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Differentiation of *Clavibacter michiganensis* subsp. *michiganensis* from seed-borne saprophytes using ELISA, Biolog and 16S rDNA sequencing

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Abstract

Specificity of a monoclonal antibody (MAb), Cmm1, to geographically diverse strains of the seed-borne tomato pathogen, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), was assessed and the MAb was tested for its usefulness as a tool to separate the pathogen from saprophytes in naturally infested tomato seed. Of the 236 international *Cmm* strains tested, 99% reacted with MAb Cmm1. MAb Cmm1 was also strongly reactive with an additional 32 strains isolated from seed that were later identified as *Cmm* by the Biolog MicroLog™ microbial identification system (Biolog, Inc., Hayward, CA) and 16S rDNA sequence analysis. It correctly differentiated these strains from 12 MAb Cmm1-negative seed strains that possessed similar colony morphology but were later identified as other Gram-positive genera and species. The specificity of MAb Cmm1 to the pathogen and the near universality of the MAb Cmm1-reactive antigen among diverse *Cmm* strains make this antibody a useful detection and identification tool. The finding that a large proportion of the *Cmm* strains associated with naturally infested tomato seed were putatively hypovirulent or non-virulent indicates that such populations cannot be ignored and points to a need for studies to determine their significance in host-pathogen interactions.

Introduction

Bacterial canker of tomato (*Lycopersicon esculentum*), caused by the Gram-positive coryneform bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) Davis et al. (1984), is an economically important disease that affects both greenhouse and field tomato crops. *Cmm* enters a production area mainly through infested seed (Strider, 1969) or latently infected tomato transplants (Chang et al., 1991; Gitaitis et al., 1991). Because of this, propagule screening for the pathogen prior to planting can be highly effective in preventing outbreaks. In the case of seed health testing, identification of the pathogen is complicated by the presence of fast-growing saprophytes or other contaminating bacteria that resemble *Cmm* in colony morphology. Rapid and accurate methods are continually sought to simultaneously identify and differentiate the pathogen, which is a quarantine pest in the United States and an A2 quarantine organism in Europe, from bacterial contaminants similar in appearance.

In order to fill the need for rapid diagnostic tools, monoclonal antibodies (MAbs) for *Cmm* were produced in the late 1980s and tested first for reactivity with other plant, human and environmental bacteria, and then against 88 virulent strains and 13 non-virulent strains that were previously identified by their providers as *Cmm*

(Alvarez et al., 1993). Based on these initial studies, one MAb, Cmm1 (clone 103–142), was selected for its specific reactions with virulent *Cmm* strains. However, strains representing a broad range of contaminants and saprophytes of tomato seed were unavailable at the time, and so reactivity of this MAb with other bacteria that would probably be encountered during a seed assay were not determined. Moreover, as bacterial identification methods improved, it became clear that most of the MAb Cmm1-negative, non-pathogenic strains, originally acquired as *Cmm* and subsequently used for antibody screening, had been misidentified and were, in fact, not *Cmm* but other yellow-pigmented bacteria. Further testing on an additional subset of strains confirmed to be *Cmm* by partial 16S rDNA sequence analysis and the Biolog MicroLog™ microbial identification system revealed subpopulations of putative hypovirulent (i.e., producing a canker but no wilting on inoculated plants) and non-virulent strains that reacted strongly with MAb Cmm1, a finding which warranted further study since it implied that MAb Cmm1 reacted universally with all *Cmm* rather than just virulent strains as originally believed.

The first part of this work focused on characterization of the Cmm1 MAb followed by screening against 236 *Cmm* strains isolated from around the world to determine the universality of the MAb as an identification tool. The second part focused on determining the specificity of MAb Cmm1 for the pathogen among other unidentified bacteria isolated from naturally infested tomato seed to assess the usefulness of MAb Cmm1 for identification of *Cmm* in a seed health assay.

Materials and methods

Characterization of MAb Cmm1

MAb Cmm1 (clone 103–142, subclass IgG2a) was generated following immunization with virulent *Cmm* strain CM761 and selected for characterization after screening with 101 virulent and non-virulent strains identified by their providers as *Cmm* and with 89 strains from other bacterial genera (Alvarez et al., 1993). Two additional MAbs, Cmm2 (clone 103–148, subclass IgG2a) and Cmm3 (clone 136–61, subclass IgG2a), were also screened using the above panel of strains. Immunofluorescence and immunoelectron microscopy were performed following the protocols of Benedict et al. (1989). A modified version of the immunofluorescence colony staining (IFC) technique of van Vuurde (1990) was used, employing preparations of fluorescein isothiocyanate (FITC)-conjugated MAb Cmm1. Briefly, 100 μl aliquots of a diluted *Cmm* suspension were distributed to individual wells in 24-well Costar tissue culture plates (Corning Inc. Life Sciences, Acton, MA), immediately followed by 100 μl per well of 5% trypticase soy agar cooled to approximately 50 °C. Plates were gently rotated to suspend the cells throughout the agar and then incubated for 24 h at 28 °C to facilitate the development of microcolonies. Enriched samples were dried overnight in a circulating air incubator. MAb Cmm1-FITC conjugate was added to the agar films at 100 μl per well and the plates incubated overnight in the dark at room temperature. After washing treated wells with phosphate buffered saline (0.01 M, pH 7.4), samples were viewed using an Olympus BHA compound microscope with a BH2-RFL reflected light fluorescence attachment. Extracellular polysaccharides (EPS) were precipitated by the method of Brumbley and Denny (1990).

Bacterial strains

Two hundred and thirty-six strains of *Cmm* were provided by donors from numerous geographical locations or were isolated prior to this study in Hawaii (Table 1). Strains were initially characterized using biochemical tests and a tomato bioassay. Cultures were stored at −80 °C in Luria-Bertani broth amended with 25% (v/v) glycerol and subcultured to yeast sucrose calcium carbonate medium (YSC; a modified YDC medium with sucrose replacing dextrose; Wilson et al., 1967) prior to testing.

Bacterial colonies recovered from seed assays on semi-selective media were lifted off plates and transferred to YSC medium to observe colony morphology. Additional purification was done on TZC-S medium (TZC medium with sucrose replacing dextrose; Norman and Alvarez, 1989), streaked one additional time to YSC medium and stored at −80 °C and in water vials at 4 °C.

Table 1. Two hundred and thirty-six strains of *Cmm* used for characterization of monoclonal antibody Cmm1 and 32 additional *Cmm* strains isolated from infested seed

Strains (no.)	Strain designations	Origin	Provider[a]
44	C12, C19, S44, S47, S51, S52, S53	California	2
	fruit, leaf, stem		9
	88, 213, 214, 1084, 1085, 1086, 1087, 1088, 1089, 1090, 1091, 1092, 1115, 1116, 1117, 1178, 7089, 7119A, 7119B, 7119C, 7148B, 7250B, C388D		11
	CMM 197A, CMM 198, N 197, N 198, N 7902		12
	STA-1A, STA-1B		20
	B-125, CM761, F1, F2		21
8	IPO 1799	Chile	8
	CMM 199, CMM 202A, N 202A, N 202B, N 213, N 71033		12
	ZUM 3036		22
26	71168, 71182, 71196, 71235, 71290, 71335, 71335B, 71341, 71412, 71421, 71427, 71428, 71430, 71431, 71450, 71472, 71474, 71479, 71564	China	5
	980829, 980806, 980834, N 211, N 212, N 71127, N 71176		12
3	A438-1, A518-1, A518-5	Hawaii	4
2	IPO 501, IPO 544	Hungary	8
2	H-160, H-171	Idaho	1
1	N 7137P	India	12
5	DR73, DR60-R1, DR59, BR4, CMM1	Iowa	3
1	IPO 542	Italy	8
2	IPO 543	Kenya	8
	ZUM 2305		22
20	25, 56, 67, 68, 115, 126, 127, 129, 131, 208, 280, 282, 285, 287, 291, 294, 303	Michigan	10
	F 290, F 293		12
	80		13
7	N 7219, N 7219P, N 7388, N 7388B, N 7388C, N 7388D, N 7904	Morocco	12
5	CM36, CM33, CM Finley, CM Kuykendall, CM Leatherwood	North Carolina	18
79	CM5, CM7	Ohio	6
	26, 29, 76, 123, 268		10
	A4504, A4505, A4506		14
	E3, E4, E5, E7, E10		15
	CM95, CM97, CM98, CM99, CM100		16
	OSURSC76, 1(A), 1(C), 1(H), 2(A), 2(C), 4, 5, 6(A), 6(C), 7, 8(A-B), 8(C), 9, 10, 11, 12, 14, 16, 17, 18(D), 18(E), 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34C, 34(E), 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 47, 57, 69, 70, 72A, 73, 74, 75, 76, 86		17
2	CMM 461, CMM 462	Portugal	19
1	IPO 545	South Africa	8
2	IPO 500, IPO 541	United Kingdom	8
19	cmm014, cmm015, cmm016, cmm018, cmm023, cmm024, cmm025, cmm029, cmm033, cmm035, cmm037, cmm038, cmm042, cmm043, cmm052, cmm054, cmm055, cmm066, cmm070	Washington	7
7	IPO 630	Unknown	8
	ZUM 2262-8, ZUM 3069		22
	C1-1, C1-3, C1-4, C1-5		23
7	C6A, C55, C59A, C63, C74A, C90A, C91A	California	This study
3	C117, C126B, C131A	Morocco	This study
10	C202, C203, C204, C205, C206, C207, C208, C209, C210, C211	Ohio	This study
12	C215, C216, C217, C218, C219, C220, C221, C222, C223, C224, C225, C226	Oregon	This study

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Tomato bioassays

Prior to large-scale bioassays, three tomato cultivars (*Lycopersicon esculentum* cvs Bonny Best, Healani and Kewalo) were evaluated for susceptibility to bacterial canker using three virulent *Cmm* strains (CM761, A518-5 and H-160). No differences in rate of wilt or canker formation were observed for these cultivars. Based on plant vigour under existing greenhouse conditions and availability of seed, cv. Kewalo was selected for all subsequent testing. Tomato plants (cv. Kewalo) between the 3 and 4 leaf stages were then used to test pathogenicity of the *Cmm* strains from the culture collection and additional bacterial strains isolated from tomato seed. Strains were revived from −80 °C storage and grown on YSC at 28 °C for 3–5 days. Plants were inoculated by stabbing directly into the stem between the cotyledons with a sterile scalpel dipped in a YSC medium culture of the appropriate test strain. Dilution plate counts of *Cmm* recovered from plants immediately after inoculation showed that this method delivered ca. 10^6–10^8 colony forming units (CFU) into the stem. Inoculated plants were maintained on a greenhouse bench and assessed for symptom development for at least 21 days. Strains were considered virulent if inoculated plants exhibited a canker at the site of inoculation and some degree of leaf wilting. Strains considered hypovirulent caused a stem canker like those produced by virulent strains, but did not produce leaf wilting by the end of the incubation period. Non-virulent strains caused no symptoms in inoculated plants. Both hypovirulent and non-virulent strains from the culture collection were inoculated into a minimum of four plants, and most were tested in at least two replicate experiments in order to establish the reproducibility of the reactions.

For the strains isolated from seed, virulence was additionally rated on a scale of 0–7, where '0' meant no symptom development and '7' was a plant exhibiting a canker and complete wilting of the leaves at the end of the incubation period. Plants with cankers and partial leaf wilt were assigned a base value of '1' (indicating canker development). Additional normalized values from 2 to 7 were based on the numbers of leaves exhibiting wilt. On this scale, strains rated '1' were considered hypovirulent and strains with values of 2 or more were considered virulent. Non-virulent and hypovirulent strains from seed were tested at least twice on a minimum of seven plants to confirm the reproducibility of the reactions.

Enzyme-linked immunosorbent assays (ELISA)

The indirect ELISA technique of Benedict et al. (1989) was used on pure, formalinized cultures of the test strains. Ascitic fluids were used at a 1:8000 dilution (1 μg protein ml^{-1}). Strains were run in replicate wells and were considered positive if ELISA values exceeded two standard deviations above the averaged value of the negative control wells. Selected ELISA-positive strains were re-tested to confirm reactivity. Negative and weakly positive strains were tested at least twice on separate days to confirm results.

A modified 'toothpick' ELISA protocol was also used for larger-scale screening of potential *Cmm* strains isolated from seed assays. Samples of the suspect colonies were lifted from YSC medium using sterile toothpicks, deposited into microtiter wells containing 100 μl of carbonate–bicarbonate buffer (pH 9.6), and mixed vigorously until a uniform cell suspension was obtained. Samples were then dried overnight at 37 °C. Dried plates were stored at 4 °C until assayed.

DNA extraction

Bacterial strains were revived from −80 °C storage and grown on YSC to confirm purity. Single colonies were subcultured to additional YSC plates and grown for another 2–3 days, after which a loopful of cells was transferred to yeast glycerol broth tubes (YG broth, containing per litre: 5 g yeast extract, 1 g dibasic potassium phosphate, 0.5 g magnesium sulphate and 17 ml glycerol) and incubated overnight on a rotary shaker (200 rpm at 30 °C). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), following the manufacturer's protocol for Gram-positive bacteria. DNA quantity was estimated using the Rapid Agarose Gel Electrophoresis (RAGE) apparatus (RGX-100 system; Cascade Biologics, Inc., Portland, OR), and working solutions of 50 ng μl^{-1} were prepared.

16S rDNA sequence analysis

PCR for 16S rDNA sequence analysis was run on subsets of virulent, hypovirulent and non-virulent

strains using primers 264F (5′-GAT GAT CAG CCA CAT TGG GAC-3′) and 1078R (5′-CCC AAC ATC TCA CGA CAC GAG-3′). Each 30 μl reaction consisted of 27 μl Platinum PCR SuperMix high fidelity (Invitrogen Corp., Carlsbad, CA), 1 μl each primer (10 μM each), and 1 μl genomic DNA (50 ng), and was amplified under the following conditions: initial denaturing at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, and ending with a final extension of 72 °C for 5 min. PCR products were purified using Montage PCR centrifugal filter devices (Millipore Corp., Bedford, MA), quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and then sequenced on an Applied Biosystems 377XL DNA sequencer at the Greenwood Molecular Biology Facility (University of Hawaii at Manoa). Most significant alignments of the returned sequences were obtained through BLASTN search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

Biolog identification

Closest identifications were obtained using the Biolog MicroLog3™ Microbial Identification System (release 4.20.04, Biolog, Inc., Hayward, CA), following the manufacturer's instructions. Gram reactivity of each strain was confirmed prior to plate inoculation using the potassium hydroxide test for Gram stain differentiation (Suslow et al., 1982). Inoculum densities were adjusted to a value equivalent to the recommended turbidity using a Klett–Summerson colorimeter. At 16–24 h, plates were read in a Biolog MicroStation microplate reader (Bio-Tek Instruments, Inc., Winooski, Vermont) and identifications obtained using the GP database (release 6.11).

Seed assays

Tomato seed samples were assayed using a modified version of the semi-selective media plating method of Fatmi and Schaad (1989). Ten to 25 g of seed from individual seed lots were placed in mesh-lined plastic Stomacher laboratory blender bags. Extraction buffer (0.01 M PBS containing 100 mg l^{-1} cycloheximide and 0.02% Tween 20, pH 7.4) was added to each bag at a 1:3 seed-to-buffer ratio. Samples were incubated at room temperature for 4 h and then processed for 15 min in a Stomacher 400 laboratory blender at normal speed (Seward, Norfolk, UK). Seed extracts were spread-plated in 100 μl subsamples to at least five Petri plates containing CMM1 semi-selective medium (Alvarez et al., 2005) and incubated at 28 °C for 7 days. CMM1 was modified from D2anx medium (Chun, 1982) with regard to carbon source and antibiotic components, and contained the following ingredients per litre: 10 g sucrose, 1.2 g Tris base, 250 mg magnesium sulphate heptahydrate, 5 g lithium chloride, 2 g yeast extract, 1 g ammonium chloride, 4 g casamino acids, 15 g agar, 200 mg cycloheximide, 28 mg nalidixic acid (free acid), and 10 mg polymyxin B sulphate. To avoid cloudiness during preparation, the pH was adjusted to 8.0 before adding the casamino acids, and readjusted to pH 7.4 before autoclaving. Antibiotics were added after autoclaving as filter-sterilized solutions.

All colonies were observed under a dissecting microscope, and those with *Cmm*-like colony characteristics (i.e., yellow to yellow-orange pigmentation, fluidal, domed, entire margins) were selected for further characterization.

In two lots, *Cmm* recovery was so high that individual seeds needed to be assayed in order to recover individual colonies. Seeds were macerated in PBS buffer in a porcelain spot plate using a sterile glass rod. All the seed extract generated in this manner was spread-plated to CMM1 medium and incubated as above.

Results

Characteristics of the MAb Cmm1-specific epitope

Individual *Cmm* cells produced a strong immunofluorescent signal when tested with MAb Cmm1, and positive reactions with heat-killed cells and partially purified EPS from culture filtrates indicated that the MAb Cmm1 epitope was associated with an abundant heat-stable extracellular antigen, most likely EPS.

MAb Cmm2 possessed nearly identical specificity to MAb Cmm1 but produced a consistently weaker positive signal. MAb Cmm3, generated after immunization with *Cmm* strain CM78, did not react to all *Cmm* strains and exhibited greater cross-reactivity with non-*Cmm* strains. However, it

did reliably react to one *Cmm* strain (N 212) that failed to react with MAb Cmm1, typically giving ELISA values of 1.0 OD_{A450} or greater, compared to values of 0.1 OD_{A450} or less with MAb Cmm1.

Immunoelectron microscopy indicated that MAb Cmm1 reacted with an antigen that was evenly distributed on the cell surface (Figure 1a and b). Purified, undiluted EPS contained the reactive antigen, which was expressed when cultures were grown on media containing different carbon and nitrogen sources. Binding curves for EPS were similar to those generated for whole cells. Reactivity of MAb Cmm1 to *Cmm* microcolonies was evident using direct IFC, where a strong reaction at the edge of the colony was observed. Diffuse halos of EPS surrounded the brighter reactive colonies (Figure 1c).

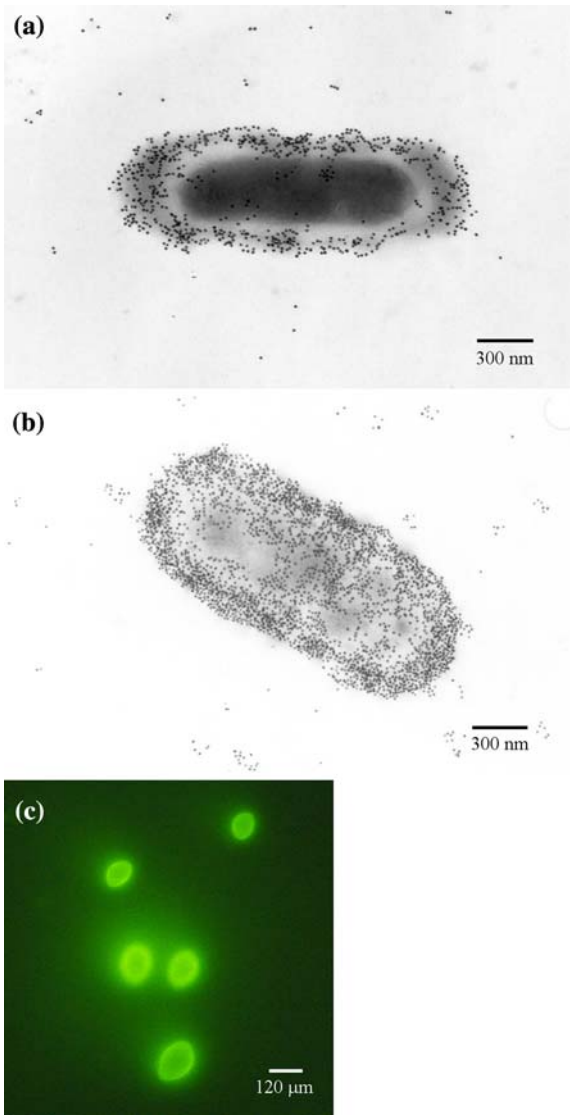

Figure 1. Reactions of MAb Cmm1 with surface antigens of *Cmm* cells (a and b) and microcolonies (c). Electron micrographs of *Cmm* strain CM761 incubated with colloidal gold-labelled MAb Cmm1 (a and b) and immunofluorescence colony staining of *Cmm* microcolonies using FITC-conjugated MAb Cmm1 (c).

Reactivity of geographically diverse Cmm strains to MAb Cmm1

Specificity of the Cmm1 MAb was determined by testing 236 *Cmm* strains from diverse geographical locations using indirect ELISA. MAb Cmm1 did not react with *Cm* subsp. *insidiosus* (*Cmi;* ATCC 10253), *Curtobacterium flaccumfaciens* (ATCC 6887), *Cf* subsp. *poinsettiae* (ATCC 9682), *Rathayibacter rathayi* (ATCC 13659) or *Rhodococcus fascians* (ATCC 12975). However, it reacted with *Cm* subsp. *sepedonicus* (*Cms*; ATCC 9850, A2041, A2042, A2043 and A2051) and a few other Gram-positive strains using indirect ELISA when ascitic fluids were used at higher concentrations. For current experiments, ascites were applied at a 1:8000 dilution (1 μg protein ml^{-1}), which reduced these cross-reactions (Figure 2). Most strains that were positive with the MAb gave very strong ELISA values (greater than 1.0 OD_{A450}), which could be easily differentiated from negative strains (values of 0.15 OD_{A450} or less).

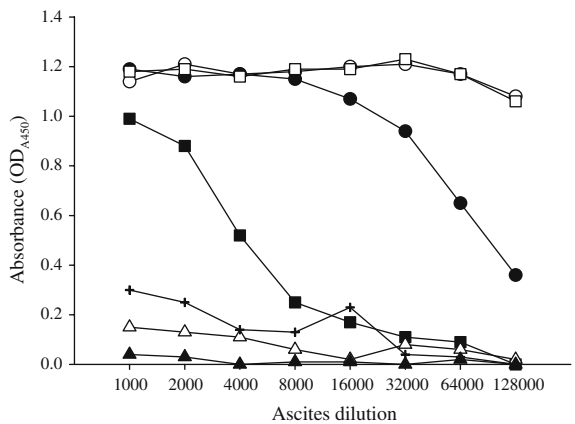

Figure 2. Titration curves for MAb Cmm1. Data for *Cmm* strains CM761 (○), A518-1 (□) and B100 (●), *Cms* strain ATCC 9850 (■), *Curtobacterium flaccumfaciens* strain ATCC 6887 (Δ), *Pantoea herbicola* strain Eh-1 (▲) and an unidentified, non-pathogenic seed saprophyte A518-8A (+) were generated using formalinized cells in an indirect ELISA.

The majority of the *Cmm* strains from the culture collection (173) reacted strongly with MAb Cmm1 and induced typical bacterial canker symptoms (canker formation and leaf wilt) when inoculated into tomato plants at estimated concentrations of 10^6–10^8 CFU (Table 2). Four strains (CM Finley, 8(C), CM99 and CM100) consistently gave low ELISA values (0.2–0.3 OD $_{A450}$), but were still considered positive compared to completely non-reactive strains, and elicited typical symptoms in inoculated plants. Most strains had typical colony morphology (i.e., yellow pigmentation, fluid, domed, smooth margins) on YSC medium. Seventeen strains exhibited atypical colony morphology: 13 were yellow but drier than typical, two were white and mucoid and two were pink and mucoid. Testing of selected strains using the Biolog system identified them as *C. michiganensis*.

Of the 236 international *Cmm* strains tested, only two (fewer than 1%) were non-reactive with MAb Cmm1 using the indirect ELISA assay (Table 2). Strain 86 was obtained from Ohio, while strain N 212 was isolated from seed originating in China. Both strains caused cankers and leaf wilt on inoculated plants, had atypical colony morphology (i.e., both were drier than typical, and one was orange), and both were identified as *C. michiganensis* by the Biolog database and by 16S rDNA sequencing.

Sixty-one strains that reacted with the Cmm1 MAb produced only cankers at the inoculation site (29), or did not elicit any symptoms when inoculated into cv. Kewalo (32), and were therefore considered putative hypovirulent or putative non-virulent strains, respectively (Table 2). The non-virulent strains gave consistent results on all tested plants. In comparison, the hypovirulent strains gave variable results in that some plants did not develop any symptoms, but those that did formed large cankers at the site of inoculation. All but 10 strains had typical *Cmm* colony morphology on YSC. Of the 10 atypical strains, one was white with mucoid consistency, one was pink and mucoid, six were yellow and less fluid than typical (dry or sticky), and two were orange and sticky. Biolog results from 26 strains and 16S rDNA sequence analysis on six strains from different geographic areas confirmed that representative strains were *C. michiganensis*.

Table 2. Characterization of 236 geographically diverse *Cmm* strains by ELISA and bioassays on tomato (cv. Kewalo)

Virulence	Symptoms produced on tomato	Strain reactivities with MAb Cmm1 in ELISA	
		Positive	Negative
Virulent	Stem cankers plus leaf wilting	173	2
Hypovirulent	Stem cankers at inoculation site only	29	0
Non-virulent	No symptoms	32	0

Detection and identification of Cmm *in infested seed lots*

Five tomato seed lots were assayed for *Cmm* infestation: CA-00 and MC-00, which originated from naturally infected tomato fields, and OH-01, OR-01 and OR-02, which were generated by spray-inoculating plants in the field with single virulent *Cmm* strains (Table 3). Over 15,000 bacterial colonies from the five lots were isolated on CMM1 semi-selective medium and observed for typical *Cmm*-like colony morphologies. Many of the colonies isolated from lots CA-00, MC-00 and OH-01 did not resemble *Cmm* on CMM1 medium, and were not studied further. Most colonies from lots OR-01 and OR-02 possessed *Cmm*-type colony morphology, and a few were chosen from each of these lots for further testing. Of the colonies selected for additional analysis, 60 possessed both typical *Cmm*-type characteristics and were strongly reactive with MAb Cmm1 in both the 'toothpick ELISA' screen and the standard indirect ELISA confirmation assay. Thirty-two of these colonies were tested further using the Biolog system and 16S rDNA sequencing to establish their identities: seven from CA-00, three from MC-00, 10 from OH-01, five from OR-01 and seven from OR-02 (Table 1). Eleven colonies from lot CA-00 and one colony from MC-00 (C140) that did not react with MAb Cmm1 but closely resembled the pathogen on the semi-selective medium were also identified further to determine whether the antibody gave false-negative results with a subpopulation of *Cmm* seed strains.

All 12 MAb Cmm1-negative seed strains were non-virulent on tomato cv. Kewalo (Table 4). Of

Table 3. Seed lots tested for *Cmm* infestation

Seed lot	Grams tested (assays)	Total colonies[a]	Colonies tested		Colonies reacting with MAb Cmm1[d]
			YSC[b]	ELISA[c]	
CA-00	395 (10)	10,522	427	39	7
MC-00	175 (7)	3615	1600	1161[e]	3
OH-01	100 (4)	139	100	100	12
OR-01	10 (2)[f]	730	10	9	7
OR-02	0.027[g]	156	42	36	31

[a]Recovered on CMM1 semi-selective medium.
[b]Transferred from CMM1 medium and observed for colony morphology on YSC.
[c]Transferred from YSC medium and tested by ELISA.
[d]MAb Cmm1 = clone 103–142; tested using an indirect ELISA.
[e]Only 205 colonies exhibited *Cmm*-type morphology on CMM1 medium. A large number of colonies from seed lot MC-00 were tested even when they did not have typical *Cmm* morphology. This was done to determine whether any additional colonies would react with MAb Cmm1.
[f]In addition to 10 g, 10 seeds were also individually assayed.
[g]Ten seeds were individually assayed.

the 32 MAb Cmm1-positive strains, 15 produced typical canker symptoms on cv. Kewalo and were considered virulent. An additional 15 strains did not elicit canker or wilt symptoms, and were considered putatively non-virulent. The remaining two strains produced large cankers but no leaf wilt, and were considered putatively hypovirulent. Replicate inoculations of the virulent strains showed variability in virulence ratings between replicate plants and among experiments; therefore all strains were given an average virulence rating and grouped into broader categories (non-virulent, hypovirulent and virulent). Putatively non-virulent ('0') and hypovirulent ('1') strains gave consistent results in multiple experiments, and virulent strains always gave average values of 2 or more.

All virulent strains were identified as *C. michiganensis* by the Biolog database (Table 4). All hypovirulent strains and the 15 MAb Cmm1-positive non-virulent strains were identified as either *C. michiganensis*, with similarity index values of 0.5 or above, or *Clavibacter* sp., with *Cmm* or *Cm* subsp. *tessellarius* (*Cmt*) as the top two likely identifications. The 12 MAb Cmm1-negative, non-virulent strains were identified as *Sanguibacter keddieii*, various *Microbacterium* or *Curtobacterium* species, or were not identified at all due to the dissimilarity of their metabolic profiles (similarity index values of less than 0.5) to any profile in the GP database.

A representative sampling of non-virulent (8), hypovirulent (1) and virulent (4) seed strains identified as *C. michiganensis* by the Biolog system were also identified as *C. michiganensis* by 16S rDNA sequencing (Table 4). The 12 seed strains that were not identified by the Biolog database or were identified as species other than *C. michiganensis* were identified by 16S rDNA sequencing as other coryneform species, including some in the *Microbacterium* and *Plantibacter* genera. In only two of the 12 cases did Biolog identifications of the MAb-negative seed strains match 16S rDNA identifications at the genus level; the remaining 10 strains were identified as different genera by the two tests.

Discussion

MAb Cmm1, an antibody that was specific for *Cmm* in initial assays (Alvarez et al., 1993), reacted to 99% of 236 *Cmm* strains representing populations from around the world. Characterization of the MAb Cmm1 antigen indicated that the epitope is associated with EPS, heat-stable and present among most of the *Cmm* strains tested. Previously, van den Bulk et al. (1991) found *Cmm* to possess EPS with a tetrasaccharide-repeating unit most likely identical to the EPS of *Cmi*, but with additional *O*-acetyl and succinyl substituent groups that the *Cmi* EPS lacked. MAb Cmm1 did not react with the single *Cmi* strain during initial screenings, implying that the *Cmm*-specific epitope is not associated with the major repeating unit of EPS, but could be associated with the substituent groups. MAb Cmm1 reactivity is identical for both

Table 4. Comparison of identifications obtained for bacteria isolated from seed using the Biolog system and 16S rDNA sequence analysis

Strain	MAb Cmm1 reactivity	Virulence	Biolog identification[a]	16S rDNA identification[b]
C117, C131A, C202, C203, C204, C205, C206, C207, C219, C220, C221, C222, C224, C225, C226	+	Virulent	*C. michiganensis* (0.51–0.83)	*C. michiganensis* (99–100%)
C126B, C215	+	Hypovirulent	*Clavibacter* sp./*C. michiganensis* (0.46–0.69)	*C. michiganensis* (99%)
C6A, C55, C59A, C63, C74A, C90A, C91A, C208, C209, C210, C211, C216, C217, C218, C223	+	Non-virulent	*Clavibacter* sp./*C. michiganensis* (0.3–0.95)	*C. michiganensis* (98–100%)
C2	–	Non-virulent	No ID	*Plantibacter agrosticola* (98%)
C24-A	–	Non-virulent	*Microbacterium saperdae* (0.58)	*Microbacterium* sp. (98%)
C25-A	–	Non-virulent	No ID	*Microbacterium arborescens* (99%)
C26	–	Non-virulent	No ID	*Subtercola pratensis* (100%)
C28	–	Non-virulent	No ID	*Plantibacter agrosticola* (99%)
C44	–	Non-virulent	*Microbacterium laevaniformans* (0.64)	*Curtobacterium* sp. (99%)
C49	–	Non-virulent	*Sanguibacter keddieii* (0.69)	*Subtercola pratensis* (99%)
C57	–	Non-virulent	*Curtobacterium citreum* (0.63)	*Plantibacter agrosticola* (99%)
C65	–	Non-virulent	*Curtobacterium pusillum* (0.64)	*Plantibacter agrosticola* (99%)
C88	–	Non-virulent	No ID	*Plantibacter agrosticola* (98%)
C92	–	Non-virulent	*Curtobacterium pusillum* (0.55)	*Rathayibacter rathayi* (99%)
C140	–	Non-virulent	*Microbacterium laevaniformans* (0.66)	*Microbacterium* sp. (99%)

[a]Identifications obtained using the Biolog MicroLog3™ microbial identification system (Biolog, Inc., release 4.20.04) with GP database release 6.11. Numbers in parentheses indicate similarity index values (for single strains) or ranges in values (for multiple strains) of the test strains to closest reference metabolic patterns.
[b]Most significant alignment obtained using BLASTN search of the NCBI database. Numbers in parentheses indicate percentage sequence homology (for single strains) or ranges in homology (for multiple strains) to the closest database matches.

young and old cultures, and the antigen is produced when cells are grown on any of the media tested or when repeatedly subcultured on different media (data not shown). This differs from the antigen of *Xanthomonas campestris* pv. *oryzae* recognized by MAb Xco-5, which is differentially expressed on various media and in cultures of different ages (Benedict et al., 1989). Boiling *Cmm* cultures prior to assay enhances reactivity to MAb Cmm1 and this property can be exploited when developing protocols using this MAb, as amplification of the positive signal would enhance reactivity for detection of the pathogen at low concentrations.

Expression of the MAb Cmm1 epitope is not linked to pathogenicity, as consistent differences were not seen between ELISA data and bioassay results. Thirty-two strains from the worldwide culture collection reacted with MAb Cmm1, but were found to be non-virulent on tomato cv. Kewalo. Further characterization with the Biolog system and 16S rDNA sequencing identified them as *C. michiganensis*. It is interesting to note that both Biolog analysis and 16S rDNA sequence analysis only accurately identified strains of *Cmm* down to the species level. Subspecific identifications varied, with Biolog identifying even virulent *Cmm* as *Cmt* a closely related subspecies pathogenic on winter wheat but not on tomato (Carlson and Vidaver, 1982). Identifications done with 16S rDNA sequencing also indicated that this method occasionally gave variable subspecies identifications, and, like the Biolog system, was also considered accurate only at the species level.

As noted in the results, a few strains had pigmentation differences or atypical (dry or sticky) colony consistencies, indicating a difference in EPS composition from the typical fluidal strains. Bermpohl et al. (1996) classified seven *Cmm* strains based on differences in EPS composition: strains that fell into Group I possessed EPS similar to that found by van den Bulk et al. (1991) and were capable of colonizing the tomato host. Strains in Group II, on the other hand, possessed EPS that varied from typical and were incapable of efficient plant colonization. Based on preliminary *in planta* experiments, at least a few of the putative non-virulent isolates possessing atypical EPS are incapable of *in planta* colonization. These strains would most likely fall into Group II, and this would suggest that MAb Cmm1 reacts with a moiety common among the EPS types.

It is possible that the non-virulent strains possessing typical EPS may cause symptoms on different tomato cultivars and thus actually be virulent; however, a few have been tested elsewhere on other cultivars with identical results (Louws et al., 1998). Indeed, natural populations of non-virulent *Cmm* have been noted previously (Fulbright et al., 1998; Werner, 2001). In some cases, the non-virulent state corresponds to loss of one or both of the known *Cmm* pathogenicity plasmids (Meletzus et al., 1993). However, this is not always true, and additional genetic factors required for pathogenicity are slowly becoming understood (Gartemann et al., 2003).

Only two of the original 236 *Cmm* strains from around the world were non-reactive with MAb Cmm1, representing less than 1% of the total strains tested. Both were virulent on tomato, but exhibited atypical dry colony phenotypes on solid media, which indicated greatly reduced or a complete lack of EPS production *in vitro*. In other studies, MAbs produced against typical, mucoid strains of *Cms* also failed to react optimally with non-mucoid strains of the subspecies (Baer and Gudmestad, 1993). The frequency of these MAb Cmm1-negative, atypical virulent strains in natural *Cmm* populations is unknown, but is probably low, based on results from this study. It is also important to note that while these two atypical virulent strains were non-reactive with MAb Cmm1, 17 other virulent strains also possessing atypical colony morphology did react with the antibody. Since MAb Cmm1 was successful at identifying *Cmm* strains exhibiting a range of atypical colony morphologies, the specificity of the antibody should be adequate for most detection and identification purposes.

In particular, MAb Cmm1 is quite suited for use in seed health assays as a confirmatory tool for colonies that have been isolated on semi-selective media and chosen for further analysis based on colony morphology (e.g., European and Mediterranean Plant Protection Organization [EPPO] phytosanitary standards [EPPO, 2005]; International Seed Health Initiative [ISHI] seed health testing method [http://www.worldseed.org/ISHI-Veg_Manual.htm]). In this study, MAb Cmm1 correctly identified 32 *Cmm* strains isolated from

seed that were later confirmed to be *C. michiganensis* by the Biolog system and 16S rDNA sequencing. It also accurately differentiated them from 12 other strains that possessed similar colony morphologies on CMM1 semi-selective medium but were identified as species other than *C. michiganensis* by Biolog or 16S rDNA sequencing. Based on these results, it appears that MAb Cmm1 can accurately differentiate *Cmm* from seed saprophytes possessing similar colony morphologies and would be useful for confirming the identities of presumptive *Cmm* colonies from seed.

MAb Cmm1 did not react to numerous bacteria from seed assays that did not possess *Cmm*-type morphology on semi-selective media. Systematic ELISA testing of 956 colonies from the naturally infested seed lot, MC-00, none of which exhibited *Cmm*-type morphology, revealed no MAb Cmm1-positive colonies among those not considered 'typical *Cmm*' types. However, screening of the same colonies with MAb Cmm3 revealed 21 reactive colonies that did not possess typical *Cmm*-type morphology. This is consistent with earlier results that indicated MAb Cmm3 showed less specificity for *Cmm* than MAbs Cmm1 and Cmm2.

The stability and abundance of the reactive epitope makes MAb Cmm1 amenable for use in a number of immunoassay formats. Unconjugated MAb Cmm1 has been successfully integrated into indirect ELISA and IF procedures in our laboratory. Unlike some *Cms* MAbs (Halk and De Boer, 1985), MAb Cmm1 remains active after conjugation to FITC, and has worked well in direct immunofluorescence (IF) and immunofluorescence colony (IFC) staining. Veena and van Vuurde (2002) also found success with this MAb in an indirect IFC. This MAb is available in a *Cmm* bacterial reagent set (previously the *Cmm* Bacterial ID test kit) and the *Cmm* ImmunoStrip (both from Agdia, Inc., Elkhart, IN), and these formats have been compared for use in detection of *Cmm* in infected plants (Sally Miller, unpublished data).

Additional *Cmm* identification tools include PCR primer sets CMM-5/CMM-6 (Dreier et al., 1995) and Cm_3/Cm_4 (Sousa Santos et al., 1997). Early findings indicate that the PCR reactions using these primers had a larger percentage of false negative results than tests with MAb Cmm1 (Alvarez and Kaneshiro, 1999; Kaneshiro and Alvarez, 2001). A more comprehensive comparison of DNA-based methods using international strains from the present culture collection as well as the seed strains isolated from this study is in progress. Further characterization of the 15 presumptive non-virulent *Cmm* strains from seed is also being done to determine whether the non-virulent phenotype correlates to the presence or absence of described *Cmm* pathogenicity factors.

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