Colonization and Movement of GFP-Labeled *Clavibacter michiganensis* subsp. *michiganensis* During Tomato Infection

L. Chalupowicz, E.-M. Zeller mann, M. Fluegel, O. Dror, R. Eichenlaub, K.-H. Gartemann, A. Savidor, G. Sessa, N. Iraki, I. Barash, and S. Manulis-Sasson

First, fourth, and eleventh authors: Department of Plant Pathology and Weed Research, ARO, the Volcani Center, Bet Dagan, Israel; second, third, fifth, and sixth authors: Department of Microbiology/Genetechnology, Faculty of Biology, University of Bielefeld, Bielefeld, Germany; seventh, eighth, and tenth authors: Department of Molecular Biology and Ecology of Plants, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel; and ninth author: UNESCO Biotechnology Center, Bethlehem University, Bethlehem, Palestinian Authority. Accepted for publication 18 August 2011.

ABSTRACT

Chalupowicz, L., Zeller mann, E.-M., Fluegel, M., Dror, O., Eichenlaub, R., Gartemann, K.-H., Savidor, A., Sessa, G., Iraki, N., Barash, I., and Manulis-Sasson, S. 2012. Colonization and movement of GFP-labeled *Clavibacter michiganensis* subsp. *michiganensis* during tomato infection. *Phytopathology* 102:23-31.

The vascular pathogen *Clavibacter michiganensis* subsp. *michiganensis* is responsible for bacterial wilt and canker of tomato. Pathogenicity of this bacterium is dependent on plasmid-borne virulence factors and serine proteases located on the chromosomal *chp/tomA* pathogenicity island (PAI). In this study, colonization patterns and movement of *C. michiganensis* subsp. *michiganensis* during tomato infection was examined using a green fluorescent protein (GFP)-labeled strain. A plasmid expressing GFP in *C. michiganensis* subsp. *michiganensis* was constructed and found to be stable in planta for at least 1 month. Confocal laser-

scanning microscopy (CLSM) of inoculated stems showed that the pathogen extensively colonizes the lumen of xylem vessels and preferentially attaches to spiral secondary wall thickening of the protoxylem. Acropetal movement of the wild-type strain *C. michiganensis* subsp. *michiganensis* NCPPB382 (*Cmm382*) in tomato resulted in an extensive systemic colonization of the whole plant reaching the apical region after 15 days, whereas *Cmm100* (lacking the plasmids pCM1 and pCM2) or *Cmm27* (lacking the *chp/tomA* PAI) remained confined to the area surrounding of the inoculation site. *Cmm382* formed biofilm-like structures composed of large bacterial aggregates on the interior of xylem walls as observed by CLSM and scanning electron microscopy. These findings suggest that virulence factors located on the *chp/tomA* PAI or the plasmids are required for effective movement of the pathogen in tomato and for the formation of cellular aggregates.

Clavibacter michiganensis subsp. *michiganensis* is the causal agent of bacterial wilt and canker of tomato. It is the most destructive bacterial disease of tomato worldwide and is subjected to strict national and international quarantines (11,12). Contaminated seed, infected plant residues in the soil, or symptomless *Solanum* spp. are common sources for *C. michiganensis* subsp. *michiganensis* inoculum (30). Bacteria enter the host plant via natural openings and wounds. *C. michiganensis* subsp. *michiganensis* colonizes primarily the xylem and leads to a systemic infection characterized by unilateral leaf wilting and canker development on the tomato stem that eventually culminates in plant death. The pathogen can survive as an effective endophyte in tomato but apparently has to establish an endophytic population of $>10^8$ CFU/g of plant tissue in order to induce disease symptoms (15).

The sequenced genome of the wild-type strain *C. michiganensis* subsp. *michiganensis* NCPPB382 (*Cmm382*) represents a single circular chromosome of 3.3 Mb with high G+C content (72.6%). It carries a pathogenicity island (PAI) of 129 kb with a lower G+C content (65.5%) designated as the *chp/tomA* region (14). This PAI can be divided into two subregions: the *chp* region, which harbors several genes encoding serine proteases, and the

tomA region, which includes *tomA*, encoding a tomatinase, as well as genes encoding proteins involved in sugar uptake and metabolism (14,19). A *chp/tomA* PAI deletion mutant (i.e., *Cmm27*) does not induce disease symptoms and colonizes the tomato xylem at a considerably lower titer than the wild type (4).

Cmm382 harbors two circular conjugative plasmids, pCM1 (27.4 kb) and pCM2 (70 kb), each of which carries a major virulence gene (24). pCM1 contains *celA*, encoding an endo- β -1,4-glucanase, and pCM2 harbors *pat-1*, encoding a putative serine protease. Both are involved in the induction of wilting in infected tomato plants (9,17). Curing *Cmm382* of either pCM1 (i.e., *Cmm102*) or pCM2 (i.e., *Cmm101*) results in a reduction of wilt symptom. The loss of both plasmids (i.e., *Cmm100*) still allows considerable endophytic growth but leads to lack of symptom induction (15,24). Additionally, *Cmm382* secretes a wide range of cell-wall-degrading enzymes (i.e., polygalacturonase, pectate lyases, xylanases, and endoglucanases) and a large group of serine proteases of different families (i.e., Chp, Ppa, and subtilase) (14). These serine proteases were induced in planta and identified in the sap of infected tomato plants, suggesting their involvement in *C. michiganensis* subsp. *michiganensis* infection (2,4) (*unpublished data*).

Development of systemic infections caused by gram-negative vascular plant-pathogenic bacteria has been previously investigated (6,28). These studies were most thoroughly performed with *Pantoea stewartii* subsp. *stewartii*, causing wilt and leaf blight in sweet corn (16,23), and *Xylella fastidiosa*, a host-specific pathogen of grape that causes leaf scorching and berry desiccation (1,27). Other vascular bacteria which have been studied include *Erwinia amylovora*, the causal agent of fire blight in apple and

Corresponding author: S. Manulis-Sasson
E-mail address: shulam@volcani.agri.gov.il

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplemental figures. Figures 2 and 7 appear in color online.

<http://dx.doi.org/10.1094/PHYTO-05-11-0135>
© 2012 The American Phytopathological Society

pear (21); *Xanthomonas campestris* pv. *campestris*; the causal agent of black rot on crucifer plants (8); and *Ralstonia solanacearum*, which causes lethal wilt on many plants (18). Common features involved in systemic development and virulence among most of these vascular pathogens are (i) formation of extensive bacterial aggregates and biofilm attached to xylem surfaces, (ii) contribution of exopolysaccharide (EPS) to vascular blockage and symptom development, and (iii) involvement of quorum-sensing (QS) in regulation of xylem colonization (6). In the case of *P. stewartii* subsp. *stewartii* and *Xylella fastidiosa*, bacterial motility was also demonstrated to play a crucial role in systemic development and virulence (16,26).

In contrast to the ample information on systemic spreading of vascular gram-negative bacteria, knowledge on the movement characteristics of gram-positive vascular bacteria is very limited. In the case of *C. michiganensis*, it could be assigned to the lack of adequate molecular tools for studying the pathogen in planta. For example, fluorescent reporters, such as the green fluorescence protein (GFP), which are commonly used to track bacteria in infected plants, could not be expressed in *C. michiganensis*. Recently, bioluminescence imaging was employed to follow *C. michiganensis* subsp. *michiganensis* infection of tomato plants (35). Although the bioluminescence technique provides sensitive detection levels, it depends on the kinetics of cellular uptake of substrates and cannot be used to follow single bacterial cells or detect them in damaged tissue or dead cells (22). To circumvent these limitations, in the present study, we utilized an enhanced (E)GFP bioreporter to examine colonization patterns and movement of *C. michiganensis* subsp. *michiganensis* in infected tomato plants. To attain this goal, an EGFP reporter that could be ex-

pressed and stably maintained in *C. michiganensis* subsp. *michiganensis* was constructed to allow a direct and precise visualization of fluorescent cells by confocal laser-scanning microscopy (CLSM). This microscopic analysis provides, for the first time, significant insights into the mode of colonization and spatial adherence preferences of *C. michiganensis* subsp. *michiganensis* during disease development. Moreover, the EGFP-labeled pathogen was utilized to test the hypothesis that virulence factors encoded in the *chp/tomA* PAI or in the plasmids are important for biofilm formation as well as for acropetal and basipetal movement within the tomato plant. Results of this study shed new light on the essential role of the systemic movement of *C. michiganensis* subsp. *michiganensis* in pathogenicity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *C. michiganensis* subsp. *michiganensis* strains used in the present study were the wild-type pathogenic strain NCPPB382 (*Cmm*382) and its derivatives, *Cmm*100 (lacking the two virulence plasmids pCM1 and pCM2), *Cmm*101 (lacking pCM2), and *Cmm*102 (lacking pCM1) (25); *Cmm*27, a deletion mutant of *Cmm*382 lacking the *chp/tomA* region (4); and *CmmchpC*⁻, a mutant in *chpC*, a gene encoding serine protease located in the *chp/tomA* PAI (29). Depending on the analysis, the *C. michiganensis* subsp. *michiganensis* strains were grown at 25°C for 48 to 72 h in Luria-Bertani (LB) broth or agar (Difco, Le Pont de Claix, France), minimal medium (M9) broth (4), or CNS broth or agar plates (20). When required, the antibiotics neomycin or chloramphenicol were added to the growth media at concentrations of 50 and 10 µg/ml, respectively.

Construction of the plasmid pK2-22. The complete coding region of *egfp* flanked by *ScaI* restriction sites was amplified using the primers EGFP-fwd (CGAAAAGTACTACCATGGT GAGCAAGGGCGAGGAGCTGTTC) and EGFP-rev (GCAAA AGTACTACAGCCAAGCTTACTTGTACAGC) and pTST101 as template. pTST101 contains a fusion of *malE* (encoding a maltose-binding protein) and *egfp*, which encodes the EGFP (31). The 755-bp polymerase chain reaction (PCR) product was cloned into pGEM-T Easy vector (Promega Corp., Madison, WI) to generate the plasmid pGemEGFP. A DNA fragment of 739-bp *ScaI* carrying the *egfp* gene from the latter plasmid was inserted into the *C. michiganensis* subsp. *michiganensis* shuttle vector pDM302 (25) by replacing the chloramphenicol resistance cassette (the 941-bp *ScaI* fragment). Afterward, the *ScaI* site in the 3' untranslated region of *egfp* was deleted by substitution of the 696 *HindIII* fragment with a *HindIII*-digested PCR fragment amplified with primers H1: GTAAGCTTGGCTGTAGTGCTC and H2: GTGAAGCTTATCGATCGAGC (plasmid pGemEGFP_H). Finally, a ribosomal-binding site was introduced by cloning annealed oligonucleotides OSpeA CTAGTCAGTACTGATGC ATCAGGCCTCAGGAGG and OSpeB CCTCCTGAGGCCTGA TGCATCAGTACTGA (ribosomal-binding site is underlined) into *SpeI/ScaI*-digested pGemEGFP_H. Transformation of *Escherichia coli* and *C. michiganensis* subsp. *michiganensis* with this plasmid (pDM302EGFP-RBS) resulted in nonfluorescent colonies. Hence, *RsaI* fragments of the *C. michiganensis* subsp. *michiganensis* bacteriophage CMP1 (10,34) (GenBank accession number Gq241246) were cloned into the *StuI* site 5 bp upstream of the ribosomal-binding site to find an appropriate promoter-region for expression of *egfp* in *C. michiganensis* subsp. *michiganensis*. Transformation of *E. coli* with plasmid pK2-22 (Fig. 1), which carries a partially digested 254-bp *RsaI* DNA fragment of CMP1 (base pair position 50,302 to 50,048 in the CMP1 genome) led to a green fluorescent phenotype. pK2-22 was transferred into the wild-type strain *Cmm*382 and its derivatives as described by Stork et al. (29). Briefly, DNA of plasmid pK2-22 was extracted from *E. coli* JM109 and subsequently electroporated into *Cmm*382,

Fig. 1. Physical map of the green fluorescent protein (*gfp*)-expressing plasmid pK2-22. A 739-bp *ScaI* DNA fragment carrying the enhanced (*e*)*gfp* gene from pGem EGFP was inserted into *Clavibacter michiganensis* subsp. *michiganensis* shuttle vector pDM302. The *StuI* and ribosomal-binding site were artificially generated by cloning annealed oligonucleotides upstream of the start codon of *egfp*. A 254-bp *RsaI* DNA fragment (pCMP1) derived from phage CMP1 (50,302–50,048) was then inserted, by blunt-end ligation, into a *StuI*-predigested plasmid to generate pK2-22. Neo = neomycin phosphotransferase, ori pBR = origin of replication of the vector pBR322, pCM1-Rep = replication region of the plasmid pCM1, par = partitioning region of pCM1, stability = region from pCM1 necessary for stable maintenance of the vector.

Cmm27, and *Cmm100*, respectively. Recombinant bacteria were selected on SB agar plates (1% tryptone, 0.5% yeast extract, 9.1% sorbitol, 34 mM NaCl, 20 mM MgCl₂, 20 mM CaCl₂, and agar at 15 g/liter) containing neomycin (50 µg/ml) after 7 days of incubation at 28°C. Twenty neomycin-resistant colonies were randomly collected and screened for fluorescence emission by a fluorescence-read microplate reader. Although all transformants displayed fluorescence, they varied in their signal intensity; therefore, clones displaying fluorescence signals above 18,000 fluorescence units (FUs) were selected for further analysis.

Plant inoculation and colonization test. Seedlings of tomato (*Solanum lycopersicum*) cv1125 (Hishtil Ltd., Moshav Nehalim, Israel) were grown in nethouse conditions. Three-week-old tomato plants were stem punctured with a needle at the junction of the first two leaves and 10 µl of bacterial suspension of 10⁸ cells/ml was placed at the wound. Bacterial inoculum was prepared from cultures of *Cmm382* or its derivatives after growth of 48 h in LB broth. At various time points (0, 3, 7, 15, and 21 days after infection), 1 cm of stem was cut at different distances above and below the inoculation site and macerated in 1 ml of distilled water, and serial 10-fold dilutions were plated on CNS plates. The numbers of CFU per gram of stem were determined and the mean of three plants was calculated. The experiments were repeated three times.

Fluorescence measurement. Fluorescence emission of *C. michiganensis* subsp. *michiganensis* strains harboring plasmid pK2-22 was measured from cultures grown for 48 h in LB broth amended with neomycin. Aliquots of 100 µl of each culture suspension (optical density at 595 nm [OD₅₉₅] = 0.5) were added to the wells of a black microtiter plate which was then read in a fluorescence microplate reader (Synergy 2 Multi-Mode Microplate Reader; Bio-Tek, Winooski, VT) using an excitation of 485 and emission at 528 nm. FUs of each well were normalized by subtracting the background fluorescence value of control wells containing LB broth.

Stability of pK2-22 in *Cmm382* and mutant derivatives. Stability of plasmid pK2-22 in vitro was determined with EGFP-labeled *Cmm382*, *Cmm100*, and *Cmm27* grown on CNS agar plates without antibiotic at 28°C for 48 h. Cells were harvested from the plates and diluted to OD₅₉₅ = 0.5, and aliquots of 100 µl were plated on fresh CNS plates. Successive transfers at 2-day intervals were repeated eight times. One hundred colonies were randomly collected and streaked on CNS agar plates containing neomycin. Colonies resistant to neomycin were further examined for fluorescence intensity as described above.

Stability assay of plasmid pK2-22 in strains grown in tomato plants was essentially carried out as described above for plant colonization. At various time points (0, 3, 7, 15, and 30 days), stem samples of three plants, inoculated with EGFP-labeled *Cmm382*, were removed 2 cm above the inoculation site and macerated with sterile water, and serial 10-fold dilutions were plated on CNS plates with or without neomycin. The CFU per gram of stem was determined and the mean of three independent experiments was calculated. At each time point, 20 single colonies were recovered from plates without antibiotic and diluted in phosphate buffer to OD₅₉₅ = 0.5. The fluorescence of each colony was determined and the percentage of EGFP-expressing colonies, with fluorescence >18,000 FUs, was calculated.

CLSM. For in vivo examination, stems of tomato plants were inoculated with EGFP-labeled *Cmm382*, *Cmm27*, and *Cmm100* as previously described. At each time point (3, 7, 15, and 30 days), plant tissues were collected from different sites above the inoculation site or from the apical region. The plant samples were transversally or longitudinally sectioned. At least 150 images taken from 50 plants were examined with a confocal laser-scanning microscope (model CLSM FluoView 500a; Olympus, Tokyo).

For in vitro studies, EGFP-labeled *C. michiganensis* subsp. *michiganensis* strains were grown in 3 ml of LB broth for 48 h at

28°C. Bacterial cultures were centrifuged and the pellet was washed once with sterile water and resuspended in the original volume of pure xylem sap (XS). XS for growth of *C. michiganensis* subsp. *michiganensis* was collected as follows. Stems of 14-week-old tomato plants were cut 10 cm above the ground. The stems were then placed horizontally to facilitate dripping of the sap into a 50-ml sterile tube for a 2-h period. The collected sap (from five plants) was filtered through a 0.22-µm membrane filter (Minisart; Sartorius Stedim Biotech, Aubagne, France) prior to use as a pure growth medium. The cell suspension (200 µl of 10¹⁰ cells/ml) in XS was placed on the polystyrene cover of 24-well plates and incubated at 28°C inside hermetic bags to avoid evaporation for 4 days followed by examination with CLSM.

Scanning electron microscopy. Tomato plants were stem inoculated as described above. Stems were collected at 3 and 15 days postinoculation (dpi) and longitudinally cut. The sections were fixed in 2.5% glutaraldehyde in sterile phosphate-buffered saline. After washing, the tissue samples were dehydrated by successive ethanol treatment. Solvents were removed by critical point drying. The samples were mounted on aluminum stubs and sputter-coated (E5100, Polaron; Quorum Technologies Ltd., West Sussex, UK) with gold. Images were captured with a scanning electron microscope (JSM 840A; Jeol Ltd., Tokyo) using a Scandium scanning electron microscope imaging platform (Olympus).

Crystal violet assay for bacterial attachment. The assay for bacterial attachment was carried out following the procedure described by Davey and O'Toole (7). Culture of *Cmm382* was grown in LB broth for 49 h at 28°C and then transferred to the same medium at 1:1000 dilution. After reaching an OD₅₉₅ = 0.5, the bacterial suspension was centrifuged and the pellet was washed once and resuspended in 3 ml of sterile water. The suspension (50 µl) was added to individual wells of 24-well multidishes containing 150 µl of XS, M9, or LB medium. The multidishes were incubated for 72 h at 28°C without agitation. After incubation, the culture was gently removed using a pipette, and wells were washed twice with 150 µl of sterile water. Following fixation at 60°C for 20 min, surface-attached bacteria were stained with 0.1% crystal violet solution for 1 h at room temperature. The multidishes were then washed gently three times with 150 µl of sterile water and air dried for 1 h for visual qualitative analysis. For quantitative analysis, crystal violet in each well was solubilized by adding 100 µl of 95% ethanol and absorption of the solution was then measured at 595 nm using a microtiter plate reader.

Statistical analysis. Statistical analyses were performed using the Excel software (Microsoft, Seattle) and JMP V7 (SAS Institute, Cary, NC).

RESULTS

Generation of EGFP-labeled *C. michiganensis* subsp. *michiganensis*. To generate a GFP-tagged *C. michiganensis* subsp. *michiganensis*, the EGFP was chosen because this protein was successfully used as reporter in related actinomycete *Streptomyces coelicolor* and for visualization of an endophytic *Streptomyces* sp. (5). The altered codon usage of the gene corresponds more closely to that of high GC organisms (5,32) and exhibits a stronger fluorescence compared with the wild-type GFP. The *egfp* was amplified from the expression plasmid pTST101 (31) and cloned into the *C. michiganensis* subsp. *michiganensis* shuttle vector pDM302, which confers neomycin resistance (24). An artificial ribosomal-binding site (GGAGG) was inserted 7 bp upstream of the start codon. Because this construct showed no fluorescence, both in *E. coli* and in *C. michiganensis* subsp. *michiganensis*, the *C. michiganensis* subsp. *michiganensis* bacteriophage CMP1 (10,34) was employed for isolation of a random 254-bp *RsaI* DNA-fragment to serve as a promoter (Fig. 1).

Transfer of the resulting plasmid, designated as pK2-22, into the wild-type strain *Cmm382* and its derivatives, as well as into *E. coli*, resulted in fluorescent clones. Twenty neomycin-resistant colonies were randomly collected and screened for fluorescence emission by a fluorescence-read microplate reader. Although all transformants displayed fluorescence, they varied in their signal intensity. Five colonies that displayed fluorescence above 18,000 FUs were selected for further analysis.

Characterization of the EGFP-labeled *C. michiganensis* subsp. *michiganensis*. EGFP-labeled *Cmm382*, *Cmm100*, and *Cmm27* showed a growth rate similar to that of the wild-type strain when grown in liquid LB at 28°C, indicating that the presence of pK2-22 or expression of *egfp* did not impair growth in vitro (data not shown). Stability of pK2-22 in *Cmm382*, *Cmm100*, and *Cmm27* was evaluated after eight successive transfers of 2-day intervals on selective media (CNS) plates without neomycin. One hundred randomly selected colonies showed resistance to neomycin and displayed fluorescence intensity similar to that of the EGFP-labeled parent strain ($P < 0.05$). Plasmid stability was also evaluated in tomato plants by monitoring the population of EGFP-labeled *Cmm382* in stem tissue for a period of 30 days (Table 1). Tissue samples taken from 2 cm above the inoculation site exhibited a similar number of colonies when cultured on CNS plates with or without neomycin, indicating that the plasmid was stable in *Cmm382*-infected tomato plants. The lower number of colonies that were recovered after 30 dpi (Table 1) could result from bacterial cell death during advanced stages of infection. However, no significant differences in colony number recovered on plates with or without neomycin were observed, indicating stability of the plasmid. EGFP expression by *Cmm382* in planta was examined by monitoring the fluorescence of 20 colonies grown on CNS without neomycin. An EGFP signal >18,000 FUs was detected in all of the monitored colonies at 3, 7, and 15 dpi (Table 1) whereas, at 30 dpi, only 70% of the colonies were fluorescent. The latter result suggests that the level of EGFP expression and resistance to neomycin are not entirely correlated. It could result from a loss of plasmid copies under nonselective conditions or attenuation of the GFP expression.

Wilt symptoms of tomato plants inoculated with EGFP-labeled *Cmm382* or the wild-type *Cmm382* appeared after 12 days (Supplemental Figure 1), suggesting that virulence was not impaired by the presence of the EGFP-expressing plasmid.

Colonization patterns of EGFP-labeled *Cmm382* during tomato infection. The ability of EGFP-labeled *Cmm382* to colonize tomato plants during infection was examined by CLSM. Longitudinal section images of the tomato stem taken at 3 dpi showed extensive multiplication and aggregates of *C. michiganensis* subsp. *michiganensis* that filled the lumen of xylem

vessels (Fig. 2A). Images taken at 7 dpi showed that *C. michiganensis* subsp. *michiganensis* cells adhered to the spiral secondary wall thickening of protoxylem, although an abundance of aggregated cells could still be observed within the lumen of the vessels (Fig. 2B). The specific and strong affinity of *C. michiganensis* subsp. *michiganensis* to the spiral secondary wall thickening was also evident when spiral elements were separated from damaged protoxylem vessels in images taken at 15 dpi (Fig. 2C). It is noteworthy that a screen of 150 images revealed that *C. michiganensis* subsp. *michiganensis* preferentially colonized the protoxylem and was not detected in adjacent metaxylem vessels (Fig. 2B), suggesting that horizontal passage of cells does not or rarely occurs at this stage. However, in damaged tissue, at later stages of infection and symptom production (e.g., 30 dpi), *C. michiganensis* subsp. *michiganensis* cells were dispersed from the vessels into adjacent parenchymal cells (Fig. 2D). Transversal sections of tomato stems revealed biofilm-like structures composed of large aggregates of bacterial cells which appeared attached to the xylem wall (Fig. 2E). Highly colonized tracheary elements were also observed in transversal mesophyll-sections in a wilted leaf taken from the apical region (Fig. 2F). The EGFP-labeled mutants *Cmm27* and *Cmm100* were examined by CLSM after 7 dpi and showed dispersed bacterial cells compared with the massive colonization and aggregate formation of the wild-type strain (Supplemental Figure 2).

Longitudinal sections of tomato stems inoculated with *C. michiganensis* subsp. *michiganensis* taken at 3 cm above the inoculation site were also examined by scanning electron microscopy (SEM). *C. michiganensis* subsp. *michiganensis* cells were attached to the xylem wall and developed aggregates among the spiral elements at 3 dpi (Fig. 3A). At 15 dpi, *C. michiganensis* subsp. *michiganensis* cells densely covered the spiral rings of xylem vessels (Fig. 3B). Furthermore, close-up images of infected xylem sections sampled at 15 dpi showed the presence of *C. michiganensis* subsp. *michiganensis* cells on perforated cell wall remnants (Fig. 3C), whereas continuous secondary wall thickening was generally observed in noninoculated xylem vessels (Fig. 3D).

Movement of *C. michiganensis* subsp. *michiganensis* in tomato. Movement of *C. michiganensis* subsp. *michiganensis* in the xylem vessels is crucial for tomato systemic infection (15). Confocal images of tomato plants infected with EGFP-expressing *Cmm382* showed that the pathogen colonized the whole plant and reached the apex within 15 dpi (Fig. 2C, D, and F). However, screening of 50 confocal images of tomato plants infected by EGFP-labeled *Cmm100* (lacking pCM1 and pCM2) and *Cmm27* (lacking the *chp/tomA* region) did not reveal the presence of the mutant strains in the apical parts of the stem. Therefore, it was of interest to examine further whether the inability of the non-pathogenic strains *Cmm100* and *Cmm27* to induce symptoms in tomato correlates to lower colonization and delayed spread within the plant. Stems of 3-week-old tomato plants were puncture inoculated with suspensions of *Cmm382*, *Cmm27*, and *Cmm100*, and the bacterial colonization at different locations above the inoculation site up to the apical region was monitored after 3, 7, 15, and 21 dpi (Fig. 4). At 3 dpi, all the examined strains showed similar bacterial titers of approximately 10^9 CFU/g at the inoculation site. However, whereas *Cmm382* could colonize the first 4 cm, *Cmm27* and *Cmm100* were only detected at the 2-cm distance. At 7 dpi, *Cmm382* reached the 6-cm site with a titer of 10^6 CFU/g whereas *Cmm27* and *Cmm100* could be isolated only from the initial 4 cm with a lower titer of 10^5 and 10^3 CFU/g, respectively. At 15 dpi, when disease symptoms appeared in stems and leaves of the wild-type inoculated plants, the pathogen could be isolated from the apical region (30 cm) of the plant with a titer of 10^9 CFU/g. In contrast, *Cmm27* and *Cmm100* were not detected beyond 4 cm above the inoculation site (Fig. 4). At 21 dpi, *Cmm382* reached a high population level of 10^{10} CFU/g at

TABLE 1. Stability of pK2-22 in strain *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 (*Cmm382*) in tomato plant

Days	Log (CFU/g of tissue) ^a		<i>egfp</i> -expressing colonies (%) ^b
	Neo+	Neo–	
0	5.5 ± 0.2	5.8 ± 0.2	100 ± 0
3	10.1 ± 0.2	10.0 ± 0.1	100 ± 0
7	10.2 ± 0.2	10.4 ± 0.4	100 ± 0
15	9.4 ± 0.9	9.8 ± 1.0	100 ± 0
30	4.6 ± 0.9	5.3 ± 0.8	70 ± 8

^a Stems of 3-week-old tomato plants were inoculated with 10 µl of suspension (10^8 cells/ml) of enhanced green fluorescent protein (EGFP)-labeled *Cmm382*. Tissue samples at different days postinoculation were taken from 2 cm above the inoculation site and plated on CNS agar plates with (Neo+) or without (Neo–) neomycin.

^b Fluorescence of 20 single colonies, collected from nonselective plates, was measured at each time point, and the percentage was calculated from the number of colonies with fluorescence above 18,000 fluorescence units. Results represent the means and standard deviation (±) of three independent experiments.

the apex of the plant (40 cm above the infection site) whereas *Cmm27* and *Cmm100* remained confined to a 6-cm region above the inoculation site with a significantly lower titer of 10^3 and 2×10^2 CFU/g, respectively. The low titer (10^5 CFU/g) of *Cmm382* 21 dpi in the first 2 cm could be assigned to cell death due to canker development in this area. The possibility that passive movement is involved in the migration of the mutants for short distances cannot be excluded.

The basipetal movement of *Cmm382*, *Cmm27*, and *Cmm100* was also monitored (Fig. 5). Results indicate that only *Cmm382* could reach 10^3 and 10^7 CFU/g at 4 cm below the inoculation site after 3 and 7 days, respectively. Only after 15 and 21 dpi did *Cmm27* and *Cmm100* also colonize the 4-cm site but with a titer of approximately four orders of magnitude lower compared with the wild-type strain.

The foregoing results support the possibility that genes located on the pCM1 and pCM2 plasmids and the *chp/tomA* PAI might play a significant role in effective acropetal and basipetal movement as measured by colonization of the pathogen during systemic infection of tomato. To further examine this premise, acropetal movement of *Cmm100*, *Cmm101* (lacking pCM2), *Cmm102* (lacking pCM1), *Cmm27*, *CmmchpC⁻* (a mutant in *chpC*), and *Cmm382* were compared (Fig. 6). *CmmchpC⁻* was selected for this experiment because *chpC* has been previously implicated in virulence (29). Bacterial titers were measured in the apical region at 15 dpi as described earlier. Results showed that *Cmm101*, *Cmm102*, and *CmmchpC⁻* colonized the apical region (20 cm) but with a titer 0.9 to 3.7 orders of magnitude lower compared with the wild-type strain, whereas *Cmm100* and *Cmm27* were not detected (Fig. 6). These findings suggest that genes located on either pCM1 or pCM2 contribute to colonization in the apex.

Effect of XS on attachment and aggregate formation by *C. michiganensis* subsp. *michiganensis*. The possibility that substances present in the XS might cause *C. michiganensis* subsp. *michiganensis* to incite attachment, aggregation, and biofilm formation was examined by employing an in vitro assay for biofilm detection in which the effect of XS, LB, and minimal M9 media were compared. *Cmm382* grown in XS developed an apparent biofilm that consisted of large aggregate of adherent cells (Fig. 7A). In contrast, cells grown in minimal M9 medium formed a very weak biofilm with no signs of aggregation and those grown in LB showed only few aggregates distributed over the polystyrene well surface (Fig. 7A). Spectrophotometric determination (OD_{590}) of the adherent cells assayed by crystal violet staining also showed that biofilm formation in XS was 2.6 compared with 0.9 and 0.2 in minimal M9 and LB media, respectively.

Confocal microscopy was performed to examine aggregate formation of *C. michiganensis* subsp. *michiganensis* grown in XS. Suspension of EGFP-labeled *Cmm382*, *Cmm27*, and *Cmm100* were grown in fresh XS. After 4 days, cells of *Cmm382* readily formed large aggregates on a polystyrene surface whereas *Cmm27* cells did not form aggregates (Fig. 7B). *Cmm100* showed a significant decrease in aggregation compared with *Cmm382* but small aggregates were still observed (Fig. 7B).

DISCUSSION

GFP-tagged cells provide a most useful tool to monitor bacterial pathogens during disease development (22). The powerful advantage of GFP labeling is that the localization of individual cells or clusters of bacterial cells can be studied over a long

Fig. 2. Colonization of tomato plants by enhanced green fluorescent protein (EGFP)-labeled *Clavibacter michiganensis* subsp. *michiganensis*. Tomato seedlings were puncture inoculated with a bacterial suspension at the junction of the first two leaves and localization of EGFP-labeled strain *C. michiganensis* subsp. *michiganensis* NCPB382 was examined by confocal laser scanning microscopy at **A**, 3; **B**, 7; **C**, 15; **D**, 30; **E**, 7; and **F**, 15 days postinoculation. Images were taken from longitudinally (**A** to **D**) and transversally cut (**E**) tomato stems and a wilted leaf (**F**). Images were captured at 3 cm above the inoculation site (**A**, **B**, and **E**) and at the apical region (**C**, **D**, and **F**). White arrows indicate noncolonized vessels. Red dots are plastids. Scale bars: 20 μ m in **A**, **C**, and **E**; 50 μ m in **B**, **D**, and **F**.

period of time in living tissues and even after cell death. Although the GFP reporter has been commonly used to determine movement and localization of many plant-associated bacteria (6), this is the first report in which GFP has been stably expressed in *C. michiganensis* subsp. *michiganensis* and applied for studying bacterial wilt and canker of tomato.

The interaction of *C. michiganensis* subsp. *michiganensis* with tomato has been divided into two phases, an endophytic stage and a symptom-inducing stage (15). The transition into the second stage relied on extensive multiplication of the pathogen in the xylem to reach a high population that is clearly reflected by the dense bacterial cells and aggregates filling the vessel lumen (Fig. 2A and B). A cluster of *C. michiganensis* subsp. *michiganensis* cells forming a biofilm-like structure was revealed in a cross section of the xylem vessels in stems (Fig. 2E) and, together with bacterial aggregates observed by SEM (Fig. 3), it can be concluded that *C. michiganensis* subsp. *michiganensis* produces biofilm-like structures in planta similar to other vascular bacterial pathogens (6). It is reasonable to expect that the severity of the disease caused by *C. michiganensis* subsp. *michiganensis* would increase with a larger proportion of vessels that become heavily colonized leading to a progressively lower water conduction capability.

A most striking phenomenon is the specific and firm adherence of *C. michiganensis* subsp. *michiganensis* to the spiral secondary wall thickening of xylem vessels (Fig. 2B to D). The bacterial surface adhesion to the spiral thickening was maintained even in

isolated spirals resulting from vessel deterioration (Fig. 2C). Damaged vessels in advanced stages of infection also facilitated the migration of *C. michiganensis* subsp. *michiganensis* into adjacent parenchymal cells (Fig. 2D). The preferential adhesion of *C. michiganensis* subsp. *michiganensis* to the secondary wall thickening is not understood. Occurrence of specific adherence to annular secondary cell wall thickening was reported for the gram-negative *P. stewartii* subsp. *stewartii* (23). Bacterial surface-associated structures, including polysaccharides and proteinaceous pili or fimbriae, are often involved in attachment to inert surfaces, to plants as well as to other bacterial cells and biofilm formations (6). EPS was implicated in biofilm formation and virulence of gram-negative vascular pathogens such as *P. stewartii* subsp. *stewartii*, *X. fastidiosa*, or *Erwinia amylovora* (6,21,33). EPS could create a physical barrier between the bacterial adhesion factors and a given substrate (23). Although EPS is produced by *C. michiganensis* subsp. *michiganensis* (12), its significance in *C. michiganensis* subsp. *michiganensis* virulence was ruled out because mutants containing reduced amounts of EPS or changed sugar composition were not affected in symptom development or colonization (3). However, the possibility that specific adhesion of *C. michiganensis* subsp. *michiganensis* to the spiral thickening of the xylem vessels might be mediated by adhesins such as fimbriae or pili cannot be excluded.

A clear preference for colonization of protoxylem as opposed to the metaxylem vessels was established for *C. michiganensis* subsp. *michiganensis*. The metaxylem vessels were generally ob-

Fig. 3. Visualization of *Clavibacter michiganensis* subsp. *michiganensis* in tomato plants by scanning electron microscopy. Longitudinal sections of xylem vessels taken 3 cm above the inoculation site at **A**, 3 and **B** and **C**, 15 days postinoculation following inoculation with suspensions of strain *C. michiganensis* subsp. *michiganensis* NCPPB382 or **D**, mock inoculation. Solid, dashed, and short arrows indicate bacterial cells, xylem wall, and spiral secondary wall thickening, respectively. Scale bars: 2 μ m in **A**, **C**, and **D**; 5 μ m in **B**. Images are representative of at least 25 sections for each time point.

Fig. 4. Colonization of tomato plants by *Clavibacter michiganensis* subsp. *michiganensis* strains NCPPB382 (*Cmm382*), *Cmm27*, and *Cmm100* at different times and distance from the inoculation site. Stems of 3-week-old tomato plants were puncture inoculated with suspensions of *Cmm382*, *Cmm27*, and *Cmm100*. Stem segments (1 cm) were individually collected and homogenized with 1 ml of distilled water, and serial dilutions were plated on CNS medium. Tomato apex segments were taken at distances of approximately 12, 15, 30, and 40 cm from the inoculation site (IS) after 3, 7, 15, and 21 days, respectively. Results represent the means of three independent experiments with standard deviations. Asterisks denote significant differences ($P < 0.05$) in *Cmm27* and *Cmm100* compared with *Cmm382* at each distance and time point according to Student's *t* test.

Fig. 5. Basipetal colonization of tomato plants by *Clavibacter michiganensis* subsp. *michiganensis* strains NCPPB382 (*Cmm382*), *Cmm27*, and *Cmm100*. Stem segments (1 cm) of tomato plants were collected at 4 cm below the inoculation site. Results represent the means of three independent experiments with standard deviations. Colonization of *Cmm27* and *Cmm100* was statistically different ($P < 0.05$) compared with *Cmm382* after 15 and 21 days according to Student's *t* test. Asterisks denote significant differences ($P < 0.05$) in *Cmm27* and *Cmm100* compared with *Cmm382*.

served to be free of bacteria even when located adjacent to heavily infected protoxylem vessels (Fig. 2B). This phenomenon is not understood. Additionally, transversal movement among vessels via bordered pits, as observed for *X. fastidiosa* (27), could not be detected. The protoxylem vessels differentiate in the primary plant body that have not completed their growth and differentiation, whereas the metaxylem matures largely after elongation is completed (13). Therefore, it might be postulated that *C. michiganensis* subsp. *michiganensis* migrates through the apoplast and invades the protoxylem but not the metaxylem during early development of the vessels. The observation that many vessels are not infected might explain the unilateral wilting observed with *C. michiganensis* subsp. *michiganensis*, as is also

Fig. 6. Colonization of tomato apex by *Clavibacter michiganensis* subsp. *michiganensis* strains NCPPB382 (*Cmm382*), *Cmm100*, *Cmm101*, *Cmm102*, *Cmm27*, and *CmmchpC⁻*. Stems of 3-week-old tomato plants were puncture inoculated with suspensions of the wild-type *Cmm382*, *Cmm100* (lacking pCM1 and pCM2), *Cmm101* (lacking pCM2), *Cmm102* (lacking pCM1), and *CmmchpC⁻* (a mutant of *chpC*). Bacterial growth in the apex was measured at 15 days postinoculation. Results represent the means of three independent experiments with standard deviations. Asterisks denote significant differences ($P < 0.05$) in *Cmm101*, *Cmm102*, and *CmmchpC⁻* population compared with *Cmm382* according to Student's *t* test.

Fig. 7. Aggregation and biofilm formation by *Clavibacter michiganensis* subsp. *michiganensis*. **A**, Crystal violet staining assay with *C. michiganensis* subsp. *michiganensis* strain NCPPB382 (*Cmm382*) grown in different culture media. *Cmm382* was grown in wells of microtiter plates in xylem sap (XS), minimal M9 (M9), and Luria-Bertani broth (LB) media and stained with crystal violet after 3 days as described in Materials and Methods. **B**, Enhanced green fluorescent protein (EGFP)-labeled *Cmm382*, *Cmm27*, and *Cmm100* grown in xylem sap. EGFP-labeled *C. michiganensis* subsp. *michiganensis* (10^{10} cells/ml) grown on polystyrene surfaces were visualized by confocal laser-scanning after 4 days. Images on the bottom are sagittal (*xz*) planes. Scale bars, 200 μ m. A minimum of five independent experiments was carried out for A and B with similar results.

the case in other bacterial and fungal wilt diseases. This might be viewed as a strategy of the pathogen to keep the plant alive as a nutritional source.

C. michiganensis subsp. *michiganensis* can readily colonize tomato xylem in both acropetal and basipetal directions (Figs. 4 and 5). Translocation in both directions has been previously shown by a bioluminescent *C. michiganensis* subsp. *michiganensis* mutant in grafted tomato seedlings (35). The colonization pattern is largely dependent on expression of genes located on the *chp/tomA* PAI and the two pathogenicity plasmids, pCM1 and pCM2 (Fig. 4). In the presence of *chp/tomA* PAI but absence of either pCM1 or pCM2, *C. michiganensis* subsp. *michiganensis* could be detected in the apex region but with a significantly lower rate (Fig. 6). It might be hypothesized that conversion of *C. michiganensis* subsp. *michiganensis* from an endophyte into a pathogen was achieved by horizontal gene transfer of genes located primarily on mobilizable elements (i.e., the *chp/tomA* PAI and plasmids pCM1 and pCM2). The dominating genes on these elements are the multiple serine proteases of different families (14). Thus, pCM1 harbors one serine protease of the Ppa family, pCM2 harbors three serine proteases of the Chp family, and the *chp/tomA* PAI contains members of three serine protease families (namely, Chp, Ppa, and subtilase). Most of these serine proteases were shown to be secreted into the XS during initial stages of infection (*unpublished data*). Results obtained can be best explained by the requirement of multiple serine proteases for *C. michiganensis* subsp. *michiganensis* colonization and movement in the plant. Thus, *Cmm100* could not be detected in the apical region, whereas *Cmm101* or *Cmm102* could (Fig. 6). Similarly, *Cmm27* did not move up to the apex whereas the mutant in *chpC* could (Fig. 6). Therefore, it might be postulated that a combination of serine proteases located on both the PAI and the plasmids are necessary for optimal movement and virulence. The observation that only *Cmm382* formed aggregates in XS as opposed to *Cmm100* and *Cmm27* (Fig. 7B) also might support their involvement in biofilm formation. The presence of partial serine proteases was sufficient for endophytic multiplication of *C. michiganensis* subsp. *michiganensis* at the infection site (Fig. 4) but not for progressive colonization in remote locations. It could be postulated that serine proteases secreted by the pathogen fulfill two roles, presumably via signal transduction: (i) suppression of defenses and (ii) release of nutrients from surrounding xylem parenchymal cells to support the extensive bacterial growth and disease development.

ACKNOWLEDGMENTS

This work was supported by the DGF program for Trilateral Cooperation among Israel, the Palestinian Authority, and Germany (grant number EI 535/12-2) and the United States–Israel Binational Agricultural Research and Development Fund (grant number IS-4403-11C). We thank E. Blausov and Vered H. for their helpful assistance in CLSM and SEM.

LITERATURE CITED

- Baccari, C., and Lindow, S. E. 2011. Assessment of the process of movement of *Xylella fastidiosa* with susceptible and resistant grape cultivars. *Phytopathology* 101:77-84.
- Balaji, V., Mayrose, M., Sherf, O., Jacob-Hirsh, J., Eichenlaub, R., Iraki, N., Manulis-Sasson, S., Rechavi, G., Barash, I., and Sessa, G. 2008. Tomato transcriptional changes in response to *Clavibacter michiganensis* subsp. *michiganensis* reveal a role for ethylene in disease development. *Plant Physiol.* 146:1797-1809.
- Bermpohl, A., Dreier, J., Bahro, R., and Eichenlaub, R. 1996. Exopolysaccharides in the pathogenic interaction of *Clavibacter michiganensis* subsp. *michiganensis* with tomato plants. *Microbiol. Res.* 151:391-399.
- Chalupowicz, L., Cohen-Kandli, M., Dror, O., Eichenlaub, R., Gartemann, K.-H., Sessa, G., Barash, I., and Manulis-Sasson, S. 2010. Sequential expression of bacterial virulence and plant defense genes during infection of tomato with *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology* 100:252-261.
- Coombs, J. T., and Franco, C. M. M. 2003. Visualization of an endophytic *Streptomyces* species in wheat seed. *Appl. Environ. Microbiol.* 69:4260-4262.
- Danhorn, T., and Fuqua, C. 2007. Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* 61:401-422.
- Davey, M. E., and O'Toole, G. A. 2000. Microbial biofilms: From ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* 64:847-867.
- Dow, J. M., Crossman, L., Findlay, K., He, Y. Q., Feng, J. X., and Tang, J. L. 2003. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc. Natl. Acad. Sci. USA* 100:10995-11000.
- Dreier, J. D., Meletzus, D., and Eichenlaub, R. 1997. Characterization of the plasmid encoded virulence region *pat-1* of the phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. *Mol. Plant-Microbe Interact.* 10:195-206.
- Echandi, E., and Sun, M. 1973. Isolation and characterization of a bacteriophage for the identification of *Corynebacterium michiganense*. *Phytopathology* 63:1398-1401.
- Eichenlaub, R., and Gartemann, K.-H. 2011. The *Clavibacter michiganensis* subspecies, molecular investigation of gram-positive bacterial plant pathogens. *Annu. Rev. Phytopathol.* 49:7.1-7.20.
- Eichenlaub, R., Gartemann, K.-H., and Burger, A. 2006. *Clavibacter michiganensis*, a group of gram-positive phytopathogenic bacteria. Pages 385-422 in: *Plant-Associated Bacteria*. S. S. Gnanamanickam, ed. Springer, Dordrecht, The Netherlands.
- Evert, R. F. 2006. *Esau's Plant Anatomy*. John Wiley and Sons Inc., Hoboken, NJ.
- Gartemann, K.-H., Abt, B., Bekel, T., Burger, A., Engemann, J., Flügel, M., Gaigalat, L., Goesmann, A., Grafen, I., Kalinowski, S., Kaup, O., Kirchner, O., Krause, L., Linke, B., McHardy, A., Meyer, F., Pohle, S., Ruckett, C., Schneiker, S., Zellerman, E.-M., Puhler, A., Eichenlaub, R., Kaiser, O., and Bartels, D. 2008. The genome sequence of the tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 reveals a large island involved in pathogenicity. *J. Bacteriol.* 190:2138-2149.
- Gartemann, K.-H., Kirchner, O., Engemann, J., Gräfen, I., Eichenlaub, R., and Burger, A. 2003. *Clavibacter michiganensis* subsp. *michiganensis*: First steps in understanding of virulence of a gram-positive phytopathogenic bacterium. *J. Biotechnol.* 106:179-191.
- Herrera, C. M., Koutsoudis, M. D., Wang, X., and von Bodman, S. B. 2008. *Pantoea stewartii* subsp. *stewartii* exhibits surface motility, which is a critical aspect of stewart wilt disease development on maize. *Mol. Plant-Microbe Interact.* 21:1359-1370.
- Jahr, H., Dreier, J., Meletzus, D., Bahro, R., and Eichenlaub, R. 2000. The endo-beta-1,4-glucanase CelA of *Clavibacter michiganensis* subsp. *michiganensis* is a pathogenicity determinant required for induction of bacterial wilt of tomato. *Mol. Plant-Microbe Interact.* 13:703-714.
- Kang, Y., Liu, H., Genin, S., Schell, M. A., and Denny, T. P. 2002. *Ralstonia solanacearum* requires type IV pili to adhere to multiple surfaces and for natural transformation and virulence. *Mol. Microbiol.* 46:427-437.
- Kaup, O., Gräfen, I., Zellerman, E. M., Eichenlaub, R., and Gartemann, K.-H. 2005. Identification of tomatinase in tomato pathogenic actinomycete *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. *Mol. Plant-Microbe Interact.* 18:1090-1098.
- Kleitman, F., Barash, I., Burger, A., Iraki, N., Sessa, G., Weinthal, D., Chalupowicz, L., Gartemann, K.-H., Eichenlaub, R., and Manulis-Sasson, S. 2008. Characterization of a *Clavibacter michiganensis* subsp. *michiganensis* population in Israel. *Eur. J. Plant Pathol.* 121:463-475.
- Koczan, J. M., McGrath, M. J., Zhao, Y., and Sundin, G. W. 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: Implications in pathogenicity. *Phytopathology* 99:1237-1244.
- Kohlmeier, S., Mancuso, M., Tecon, R., Harms, H., van der Meer, J. R., and Wells, M. 2007. Bioreporters: *gfp* versus *lux* revisited and single-cell response. *Biosens. Bioelectron.* 22:1578-1585.
- Koutsoudis, M. D., Tsaltas, D., Minogue, T. D., and von Bodman, S. 2006. Quorum-sensing regulation governs bacterial adhesion, biofilm development and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proc. Natl. Acad. Sci. USA* 103:5983-5988.
- Meletzus, D., Bermpohl, A., Dreier, J., and Eichenlaub, R. 1993. Evidence for plasmid-encoded virulence factors in the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. *J. Bacteriol.* 175:2131-2136.
- Meletzus, D., and Eichenlaub, R. 1991. Transformation of the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* by electroporation and development of a cloning vector. *J. Bacteriol.* 173:184-190.
- Meng, Y., Li, Y., Galvani, C. D., Hao, G., Turner, J. N., Burr, T. J., and Hoch, H. C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-

- driven twitching motility. *J. Bacteriol.* 187:5560-5567.
27. Newman, K. L., Almeida, R. P., Purcell, A. H., and Lindow S. E. 2003. Use of green fluorescence stain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Appl. Environ. Microbiol.* 69:7319-7327.
 28. Ramey, B. E., Koutsoudis, M., von Bodman, S. B., and Fuqua, C. 2004. Biofilm formation in plant-microbe interactions. *Curr. Opin. Microbiol.* 7:602-609.
 29. Stork, I., Gartemann, K.-H., Burger, A., and Eichenlaub, R. 2008. A family of serine proteases of *Clavibacter michiganensis* subsp. *michiganensis*: *chpC* plays a role in colonization of the host plant tomato. *Mol. Plant Pathol.* 9:599-608.
 30. Strider, D. L. 1969. Bacterial canker of tomato caused by *Corynebacterium michiganense*. A literature review and bibliography. N.C. Agric. Exp. Stn. Tech. Bull. 193.
 31. Stumpp, T., Wilms, B., and Altenbuchner, J. 2000. Ein neues L-rhamnose-induzierbares expressions system für *Escherichia coli*. *BIOspektrum* 1:33-36.
 32. Sun J., Kelemen G. H., Fernández-Abalos J. M., and Bibb M. J. 1999. Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* 145:2221-2227.
 33. von Bodman, S. B., Bauer, W. D., and Coplin, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. *Annu. Rev. Phytopathol.* 41:455-482.
 34. Wittmann, J., Gartemann, K.-H., Eichenlaub, R., and Dreiseikelmann, B. 2011. Genomic and molecular analysis of phage CMP1 from *Clavibacter michiganensis* subspecies *michiganensis*. *Bacteriophage* 1:1-8.
 35. Xu, X., Miller, S. A., Baysal-Gurel, F., Gartemann, K.-H., Eichenlaub, R., and Rajashekara, G. 2010. Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* infection of tomato seeds and plants. *Appl. Environ. Microbiol.* 76:3978-3988.