Genomic Analysis of *Clavibacter michiganensis* Reveals Insight Into Virulence Strategies and Genetic Diversity of a Gram-Positive Bacterial Pathogen

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Clavibacter michiganensis subsp. *michiganensis* is a gram-positive bacterial pathogen that proliferates in the xylem vessels of tomato, causing bacterial canker disease. In this study, we sequenced and assembled genomes of 11 *C. michiganensis* subsp. *michiganensis* strains isolated from infected tomato fields in California as well as five *Clavibacter* strains that colonize tomato endophytically but are not pathogenic in this host. The analysis of the *C. michiganensis* subsp. *michiganensis* genomes supported the monophyletic nature of this pathogen but revealed genetic diversity among strains, consistent with multiple introduction events. Two tomato endophytes that clustered phylogenetically with *C. michiganensis* strains capable of infecting wheat and pepper and were also able to cause disease in these plants. Plasmid profiles of the California strains were variable and supported the essential role of the pCM1-like plasmid and the CelA cellulase in virulence, whereas the absence of the pCM2-like plasmid in some pathogenic *C. michiganensis* subsp. *michiganensis* strains revealed it is not essential. A large number of secreted *C. michiganensis* subsp. *michiganensis* proteins were carbohydrate-active enzymes (CAZymes). Glycome profiling revealed that *C. michiganensis* subsp. *michiganensis* but not endophytic *Clavibacter* strains is able to extensively alter tomato cell-wall composition. Two secreted CAZymes found in all *C. michiganensis* subsp. *michiganensis* strains, CelA and PelA1, enhanced pathogenicity on tomato. Collectively, these results provide a deeper understanding of *C. michiganensis* subsp. *michiganensis* diversity and virulence strategies.

Plants can be infected by both gram-negative and gram-positive bacteria. Gram-positive bacterial pathogens can cause plant diseases that are responsible for substantial crop losses, including species of the genera *Clavibacter*, *Streptomyces*, and *Rhodococcus* (Francis et al. 2010). The genus *Clavibacter* contains one species, *Clavibacter michiganensis*, which proliferates in the xylem and is able to infect a wide range of plants (Eichenlaub et al. 2006). Subspecies of *C. michiganensis* differentially infect various dicot and monocot hosts, including: tomato, potato, pepper, bean, wheat, maize, and alfalfa (Eichenlaub

and Gartemann 2011; González and Trapiello 2014). Despite the importance of these pathogens, there are no commercial cultivars with high levels of resistance or effective chemical control strategies. Plant-pathogenic *Clavibacter* strains initially establish latent infections, which facilitate pathogen colonization of plants as well as seed contamination (Gitaitis et al. 1991). Furthermore, endophytic *Clavibacter* strains, which are capable of colonizing vascular tissue without causing disease symptoms, have been frequently described (Ding et al. 2011; Eichenlaub and Gartemann 2011).

C. michiganensis subsp. *michiganensis* causes bacterial canker, which is one of the most important bacterial diseases of tomato (de León et al. 2011; Mansfield et al. 2012). Bacterial canker can cause substantial losses in greenhouse and field production systems, including losses in processing tomato production in California. *C. michiganensis* subsp. *michiganensis* gains entry into the plant through contaminated seeds, wounds, broken trichomes, or hydathodes and then proliferates in xylem vessels (Eichenlaub et al. 2006; Sen et al. 2015). Systemic infection results in unilateral leaf wilting, formation of stem canker and, in severe cases, wilting, necrosis, and death of the entire plant (de León et al. 2011). Inside stems, vascular discoloration and degradation of pith tissues is typically observed. The economic threat and the challenges to manage this disease have led to the inclusion of this pathogen as a quarantine agent in Europe, Asia, Africa, and the Caribbean (Eichenlaub and Gartemann 2011; Official Journal of the European Communities 2000). On tomato, nonpathogenic *Clavibacter* strains are frequently seed-associated and can be phylogenetically distinct from pathogenic *C. michiganensis* subsp. *michiganensis* strains based on multilocus sequencing (Jacques et al. 2012; Yasuhara-Bell and Alvarez 2015; Zaluga et al. 2013).

Several gram-negative bacterial plant pathogens, such as *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas euvesicatoria*, have been extensively studied, and scientists possess a detailed understanding of the molecular mechanisms regulating virulence (Cunnac et al. 2011; Dou and Zhou 2012; Toruño et al. 2016). A major virulence component of most gram-negative bacterial pathogens is the type III secretion system (T3SS), which enables the delivery of effector proteins into host cells to facilitate colonization and disease (Cornelis 2010; Cunnac et al. 2011). In contrast, gram-positive bacteria lack the T3SS and a mechanistic understanding of how they cause disease and suppress host defenses is largely unknown. Analyses of *C. michiganensis* subsp. *michiganensis* strains collected from diverse geographic origins have revealed diversity with respect to plasmid composition, plasmid number, and the presence of putative chromosomally

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encoded pathogenicity genes (Jacques et al. 2012; Milijašević-Marčić et al. 2012; Quesada-Ocampo et al. 2012; Tancos et al. 2015).

Investigations of the molecular basis of pathogenicity have been primarily performed on *C. michiganensis* subsp. *michiganensis* NCPPB382 (Eichenlaub and Gartemann 2011; Sen et al. 2015). The NCPPB382 genome has been sequenced and contains two plasmids, pCM1 and pCM2 (Gartemann et al. 2008; Meletus et al. 1993). The virulence factors *celA* and *pat-1* are encoded on pCM1 and pCM2, respectively. Loss of either plasmid decreases virulence, whereas a plasmid-free derivative was unable to induce disease symptoms but was still able to colonize tomato (Gartemann et al. 2003; Meletus et al. 1993). NCPPB382 possesses the *chp/tomA* pathogenicity island, which contains multiple glycosidases and proteases as well as a tomatinase involved in detoxification of the tomato alkaloid α -tomatine (Gartemann et al. 2008; Kaup et al. 2005). Deletion of the entire *chp/tomA* region rendered NCPPB382 unable to induce disease symptoms (Chalupowicz et al. 2012). Recently, several genes have been identified that are important for localized infections, including serine proteases and two cell wall-degrading enzymes (PCWDEs) (Chalupowicz et al. 2017).

In the present study, we sequenced and assembled high-quality genomes of 11 pathogenic *C. michiganensis* subsp. *michiganensis* strains isolated from tomato plants in California. In addition, we sequenced the genomes of five *Clavibacter* strains that were isolated from surface-disinfested tomato parts and colonized tomato endophytically but were not pathogenic on this host. We refer to these strains as tomato endophytes, as this term most commonly refers to microorganisms that can be isolated from surface-disinfested plant tissues in the absence of visible disease symptoms (Hardoim et al. 2015). We performed a comparative genomics analysis with the complete sequences of these strains, coupled with functional analyses of conserved genomic regions. Taken together, this study provides novel insights into genetic diversity and virulence strategies of diverse *C. michiganensis* subsp. *michiganensis* strains.

RESULTS

Phenotypic analyses, phylogeny, and genome sequencing of *Clavibacter* strains.

Over 95% of the United States processing tomatoes are grown in California (Geisseler and Horwath 2014). In California, *C. michiganensis* subsp. *michiganensis* infection is a reoccurring problem in greenhouse production of transplants and in the field. In order to investigate pathogenicity and aggressiveness of

different strains, the tomato cultivar Early Pak 7 was inoculated by stem prick with 12 *Clavibacter* strains isolated from tomato plants in California over different years and locations as well as the reference *C. michiganensis* subsp. *michiganensis* strain NCPPB382 (Table 1). Bacterial titers, leaf wilting, and canker formation were analyzed. Bacterial titers were analyzed from infected stem material 14 days postinoculation (dpi). With the exception of strain CASJ009, all strains grew to high titers and were pathogenic in tomato, although there was up to an eight-fold difference in bacterial growth among pathogenic strains (Fig. 1A to D). These results indicated that these 11 California strains are *C. michiganensis* subsp. *michiganensis*. In contrast, the *Clavibacter* strain CASJ009 did not cause disease symptoms (Fig. 1A to D). However, by 14 dpi, CASJ009 was recovered from vascular tissues above and below the site of inoculation (Supplementary Fig. S1C) and as far as 9 cm above the inoculation site. Taken together, these results indicate that CASJ009 is not a strain of *C. michiganensis* subsp. *michiganensis* but is an endophyte in tomato.

In order to investigate the genetic relationship of the *C. michiganensis* subsp. *michiganensis* strains from California with previously characterized *C. michiganensis* subsp. *michiganensis* strains, other *Clavibacter* subspecies, and nonpathogenic *Clavibacter* strains, we used a multilocus sequence analysis (MLSA) approach. We first verified that strains described as nonpathogenic on tomato do not cause disease symptoms on tomato and can act as endophytes. These analyses included many strains whose genomes have not been sequenced, but MLSA data are available (Jacques et al. 2012). Pathogenic strains of *C. michiganensis* subspecies included in the analysis were *C. michiganensis* subsp. *tessellarius* (NZ_LQXA00000000, wheat), *C. michiganensis* subsp. *insidiosus* (NZ_CP011043, alfalfa), *C. michiganensis* subsp. *nebraskensis* (NC_020891, corn), *C. michiganensis* subsp. *capsici* (NZ_CP012573, pepper), and *C. michiganensis* subsp. *sepedonicus* (NC_010407, potato) (Bentley et al. 2008; Lu et al. 2015b). Concatenated sequences from six housekeeping genes (*atpD*, *dnaK*, *gyrB*, *ppk*, *recA*, and *rpoB*) were used to generate a maximum likelihood phylogeny. The 11 pathogenic strains from California were placed in a large monophyletic clade with other *C. michiganensis* subsp. *michiganensis* strains, confirming that these are strains of *C. michiganensis* subsp. *michiganensis* (Fig. 1E). However, this analysis revealed that the California *C. michiganensis* subsp. *michiganensis* strains are not clonal, as they were placed in different subclades within the *C. michiganensis* subsp. *michiganensis* clade. This level of genetic diversity suggests multiple introduction events in California.

Table 1. *Clavibacter* strains sequenced in this study^a

Strains	Year isolated	Path ^b	Seq platform	Contigs	N50 value	Size (bp)	Accession no.	Plasmids
CASJ001	1999	+	MiSeq, PacBio	4	1,764,894	3,423,339	MDHB000000000	pCM1
CASJ002	1999	+	MiSeq, PacBio	3	3,284,808	3,423,641	MDHC000000000	pCM1/pCM2
CASJ003	1999	+	MiSeq	315	17,464	3,294,503	MDHD000000000	pCM1/pCM2
CASJ004	1999	+	MiSeq	477	14,451	3,351,833	MDHE000000000	pCM1/pCM2
CASJ005	2001	+	MiSeq	321	16,819	3,284,480	MDHF000000000	pCM1/pCM2
CASJ006	2002	+	MiSeq, PacBio	4	3,215,422	3,499,162	MDHG000000000	pCM1/pCM2
CASJ007	2011	+	MiSeq, PacBio	11	480,089	3,400,049	MDHH000000000	pCM1
CASJ008	2002	+	HiSeq	39	2,059,966	3,392,817	MDHI000000000	pCM1/pCM2
CAYO001	2001	+	MiSeq, PacBio	3	3,331,437	3,481,307	MDHL000000000	pCM1/pCM2
CA00001	2000	+	HiSeq	120	90,378	3,625,805	MDHK000000000	pCM1/pCM2
CA00002	2000	+	HiSeq, PacBio	4	3,281,486	3,371,744	MDHM000000000	pCM1/pCM2
CASJ009	2011	–	MiSeq, PacBio	2	3,279,109	3,422,081	MDHJ000000000	pSap1
CFBP7494	1999	–	HiSeq	15	439,451	3,425,817	MDJW000000000	–
CFBP7576	1997	–	HiSeq	372	20,825	3,399,000	MDJX000000000	pCM1/pCM2
CFBP8017	2006	–	HiSeq	65	95,162	3,284,576	MDJY000000000	–
CFBP8019	–	–	HiSeq	18	339,261	3,076,288	MDJZ000000000	–

^a Pathogenicity was verified on tomato and all pathogenic strains are *Clavibacter michiganensis* subsp. *michiganensis*.

^b Path = pathogenicity on tomato.

Fig. 1. Pathogenicity and phylogenetic analyses of *Clavibacter* strains. Three-week-old tomato plants (*Solanum lycopersicum* cv. Early Pak 7) were inoculated with wild-type *Clavibacter michiganensis* subsp. *michiganensis* CASJ001, CASJ002, CASJ003, CASJ004, CASJ005, CASJ006, CASJ007, CASJ008, CAYO001, CA00001, CA00002, NCPBP382, and the endophyte CASJ009. Tomato stems were stabbed with a needle and inoculated with 5 μ l of *C. michiganensis* subsp. *michiganensis* at 10^7 CFU/ml. **A**, Bacterial titers in infected tomato stem tissue 14 days postinoculation (dpi). Stem sections were taken 1 cm above the inoculation point to determine bacterial titers. No significant differences were detected by Tukey's honest significant difference, $P < 0.05$, $n = 18$. **B**, Time course of the appearance of wilting of the first leaflet above the inoculation point was monitored over 12 days. Plants were inoculated as described above, $n = 18$. The x axis indicates days postinoculation. **C**, Graph of wilting severity across all strains at 7 dpi, $n = 18$. **D**, Representative tomato stems exhibiting canker symptoms after inoculation with different *C. michiganensis* subsp. *michiganensis* strains. Images were taken 14 dpi. CASJ009 is an endophyte. **E**, Phylogeny of strains of *C. michiganensis* subspecies based on concatenated sequences of six housekeeping genes (*atpD*, *dnaK*, *gyrB*, *ppk*, *recA*, and *rpoB*). A maximum likelihood approach was used to obtain the phylogenetic tree, using MEGA software version 6 with 1,000 bootstrap replicates. E = endophyte/nonpathogen, P = pathogen, no color = strains sequenced in the present study, Cmt = *Clavibacter michiganensis* subsp. *tessellarius*, Cmi = *Clavibacter michiganensis* subsp. *insidiosus*, Cmn = *Clavibacter michiganensis* subsp. *nebraskensis*, Cm sp. tomato seed = *Clavibacter* isolated from tomato seeds, Cmc = *Clavibacter* isolated from pepper, and Cms = *Clavibacter michiganensis* subsp. *sepedonicus*. Unless otherwise noted, all pathogenic strains are *Clavibacter michiganensis* subsp. *michiganensis*. **F**, Representative wheat leaves exhibiting symptoms after inoculation with CFBP8017 and representative pepper stems exhibiting canker symptoms after inoculation with CFBP7576 strains. Stem and leaves images were taken 14 dpi. CASJ009 is a nonpathogenic strain.

Endophytic *Clavibacter* strains were distributed in different clades and three clustered with other *C. michiganensis* subspecies, which are known pathogens of wheat, pepper, and alfalfa (Fig. 1E). In order to determine if these endophytic strains could infect and cause disease in the crops infected by the *C. michiganensis* subspecies that they clustered with, inoculations were performed on wheat and pepper. *Clavibacter* isolate CFBP8017 clustered with *C. michiganensis* subsp. *tessellarius* strains that infect wheat and it induced mosaic disease symptoms on wheat plants (Fig. 1F). *Clavibacter* isolate CFBP7576 clusters with *C. michiganensis* subsp. *capsici* strains that infect pepper and it induced stem canker symptoms on pepper plants (Fig. 1F). In contrast, the *Clavibacter* isolate CASJ009, which does not cluster with any known pathogens, is unable to cause disease symptoms on either wheat or pepper.

In order to further investigate the genetic diversity of the *Clavibacter* strains able to colonize tomato, we sequenced and assembled 16 genomes using sequencing by synthesis on the Illumina HiSeq 2500 or Miseq platforms. We included the 11 *C. michiganensis* subsp. *michiganensis* strains collected in California as well as the five tomato endophytes collected from tomato plant materials, i.e., CASJ009 isolated from a tomato stem in California and four strains from the CFBP (French Collection of Plant-Associated Bacteria) (Table 1). CFBP7494 was isolated from seeds produced in Chile, whereas CFBP8017 and CFBP8019 were isolated from seeds in The Netherlands. We were unable to obtain information about the origin of CFBP7576. SMRT (single molecule, real-time) sequencing on the PacBio platform was also employed for seven strains in order to facilitate more complete genome assemblies. To generate draft genomes for each strain, the assemblies with the highest N50 value and assembly size were selected and were merged by Mix pipeline (Soueidan et al. 2013). The genome sizes of the *Clavibacter* strains ranged from 3.27 to 3.48 Mb, with an average mean coverage of 100x. Assemblies using Illumina and PacBio sequence data enabled the generation of complete or near-complete genome assemblies. General properties of all the genomes are summarized in Table 1.

To facilitate a high resolution comparison of the sequenced *Clavibacter* species, core orthologous proteins were identified. The core genome common to all 16 strains sequenced in this study as well as *C. michiganensis* subsp. *michiganensis* NCPPB382, *C. michiganensis* subsp. *sepedonicus* ATCC33113, *C. michiganensis* subsp. *nebraskensis* NCPPB2581, *C. michiganensis* subsp. *insidiosus* R1-1, *C. michiganensis* subsp. *tessellarius* DOAB609, and *C. michiganensis* subsp. *capsici* PF008 was determined. A total of 1,358 orthologous proteins were identified. The orthologous proteins were concatenated for each strain (476,583 amino acid residues) and a maximum likelihood approach was employed to generate a phylogenetic tree. Similar to the results obtained with the MLST analysis, the California *C. michiganensis* subsp. *michiganensis* strains and the reference strain were placed into a monophyletic clade (Fig. 2A). The phylogenetic analyses with the core orthologous sequences further refined the genetic relationship among the California *C. michiganensis* subsp. *michiganensis* strains by revealing five subclades. Several strains clustered together in these subclades, including strains CASJ002, CA00002, and CASJ003, were isolated in 1999 and 2000; CASJ005 and CAYO001 were isolated in 2001; and CASJ006 and CASJ008 were isolated in 2002. These results suggest an association between the subclade and the years in which the strains were isolated. The California strains CASJ001 and CASJ007 were most closely related to the reference strain NCPPB382. Collectively, these data demonstrate that California *C. michiganensis* subsp. *michiganensis* strains are not clonal and support the concept of multiple introductions with seed. Also consistent with the results of the MLST analysis, the endophytic strains isolated from tomato were not monophyletic and were more diverse compared with

C. michiganensis subsp. *michiganensis* strains (Fig. 2A). These results clearly show that genetically diverse *Clavibacter* strains can colonize tomato endophytically.

Comparative analyses.

We next compared the genomes of a pathogenic California *C. michiganensis* subsp. *michiganensis* strain (CASJ002), the California tomato endophyte (CASJ009), and the *C. michiganensis* subsp. *michiganensis* reference strain NCPPB382. The three genomes were each predicted to encode an average of 3,200 protein-coding sequences. Using a value of >90% amino acid identity to indicate common genes, the two *C. michiganensis* subsp. *michiganensis* strains (CASJ002 and NCPPB382) shared 93% (2,955) of their predicted genes. In contrast, the endophyte (CASJ009) shared only around 70% (2,264) of its predicted genes with the pathogenic *C. michiganensis* subsp. *michiganensis* strains. Bacterial chromosomes were visualized using Circos plots. There was a higher level of conservation in gene content and organization between the two *C. michiganensis* subsp. *michiganensis* strains (CASJ002 and NCPPB382) compared with the endophyte CASJ009 (Fig. 2B and C). The genome sequences of *C. michiganensis* subsp. *michiganensis* strains CASJ002 and NCPPB382 also display a higher levels of colinearity and genome-wide sequence similarity, compared with the tomato endophyte CASJ009 (Fig. 2B and C).

Genomic islands (GIs) are clusters of genes acquired via horizontal transfer. GIs have been demonstrated to significantly impact bacterial adaptation, evolution and pathogenicity (Bellanger et al. 2014). To identify GIs, IslandViewer, which integrates multiple GI prediction methods (Dhillon et al. 2015), was used. The largest GI present in all pathogenic *C. michiganensis* subsp. *michiganensis* strains was the approximately 129-kb *chp/tomA* region (Fig. 2B and C; Supplementary Fig. S2). *Chp/tomA* is required for NCPPB382 pathogenicity on tomato, as a mutant having a deletion of the entire *chp/tomA* GI exhibited reduced growth in tomato and was unable to cause disease (Gartemann and Eichenlaub 2001; Gartemann et al. 2008). Consistent with the inability of CASJ009, CFBP7494, CFBP7576, CFBP8017, and CFBP8019 to cause disease symptoms in tomato, the *chp/tomA* GI is lacking in these strains, whereas it was present in *C. michiganensis* subsp. *michiganensis* strains. Indeed, *Chp/tomA* is the only GI universally found in *C. michiganensis* subsp. *michiganensis* strains. Most of the other GIs present in sequenced *C. michiganensis* subsp. *michiganensis* strains are variable, and hallmarks of GI loss were also detected. The GIs present in the California *C. michiganensis* subsp. *michiganensis* strains and their locations are provided in Supplementary Table S3.

Plasmid presence uncouples pathogenicity and growth in tomato.

Bacterial plasmids play an important role in acquisition of new traits and can impact virulence. We investigated the presence and composition of plasmids in the 16 *Clavibacter* strains sequenced in this study. The presence of plasmids with similarity to pCM1 and pCM2 were detected based on similarity in the plasmid backbone (replication and partitioning genes) and conjugal transfer genes as well as the presence of *celA* (pCM1) or *pat-1* (pCM2). We detected a range in plasmid sizes for both pCM1- and pCM2-like plasmids, 31 to 59 kb and 64 to 109 kb, respectively. All pathogenic *C. michiganensis* subsp. *michiganensis* strains possessed a plasmid with similarity to pCM1 (Supplementary Fig. S3). For example, the plasmid present in strain CASJ001 was most closely related to NCPPB382's pCM1, consistent with their close relationship in the phylogenetic analyses. However, the pCM1-like plasmids in the other California *C. michiganensis* subsp. *michiganensis* strains differed substantially from that in NCPPB382 in terms of their overall size and gene content.

Importantly, *C. michiganensis* subsp. *michiganensis* strains CASJ001 and CASJ007 lacked the pCM2-like plasmid but were still pathogenic in tomato to a similar extent as strains possessing pCM2 (Table 1; Figs. 1 and 3A). The absence of the pCM2-like plasmid in these strains clearly indicates that the presence pCM2, which is required for symptom development in NCPPB382 (Dreier et al. 1997), is not universally required for pathogenicity (Fig. 3A). A pCM2-like plasmid was present in other sequenced *C. michiganensis* subsp. *michiganensis* strains, but its overall size and gene content differed substantially among strains. pCM1- and pCM2-like plasmids were also present in the endophyte CFBP7576, consistent with it being pathogenic on pepper,

whereas none of the other endophytic *Clavibacter* strains possessed plasmids with similarity to pCM1 or pCM2 (Table 1). The endophyte CASJ009 possesses a novel plasmid, pCMsap1, which lacks sequence similarity with pCM1 and pCM2 (Table 1). These data reveal considerable diversity in the presence and composition of plasmids in *C. michiganensis* subsp. *michiganensis* and, further, highlight the importance of investigating virulence mechanisms in diverse *C. michiganensis* subsp. *michiganensis* strains.

In order to investigate the relative importance of pCM1 and pCM2-like plasmids, we sought to remove (cure) them and then to assess disease development and pathogen multiplication in

Fig. 2. Genome-wide comparison of *Clavibacter* strains. **A**, Phylogeny of *Clavibacter michiganensis* subsp. *michiganensis* and other *Clavibacter* species based on orthologous genes. In total, 1,358 orthologous proteins were concatenated in an alignment of 476,583 amino acids. A maximum likelihood approach was used to generate the phylogeny with 1,000 bootstrap replicates. Bootstrap values are indicated at each node. E = endophyte. **B** and **C**, Circos plots depicting genome-wide comparisons of pathogen CASJ002, the NCPPB382 reference strain, and the CASJ009 endophyte. The Circos plot illustrates regions of significant similarity between CASJ002 to NCPPB382. Chromosomes are depicted in arbitrary shades (outer ring). The two pathogens are more closely related to each other than to the endophytic strain. The endophyte *Clavibacter* sp. strain CASJ009 is lacking many genes associated with virulence and has a larger number of unique genes.

Fig. 3. Plasmid presence can uncouple bacterial virulence and growth in tomato. **A**, Representative bacterial wilt symptoms for the endophyte CASJ009 and the pathogens CASJ001, NCPBP382, and CASJ002 7 days postinoculation (dpi). **B**, Wilt and canker disease symptoms on tomato leaves and stems mock-inoculated and infected with CASJ001 and CASJ001ΔpCM1 7 dpi. Tomato stems (*Solanum lycopersicum* cv. Early Pak 7) were stabbed with a needle and were inoculated with 5 μ l of *Clavibacter michiganensis* subsp. *michiganensis* at 10^7 CFU per milliliter. Strains were dilution-plated at day 0 to verify equal inoculation concentrations. CASJ001ΔpCM1 did not cause disease symptoms 7 dpi. **C**, Representative disease symptoms in CASJ002, CASJ002ΔpCM2, and CASJ002ΔpCM1ΔpCM2 at 7 dpi. Inoculations were performed as described in **B**. **D**, Bacterial titers in stem tissue 7 dpi with CASJ002 and CASJ002ΔpCM2. Inoculations were performed as described in **B**. Stem sections were taken 1 cm above the inoculation point to determine bacterial titers. Significant differences were detected by a two-tailed *t* test, $P < 0.001$. **E**, Bacterial titers in stem tissue 7 dpi with CASJ002, CASJ002ΔpCM1, and CASJ002ΔpCM1pCM2. No significant differences were detected between treatments (Tukey's honest significant difference, $P < 0.05$).

tomato. Experiments focused on two *C. michiganensis* subsp. *michiganensis* strains: CASJ001, which only possesses a pCM1-like plasmid, and CASJ002, which possesses pCM1- and pCM2-like plasmids (Table 1). We cured pCM1 from CASJ001 (CASJ001Δp1), pCM2 from CASJ002 (CASJ002Δp2), and both plasmids from CASJ002 (CASJ002Δp1Δ2). The plasmid status of these strains was confirmed by polymerase chain reaction (PCR) and their differential capacity to degrade cellulose.

The plasmid-cured strains were inoculated onto tomato stems, and disease symptoms as well as bacterial titers were analyzed at 7 dpi. The plasmid-cured derivatives CASJ001Δp1 and CASJ002Δp1Δ2 failed to induce bacterial canker symptoms on tomato, indicating the essential role of pCM-1 in pathogenicity (Fig. 3B and C). Bacterial titers of CASJ001Δp1 were significantly lower (fivefold) compared with the wild-type CASJ001 strain (Fig. 3D), although CASJ001Δp1 was still able to multiply in planta and reach relatively high titers (Fig. 3D). In contrast, the bacterial titers of CASJ002, CASJ002Δp2, and CASJ002Δp1Δ2 were not significantly different (Fig. 3E). Curing pCM2 from CASJ002 significantly decreased wilting symptoms and rendered this strain weakly pathogenic (Fig. 3E). This indicated that pCM2 is important for the virulence of this strain. Together, these results demonstrate the essential role of pCM1 in pathogenicity and a differential role of pCM2 plasmids that is strain-dependent. In both CASJ002 and NCPBB382, pCM2 significantly enhances pathogenicity. However, pCM2 is absent in CASJ001 and this strain is able to grow to high titers and cause typical bacterial canker disease symptoms, indicating that pCM2 is not always essential for pathogenicity (Figs. 1 and 3).

The *CelA* cellulase is present in pCM1-like plasmids and was conserved in all the sequenced *C. michiganensis* subsp. *michiganensis* strains. Hence, we sought to investigate the capacity of *celA* to complement pathogenicity of the plasmid-cured derivatives of CASJ001 and CASJ002. *CelA* was cloned into the pHN216 vector, which stably replicates in *Clavibacter* spp. (Laine et al. 1996). pHN216 carrying *celA* was transformed into CASJ001Δp1 and CASJ002Δp1Δp2. The presence of *celA* in CASJ001Δp1 partially restored pathogenicity, but the symptoms were not as severe as the wild-type strain (Fig. 4A). Bacterial growth in the *celA* CASJ001Δp1 complemented strain was not significantly different compared with CASJ001Δp1(EV) (Fig. 4B). The presence of *celA* in CASJ002Δp1Δp2 also partially restored pathogenicity in this strain lacking both pCM1 and pCM2, with the development of wilt and canker symptoms (Fig. 4C). However, there was no significant difference in bacterial titers in planta in comparison with wild-type CASJ002 (Fig. 4D). It is striking that a single gene, *celA*, partially restored pathogenicity of the plasmid-cured CASJ001Δp1 and CASJ002Δp1Δp2 and further supports the critical role this cellulase plays in bacterial canker disease.

Prediction and analyses of secreted *Clavibacter* proteins.

During infection, many pathogens secrete proteins, termed effectors, that are collectively required for virulence (Dou and Zhou 2012; Toruño et al. 2016). Gram-negative plant pathogenic bacteria inject effectors into plant cells via the T3SS. However, *C. michiganensis* subsp. *michiganensis* is a gram-positive bacterium and, thus, does not possess a T3SS, nor is there evidence that there is an alternative mechanism to secrete effectors directly into host cells (Chang et al. 2014). *C. michiganensis* subsp. *michiganensis* does possess general secretory pathway (Sec) and twin arginine translocation (Tat) systems for protein transport across the cell membrane and wall (Chang et al. 2014). Identification and functional annotation of a pathogen's secretome are particularly helpful for detecting potential virulence factors. We predicted the Sec and Tat secretomes in all the *C. michiganensis* subsp. *michiganensis* strains sequenced in the present study in

addition to the reference genome NCPBB382. Note that lipoproteins or other proteins predicted to be anchored to the bacterial cell were not included.

The number of Sec-dependent secreted proteins in *C. michiganensis* subsp. *michiganensis* genomes ranged from 62 to 89 (Fig. 5A), whereas Tat-dependent proteins ranged from 32 to 44 (Fig. 5B). Interestingly, CASJ007 and CASJ001, which both lack the pCM2 plasmid, possess very similar Sec and Tat secretomes. Thirteen core Sec-dependent proteins were identified in all the sequenced *C. michiganensis* subsp. *michiganensis* strains (Supplementary Table S4). In order to determine if these core proteins are conserved in *C. michiganensis* subsp. *michiganensis* and tomato endophytes, we performed clustering analyses based on sequence identity using CD-HIT software with an identity threshold set to 95% (Li and Godzik 2006). There was little overlap in the secretome of *C. michiganensis* subsp. *michiganensis* and endophytic *Clavibacter* strains. With respect to Sec secreted proteins, the number of homologous proteins shared between *C. michiganensis* subsp. *michiganensis* and endophytes ranged from one to nine (Fig. 5A). Similarly, few Tat-dependent proteins were shared between *C. michiganensis* subsp. *michiganensis* and endophytes (Fig. 5B).

The function of Sec and Tat secreted proteins were predicted using InterProScan (Quevillon et al. 2005). Most of the Sec secreted proteins were predicted α/β -hydrolases, peptidases, and carbohydrate-degrading enzymes (Fig. 5C). The Sec secreted proteins also included lipolytic enzymes, cutinases, peptidoglycan binding proteins, and pectate lyases (Fig. 5C). The Tat secreted proteins included carboxypeptidases, Dyp type peroxidases, and some carbohydrate-degrading enzymes (Fig. 5D). These analyses indicate that *C. michiganensis* subsp. *michiganensis* strains secrete a wide range of proteins during infection. These secreted proteins include a range of degradative enzymes that could alter host physiology and facilitate colonization as well as degrade the host cell wall to provide nutrients and manipulate defense responses.

Annotation of carbohydrate-active enzymes (CAZymes) in *Clavibacter*.

CAZymes are involved in the degradation and metabolism of diverse glycoconjugates, oligosaccharides, and polysaccharides (Cantarel et al. 2009). Plant-pathogenic CAZymes have a central role in the ability to breakdown the plant cell wall and facilitate bacterial colonization and nutrient acquisition (Cantarel et al. 2009). To assess the capability of *C. michiganensis* subsp. *michiganensis* to degrade and metabolize different polysaccharides, all putative CAZymes present in each genome were identified based on similarity to signature domains present in the four catalytic classes of these proteins (Cantarel et al. 2009). Five percent (154 to 173) of *C. michiganensis* subsp. *michiganensis* genes encoded predicted CAZymes (Supplementary Fig. S4; Supplementary Table S5). The five tomato endophytic *Clavibacter* strains were also analyzed and had fewer total CAZymes (138 to 154). Comparison of glycoside hydrolases (GHs), glycosyltransferases, polysaccharide lyases (PLs), and carbohydrate esterases revealed that *C. michiganensis* subsp. *michiganensis* exhibits substantial variation in CAZyme classes when compared with endophytes and pathogenic *Clavibacter* subspecies (Supplementary Table S6). Furthermore, there was a higher overall number of CAZymes in *C. michiganensis* subsp. *michiganensis* compared with other pathogenic *C. michiganensis* subspecies. These differences may reflect the host specificity of different *C. michiganensis* subspecies as well as the tomato endophytes that are pathogenic on other hosts.

Plant PCWDEs are a subset of CAZymes produced by plant pathogens to degrade plant cell walls (Cantarel et al. 2009). Plants possess primary and secondary cell walls that serve as a structural

barrier. Specific classes of CAZymes are involved in degrading cellulose, hemicellulose, xylans, and pectins in the plant cell walls. We compared the PCWDEs found in *C. michiganensis* subsp. *michiganensis* strains with other *C. michiganensis* subspecies and xylem-restricted bacteria with different host specificities. Interestingly, *C. michiganensis* subsp. *michiganensis* has a greater number of PCWDEs (Fig. 6). Overall, the GH1 and GH3 families, which have members that are able to degrade cellulose, were most abundant in *C. michiganensis* subsp. *michiganensis*. Four copies of GH1 were found in each *C. michiganensis* subsp. *michiganensis* genome, whereas zero to two copies were present in the other analyzed bacteria (Fig. 6). Each *C. michiganensis* subsp. *michiganensis* strain possessed a minimum of eight GH3 members, whereas other *C. michiganensis* subspecies and tomato endophytes had no more than four (Fig. 6). Proteins involved in hemicellulose decomposition (GH31, GH35) were more commonly found in the *C. michiganensis* subsp. *michiganensis* genome (Fig. 6). GH43, which encodes for endo-1,4- β -xylanases, was found in all *C. michiganensis* subsp. *michiganensis* strains as well as *C. michiganensis* subsp. *sepedonicus* and the endophyte CASJ009 (Fig. 6). However, GH43 members were not found in any other *Clavibacter* genomes (Fig. 6). Multiple classes of PLs degrade pectin (Abbott and Boraston 2008). Interestingly, PL3 was the only PL family present in *C. michiganensis* subsp. *michiganensis* strains, and most of the pathogenic *C. michiganensis* subsp. *michiganensis* strains contained two copies. The genomes of CASJ009, *C. michiganensis* subsp. *sepedonicus* and *C. michiganensis* subsp. *tessellarius* contained one copy of PL3, whereas it was absent in all other

Clavibacter species and xylem-restricted bacteria. Based on their overall arsenal of CAZymes, *C. michiganensis* subsp. *michiganensis* seems to possess unique capabilities to break down and metabolize different types of tomato cell-wall components, consistent with a lifestyle in the vasculature of this host plant.

Glycome profiling reveals differences in tomato cell-wall chemistry after infection.

To investigate the ability of *C. michiganensis* subsp. *michiganensis* to break down and metabolize different types of plant cell-wall material, glycome profiling was performed. Glycome profiling employs an enzyme-linked immunoabsorbent assay (ELISA), using 149 monoclonal antibodies recognizing diverse epitopes in plant cell-wall matrix polymers to evaluate cell-wall composition (Pattathil et al. 2012). To identify differences in overall composition and extractability of major noncellulosic cell-wall glycans, glycome profiling was performed on tomato stem segments 14 dpi with *C. michiganensis* subsp. *michiganensis* CASJ002, the tomato endophyte CASJ009, and an uninfected control. Cell-wall extracts were prepared using increasingly harsh reagents (ammonium oxalate, sodium carbonate, 1 M KOH, and 4 M KOH), and glycome profiles were generated after pooling three biological replicates from each treatment.

Overall, glycome profiles revealed clear differences among treatments, demonstrating that cell walls are affected in a significant way after infection (Fig. 7; Supplementary Table S7). The most striking differences (Fig. 7, outlined with white boxes) were observed in the 1 M KOH extract. In plants infected with *C. michiganensis* subsp. *michiganensis* CASJ002, the most notable

Fig. 4. The *celA* gene can partially complement *Clavibacter michiganensis* subsp. *michiganensis* virulence in plasmid-cured strains. **A**, Bacterial canker disease symptoms in tomato leaves and stems infected with CASJ001ΔpCM1, CASJ001ΔpCM1 carrying the vector pHN16(EV), and CASJ001ΔpCM1 carrying pHN16 with *celA* at 7 days postinoculation (dpi). Tomato stems (*Solanum lycopersicum* cv. Early Pak 7) were stabbed with a needle and were inoculated with 5 μ l of *C. michiganensis* subsp. *michiganensis* at 10^7 CFU per milliliter. CASJ001ΔpCM1 is unable to cause disease symptoms. **B**, Bacterial titers in stem tissue 7 dpi with strains described in A. Inoculations were performed as described in A. Stem sections were taken 1 cm above the inoculation point to determine bacterial titers. Statistical differences were detected by Tukey's honest significant difference, $P < 0.05$, $n = 18$. **C**, Representative disease symptoms in CASJ002, CASJ002ΔpCM1ΔpCM2(EV), and CASJ002ΔpCM1ΔpCM2 pHN16 (*celA*) at 7 dpi. Inoculations were performed as described in A. **D**, Bacterial titers in stem tissue 7 dpi with strains described in C. Inoculations and statistical analyses were performed as described above.

effect observed was a reduction in the amounts of diverse arabinogalactan epitopes in the 1 M KOH extracts. Here, the pectic arabinogalactan epitopes (RG-I/AG) were almost completely absent from the 1 M KOH extract, as were several other AG epitopes (AG-3 and AG-4). Other changes observed in the glycome profiles reflected more a change in epitope extractability rather than epitope removal. For example, there appeared to be higher levels of RG-I backbone epitopes and diverse xylan epitopes (Xylan 3-7) in the 1 M KOH extracts from *C. michiganensis* subsp. *michiganensis*-infected plants, as compared with plants colonized with the endophyte and uninfected controls. Furthermore, trace amounts of xyloglucan epitopes (nonfuc XG 1-6 and fuc XG monoclonal antibody [mAbs]) were detected in the 1 M KOH extract, which indicates some loosening of xyloglucan epitopes from the plant cell wall after infection. Uninfected control plants showed the highest overall abundance of epitopes in the glycome profiles, further suggesting that infection leads to actual degradation of wall

structures. This is substantiated by the trends in the amounts of extracted carbohydrates (Fig. 7, top bar graphs). These results clearly demonstrate severe modification of cell-wall integrity and architecture in tomato plants infected with *C. michiganensis* subsp. *michiganensis* CASJ002 compared with endophytic CASJ009 and control treatments.

The *pelA1* pectate lyase is important for symptom development.

Multiple Sec and Tat secreted proteins were predicted to be CAZymes. The *chp/tomA* cluster contains many virulence genes essential for effective colonization, which includes secreted proteases and CAZymes (Fig. 8A). The *chp/tomA* cluster is conserved among *C. michiganensis* subsp. *michiganensis* strains, and several core effectors are present in this GI. Our investigation of glycome profiles revealed significant differences in tomato cell-wall composition upon infection with *C. michiganensis* subsp. *michiganensis* (CASJ002). In order to gain a greater understanding

Fig. 5. Prediction of the *Clavibacter* secretome. **A** and **B**, General secretory pathway (Sec)-secreted and twin arginine translocation (Tat)-secreted effectors were predicted from genome sequences and were analyzed for their conservation across strains. Identified proteins were subsequently scanned for the presence of transmembrane regions and lipoproteins. Predicted transmembrane and lipoproteins were removed. **C** and **D**, Functional annotation of Sec- and Tat-secreted effectors performed using InterProScan. Representation of predicted function of Sec- and Tat-secreted proteins.

of the importance of individual PCWDE members, we investigated the roles of two pectate lyases belonging to the PL3 family. Within the *chp/tomA* GI, there are two highly conserved genes, with homology to pectate lyases *pelA1* and *pelA2*, that are predicted to be secreted by the Sec system (Fig. 8A). We deleted each of these genes to generate $\Delta pelA1$ and $\Delta pelA2$ in *C. michiganensis* subsp. *michiganensis* CASJ002. Two independent deletions were generated and analyzed for each gene, with deletion of each gene verified by PCR and DNA sequencing. Three-week-old tomato plants were inoculated with wild-type *C. michiganensis* subsp. *michiganensis* CASJ002, CASJ002 $\Delta pelA1$, and CASJ002 $\Delta pelA2$. Development of disease

symptoms was monitored up to 14 dpi. Plants infected with CASJ002 $\Delta pelA1$ exhibited a reduction in wilting, compared with plants infected with wild-type CASJ002 (Fig. 8B). In contrast, disease symptoms induced by CASJ002 $\Delta pelA2$ were indistinguishable from those induced by wild-type CASJ002 (Fig. 8B). To examine the ability of CASJ002 $\Delta pelA1$ and CASJ002 $\Delta pelA2$ to grow in planta, bacterial titers were determined in tomato plants 14 dpi. Bacterial titers were not significantly different among CASJ002, CASJ002 $\Delta pelA1$, or CASJ002 $\Delta pelA2$ (Fig. 8C).

Given the high degree of amino acid similarity between the two proteins (90%), it was unexpected that deletion of *pelA1* but

Fig. 6. Comparison of plant cell wall-degrading enzymes (PCWDEs) across *Clavibacter* spp., related gram-positive bacteria, and other xylem-colonizing bacteria. Pathogenic *C. michiganensis* subsp. *michiganensis*, five endophytes, five *Clavibacter michiganensis* strains pathogenic on plants other than tomato, four gram-positive actinomycetes, and three gram-negative xylem-infecting pathogens were analyzed. Families of PCWDEs are grouped based on their major targeted substrates including cellulose, hemicellulose, xylans, and pectin (gray blocks in the right). GH = glycoside hydrolases, CE = carbohydrate esterase, and PL = polysaccharide lyase. The stacked bar charts indicate different types of PCWDEs in different strains. On the left are bacterial strains, gram-positive xylem-infecting bacteria, other pathogenic *Clavibacter* subspecies, endophytic *Clavibacter* strains, and *C. michiganensis* subsp. *michiganensis*.

not *pelA2* reduced disease symptom severity. To gain a greater understanding of this phenomenon, we investigated the expression of *pelA1* and *pelA2*. CASJ002 was grown in rich Luria broth (LB) medium to midexponential phase and was then

transferred to M9 minimal media. Total RNA was extracted and expression of *pelA1* and *pelA2* was monitored by quantitative PCR. Consistent with a role in pathogen virulence in planta, *pelA1* expression was significantly higher than that of *pelA2*

Fig. 7. Glycome profiling reveals extensive disruption of tomato cell-wall components after *Clavibacter* infection. Tomato stems (*Solanum lycopersicum* cv. Early Pak 7) were stabbed with a needle and were inoculated with 5 μ l of *Clavibacter* CASJ002 and CASJ009 at 10^7 CFU per milliliter. Glycome profiling was performed on tomato stems 14 days postinoculation. The enzyme-linked immunoabsorbent assay (ELISA) response values (with background values subtracted from the mean value of two technical replicates) are represented as heat maps. The monoclonal antibodies are grouped based on the cell-wall glycans they detect, as depicted in the panel at the right of the heat map. The gravimetric amounts of carbohydrate materials extracted from the walls by each extraction reagent are shown as bar graphs at the top of the figure. CASJ002 = pathogenic *C. michiganensis* subsp. *michiganensis*, CASJ009 = endophytic *Clavibacter* spp. isolated from a tomato plant with bacterial canker symptoms in California; control = no infection.

in M9 minimal media (Fig. 8D). We also examined *pelA1* and *pelA2* expression in *C. michiganensis* subsp. *michiganensis* NCPPB382 and CA00002 and observed similar results (Supplementary Fig. S5). These data revealed similar expression patterns of the *pelA1* CAZyme across diverse *C. michiganensis* subsp. *michiganensis* strains as well as another level of complexity in *C. michiganensis* subsp. *michiganensis* virulence, i.e., different levels of virulence gene expression.

DISCUSSION

Bacterial canker of tomato is a disease of worldwide importance, particularly in greenhouse production and in regions with Mediterranean and temperate climates, e.g., processing tomato production in California (de León et al. 2011; Sen et al. 2015). Outbreaks of bacterial canker can be devastating, but, like many diseases, tend to occur sporadically (de León et al. 2011).

Here, we utilized recent advancements in genome sequencing and long-read technology to obtain high-quality genomes for multiple pathogenic *C. michiganensis* subsp. *michiganensis* strains collected at different times and in different geographical locations in California as well as for *Clavibacter* strains that are endophytes in tomato. The 11 *C. michiganensis* subsp. *michiganensis* strains isolated from tomato plants with bacterial canker symptoms from commercial production fields in California in different years and geographical locations were placed in a single clade with other *C. michiganensis* subsp. *michiganensis* strains from worldwide locations is consistent with the monophyletic nature of this pathogen. However, the finding that the California *C. michiganensis* subsp. *michiganensis* strains were not clonal and exhibited a high degree of genetic diversity when compared with each other and other *C. michiganensis* subsp. *michiganensis* strains is in agreement with recent studies showing considerable genetic diversity in *C. michiganensis* subsp. *michiganensis* strains from various

Fig. 8. Deletion of the *PelA1* pectate lyase but not *PelA2* decreases bacterial virulence. **A**, *Chp/tomA* is present in all pathogenic *Clavibacter michiganensis* subsp. *michiganensis* but is absent in endophytic *Clavibacter* strains. Gene content in the *chp/tomA* pathogenicity island of CASJ002 and NCPPB382. Top panel, *chp* cluster and bottom panel, *tomA* cluster. **B**, Unilateral wilting symptoms in infected tomato plants (*Solanum lycopersicum* cv. Early Pak 7) 14 days postinoculation (dpi). Tomato stems were stabbed with a needle and were inoculated with 5 μ l of *C. michiganensis* subsp. *michiganensis* at 10^7 CFU per milliliter. Strains were dilution-plated at day 0 to verify equal inoculation concentrations. The fraction on the side of each image indicates the number of plants with similar disease symptoms relative to the 18 inoculated plants. The *pelA1* deletion exhibits decreased disease symptoms. **C**, Bacterial titers of wild-type *C. michiganensis* subsp. *michiganensis* compared with the *pelA1* and *pelA2* deletions 14 dpi. Plants were inoculated as described in B. No significant differences were detected, using Tukey's honest significant difference, $P < 0.05$, $n = 18$. **D**, Expression of *pelA1* and *pelA2* by quantitative polymerase chain reaction. *C. michiganensis* subsp. *michiganensis* CASJ002 was grown in M9 mimicking media, RNA was extracted, and gene expression was normalized to the *ITS* gene. Significant differences were detected by a two-tailed *t* test, $P < 0.001$.

geographical regions (Jacques et al. 2012; Milišašević-Marčić et al. 2012; Quesada-Ocampo et al. 2012; Tancos et al. 2015). Thus, the California *C. michiganensis* subsp. *michiganensis* strains likely represent multiple introductions over time, most likely via contaminated seed.

Moreover, the endophytic *Clavibacter* strains exhibited even more genetic diversity than the *C. michiganensis* subsp. *michiganensis* strains, with three strains, CFPB7494, CFPB8017, and CFPB7576, placed in monophyletic clades with *C. michiganensis* subspecies pathogenic on alfalfa, wheat, and pepper, respectively. Evidence that some of the tomato endophytes are strains of pathogenic subspecies came from the finding that CFPB7494 and CFPB7576 caused disease on wheat and pepper, respectively. Thus, these *Clavibacter* strains are pathogenic on other plant species but are capable of colonizing tomato as endophytes. This finding reveals an additional mechanism by which plant pathogenic *C. michiganensis* subspecies can be disseminated, i.e., as endophytes associated with nonhost plants. The ability to colonize plants as a symptomless endophyte on one plant species but act as a pathogen on other plant species has been observed in several pathogenic fungi (Barnes and Shaw 2003; Ploch and Thines 2011; Sowley et al. 2010) and bacteria (Darrasse et al. 2010).

The reference strain *C. michiganensis* subsp. *michiganensis* NCPPB382 carries the pCM1 and pCM2 plasmids, which are both required for wilting and canker formation (Dreier et al. 1997; Chalupowicz et al. 2012; Meletzus et al. 1993). *C. michiganensis* subsp. *sepedonicus* is unable to infect and cause disease in tomato but is able to infect another solanaceous plant, potato, causing bacterial ring rot disease. Whereas the chromosomes of *C. michiganensis* subsp. *michiganensis* NCPPB382 and *C. michiganensis* subsp. *sepedonicus* ATCC33113 exhibit significant similarity, there are clear differences in their plasmid composition (Bentley et al. 2008; Brown et al. 2002; Eichenlaub and Gartemann 2011; Mogen and Oleson 1987). The plasmid-free derivative of *C. michiganensis* subsp. *michiganensis* NCPPB382 causes localized lesions but not systemic disease symptoms in tomato (Chalupowicz et al. 2017). Thus, plasmid composition may play an important role in *C. michiganensis* host specificity and the type of disease symptoms induced. All *C. michiganensis* subsp. *michiganensis* strains sequenced in the present study possessed a pCM1-like plasmid, whereas two strains lacked the pCM2 plasmid and retained pathogenicity on tomato. Curing of pCM1 from *C. michiganensis* subsp. *michiganensis* CASJ001 and CASJ002 rendered these strains unable to cause disease, which is fully consistent with the essential role for the pCM1 plasmid in induction of disease symptoms and bacterial virulence. In contrast, curing of pCM2 reduced but did not abolish virulence and, taken together with the finding that two pathogenic California *C. michiganensis* subsp. *michiganensis* strains lacked pCM2, indicates that this plasmid is not essential for virulence. Stress conditions can induce *C. michiganensis* subsp. *michiganensis* plasmid loss, and the pCM1/pCM2 plasmids are conjugative (Gartemann et al. 2003; Meletzus et al. 1993). This may partly explain the diversity of the plasmid profiles found for the California *C. michiganensis* subsp. *michiganensis* strains and the possibility that endophytic *Clavibacter* species or strains could be rendered pathogenic through acquisition of pCM1 or pCM2.

The capacity of *C. michiganensis* subsp. *michiganensis* to cause bacterial canker disease symptoms and to grow as an epiphyte in tomato plants can be uncoupled. The plasmid-free derivative CASJ001Δp1Δp2 no longer induced disease symptoms in tomato without a corresponding decrease in bacterial growth. The secreted endocellulase *celA*, present in all pCM1-like plasmids, partially restored pathogenicity but not bacterial titers of CASJ001Δp1, possibly indicating a role for other genes on the pCM1 plasmid. Disease symptoms and bacterial growth were also uncoupled in NCBBP382 in multiple cases, including

plasmid cured derivatives, complementation of pCM1 with a large DNA fragment containing *celA*, and deletions of the *vatr1* and *vatr2* transcriptional regulators (Chalupowicz et al. 2012; Meletzus et al. 1993; Savidor et al. 2014). In other plant species, *C. michiganensis* subsp. *michiganensis* elicits cell death in some nonhost plants, consistent with the recognition of conserved microbial features or secreted pathogen proteins (Alarcón et al. 1998; Gitaitis 1990). In *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus*, some members of the secreted protease family *pat-1* can induce cell death in nonhosts, indicating it might be recognized by the host defense system (Lu et al. 2015a; Nissinen et al. 2009; Stork et al. 2008). The observation that plasmid-free *C. michiganensis* subsp. *michiganensis* strains can act as endophytes in tomato suggests that presumed genes necessary for endophytic growth in tomato, e.g., for host recognition, colonization, and defense suppression, are chromosomally encoded.

Pathogens secrete a variety of effector proteins, and expression of effectors may occur spatially and temporally, depending on the stage of infection (Toruño et al. 2016). After entry into the plant, *Clavibacter michiganensis* strains systemically infect vascular tissue and, initially, have a biotrophic lifestyle (Eichenlaub and Gartemann 2011). The *C. michiganensis* subsp. *michiganensis* strains characterized in the present study exhibited significant differences in their secretomes compared with endophytic strains, indicating a degree of specialization with the tomato host. During later stages of disease, *C. michiganensis* subsp. *michiganensis* is an aggressive pathogen that induces formation of stem cankers and degradation of pith tissues (de León et al. 2011; Eichenlaub and Gartemann 2011). Secreted proteins included many CAZymes involved in plant cell-wall breakdown and metabolism of cell-wall components. Indeed, *C. michiganensis* subsp. *michiganensis* strains possessed more predicted CAZymes compared with other gram-positive or xylem-limited bacterial plant pathogens. This higher number of CAZymes is consistent with *C. michiganensis* subsp. *michiganensis* inducing extensive tissue damage during later stages of disease. Using glycome profiling, we detected significant alterations of plant cell-wall glycans, particularly among diverse arabinogalactans, indicating severe modification of tomato cell-wall architecture during *C. michiganensis* subsp. *michiganensis* infection. The predicted pectate lyase CAZyme *pelA1* was also required for robust induction of wilting and canker symptoms, highlighting the importance of specific secreted degradative enzymes in the pathogenesis of *C. michiganensis* subsp. *michiganensis*.

In the last decade, rapid technological advances have facilitated the sequencing of many different organisms. Here, we used a combination of short- and long-read technologies to obtain high-quality sequences of multiple *Clavibacter* genomes for subsequent comparative and functional analyses. We confirmed the importance of some virulence factors present in the *C. michiganensis* subsp. *michiganensis* NCPPB382 reference strain, such as *pelA1*, the *chp/tomA* GI, and the pCM1 plasmid. We also identified 13 core secreted proteins present in all pathogenic *C. michiganensis* subsp. *michiganensis* strains, which warrant future investigation for their contribution to pathogen virulence and represent promising targets for disease control. However, clear differences also were found among *C. michiganensis* subsp. *michiganensis* strains with respect to plasmid composition, bacterial aggressiveness, and gene content. This highlights the importance of comparative genomics and investigation of virulence mechanisms of various agriculturally relevant *C. michiganensis* subsp. *michiganensis* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 1 and Supplementary Table S1. *C. michiganensis* subsp.

michiganensis strains were grown in tryptone broth with yeast (TBY), D2, C medium, sorbitol broth (SB) medium at 28°C (Kirchner et al. 2001), or minimal M9 medium (Chalupowicz et al. 2010). *Escherichia coli* strains (DH5 α and GM48) were grown in LB at 37°C. The growth medium was supplemented with the antibiotics chloramphenicol (20 μ g/ml for *E. coli* and 10 μ g/ml for *C. michiganensis* subsp. *michiganensis*), gentamicin (100 μ g/ml for *E. coli* and *C. michiganensis* subsp. *michiganensis*), and neomycin (50 μ g/ml for *E. coli* and *C. michiganensis* subsp. *michiganensis*), where necessary.

Plant growth and pathogenicity assays.

The tomato cultivar Early Pak No. 7 (EP-7) was used for all assays. Tomato plants were grown in a greenhouse at 23°C with a 14-h photoperiod and 70% relative humidity. All plant inoculations were performed with at least six biological replications and were repeated three times. Three- to four-week-old tomato plants were used for inoculation assays. *C. michiganensis* subsp. *michiganensis* strains were plated on TBY plates and were incubated for 2 to 3 days at 28°C. Inoculum was prepared by diluting cells in 10 mM MgCl₂ to a concentration of approximately 10⁷ CFU per milliliter. Strains were dilution-plated at day 0 to confirm equal inoculum concentrations. Tomato plants were inoculated by stabbing the stem with a needle and pipetting 5 μ l of a 10⁷-CFU/ml suspension of *C. michiganensis* subsp. *michiganensis* into the wound site. Plants were grown in the greenhouse as previously described (Thapa et al. 2015). To assess percent wilting after inoculation, plants were monitored every day for the appearance of wilting symptoms (n = 18 plants per strain). Disease symptoms were monitored for up to 14 days. Virulence was assessed by determining the number of plants that exhibited wilting symptoms 2 weeks postinoculation. For a plant to be rated as wilted, at least one leaflet had to exhibit unilateral or complete wilting. Wilting first appeared 5 dpi. Three-week-old pepper ('Numex Joe E Parker') and wheat plants ('Bobwhite') were inoculated as described above for tomato.

To determine bacterial titers in the tomato stem, 1-cm stem segments were cut 1 cm above and below the inoculation site at 7 and 14 dpi. To determine movement in tomato, samples were also collected 3, 6, and 9 cm above the infection site. Stem segments were surface-disinfected in 75% ethanol, the outer epidermis was removed, and the remaining segment was weighed and ground in 900 μ l of 10 mM MgCl₂. Serial dilutions of the homogenate were plated on D2 medium (Kado and Heskett 1970) supplemented with 20 mg of cycloheximide and were incubated for 4 to 6 days at 28°C. Statistical differences between genotypes were detected using Tukey's honest significant difference (P < 0.05) or a two-tailed t test.

Genomic DNA sequencing, assembly, and annotation.

We sequenced the genomes of 11 *C. michiganensis* subsp. *michiganensis* strains and one tomato endophyte (CASJ009) isolated from tomato plants with bacterial canker symptoms in fields in California from 1999 to 2011 (Table 1). Four endophytes from the French Collection for Plant-associated Bacteria (CFBP) culture collection were also included. Total genomic DNA was isolated with the Promega Wizard Genomic DNA purification kit (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. For library construction for the Illumina platform, genomic DNA was sheared with a Covaris E220 sonicator, followed by end repair and phosphorylation. Fragmented DNA was used to construct paired-end libraries. Libraries were checked for size with a High-Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, U.S.A.) in the Bioanalyzer 2100, with an expected average size of 250 bp. Libraries were pooled and sequenced with paired-end reads on MiSeq and HiSeq 2500 systems (Illumina, San Diego, CA, U.S.A.). Seven *Clavibacter* strains were also sequenced

with the Pacific Biosciences RSII sequencing platform (Pacific Biosciences, Menlo Park, CA, U.S.A.) (Table 1). Genomic DNA was prepared as a 20-kb library for P6-C4 chemistry, followed by BluePippin size selection at 15 kb. Genome sequencing was performed at the Genome Center at the University of California Davis DNA Technologies Core Facility.

Genomes sequenced on Illumina were de novo assembled with the SPAdes (Bankevich et al. 2012) and the genomes sequenced on PacBio were de novo assembled with Hierarchical Genome Assembly Process 3 (Chin et al. 2013). The assembly with the highest assembly qualities in terms of N50 value and assembly size was selected and merged by the Mix pipeline, to obtain a consensus assembly for each species (Soueidan et al. 2013). The draft genomes were annotated with Prokka and the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (Seemann 2014; Tatusova et al. 2016).

Phylogenetic analyses.

We constructed a multigene tree of 59 *Clavibacter* isolates. Sequences for the maximum-likelihood MLSA tree were gathered from the NCBI nt and wgs databases as well as the strains sequenced in the present study, for six housekeeping genes (*atpD*, *dnaK*, *gyrB*, *ppk*, *recA*, and *rpoB*). Alignments were produced with the PRANK alignment tool (Löytynoja and Goldman 2005). A maximum likelihood approach was used to construct the phylogenetic tree using MEGA software version 6 and a bootstrap analysis of 1,000 replicates (Tamura et al. 2013). The resulting phylogeny was visualized using iTOL (Letunic and Bork 2007).

Orthologous genes of 22 *Clavibacter* strains were predicted, using the OrthoMCL pipeline (Li et al. 2003). All-versus-all BLASTP (E value < 1×10^{-5} , alignment coverage >50%) comparison of all protein sequences for each species was performed, and orthologous genes were clustered by OrthoMCL. The normalized scores were fed into the MCL algorithm to classify the genes into predicted orthologous gene families with a default inflation parameter of 1.5. We performed multiple alignments of protein sequences with PRANK (Löytynoja and Goldman 2005). All the alignments were concatenated by FASconCAT, yielding a protein supermatrix (Kück and Meusemann 2010). A maximum likelihood approach was used to reconstruct the phylogenetic tree using RAxML software (Stamatakis 2006). The bootstrap was performed with 1,000 replicates. The resulting phylogeny was visualized using FigTree (version 1.4.0).

Bioinformatics analyses.

Circularized genome visualization was performed with Circos version 0.69 (Krzywinski et al. 2009). GIs were detected using IslandViewer (Dhillon et al. 2015). IslandViewer integrates the sequence composition prediction methods IslandPath-DIMOB and SIGI-HMM as well as the single comparative GI prediction method IslandPick (Hsiao et al. 2005; Langille et al. 2008; Waack et al. 2006). CAZymes were identified with the Hmmscan program in the HMMER 3.0 package, using the family-specific hidden Markov model (HMM) profiles of CAZymes from the dbCAN database (Eddy 2009; Yin et al. 2012). Predicted protein sequences were screened for Sec and Tat signal peptides. Proteins possessing signal peptides for the Sec-dependent pathway were identified using the SignalP 3.0 server (Petersen et al. 2011). Proteins possessing signal peptides for the Tat secretory pathway were analyzed by TatP 1.0 (Bendtsen et al. 2005). Predicted lipoproteins and transmembrane proteins were filtered from the Sec and Tat secretomes. Transmembrane topology was predicted using TMHMM 2.0 (Sonnhammer et al. 1998). LipoP1.0 was used to predict lipoprotein signal sequence features (Juncker et al. 2003).

Cell-wall extraction and glycome profiling.

C. michiganensis subsp. *michiganensis* strains were cultured for 2 to 3 days on TBY plates at 28°C. Inoculum was prepared by diluting cells in 10 mM MgCl₂ to a concentration of approximately 10⁷ CFU/ml. Four-week-old tomato plants were inoculated by stabbing the stem with a needle and pipetting 5 µl of a 10⁷–CFU/ml suspension of buffer or *Clavibacter* strains CASJ002 and CASJ009 into the wound site. Stem samples above inoculation sites were collected 14 dpi. For each treatment, stem samples from 12 individual tomato plants were pooled for glycome profiling. Profiling was repeated twice with raw data. Alcohol-insoluble residues were prepared and glycome profiling were performed as described previously (Pattathil et al. 2012). Briefly, cell-wall extracts were prepared, using increasingly harsh reagents (ammonium oxalate, sodium carbonate, 1 M KOH, 4 M KOH), and subsequent ELISA screening of these extracts was performed with a comprehensive suite of plant cell-wall glycan-directed mAbs (Pattathil et al. 2010). The mAbs were obtained from laboratory stocks (CCRC, JIM, and MAC series) at the Complex Carbohydrate Research Center (available through CarboSource Services) or from BioSupplies (Bundoora, Australia) (BG1, LAMP). Details about individual mAbs used for profiling are provided in Supplementary Table S8 and on the *WallMabDB* website.

Recombinant DNA techniques.

DNA manipulations and PCR were conducted according to standard protocols (Joseph and David 2001). Primers and primer sequences used for cloning and molecular assays are listed in Supplementary Table S2. For construction of the $\Delta pelA1$ and $\Delta pelA2$ deletion constructs, 1.5-kb upstream and 1-kb downstream regions flanking the *pelA1* and *pelA2* genes were PCR-amplified from *C. michiganensis* subsp. *michiganensis* CASJ002. PCR fragments were digested with *HindIII* and *BamHI*. Next, a 1.5-kb fragment of the chloramphenicol cassette was digested with *BamHI* from the pEC70 plasmid (Tauch et al. 1998). The digested *C. michiganensis* subsp. *michiganensis* PCR fragments were ligated with the *cmx* fragment in the pUC19 vector with T4 DNA ligase. The pUC19*pelA1cmx* and pUC19*pelA2cmx* were transformed into *E. coli* (DH5 α) and were selected on LB medium containing chloramphenicol (10 µg/ml). The presence of the cloned fragments in recombinant plasmids was confirmed by PCR and sequencing. The pUC19*pelA1cmx* and pUC19*pelA2cmx* DNA plasmids were extracted from *E. coli* DH5 α cells with Qiagen mini plasmid kits (Qiagen, Hilden, Germany). Plasmid DNA was then transformed into the nonmethylating *E. coli* GM48 (Palmer and Marinus 1994). The plasmids were extracted from *E. coli* GM48 cells and were subsequently electroporated into *C. michiganensis* subsp. *michiganensis* CASJ002. *C. michiganensis* subsp. *michiganensis* electrocompetent cells were prepared as previously described (Kirchner et al. 2001). Electroporation was performed with 100 µl of competent cells and 1 µg of the purified pUC19*pelA1cmx* and pUC19*pelA2cmx* in 0.1-cm electroporation cuvettes, using an Eppendorf electroporator model 2510 with the following settings: 12.5 Kv/cm (Table 1), 25 µF, and 600 Ω with a time constant between 12 and 16 ms. Cells were immediately mixed into SB medium and were regenerated for 3 h at 28°C. Cells were spread onto SB agar plates containing chloramphenicol (10 µg/ml) and were incubated for 5 to 7 days. The presence of the $\Delta pelA1$ and $\Delta pelA2$ deletions in the selected transformant was confirmed by PCR and sequencing.

Quantification of gene expression by quantitative reverse transcription (qRT)-PCR.

C. michiganensis subsp. *michiganensis* strains were grown overnight in TBY medium and an aliquot was transferred into minimal media. Cells were harvested at optical densities between 0.35 and 0.55. RNA was extracted from 5 ml of the culture with Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.),

according to the manufacturer's instructions. The total RNA was incubated for 30 min at 37°C with 10 units of DNase I (Promega) to remove residual genomic DNA. One microgram of total RNA was used to make cDNA (in a total volume of 20 µl) with M-MLV reverse transcription (Promega), using oligo dT₁₈ primer. qRT-PCR was performed with a CFX96 touch real-time PCR detection system with the SsoFast EvaGreen Supermix kit (Bio-Rad, Munich). qRT-PCR conditions consisted of an initial incubation step at 95°C for 30 s, followed by 40 cycles of 10 s of denaturation at 95°C and 15 s of annealing at 60°C. Primers based on the internal transcribed spacer sequence between 16S and 23S rDNA were used as an internal standard for normalization. All reactions were performed in triplicate and were repeated three times.

Plasmid curing.

Plasmids present in *C. michiganensis* subsp. *michiganensis* CASJ001 and CASJ002 were cured by a modified procedure described by Meletzus et al. (1993). *C. michiganensis* subsp. *michiganensis* was grown in C medium (per liter: peptone at 10 g, yeast extract at 5 g, glucose at 5 g, NaCl at 5 g, pH 7.2 to 7.4) at 28°C until late log phase (optical density at 600 nm = 1). One milliliter of late log phase culture was transferred into 50 ml of C medium in a 500-ml flask and was grown at 34°C to approximately 10⁸ cells per milliliter. Dilutions were spread on C medium agar plates and were incubated at 24°C. Colonies were then tested for endocellulase activity, in order to identify those that lacked pCM1. Briefly, colonies from these plates were picked and replica-plated onto M9 CMC agar plates (consisting of M9 medium supplemented with 0.1% [wt/vol] yeast extract and 0.5% [wt/vol] carboxymethylcellulose [Joseph and David 2001] and C medium). Plates were incubated for 5 days at 25°C, were then stained with 0.1% (wt/vol) Congo red for 20 min, and were bleached with 1 M NaCl. Endocellulase activity was detected as a clear, yellowish halo around the colonies. Plasmid curing was confirmed by PCR and sequencing with primers targeting the *celA* gene, in plasmid pCM1, and *pat-1* gene, in plasmid pCM2.

CelA expression and cloning.

The *celA* gene was amplified with the *EcoR*_{celAF1/R1} primer pair from *C. michiganensis* subsp. *michiganensis* CASJ001 and CASJ002. The amplified PCR product was digested with *EcoRI* (Promega). The pHN216 vector (Laine et al. 1996) was digested with *EcoRI* overnight at 37°C, was treated with Antarctic phosphatase, and was ligated with *EcoRI*-digested *CelA* using T4 DNA ligase. The pHN216*celA* was transformed into *E. coli* DH5 α and was selected on LB medium containing neomycin (50 µg/ml). The presence of the *CelA* gene in the recombinant plasmid clone was confirmed by PCR and sequencing. Plasmid DNA isolation and *C. michiganensis* subsp. *michiganensis* transformation was performed as described above. After electroporation, cells were incubated on SB agar plates containing neomycin (50 µg/ml) for 5 to 7 days.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

CarboSource Services: <http://www.carbosource.net>
 FigTree program (version 1.4. 0): <http://tree.bio.ed.ac.uk/software/figtree/>
 WallMabDB website: <http://www.wallmabdb.net>