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Characterization of a *Clavibacter michiganensis* subsp. *michiganensis* population in Israel

Frida Kleitman · Isaac Barash · Annette Burger · Naim Iraki · Yunis Falah · Guido Sessa · Dan Weinthal · Laura Chalupowicz · Karl-Heinz Gartemann · Rudolf Eichenlaub · Shulamit Manulis-Sasson

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Abstract *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) strains, collected during the last decade from different locations in Israel, were analyzed by macrorestriction pulsed-field gel electrophoresis (PFGE). Fifty-eight strains from Israel and 18 from other sources were differentiated into 11 haplotypes with either *Vsp*I or *Dra*I restriction enzymes. The strains from Israel formed four distinct groups among which groups A (16 strains) and B (32 strains) constituted the major clusters. These two groups originated from the Besor region, which is the main area for growing tomatoes under cover. Rep-PCR, with either ERIC or BOX primers, confirmed results obtained by PFGE. PCR with primers based on three genes – *ppaA*, *chpC* and *tomA* – that spanned the pathogenicity island of the reference strain NCPPB382, produced the expected products with the tested pathogenic strains. Plasmid analysis of representative strains revealed different profiles of one or two plasmids. However all the strains, including five non-pathogenic ones, reacted positively in PCR with primers based on *celA* gene, which resides on the plasmid pCM1 of NCPPB382. Southern hybridization of total DNA with a 3.2-kb *Bgl*II-fragment of pCM1 containing the *celA* gene was positive when carried out with 31 strains, but the size of the reacting band was not always the same as that of pCM1, suggesting that the plasmids carrying *celA* may differ in size. Comparison between the colonization rates of strain *Cmm*42 (group A) and of *Cmm*32 (group B) did not show any significant differences. The high diversity of the *Cmm* strains, on the one hand, and the presence of two persistent groups in the Besor region, on the other hand, suggests that the primary inoculum originated each year from residual plants in the soil rather than from infested seeds, in spite of extensive control measures taken by the growers in this area.

F. Kleitman · D. Weinthal · L. Chalupowicz · S. Manulis-Sasson (✉)
Department of Plant Pathology and Weed Research, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel
e-mail: shulam@volcani.agri.gov.il

I. Barash · G. Sessa · D. Weinthal · L. Chalupowicz
Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel

A. Burger · K.-H. Gartemann · R. Eichenlaub
Fakultät für Biologie, Gentechnologie/Mikrobiologie, Universität Bielefeld, D-33501 Bielefeld, Germany

N. Iraki
UNESCO Biotechnology Center, Bethlehem University, Bethlehem, Palestine Authority

Y. Falah
Agricultural Experimental Station of the Palestinian Ministry of Agriculture, Gaza, Palestine Authority

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Abbreviations

Cmm	*Clavibacter michiganensis* subsp. *michiganensis*
PFGE	pulsed-field gel electrophoresis
PAI	pathogenicity island

Introduction

The Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is the causal agent of bacterial wilt and canker of tomato (*Solanum lycopersicum)* (Strider 1969), and it is a quarantine organism under the European Union Plant Health legislation (Anonymous 1995). It is a most important bacterial disease of tomato, causing substantial economic losses in all major tomato growing areas worldwide (Davis et al. 1984; Strider 1969). At present, neither effectively resistant tomato cultivars nor effective chemical controls are available; therefore, disease prevention is achieved mainly by using certified pathogen-free seeds and transplants. However, severe outbreaks of the disease occurred in Israel during recent years in spite of extensive seed testing. The disease has been known in Israel since 1963 and has appeared sporadically since then (Volcani 1985). A severe epidemic occurred in 2000 in the southern part of the country, which is the main area for growing tomatoes under cover (G. Kritzman, ARO, Israel, personal communication). Since then, the disease has been found in most of the greenhouses in this area, including the Gaza Strip, and has also spread to tomato fields in northern locations.

In recent years substantial progress in elucidating the molecular mechanism of *Cmm* pathogenicity has been achieved (Gartemann et al. 2003), and sequencing of the genome of strain NCPPB382 has been completed (Eichenlaub, unpublished data). This strain contains two circular plasmids, pCM1 (27 kb) and pCM2 (70 kb), that carry genes essential for virulence on tomato. A large region (~129 kb) with low G + C content was detected in the chromosome. It can be divided into two regions: the *chp*-region containing several serine proteases, and the *tomA*-region containing genes encoding for proteins involved in sugar uptake and the *tomA* gene encoding for a tomatinase. This *chp/tomA* region can be considered as a 'pathogenicity island', in the light of the finding that a derivative strain of NCPPB382 that lacked most of this region was non-virulent and unable to colonize tomato effectively. The development of the wilting symptom on tomato was shown to be induced by the genes, *celA* and *pat*-1, which are located on the plasmids, pCM1 and pCM2, respectively (Jahr et al. 2000; Meletzus et al. 1993). Dreier et al. (1995, 1997) used *pat*-1 for detection of *Cmm* by Southern hybridization and PCR. However, false negative results were obtained (Alvarez and Kaneshiro 1998; Kaneshiro and Alvarez 2001) probably because of the loss of pCM2. Such a phenomenon has been observed in *Cmm*, especially when grown at temperatures of 30–32°C. Derivatives cured of the plasmids have a reduced virulence (Meletzus et al. 1993). If such plasmid curing occurred in nature also, it could cause problems in detection of *Cmm* when testing only for the presence of the plasmid pCM2 encoded *pat*-1 gene. Therefore, additional primers based on *celA* and other genes from the pathogenicity island are necessary.

The aims of the present study were to characterize the population of *Cmm* in Israel by means of DNA-based typing procedures, and to determine the presence of pathogenicity genes residing on the chromosome and plasmids.

Materials and methods

Bacterial strains and growth conditions

The *Cmm* strains used in this study are listed in Table 1. The bacteria were maintained on plates containing nutrient agar (NA) (Difco, Le Pont de Claix, France) and incubated at 28°C for 48–72 h. They were stored for short periods at 4°C and for long periods at −80°C, in 40% glycerol. The strains were isolated from tomato plants showing disease symptoms and from seeds on three media; CNS, mSCM and D_2ANX, as described previously (Hadas et al. 2005). Gram staining was carried out according to Schaad et al. (2001), and ELISA was carried out with antibodies purchased from Neogen Europe Ltd., Scotland, UK.

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

Strain number	Strain designation	Origin	Isolation date	Host/cultivar	Source	PFGE group
10	4yathed	Yated	1998	Tomato plant	E. Hadar	A
11	6yathed	Yated	1998	Tomato plant	E. Hadar	A
15	CBM-3	Prigan	2000	Tomato plant	E. Hadar	A
16	CBM-7	Prigan	2000	Tomato plant	E. Hadar	A
17	CBM-8	Prigan	2000	Tomato plant	E. Hadar	A
18	Z-1	Zipori	1997	Tomato plant	D. Zutra	C
19	Z-2	Zipori	1997	Tomato plant	D. Zutra	C
24	Z-7	Zipori	1997	Tomato plant	D. Zutra	C
25	Z-8	Zipori	1997	Tomato fruit	D. Zutra	C
29	Cmm-34	Gush Katif (Gaza Strip)	1994	Tomato seeds	R. Hadas	L
30	602/8	Ahitov	1996	Tomato fruit	R. Hadas	M
31	3699/2	P. Rafiha (Gaza Strip)	2000	Tomato plant	R. Hadas	B
32	189/1-1	Talmi Eliyahu	2001	Plant cv. 189	S. Manulis	B
33	189/1-2	Talmi Eliyahu	2001	Plant cv. 189	S. Manulis	B
34	189/2-2	Talmi Eliyahu	2001	Plant cv. 189	S. Manulis	B
38	189/5-1	Talmi Eliyahu	2001	Plant cv. 189	S. Manulis	B
39	189/5-2	Talmi Eliyahu	2001	Plant cv. 189	S. Manulis	B
40	189/3-1	Talmi Eliyahu	2001	Plant cv. 189	S. Manulis	B
42	189/6-1	Prigan	2001	Plant cv. 189	S. Manulis	A
44	189/7-1	Prigan	2001	Plant cv. 189	S. Manulis	A
46	1402/81	Emi'oz	2001	Plant cv. 1402	S. Manulis	B
48	189/9-1	Prigan	2001	Plant cv. 189	S. Manulis	A
50	189/10-1	Prigan	2001	Plant cv. 189	S. Manulis	A
52	189/11-1	Prigan	2001	Plant cv. 189	S. Manulis	A
54	13-1	Yesha	2001	Plant cv. 189	S. Manulis	B
56	1309/14-1	Gani Tal (Gaza Strip)	2001	Plant Cherri 1309	S. Manulis	B
58	189-12-1	Prigan	2001	Plant cv. 189	S. Manulis	A
60	189-18-1	Talmi Eliyahu	2001	Plant cv. 189	S. Manulis	B
62	Cmm-31	Gush Katif (Gaza Strip)	1994	Tomato plant	R. Hadas	N
63	Cmm-43	Bikat Hayarden	1996	Tomato plant	R. Hadas	O
64	3700/2	Gush Katif (Gaza Strip)	2000	Tomato plant	R. Hadas	B
65	3713/2	Prigan	2000	Tomato plant	R. Hadas	A
67	CMM19-8/2	Yesha	2002	Fruit cv. 189	S. Manulis	B
72	20-1	Emi'oz	2002	Plant cv. 189	S. Manulis	B
79	23-2/1	Prigan	2002	Plant cv. 189	S. Manulis	A
102	189-4	Emi'oz	2002	Fruit cv. 189	S. Manulis	B
103	189-6	Emi'oz	2002	Fruit cv. 189	S. Manulis	B
104	189-11	Emi'oz	2002	Plant cv. 189	S. Manulis	B
109	492/4	Emi'oz	2003	Cheri 492	S. Manulis	B
110	189/10	Mivthaim	2003	Plant cv. 189	S. Manulis	B
112	870/10	Mivthaim	2003	Plant cv. 870	S. Manulis	B
113	870/12	Mivthaim	2003	Plant cv. 870	S. Manulis	B
116	Yt-1	Yated	2003	Plant cv. 189	S. Manulis	A
117	Yt-2	Yated	2003	Plant cv. 189	S. Manulis	A
120	Mvt-1	Mivthaim	2003	Plant cv. 189	S. Manulis	B
136	Ohd-870	Ohad	2004	Plant cv. 870	S. Manulis	B
139	Te-11-41-1	Talmi Eliyahu	2004	Plant cv.11–41	S. Manulis	B
140	Te-11-41-2	Talmi Eliyahu	2004	Plant cv.11–41	S. Manulis	B
142	Te870-2	Talmi Eliyahu	2005	Plant cv. 870	S. Manulis	B
147	AM1335-2	Emi'oz	2005	Cheri 1335	S. Manulis	B
148	Am-522-A	Emi'oz	2005	Plant cv. 522	S. Manulis	B

Table 1 (continued)

Strain number	Strain designation	Origin	Isolation date	Host/cultivar	Source	PFGE group
150	AA189-1	Avni Eithan	2005	Plant cv. 189	S. Manulis	D
151	AA189-2	Avni Eithan	2005	Plant cv. 189	S. Manulis	D
162	Aza2-3	Gaza Strip	2006	Plant cv. 593	F. Younis	B
164	Aza8-2	Gaza Strip	2006	Plant cv. 593	F. Younis	B
168	Cmm870-R	Talmi Eliyahu	2006	Roots cv. 870	S. Manulis	B
174	Cmm-Ch	Emi'oz	2006	Cheri 1335	S. Manulis	B
179	KB1912/3	Kadesh Barnea	2007	Plant cv. 1912	S. Manulis	A
1	CMM	USA		Tomato	S. Miller	E
2	C21	USA		Tomato	S. Miller	E
3	A300	USA		Tomato	S. Miller	G
4	E3	USA		Tomato	S. Miller	E
5	E7	USA		Tomato	S. Miller	E
6	E10	USA		Tomato	S. Miller	E
382	NCPPB382	UK	1953		R. Eichelaub	F
100	*Cmm*100	Derivative of NCPPB382 without pCM1 and pCM2			R. Eichelaub	F
101	*Cmm*101	Derivative of NCPPB382 without pCM2			R. Eichelaub	F
82	DR60-R1/1	USA			ISHI	D
86	Fr	France			ISHI	E
88	IPO-150	The Netherlands			ISHI	G
89	542	USA			ISHI	D
127	4775	Michigan			A. Alvarez	H
128	4829	Ohio			A. Alvarez	H
130	4866	Gilroy CA			A. Alvarez	D
131	4598	WA State			A. Alvarez	I
132	2701	N. Carolina			A. Alvarez	J
133	4592	WA State			A. Alvarez	I
134	4011	Ohio			A. Alvarez	K

Pathogenicity and colonization tests

All the strains were subjected to pathogenicity tests on tomato seedlings, *Solanum lycopersicum* cv. 189. The stems of 4 week-old plants were punctured three times with a needle that had been dipped in a suspension of bacteria containing 10^8 cells ml^{-1}. The inoculum was prepared from a culture grown overnight on NA and suspended in distilled water. The plants were maintained at 29°C, and symptoms were recorded after 7–21 days. For each strain, five plants were inoculated and the test was conducted twice.

The colonization rates of two strains, *Cmm*42 (group A) and *Cmm*32 (group B), on tomato plants, *S. lycopersicum* cv. 189, were compared. The plants were inoculated by puncturing the stem at the axil of the first leaf three times with a needle, and applying 10 µl of *Cmm* suspension containing 10^6 cells ml^{-1}. The colonization of the stem was determined at 0, 7, 14, 21 and 28 days after inoculation, and at distances of 5, 10, 20, 30, 40, 50, 60 cm from the infection site. One centimeter of tissue was removed, macerated in 2 ml of sterile water, subjected to tenfold serial dilution and plated on CNS plates. After incubation at 28°C for 5–6 days the colonies were counted. Three plants for each time point were sampled in each of two independent experiments.

DNA isolation and Southern hybridization

Total DNA from selected *Cmm* strains (16, 18, 19, 24, 25, 29, 31, 101, 382, 58, 62, 63, 64, 65, Table 1) was isolated according to Hopwood et al. (1985). Plasmid

DNA of *Cmm* was isolated according to Birnboim and Doly (1979). Labelling the DNA and Southern hybridization was performed with DIG DNA Labelling kit from Roche Diagnostics (Mannheim, Germany), according to the manufacturer's instructions. The DNA probes for detection of *pat*-1 and *celA* were the 3.75-kb *Bgl*II-fragment of pCM2 (*pat*-1) (Dreier et al. 1997) and the 3.2 kb *Bgl*II-fragment of pCM1 (*celA*) (Jahr et al. 2000), respectively. Total DNA for Southern hybridization was restricted with *Bgl*II.

Cmm plasmids were separated by electrophoresis on 0.8% agarose gel at 80–100 V for 4 h. The gels were subsequently stained in a EtBr solution (1 μg ml^{-1}).

Polymerase chain reaction

The primers used in this study are listed in Table 2. Primers for amplification of *celA*, *pat*-1, *repA*, *parA*, *ppaA*, *chpC* and *tomA* were based on the sequence of the NCPPB382 strain. DNA fragments were amplified with *Taq* polymerase (Super-Therm Polymerase JMR-801; Roche, Mannheim, Germany), and synthetic oligonucleotides were synthesized according to specification by Sigma-Aldrich, Rehovot, Israel. The amplified products were subjected to electrophoresis on 1.2% agarose gel, and were stained with ethidium bromide.

DNA-based typing procedures

The protocol for pulsed-field gel electrophoresis (PFGE) was adapted from Ribot et al. (2001). Cultures were grown overnight in LB broth at 28°C. One milliliter of each culture was transferred into an Eppendorf tube and centrifuged at 14,000 rpm for 1 min. The pellet was resuspended in 1 ml of suspension buffer (100 mM Tris–HCl pH 7.5, 100 mM EDTA, pH 8). Each cell suspension was adjusted to an optical density of OD_{600}=1.33, and 250 μl of this suspension were mixed with 250 μl of

Table 2 Sequence of primers used in this study

Primer	Sequence 5′ to 3′	Used for amplification of
PFC3	GGTACGAAGTTCGAGACGAC	*celA* CB domain
PFC5	TGTAGCGGTGAGTCGTGGTGA	
P5	GCGAATAAGCCCATATCAA	*pat*-1
P6	CGTCAGGAGGTCGCTAATA	
repA-pCM1-F	GTCAGAACGTGAGCAAGCG	*repA* of pCM1
repA-pCM1-R	GTCATCAACCAGGAGCACC	
repA-pCM2-F	CCTGTGCTTCGACTTCGAC	*repA* of pCM2
repA-pCM2-R	GGAACGACGTCAGGATGG	
parA-pCM2-F	CGTATCGAAGACGACTACC	*parA*
parA-pCM2-R	GGTATATGCGGCCAAGACC	
ppaA-F	CATGATATTGGTGGGGAAAG	*ppaA*
ppaA-R	CCCCGTCTTTGCAAGACC	
chpC-F	GCTCTTGGGCTAATGGCCG	*chpC*
chpC-R	GTCAGTTGTGGAAGATGCTG	
tomA-F	CGAACTCGACCAGGTTCTCG	*tomA*
tomA-R	GGTCTCACGATCGGATCC	
CM3	CCTCGTGAGTGCCGGGAACGTATCC	(Santos et al. 1997)
CM4	CCACGGTGGTTGATGCTCGCGAGAT	
ERIC-1R	ATGTAAGCTCCTGGGGATTCAC	rep-PCR (Versalovic et al. 1991)
ERIC-2	AAGTAAGTGACTGGGGTGAGCG	
BOX A1R	CTACGGCAAGGCGACGCTGACG	rep-PCR (Versalovic et al. 1994)
CMR16F1	GTGATGTCAGAGCTTGCTCTGGCGGAT	16S RNA (Lee et al. 1997)
CMR16R1	GTACGGCTACCTTGTTACGACTTAGT	
CMR16F2	CCCCGACTCTGGGATAACTGCTA	
CMR16R2	CGGTTAGGCCACTGGCTTCGGGTGTTACCGA	

1% SeaKem Gold agarose (SKG) (F50152; FMC, Rockland, ME, USA) in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) at 50°C. The agarose cell suspension mixture was dispensed immediately into the wells of reusable plug molds (Bio-Rad Laboratories, Hercules, CA) and allowed to solidify at room temperature for 15 min. The agarose plugs were then cut into three pieces with a sterile spatula, and one piece was transferred into a 2-ml Eppendorf tube containing 1 ml of 2 mg lysozyme (Sigma-Aldrich, Switzerland) in suspension buffer. After overnight incubation at 37°C in an orbital shaker the lysozyme solution was removed and 1 ml of lysis solution containing 1 ml of NDS buffer (0.5 M EDTA, pH 8, and 1% sarcosyl) and 50 µl of 20 mg ml^{-1} proteinase K (Sigma-Aldrich) was added.

The lysis was allowed to proceed overnight at 50°C, with agitation in an orbital shaker. The plugs were then washed twice in double-distilled water, and four times in TE buffer at 50°C. The water and TE buffer were pre-warmed to 50°C before each washing step. The plugs were either used for restriction digestion or stored at 4°C for up to 6 months. Restriction digestion was applied to a 3 mm-wide slice from each plug, with 30 units of either *Vsp*I or *Dra*I restriction enzymes in 150 µl buffer for 16 h at 37°C. The plug slices were loaded into appropriate wells of a 1% SKG agarose gel prepared in 0.5×TBE (Tris–borate-EDTA buffer, Cat No.T4415; Sigma). Lambda concatemer (Cat. 1703635; BioRad Laboratories, Hercules, CA, USA) was used as a size marker in the range of 50 to 1000 kb. Electrophoresis was performed in a CHEF-DR III system (BioRad). The electrophoresis conditions were: an initial switch time of 0.2 s and a final switch time of 54.2 s (with a gradient of 6 V/cm and an angle of 120° at 14°C) for 22 h. After electrophoresis, the gels were stained with ethiduim bromide for further analysis. The PFGE patterns were analyzed with the Molecular Analysts Fingerprinting II software package, version 3 (BioRad). Matching and dendrogram UPGMA analysis of the PFGE patterns were performed with the Dice coefficient, with a 1.0–1.5% tolerance window.

Rep-PCR was performed according to Louws et al. (1998), with primers ERIC-1R, ERIC-2 and BOX A1R (Table 2).

Results

Virulence of *Cmm* strains

Fifty-eight *Cmm* strains were collected from various locations in Israel during 1994 to 2007 (Table 1); they were isolated from various tomato cultivars and from different parts of the plant (stem, petiole, fruit or seed). Eighteen strains were obtained from other sources (Table 1). All the strains were subjected to pathogenicity tests on tomato and were found to induce disease symptoms (Table 3). However, certain strains (29, 30, 33, 62 and 63) were non-pathogenic. These strains were confirmed as *Cmm* by PCR with primers based on the 16S ribosomal RNA (Table 2), Gram staining and ELISA.

Genomic fingerprints of *Cmm* strains

PFGE of macrorestricted genomic DNA with *Vsp*I or *Dra*I produced several fragments suitable for fingerprint analysis. All the pathogenic strains listed in Table 1 were differentiated into 11 haplotypes by either *Vsp*I or *Dra*I (Fig. 1; Table 3). The Israeli strains were separated into four groups: group A contained 16 strains; B, 32; C, 4; and D, 2 (Table 3). Whereas the similarity among the strains within each group was 92 to 100%, the similarity between the two major Israeli groups, namely, A & B, was 58%. Strains of group C were isolated from tomato plants from a single location in northern Israel in 1997, and since then have not been detected in any other sites. Group D was the only one that contained both Israeli strains and strains isolated from the USA (Table 3). Four of the five nonpathogenic strains – 29, 30, 62 and 63 – were not grouped into any of the 11 haplotypes and were assigned to different groups. Interestingly, the non-pathogenic strain 33 was clustered into group B.

Rep-PCR carried out with either ERIC or BOX primers with two-four representative strains from each of the Israeli PFGE groups, confirmed the similarity among the strains within each group, as obtained for PFGE (Fig. 2).

PCR with primers based on three genes spanning the PAI of NCPPB382 were carried out with 20 representative strains. All the tested pathogenic strains generated PCR products of 587, 638 and 528 bp with

Table 3 Characterization of *Clavibacter michiganensis* subsp. *michiganensis* strains

Character	Strain No.
Pathogenicity[a]	All the strains listed in Table 1 except **29**,**30**,**33**,**62**,**63**
PFGE group[b]	
A	10,11,15,16,17,42,44,48,50,52,58,65,79,116,117,179
B	31,32,**33**,34,38,39,40,46,54,56,60,64,67,72,102,103,104,109,110, 112,113,120,136,139,140,142,147,148,162,164,168,174
C	18,19,24,25
D	150,151,82,89,130
E	1,2,4,5,6,86
F	382
G	3,88
H	127,128
I	131,133
J	132
K	134
L	29
M	30
N	62
O	63
PCR with PAI-based primers[c]	
ppaA	15,18,25,32,42,46,112,116,150,82,130,2,3,6,382, (**33**,**29**,**30**,**62**,**63**)
chpC	15,18,25,32,**33**,42,46,112,116,150,82,130,2,3,6,382 (**29**,**30**,**62**,**63**)
tomA	15,18,25,**29**,**30**,**33**,32,42,46,**62**,**63**,112,116,150,82,130,2,3,6,382
PCR with plasmids-based primers	
celA	All the strains listed in Table 1
pat-1	All the strains listed in Table 1 except **29**,**30**
Hybridization with[d]	
celA	10,11,15,16,17,42,44,48,50,52,58,65,31,32,34,38,39,40,46,54,56, 64,18,19,24,25,382,**29**,**30**,**33**,**62**,**63**
pat-1	10,11,15,16,42,44,48,50,52,58,31,32,34,38,39,40,46,54,56, 64,18,19,24,25,382,**33**,**62**,**63**, (**29**,**30**)
PCR with[e]	
repA	382,42,116,3,89,127,131,132,134,**63**, (15,32,46,112,150,130,82,18,25,2,6,131,**62**)
parA	382, (42,116,3,89,127,131,132,134,15,32,46,112,150,130,82,18,25,2,6,131,**62**,**63**)

[a] Pathogenicity test on tomato plants was carried out as described in Materials and methods. Non-pathogenic strains are printed in bold letters.

[b] PFGE groups were determined by macrorestriction with *Vsp*I and *Dra*I.

[c] PAI-pathogenicity island. Numbers in parentheses were negative in PCR.

[d] Hybridization with a 3.2-kb *Bgl*II-fragment of pCM1 and a 3.75 kb *Bgl*II-fragment of pCM2 on total DNA. Numbers in parentheses were negative.

[e] *repA* of pCM1 and *parA* of pCM2. Numbers in parentheses were negative in PCR.

ppaA, *chpC* and *tomA* primers, respectively (Table 3). In contrast, the non-pathogenic strains generated a PCR product only with *tomA*, with the exception of strain 33, which reacted positively also with *chpC* primers. These results might suggest that the loss in pathogenicity was associated with deletions in the chromosomal PAI.

Presence of plasmids and the pathogenicity determinants, *celA* and *pat-1*, in the *Cmm* strains

Plasmid profiles of 14 representative strains revealed different patterns with one or two plasmids (Fig. 3). All strains tested, including the five non-pathogenic ones, were positive in PCR with *celA* primers

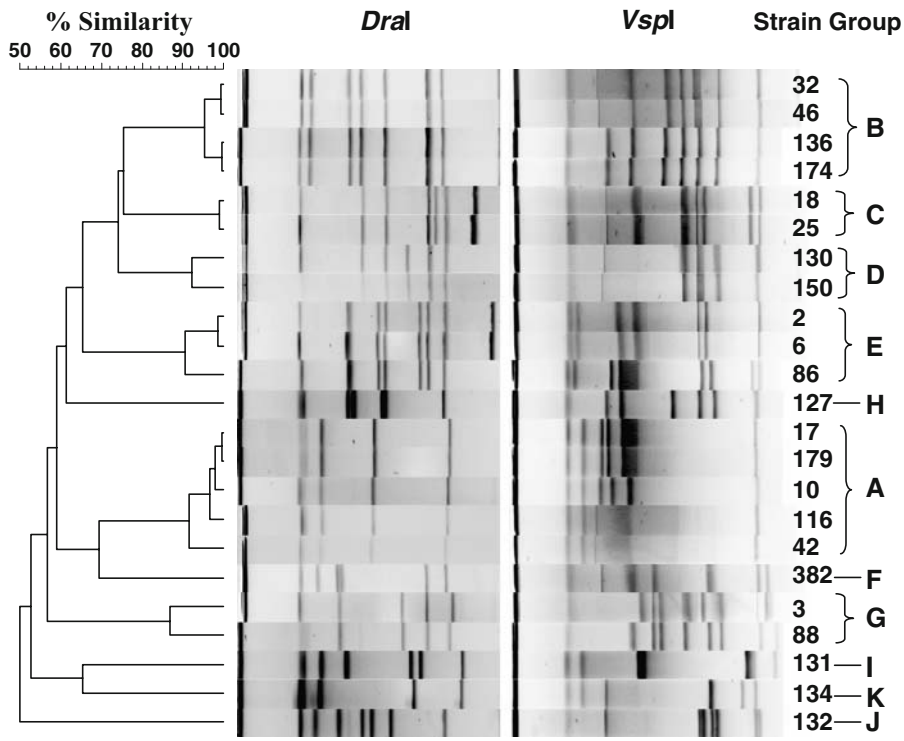

Fig. 1 PFGE patterns of *Clavibacter michiganensis* subsp. *michiganensis* strains after macrorestriction with *Vsp*I and *Dra*I. PFGE conditions were as described in Materials and methods. Unweighted average linkage dendrogram of the cluster analysis on the basis of PFGE patterns with the two restriction enzymes is shown on the left side. Numbers on the horizontal axis indicate percentage similarity as determined by Dice coefficient. Columns on the right-hand side indicate the strain number and the PFGE-group

(Table 3). Strain *Cmm*100, a derivative of NCP PB382 lacking plasmids pCM1 and pCM2, was used as a negative control. Southern hybridization with a 3.2-kb *Bgl*II-fragment of pCM1 containing the *celA* gene was positive when carried out with 31 strains (Table 3). However, the size of the hybridizing band was not always similar to that of pCM1 from strain NCPPB382, suggesting that the plasmids carrying the *celA* may differ (results not shown). In addition, PCR with primers based on *repA* of pCM1 produced the expected product of 562 bp with only nine of 21 pathogenic strains (Table 3) suggesting the possible presence of different replicons. When all the *Cmm* strains were analyzed for the presence of the pCM2-encoded pathogenicity gene *pat*-1 using PCR with suitable primers (Table 2), only two non-pathogenic strains (29 and 30) did not produce the expected product of 614 bp (Table 3). These results suggested that *pat*-1 was widespread among *Cmm* strains. Hybridization with a 3.75-kb *Bgl*II-fragment of pCM2 was correlated with the PCR results (Table 3). Interestingly, PCR with *parA* of pCM2 did not produce the expected 708-bp product with any of the tested strains, suggesting that *pat*-1 might reside either on other plasmids or on the chromosome.

Colonization of tomato plants

In order to determine whether the two major groups A and B might differ in their virulence or their colonization rates, two representative strains, namely, *Cmm*42 (group A) and *Cmm*32 (group B), were compared. The results presented in Fig. 4 showed no significant differences between the colonization rates of these strains at various time points and at several different distances from the infection site. At 7 days post-infection (dpi), a large population – of 10^8 CFU g^{-1} tissues – was observed at the infection site, and 10^5–10^6 CFU g^{-1} at 5 cm from the infection site. Disease symptoms appeared after 14 dpi in plants infected with either strain and their populations reached 10^9–10^{10} CFU g^{-1} at 10 cm and 10^8 CFU g^{-1} at 20 cm. Large populations of *Cmm* could be detected after 21 and 28 days at 40, 50 and 60 cm from the infection site, which suggests that *Cmm* spread rapidly through the xylem vessels.

Discussion

Pulse field analysis of *Cmm* strains obtained from different geographic locations in Israel and other

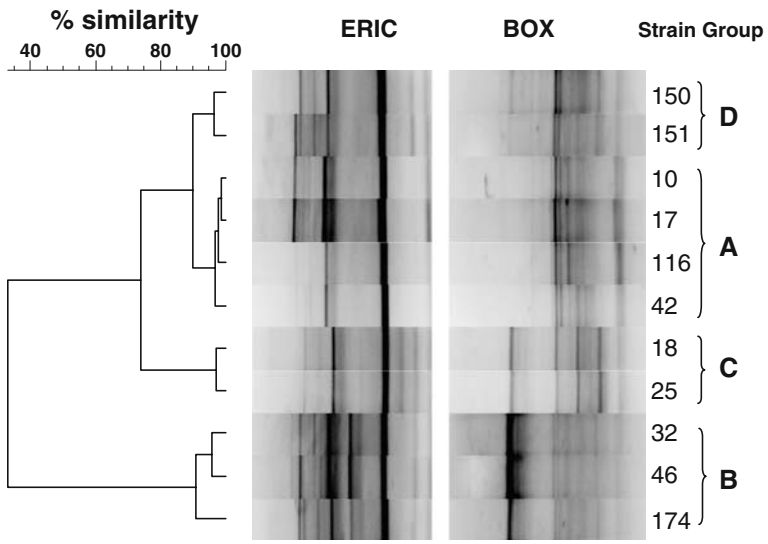

Fig. 2 rep-PCR analysis of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated in Israel. Conditions were as described in Materials and methods. Numbers on the horizontal axis indicate percentage similarity as determined by Dice coefficient. Columns on the right-hand side indicate the strain number and the group according to PFGE

countries revealed high diversity, as expressed in the presence of 11 haplotypes, with a lowest similarity of 50% (Fig. 1). The Israeli strains, which were collected during more than 10 years, were divided into four groups. The two major groups – A and B – originated from the main area for growing tomatoes undercover, i.e., the Besor region (Fig. 5a,b). Strains from group A were first isolated in 1998 and since then were repeatedly found in three different sites of this region. Strains from group B were first isolated in 2000 and since then have been repeatedly isolated from six different sites in the same region. It is noteworthy that the areas in which the two groups were established are separated by only 10 to 15 km. Recently, strains from group A were detected, for the first time, in a new site about 50 km south of the Besor region (Fig. 5a). Presumably they were transferred in infested plant residues.

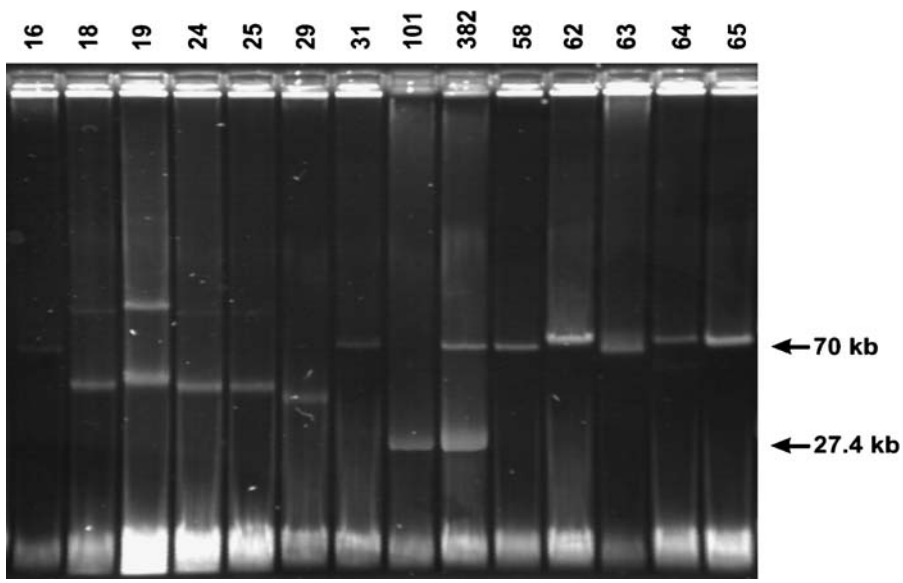

Fig. 3 Plasmids profile of representative *Clavibacter michiganensis* subsp. *michiganensis* strains. Conditions were as described in Materials and methods. Arrows and sizes refer to the plasmids pCM1 and pCM2 of strain NCPPB382

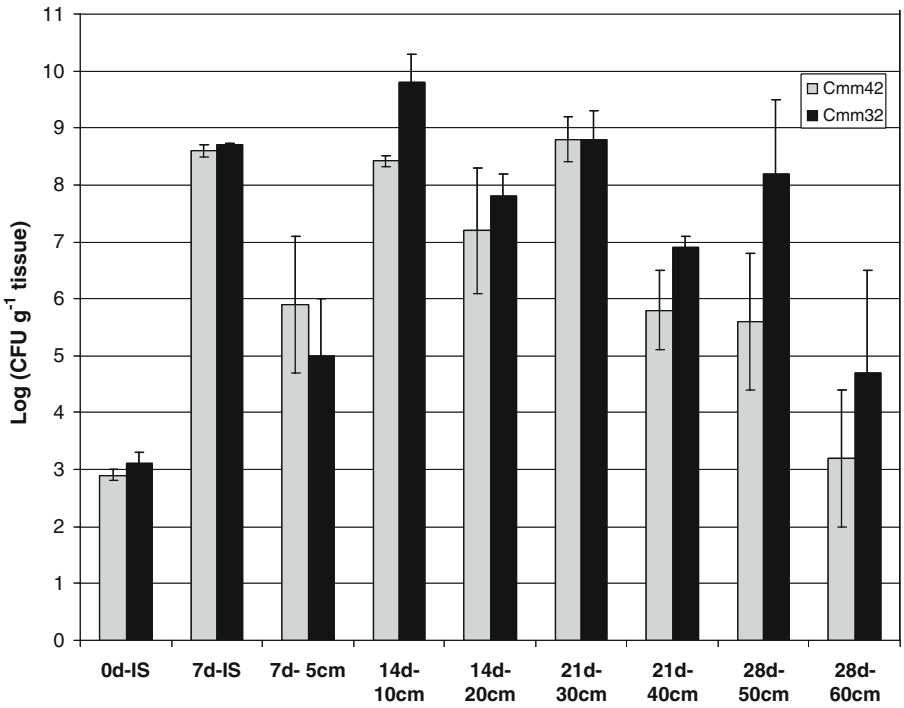

Fig. 4 Comparison between growth rates of strains *Cmm*42 (group A) and *Cmm*32 (group B), as a function of time and distance from the infection site. Results are an average of three plants for each time point with standard deviation. IS – infection site, d – days post-infection

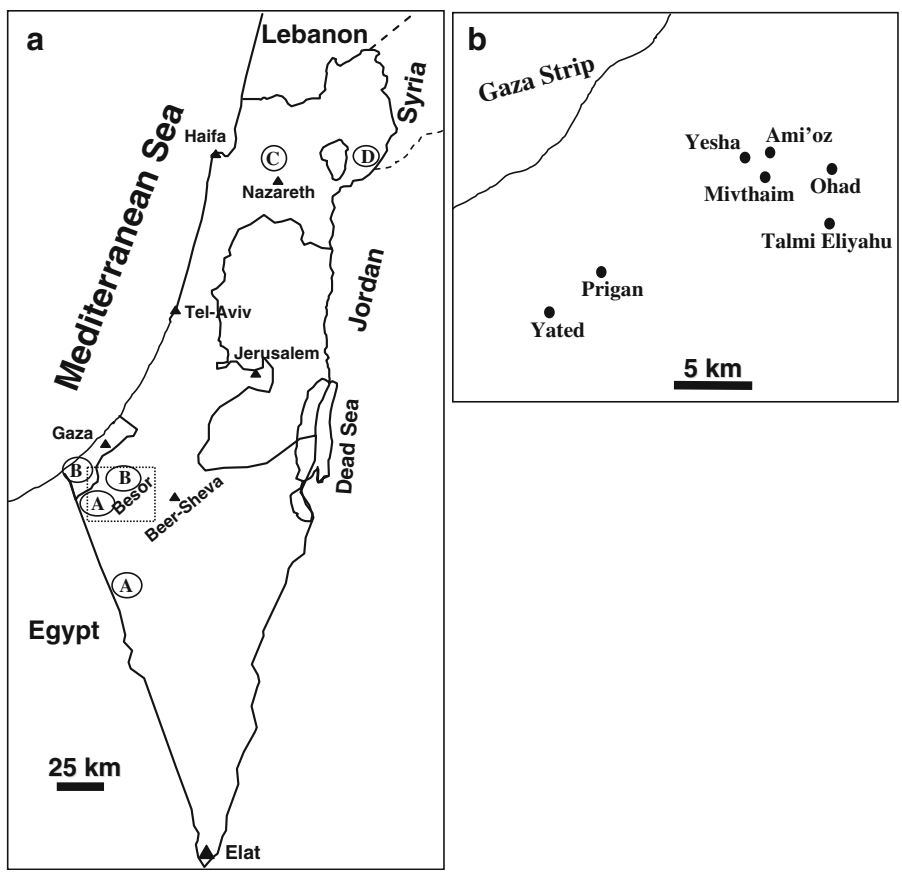

Fig. 5 Distribution of *Cmm* groups as determined by PFGE in Israel. (**a**) Schematic map of Israel illustrating the four different groups (letters in circle).(**b**) Enlargement of the Besor area (the dotted square in (**a**) indicating the locations in which strains of groups *A* and *B* were isolated

The repeated isolation of strains from groups A and B suggests that they persist in this area in spite of extensive control measures taken by the growers to eliminate the inoculum. Tomatoes grown in greenhouses and under net are usually planted twice a year (autumn and spring) with transplants originated mainly from the same area. The recommendations for the tomato growers are to discard the infected plants and the trellising materials, and to disinfect the soil by formalin treatment. However, these control measures might not be sufficient to eradicate the pathogen. Although *Cmm* does not survive well in the soil, it can survive on plant debris for several years (Fatmi and Schaad 2002; Gleason et al. 1993). The possibility that the pathogen can reside on alternative hosts or on volunteer wild plants cannot be excluded (Chang et al. 1992; Tsiantos 1987), but this might not explain the severe outbreaks of the disease that occurred during recent years.

Cmm generally enters a production area mainly through infested seed or latently infected tomato transplants (Gleason et al. 1993). The source of inoculum in the Besor region might have originated from contaminated tomato seeds or seedlings. It is surprising that strains representing other groups have not been isolated in these areas during the long period of more than 10 years. It could be assumed that the residual infected tomato plants, which serve as the major inoculum in each growing season, are heavily infested by the dominant strains of either group A or group B, which are equivalent in their colonization rates and virulence. Moreover, it appears that new strains, that could have arrived via contaminated tomato seeds or transplants, apparently could not be established in this area. In addition, the lack of crop rotation is most likely to be responsible for the accumulation of *Cmm* inoculum in this region.

The high heterogeneity among the *Cmm* strains, as determined by PFGE, contrasts with the relative homogeneity of strains of the related pathogen, *Clavibacter michiganensis* subsp. *sepedonicus* (Brown et al. 2002). It is possible that the observed difference was enhanced by the use of different restriction enzymes. Sequence analysis of *Cmm*382 showed that most of the *Vsp*I/*Dra*I sites are located in the low-GC regions, which, except for the ribosomal RNA operons, might exhibit higher genome variability among the strains. However, the Israeli *Cmm* strains were highly homogenous (> 92% similarity) within each of the four groups, as shown in Fig. 1. These findings were also confirmed by Rep-PCR (Fig. 2). The possibility that strains within each group are clonal remains to be determined by multi-locus sequence typing (MLST) (Achtman 2004; Maiden 2006).

PCR-based detection of three genes located on different sites of the pathogenicity island revealed that all the tested pathogenic strains were positive for the presence of *ppaA*, *chpC* and *tomA*, whereas the non-pathogenic strains lacked *ppaA*, *chpC* but not *tomA* (Table 3). These results may imply that *ppaA* and *chpC* are mandatory for virulence, as previously demonstrated for NCPPB382 (Eichenlaub, unpublished data) whereas *tomA* is not (Kaup et al. 2005). Moreover, the presence of these genes can support a reliable diagnosis of pathogenic *Cmm* strains. Interestingly, the non-pathogenic strain 33 did harbour the *chpC* but not *ppaA*, and it is the only non-pathogenic strain that was clustered into group B. This could be attributed to a small deletion of the PAI, which was not detected by PFGE.

The plasmid-borne virulence gene *celA*, originally characterized on pCM1, was detected by PCR in all the pathogenic and non-pathogenic strains, whereas the *pat-1* of pCM2 was similarly detected in all the strains, with the exception of two non-pathogenic ones (Table 3). Hybridization with total DNA of representative strains confirmed the PCR results (Table 3). These data suggest that the presence of *celA* and *pat-1* is conserved in the *Cmm* population and that these genes may reside either on plasmids or on the chromosome. The plasmids that harbour these two genes may not necessarily be identical to the originally characterized pCM1 or pCM2 (Jahr et al. 2000; Meletzus et al. 1993). This conclusion derives from the observation that the plasmid profiles of some of the tested strains varied in size and, more importantly, PCR with primers based on the *repA* of pCM1 reacted with only a limited number of the strains, and primers based on *parA* of pCM2 reacted only with the strain NCPPB 382. It is noteworthy that the two genes that were originally described in NCPPB 382, which was isolated in 1954 in the UK, were present in most of the recently isolated pathogenic strains, and were still surrounded by conserved nucleotide sequences.

The diversity of the *Cmm* stains, as revealed by PFGE, and its reproducibility, suggests that it is the

preferred method for DNA typing and is suitable for epidemiological studies. In contrast, a limited DNA polymorphism was revealed by rep-PCR fingerprinting of *Cmm* strains (Louws et al. 1998). The different groups obtained in the present study could not be correlated with plasmid profiles, which suggest that it cannot be used for diagnostic purposes and, furthermore, indicates that the plasmids and the chromosome evolved independently. Nevertheless, PCR with primers *celA* or *pat*-1, from plasmids pCM1 and pCM2, respectively, were strongly correlated with pathogenic *Cmm* strains, and no false negative results were obtained. However, since several non-pathogenic strains reacted positively with *celA* and *pat*-1 primers in PCR, causing false positive results, it is suggested that multiplex PCR, conducted with primers based on genes from the PAI like *chpC* or *ppaA*, could be useful for diagnostic purposes.

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