomato seed lot in alpha order per appearance							pH
		1	2	3	4	5	6	7	
Distilled water	5.89	2.29	2.91	1.62	5.70	3.87	2.23	2.31	2.99 ± 1.39 b[d]
PBS-3	7.20	3.44	4.37	2.22	6.28	5.00	2.78	2.85	3.85 ± 1.44 b
PB-2	7.44	7.06	7.14	6.80	7.30	7.12	6.61	6.67	6.96 ± 0.26 a

[a] The pH of the different tomato seed lots (one sample per lot) was recorded 5 min after the seeds were added and mixed in the extraction solution.
[b] Mean pH for the seven tomato seed lots and standard deviation.
[c] The solutions tested were: distilled water, 0.005 M phosphate-buffered saline (0.85% NaCl) (PBS-3), and 0.05 M phosphate buffer PB-2.
[d] Means followed by the same letter are not significantly different from one another ($P = 0.05$) by Duncan's multiple range test.

TABLE 6. Effect of time on extracting *Clavibacter michiganense* subsp. *michiganense* from tomato seed by a Stomacher laboratory blender

Time (min)	Mean number of bacteria recovered per milliliter[a]	Increase over washing for 72 hr (%)[b]
0.5	61 e[c]	−96.9
3.0	826 de	−57.3
5.0	1,396 cd	−28.0
8.0	2,084 c	+8.0
10.0	4,067 b	+110.0
12.0	4,711 ab	+144.0
15.0	5,078 a	+163.0
20.0	400 de	−79.0

[a] Assayed by plating serial dilutions onto *C. michiganense* semiselective agar medium (three replicates per treatment).
[b] Based on a recovery of 1,933 colony-forming units of *C. michiganense* after shaking seeds in a flask at 2 C for 72 hr.
[c] Means followed by the same letter are not significantly different from one another ($P = 0.05$) by Duncan's multiple range test.

bag containing 150 ml of PBT-2, pH 7.5, at 4 C. Place the bag and its contents in a laboratory blender and blend for 15 min at room temperature. Remove 50 ml and concentrate by centrifugation at 12,000 × *g* for 10 min. Suspend the pellet in PBT-2 and plate three samples of 0.1 ml of nondiluted and 10^{-1} and 10^{-2} dilutions onto each of three plates of SCM medium (nine plates total). Incubate plates at 26 C for 7 days. Mark suspected convex, irregular, mucoid, and speckled gray-to-black colonies (Fig. 1). Streak suspected colonies onto NBY medium and observe yellow pigmentation after 4 days of incubation at 26 C. Save original plates and observe again after 2 days at 26 C. Confirm identity by pathogenicity tests, as described (19).

DISCUSSION

The only test for routine assays of tomato seeds for *C. michiganense* is the immunofluoresence test developed in France (11,34). However, this test has several disadvantages, including the inability to differentiate viable and nonviable cells, background staining problems, low antibody specificity, sensitivity limitations, and lack of a viable culture for pathogenicity tests. The inoculation of tomato plants with ground seed filtrate (30) and the grow-out tests (1,14) have been used, but with little success. Semiselective agar media and washing techniques have been useful for many seedborne bacteria, but none are available for *C. michiganense*. Some semiselective agar media, such as D2 (17), CNS, and YDS (15), have been reported to be selective for *C. michiganense*, but we found these media inadequate for tomato seed assays. None of the four strains we tested grew on D2 medium. With CNS medium, the recovery of *C. michiganense*, in comparison to NBY medium, varied from 11.0 to 62.4%, with a mean of 47.8%. CNS medium contains 10 g/L of LiCl, which we found to inhibit growth of most strains of *C. michiganense*. On YDS medium, the recovery of four strains of *C. michiganense* ranged from only 6.7 to 45.1% of that of NBY medium. YDS medium did, however, give very good control of saprophytic bacteria associated with tomato seed. Another problem with both CNS and YDS media was the difficulty in differentiating the yellow colonies of *C. michiganense* from other yellow saprophytic colonies. Therefore, CNS medium was not tested for recovering *C. michiganense* from naturally contaminated seed. On the other hand, SCM medium controls growth of most seed-associated saprophytes and results in high recoveries of *C. michiganense*. Furthermore, colonies of *C. michiganense* are distinct, due to the mucoid colony morphology and speckled gray-to-black color. Most saprophytic bacteria either take up more potassium tellurite and become black or take up none and remain colorless. Another advantage of SCM medium is that colonies of *C. michiganense* continue to grow, reaching 5–6 mm in diameter after 8–10 days, whereas most saprophytic colonies do not continue to grow after 6 days. This permits easy identification of *C. michiganense* even when the plate is nearly covered with saprophytic bacterial colonies. The high selectivity of SCM medium is the result of the combination of the basal sucrose medium and the added inhibitors, nalidixic acid, boric acid, and potassium tellurite. The sucrose-nicotinic acid basal medium alone reduced the saprophytic bacteria associated with tomato seed by 38% in comparison to NBY medium. Our results agree with Starr's (27) report that nicotinic acid is needed as a growth factor for *C. michiganense*. In our study, none of the other nitrogen sources tested was found to be used by *C. michiganense*. In addition, no increase of growth of *C. michiganense* occurred when nicotinic acid was used with the different nitrogen sources studied. Thiamine, biotin, or pyridoxin had no effect on growth of *C. michiganense*. All of the strains of *C. michiganense* tested grew on the sucrose-nicotinic acid basal medium. However, three strains (H111, H114, H115) failed to grow when nalidixic acid (30 mg/L), boric acid (1.5 g/L), and potassium tellurite (10 mg/L) were supplemented to the sucrose-nicotinic acid basal medium. These strains were originally from the laboratory collections of E. Echandi (North Carolina State University) and C. I. Kado (University of California, Davis). Their failure to grow could be due to changes in storage, as all freshly isolated strains grew well. Furthermore, the strains H114 and H115 were yellow but not viscous on NBY and produced atypical symptoms in tomato. Rather than causing general wilting, these strains induced a limited brown-to-black lesion around the site of inoculation. These two strains may not be *C. michiganense* or may have lost their virulence. On the other hand, these two strains appear typical on SCM medium and cannot be differentiated from *C. michiganense*. Yeast extract (0.1 g/L) was added to SCM medium to increase growth of strains H111, H114, and H115.

Thiamine-HCl was reported to be responsible for production of yellow pigmentation by *C. michiganense* (4). However, in our study, the addition of thiamine-HCl to SCM medium at a final concentration of 2, 20, 100, or 200 mg/L failed to induce yellow pigmentation production by *C. michiganense*. On the other hand, pigment production can be induced by adding 1 g/L of yeast extract to SCM medium.

C. michiganense survives for 24 hr or longer in distilled water, saline, PB-2, PBS-3, and PB-2 plus $CaCO_3$. But when tomato seeds are added, distilled water, saline, and PBS-3 fail to support survival of *C. michiganense*. This is apparently due to a decrease in pH that occurs immediately after the seeds are added. PB-2 was the only extract solution in which *C. michiganense* survived for long periods and the only one tested that maintained the pH level.

Results of assaying seed by washing and blending in a laboratory blender showed that the highest recoveries of *C. michiganense* (for each method) occurred after 72 hr and 15 min, respectively. These results with washings of seed agree with previous results using immunofluorescence as a direct assay (11). The increased recovery of *C. michiganense* with time is likely a result of the opening of the integument allowing internally borne bacteria to escape. In liquid, tomato seeds do begin germination after 48 hr.

The relatively high percentage of infection of seeds obtained after spray inoculation of flowers and needle inoculation of peduncles agrees with earlier results (11,36). Our success in obtaining infection of seeds of greenhouse-grown plants by flower inoculation agrees with reports that *C. michiganense* invades seed through the calyx scar and/or the vascular bundle of fruits (36).

SCM medium should prove useful in the routine detection of *C. michiganense*-contaminated tomato seed lots and isolation from leaf and stem tissues. The medium should also be useful in monitoring the primary inoculum population in residue, soil, or water.

TABLE 7. Recovery of *Clavibacter michiganense* subsp. *michiganense* from commercial tomato seed lots on NBY and SCM media[a]

Origin of seed lot	No. of lots tested	Mean colony-forming units of *C. michiganense*/10,000 seeds	
		NBY	SCM
Canada	1	0.0	1.5×10^7
Canada	1	0.0	1.0×10^6
USA	1	0.0	1.6×10^5
USA	9	0.0	0.0
Canada	1	0.0	3.3×10^4
China	1	0.0	0.0
France	1	0.0	0.0
Morocco	2	0.0	0.0

[a] The media used contained a nutrient broth yeast extract (NBY) medium and a semiselective *Clavibacter michiganense* subsp. *michiganense* agar (SCM) medium.

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