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TESIS:

"Análisis comparativo de la diversidad morfológica, patogénica y genética de *Clavibacter michiganensis* subsp. *michiganensis* aisladas de México."

Que para obtener el grado de
Doctor en Ciencias Agropecuarias

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DEDICATORIA

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RESUMEN

Clavibacter michiganensis subsp*. michiganensis* (*Cmm*) es un actinomiceto patógeno de plantas que causa el marchitamiento y el cáncer bacteriano del tomate (*Solanum lycopersicum* L.); es una bacteria difícil de controlar a nivel mundial y año con año causa grandes pérdidas económicas. El objetivo de la investigación fue determinar la variabilidad morfológica, patogénica y genética de cepas de *Cmm* colectadas en diferentes zonas productoras de tomate en México, para lo cual se llevó a cabo un estudio de tipo descriptivo durante el periodo de septiembre de 2019 a agosto de 2023, que incluyó el análisis comparativo de 60 cepas de *Cmm* considerando características de color, mucosidad y tamaño de colonia; así como, la presencia/ausencia de los genes relacionados a la capacidad patogénica de la bacteria como: celA, pat-1, Chpc, ppaA, y tomA; además del gen altamente conservado 16S, encontrando escasa diferencia en cuanto a genes de patogenicidad, pero una gran variabilidad en características morfológicas y en el análisis filogenético del gen 16S. La capacidad patogénica de la bacteria también fue analizada donde se inocularon además de tomate, otras plantas solanáceas como: petunias, papa, berenjena, chile y tabaco; encontrando que, la bacteria tiene la capacidad de reproducirse en todas ellas; sin embargo, ésta puede o no ocasionar síntomas, lo último observado en berenjena y tabaco, por lo que se sugiere que la tasa de replicación de la bacteria se mantiene en niveles bajos como para no ocasionar daño. Finalmente, se llevó a cabo la secuenciación de los genomas de cinco cepas de *Cmm* clasificadas en base a su virulencia desde muy baja a muy alta y se realizó un análisis bioinformático genómico comparativo con el programa Geneious y el algoritmo MAUVE progressive. Se obtuvieron para las cinco cepas las secuencias de un cromosoma y dos plásmidos que mostraron una alta similitud en tamaño y contenido de GC. A nivel de cromosoma se observó una zona altamente variable entre cepas y que codifican para enzimas relacionadas al metabolismo celular y de respuesta a condiciones adversas. Lo anterior pudiera estar relacionado con las diferencias observadas en las cinco cepas en cuanto a virulencia. Se observó también alta variabilidad en la secuencia pCM2U de la cepa CMM09 altamente virulenta con respecto a las otras y escasa variabilidad en las secuencias del plásmido pCM1. Debido a lo anterior, se recomienda analizar la expresión directa de estos genes diferenciales para corroborar su función en la virulencia de la bacteria.

Palabras clave: Genoma, Cromosoma, Plásmidos, Genes de patogenicidad, Secuenciación.

ABSTRACT

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is an actinomycete plant pathogenic that causes wilting and bacterial cancer of tomatoes (*Solanum lycopersicum* L.); it is a bacterial that is difficult to control worldwide and causes great economic losses year by year. The objective of the research was to determine the morphological, pathogenic, and genetic variability of *Cmm* strains collected in different tomato-producing areas in Mexico, for which a descriptive study was carried out from September 2019 to August 2023, which included the comparative analysis of 60 *Cmm* strains considering color, mucus and colony size characteristics; as well as the presence/absence of genes related to the pathogenic capacity of the bacteria such as: celA, pat-1, Chpc, ppaA, and tomA; in addition to the highly conserved 16S gene, finding little difference in terms of pathogenicity genes but great variability in the morphological characteristics and the phylogenetic analysis of the 16S gene. The pathogenic capacity of the bacteria was also analyzed where, in addition to tomatoes, other solanaceous plants such as petunias, potatoes, eggplant, chili, and tobacco were inoculated; finding that the bacteria can reproduce in all of them; however, it may or may not cause symptoms, the latest observed in eggplant and tobacco, which is why it is suggested that the replication rate of the bacteria remains at low levels so as not to cause damage. Finally, the genomes of five *Cmm* strains classified based on their virulence from very low to very high were sequenced, and comparative genomic bioinformatics analysis was performed with the Geneious program and the MAUVE progressive algorithm. Sequences of one chromosome and two plasmids that showed high similarity in size and GC content were obtained for the five strains. At the chromosome level, a highly variable area was observed between strains that encode enzymes related to cellular metabolism and response to adverse conditions. The above could be related to the differences observed in the five strains at the virulence level. High variability was also observed in the pCM2U sequence of the highly virulent CMM09 strain concerning the others and scarce variability in the sequences of the pCM1 plasmid. Due to the above, it is recommended to analyze the direct expression of these differential genes to corroborate their function in the virulence of the bacteria.

Keywords: Genome, Chromosome, Plasmids, Pathogenicity genes, Sequencing

CAPÍTULO 1. INTRODUCCIÓN Y REVISIÓN DE LITERATURA

1.1 INTRODUCCIÓN

Las plantas solanáceas pueden ser afectadas por un gran número de enfermedades ocasionadas por bacterias, las cuales pueden afectar varios órganos; por ejemplo: la pierna negra causada por *Pectobacterium carotovorum*; la mancha bacteriana causada por *Xanthomonas axonopodis* pv. *vesicatoria* (chile y tomate), la pudrición anular de la papa por *Clavibacter michiganensis* subsp. *sepedonicus*, la necrosis de médula por *Pseudomonas corrugata* (chile y tomate), el ojo rosa por *Pseudomonas fluorescens* (papa), el cáncer bacteriano por *Clavibacter michiganensis* subsp. *michiganensis* (tomate) y *Clavibacter michiganesis* subsp. *capsici* (chile), la marchitez bacteriana por *Ralstonia solanacearum* (chile, tomate, papa y berenjena), la zebra chip por *Candidatus Liberibacter solanacearum* (papa, tomate y chile) y la pudrición blanda causada por *Pectobacterium caratovorum* subsp. *caratovorum* (chile, tomate y papa) (Agrios, 2005). En las últimas décadas, el cáncer bacteriano ocasionado por *Clavibacter michiganensis* subsp. *michiganensis* se ha convertido en una de las enfermedades más devastadoras del cultivo de tomate a nivel mundial reportándose perdidas en producción de hasta un 70% si no se toman las medidas adecuadas (García, 2009). Cabe mencionar que, la semilla infectada con la bacteria es considerada la principal fuente de inóculo que conduce a brotes de cáncer bacteriano (Gartemann *et al.*, 2003). En México, existen reportes de pérdidas económicas calculadas en 40 millones de dólares solamente en el estado de Sinaloa debido a la introducción de plantas de tomate injertadas provenientes

del estado de Michoacán. A pesar del intento por mejorar el manejo y control de la enfermedad del cáncer bacteriano hasta el momento no se ha encontrado un protocolo adecuado para lograr dicho objetivo (García, 2009; Lara-Avila, 2012). Dicha dificultad se ha asociado principalmente, a la naturaleza Gram (+) de la bacteria y a que se encuentra internamente en el embrión de la semilla; además de, que se trasmite mecánicamente por las labores culturales. Aunado a lo anterior, cabe mencionar que hasta el momento, no hay un material vegetal que tenga tolerancia o resistencia a la infección por *Cmm* y a que tampoco hay algún producto químico que se considere específico para su control.

1.2 REVISIÓN DE LITERATURA

1.2.1 Enfermedades del cultivo del tomate.

El cultivo de tomate es altamente susceptible de sufrir enfermedades que afectan su desarrollo y producción, las cuales pueden clasificarse como enfermedades abióticas que se asocian a factores medio ambientales como: humedad, altas o bajas temperaturas, deficiencias de nutrientes y daños por fertilizantes y plaguicidas. Por otra parte, las enfermedades bióticas se presentan debido al ataque en el cultivo por organismos vivos como las ocasionadas por hongos, bacterias, virus, geminivirus, fitoplasmas, nematodos, insectos y plantas parasitas (Salas *et al*., 2022). Dentro de las enfermedades ocasionadas por bacterias, el cáncer bacteriano es considerado como la más importante, debido a las pérdidas que ocasiona. Las pérdidas en producción pueden variar con los años, localidad, el cultivo y la edad fenológica del hospedero. Como resultado de la severidad en las pérdidas en producción y económicas, *Cmm* es considerado como un organismo cuarentenado por la Unión Europea y muchos otros países (De León *et al*., 2008). De acuerdo con diversos trabajos de investigación se ha reportado que *Cmm* tiene la capacidad de causar daños en otros cultivos, además de su hospedero principal que es el tomate. En este sentido, variantes fenotípicas de esta bacteria se aislaron de plantas y semillas de pimiento durante las inspecciones de cuarentena. Los resultados de ELISA, análisis de ácidos grasos, secuenciación del gen rDNA 16S y el análisis de PCR mostraron que todos los aislados de pimiento eran lo suficientemente similares como para ser identificados como *Cmm*; sin embargo, los aislados de pimiento no causaron marchitamiento, incluso en el tomate (Kyu-Ock *et al*., 2011). Ignatov *et al.* (2019) aislaron a *Cmm* de plantas de pimiento amarillo y tubérculos de papa enfermos de 5 regiones de Rusia durante los años 2011 y 2017. Las plantas exhibieron coloración amarillenta, necrosis de la hoja, marchitamiento de las hojas y plantas enteras. Se identificaron aislamientos de cada planta de papa enferma (35 aislamientos) como *Clavibacter michiganensis* subsp. *michiganensis*. En este estudio se utilizó la técnica de reacción en cadena de la polimerasa (PCR) con los cebadores

CMM5/CMM6. La secuenciación del gen 16S rRNA (números de acceso de GenBank MH035728.1 a MH035762.1) mostró una relación > 99% con la cepa tipo de *Cmm* NCPPB2979T. Este es el primer informe de *Cmm* causando síntomas de enfermedad en papa. Aunado a lo anterior Rokiene *et al*., (2005) estudiaron la diversidad genética de 14 cepas de *Cmm* en Lituania mediante dos métodos basados en el ADN: PCR-RFLP de los genes 16S-23S rARN y por RAPD. En este estudio se reportó alta heterogeneidad entre los aislados y se obtuvo por ambos métodos la agrupación de los aislados en dos grupos conocidos como clústers; sin embargo, no se encontró relación entre la diversidad genética y el origen de los aislados, hospedero y virulencia. Cabe mencionar que, debido a la variabilidad patogénica y genética de la bacteria *Cmm* se han originado múltiples estudios encaminados a mejorar nuestro entendimiento y comprensión acerca de la biología de este importante patógeno.

1.2.2 Secuenciación y análisis comparativo del genoma de cepas de *Cmm*

La diversidad genética de *Cmm* en brotes de enfermedades se ha investigado en los últimos años en diversas regiones del mundo y los primeros estudios de secuenciación y caracterización genómica de la especie dieron pie a diversas investigaciones sobre la caracterización fenotípica y genotípica; en ese sentido, Baysal *et al*. (2010) determinaron la secuencia de nucleótidos del genoma de la cepa NCPPB382 encontrando un cromosoma circular (Fig. 1) que consta de 3298 Mb y tiene un alto contenido en G+C (72.6%). La anotación reveló 3080 secuencias putativas que codifican para proteínas y sólo se detectaron 26 pseudogenes; además, se encontraron dos operones rrn, 45 ARNt y tres pequeños genes de ARN estables. Los dos plásmidos circulares, pCM1 (27,4 kpb) y pCM2 (70,0 kpb) que portan genes de patogenicidad y, por tanto, son esenciales para la virulencia, tienen contenidos de G+C más bajos (66.5 y 67.6%, respectivamente). A diferencia del genoma del organismo estrechamente relacionado de *Clavibacter michiganensis* subsp. *sepedonicus*, el genoma de *C. michiganensis* subsp. *michiganensis* carece de transposones y elementos de

inserción completos. Se demostró que la región chp / tomA de 129 kb (Fig. 2) con un bajo contenido de G+C cerca del origen cromosómico de replicación era necesaria para la patogenicidad. Esta región contiene numerosos genes que codifican proteínas implicadas en la captación y el metabolismo de azúcares y varias serinas proteasas. Existe evidencia de que genes únicos ubicados en esta región, especialmente genes que codifican serina proteasas, son necesarios para una colonización eficiente del huésped. Aunque *C. michiganensis* subsp. *michiganensis* crece principalmente en el xilema de las plantas de tomate, no se encontró evidencia de una reducción pronunciada del genoma. Esta bacteria parece tener tantos transportadores y reguladores como las bacterias típicas que habitan en el suelo; sin embargo, la aparente falta de una vía de reducción de sulfatos hace que *Cmm* dependa de compuestos reducidos de azufre para su crecimiento, y es probablemente la razón de la escasa supervivencia de la bacteria en el suelo.

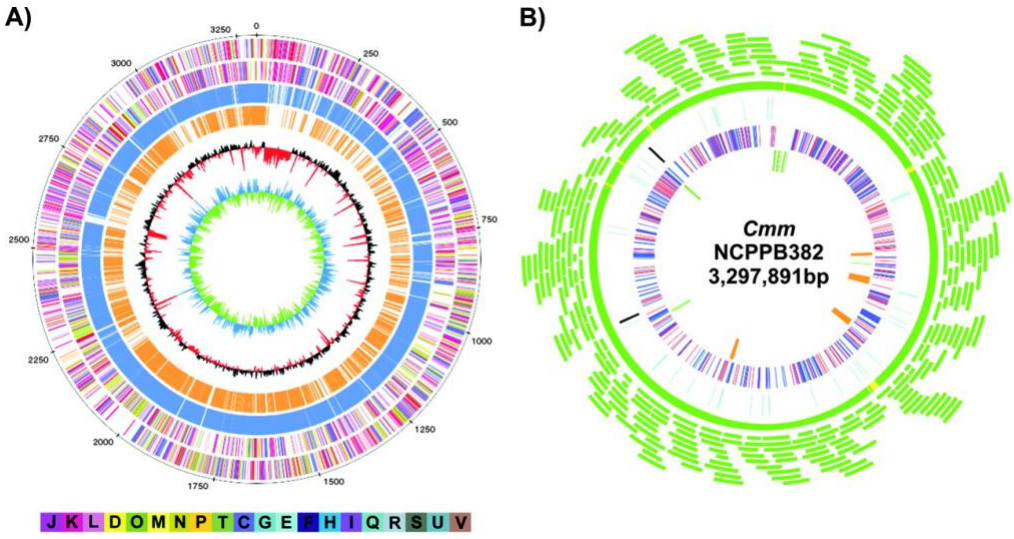

Figura 1. Validación del ensamblaje de secuencia mediante un mapa BAC. (A) Círculos que proporcionan la siguiente información: círculo 1, posición (en kilobases); círculos 2 y 3, CDS predicho en los hilos delantero (círculo 2) y reverso (círculo 3), con los colores indicando las clases COG asignadas; círculos 4 y 5, CDS con homólogos en los cromosomas de *C. michiganensis* subsp. *sepedonicus* (azul) y (naranja), respectivamente; círculo 6, contenido de G y C, que muestra desviaciones del valor medio (72.66%); círculo 7, GC sesgado. El bar en la parte inferior se explican los colores utilizados para indicar los grupos COG funcionales (C, producción y conversión de energía; D, control del ciclo celular, mitosis, y meiosis; E, transporte y metabolismo de aminoácidos; F, transporte y metabolismo de nucleótidos; G, transporte y metabolismo de carbohidratos; h, transporte y metabolismo de coenzimas; I, transporte y metabolismo de lípidos; J, traducción; K, transcripción; L, replicación, recombinación y reparación; M, biogénesis de pared y

membrana celular; N, motilidad celular; O, modificación postraduccional, recambio de proteínas y chaperonas; P, ion inorgánico transporte y metabolismo; Q, biosíntesis, transporte y catabolismo de metabolitos secundarios; R, predicción de funciones generales; S, función desconocida; T, mecanismos de transducción de señales; U, tráfico y secreción intracelular; V, mecanismos de defensa). cromosoma *michiganensis* (*Cmm*). Cada arco verde representa un único clon de BAC mapeado en la secuencia ensamblada. Desde el círculo exterior hasta el círculo interior: círculo 1, cobertura de C. *michiganensis* subsp. *michiganensis* con clones BAC (verde, cubierto por más de un clon BAC; amarillo, cubierto por un clon BAC); círculo 2, genes que codifican ARNt (cian) y ARNr (negro); círculo 3, proteínas transportadoras predichas (azul) y reguladores transcripcionales (rojo); círculo 4, genes supuestamente implicados en la patogenicidad (verde) y grupos de EPS (naranja).

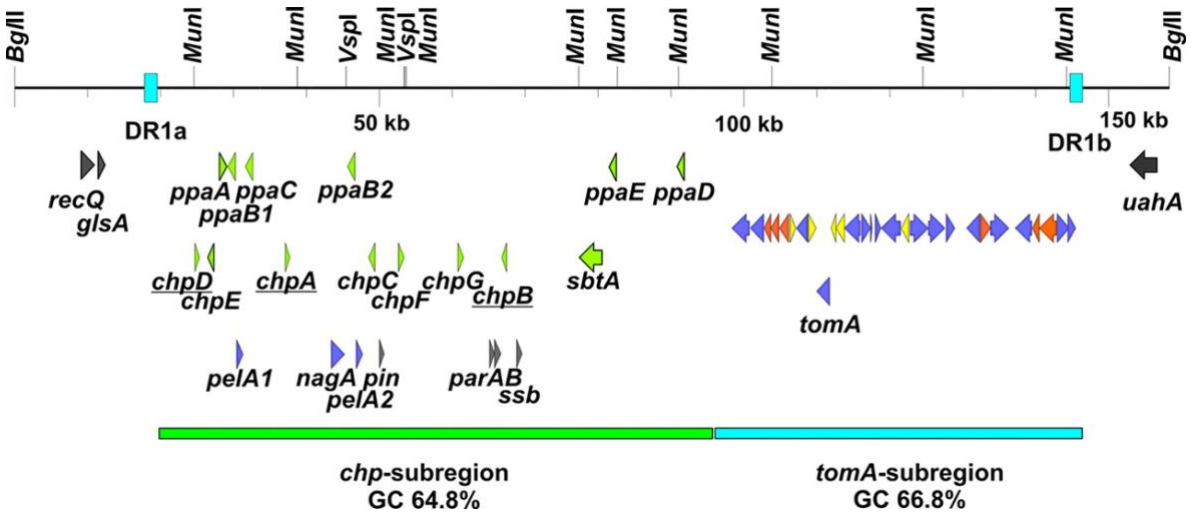

Figura 2. Representación esquemática de la región chp/tomA de *C. michiganensis* subsp. *michiganensis* de la cepa NCPPB382. Las repeticiones directas de 1,9 kb que flanquean la región están indicadas por cuadros azules. Los genes seleccionados se indican mediante los siguientes colores: verde, genes que codifican serina proteasas; amarillo, genes que codifican, reguladores; naranja, genes que codifican transportadores; violeta, genes que codifican glicosidasas. Los pseudogenes están subrayados.

1.2.3 Comparación de los plásmidos y genomas de *Clavibacter michiganensis* subsp. *michiganensis.*

Méndez *et al*. (2020) realizaron el estudio de secuenciación del genoma de tres cepas de *Cmm*, VL527, MSF322 y OP3, aisladas de tres regiones del centro del país de Chile con la finalidad de conocer los diferentes niveles de virulencia mediante la genómica; así mismo, la comparación de los genomas para encontrar la ubicación filogenómica de las tres cepas y el análisis comparativo de los genes de virulencia (Fig. 3). Al respecto, las tres cepas mostraron una alta conservación de factores de virulencia. Por el contrario, otros cromosomas menos conservadores de los factores de virulencia codificados fuera de la isla de

patogenicidad (PAI) también estaban típicamente presentes en cepas no patógenas; esto nos dice que es muy posible que no desempeñen un papel crítico en la patogenicidad. Si bien las cepas chilenas VL527, MSF322 y OP3 poseían una baja diversidad genómica entre ellas, se encontraron diferentes niveles de virulencia en las plantas de tomate. Esto puede estar relacionado con genes de virulencia, como el gen pelA1 de la región chp/tomA y genes que codifican proteasas e hidrolasas exclusivos de cada una de las cepas chilenas.

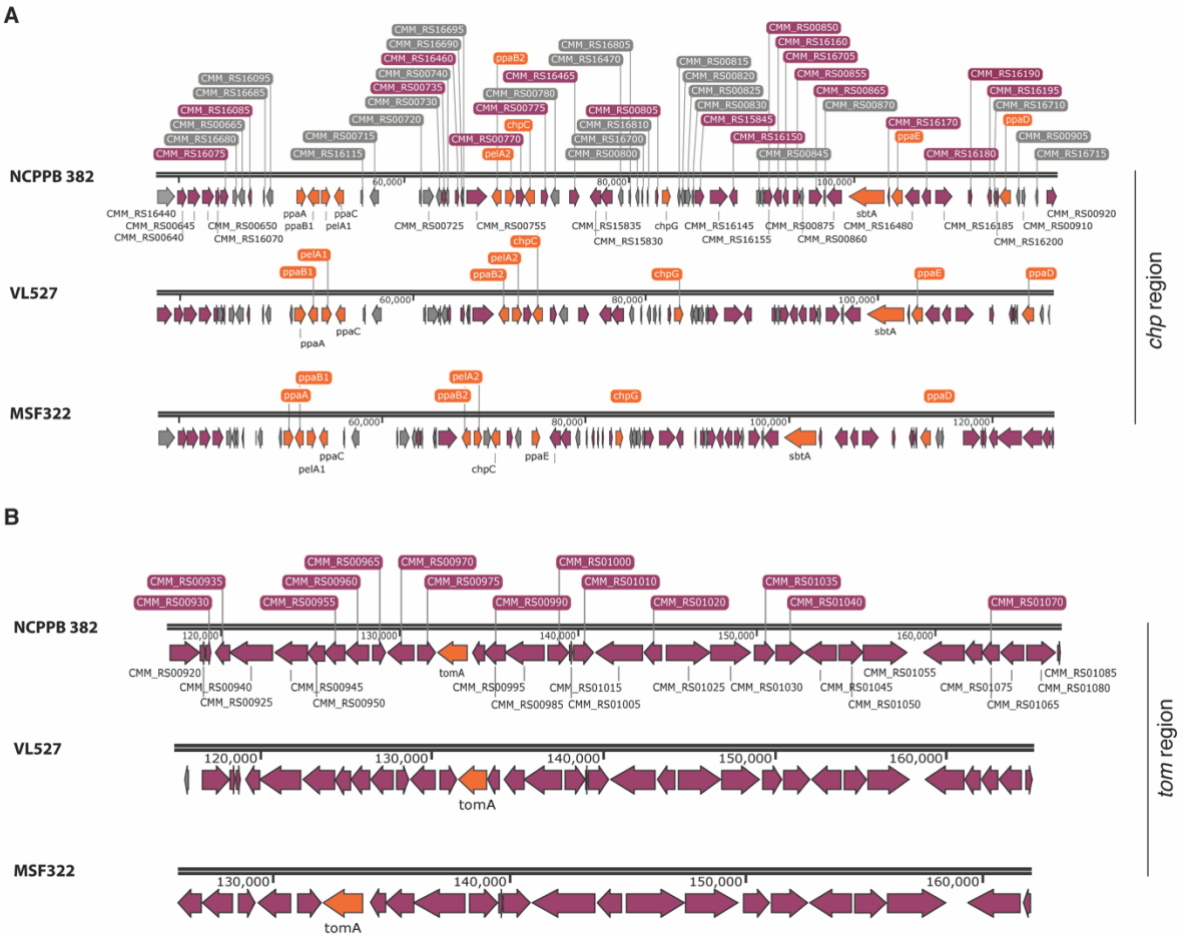

Figura 3. Representación esquemática de las regiones de patogenicidad de las cepas chilenas VL527 y MSF322. Las regiones chp (A) y tom (B) de C. *michiganensis* subsp. *michiganensis* se muestran las cepas NCPBB 382, VL527 y MSF322. Los genes de virulencia (p. ej., pelA, chpC, chpG, ppaA—ppaE, sbtA, tomaA están representados en naranja. Los genes con otras funciones se representan en violeta y los genes hipotéticos se ilustran en gris.

Así mismo, Oh *et al*. (2022), realizaron el estudio de la secuenciación completa del genoma y el análisis comparativo de 3 cepas de *Clavibacter michiganensis* subsp. *michiganensis*: cepas LMG7333T, UF1 y NCPPB382; demostrando que, las tres cepas cuentan con un cromosoma y dos plásmidos: pCM1 y pCM2 y, que las tres

secuencias de ADN cromosómicos son muy parecidos; sin embargo, las secuencias de ADN de los plásmidos de las cepas LMG7333T y UF1 son muy parecidos entre sí, pero muy diferentes a los plásmidos de la cepa NCPPB382, eso quiere decir que los plásmidos tienen genes diferentes entre cepas de *Clavibacter michiganensis* subsp. *michiganensis*; además, 216 secuencias codificaron para proteínas (CDS) que solo se encontraron en el genoma de la cepa LMG7333T. En comparación con cepas de otras especies de *Clavibacter*, entre estos 216 CDs, aproximadamente el 83% estaban en el cromosoma mientras que otros estaban en ambos plásmidos (6% en pCM1 y 11% en pCM2). La proporción de CDS único del total de CDS en ambos plásmidos fueron de aproximadamente 38% en pCM1 y 30% en pCM2, lo que indica que el alto porcentaje del gen en ambos plásmidos de *Clavibacter michiganensis* subsp. *michiganensis*, son diferentes a los de otras especies de *Clavibacter* y los ADN plasmídicos podrían variar dependiendo del origen.

CAPÍTULO 2

Morphological characterization and genetic diversity of *Clavibacter michiganensis* subsp. *michiganensis* in tomato producing areas of Mexico.

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SUMMARY

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.

2.1 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.* 1998). 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.

2.2 MATERIALS AND METHODS

2.2.1 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.

2.2.3 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.

2.2.4 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.

2.2.5 Virulence test

The virulence of the strains was analyzed 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 9×10^8 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 analyzed in the SAS program.

2.2.6 Morphological characteristics

Morphological characteristics of the colonies that developed from the *Cmm* strains were recorded; for example, coloration 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.

2.2.7 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.

2.2.8 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.

2.2.9 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.

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

Oligonucleotides	Sequences (5′ to 3′)	Genes	Amplicon sizes
CM3	CCTCGTGAGTGCCGGGAACGTATCC	*Cmm* confirmation	645 pb
CM4	CCACGGTGGTTGATGCTCGCGAGAT		
PFC3	GGTACGAAGTTCGAGACGAC	CelA	551 pb
PFC5	TGTAGCGGTGAGTCGTGGTGA		
P5	GCGAATAAGCCCATATCAA	Pat-1	614 pb
P6	CGTCAGGAGGTCGCTAATA		
chpcF	GCTCTTGGGCTAATGGCCG	chpC	639 pb
chpcR	GTCAGTTGTGGAAGATGCTG		
ppaAF	CATGATATTGGTGGGGAAAG	ppaA	587 pb
ppaAR	CCCCGTCTTTGCAAGACC		
tomAF	CGAACTCGACCAGGTTCTCG	toma	529 pb
tomAR	GGTCTCACGATCGGTCC		

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).

2.2.10 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 GelRed at a concentration of 1X at 80 V and 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).

2.3 RESULTS AND DISCUSSIONS

2.3.1 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).

Figure 4. Geographic distribution of the *Cmm* strains isolated from the tomato fruit and plants collected in this study.

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, Culiacan unit.

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

Strains	Location origin	Isolated from	Year of collection
Cmm01	Culiacán, Sinaloa	Fruit	2015
Cmm02	Culiacán, Sinaloa	Stem	2015
Cmm03	Culiacán, Sinaloa	Stem	2015
Cmm04	Culiacán, Sinaloa	Stem	2015
Cmm05	Culiacán, Sinaloa	Stem	2015
Cmm06	Culiacán, Sinaloa	Stem	2015
Cmm07	Culiacán, Sinaloa	Stem	2015
Cmm08	Numarán, Michoacán	Stem	2015

Cmm09	Numarán, Michoacán	Stem	2015
Cmm12	Culiacán, Sinaloa	Fruit	2015
Cmm13	Mexicali, Baja California	Stem	2015
Cmm14	Sayula, Jalisco	Stem	2015
Cmm15	Sayula, Jalisco	Stem	2015
Cmm16	Guaymas, Sonora	Stem	2015
Cmm19	Puebla, Puebla	Stem	2015
Cmm20	Torreón, Coahuila	Stem	2015
Cmm21	Villa de Arista, San Luis Potosí	Stem	2015
Cmm26	Compostela, Nayarit	Stem	2015
Cmm36	Compostela, Nayarit	Stem	2015
Cmm38	Torreón, Coahuila	Stem	2015
Cmm39	Sayula, Jalisco	Stem	2015
Cmm41	Numarán, Michoacán	Stem	2016
Cmm42	Zacatecas, Zacatecas	Stem	2016
Cmm43	Ensenada, Baja California	Stem	2016
Cmm48	Villa de Arista, San Luis Potosí	Stem	2016
Cmm49	Rio Verde, San Luis potosí	Stem	2016
Cmm67	Betulia, Jalisco	Stem	2017
Cmm68	Ciudad Obregón, Sonora	Stem	2017
Cmm69	Rio Verde, San Luis Potosí	Stem	2017
Cmm70	San Miguel de Allende, Guanajuato	Stem	2017
Cmm71	Jalisco, Jalisco	Stem	2017
Cmm72	Estado de México, Estado de México	Stem	2017
Cmm74	Villa de Arista, San Luis Potosí	Stem	2017
Cmm76	Durango, Durango	Stem	2017
Cmm78	San Miguel de Allende, Guanajuato	Stem	2017
Cmm79	Culiacán, Sinaloa	Stem	2018
Cmm80	Culiacán, Sinaloa	Stem	2018
Cmm81	Culiacán, Sinaloa	Stem	2018
Cmm82	Culiacán, Sinaloa	Stem	2018
Cmm83	La Cruz de Elota, Sinaloa	Stem	2018
Cmm84	Culiacán, Sinaloa	Stem	2018
Cmm85	Culiacán, Sinaloa	Stem	2018
Cmm92	San Miguel de Allende, Guanajuato	Stem	2018
Cmm93	San Miguel de Allende, Guanajuato	Stem	2018
Cmm94	La Cruz de Elota, Sinaloa	Stem	2018
Cmm95	Culiacán, Sinaloa	Stem	2018
Cmm97	La Palma, Navolato, Sinaloa	Stem	2018
Cmm98	La Palma, Navolato, Sinaloa	Stem	2018

Cmm101	Carr. Las puentes, Sinaloa	Stem	2018
Cmm102	Carr. A Eldorado, Sinaloa	Stem	2018
Cmm105	Culiacán, Sinaloa	Stem	2018
Cmm106	Numarán, Michoacán	Stem	2018
Cmm107	El Huizache, San Luis Potosí	Stem	2018
Cmm108	Altata Navolato, Sinaloa	Stem	2018
Cmm110	Villa de Arista, San Luis Potosí	Fruit	2018
Cmm111	Altata Navolato, Sinaloa	Stem	2018
Cmm113	Jalisco, Jalisco	Stem	2018
Cmm114	San Ignacio, Sinaloa	Stem	2019
Cmm115	Altata Navolato, Sinaloa	Stem	2019
Cmm116	Jalisco, Jalisco	Stem	2019

2.3.2 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).

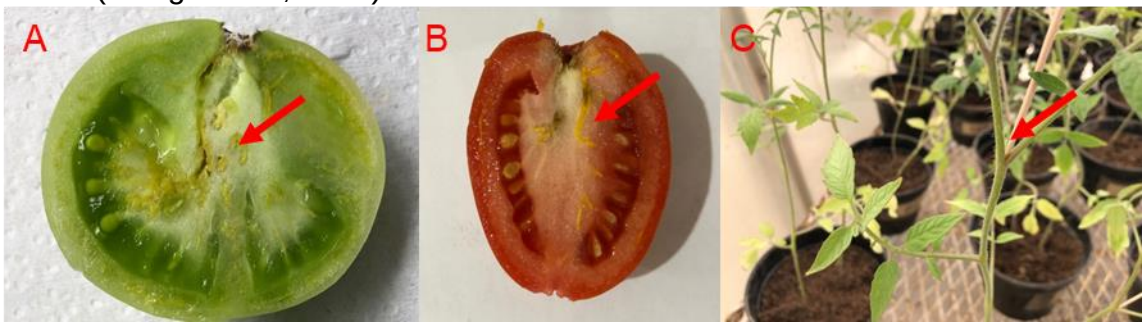

Figure 5. Bacterial canker symptoms in inoculated tomato plants. A) and B) green and mature fruit with the bacteria on the placenta, C) Plant inoculated with the bacteria with a wooden stick.

2.3.3 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.

Figure 6. Tomato plant with *Cmm* symptoms. A): Plant inoculated with *Cmm* by infiltration. B): Plant with initial symptoms of *Cmm*. C): Plant killed by *Cmm*.

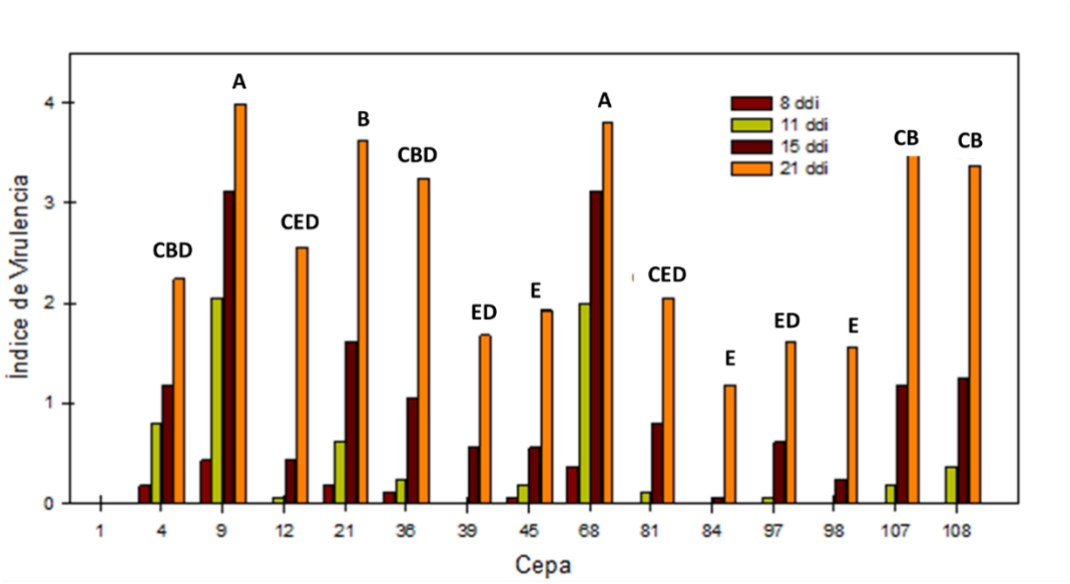

Figure 7. The virulence index values of the strains of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seedlings at 21 days after inoculation (DAI). A value of 1 on the X axis represents the negative control (water). The other values represent the identification code number of each isolate. 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.

2.3.4 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 (Jahr *et al*., 2000). 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.

Table 3. Characteristics of the *Cmm* colonies in the study

Strain	Consistency	Size	Coloration (Hue degrees)	Strain	Consistency	Size	Coloration (Hue degrees)
Cmm1	-	L	93.18	Cmm71	-	L	90.52
Cmm2	-	M	88.61	Cmm72	-	S	85.57
Cmm3	-	M	90.03	Cmm74	-	L	81.27
Cmm4	-	M	88.08	Cmm76	-	L	87.59
Cmm5	-	M	94.97	Cmm78	-	L	92.74
Cmm6	-	M	91.50	Cmm79	-	L	92.93
Cmm7	-	M	93.15	Cmm80	-	M	87.54
Cmm8	+	M	86.86	Cmm81	-	M	89.70
Cmm9	-	L	87.17	Cmm82	-	L	90.70
Cmm12	-	M	86.50	Cmm83	-	L	91.78
Cmm13	+	M	87.67	Cmm84	-	M	94.55
Cmm14	-	L	88.62	Cmm85	-	L	80.15
Cmm15	-	M	88.05	Cmm92	-	M	93.39
Cmm16	+	M	87.28	Cmm93	+	M	87.79
Cmm19	+	L	89.61	Cmm94	-	L	86.75
Cmm20	+	M	85.97	Cmm95	-	L	95.18
Cmm21	+	L	90.44	Cmm97	-	S	85.71
Cmm26	-	M	88.77	Cmm98	-	L	85.73
Cmm36	-	M	91.91	Cmm101	-	L	84.93
Cmm38	+	M	93.56	Cmm102	-	L	85.29
Cmm39	+	S	90.00	Cmm105	-	L	84.16
Cmm41	+	L	92.58	Cmm106	-	M	90.27
Cmm42	-	L	90.31	Cmm107	-	M	89.00
Cmm43	-	L	91.82	Cmm108	-	M	86.79
Cmm48	-	M	91.64	Cmm110	-	M	89.14
Cmm49	-	M	88.56	Cmm111	-	S	80.79
Cmm67	-	S	92.92	Cmm113	-	S	87.32
Cmm68	-	L	90.39	Cmm114	-	M	87.04
Cmm69	-	L	90.25	Cmm115	-	S	88.76
Cmm70	-	L	87.82	Cmm116	-	S	89.72

Characteristics such as the colour, size and consistency of *Cmm* colonies can vary depending on the medium on which it is grown and the number of days of incubation. The *Cmm* strains grown in the Mueller Hinton medium showed diversity in colour, size and consistency. However, there is no correlation of the morphological characteristics of the strains with virulence since both the more virulent strains (Cmm9 and Cmm68) and less virulent strains, which are lacking the *pat*-1 gene (Cmm84 and Cmm98), were nonmucoid and had a large size (L). Colony size was determined visually and classified as small, medium or large. According to this classification scheme, 8 small strains, 27 medium strains and 25 large strains were observed.

2.3.5 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).

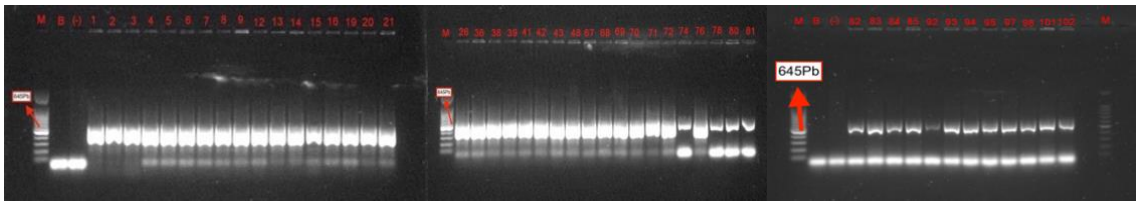

Figure 8. PCR results with primers Cm3/Cm4. 1% agarose gel stained with RedGel. A. Lane 1: 100 bp molecular marker. Lane 2: blank. Lane 3: Negative control. Lanes 4 to 20: DNA of strains isolated from tomato plants and seeds. B. Lane 1: 100 bp molecular marker. Lanes 2 to 20: DNA of strains isolated 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.

2.3.6 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 analyzed 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.

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

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 (+) means that amplification was observed for that gene and (-) means that amplification was not observed.

2.3.7 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 michiganensis* 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.


Cmm 97 (MK816914)
Cmm 36 (MK816890)
Cmm 20 (MK816887)
Clavibacter michiganensis subsp. michiganensis Cmm VT3 (HQ144242)
Cmm 110 (MK816920)
Cmm 106 (MK816918)
Cmm 83 (MK816907)
Cmm 76 (MK816901)
Cmm 78 (MK816902)
Cmm 26 (MK816889)
Cmm 15 (MK816884)
Cmm 81 (MK816905)
Cmm 74 (MK816900)
Cmm 14 (MK816883)
Cmm 39 (MK816892)
Cmm 38 (MK816891)
Cmm 06 (MK816877)
Cmm 101 (MK816915)
Cmm 69 (MK816896
Cmm 21 (MK816888)
Cmm 82 (MK816906)
Cmm 49 (MK816894)
Cmm 42 (MK816893)
Cmm 71 (MK816898)
Cmm 93 (MK816911)
Cmm 16 (MK816885)
Cmm 70 (MK816897)
Cmm 68 (MK816895)
Cmm 12 (MK816881)
Cmm 09 (MK816880)
Cmm 08 (MK816879)
Cmm 07 (MK816878)
Cmm 02 (MK816873)
Cmm 04 (MK816875)
Cmm 95 (MK816913)
Cmm 92 (MK816910)
Cmm 13 (MK816882)
Cmm 05 (MK816876)
Cmm 85 (MK816909)
Clavibacter michiganensis subsp. michiganensis FQ 2506 (HM189671)
Cmm 113 (MK816921)
Cmm 79 (MK816903)
Cmm 19 (MK816886)
Cmm 01 (MK816872)
Cmm 108 (MK816919)
Cmm 102 (MK816916)
Cmm 80 (MK816904)
Cmm 94 (MK816912)
Cmm 84 (MK816908)
Clavibacter michiganensis subsp. michiganensis BC2643 (EU685335)
Clavibacter michiganensis subsp. michiganensis 1232 (MH035729)
Clavibacter michiganensis subsp. michiganensis (AB299158)
Clavibacter michiganensis subsp. californiensis CFBP 8216 (OM457009)
Clavibacter michiganensis subsp. nebraskensis II296 (NR 037015)
Clavibacter michiganensis subsp. tessellarius (AM410693)
Clavibacter michiganensis subsp. chilensis ZUM3936 (NR 134713)
Clavibacter michiganensis subsp. phaseoli LPPA 982 (NR 133729)
Clavibacter michiganensis subsp. capsici PF008 (KX373437)
Clavibacter michiganensis subsp. sepedonicus (LN774178)
Clavibacter michiganensis subsp. insidiosus DSM 20157 (NR 115039)
Clavibacter michiganensis subsp. insidiosus Burkholder Pb (NR 036947)
Cmm 72 (MK816899)
Cmm 105 (MK816917)
Cmm 03 (MK816874)
Cmm 116 (MK816922)
0.0050

Figure 9. 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).

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 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.

2.4 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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CAPÍTULO 3

Pathogenic capacity of *Clavibacter michiganensis* subsp. *michiganensis isolated* from tomato on different solanaceae*:* tomato (*Solanum lycopersicum),* potato *(Solanum tuberosum),* eggplant *(Solanum melongena),* pepper *(Capsicum annuum),* tobacco *(Nicotiana tabacum)* and petunia *(Petunia hybrida).*

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ABSTRACT

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), is a plant pathogenic actinomycete that causes wilting and bacterial canker of tomato (*Solanum lycopersicum* L.). The *Clavibacter* genus includes bacteria of economic importance, causing great losses because they infect crops such as corn, alfalfa, potatoes, peppers, tomatoes and wheat and are quarantined worldwide. The objective of this research was to determine the pathogenic capacity of *Cmm* in different *Solanaceae* species of economic importance in Mexico. For this reason, inoculations with *Cmm* were carried out on tomato (*Solanum lycopersicum*), petunias (*Petunia hybrida*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*) and tobacco (*Nicotiana tabacum*) plants. The TM 68 strain conserved in the strain collection of the Phytopathology laboratory of CIAD, A.C. Culiacán was used, which was isolated from tomato plants from Ciudad Obregón Sonora, Mexico, and characterized morphologically and molecularly. The results indicate that, in inoculated tomato plants, the presence of symptoms was observed 13 days after inoculation (dai), showing characteristic symptoms such as yellow and rolled leaves, which at 24 dai were dry. In addition, general wilting is observed until finally the plant dies. Regarding the other solanaceous plants, the petunia plants presented symptoms of yellowing at the tips of the leaves and curling at 67 dai. On the other hand, the potato plants showed yellowing at 77 days and the tubers showed a brown ring; and the pepper plants showed strong defoliation. Finally, the eggplant and tobacco plants did not show any symptoms. At 60 days after specific detection of the bacteria using the PCR technique, it was determined positive in the six Solanaceae species. Therefore, it is concluded that the bacterium *Clavibacter michiganensis* subsp. *michiganensis* has the ability to reproduce in other solanaceous plants other than tomatoes, although it may or may not cause disease symptoms.

Keywords: *Cmm*, Solanaceae, Bacterial canker, Pathogenicity

3.1 INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), is a gram-positive aerobic bacterium and is the causal agent of bacterial canker in tomato (*Solanum lycopersicum* Mill.). This disease was first described in 1910 in Michigan, USA (Eichenlaub et al., 2006). As a result of the severity of the disease and due to economic losses due to the decrease in production, *Cmm* is considered a quarantined organism by the European Union and many other countries (De León et al., 2008). *Cmm* infection begins through wounds and natural openings such as stomata, causing a decrease in the yield of tomato production worldwide (Carlton et al., 1998). Secondary infection can take place once the crop is established and occurs mainly due to cultural practices such as pruning, tutoring, contact between diseased and healthy plants, splashes during pesticide applications, irrigation, etc. (Ricker and Riedel, 1993). The symptom of wilting on the leaves is the most commonly observed; the damage begins in the oldest leaves, where yellowing of the margins towards the leaf blade appears, the affected tissues gradually die and the leaf blade dries out. The disease progresses from the lower part upwards and the foliage becomes burned or blackened. Darkening can develop on the petioles of the leaves as well as on the stems (Vega and Romero, 2016). When cutting the stems longitudinally, the vascular tissues can be seen to acquire a moist, yellow or orange appearance, and in some cases even a dark color. It is worth mentioning that, when the bacteria are found in high concentrations in these tissues, the marrow can be easily separated (Jahr et al., 2000). In infected fruits, a spot known as “bird's eye” is occasionally observed, which appears as a small dark spot, surrounded by a white halo (Chang et al., 1992; Werner et al., 2002). Another characteristic symptom in fruits is the presence of yellow and/or orange dots in the area of the vascular tissues of the peduncle when the fruit is separated from diseased plants by *Cmm*. In this case, the points (xylem and phloem tissues) have a wet appearance and are deep yellow and in some cases orange. In this regard, great variability has been observed in the symptoms caused by *Cmm* in solanaceous plants and this depends mainly on the susceptibility of the host cultivar, the virulence of the strain and the environmental conditions that favor or

do not favor the development of the disease (Smirnov et al., 2023). The bacteria moves through the vascular tissues of the fruit, reaches and enters the internal part of the seeds, these being the main source of dissemination over long distances where it is transmitted to the plant that gives rise and from there to the crop in general. In the final stage of the disease, the entire plant wilts and dies (Sen et al., 2015). The genus *Clavibacter* has been grouped into five subspecies which cause diseases in different crops of economic importance: in tomato (*C. michiganensis* subsp. *michiganensis*, *Cmm*), in corn (*C. michiganensis* subsp. *nebraskensis*, *Cmn*), in potato (*C. michiganensis* subsp. *sepedonicus*, *Cms*) in alfafa (*C. michiganensis* subsp, *insidiosus*, *Cmi*) and in peppers (*C. michiganensis* subsp. *capsici*, *Cmc*) (Tambong, 2017).According to various research works, it has been reported that *Cmm* has the capacity to cause damage to other crops in addition to its main host, which is tomato. In this sense, phenotypic variants of *Cmm* were isolated from pepper fields and pepper seeds during quarantine inspections in the United States, Europe, and other countries. All strains isolated from this crop produced orange colonies with a less mucoid consistency than typical *Cmm* strains isolated from tomato; however, the results of ELISA, fatty acid analysis, 16S rDNA sequencing, and PCR analysis showed that all pepper isolates were similar enough to be identified as *Cmm*. Likewise, inoculation tests of the tomato and pepper isolates showed that the former caused severe wilting and canker in the tomato, but they only caused canker and did not wilt the pepper and bell pepper; however, pepper isolates did not cause wilting, even in tomato. In that sense, Yim et al. (2012) suggest that pepper isolates may represent a separate *Cmm* population that has evolved within the confines of this host. In 5 regions of Russia during the years 2011 to 2017 Ignatov et al. (2019) isolated *Clavibacter* from yellow pepper plants and diseased potato tubers. Potato plants exhibited yellowing, leaf necrosis, wilting of leaves and whole plants, and brown veins around the eyes of the tuber were observed in cross sections. Isolates from each diseased potato plant (35 isolates) were identified as *Clavibacter michiganensis* subsp. *Michiganensis* (*Cmm*). In this study, the taxon-specific polymerase chain reaction (PCR) technique was used with primers CMM5/CMM6. Sequencing of the 16S

rRNA gene (GenBank accession numbers MH035728.1 to MH035762.1) showed a >99% relationship to the *Cmm* type strain NCPPB2979T. This was the first report of *Cmm* affecting potatoes in this country, so there is a need to know the ability of the bacteria to infect other species of solanaceous plants, which will improve the understanding of its pathogenesis.

3.2 MATERIALS AND METHODS

3.2.1 *Cmm* strain used for pathogenicity testing.

The *Cmm* strain isolated from tomato from Mexico identified as TM 68, which showed a high degree of pathogenicity in this crop, and which was previously characterized morphologically and molecularly (GenBank accession number: MK816895), was reactivated in Mueller Hinton artificial medium.

3.2.2 Seedling production

The seeds of the six nightshades were sown: tomato (*Solanum lycopersicum*), petunias (*Petunia hybrida*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*) and tobacco (*Nicotiana tabacum*), for which soil previously sterilized at 121°C for one hour.

3.2.3 Inoculation of seedlings and visualization of disease symptoms

Two pots containing 5 seedlings each were used for each solanaceous species evaluated. Uninoculated control seedlings were included in the analysis. For inoculation, a sterile wooden stick was used, the tip of which was impregnated with bacterial cells of the *Cmm* strain and placed in the axil of the seedlings (Figure 1). The pots were maintained under greenhouse conditions with the agronomic management of irrigation and fertilization required for the different species. Symptom assessment was performed daily after inoculation.

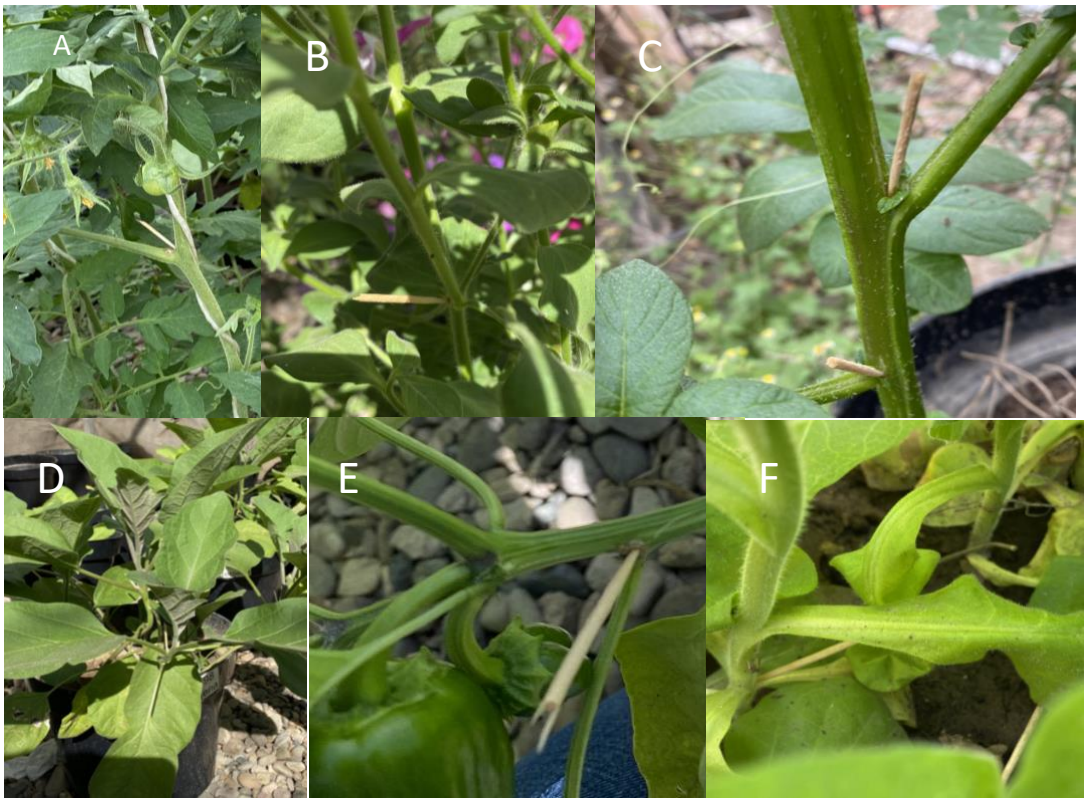

Figure 10. Inoculated seedlings of the different solanaceous crops A): tomato, B): petunia, C): potato, D): eggplant, E): pepper and F): tobacco.

3.2.4 Rapid detection of *Cmm* bacteria with the use of immunostrips and reisolation in Mueller Hinton Culture medium.

A tissue sample was taken from each of the 6 solanaceous crops and the test was performed with the *Cmm*-specific immunostrips from the Agdia brand, according to the instructions suggested by the manufacturer. To verify that the bacteria was viable, it was reisolated from the immunostrip buffer from which a sample was taken and sown in Mueller Hinton culture medium.

3.2.5 Detection of *Cmm* by PCR in inoculated plants.

In order to confirm the presence/absence of *Cmm* and verify the detection sensitivity of the immunostrips, an endpoint PCR was run in all evaluations. To this end, DNA extraction was carried out from plants inoculated with the TM 68 strain of the six Solanaceae species and from the control plants using the method using CTAB (Cetyltrimethylammonium Bromide), following the protocol described by

Voigt et al. (1999). Subsequently, specific detection was carried out by PCR with the primers Cm3 (5'-CCTCGTGAGTGCCGGGAACGTAT-3') and Cm4 (5'-CCACGGTGGTTGATGCTCGCGA-3') under the amplification conditions proposed by Sousa et al. (1997), with modification of the annealing temperature and time in the three phases of PCR (denaturation, annealing and elongation) as described below: initial denaturation of 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 30 s and a final extension of 72°C for 5 min. The PCR was carried out in a Bio-Rad model T-100 thermocycler. Finally, the PCR products were visualized on 1% agarose gel in an electrophoresis chamber (BioRad) with the conditions of 80 V, 400 mA for 80 min.

3.3 RESULTS AND DISCUSSIONS

The first symptoms recorded were observed in the inoculated tomato plants at 13 dai, where the presence of yellow and rolled leaves was manifested. Subsequently, at 24 dai the plants showed general wilting and the foliage showed blighting which was more intense in the older leaves and increased as the days went by. The detection of the bacteria was corroborated with the use of immunostrips, which gave a positive result (Figure 2A) and from the buffer, seedings were carried out in Mueller Hinton culture medium and the bacteria were correctly re-isolated (Figure 3A). Finally, when making longitudinal cuts in the stems, the vascular tissues were observed to be moist and yellow in color, where the stem cover was easily detached between nodes. The symptoms observed in tomato plants corresponded to those reported by various authors such as Jahr et al. (2020), Vega and Romero (2016), Chalupowicz et al. (2012) and Sen et al. (2015). With respect to the symptoms associated with bacterial canker disease, it has been reported that the *Cmm* bacteria require the active secretion of serine proteases from the early stages of the infection and that they facilitate its movement to make bacterial aggregates in the xylem of the plant tomato, and this way obstructs the vascular bundles, preventing free access of essential nutrients for the normal development of plants (De león et al., 2011). In pepper plants

inoculated with the *Cmm* bacteria, the characteristic symptoms that commonly occur in tomatoes were not observed; however, at 24 dai, a positive result was obtained in the rapid tests with immunostrips (Figure 2E) from which the strain inoculated in Mueller Hinton culture medium was reisolated (Figure 3B). At 67 dai the plants showed severe defoliation, which differs from the results reported by Yim et al. (2012) where they observed in pepper plants inoculated with a *Cmm* strain isolated from tomato a minimal development of canker on the stems and leaf blight without wilting; however, it is worth mentioning that its evaluation period was limited to 25 days and that the presence of symptoms is associated with the susceptibility of the host cultivar, the virulence of the strain and the environmental conditions that favor or do not favor the development of the disease (Smirnov et al., 2023). For the other inoculated solanaceae, rapid detection was performed with immunostrips at 24 dai; however, these gave negative results (Figures 1B, 1C, 1D and 1F) corresponding to petunia, potato, eggplant and tobacco plants, respectively.

Figure 11. Rapid immunological test for *Cmm* at 24 dai A): Positive tomato plant (indicated by two intense red bands including the positive control). B): Negative petunia plant. C): Negative potato plant. D): Negative eggplant plant. E): Positive pepper plant, and F): Negative tobacco plant.

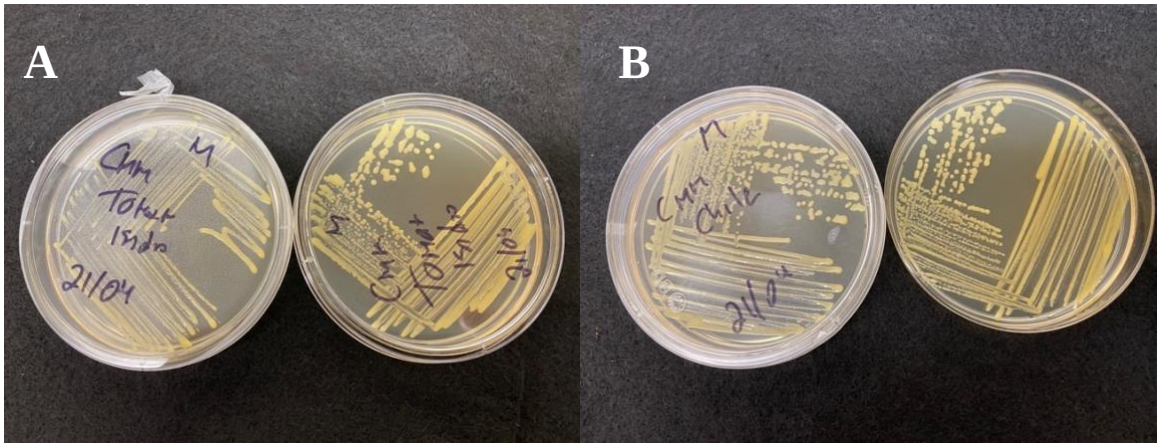

Figure 12. *Cmm* seeding of immunostrip buffer. A) isolated from tomato plants with symptoms and B) isolated from pepper plant without symptoms of bacterial canker.

At 60 dai, samples were taken from the 6 different solanaceae inoculated with the TM 68 strain, and DNA extraction was carried out to perform a PCR with the specific primers for *Cmm* (Cm3 and Cm4) that generate a 640 bp amplicon. As a result, positive detection was observed for all inoculated solanaceous plant species. The above confirms the positive result previously obtained for tomato, and chili plants and suggests that petunia, potato, eggplant and tobacco plants cover up the *Cmm* infection showing a certain degree of tolerance, a phenomenon defined by Pagán and García-Arenal (2018) as the host's ability to cope with infections caused by pathogens where the plant moderates the level of their replication and reduces the damage caused by its infection. Subsequently, seven days after the positive confirmation of the bacteria by PCR, yellowing symptoms were observed on the tips of the leaves towards the stem and flowers in the inoculated petunia plants. In addition, detection by immunological strips and seeding in Mueller Hinton culture medium were also carried out (Figure 4). The above allows us to indicate that petunia plants have an intermediate degree of tolerance to *Cmm* infection, but they do show characteristic symptoms, although in a much longer time than that observed for tomatoes and peppers, the former being their main host and the second with the least affectation, a host already reported (Burokiene et al., 2005 ; Latin et al., 1995 ; Lewis-Ivey and Miller, 2000).

Figure 13. Detection of *Cmm* in petunia plant. A) Plant with symptoms caused by the bacteria, B) Positive detection with the use of immunostrips, and C) Growth of the bacteria in culture medium.

At 77 dai, the final evaluation of the potato, eggplant and tobacco plants was carried out, where the potato plants showed yellowing and dry leaves; as well as, a brown ring when making longitudinal cuts in the tubers (Figures 5A and 5B), which agrees with what was reported by Ignatov et al. (2019) for the symptoms developed by *Cmm* infection in this crop. Subsequently, a rapid immunological test was performed and this gave a positive result. Furthermore, from the immunostrip buffer, the seeding was carried out in Mueller Hinton culture medium (Figures 5C and 5D). The above confirms the positive result obtained by the PCR technique and also corresponds to the degree of intermediate tolerance described above for petunia plants.

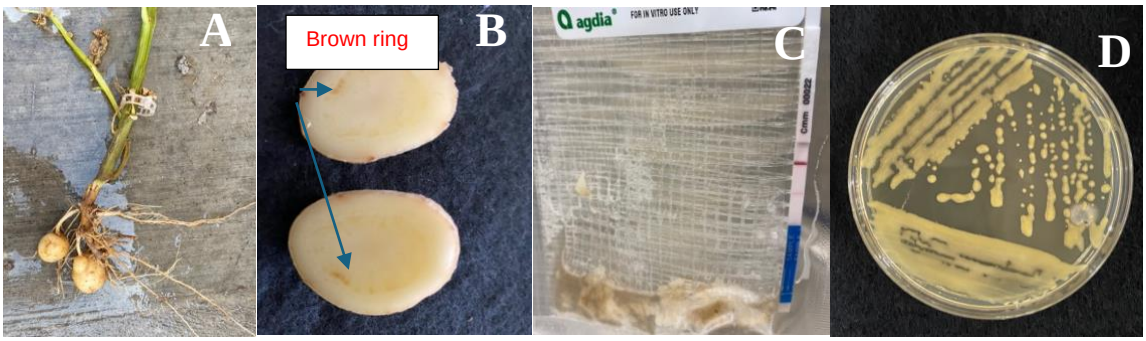

Figure 14. Detection of *Cmm* in potato fruit and potato plant. A) Potato plant with fruits and necrotic stem base, B) Potato fruit that has brown rings in the center, C) Rapid test for the detection of the bacteria with a positive result, and D) Growth of the bacteria in culture medium.

At the end of the evaluation period at 77 dai, the eggplant and tobacco plants inoculated with the *Cmm* bacteria showed no differences with the non-inoculated plants (negative controls) and although both species gave positive results for the detection of the bacteria by PCR, they did not showed symptoms and the strain was not recovered in culture medium as in the other four solanaceae. The above may indicate that these two species have developed a high degree of tolerance to the *Cmm* infection process in such a way that the bacterial replication thresholds are conserved at such low levels that they are not manifested visually since they are not capable of causing damage.

3.4 CONCLUSIONS

In the present work was determined that the bacteria *Clavibacter michiganensis* subsp. *michiganensis* is capable of causing disease in other solanaceous plants such as pepper, petunias and potatoes; in addition to tomato plants, where the symptoms develop in a shorter period of time and with greater severity. Although the common symptoms caused by the bacteria did not occur in the pepper plants, defoliation was observed at the end of the experiment. On the other hand, the eggplant and tobacco plants did not show any symptoms; however, they were positive in PCR detection, so this phenomenon may indicate that these species show a very high degree of tolerance to *Cmm* infection; however, they can be considered hosts and could contribute to the spread of the disease. Of the 6 solanaceae inoculated, only the TM 68 strain was recovered from tomato, pepper, petunia and potato plants, so it could be suggested that in eggplant and tobacco plants the replication of the bacteria is maintained at levels lower than the other solanaceous plants evaluated.

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CAPÍTULO 4.

Análisis genómico comparativo de cepas de *Clavibacter michiganensis* subsp. *michiganensis* con diferente grado de virulencia en plantas de tomate.

RESUMEN

El cultivo de tomate (*Solanum lycopersicum* L.) es fuertemente atacado por la bacteria *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), que es la causante del cáncer bacteriano. Esta bacteria causa grandes pérdidas en producción y por tal motivo se encuentra cuarentenada a nivel mundial. El objetivo de la presente investigación fue realizar el análisis comparativo de los genomas de cinco cepas de *Cmm* que se aislaron de plantas de tomate de México y que previamente se clasificaron en un rango de menor a mayor virulencia como: Cmm84, Cmm39 Cmm04, Cmm21 y Cmm09. La obtención de ADN bacteriano se realizó mediante el método de lisis por calor y la secuenciación del genoma se realizó por servicio en el laboratorio CD Genomics (45-1 Ramsey Road, Shirley, NY 11967, USA) con la plataforma Sanger / Illumina 1.9. El análisis comparativo de los genomas y los plásmidos se realizó con el programa Geneious Prime® 2023.2.1 utilizando el algoritmo MAUVE Progressive. Se concluye que la mayor diferencia encontrada entre los genomas se ubica en una región del cromosoma y en diversas regiones del plásmido pCM2U, las cuales codifican para genes involucrados principalmente en el metabolismo celular y en factores de respuesta a estrés, que por lo general se consideran factores de virulencia en otros microorganismos patógenos de plantas. Con la finalidad de corroborar la significancia de estos hallazgos bioinformáticos, se recomienda realizar estudios posteriores de expresión génica.

Palabras claves: *Cmm*, Virulencia, Cromosomas, Plásmidos, Genomas.

4.1 INTRODUCCIÓN

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), es una bacteria aerobia Gram positiva y es el agente causal del cáncer bacteriano en tomate (*Solanum lycopersicum* Mill.). Esta enfermedad fue descrita por primera vez en 1910 en Michigan, USA (Eichenlaub *et al*., 2006). Debido a la severidad de la enfermedad se generan pérdidas económicas consecuencia de la disminución en producción y por tal motivo *Cmm* es considerada como un organismo cuarentenado por la Unión Europea y muchos otros países (De León *et al*., 2008). La diversidad genética de *Cmm* en brotes de enfermedades se ha investigado en los últimos años en diversas regiones del mundo y los primeros estudios de secuenciación y caracterización genómica de la especie dieron pie a diversas investigaciones sobre la caracterización fenotípica y genotípica de *Cmm* utilizando métodos de tipificación genómica (Baysal *et al*., 2010). Recientemente, los estudios para entender el mecanismo de la patogenicidad de *Cmm* y la interacción con su hospedero durante este proceso tienen avances sustanciales y hoy se conoce que *Cmm* cuenta con dos plásmidos circulares descritos como pCM1 y pCM2, los cuales contienen genes esenciales para la patogenicidad en su hospedero. También se ha informado que en el cromosoma bacteriano se encuentra una "Isla de patogenicidad" que cuenta con dos regiones: la región *chp* que incluye varias serin proteasas y la región *tomA*. Cabe mencionar que existen varios estudios enfocados en la descripción de la patogénesis y virulencia de *Cmm* que han ayudado a la caracterización de poblaciones de esta bacteria en diferentes regiones productoras de tomate en el mundo, permitiendo conocer su potencial mediante el análisis de genes asociados a esta función. Por lo anteriormente descrito, el objetivo de esta investigación fue realizar el análisis genómico comparativo de cinco cepas de *Cmm* que mostraron diferencias en grados de virulencia con la finalidad de mejorar nuestra comprensión sobre los factores que podrían ser determinantes sobre tal actividad.

4.2 MATERIALES Y METODOS

4.2.1 Selección de cepas de *Cmm* para secuenciación de los genomas

Se realizaron pruebas de análisis de virulencia de 14 cepas de *Cmm* que se encuentran caracterizadas morfológica y molecularmente mediante secuenciación del gen 16S, las cuales se identificaron como: Cmm04, 09, 12, 21, 36, 39, 45, 68, 81, 84, 97, 98, 107 y 108.

Las cepas se encuentran preservadas en el cepario del Laboratorio de Fitopatología del Centro de Investigación en Alimentación y Desarrollo, A. C. subsede Culiacán y se reactivaron en medio de cultivo Mueller-Hinton para luego ser inoculadas con el uso de un palillo de madera estéril en 16 plántulas de tomate de la variedad Moctezuma por cada cepa bajo estudio. El testigo negativo se conformó de 16 plántulas de tomate sin inocular.

El registro de presencia y severidad de los síntomas causados por las diferentes cepas de *Cmm* se realizó diariamente hasta los 21 días después de inoculación. La evaluación del daño se realizó de acuerdo con la escala propuesta por Foster y Echandi (1973), dónde:

0= Planta sana

1= Planta con 1/3 de hojas marchitas

2= 1/3-2/3 de hojas marchitas

3= más de 2/3 de hojas marchitas y,

4= Planta muerta.

El análisis de los datos se realizó con el programa Minitab 19 mediante la prueba de ANOVA de un solo factor y comparación de medias de Tukey.

4.2.2 Procesamiento de las cepas de *Cmm* con diferente grado de virulencia para la secuenciación de los genomas.

De acuerdo a los resultados obtenidos de la prueba de virulencia se seleccionaron cinco cepas que se encontraron en el rango entre virulencia alta y baja a las cuales se les realizó la extracción del ADN genómico mediante el método de lisis por calor, donde se colocaron las cepas bacterianas a 95ºC por 15 min. Después del tiempo se colocaron en hielo por un periodo de 7 min. Se centrifugaron a 16000 *x g* por 10 min. Se decantó el sobrenadante y se resuspendió la pastilla de ADN en 40 µL de agua destilada estéril grado molecular.

El ADN genómico obtenido se revisó en cuanto a parámetros de calidad y pureza en un NanoDrop One (Thermoscientific) para su envió a secuenciación por servicio al Laboratorio CD Genomics (45-1 Ramsey Road, Shirley, NY 11967, USA), con la tecnología Sanger / Illumina 1.9.

Se realizó el depósito de las secuencias de los genomas en la base de datos del GenBank para obtener los números de acceso correspondientes. El análisis comparativo de los cinco genomas; así como, de los plásmidos bacterianos se realizó con el programa Geneious Prime® 2023.2.1., utilizando para el alineamiento múltiple el algoritmo Mauve Progressive.

4.3 RESULTADOS Y DISCUSIONES

4.3.1 Selección de cinco cepas de *Cmm* de acuerdo a su nivel de virulencia

Con base en el grado de virulencia observado en plantas de tomate inoculadas con 14 cepas de *Cmm* se realizó la selección de las cepas Cmm09, 21, 04, 39 y 84 (remarcadas en letras negritas) cuyo rango de virulencia varía de mayor a menor virulencia de acuerdo con los datos incluidos en el cuadro 5.

Cuadro 5. Prueba de Tukey para análisis de medias de virulencia.

Cepa	Media	Agrupamiento
09	3.0625	A
68	2.9792	A
21	1.9583	B
108	1.6667	BC
107	1.6250	BC
36	1.5209	BCD
04	1.4267	CD
12	1.0208	CDE
81	1.000	CDE
45	0.8958	CDE
97	0.7709	DE
39	0.7500	DE
98	0.6042	E
84	0.4167	E

Las cinco cepas seleccionadas se enviaron a secuenciación dónde se recibió para cada cepa las secuencias de un cromosoma que varió de 3,271,828 a 3,271,848 pb, de un plásmido pCM1 de 20,560 pb y de un plásmido pCM2U que varió de 73,135 a 73,141 pb y altos porcentajes de Guanina-Citocina (%GC) que variaron de 71.12 a 71.75, lo cual es similar a lo observado por Gartemann *et al*. (2008) para el genoma de la cepa NCPPB382. Los números de accesos proporcionados por el GenBank de las secuencias de los genomas de las cinco cepas de *Cmm* seleccionadas se encuentran en el cuadro 6.

Cuadro 6. Números de Acceso del GenBank para las cepas de *Cmm*

Cepa de *Cmm*	N° de Acceso cromosoma (Tamaño bp)	N° de Acceso plásmido pCM1 (Tamaño bp)	N° de Acceso plásmido pCM2U (Tamaño bp)	%GC
CMM04	NZ_CP085140.1 (3,271,831 pb)	NZ_CP085141.1 (20,560 pb)	NZ_CP085142.1 (73,141 pb)	71.65%
CMM09	NZ_CP085137.1 (3,271,828 pb)	NZ_CP085138.1 (20,560 pb)	NZ_CP085139.1 (73,139 pb)	71.12%
CMM21	NZ_CP117776.1 (3,271,848 pb)	NZ_CP117777.1 (20,560 pb)	NZ_CP117778.1 (73,141 pb)	71.64%
CMM39	CP085140.1 (3,271,831 pb)	CP085141.1 (20,560 pb)	CP085142.1 (73,141 pb)	71.75%
CMM84	NZ_CP085106.1 (3,271,832 pb)	NZ_CP085107.1 (20,560 pb)	NZ_CP085108.1 (73,135 pb)	71.70%

Al realizar el análisis comparativo de la información genética de los cromosomas y de los dos plásmidos obtenidos de las cinco cepas de *Cmm* se obtuvo la siguiente información:

4.3.2 Análisis comparativo de los cinco cromosomas con el programa MAUVE PROGRESSIVE.

Al realizar el análisis bioinformático se observó una región muy variable entre las secuencias de los cinco cromosomas de las cepas de *Cmm* (Figura 15), por lo que se enfocó la búsqueda en las diferencias presentes en esta zona (Figura 16). Cabe mencionar que la diversidad genética entre cepas de *Cmm* ha sido reportada con anterioridad (Tancos *et al*., 2015; Thapa *et al*., 2017) y en este estudio se confirma este hallazgo lo que sugiere la existencia de múltiples eventos de introducción de material genético a lo largo del tiempo (Thapa *et al*., 2017).

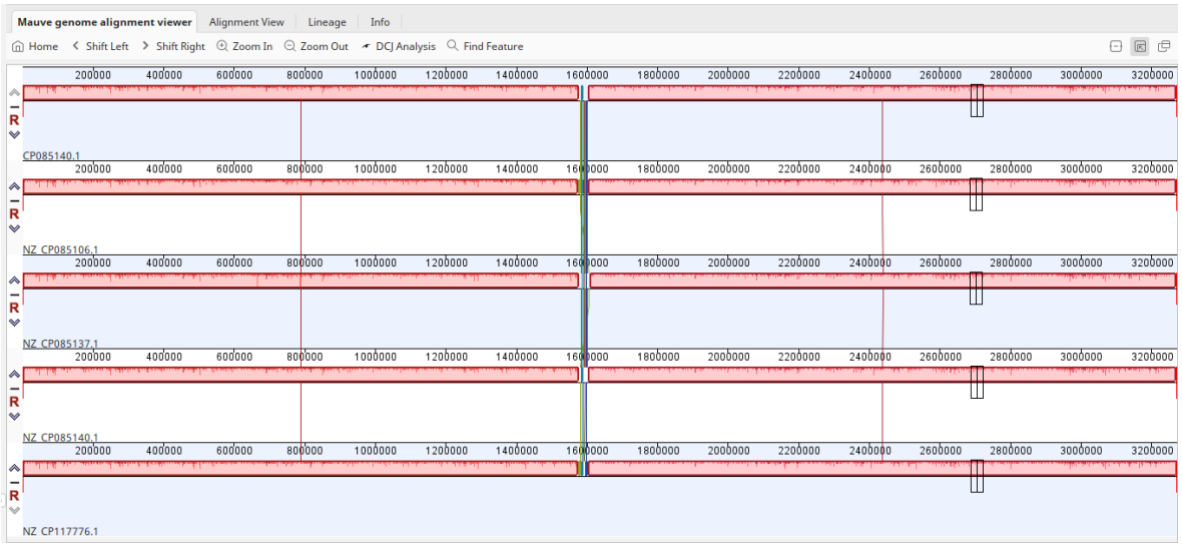
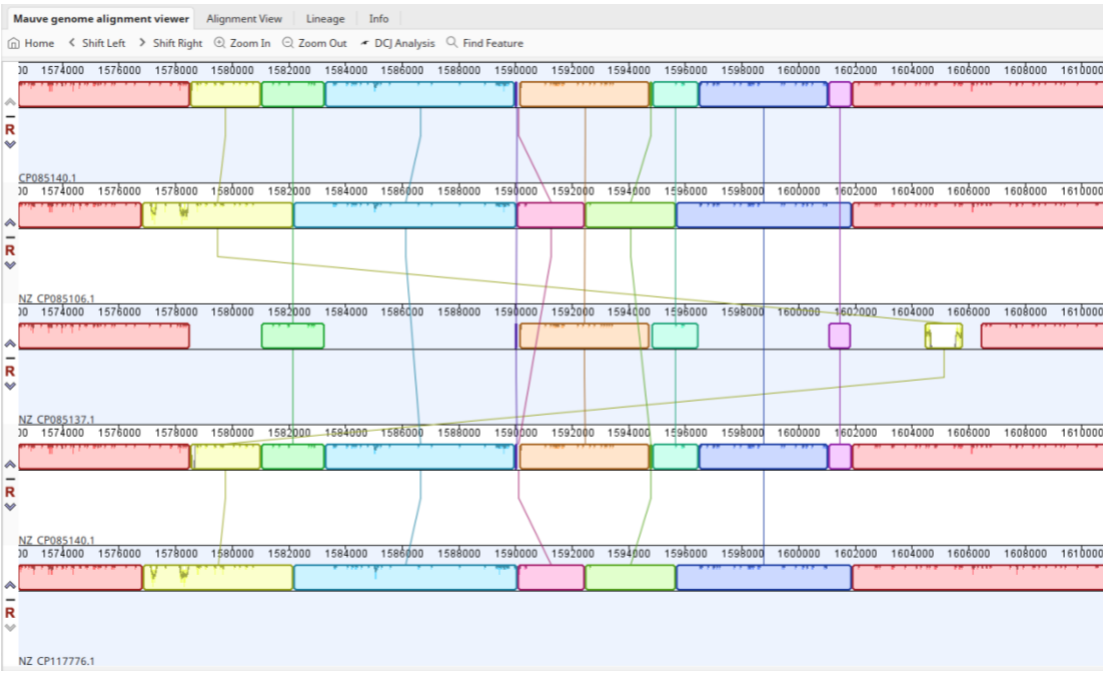


Figura 15. Análisis comparativo de los cinco cromosomas de las cepas de *Cmm*.



El análisis bioinformático de dicha región hipervariable mostró que ésta codifica para diversos genes cuyas funciones se describen a continuación en el cuadro 7.

Cuadro 7. Genes asociados a regiones hipervariables dentro de los cinco cromosomas de las cepas de *Cmm*.

Gen	Función
Alpha-glucan family phosphorylase	Regulación del metabolismo del

	glucógeno endógeno en períodos de inanición, respuesta al estrés o adaptación rápida a entornos cambiantes.
Isoamylase	Efectores citosólicos que manipulan el metabolismo del glucógeno.
ABC-F family	Reparación del DNA y modulación de la regulación de la traducción del RNA mensajero (mRNA).
Long-chain-fatty-acid--CoA ligase	Juega un rol importante en la degradación de ácidos grasos.
Malto-oligosyltrehalose synthase	La síntesis de trehalosa protege a las células bacterianas de sufrir desecación, congelación y estrés osmótico lo que explica la sobrevivencia tanto dentro como fuera del hospedero. Esto es de especial relevancia en bacterias que crecen en la savia del xilema de las plantas y cuya fuerza osmótica puede variar dependiendo de los niveles de azúcares.
Glycogen debranching protein GlgX	Promueve la degradación del glucógeno que se considera una fuente de carbono para la bacteria.
Aminopeptidase N	Son exopeptidasas que liberan selectivamente residuos de aminoácidos N-terminales de polipéptidos y proteínas.

4.3.3 Comparación de los plásmidos pCM1 de las cinco cepas de *Cmm*

El análisis comparativo realizado sobre los plásmidos pCM1 de las cinco cepas de *Cmm* no mostró diferencias significativas (Fig. 16). De acuerdo con Thapa *et al*. (2017), el plásmido pCM1 juega un rol esencial en la capacidad patogénica de *Cmm* en plantas de tomate; sin embargo, los resultados obtenidos en este estudio no muestran algún dato diferencial relevante que nos permita explicar la diferencia observada en cuanto a datos de virulencia de las cepas bajo estudio.

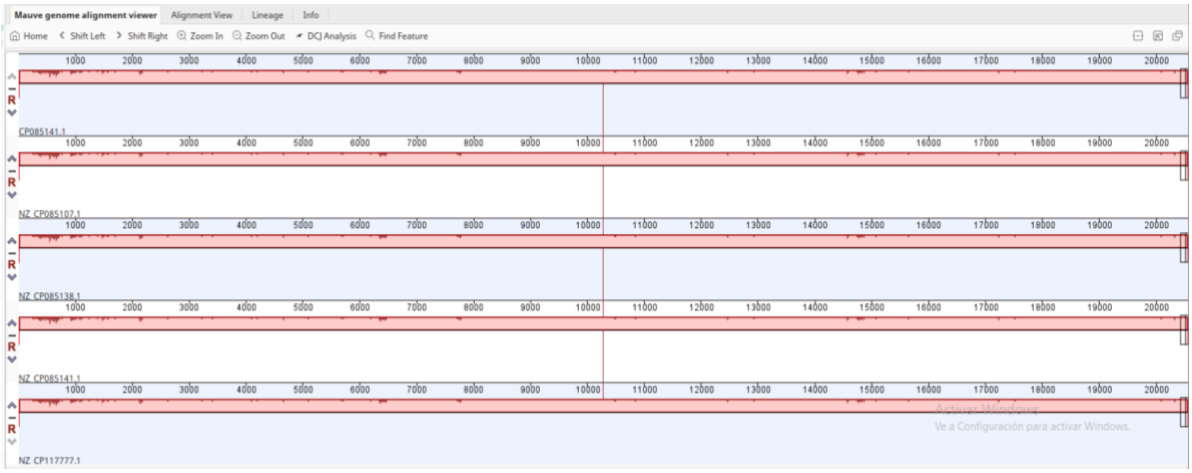


Figura 16. Comparación de los plásmidos pCM1

4.3.4 Comparación de los plásmidos pCM2U de las cinco cepas de *Cmm*

Al realizar el análisis comparativo de los plásmidos pCM2U de las cinco cepas de *Cmm* bajo estudio, se observaron zonas de gran variabilidad que se hacen evidentes por la presencia de líneas blancas dentro del alineamiento principalmente en la cepa CMM09 (NZ_CP085139.1) (Figura 17), que es considerada la de más alta virulencia. Al respecto, Thapa *et al*. (2017) observaron que, el contenido genético del plásmido pCM2U difiere sustancialmente entre cepas de *Cmm* y confirmaron que el rol de este plásmido en la virulencia es dependiente de cada cepa.

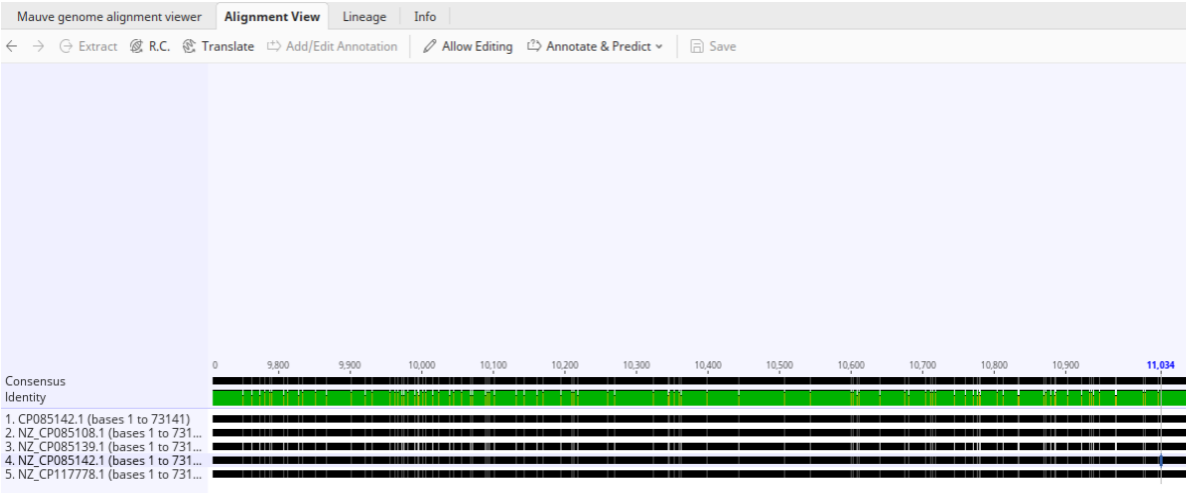


Figura 17. Comparación de los plásmidos pCM2U

Al hacer el alineamiento en BLAST (Figura 18) se confirma la variabilidad existente de estas regiones entre diferentes cepas de *Cmm* lo que puede indicar cierta relevancia. Se toma como ejemplo un fragmento del gen RHS (repeat-associated core domain) que cumple con la función de mediación de la liberación de toxinas para competencia intercelular.

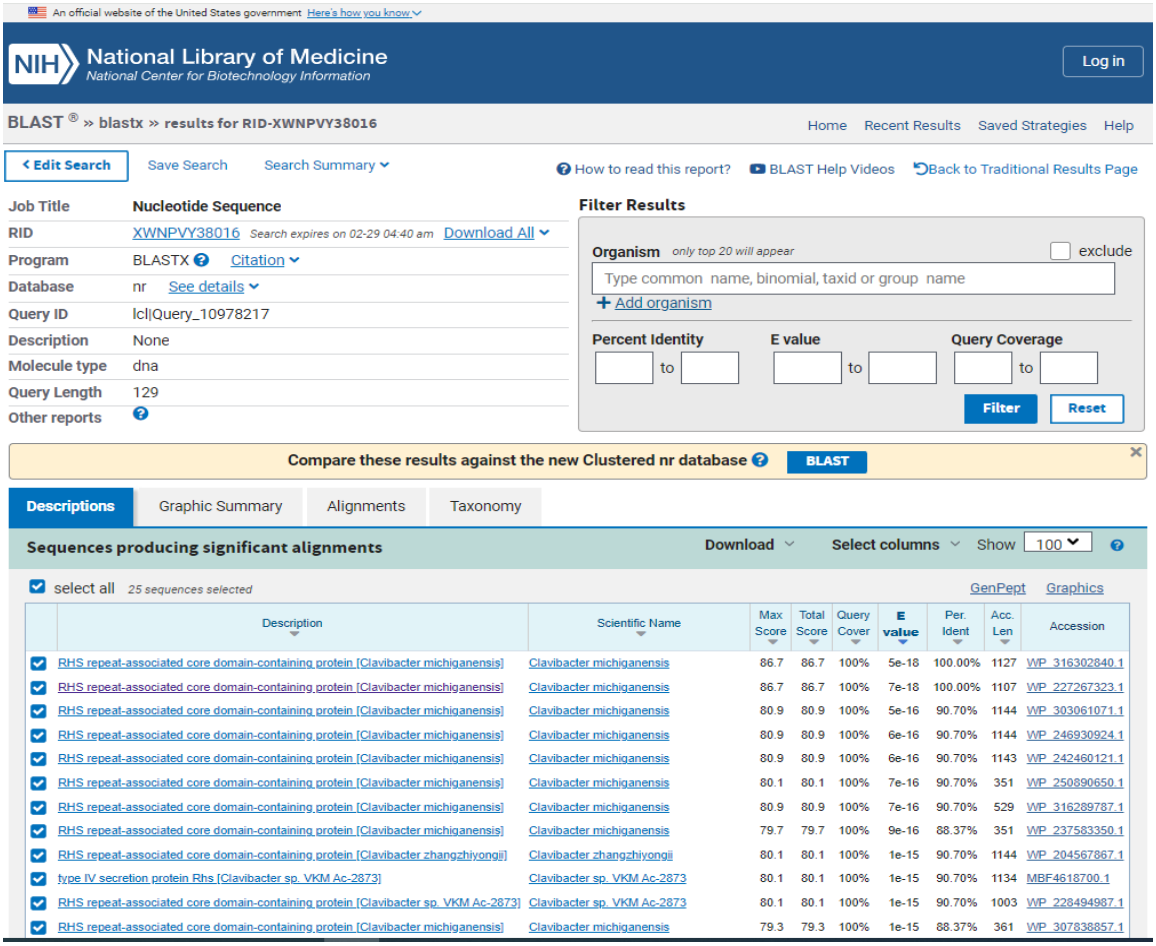


Figura 18. Alineamiento en BLAST de un fragmento del plásmido pCM2U que codifica para el gen RHS. Se observa una gran variabilidad en el porcentaje de identidad para diferentes cepas depositadas en el GenBank.

En el cuadro 8 se anexa lista de regiones hipervariables donde la mayor variabilidad se concentra en la cepa CMM09.

Cuadro 8. Genes asociados a regiones hipervariables dentro de los plásmidos pCM2U de las cepas de *Cmm*.

Gen	Función
SOS response-associated peptidase	La respuesta SOS implica la inducción de múltiples proteínas que sirven para promover la integridad del ADN; también incluye factores propensos a errores que permiten una mejor supervivencia y una replicación continua en presencia de daño extenso en el ADN.
MHS transporter (Metabolite H+ symporters)	Son transportadores secundarios de un sólo polipéptido, capaces de transportar pequeños solutos en respuesta a gradientes de iones quimiosmóticos.
RHS repeat-associated core domain (Rearrangement hotspot)	Medían la liberación de toxinas para competencia intercelular.
ParA family protein	ParA medía la segregación al interactuar con ParB unido al centrómero, pero cuando se une a ADP, ParA cumple una función diferente: la autorregulación de la transcripción de unión al ADN.
MobF family relaxase	Mob (for mobilization): frecuentemente confieren resistencia a antibióticos.
AAA family ATPase	Promueven el ensamble, operación o desensamble de complejos proteicos.

4.4. CONCLUSIONES

El análisis genómico comparativo de cinco cepas de *Cmm* con diferente grado de virulencia mostró a nivel de cromosoma una zona altamente variable y que codifica para enzimas relacionadas al metabolismo celular y de respuesta a condiciones adversas; así mismo, también se observó alta variabilidad en la secuencia pCM2U de la cepa CMM09 altamente virulenta con respecto a las otras y, escasa variabilidad en las secuencias del plásmido pCM1. Aunque el análisis bioinformático nos muestra hallazgos significativos, se recomienda analizar la

expresión directa de estos genes diferenciales para corroborar su función en la virulencia de la bacteria.

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5. CONCLUSIONES

En el presente estudio se generó información acerca de la diversidad morfológica, patogénica y genética de cepas de *Clavibacter michiganensis* subsp. *michiganensis* colectadas en diferentes zonas productoras de tomate en México, encontrando una gran variabilidad en características morfológicas macroscópicas; así como, en el análisis filogenético del gen 16S; así mismo, se observó que la bacteria *Cmm* tiene la capacidad de infectar a otras plantas solanáceas además del tomate; entre ellas, petunias, papa, berenjena, chile y tabaco; sin embargo, ésta puede o no ocasionar síntomas, lo último observado en berenjena y tabaco, por lo que se sugiere que la tasa de replicación de la bacteria se mantiene en niveles bajos como para no ocasionar daño. Finalmente, derivado del análisis de los genomas de cinco cepas de *Cmm* con diferente grado de virulencia se observó a nivel de cromosoma una zona altamente variable y que codifica para enzimas relacionadas al metabolismo celular y de respuesta a condiciones adversas. Se observó también alta variabilidad en la secuencia pCM2U de la cepa CMM09 altamente virulenta con respecto a las otras y escasa variabilidad en las secuencias del plásmido pCM1. Aunque el análisis bioinformático nos muestra hallazgos significativos, se recomienda analizar la expresión directa de estos genes diferenciales para corroborar su función en la virulencia de la bacteria.

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