Characterization of Fatty Acid Methyl Ester Content of *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

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Profiles of fatty acid methyl esters (FAMEs) obtained from strains of *Clavibacter michiganensis* subsp. *michiganensis* from Canada, the People's Republic of China, Thailand, Taiwan, and the United States were analyzed by gas-liquid chromatography. The FAMEs of 45 such strains were highly similar and were characterized by the presence of saturated, branched-chain C12 to C17 fatty acids. Other FAMEs occurring in significant amounts were the saturated straight-chain fatty acids 12:0 and 16:0. The presence of unsaturated branched-chain a15:1, anteisopentadecenoic acid, was highly specific for *C. m. michiganensis*. When the amount of a15:1 exceeded 2.56% of the total profile, the confidence level for identification was $P = 0.10$. Other qualitative and quantitative differences in FAMEs also were used to distinguish *C. m. michiganensis* from morphologically similar gram-positive bacteria that grew on semiselective media. When plotted three dimensionally, FAMEs defined a spatial relationship among strains of *C. m. michiganensis*. The technique was used as an accurate and rapid method for the characterization of bacteria recovered from irrigation ponds, seed, soil, transplants, and weeds.

Additional keywords: bacterial canker of tomato, *Lycopersicon esculentum*.

The ability to identify microorganisms with a high degree of confidence is essential for most epidemiologic studies. The use of semiselective media has been valuable for the recovery of certain plant pathogens from the environment (1,14,16,21,26); nonetheless, a diverse array of microorganisms is capable of growth on most of these media. This is especially true from sources such as pond water or soil. Most conventional tests and procedures (7) used for the characterization of bacteria are time-consuming, limited by the number of samples that can be processed, and often require long periods of incubation (7–21 days). It has been our experience that traditional microbiological tests are of limited value for the identification of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato (*Lycopersicon esculentum* Mill.). Gram-positive bacteria that appear similar on semiselective media, such as SCM (5), KBST (4), and CNS (12), are particularly difficult to differentiate from *C. m. michiganensis*. Vidaver and Starr (31) stated the need for pathogenicity tests in the identification of most phytopathogenic coryneform bacteria; we concur with that opinion. However, the need for a constant supply of plants, the long latent period of bacterial canker, and the danger of contamination from an outside source of inoculum or from other test strains, make identification by pathogenicity testing laborious, time consuming, and subject to error. It is particularly difficult to identify *C. m. michiganensis* from samples other than host material or from plants or plant parts that have no symptoms. This has been a problem for the Georgia Department of Agriculture, which conducts a Plant Certification Program for the vegetable transplant industry. To date, the Plant Certification Program has had to deal with separating *C. m. michiganensis* from nonpathogenic bacteria associated with either seed, symptomless plants, plants with multiple infections, or soil. Test results are used for certification of seed and plants or for land management decisions. Plant certifications affect the interstate and international shipment of plants to the northern United States and Canada. Hence, there is a need for an accurate and reliable method for the rapid identification of this organism.

Lipid analysis has been used to classify various microorganisms in numerous studies (2,13,15,20,22,23). In particular, the analysis of fatty acid methyl esters (FAMEs) has been used for the identification and classification of bacteria (3,24,29,30). Sasser et al (25) have constructed a vast library of fatty acid profiles, including a profile of *C. m. michiganensis*. Fieldhouse and Miller (6) reported on the taxonomic importance of FAME content in coryneform bacteria, including *Clavibacter*. However, these analyses were based primarily on a small number of samples from recognized culture collections and on a medium (trypticase-soy-agar) typically not used for culture of *C. m. michiganensis*. Consequently, there was a need to evaluate the technique on a larger number of samples from diverse sources and for samples grown on CNS (12) semiselective medium, which is commonly used for the culture of *C. m. michiganensis*. In this paper the FAMEs of 45 strains of *C. m. michiganensis* from various geographical locations are characterized to create a data base for comparison with bacteria of unknown identity. The quantity and pattern of FAMES of *C. m. michiganensis* then were contrasted with FAME profiles for morphologically similar appearing bacteria of unknown identity recovered from diseased tomatoes, symptomless transplants, irrigation ponds, seeds, soil, or weeds. Finally, the technique was assessed for its usefulness in the identification of the tomato canker bacterium in the Georgia Plant Certification Program. An abstract of this work has been published (10).

MATERIALS AND METHODS

Bacterial strains. The origin of strains of *C. m. michiganensis* (CM) was as follows: CM-0, D. Emmatty, Heinz, U.S.A., Ohio; CM-1, CM-2, and CM-3, D. Cuppels, Ontario, Canada; CM-4, CM-5, and CM-6, G. Bonn, Ontario, Canada; CM-7 through CM-18, M. Ricker, Campbell Institute of Research and Technology, Ohio; CM-19, CM-20, and CM-21, H. Bolkan, Campbell Institute for Research and Technology, Davis, CA (strains originated in People's Republic of China); and CM-22 through CM-44, R. Gitaitis, Georgia (strains originated in Florida, Georgia, North Carolina, Ohio, Taiwan, and Thailand). A total of 341 test strains were isolated and compared to the 45 known

strains of *C. m. michiganensis* listed above. Seventeen, 37, 38, 42, and 108 bacterial test strains were isolated from commercial fields from roots and leaves of the rye cover-crop (R1–R17), soil (D1–D37), irrigation water (I1–I38), weeds (W1–W42), and tomato plants (P1–P108), respectively. Plant samples included specimens from commercial fields of either staked or processing tomatoes in Florida, Georgia, North Carolina, and Ohio as well as from transplants in the Tifton area. An additional 99 bacterial test strains (S1–S99) were isolated from more than 400 tomato seed lots tested by the methods of Fatmi and Schaad (5). A working culture of each strain was maintained in 2 ml of sterile tap water in a screw cap vial at room temperature. Long-term storage of strains of *C. m. michiganensis* was done by freezing turbid bacterial suspensions in 15% glycerol at −73 C and by lyophilization in commercially available evaporated milk (8).

Preparation of FAME samples. Previously reported methods for preparation and analysis of FAMEs from cellular fatty acids were used (11,17–19). Bacteria were grown routinely on CNS (12), with the modification that lithium chloride was deleted (27), at 30 C for 48–72 hr. A loopful of bacteria was added to 1 ml of 1.2 N NaOH in 50% aqueous methanol in a screw cap tube and saponified for 30 min at 100 C. After cooling, samples were sonicated in a sonic water bath for 5 min and then acidified with 0.5 ml of 6 M HCl (final pH 2). Samples were methylated with 1 ml of 12% BCl_3 in methanol and incubated at 100 C for 4 min. After cooling, FAMEs were extracted with 1 ml of a hexane and diethyl ether mixture (1:1). After gentle mixing (3 min), the lower aqueous phase was removed. The organic phase was washed by gentle agitation (end-over-end five times) with 3 ml of 0.3 N NaOH. After separation of phases, the upper organic phase was removed for analysis. FAMEs were analyzed by gas-liquid chromatography with a Hewlett-Packard model 5710 gas chromatograph equipped with a 30-m × 0.25-mm phenyl methyl silicone fused silica capillary column. Before analysis of samples, the gas chromatograph was calibrated with a commercial FAMEs mix (Supelco, Bellefonte, PA).

Biochemical and physiological tests. All strains were tested for gram reactions (28) and responses in litmus milk (7). In addition, appearance on CNS semiselective medium (12) modified by omission of lithium chloride (27) was used for preliminary characterization of all test strains.

Pathogenicity tests. Bacteria were grown on CNS for 48–72 hr at 30 C. Suspect colonies were picked with the point of a sterile toothpick that was used to stab into the leaf axil of a tomato seedling grown in the greenhouse. Signs of unilateral wilting of leaflets in 2–3 wk and internal discolorations of the vascular tissues and/or pith in stems split lengthwise after 4–5 wk were recorded as positive for pathogenicity. In addition, all strains were evaluated for the ability to elicit a hypersensitive reaction (HR) in leaves of four-o'clock (*Mirabilis jalapa* L.), an indicator plant for *C. m. michiganensis* (9). Intercellular areas of mature leaves of greenhouse-grown *M. jalapa* were infiltrated with an aqueous suspension (= 10^8 cfu/ml) of each bacterial strain by injection with a hypodermic needle (27 gauge) and syringe. Plants were incubated for 24–48 hr at 22–38 C and results were recorded at that time.

RESULTS

All 45 strains of *C. m. michiganensis* from various geographical locations were characterized by the presence of isopentadecanoic acid (i15:0), anteisopentadecanoic acid (a15:0), isopalmitic acid (i16:0), and anteisoheptadecanoic acid (a17:0), which are saturated branched-chain fatty acids. The prefixes of iso (i) and ante (a) indicate the attachment of a methyl group to the second and third carbon atom of the carbon chain, respectively, when numbering at the end opposite the carboxyl group. Other FAMEs occurring in significant amounts were the saturated straight-chain fatty acids lauric acid (12:0) and palmitic acid (16:0). The presence of anteisopentadecenoic acid (a15:1), an unsaturated branched-chain fatty acid with 15 carbon atoms and one double bond, was highly diagnostic for *C. m. michiganensis*; its presence alone could be used for tentative identification. When FAME profiles of *C. m. michiganensis* were compared with those of other microflora encountered in this study, the confidence level for identification was $P = 0.10$ if a15:1 exceeded 2.56% of the total FAME content. All 45 strains produced an HR in leaves of *M. jalapa* and typical bacterial canker symptoms when inoculated into tomato.

Out of the total of 341 test strains recovered on semiselective media from various habitats and categorized as "suspects," none from rye, soil, irrigation water, or weeds were identified as *C. m. michiganensis*. Twenty-six and nine strains recovered from tomato plants and seed, respectively, had a FAME profile where all FAMEs fell within ± two standard deviations for the population ($n = 45$) of *C. m. michiganensis*. All 35 of these produced an

TABLE 1. Values (percentage of total fatty acid methyl esters [FAMEs]) for *Clavibacter michiganensis* subsp. *michiganensis* contrasted with values generated for various microflora with similar colony characteristics on semiselective media

Strain	Relative % fatty acid methyl esters present								
	12:0	a15:1	i15:0	a15:0	i16:0	16:0	a17:0	Others[a]	HR[b]	Path.[c]
CMM[d]	1.8 ± 3.2	8.5 ± 13.2	0.9 ± 1.2	40.9 ± 13.8	13.9 ± 7.0	3.7 ± 3.5	21.3 ± 10.0	0.0	+	+
W-1	0.7	0.0	7.6	41.3	31.2	1.9	10.9	0.0	−	−
W-5	0.6	1.3	3.2	46.5	22.7	1.3	24.7	0.0	−	−
W-9	1.0	0.0	0.0	34.3	33.2	5.4	2.2	0.0	−	−
W-11	0.7	0.0	8.9	41.0	17.0	2.0	24.7	0.0	−	−
W-17	1.2	0.0	3.5	38.7	30.2	1.4	19.3	0.0	−	−
W-19	5.2	0.0	3.2	40.0	23.9	2.1	26.9	0.0	−	−
S-3	31.7	0.0	0.0	0.0	0.0	8.4	0.0	49.7	−	−
S-6	0.7	0.3	4.5	47.1	14.3	1.9	26.2	0.0	−	−
S-10	0.4	0.0	1.0	39.5	32.9	1.4	21.6	0.0	−	−
S-15	0.5	0.0	4.2	38.5	15.7	1.5	26.2	0.0	−	−
S-19	0.5	0.0	0.0	0.0	0.2	14.9	0.2	61.1	−	−
S-22	6.4	0.0	0.0	0.0	0.0	9.4	0.0	76.3	−	−
S-31	20.2	0.0	0.0	0.0	0.0	8.7	0.0	49.1	−	−
S-35	1.0	3.1	0.8	54.3	10.7	2.7	13.6	0.0	+	+
S-38	1.6	5.8	2.0	32.6	8.6	3.8	16.6	0.0	+	+
P-4	3.2	6.2	0.8	45.6	15.6	2.6	24.6	0.0	+	+
P-25	0.8	15.3	1.3	39.6	15.4	2.5	22.5	0.0	+	+
P-42	2.7	1.6	0.0	40.9	12.4	4.7	17.9	0.0	+	+
P-44	1.9	2.5	1.1	34.0	9.1	4.2	12.1	0.0	+	+

[a] Represents a variety of other FAMEs that are not commonly found in *C. m. michiganensis*.
[b] + indicates strain induced hypersensitive reaction in four-o'clock within 48–72 hr, − indicates that strain failed to induce any visible reaction.
[c] Pathogenicity test in tomato; + indicates strain induced typical systemic symptoms associated with bacterial canker, − indicates no reaction.
[d] Values represent the mean ± two standard deviations of 45 reference strains of *C. m. michiganensis*.

HR in leaves of *M. jalapa* and produced typical symptoms of bacterial canker when inoculated in tomato. Twenty-three of the positive strains recovered from plants were isolated from mature plants (cultivars for either fresh market or processing use) from Florida, Georgia, North Carolina, and Ohio that displayed typical bacterial canker symptoms. Three strains identified as *C. m. michiganensis* were isolated from symptomless transplants from Georgia after plants had been inspected and certified. The remaining 306 strains from all habitats had one or more FAMEs fall outside the ± two standard deviations established for the 45 reference strains, failed to elicit an HR in leaves of *M. jalapa*, and were not pathogenic on tomato. FAME profiles of representative examples are contrasted with the FAME profile of *C. m. michiganensis* in Table 1. In addition, the graphic representation of the percent content of a15:1, i16:0, and i15:0 and the ratios of the % a15:1/% i15:0, % i16:0/% a15:1, and % 12:0/% i15:0 indicate a relationship among strains of *C. m. michiganensis* and no apparent relationship with saprophytic strains (Fig. 1).

DISCUSSION

Certain profiles of various test strains (examples: S-3, S-19, S-22, and S-31) contained high percentages of FAMEs not found in *C. m. michiganensis* and were vastly different from those of the canker bacterium. A glance at their profiles would readily identify those strains as being different from *C. m. michiganensis*. Other strains (examples: W-1, W-5, W-9, W-11, W-17, W-19, S-6, S-10, and S-15) were related more closely to *C. m. michiganensis* in terms of their FAME profile. However, all of these strains had one or more FAME values that fell outside the ± two standard deviations established for the pathogen. These differences used in conjunction with the confidence level established for a15:1 ($P = 0.10$ if a15:1 exceeded 2.56% of the total FAME content) were used to make tentative identifications.

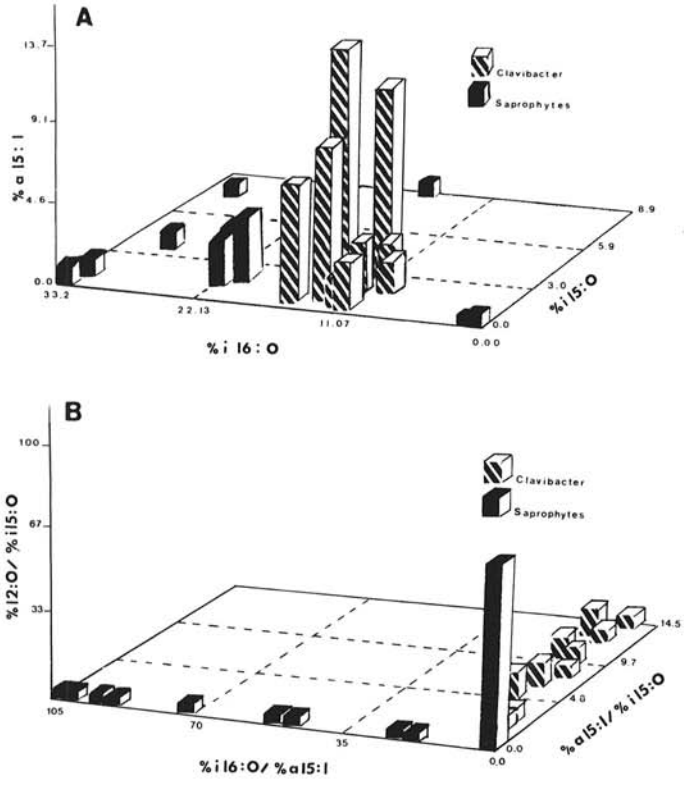

Fig. 1. Three-dimensional plot of relationships of *Clavibacter michiganensis* subsp. *michiganensis* and saprophytic *Clavibacter*-like bacteria based on *x*, *y*, and *z* coordinates of **A,** percent content of certain fatty acid methyl esters (FAMEs) and, **B,** ratios of percent content of selected FAMEs.

Furthermore, the clustered-distribution of the spatial coordinates of the percent content of a15:1, i16:0, and i15:0 in *C. m. michiganensis* demonstrates a relationship among these strains compared with saprophytic strains. When ratios of key FAMEs were plotted, the distribution of spatial coordinates again indicated a relationship among strains of *C. m. michiganensis* as they plotted along the *X* axis (% a15:1/% i15:0). In contrast, all saprophytic strains plotted along the *Y* axis (% i16:0/% a15:1).

Until now, the lack of a rapid and reliable method for identification of *C. m. michiganensis* has been a problem for certification of both seed and transplants in the Georgia plant certification program. Analysis of FAMEs by gas-liquid chromatography of suspect bacteria recovered from semiselective media appears to be a promising method for reliable identification of the bacterial canker pathogen. The technique is currently recommended for identification of plant pathogenic bacteria in the Georgia plant certification program. It has proven highly reliable and all identifications to date by this technique have proven valid when supported by HR and pathogenicity tests.

Although other species of *Clavibacter* and subspecies of *C. michiganensis* were not analyzed in this study, it is unlikely that they would be associated with tomato transplants or seed and result in misidentification of the pathogen. Generally the other recognized subspecies of *C. michiganensis* are considered unimportant in Georgia; if they ever were to present a problem it is most probable that these subspecies would be isolated as residents from a nonhost or from soil. Until this supposition can be tested and the profiles of other pathogenic *Clavibacter* are analyzed for comparison, the identity of an organism by this method should be considered tentative and be confirmed with other methods. However, to date all bacteria tentatively identified as *C. m. michiganensis* by FAMEs analysis have been confirmed by HR and pathogenicity in tomato.

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