Eur J Plant Pathol (2012) 133:559–575
DOI 10.1007/s10658-011-9927-7

Characterization of phenotypic variants of *Clavibacter michiganensis* subsp. *michiganensis* isolated from *Capsicum annuum*

Kyu-Ock Yim · Hyok-In Lee · Jung-Hee Kim · Seung-Don Lee · Jung-Hee Cho · Jae-Soon Cha

Accepted: 12 December 2011 / Published online: 24 December 2011

Abstract Phenotypic variants of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) were isolated from pepper fields and from pepper seeds during quarantine inspections. All strains isolated from pepper (pepper isolates) produced orange-coloured colonies with lower mucoidy than typical *Cmm* strains isolated from tomato (tomato isolates). 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*. In addition to phenotypic variations, the pepper isolates showed different pathogenic and genetic characteristics from tomato isolates from the USA, Europe, or other countries. They could be clearly distinguished in terms of pathogenicity, as they showed increased pathogenicity to pepper but reduced pathogenicity to tomato. Tomato isolates caused strong wilting and canker in tomato, but caused only canker and no wilting in pepper and bell pepper. However, pepper isolates caused no wilting, even in tomato, and only caused canker in the three host plants. In addition, compared to tomato isolates, pepper isolates showed increased colonization efficiency and caused a greater reduction in shoot dry weight in pepper. Pepper and tomato isolates could be separated into two groups according to host origin on the basis of 16S rDNA and ITS sequence analysis. They also showed different rep-PCR genomic fingerprints. All pepper isolates showed higher cellulase activity than tomato isolates on M9CMC plates. However, two plasmid-borne virulence genes of *Cmm*, *pat*-1, and *cel*A, were not detected in any pepper isolates by PCR. Furthermore, PCR for pathogenicity-related genes located on a pathogenicity island (PAI) revealed that all tomato isolates were positive for these genes, whereas the pepper isolates did not show any PCR products for the *chp*C, *chp*G, *ppa*A, or *tom*A genes. Therefore, we suggest that the pepper isolates may represent a separate *Cmm* population that has evolved within the limits of this host.

Keywords Bacterial canker · *Clavibacter michiganensis* subsp. *michiganensis* · Pepper isolate

Kyu-Ock Yim and Hyok-In Lee contributed equally to the work

K.-O. Yim (✉) · H.-I. Lee · J.-H. Kim
National Plant Quarantine Service,
175 Anyangro,
Anyang, Kyoungki-do 430-016, Korea
e-mail: koyim@korea.kr

S.-D. Lee
Rural Development Administration,
150 Suinro,
Suwon, Kyoungki 441-707, Korea

J.-H. Cho · J.-S. Cha
Department of Plant Medicine,
Chungbuk National University,
52 Naesudongro,
Cheongju, Chungbuk 361-763, Korea

Introduction

Bacterial canker of tomato (*Solanum lycopersicon*), caused by the gram-positive bacterium *Clavibacter*

michiganensis subsp. *michiganensis* (Smith) Davis et al. (*Cmm*), is an important and widespread disease causing major economic losses in commercial tomato production (Gartemann et al. 2003; Gleason et al. 1993). In many countries, *Cmm* is regulated by quarantine procedures to minimize the introduction of the pathogen through commercial seeds, which are considered the most important source of inoculum; however, the pathogen has still been reported in most tomato-growing countries (Gartemann et al. 2003). In order to isolate *Cmm* from seeds and plants, various semi-selective media have been developed and improved (Gleason et al. 1993). In addition to traditional microbiological tests, various techniques, such as ELISA, fatty acid analysis, rDNA sequence analysis, PCR, and DNA fingerprinting, have been used for identification of *Cmm* (Gleason et al. 1993).

The *Clavibacter michiganensis* (*Cm*) species is divided into five subspecies on the basis of host specificity and biochemical and genetic characteristics: *Cm*. subsp. *sepedonicus* (*Cms*), *Cm*. subsp. *michiganensis* (*Cmm*), *Cm*. subsp. *insidiosus* (*Cmi*), *Cm*. subsp. *tesselarius* (*Cmt*), and *Cm*. subsp. *nebraskensis* (*Cmn*) (Gartemann et al. 2003). Although tomato is the primary host of *Cmm*, several other plants belonging to the family Solanaceae—pepper (*Capsicum annuum*), bell pepper (*Capsicum sativum*), and eggplant (*Solanum melongena*)—have been found to be naturally infected with *Cmm* in the field. However, these infections are unusual and are described in brief reports in many cases (Burokiene et al. 2005; Latin et al. 1995; Lewis-Ivey and Miller 2000).

Cmm spreads throughout the plant via the xylem vessels and induces systemic symptoms, including wilting, stem canker, vascular discolouration, and plant death (Gartemann et al. 2003; Gleason et al. 1993). *Cmm* titers in tomato may reach 10^9 bacteria per g plant tissue (Meletzus et al. 1993). The development of wilting symptoms in tomato is induced by the *cel*A and *pat*-1 genes, which are located on the pCM1 and pCM2 plasmids of *Cmm* strain NCPPB382 (Gartemann et al. 2003, 2008; Meletzus et al. 1993). In addition, a large (~129 kb) pathogenicity island (PAI) with low G+C content was discovered in the chromosome of *Cmm* NCPPB382 (Gartemann et al. 2008). Numerous genes in the PAI might be involved in *Cmm*-tomato interactions such as the colonization of the tomato plant, hypersensitive reactions, and suppression of host defence reactions (Burger et al. 2005; Gartemann et al. 2008; Stork et al. 2008).

Various atypical strains of *Cmm* with phenotypic and genotypic variations have been reported in previous studies. Strains that show variant colony morphology on nutrient-rich medium have been reported, which occasionally include dry, sticky, less mucoid, pink, red, orange, white, or colourless strains (Davis and Vidaver 2001; Hayward and Waterston 1964; Kaneshiro et al. 2006). *Cmm* strains with variations in virulence have also been isolated from seeds and plants (Kaneshiro et al. 2006; Kaneshiro and Alvarez 2001; Louws et al. 1998). Despite the presence of these variations in *Cmm* strains, their characteristics and the relationships of these to their host preferences have never been studied in detail.

Cmm isolates collected from the USA or Europe show generally conserved sequences in the internal transcribed spacer region (ITS region) and 16S rDNA gene (Lee et al. 1997b; Pastrik and Rainey 1999). It was also reported that *Cmm* can be subdivided into four or six distinct groups on basis of the diversity in repetitive sequence-based PCR (rep-PCR) fingerprint patterns (Louws et al. 1998; Nazari et al. 2007; Kleitman et al. 2008). More recently, genetic diversity related to geographical area was observed within the *Cmm* population (De Leon et al. 2009; Ignatov et al. 2004; Kaneshiro et al. 2006; Nazari et al. 2007). Among the five *Cm* subspecies, the relatively high genetic diversity of *Cmm* strains was confirmed by PCR-RFLP and PFGE analysis (Kleitman et al. 2008; Waleron et al. 2011).

The *Cmm* pathogen was first isolated from peppers in 1997–1998, during a disease outbreak in pepper fields in Korea, and most isolates showed different phenotypes from the typical description of the *Cmm* strains that had been isolated from tomato (Lee et al. 1999). In Korea, most commercial tomato and pepper seeds are imported from Asian countries, and the detection rate of *Cmm* in imported seeds during quarantine inspections has increased. In quarantine laboratory tests, *Cmm* isolates from imported pepper seeds have consistently exhibited similar morphological characteristics that are different from those of tomato isolates. This study aimed to determine the variations in morphological, molecular, and pathogenic properties of *Cmm* isolates from pepper.

Materials and methods

Bacterial strains and culture conditions

Cmm strains were obtained from the BCCM/LMG Bacteria Collection (Gent, Belgium) and Korean Agricultural Culture Collection (Suwon, Korea) (Table 1). The *Cmm* pepper isolates used in this study were isolated from imported pepper seeds during quarantine inspections and from pepper fields in Korea. A KBST semi-selective medium (Gleason et al. 1993) was used to isolate *Cmm*. After incubation at 27°C for 7 days, the colonies exhibiting *Cmm*-like morphology were selected for growth in pure culture by streaking onto plates of NBY agar medium. All the isolates were incubated on NBY or YDC medium at 27°C for 48–72 h prior to further analysis.

Identification of *Cmm*

All strains were initially identified using ELISA according to the protocol provided by the manufacturer (Agdia, Inc., Elkhart, IN, USA). PCR analysis for identification was performed using three *Cmm* primer pairs: CMR16F1/16R1, ClaF1/ClaR2, and Cm3/Cm4 (Table 2). Positive strains were identified and characterized using the Sherlock Microbial Identification System (MIDI Inc., Newark, DE, USA).

Pathogenicity and colonization tests

Ten representative *Cmm* strains were selected for further pathogenicity tests on three host plants. Tomato (var. Moneymaker), pepper (var. Manitta), and bell pepper (var. Spirit) seeds were planted in 8×8 cm trays and grown in a greenhouse at 25–30°C and 80% relative humidity. Each plant was inoculated between the 3- and 4-leaf stage of growth by cutting off the top of the youngest leaf with scissors that had been dipped into a bacterial suspension (10^8 CFU/ml) (Poysa 1993). The monitoring of symptom development was started after 10 days of inoculation, at which time unilateral wilting of the leaves was first observed in tomato seedlings. All plants were examined daily over a period of 25 days for the development of wilting and canker symptoms.

In order to measure the reduction in shoot dry weight of inoculated plants, the shoots of seedlings were harvested 25 days after inoculation. The harvested samples were dried at 80°C for 3 days, and their weight was measured. Six plants of each strain were inoculated, and all tests were conducted three times.

At 25 days after inoculation, the shoot of each plant was harvested, frozen separately in liquid nitrogen, and then ground to powder with a sterile pestle and mortar. The powder was suspended in PBST buffer (1 ml buffer/g fresh weight), and 100-µl aliquots of progressive tenfold serial dilutions ($1–10^{-4}$) were plated on KBST. Plates were incubated at 27°C for 7 days to determine the number of colony-forming units (CFU). For each strain, three plants were inoculated and the same tests were repeated three times.

DNA extraction and PCR

The primers used in this study are listed in Table 2. Total DNA of *Cmm* strains was extracted using the DNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol for gram-positive bacteria. General PCR for identification was performed using HotStart PCR PreMix (Bioneer, Daejeon, Korea). Takara Ex Taq PCR Kit (Takara, Kyoto, Japan) was used for genomic fingerprinting and sequence analysis. Rep-PCR was performed according to the method described in Louws et al. (1998), with ERIC-1R, ERIC-2, and BOX A1R primers (Table 2). PCR was performed on Gene Amp PCR System 2400 (Perkin-Elmer, Foster City, CA, USA). The amplified products were subjected to electrophoresis on a 1.2% agarose gel and then stained with ethidium bromide. The analysis of rep-PCR products was performed with a LabChip GX-DNA analysis system (Caliper Life Sciences Inc., MA, USA). Results of rep-PCR were recorded as presence (1) or absence (0) of product, and agglomerative hierarchical cluster (AHC) analysis was applied using the unweighted pair-group average method (UPGMA) with Dice dissimilarity matrix on Microsoft Excel software with the XLSTAT (T. Fahmy, Paris, France) add-in.

Sequence analysis

PCR for sequence analysis of the 16S rDNA gene and ITS region was performed on selected *Cmm* subsets from tomato and pepper isolates using the universal primer pair FGPS-6/FGPL-132 (Table 2). Each PCR product was cloned with the pGEM-T-Easy vector

Table 1 Strains of *Clavibacter michiganensis* used in this study

No.	Strain designation[a]	Cm. subspecies	Host	ELISA	MIDI	PCR				Color on YDC	Origin[c]	GenBank Accession No.	
						CMR	Cla	Cm3/4	Cmm5/6			16S rDNA	ITS
1	LMG 7333	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	Hungary	JN603277	JN603289
2	LMG 3685	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	–	Yellow	USA	JN603280	JN603292
3	LMG 3681	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	–	Yellow	England	JN603278	JN603290
4	LMG 3687	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	Italy	JN603279	JN603291
5	LMG 3683	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	Italy		
6	LMG 2891	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	Hungary		
7	LMG 3686	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	Zimbabwe		
8	LMG 3679	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	Kenya	JN603281	JN603293
9	TF2644	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	Korea	JN603282	JN603294
10	TS004	*michiganensis*	Tomato	+	Cmm/Cms	+	+	+	+	Yellow	China	JN603283	JN603295
11	PS003	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	China	JN603284	JN603296
12	PS005	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	China	JN603285	JN603297
13	PS006	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	China	JN603286	JN603298
14	PF007	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea	JN603287	JN603299
15	PF008	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea	JN603288	JN603300
16	PF009	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
17	PF010	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
18	PF011	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
19	PF013	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
20	PF014	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
21	PF015	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
22	PF016	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
23	PF017	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
24	PF018	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
25	PF019	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
26	PF020	*michiganensis*	Pepper	+	Cmm/Cms	+	+	–	–	Orange	Korea		
27	LMG 2901	*sepedonicus*	Potato	NT[b]	NT	NT	NT	NT	NT	NT	USA		JN613837
28	KACC 20766	*insidiosus*	Alfalfa	NT	NT	NT	NT	NT	NT	NT	USA		JN613834
29	KACC 20788	*nebraskensis*	Corn	NT	NT	NT	NT	NT	NT	NT	USA		JN613835
30	KACC 20800	*tessellarius*	Wheat	NT	NT	NT	NT	NT	NT	NT	USA		JN613836

[a] *KACC* Korean agricultural culture collection

[b] *NT* not tested

[c] China: isolated from seeds imported from China; Korea: isolated from pepper fields in South Korea

Table 2 Sequences of primers used in this study

Primer	Sequence (5′–3′)	Target[a]	Reference
CMR16F1 CMR16R1	GTGATGTCAGAGCTTCCTCTGGCGGAT GTACGGCTACCTTGTTACGACTTAGT	16S-rRNA	Lee et al. (1997a)
ClaF1 ClaR2	TCATTGGTCAATTCTGTCTCCC TACTGAGATGTTTCACTTCCCC	ITS region	Peng et al. (2005)
Cm3 Cm4	CCTCGTGAGTGCCGGGAACGTATCC CCACGGTGGTTGATGCTCGCGAGAT	pCM1	Santos et al. (1997)
FGPS-6 FGPL-132	GGAGAGTTAGATCTTGGCTCAG CCGGGTTTCCCCATTCGG	16S-ITS-23S	Normand et al. (1992)
16S 23S	TTGTACACACCGCCCGTCA GGTACCTTAGATGTTTCAGTTC	ITS region	Kostman et al. (1992)
BOX A1R	CTACGGCAAGGCGACGCTGACG	rep-PCR	Louws et al. (1998)
ERIC-1R ERIC-2	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	rep-PCR	Louws et al. (1998)
Cmm-5 Cmm-6	GCGAATAAGCCCATATCAA CGTCAGGAGGTCGCTAATA	*pat-1*	Dreier et al. (1995)
P1rep P3rep	CGTACCCCGAGAACCGGGG GCGCCCGTGTCGAACATT	*pat-1rep*	Dreier et al. (1997)
cel-578up cel-2752low	ATGGCTTCCCTACGATCC ACAGGGTAGAAGCGGGAGG	*cel*A	Jahr et al. (2000)
pCRcel-593 pCRcel-1860	TCCTTATATGACATTTCGCC GCCACTTCGCTGATACAG	CD of *cel*A	Jahr et al. (2000)
PFC3 PFC5	GGTACGAAGTTCGAGACGAC TGTAGCGGTGAGTCGTGGTGA	CBD of *cel*A	Kleitman et al. (2008)
tomA-F tomA-R	CGAACTCGACCAGGTTCTCG GGTCTCACGATCGGATCC	*tom*A	Kleitman et al. (2008)
ppaA-F ppaA-R	CATGATATTGGTGGGGAAAG CCCCGTCTTTGCAAGACC	*ppa*A	Kleitman et al. (2008)
chpC-F chpC-R	GCTCTTGGGCTAATGGCCG GTCAGTTGTGGAAGATGCTG	*chp*C	Kleitman et al. (2008)
chpG-F chpG-R	GACAACATGACCCTGCACTG TCGGGGTGTAGACAAGGAAG	*chp*G	Kleitman et al. (2008)

[a] *CD* catalytic domain; *CBD* cellulose binding domain

system (Promega, Wisconsin, USA). The true clones were selected by M13 PCR amplification, and the PCR products were used as templates for sequencing. Sequencing was performed on an ABI 3730xl DNA sequencer (Applied Biosystems, CA, USA). Acquired sequences were compared with reference sequences using BLAST (http://blast.ncbi.nlm.nih.gov) and aligned using ClustalW software (Thompson et al. 1994). In addition, ITS region of four *Cm* subspecies were sequenced using the primer pair 16S/23S by the same method because there is not enough ITS sequence data in GenBank (Table 2). Prior to construction of phylogenetic trees, the sequences of the 16S rDNA gene and ITS region were separated from full alignments and adjusted to equal lengths. Phylogenetic analysis was performed using the MEGA 4.0 program (Kumar et al. 2004), and the phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei 1987).

Assay for cellulase activity

Cellulase activity of *Cmm* isolates was detected on M9CMC agar plates (Meletzus et al. 1993). A single colony grown on NBY was inoculated onto an M9CMC plate using a sterilized tip and incubated for 4 days at 26°C. Plates were stained with 0.1% (wt/vol) Congo red for 10 min and then bleached three times with 1 M NaCl. Cellulase activity was detected

by clear zone formation. The same tests were repeated three times.

Results

Identification and characterization of *Cmm* strains

All *Cmm* strains isolated from tomato showed typical colony morphology (convex, mucoid, and pale yellow to yellow) on NBY and YDC media. In contrast, all strains isolated from pepper produced orange-coloured, less mucoid colonies (Fig. 1). Nevertheless, both tomato and pepper isolates gave positive results in commercial ELISA for detection of *Cmm* (Table 1). In fatty acid analysis using the MIDI system, all ELISA-positive strains showed high similarity with the *Cmm*/*Cms* group (Table 1). The fatty acid composition of strains in the two groups of isolates were very similar to each other, although there are slight differences in levels of anteiso-heptadecanoic acid (a17:0) and palmitic acid (16:0), which were higher in pepper isolates (data not shown).

PCR was carried out with three primer pairs for identification of *Cmm*, and the PCR patterns of the two groups were compared. All tested strains showed positive PCR results with the CMR16F1/CMR16R1 primer pair, which was designed from the 16S rDNA region for detection of *Cm* at the species level (Fig. 2-a and Table 1). With a *Cmm*-specific primer pair, ClaF1/ClaR2, derived from the ITS region, all collected strains were identified as *Cmm* by amplification of PCR products of the expected sizes. All tomato isolates gave strong bands of products with the ClaF1/ClaR2 primer pair, whereas only faint bands were observed with the pepper isolates (Fig. 2-b and Table 1). However, two of the 10 tomato isolates did not react with Cmm5/Cmm6, which is also known to be a *Cmm*-specific primer pair (Table 1). In addition, none of the pepper isolates gave a PCR product with the Cm3/Cm4 primer pair, derived from the pCM1 plasmid, while all tomato isolates produced strong product bands with the same primer (Fig. 2-c and Table 1).

Fig. 1 Phenotypes of *Clavibacter michiganensis* subsp. *michiganensis* strains on YDC agar. T: LMG7333; T1: LMG3681; T2: LMG3685; P1: PF008; P2: PF007

Pathogenicity and colonization in the 3 different hosts

The tomato and pepper isolates varied in pathogenicity and colonization efficiency in tomato, pepper, and bell pepper plants. All tested tomato isolates appeared to be very highly pathogenic to tomato seedlings. After 25 days of inoculation, all infected tomato seedlings showed strong wilting and canker symptoms, and over 80% of them were dead (Fig. 3; Table 3). In addition, the shoot dry weight of infected tomato plants was significantly reduced by 56% with the LMG7333 strain. However, in pepper plants, the tomato isolates caused minimal stem canker and leaf blight without wilting (Fig. 3; Table 3), and reduced the shoot dry weight by only 13% after 25 days (Table 4).

When pepper isolates were inoculated on tomato seedlings, wilting symptoms did not appear, although slight stem canker was observed in 50% of tomatoes, and leaf blight was seen on only 30% of seedlings. Shoot dry weight was reduced by approximately 15%. When pepper seedlings were inoculated with pepper isolates, most of the seedlings showed stem and leaf blight, and the shoot dry weight was reduced by approximately 30% (Fig. 3; Table 4). In summary, we found that in comparison with tomato isolates, the pepper isolates showed reduced pathogenicity on tomato and increased pathogenicity on pepper.

When inoculated on bell pepper seedlings, the tomato and pepper isolates caused similar symptoms (Table 3) and a similar reduction in shoot dry weight (Table 4). These differences in symptoms were also observed in other commercial varieties of tomato, pepper, and bell pepper (data not shown).

The population sizes of tomato and pepper isolates in different hosts were compared in order to identify differences in colonization efficiency. In tomato, the tomato isolates were detected at levels over 10^8 CFU/g after 25 days of inoculation, whereas pepper isolates showed population sizes under 10^6 CFU/g. In contrast, the population size in pepper reached approximately 10^6 CFU/g for tomato strains and over 10^8 CFU/g for

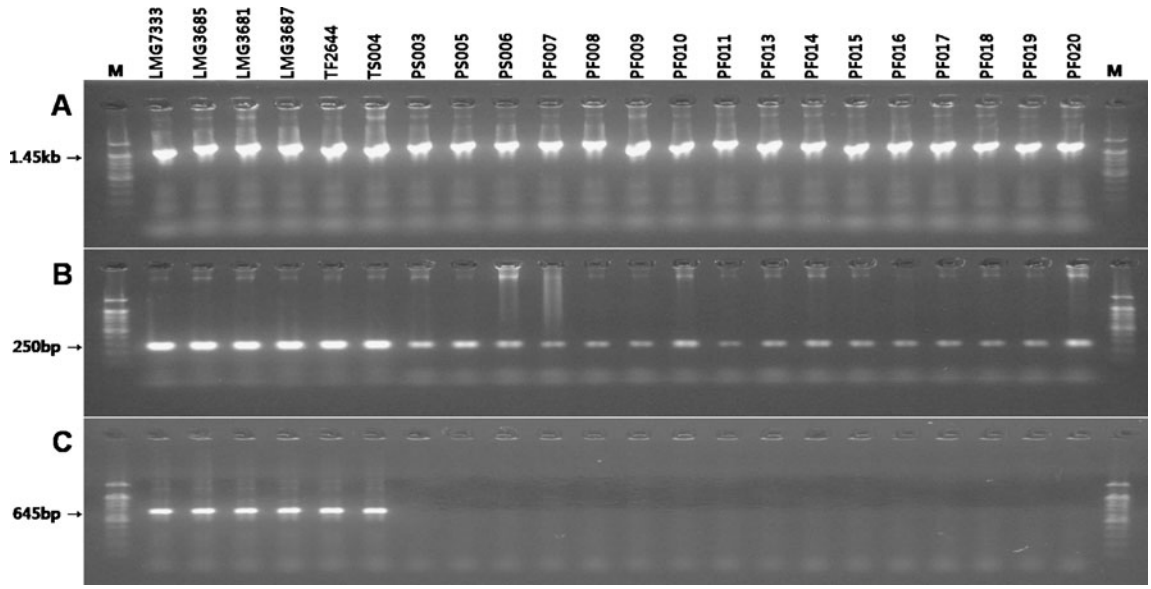

Fig. 2 PCR assay for detection *Clavibacter michiganensis* subsp. *michiganensis* using specific primers. **a** CMR16F1/CMR16R1; **b** ClaF1/ClaR2; **c** Cm3/Cm4; M: molecular marker

pepper strains. The 2 *Cmm* groups showed a similar population size in bell pepper (Table 4). These results showed that pepper isolates have higher colonization efficiency in pepper plants, whereas that of tomato isolates was higher in tomatoes.

Genomic fingerprints of the 2 *Cmm* groups

Genomic fingerprinting analysis using BOX and ERIC primers showed that *Cmm* isolates could be separated into two groups according to the host origin. The distinct differences between the two *Cmm* groups were easily observed from the overall patterns observed in BOX PCR. The polymorphisms of BOX PCR bands in the region around 1,500 bp to 2,000 bp allowed differentiation between the two *Cmm* groups. In addition, all pepper isolates showed common bands in the 800 bp and 900 bp regions (Fig. 4-a). ERIC PCR fingerprinting showed more polymorphic bands for each *Cmm* strain. However, tomato and pepper isolates could still be distinguished on the basis of the ERIC PCR banding patterns in the region around 500 bp to 700 bp (Fig. 4-b). A visual inspection of the dendrogram obtained using the UPGMA method shows that *Cmm* isolates are clustered in two groups (Fig. 5). This high dissimilarity in fingerprint patterns suggested that there is significant genetic diversity between the two *Cmm* groups.

Comparison of the 16S rDNA gene and ITS sequences

Approximately 2.1-kb DNA sequences encompassing the 16S rDNA gene, ITS region, and partial 23S rDNA gene were obtained from the PCR products generated using the FGPS-6/FGPL-132 primer pair. The results of NCBI BLAST comparative analysis with these sequences showed over 98% sequence identity with the rRNA gene represented in the published *Cmm* NCPPB382 complete genome sequence (Gartemann et al. 2008). The sequences were also divided into 16S rDNA and ITS regions and subjected to individual BLAST searches to analyze the variation at each locus. The 16S rDNA sequences of all tested strains exhibited over 99% similarity with various *Cmm* strains. When comparing the 16S rDNA sequences, only 19 positions out of 1,359 nucleotides (1.4%) showed variation, and five of these positions showed grouped differences between tomato and pepper isolates (data not shown). In contrast, the sequences of the ITS region displayed relatively low similarities in the range of 94% to 98% (data not shown). In the ITS sequences, 33 positions showed variation, out of a total of 774 nucleotides (4.3%). Of the 33 nucleotide positions showing variation, 17 positions (51.5%) showed grouped differences between tomato and pepper isolates (Fig. 6).

To further characterize the genetic relationships of the pepper and tomato isolates in the context of the

Fig. 3 Symptoms induced by *Clavibacter michiganensis* subsp. *michiganensis* isolates. **a**: tomato isolates (LMG7333); **b**: pepper isolates (PF007); *Left*: tomato; *Middle*: pepper; *Right*: bell pepper

five *Cm* subspecies, a phylogenetic analysis were performed on the basis of the 16S rDNA and ITS sequences. The phylogenetic trees demonstrated that pepper isolates formed a distinct group separate from tomato isolates. Further, unexpectedly, the similarity of the 16S rDNA and ITS sequences between the two *Cmm* groups was not much higher than that seen between *Cm*. subspecies (Fig. 7).

Detection of virulence related genes

The presence of the plasmid-borne virulence genes, *cel*A and *pat*-1, was determined by PCR with various primers. We found that the two virulence genes could not be detected in any pepper isolates, whereas bands of the expected size from the *pat*-1 gene were generated in four of the six tomato isolates with Cmm5/Cmm6 and

Table 3 Symptoms of infection with *Clavibacter michiganensis* subsp. *michiganensis* isolates in 3 host plants at 25 days after inoculation

Symptoms	Tomato isolates			Pepper isolates		
	Tomato	Pepper	Bell Pepper	Tomato	Pepper	Bell Pepper
Wilting	+++[a] (100%)[b]	–	–	–	–	–
Stem canker	+++ (100%)	+ (20%)	++ (80%)	++ (50%)	++ (50%)	++ (70%)
Leaf blight	NA[c]	+ (20%)	+ (30%)	+ (30%)	+ (30%)	++ (50%)
Death	+++ (80%)	–	–	–	–	–

[a] +, ++, +++: refer to weakly, moderately and highly virulent reactions;–: absent of symptom

[b] (%): percentage of plants showing symptom

[c] *NA* not available

P1rep/P3rep primers, and all tomato isolates were also positive for the presence of *cel*A gene with three primer pairs (Fig. 8). A nonspecific PCR product of about 1 kb was amplified in all pepper isolates when using the PFC3/PFC5 primer pair for amplification of the cellulose-binding domain of *cel*A (Fig. 8-e). The sequence of this nonspecific product from pepper isolates PS003 (Accession no: JN603301), PS010 (Accession no: JN603302), and PS018 (Accession no: JN603303) showed a high similarity (94%) with the *uvr*B gene of *Cmm* NCPPB382 (data not shown). PCR detection of four pathogenicity-related genes located on the PAI revealed that all tomato isolates were positive for these genes, whereas all pepper isolates lacked *chp*C, *chp*G, *ppa*A, and *tom*A (Fig. 9). These results might suggest that the pathogenic differences between the two *Cmm* groups are associated with variations in these virulence-related genes.

Cellulase activity

The endoglucanase CelA of *Cmm* is known to be an important virulence factor required for wilt-induction capability on tomato. In this study, *cel*A was not detected in any pepper isolates, and, consistent with this result, these isolates caused no wilting symptoms in the three hosts. To determine whether cellulase activity is absent in pepper isolates, a bioassay was carried out on M9CMC agar plates. Surprisingly, these isolates appeared to have much higher activity than tomato isolates on this medium (Fig. 10). These results suggest that another type of cellulase, which does not

Table 4 Shoot dry weight and colonization of 3 host plants infected by several tomato and pepper isolates of *Clavibacter michiganensis* subsp. *michiganensis* at 25 days after inoculation

Strain	Dry weight (g/plant)			Colonizatin (CFU/g FW)		
	Tomato	Pepper	Bell-Pepper	Tomato	Pepper	Bell-Pepper
LMG7333	0.56	0.63	0.43	1.6×10^{9}	5.5×10^{5}	2.1×10^{7}
LMG3681	0.82	0.55	0.42	7.5×10^{8}	2.6×10^{6}	3.9×10^{8}
LMG3687	0.68	0.68	0.46	3.0×10^{8}	2.6×10^{6}	3.9×10^{8}
LMG3679	0.66	0.64	0.40	3.0×10^{8}	1.6×10^{6}	2.0×10^{8}
TS004	0.69	0.62	0.40	2.8×10^{8}	4.7×10^{6}	2.9×10^{8}
PS003	1.12	0.48	0.38	1.7×10^{5}	2.1×10^{8}	3.9×10^{8}
PF007	1.04	0.54	0.43	1.1×10^{6}	2.3×10^{8}	1.8×10^{8}
PF008	1.03	0.51	0.41	1.0×10^{6}	5.0×10^{8}	1.6×10^{8}
Negative control	1.26	0.72	0.62	0	0	0

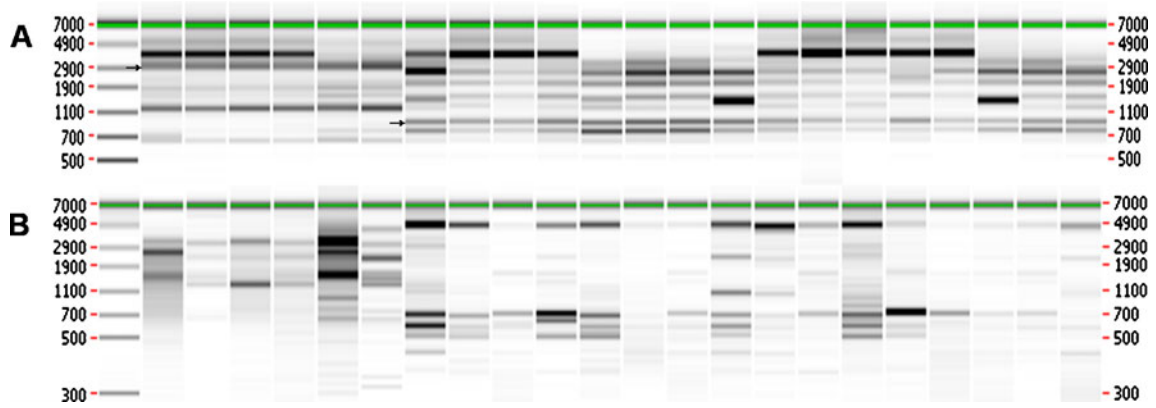

Fig. 4 Rep-PCR fingerprint patterns of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from tomato and pepper. **a**: BOX-PCR; **b**: ERIC-PCR; M: molecular marker

have any impact on wilt induction, is present in pepper isolates.

Discussion

Bacterial canker caused by *Cmm* has been reported in commercial pepper, bell pepper, and eggplant fields in Israel, Italy, Japan, Korea, Lithuania, and the USA. However, this disease has received little attention, perhaps because infected plants do not generally die, and therefore damage and economic losses are relatively limited (Burokiene et al. 2005; Buonaurio et al. 1999; Kobayashi and Kijima 1981; Lai 1976; Latin et al. 1995; Lewis-Ivey and Miller 2000; Lee et al. 1999; Medina-mora et al. 2000; Volcani et al. 1970). Although some cases of *Cmm* in pepper plants had been reported previously, analysis of characteristics such as pathogenicity and genetic diversity had not been performed sufficiently, in many cases because the inoculum source was assumed to come from nearby tomato fields.

In this study, the comparison of pepper and tomato isolates revealed a high diversity of pathogenic properties as well as colony morphology. *Cmm* isolates from pepper consistently produced orange-coloured

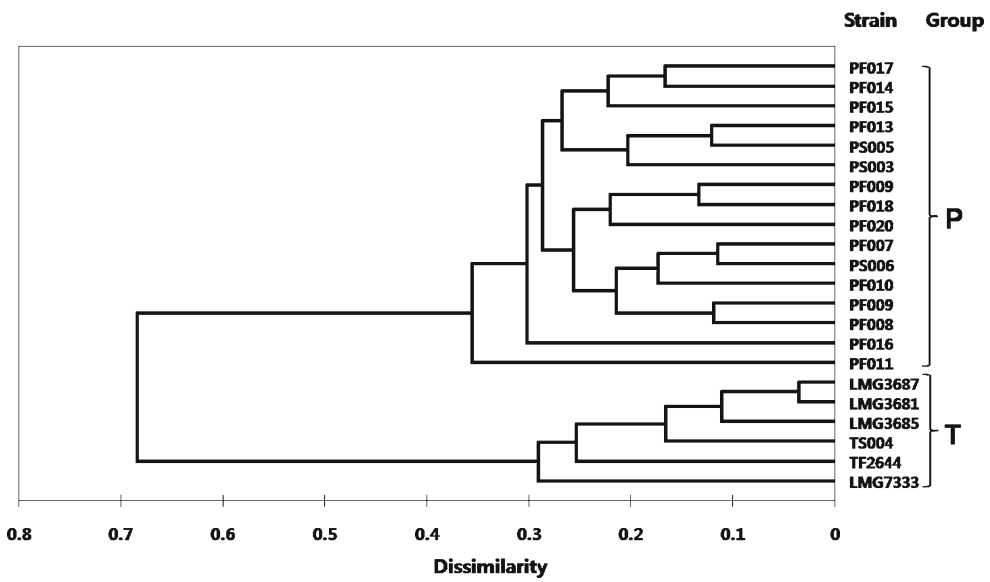

Fig. 5 Dissimilarity UPGMA tree derived from rep-PCR fingerprint patterns of *Clavibacter michiganensis* subsp. *michiganensis*. T: tomato isolates group; P: pepper isolates group

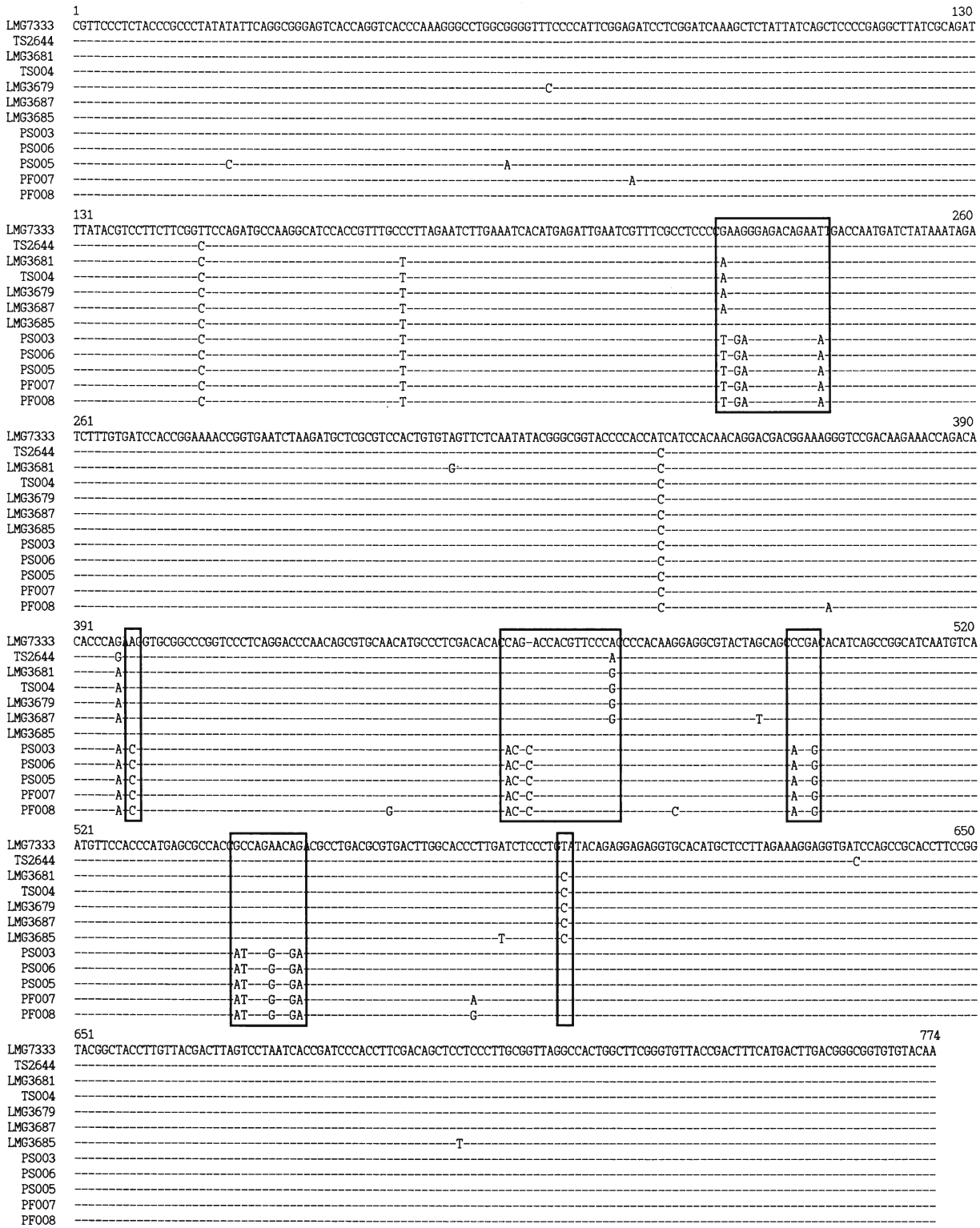

Fig. 6 Comparison of the nucleotide sequences of the ITS region between tomato and pepper isolates of *Clavibacter michiganensis* subsp. *michiganensis.* The nucleotide areas showing grouped differences are boxed

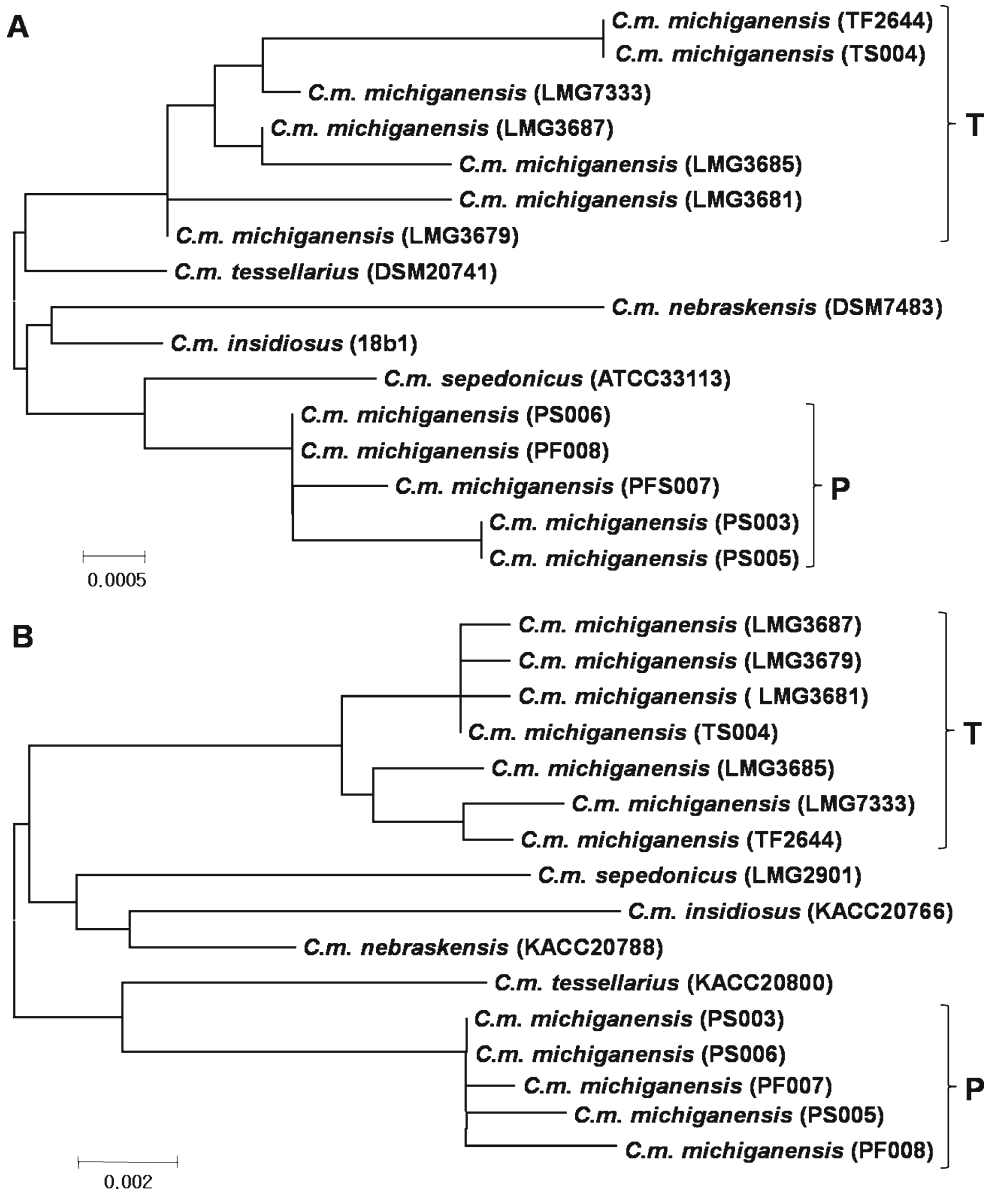

Fig. 7 Neighbour-joining tree derived from 16S rDNA (**a**) and ITS region (**b**) sequence analysis of *Clavibacter michiganensis* subspecies. T: tomato isolates; P: pepper isolates. The following 16S rDNA sequences in GenBank were used: *C.m.* subsp. *sepedonicus* ATCC33113 (Accession no. AM849034); *C.m.* subsp. *insidiosus* 18b1 (Accession no. GQ332310); *C.m.* subsp. *nebraskensis* DSM7483 (Accession no. AM410697); *C.m.* subsp. *tessellarius* DSM20741 (Accession no. AM410693)

colonies rather than yellow. However, although these morphological variations have been reported previously (Kaneshiro et al. 2006), the correlation between pathogenicity and morphology of *Cmm* was not revealed. Upon inoculation on pepper, atypical strains isolated from pepper showed higher virulence and colonization efficiency (>100 times) than tomato isolates, whereas they showed significantly reduced pathogenicity on tomato seedlings than tomato isolates. These correlations between virulence and host origin could be demonstrated by host preference, which was recognized through Korean quarantine inspection statistics. *Cmm* strains showing orange colonies have been isolated from pepper seeds but only in one case from tomato seeds,

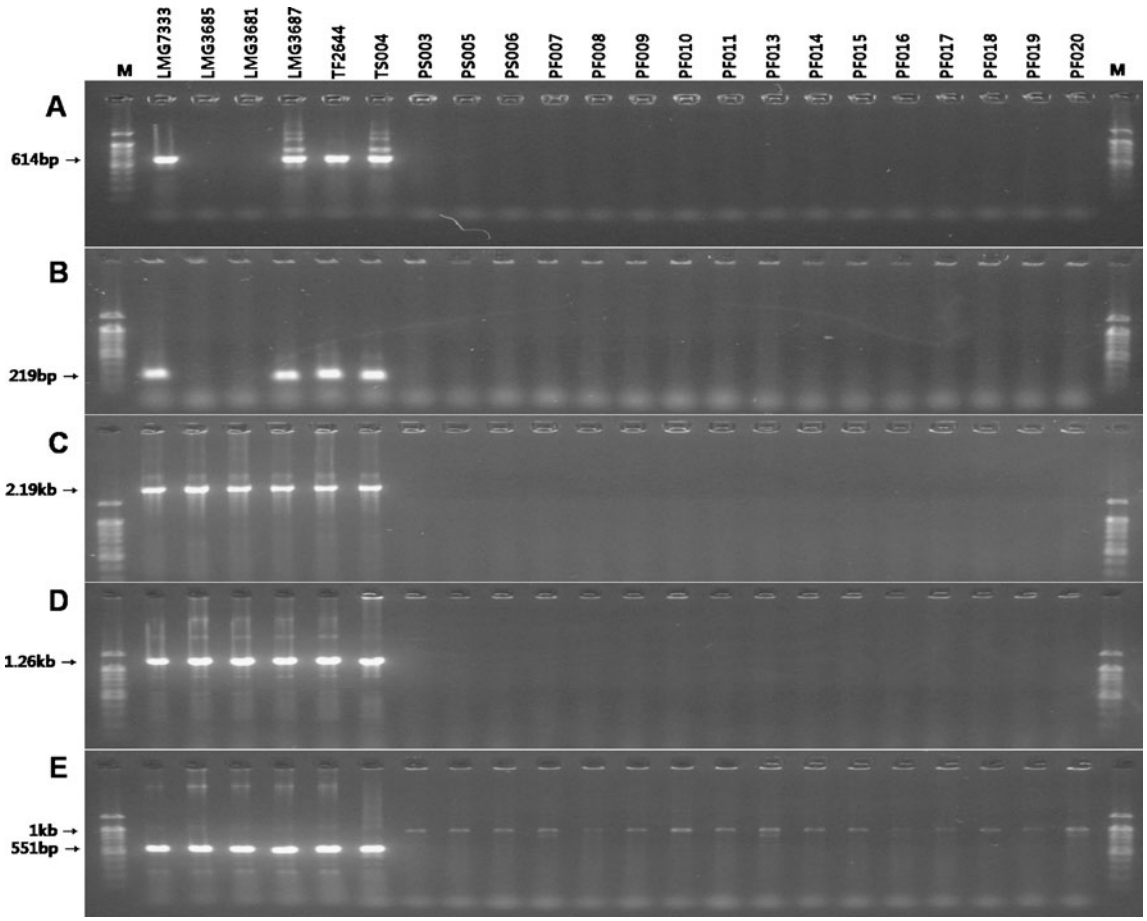

Fig. 8 PCR assay for detection of *pat*-1 and *cel*A genes from *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from tomato and pepper. **a** *pat*-1 gene with Cmm5/Cmm6 primers; **b** *pat*-1 gene with P1rep/P3rep primers; **c** *cel*A gene with 578up/2752low primers; **d** *cel*A gene with pCRcel593/pCRcel1860 primers; E: *cel*A gene with PFC3/PFC5 primers; M: molecular marker

while the typical pale yellow *Cmm* strains were isolated only from tomato seeds but never detected on pepper seeds.

The pepper isolates, which show relatively low mucoidy, did not induce wilting symptoms in either tomato or pepper, and consequently caused less reduction in shoot dry weight. The mucoid and non-mucoid strains of *Cms* are known to differ in terms of the amount and composition of their extracellular polysaccharides (EPS). The composition of EPS can affect antigenic properties, colonization of host, and induction of wilting symptoms, as well as colony morphology of *Cms* (Fousek and Mraz 2003; Gartemann et al. 2003). However, EPS content may not be a significant factor affecting the antigenic properties of *Cmm* strains, because all *Cmm* isolates in this study responded to commercial antibody at a similar range of OD_{A405} values, and no significant differences were found between the fatty acid profiles of the 2 *Cmm* groups in this study. Although two plasmid-borne genes and the chromosomal PAI were known to be involved in the pathogenicity of *Cmm*, it was also reported that some avirulent strains respond positively, while virulent strains respond negatively to PCR primers for these virulence-related genes (Bella et al. 2007; Kleitman et al. 2008). In this study, none of the pepper isolates gave PCR products with *cel*A or *pat*-1 primers. Moreover, four virulence-related genes residing on the PAI could not be detected in any pepper isolates by PCR. These results may explain the differences of pathogenicity between the two *Cmm* groups. However, all pepper isolates still

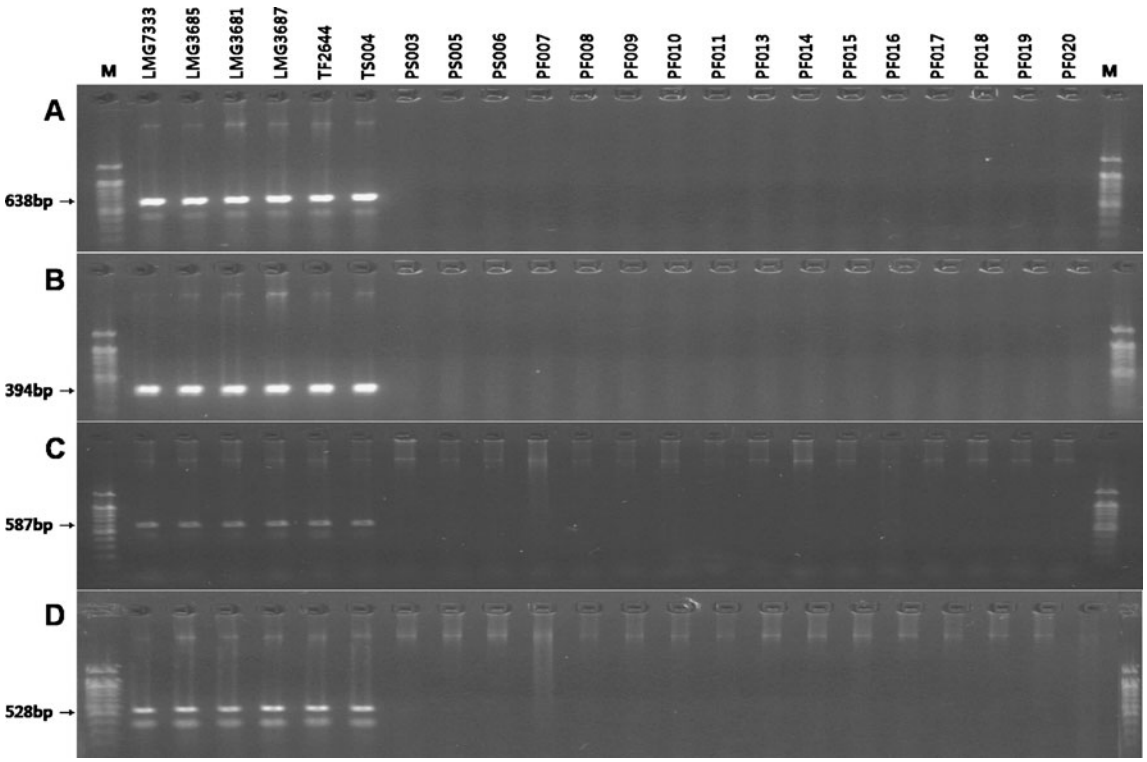

Fig. 9 PCR assay for detection of virulence-related genes residing on the pathogenicity island of *Clavibacter michiganensis* subsp. *michiganensis* strains. **a** *chp*C gene with chpC-F/chpC-R primers; **b** *chp*G gene with chpG-F/chpG-R primers; **c** *ppa*A gene with ppaA-F/ppaA-R primers; **d** *tom*A gene with tomA-F/tomA-R primers; M: molecular marker

showed high cellulase activity in bioassay. Therefore, further studies are required in order to determine whether the non-wilting of pepper isolates is associated with EPS composition, variation in virulence-related genes, or type of cellulase.

The rep-PCR method has been used to detect genetic variability even between single bacterial strains; for example, between *Cmm* and *Cms* subspecies (Fousek and Mraz 2003; Louws et al. 1998). In a previous study on *Cms*, no genetic differences

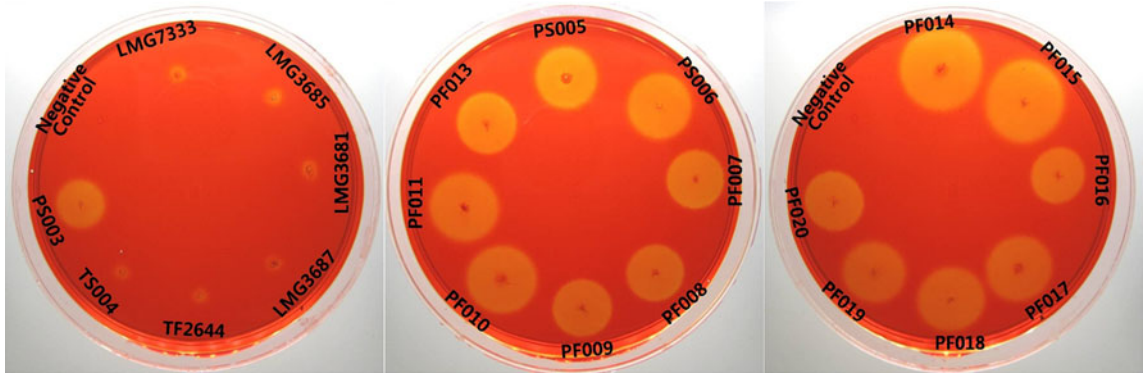

Fig. 10 Bioassay for cellulase activity of *Clavibacter michiganensis* subsp. *michiganensis* strains on M9CMC agar plates stained with 0.1% Congo red and washed with 1 M NaCl

between mucoid and non-mucoid strains could be detected by rep-PCR (Fousek and Mraz 2003). However, in this study, genetic differences between tomato isolates and pepper isolates were identified by genomic fingerprinting.

Analysis of the 16S rDNA sequence has been widely used as a taxonomic tool for bacteria, including unculturable species. In this study, the average similarity value of 16S rDNA sequences among *Cmm* strains was 99.5%, indicating that all tested isolates may be similar enough to be identified as part of a single subspecies. However, it was reported that the 16S rDNA sequence analysis cannot accurately identify strains of *Cmm* (Kaneshiro et al. 2006) due to highly similar 16S rDNA sequences between different *Cm* subspecies (Lee et al. 1997b; Suzuki et al. 1996). Considering such high similarity between the five subspecies, the presence of grouped differences in 16S rDNA between pepper and tomato isolates could give new meaning to classification of pepper isolates at the subspecies level.

The evolution of the ITS region is much faster than that of the 16S rDNA genes, probably due to reduced functional restriction. Thus, ITS sequence analysis has been used as a means to trace more recent evolutionary events at species and strain level. Pastrik et al. compared the sequences of six *Cmm* isolates from Europe and showed that *Cmm* has little variation in the ITS region (Pastrik and Rainey 1999). However, in the present study, tomato and pepper isolates could be separated into two distinct groups by ITS analysis according to their host origin. Over 50% of the variation in ITS sequences showed grouped differences between tomato and pepper isolates. These distinct dissimilarities in the ITS region between the two groups may explain why all pepper isolates were weakly amplified with the *Cmm*-specific primer, ClaF1/ClaR2, which is based on ITS sequences. In addition, these results suggest that these groups have followed individual paths of evolution within each host. Furthermore, the classification of the pepper isolates as a new subspecies could be proposed on the basis of genetic dissimilarity, since the 16S rDNA and ITS sequences between the two *Cmm* groups showed relatively low similarity in the context of that seen between the five different *Cm* subspecies.

Genetic variations between populations of *Cmm* have consistently been reported from various countries, and these results have been used to estimate their origins (De Leon et al. 2009; Ignatov et al. 2004; Kaneshiro et al. 2006; Nazari et al. 2007). Pepper isolates could have long existed in pepper fields undiscovered because they were not usually a major disease problem. In addition, bacterial canker may be hard to detect in the case of co-infection with other diseases such as bacterial leaf spot (*Xanthomonas campestris* pv. *vesicatoria*), which is one of the most common and destructive diseases of peppers. These disease symptoms are indistinguishable in mixed infection, and the different growth rates of two bacterial pathogens on an agar plate may easily cause *Cmm* to be overlooked. Therefore, it is very likely that the atypical pepper isolates may represent a separate *Cmm* population that has existed preferentially on pepper plants in Asian countries, since peppers are more commonly cultivated in Asia than in Western countries.

Acknowledgements This work was funded by the National Plant Quarantine Service of Korea.

References

Bella, P., Ialacci, G., Licciardello, G., & Catara, V. (2007). Detection of atypical strains of *Clavibacter michiganensis* subsp. *michiganensis* in greenhouse tomatoes in Sicily. *Journal of Plant Pathology, 89*(3), S30.

Buonaurio, R., Stravato, V. M., & Scorthchini, M. (1999). Le malattie batteriche del peperone. *Informatore Fitopatologico, 49*(5), 13–19.

Burger, A., Grafen, I., Engemann, J., Niermann, E., Pieper, M., Kirchner, O., Gartemann, K., & Eichenlaub, R. (2005). Identification of homologues to the pathogenicity factor *pat-1*, a putative serine protease of *Clavibacter michiganensis* subsp. *michiganensis*. *Microbiological Research, 160*, 417–427.

Burokiene, D., Sobiczewski, P., & Berczynski, S. (2005). Phenotypic characterization of *Clavibacter michiganensis* subsp. *michiganensis* isolates from Lithuania. *Phytopathologia Polonica, 38*, 63–77.

Davis, M. J., & Vidaver, A. K. (2001). Coryneform plant pathogens. In N. W. Schaad (Ed.), *Laboratory guide for identification of plant pathogenic bacteria* (3rd ed., pp. 218–235). St. Paul: The American Phytopathological Society.

De Leon, L., Rodriguez, A., Llop, P., Lopez, M. M., & Siverio, F. (2009). Comparative study of genetic diversity of *Clavibacter michiganensis* subsp. *michiganensis* isolates from the Canary Islands by RAPD-PCR, BOX-PCR and AFLP. *Plant Pathology, 58*(5), 862–871.

Dreier, J., Bermpohl, A., & Eichenlaub, R. (1995). Southern hybridization and PCR for specific detection of

phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology, 85*(4), 462–468.

Dreier, J., Meletzus, D., & Eichenlaub, R. (1997). Characterization of the plasmid encoded virulence region pat-1 of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. *Molecular Plant-Microbe Interactions, 10*(2), 195–206.

Fousek, J., & Mraz, I. (2003). Determination of genetic differences between fluid and nonfluid variants of *Clavibacter michiganensis* subsp. *sepedonicus* using rep-PCR technique. *Folia Microbiologica, 48*(5), 682–686.

Gartemann, K. H., Kirchner, O., Engemann, J., Grafen, I., Eichenlaub, R., & Burger, A. (2003). *Clavibacter michiganensis* subsp. *michiganensis*: first steps in the understanding of virulence of a Gram-positive phytopathogenic bacterium. *Journal of Biotechnology, 106*, 179–191.

Gartemann, K. H., Abt, B., Beke, T., Burger, A., Engemann, J., Flugel, M., Gaigalat, L., Goesmann, A., Grafen, I., Kalinowski, J., Kaup, O., Kirchner, O., Krause, L., Linke, B., McHardy, A., Meyer, F., Pohle, S., Ruckert, C., Schneiker, S., Zellermann, E., Puhler, A., Eichenlaub, R., Kaiser, O., & Bartels, D. (2008). The genome sequence of the tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp *michiganensis* NCPPB382 reveals a large island involved in pathogenicity. *Journal of Bacteriology, 190*(6), 2138–2149.

Gleason, M. L., Gitaitis, R. D., & Ricker, M. D. (1993). Recent progress in understanding and controlling bacterial canker of tomato in Eastern North America. *Plant Disease, 77*(11), 1069–1076.

Hayward, A. C., & Waterston, J. M. (1964). *Corynebacterium michiganense. Descriptions of pathogenic fungi and bacteria no. 19*. Wallingford: CAB International.

Ignatov, A., Matveeva, E., & Schaad, N. (2004). Genetic diversity among *Clavibacter michiganensis* subsp. *Michiganensis* and subsp. *sepedonicus* in Russian Federation. *Phytopathology, 94*, 543.

Jahr, H., Dreier, J., Meletzus, D., Bahro, R., & Eichenlaub, R. (2000). The endo-β-1,4-glucanase *Cel*A of *Clavibacter michiganensis* subsp. *michiganensis* is a pathogenicity determinant required for induction of bacterial wilt of tomato. *Molecular Plant-Microbe Interactions, 13*(7), 703–714.

Kaneshiro, W. S., & Alvarez, A. M. (2001). Specificity of PCR and ELISA assays for hypovirulent and avirulent *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology, 91*, S46.

Kaneshiro, W. S., Mizumoto, C. Y., & Alvarez, A. M. (2006). Differentiation of *Clavibacter michiganensis* subsp. *michiganensis* from seed-borne saprophytes using ELISA, Biolog and 16S rDNA sequencing. *European Journal of Plant Pathology, 116*(1), 45–56.

Kleitman, F., Barash, I., Burger, A., Iraki, N., Falah, Y., Sessa, G., Weinthal, D., Chalupowica, L., Gartemann, K. H., Eichenlaub, R., & Manulis-Sasson, S. (2008). Characterization of a *Clavibacter michiganensis* subsp. *michiganensis* population in Israel. *European Journal of Plant Pathology, 121*, 463–475.

Kobayashi, M., & Kijima, T. (1981). Occurrence of bacterial canker (temporary name) of sweet pepper in Japan. *Proceedings of the Kanto-Tosan Plant Protection Society, 28*, 54.

Kostman, J. R., Edlind, T. D., Lipuma, J. J., & Stull, T. L. (1992). Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. *Journal of Clinical Microbiology, 30*, 2084–2087.

Kumar, S., Tamura, K., & Nei, M. (2004). MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics, 5*, 150–163.

Lai, M. (1976). Bacterial canker of bell pepper caused by *Curtobacterium michiganensis*. *Plant Disease Reporter, 60*, 339–342.

Latin, R., Tikhonova, I., & Rane, K. (1995). First report of bacterial canker of pepper in Indiana. *Plant Disease, 79*(8), 860.

Lee, I. M., Bartoszyk, I. M., Gundersen, D. E., Mogen, B., & Davis, R. E. (1997a). Nested PCR for ultrasensitive detection of the potato ring rot bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*. *Applied and Environmental Microbiology, 63*(7), 2625–2630.

Lee, I. M., Bartoszyk, I. M., Gundersen-Rindal, D. E., & Davis, R. E. (1997b). Phylogeny and classification of bacteria in the genera *Clavibacter* and *Rathayibacter* on the basis of 16S rRNA gene sequence analysis. *Applied and Environmental Microbiology, 63*, 2631–2636.

Lee, S. D., Yoon, C. M., Lee, Y. K., Choi, Y. C., & Cho, Y. S. (1999). Occurrence and distribution of bacterial canker of red pepper caused by *Clavibacter michiganensis* subsp. *michignensis*. *Plant Disease and Agriculture, 5*(2), 105–110.

Lewis-Ivey, M. L., & Miller, S. A. (2000). First report of bacterial canker of pepper in Ohio. *Plant Disease, 84*(7), 810–811.

Louws, F. J., Bell, J., Medina-Mora, C. M., Smart, C. D., Opgenorth, D., Ishimaru, C. A., Hausbeck, M. K., de Bruijn, F. J., & Fulbright, D. W. (1998). rep-PCR-mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology, 88*, 862–868.

Medina-Mora, M., Hausbeck, M. K., & Fulbright, D. W. (2000). Rep-PCR analysis of *Clavibacter michiganensis* subsp. *michignensis* from peppers. *Phytopathology, 90*, S51.

Meletzus, D., Bermpohl, A., Dreier, J., & Eichenlaub, R. (1993). Evidence for plasmid-encoded virulence factors in the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michignensis* NCPPB382. *Journal of Bacteriology, 175*(7), 2131–2136.

Nazari, F., Niknam, G. R., Ghasemi, A., Taghavi, S. M., Momeni, H., & Torabi, S. (2007). An investigation on strains of *Clavibacter michiganensis* subsp. *michignensis* in north and North West of Iran. *Journal of Phytopathology, 155*, 563–569.

Normand, P., Cournoyer, B., Simonet, P., & Nazaret, S. (1992). Analysis of a ribosomal operon in the actinomycete Frankia. *Gene, 111*, 119–124.

Pastrik, K. H., & Rainey, F. A. (1999). Identification and differentiation of *Clavibacter michiganensis* subsp. *michignensis* by polymerase chain reaction-based techniques. *Journal of Phytopathology, 147*, 687–693.

Peng, F., Guo, Y. H., Shang, X. M., & Guo, J. H. (2005). Development of a PCR assay for the detection of *Cmm*. *Jiangsu Journal of Agricultural Society, 21*(2), 118–122.

Poysa, V. (1993). Evaluation of tomato breeding lines resistant to bacterial canker. *Canadian Journal of Plant Pathology, 15*, 301–304.

Santos, M. S., Cruz, L., Norskov, P., & Rasmussen, O. F. (1997). A rapid and sensitive detection of in tomato seeds by polymerase chain reaction. *Seed Science and Technology, 25*, 581–584.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*, 406–425.

Stork, I., Gartemann, K. H., Burger, A., & Eichenlaub, R. (2008). A family of serine proteases of *Clavibacter michiganensis* subsp. *michignensis*: *chp*C plays a role in colonization of the host plant tomato. *Molecular Plant Pathology, 9*(5), 599–608.

Suzuki, K., Sasaki, J., Uramoto, M., Nakase, T., & Komagata, K. (1996). *Agromyces mediolanus* sp. nov., nom. rev., comb. nov., a species for "*Corynebacterium mediolanum*" Mamoli 1939 and for some aniline-assimilating bacteria with contain 2,4-diaminobutyric acid in the cell wall peptidoglycan. *International Journal of Systemic Bacteriology, 46*(1), 88–93.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Reserch, 22*, 4673–4680.

Volcani, Z., Zutra, D., & Cohn, R. (1970). A new leaf and fruit spot disease of pepper caused by *Corynebacterium michiganense*. *Plant Disese Reporter, 54*, 804–806.

Waleron, M., Waleron, K., Kamasa, J., Przewodowski, W., & Lojkowska, E. (2011). Polymorphism analysis of housekeeping genes for identification and differentiation of *Clavibacter michiganensis* subspecies. *European Journal of Plant Pathology, 131*(2), 341–354.