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MORPHOLOGICAL CHARACTERIZATION AND GENETIC DIVERSITY OF CLAVIBACTER MICHIGANENSIS SUBSP. MICHIGANENSIS IN TOMATO-PRODUCING AREAS IN MEXICO

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Abstract: *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is the bacterium that causes bacterial canker in tomato (*Solanum lycopersicum* L.). This disease is one of the main factors limiting the production of this crop both in open fields and in greenhouses. The objective of this research was to determine the phenotypic and genotypic variability in different strains of *Cmm* isolated from the main tomato-producing areas of Mexico. A descriptive study was carried out from October 2015 to April 2019 and included samplings from different tomato-producing areas as well as the inclusion of *Cmm* strains from the Phytopathology Laboratory of the Center for Research in Food and Development, AC, collected from 2015-2016. The samples obtained were isolated in artificial culture medium; subsequently, they were phenotypically characterized by colour, size, mucus type, pathogenicity and virulence, and finally, the genes associated with the pathogenicity of the bacteria and that are located in the plasmids (*CelA* and *Pat-1*) were amplified by PCR of the pathogenicity island in the chromosome (*tomA*, *chpC*, *ppaA*). In addition, sequencing of the ITS region of the 16S rRNA gene of the isolated strains was performed to carry out phylogenetic analysis. In the study period, 60 strains of *Cmm* that showed diversity in colour, size and colonial mucus type were isolated. The strains were classified as orange yellow (5 strains), yellow (30 strains) and cream yellow (25 strains). They were classified as small (8 strains), medium (27 strains) or large (25 strains); 50 strains presented a nonmucoid consistency and 10 presented a mucoid consistency. All the strains in the study were pathogenic but with different degrees of virulence. The Cmm9 and Cmm68 strains were highly virulent. Meanwhile, the Cmm84 and Cmm98 strains showed a lower degree of virulence, presenting a delay of approximately 7 days before the appearance of the first symptoms of bacterial canker in the tomato plants. For all the strains except for Cmm84 and Cmm98, all the genes associated with pathogenicity were amplified; for the Cmm84 and Cmm98 strains, the *Pat-1* gene (located in the pCM2 plasmid), which is directly associated with the induction of disease symptoms, was not amplified. This finding could be associated with the reduction in the virulence of these strains. The phylogenetic analysis of the ITS region of the 16S rRNA gene of the *Cmm* strains shows the formation of 8 groups, corroborating the genetic diversity of this bacterium. The results of this research provide information about the phenotypic and genotypic variability in *Cmm*, which could mean that bacterial canker outbreaks can be caused by a complex of clones introduced to Mexico from different geographical locations over time.

Keywords: Tomato, *Cmm*, Genotypic variability, Phenotypic variability, Pathogenicity.

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is a gram-positive aerobic bacterium that lacks flagella and is rod shaped (Smith, 1910; Davis *et al*., 1984). It is the causal agent of bacterial canker in tomato (*Solanum lycopersicum* Mill.) In Mexico, it has become one of the devastating diseases of this crop. In recent years, it has caused production losses of up to 70% (García, 2009). In Sinaloa, *Cmm* has caused losses estimated at 40 million dollars because the control methods applied thus far have not been sufficient (Lara-Avila, 2012). Due to the severity of production and economic losses, *Cmm* is considered a quarantined organism by the European Union and many other countries (De León *et al*., 2008). This disease was first described in 1910 in Michigan, USA. *Cmm* can develop at temperatures of 20-30 °C and is able to survive

at temperatures up to 50 °C; its optimal growth has been recorded at 25 °C. The bacteria can develop in artificial media with a slow growth rate; after 3-7 days, a small colony begins to be visible. The optimal pH for the growth of *Cmm* is between 7 and 8, but it can develop in the xylem of plants at pH 5 (Eichenlaub *et al.* 2006). The main source of dissemination over great distances is seeds, from which *Cmm* is transmitted to plants and then to the crop in general. The seed-seedling transmission rate can vary from 0.25 to 85%, and a density of five bacterial cells per individual can result in a diseased seedling, which will show leaf wilting, the most common symptom, during the early stages of disease development. Later, the stems and petioles darken, and in infected fruits, a spot known as a "bird's eye" is occasionally observed; this spot appears as a small dark area surrounded by a white halo. In the final stage of the disease, the entire plant withers and dies (Sen *et al.* 2015).

(Lelis *et al.* 2014). Secondary infection can take place once the crop is established and occurs mainly by cultural practices such as pruning, guarding, contact between diseased and healthy plants, splashing during pesticide applications, and irrigation (Ricker and Riedel 1993; Carlton *et al.* 2008). In recent years, there have been great advancements in the understanding of the mechanism of pathogenicity of *Cmm* and the interaction it maintains with its host during this process. It is known that *Cmm* generally contains two circular plasmids (pCM1 and pCM2) that present essential genes for pathogenicity in tomato. In the chromosome of the bacterium, a "pathogenicity island" has been identified, characterized by two regions: the *chp* region, which conserves several serine proteases, and the *tomA* region. Studies focused on the description of the pathogenesis of *Cmm* have improved the characterization of *Cmm* populations in different tomato-producing regions globally, thus allowing us to understand the pathogenic potential of the strains through the detection and characterization of these genes. PCR techniques coupled with the development of new technologies allow the analysis of the genetic diversity of populations through bioinformatic analysis from sequencing (16S ribosomal gene), pulsed field gel electrophoresis (PFGE), and box-PCR, among others. In all the tomato-producing areas in Mexico, bacterial canker is present in each production cycle; therefore, the objective of this study is focused on the morphological characterization and genetic diversity of different strains of *Clavibacter michiganensis* subsp. *michiganensis* isolated from tomato crops (*Solanum lycopersicum* L.) in the main tomato-producing areas of Mexico.

MATERIALS AND METHODS

ISOLATION AND PURIFICATION OF *CMM*

Tomato plants with foliage that showed symptoms corresponding to bacterial canker were selected, and small cuts were made with a scalpel in the phloem and xylem tissue; then, the observation of bacterial flow was carried out under a biological microscope (Carl Zeiss Primo Star). The isolation and purification of the *Cmm* strains was performed by seeding in Mueller Hinton culture medium and subsequent incubation at 27 °C.

CONSERVATION OF THE BACTERIA

The pure strains of the isolates were preserved in phosphate buffer at pH 7.4 (NaCl, 8 g/L; KCl, 0.2 g/L; NA_2HPO_4, 1.44 g/L; and KH_2PO_4, 0.24 g/L) and stored at 4 °C for later use.

PATHOGENICITY TEST

To determine the pathogenicity of the strains isolated from the different tomato-

producing areas in Mexico, seedlings of saladette tomatoes (variety Moctezuma) were inoculated when they had 3 to 4 true leaves. The bacterial strains were obtained after 4 days of growth in Mueller Hinton culture medium with the help of a sterile wooden stick. For inoculation, a bacterial colony was transferred onto one of the leaf axils of the plant. The first evaluation was performed 26 days after inoculation (DAI), and a second evaluation was performed at 34 DAI. The reisolation of the bacterial strains was carried out based on the presence of symptoms in the inoculated plant, and the diseased tissue was transferred to Mueller Hinton culture medium.

VIRULENCE TEST

The virulence of the strains was analysed according to the morphological and molecular variability recorded. Of the total number of strains, a representative proportion (1/4 part) was selected for further analysis. For each strain, 16 tomato plants were inoculated by infiltration using an insulin syringe with a bacterial concentration of 9×108 CFU/mL, corresponding to a value of 3 on the MacFarland scale (10 µL per plant). Visual virulence assessment was performed daily until the first symptoms of the disease were observed (leaves of the upper part of the plants had yellow spots and a dry appearance and were curled). In total, evaluations were performed at 8, 11, 15 and 21 DAI. The evaluation of the incidence and severity or virulence was performed according to the severity scale proposed by Foster and Echandi (1973): 0 = healthy plant, 1 = plant with 1/3 wilted leaves, 2 = plant with 1/3-2/3 wilted leaves, 3 = plant with more than 2/3 wilted leaves and 4 = dead plant. The data generated were statistically analysed in the SAS program.

MORPHOLOGICAL CHARACTERISTICS

Morphological characteristics of the colonies that developed from the *Cmm* strains were recorded; for example, colouration was determined with a CM-700d spectrophotometer (Japan), which provided the HUE value of each of the strains. Mucus and size characteristics were also recorded depending on the diameter of the colony: small (1-2 mm), medium (2-3 mm) and large (> 3 mm). The analysis of these characteristics was performed after the strains were established in Mueller Hinton culture medium.

MOLECULAR ANALYSIS OF THE *CMM* STRAINS

The genomic DNA of the bacterial strains was extracted from pure colonies after 6 days of growth in Mueller Hinton culture medium by heat lysis of the bacterial cells according to the methodology proposed by Sousa *et al*., (1997). A total of 100 µL of sterile molecular grade SIGMA water was placed in a 2 mL microcentrifuge tube, and a bacterial colony was added to the liquid; subsequently, the solution was vortexed until it was homogeneous. The tubes were placed in a thermoblock at 95 °C for a period of 15 min and then placed on ice for 10 min. After incubating on ice, the tubes were centrifuged at 16,000 x *g* for 10 min; then, the supernatant was removed, and the pellet was resuspended in 40 µL of sterile molecular grade water. Finally, the reading was performed with a Fisher NanoDrop to verify the concentration and purity of the DNA. The final product was stored at -20 °C for later use.

DETECTION OF *CMM* STRAINS BY PCR

Specific detection by PCR of the strains isolated from the different sampled points was performed, and general primers were used for

the identification and confirmation of *Cmm*. The primers Cm3/Cm4 (Table 1) were used under the amplification conditions proposed by Sousa *et al*., (1997), with modifications of the alignment temperature and time in the three phases of the PCR (denaturation, alignment and elongation). PCR was performed with a Bio-Rad model T-100 thermocycler, and the process consisted of denaturation at 94 °C for 5 min; followed by 40 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

ANALYSIS OF PATHOGENICITY GENES OF *CMM*

The detection of the genes that are associated with the pathogenesis of *Cmm* was performed by endpoint PCR in a Bio-Rad T100 thermocycler. The oligonucleotides used in the study are shown in Table 1.

The detection of the CelA gene was performed under the conditions proposed by Kleitman *et al*. (2008) with modifications of the alignment temperature and time in each of the three phases of the PCR (denaturation, alignment and elongation). The PCR consisted of an initial denaturation of 94 °C for 1 min; followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. On the other hand, for the detection of *pat* gene-1 (wilt inducers), the protocol consisted of denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 5 min. For the PAI gene (pathogenicity island) ppaAR/ ppaAF, denaturation was performed at 94 °C for 1 min; this was followed by 35 cycles of 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 5 min. The protocol for the detection of the *chpC* gene (serine protease) included denaturation at 94 °C for 1 min; followed by 35 cycles of 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. Finally, the protocol for the detection of the *gene tomA* (tomatinase) consisted of denaturation at 94 °C for 1 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min.

All the PCR products were visualized in a 1% agarose gel in an electrophoresis chamber (BioRad) at 80 V and 400 mA for 80 min. The 100 bp molecular marker (Promega) was loaded into the first well of the gel, the next lane was loaded with the blank, and the samples of the 60 strains were loaded in the subsequent lanes. The gel was visualized in a Molecular photodocumenter (Imager Gel DOC XR + BioRad-USA).

AMPLIFICATION AND SEQUENCING OF THE 16S GENE OF THE FD2 AND RP1 REGIONS

The reconstruction of the phylogeny for the Cmm strains was carried out by amplification, sequencing and phylogenetic analysis of the 16S rRNA region according to the protocols reported by Weisburget *et al*. (1991) and McLaughlin *et al*. (2012), where the oligonucleotide pair FD2 (5'-AGAGTTTGATCATGGCTCAG-3') and RP1 (5'-ACGGTTACCTTGTTACGACTT-3') was used. The reaction mixture for the PCR (25 µL) used 12.5 µL of master mix green (Promega, USA), 1.0 µL of each oligonucleotide, 2 µL of DNA (20 ng) and 8.5 µL of molecular grade water. The PCR protocol was carried out in a BioRad T100TM thermal cycler (Singapore) with the following steps: initial denaturation at 95 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min.

To visualize the bands of the amplified DNA (approximately 1500 bp), the products were run on a 1% agarose gel stained with RedGel at a concentration of 1X at 80 V and

Oligonucleotides	Sequences de 5′ a 3′	gene	sizes of the products
CM3	CCTCGTGAGTGCCGGGAACGTATCC	Confirmation of *Cmm*	645 pb
CM4	CCACGGTGGTTGATGCTCGCGAGAT		
PFC3	GGTACGAAGTTCGAGACGAC	CelA	551pb
PFC5	TGTAGCGGTGAGTCGTGGTGA		
P5	GCGAATAAGCCCATATCAA	Pat-1	614pb
P6	CGTCAGGAGGTCGCTAATA		
chpcF	GCTCTTGGGCTAATGGCCG	chpC	639pb
chpcR	GTCAGTTGTGGAAGATGCTG		
ppaAF	CATGATATTGGTGGGGAAAG	ppaA	587pb
ppaAR	CCCCGTCTTTGCAAGACC		
tomAF	CGAACTCGACCAGGTTCTCG	toma	529pb
tomAR	GGTCTCACGATCGGTCC		
FD2	AGAGTTTGATCATGGCTCAG	16S Ribosomal	1500pb
RP1	ACGGTTACCTTGTTACGACTT		

Table 1. Oligonucleotides, sequences and sizes of the products used.

Figure 1. Geographic distribution of the *Cmm* strains isolated from the tomato fruit and plants collected in this study.

Strains	Location origin	coordenadas	Isolated from	Year of collection
Cmm01	Culiacán, Sinaloa	24.770991, -107.509072	Fruit	2015
Cmm02	Culiacán, Sinaloa	24.771477, -107.508660	Stem	2015
Cmm03	Culiacán, Sinaloa	24.766975, -107.512077	Stem	2015
Cmm04	Culiacán, Sinaloa	24.754918, -107.515113	Stem	2015
Cmm05	Culiacán, Sinaloa	24.765137, -107.509393	Stem	2015
Cmm06	Culiacán, Sinaloa	24.761590, -107.510947	Stem	2015
Cmm07	Culiacán, Sinaloa	24.760606, -107.508923	Stem	2015
Cmm08	Numarán, Michoacán	20.420180, -102.044606	Stem	2015
Cmm09	Numarán, Michoacán	20.419708, -102.044402	Stem	2015
Cmm12	Culiacán, Sinaloa	23.909094, -106.936968	Fruit	2015
Cmm13	Mexicali, Baja California	24.636801, -107.443423	Stem	2015
Cmm14	Sayula, Jalisco	19.875103, -103.602458	Stem	2015
Cmm15	Sayula, Jalisco	19.875052, -103.602466	Stem	2015
Cmm16	Guaymas, Sonora	27.923988, -110.887627	Stem	2015
Cmm19	Puebla, Puebla	18.897776, -97.787886	Stem	2015
Cmm20	Torreón, Coahuila	25.450312, -103.391641	Stem	2015
Cmm21	Villa de Arista, San Luis Potosí	22.670943, -100.914400	Stem	2015
Cmm26	Compostela, Nayarit	21.260966, -104.867463	Stem	2015
Cmm36	Compostela, Nayarit	21.256967, -104.872441	Stem	2015
Cmm38	Torreón, Coahuila	25.629024, -103.398036	Stem	2015
Cmm39	Sayula, Jalisco	19.905701, -103.563346	Stem	2015
Cmm41	Numarán, Michoacán	20.258982, -101.955276	Stem	2016
Cmm42	Zacatecas, Zacatecas	23.288335, -102.331566	Stem	2016
Cmm43	Ensenada, Baja California	30.577992, -115.925057	Stem	2016
Cmm48	Villa de Arista, San Luis Potosí	22.674644, -100.910858	Stem	2016
Cmm49	Rio Verde, San Luis potosí	22.675278, -100.911450	Stem	2016
Cmm67	Betulia, Jalisco	21.756472, -102.026218	Stem	2017
Cmm68	Ciudad Obregón, Sonora	27.392597, -109.930954	Stem	2017
Cmm69	Rio Verde, San Luis Potosí	22.010336, -100.121204	Stem	2017
Cmm70	San Miguel de Allende, Guanajuato	21.111518, -100.479824	Stem	2017
Cmm71	Jalisco, Jalisco	20.381534, -102.960134	Stem	2017
Cmm72	Estado de México, Estado de México	19.817548, -99.464687	Stem	2017
Cmm74	Villa de Arista, San Luis Potosí	22.674529, -100.910361	Stem	2017
Cmm76	Durango, Durango	25.529889, -103.530070	Stem	2017
Cmm78	San Miguel de Allende, Guanajuato	21.107810, -100.450172	Stem	2017
Cmm79	Culiacán, Sinaloa	24.885823, -107.416010	Stem	2018
Cmm80	Culiacán, Sinaloa	24.885406, -107.416871	Stem	2018
Cmm81	Culiacán, Sinaloa	24.887203, -107.416526	Stem	2018
Cmm82	Culiacán, Sinaloa	24.885953, -107.415464	Stem	2018

Cmm83	La Cruz de Elota, Sinaloa	23.901041, -106.935134	Stem	2018
Cmm84	Culiacán, Sinaloa	24.772606, -107.510822	Stem	2018
Cmm85	Culiacán, Sinaloa	24.764018, -107.509616	Stem	2018
Cmm92	San Miguel de Allende, Guanajuato	20.915314, -100.654126	Stem	2018
Cmm93	San Miguel de Allende, Guanajuato	20.915172, -100.644666	Stem	2018
Cmm94	La Cruz de Elota, Sinaloa	23.898491, -106.932044	Stem	2018
Cmm95	Culiacán, Sinaloa	24.771299, -107.512048	Stem	2018
Cmm97	La Palma, Navolato, Sinaloa	24.812939, -107.657894	Stem	2018
Cmm98	La Palma, Navolato, Sinaloa	24.812170, -107.657175	Stem	2018
Cmm101	Carr. Las puentes, Sinaloa	24.715725, -107.444918	Stem	2018
Cmm102	Carr. a Eldorado, Sinaloa	24.542092, -107.446825	Stem	2018
Cmm105	Culiacán, Sinaloa	24.769660, -107.520171	Stem	2018
Cmm106	Numarán, Michoacán	20.419168, -102.046124	Stem	2018
Cmm107	El Huizache, San Luis Potosí	22.155297, -100.984387	Stem	2018
Cmm108	Altata Navolato, Sinaloa	24.698245, -107.819331	Stem	2018
Cmm110	Villa de Arista, San Luis Potosí	22.673897, -100.911299	Fruit	2018
Cmm111	Altata Navolato, Sinaloa	24.696809, -107.834981	Stem	2018
Cmm113	Jalisco, Jalisco	20.378484, -102.960701	Stem	2018
Cmm114	San Ignacio, Sinaloa	23.946532, -106.427227	Stem	2019
Cmm115	Altata Navolato, Sinaloa	24.707974, -107.795014	Stem	2019
Cmm116	Jalisco, Jalisco	20.377374, -102.959566	Stem	2019

Table 2. Strains of *Clavibacter michiganensis* subsp. *michiganensis* coded by location, origin and year of collection.

Figure 2. seedlings with symptoms of Clavibacter michiganensis sussp. Michiganensis.

Figure 3. A): Plant inoculated with *Cmm* by infiltration. B): Plant with initial symptoms of *Cmm*. C): Plant killed by Cmm.

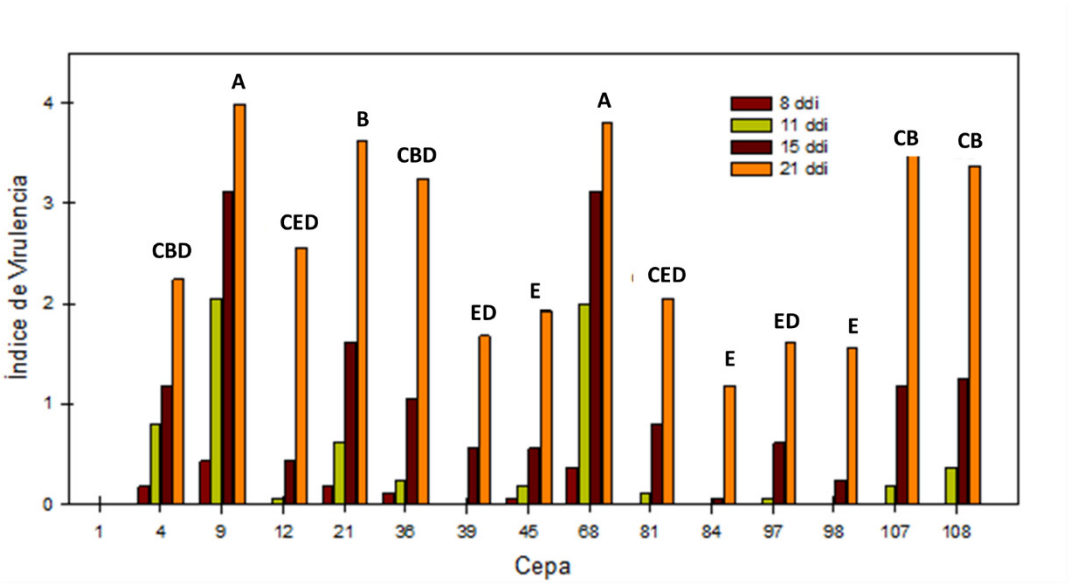

Figure 4. The virulence index values of the strains of *Clavibacter michiganensis* subs. *michiganensis* in tomato seedlings at 21 days after inoculation (DAI). A value of 1 on the X axis represents the negative control (water). The other values represent the identification code number of each isolate. The data are the average of four repetitions. Strains with the same letter are not significantly different ($P \geq 0.05$) according to the Tukey test.

Cepa	Mucoid	Size	Color	Cepa	Mucoid	Size	Color
Cmm1	-	S	Cream	Cmm71	-	M	Cream yellow
Cmm2	-	S	Yellow	Cmm72	-	L	Yellow
Cmm3	-	S	Yellow	Cmm74	-	S	Yellow
Cmm4	-	S	Cream yellow	Cmm76	-	M	Yellow
Cmm5	-	M	Cream	Cmm78	-	M	Yellow
Cmm6	-	M	Yellow	Cmm79	-	S	Cream yellow
Cmm7	-	M	Cream	Cmm80	-	S	Cream yellow
Cmm8	+	M	Cream	Cmm81	-	M	Yellow
Cmm9	-	M	Yellow orange	Cmm82	-	S	Cream yellow
Cmm12	-	S	Yellow	Cmm83	-	M	Yellow
Cmm13	-	M	Yellow	Cmm84	-	M	Yellow
Cmm14	-	M	Cream yellow	Cmm85	-	M	Yellow
Cmm15	-	P	Cream yellow	Cmm92	-	M	Cream yellow
Cmm16	-	P	Cream yellow	Cmm93	-	M	Cream yellow
Cmm19	-	M	Yellow orange	Cmm94	+	M	Yellow orange
Cmm20	+	M	Yellow	Cmm95	-	L	Yellow
Cmm21	+	M	Yellow	Cmm97	+	S	Yellow orange
Cmm26	-	M	Cream yellow	Cmm98	-	M	Yellow orange
Cmm36	+	M	Cream yellow	Cmm101	-	M	Cream yellow
Cmm38	-	L	Yellow orange	Cmm102	-	M	Cream yellow
Cmm39	+	S	Cream yellow	Cmm105	-	M	Cream yellow
Cmm41	-	M	Cream yellow	Cmm106	-	M	Yellow
Cmm42	-	L	Yellow	Cmm107	-	S	Cream yellow
Cmm43	+	S	Yellow	Cmm108	-	M	Yellow
Cmm48	+	S	Cream yellow	Cmm110	-	S	Cream yellow
Cmm49	-	L	Cream yellow	Cmm111	-	M	Cream yellow
Cmm67	+	L	Yellow orange	Cmm113	-	M	Yellow
Cmm68	-	L	Yellow	Cmm114	-	L	Cream yellow
Cmm69	-	M	Yellow	Cmm115	-	S	Cream yellow
Cmm70	-	M	Cream yellow	Cmm116	-	M	Cream yellow

Table 3. phenotypic description of Cmm strains: mucoid, color and size.

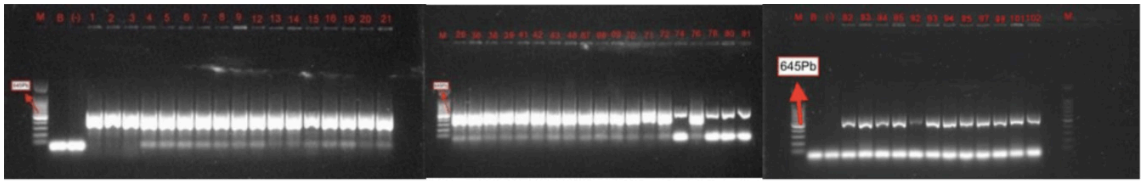

Figure 5. PCR results with primers Cm3/Cm4: 1% agarose gel stained with RedGel. A. Lane 1: 100 bp molecular marker. Lane 2: blank. Lane 3: Negative control. Lanes 4 to 20: DNA of strains isolated from tomato plants and seeds. B. Lane 1: 100 bp molecular marker. Lanes 2 to 20: DNA of strains isolated from tomato plants and seeds. C. Lane 1: 100 bp molecular marker. Lane 2: blank. Lane 3 Negative control. Lanes 4 to 15: DNA of strains isolated from tomato plants and seeds.

Strains	Cm3 / Cm4 645pb	*celA* 551pb	*pat*-1 614pb	*Chpc* 639pb	*ppaA* 587pb	*tomA* 529pb	Pathogenicity
Cmm1	+	+	+	+	+	+	+
Cmm2	+	+	+	+	+	+	+
Cmm3	+	+	+	+	+	+	+
Cmm04*	+	+	+	+	+	+	+
Cmm5	+	+	+	+	+	+	+
Cmm6	+	+	+	+	+	+	+
Cmm7	+	+	+	+	+	+	+
Cmm8	+	+	+	+	+	+	+
Cmm09*	+	+	+	+	+	+	+
Cmm12*	+	+	+	+	+	+	+
Cmm13	+	+	+	+	+	+	+
Cmm14	+	+	+	+	+	+	+
Cmm15	+	+	+	+	+	+	+
Cmm16	+	+	+	+	+	+	+
Cmm19	+	+	+	+	+	+	+
Cmm20	+	+	+	+	+	+	+
Cmm21*	+	+	+	+	+	+	+
Cmm26	+	+	+	+	+	+	+
Cmm36*	+	+	+	+	+	+	+
Cmm38	+	+	+	+	+	+	+
Cmm39*	+	+	+	+	+	+	+
Cmm41	+	+	+	+	+	+	+
Cmm42	+	+	+	+	+	+	+
Cmm43*	+	+	+	+	+	+	+
Cmm48	+	+	+	+	+	+	+
Cmm49	+	+	+	+	+	+	+
Cmm67	+	+	+	+	+	+	+
Cmm68*	+	+	+	+	+	+	+

Cmm69	+	+	+	+	+	+	+
Cmm70	+	+	+	+	+	+	+
Cmm71	+	+	+	+	+	+	+
Cmm72	+	+	+	+	+	+	+
Cmm74	+	+	+	+	+	+	+
Cmm76	+	+	+	+	+	+	+
Cmm78	+	+	+	+	+	+	+
Cmm79	+	+	+	+	+	+	+
Cmm80	+	+	+	+	+	+	+
Cmm81*	+	+	+	+	+	+	+
Cmm82	+	+	+	+	+	+	+
Cmm83	+	+	+	+	+	+	+
Cmm84*	+	+	-	+	+	+	+
Cmm85	+	+	+	+	+	+	+
Cmm92	+	+	+	+	+	+	+
Cmm93	+	+	+	+	+	+	+
Cmm94	+	+	+	+	+	+	+
Cmm95	+	+	+	+	+	+	+
Cmm97*	+	+	+	+	+	+	+
Cmm98*	+	+	-	+	+	+	+
Cmm101	+	+	+	+	+	+	+
Cmm102	+	+	+	+	+	+	+
Cmm105	+	+	+	+	+	+	+
Cmm106	+	+	+	+	+	+	+
Cmm107*	+	+	+	+	+	+	+
Cmm108*	+	+	+	+	+	+	+
Cmm110	+	+	+	+	+	+	+
Cmm111	+	+	+	+	+	+	+
Cmm113	+	+	+	+	+	+	+
Cmm114	+	+	+	+	+	+	+
Cmm115	+	+	+	+	+	+	+
Cmm116	+	+	+	+	+	+	+

* Strains used for the virulence experiment. Strains (+) for which the different genes were amplified and strains (-) for which no genes were amplified.

Table 4. Identification of the pathogenicity genes in each of the samples.

400 mA for 90 min. The estimation of the molecular weights of the amplified products was performed by comparison with a 1 Kb molecular marker (Promega, USA). The visualization of the amplification bands was performed in a photodocumentor Molecular imager Gel DOC XR+ from BioRad (USA).

The purification of the DNA product of the PCR was performed with the Wizard® SV Gel and PCR Clean-Up System Kit (Promega, USA) according to the instructions provided by the manufacturer. The purified PCR products were sent for sequencing in both directions at the LANGEBIO-CINVESTAV Genomic Services Unit, Irapuato Unit, with the FD2 and RP2 oligonucleotides (Weisburget *et al.* 1991; McLaughlin *et al.* 2012). Sequence editing was performed with the BioEdit Sequence Alignment Editor program, version 7.2.5. (Hall, 1999). Sequence alignment was carried out with the program ClustalW, and the consensus sequences obtained were compared with the basic search tool for local alignments (BLASTN) from the NCBI (National Center for Biotechnology Information) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSIONS

ISOLATION AND IDENTIFICATION OF *CMM*

In the present study, a total of 60 strains that cause bacterial canker were isolated from tomato plants of different tomato-producing states. Of these, 27 were from Sinaloa, 7 were from Jalisco, 7 were from San Luis Potosí, 4 were from Michoacán, 4 were from Guanajuato, 2 were from Baja, California, 2 were from Sonora, 2 were from Coahuila, 1 was from Nayarit, 1 was from Puebla, 1 was from Durango, 1 was from the State of Mexico and 1 was from Zacatecas (Fig. 1).

All the strains were isolated from tomato plants and fruits with symptoms of bacterial canker that were positive for *Cmm*. Most of the strains (57) of *Cmm* were isolated from the stem, and only three of the strains (Cmm1, Cmm12 and Cmm110) were isolated from tomato fruits (Table 2). After purification, the strains were classified and preserved in the strain collection of the Laboratory of Phytopathology, CIAD, Culiacán unit.

PATHOGENICITY TEST

The 60 strains isolated from tomato plants and fruits had the ability to induce bacterial canker symptoms in inoculated tomato plants (Fig. 2). Several authors have reported the presence of nonpathogenic strains of *Cmm*, which are mainly isolated from tomato fruits and seeds (Jacques *et al.*, 2012; Zaluga *et al.*, 2013). These strains have high serological and genetic similarity with *Cmm*. Most of the nonpathogenic Clavibacter strains have cells and colony morphology very similar to those of *Cmm*, which causes false-positive reports of the disease mainly in seeds (Zaluga *et al.*, 2011).

VIRULENCE TEST

The virulence test was performed on a quarter of the strains collected in the present study, so 14 of the 60 isolated strains of *Cmm* were selected. Included in this group were the two strains for which the *Pat*-1 gene (Cmm84 and Cmm98) was not amplified. The first symptoms of bacterial canker in plants were observed at 8 days DAI, when the aerial leaves showed yellowing and curling (Fig. 3). The results show that the strains for which symptoms were evident during the first evaluation (8 DAI) were the strains Cmm4, Cmm9, Cmm21, Cmm36, Cmm45 and Cmm68, with an average value (N = 16) on the severity scale of less than 1. (Fig. 4). During the second evaluation (11 DAI), only the strains Cmm39, Cmm84 and Cmm98 had a value of 0 on the severity scale. All the other strains presented different severity values

during this evaluation, with the Cmm9 and Cmm68 strains being the most virulent, with average severity values greater than 2.

PHENOTYPIC DESCRIPTION OF THE *CMM* STRAINS: MUCUS, COLOUR AND SIZE

According to the morphological descriptions of the strains isolated in this study, it was determined that 10 strains formed mucoid colonies and 50 strains formed nonmucoid colonies (Table 3). Studies of the subspecies *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) showed that the presence of mucoid and nonmucoid strains is determined by the amount and composition of extracellular exopolysaccharides (EPS). Strains with greater mucus can produce more EPS (Fousek and Mraz 2003; Gartemann *et al*., 2003). *Cmm*, like most phytopathogenic bacteria, produces EPS, which has different biological functions, such as protecting the bacteria by generating a matrix around it, thus preventing dehydration. Particularly, in the development of pathogenicity with the host plant, EPS can prevent the recognition of the pathogen by the defence system of the plant; in addition, by adhering to abiotic or biological surfaces, it can promote infection and colonization of the host plant (Jarh *et al*., 1999). Generally, subsp. *Cmm* is classified as nonmucoid; this characteristic is more typically associated with subsp. *Cms*, but in the study, we found that approximately 16% of the isolated *Cmm* strains had a mucoid consistency.

Although characteristics such as the consistency, color and size of the Cmm colonies may vary depending on the medium where it is grown and the days of incubation; the Cmm strains showed diversity in the Mueller Hinton medium both in consistency, color and size. Although there is no correlation of the morphological characteristics of the strains with virulence, since both the strains that presented greater virulence (Cmm9 and Cmm68) and those that were less virulent, which lack the pat-1 gene (Cmm84 and Cmm98), were strains non-mucoid, yellow and yellow orange in color and medium (M) and large (L) in size.

The size of the colonies was determined by measuring the small strains (1-2mm), medium (2-3mm) and the large ones (> 3mm). According to the above, 8 small strains, 27 medium strains and 25 large strains of the total strains were obtained.

ANALYSIS OF CMM STRAINS BY PCR

The identity of the strains isolated as *Cmm* was analysed with the specific primers *Cmm* Cm3/Cm4. All the strains amplified the expected 645 bp product (Fig.5).

PATHOGENIC POTENTIAL OF *CMM* STRAINS

All the *Cmm* strains were isolated from amplified PCR products of 639, 587 and 528 bp with the primers *ChpC*, *ppaA* and tomA, respectively (Table 4). These amplified fragments are associated with the pathogenicity island (PAI) of *Cmm*.

In the genetic study of the genes that reside in the two plasmids pCM1 and pCM2, all the isolated strains amplified a PCR product of 551 bp expected for the *celA gene,* which is found in the plasmid DNA pCM1. When regions were analysed for the presence of the pathogenicity gene *pat* -1 within the plasmid pCM2, two of the strains, Cmm84 and Cmm98, showed negative results (i.e., the absence of the 614 bp amplicon). The occurrence of *Cmm* strains that do not have the *pat-1* gene, as well as the possible complete absence of plasmid pCM2, where this gene is housed, has been previously reported in several studies (Alvarez and Kaneshiro, 2005; Kleitmann *et al*., 2008; Bella *et al*., 2012). The studied strains accounted for

20 to 45% of the studied population. In our study, we found that only 3% of our *Cmm* population lacked the *pat*-1 gene.

PHYLOGENETIC ANALYSIS OF THE 16S rDNA GENE

Based on the BLASTn search of the sequences of the 60 *Cmm* strains isolated in this study, 51 of the sequences showed a 99-100% similarity with the 16S rRNA sequences of other *Cmm* in the database. The sequences of the 51 strains were registered in the NCBI (National Center for Biotechnology information) database.

To characterize the genetic relationships of the *Cmm* isolates, a phylogenetic analysis was performed based on the 16S rRNA sequences. According to the analysis of the dendrogram of the sequences of all the strains, the strains were mainly sorted into eight groups (Fig. 6). In group one, we found four strains isolated in 2015 and 2018 from the states of Nayarit, Torreón, San Luis Potosí and Michoacán. Also in this group was a strain from Italy, which could suggest their place of origin. Group two included 20 strains collected in 2015, 2016, 2017 and 2018 from the states of Durango, Guanajuato, Jalisco, Nayarit, San Luis Potosí, Sinaloa, Sonora, Torreón and Zacatecas, so this group was considered more numerous. In group three, 11 strains collected in 2015, 2017 and 2018 from the states of Baja California Norte, Guanajuato, Michoacán, Sonora and Sinaloa were found. In group four, two strains isolated in Sinaloa in 2015 and 2018 were grouped with a strain from China, which could indicate their place of origin. In group five, there were four strains collected in 2015 and 2018 from the states of Jalisco, Puebla and Sinaloa. In group six, five strains collected in 2018 in the state of Sinaloa were observed. Within group seven, we found all the strains of *Clavibacter michiganensis* subsp. *michiganensis* and other subspecies of *Clavibacter michiganesis* used for the analysis. In group eight, there were two strains collected in 2017 and 2018 from the states of Mexico and Sinaloa. Finally, in the analysis, we found three strains that were not included in any of the eight groups (Cmm97 (Sinaloa, 2018), Cmm03 (Sinaloa, 2015) and Cmm116 (Jalisco, 2019)), demonstrating the high genetic variability of the strains.

In general, the grouping of the strains based on the sequencing of the 16S rRNA gene does not show a clear relationship with the place of isolation or collection year since in most of the groups, we found variability in the collection year and isolation site, which suggests different sources of inoculum. Only group 6 included strains mainly from Sinaloa and isolated in 2018.

Phylogenetic analysis techniques such as sequencing and subsequent analysis of the sequences are widely used and have resulted in the reclassification of the genus *Clavibacter*. Eom-Ji *et al.* (2016) used 16S rRNA gene sequencing to characterize a population of *Cmm* isolated from tomato and chili peppers. The analysis showed that the strains isolated from the chili peppers were grouped separately from those of tomato. As a result of the analysis of the 16S rRNA gene, this study proposes a new subspecies, *C. michiganensis* subsp. *capsici*, causing bacterial canker in chili peppers.

In our study, we did not find any direct association between the year or origin of collection and the strains since in most groups, there were strains isolated in different locations and years. This can be explained by the findings of Jacques *et al.* (2012), who attribute the majority of *Cmm* outbreaks to a complex of *Cmm* clones that can come from different countries or even continents over a period of time, leading to the occurrence of groups with strains isolated from different geographical areas and in different years. There are 3 known

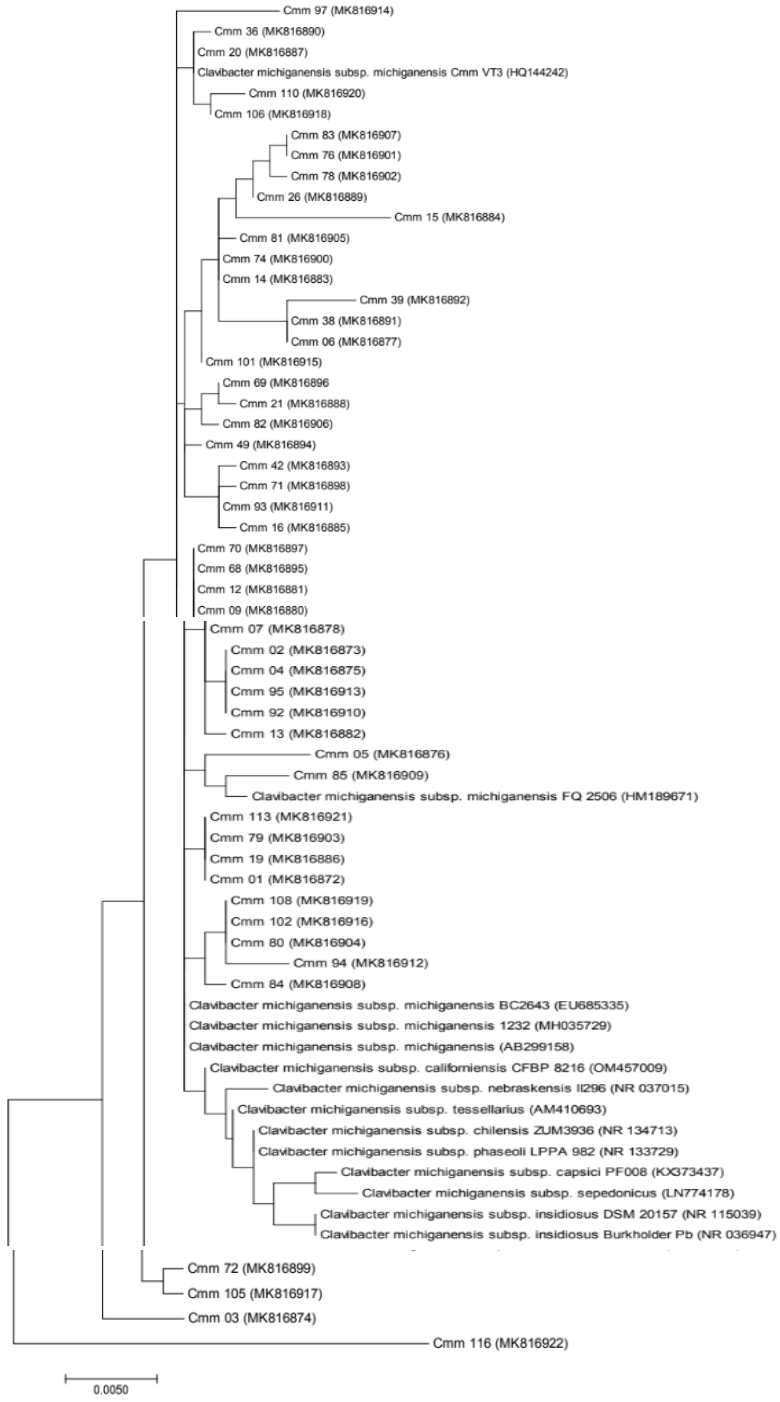

Figure 6.Dendrogram based on the maximum likelihood method of sequences obtained from the 16S rRNA gene of *Clavibacter michiganensis* subsp. *michiganensis* and sequences recorded in the NCBI database. The distances were calculated by the Tamura-Nei method, and the tree is presented graphically by the application of the neighbour-joining method. To determine the confidence values for the clades within the resulting tree, a statistical bootstrap test (1000 repetitions) was performed. The GenBank accession numbers for the reference strains are VT3 (HQ144242), FQ 2506 (HM189671), AB299158, BC2643 (EU685335), 1232 (MH035729), FQ2506 (HM189671), CFBP 8216 (OM457009), II296 (NR 037015), (AM410693), ZUM3936 (NR 134713), LPPA 982 (NR 133729), PF008 (KX373437), (LN774178), DSM 20157 (NR 115039) and Pb (NR 036947).

environments where the pathogen is present: on seeds, in greenhouses used for seedling production and in tomato production areas. In Mexico, rootstocks are widely used for the prevention of *Fusarium* spp. in the field. The production of seedlings for grafting caused one of the main outbreaks of *Cmm* in 2016 in Mexico because transmission of *Cmm* most commonly occurs mechanically. Seedlings are produced in greenhouses and are then sent to numerous states of Mexico where tomato is cultivated; therefore, it is to be expected that when *Cmm* infection is not detected in the greenhouse, it will be disseminated to different cultivation areas.

CONCLUSION

- The results of this study provide information that contributed to a better understanding of the morphological characterization and genetic diversity of *Cmm* in the main tomato-producing areas in Mexico.
- The 60 strains of Cmm under study were found to be pathogenic in tomato plants but with different degrees of virulence.
- A total of 58 strains had pathogenicity genes (*cel*-A, *pat*-1, PAI, *chp* C and *tom* A), while strains *Cmm* 84 and 98 did not present the *pat-1* gene.
- The phylogenetic analysis of the strains yielded 8 clusters; however, no clear relationship was found between the strains and the collection time (5 years) and site (13 tomato-producing states).

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