Final Scientific Report

Cover Page

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Project Title: *Clavibacter michiganensis* subsp. *michiganensis*-tomato interactions: expression and function of virulence factors, plant defense responses and pathogen movement

Investigators

**Principal Investigator (PI):
Shulamit Manulis-Sasson**

**Co-Principal Investigator (Co-PI):
Christine D. Smart**

Collaborating Investigators:

Isaac Barash

Laura Chalupowicz

Guido Sessa

Tom J Burr

Institutions

ARO, The Volcani Center

Cornell university

Tel-Aviv University

ARO, The Volcani Center

Tel-Aviv University

Cornell university

Keywords *not* appearing in the title and in order of importance. Serine proteases, cell wall degrading enzymes, blisters, transcriptional regulators, seed colonization

Abbreviations commonly used in the report, in alphabetical order: Cmm- *Clavibacter michiganensis* subsp. *michiganensis*, PAI-pathogenicity island

Budget: IS: \$ 168,000

US: \$ 112,000

Total: \$ 280,000

Signature

Principal Investigator

Shulamit Manulis-Sasson

Signature

Authorizing Official, Principal Institution

Sheenan Harpaz

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1	1	2	4
Submitted, in review, in preparation	1			1
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts	2		7	9
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		3		3
Longer Visits (Sabbaticals)				

Description Cooperation:

A close cooperation was maintained between the collaboration groups in this project. Details are in the report.

Patent Summary - none

Abstract

Clavibacter michiganensis subsp. *michiganensis* (Cmm), the causal agent of bacterial wilt and canker of tomato, is the most destructive bacterial disease of tomato causing substantial economic losses in Israel, the U.S.A. and worldwide. The goal of the project was to unravel the molecular strategies that allow Cmm, a Gram-positive bacterium, to develop a successful infection in tomato. The genome of Cmm contains numerous genes encoding for extracellular serine proteases and cell wall degrading enzymes. The first objective was to elucidate the role of secreted serine proteases in Cmm virulence. Mutants of nine genes encoding serine proteases of 3 different families were tested for their ability to induce wilting, when tomato stems were puncture-inoculated, as compared to blisters formation on leaves, when plants were spray-inoculated. All the mutants showed reduction in wilting and blister formation as compared to the wild type. The *chpC* mutant displayed the highest reduction, implicating its major role in symptom development. Five mutants of cell wall degrading enzymes and additional genes (i.e. perforin and sortase) caused wilting but were impaired in their ability to form blisters on leaves. These results suggest that Cmm differentially expressed virulence genes according to the site of penetration. Furthermore, we isolated and characterized two Cmm transcriptional activators, Vatr1 and Vatr2 that regulate the expression of virulence factors, membrane and secreted proteins.

The second objective was to determine the effect of bacterial virulence genes on movement of Cmm in tomato plants and identify the routes by which the pathogen contaminates seeds. Using a GFP-labeled Cmm we could demonstrate that Cmm extensively colonizes the lumen of xylem vessels and preferentially attaches to spiral secondary wall thickening of the protoxylem and formed biofilm-like structures composed of large bacterial aggregates. Our 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. We constructed a transposon plasmid that can be stably integrated into Cmm chromosome and express GFP, in order to follow movement to the seeds. Field strains from New York that were stably transformed with this construct, could not only access seeds systemically through the xylem, but also externally through tomato fruit lesions, which harbored high intra- and intercellular populations. Active movement and expansion of bacteria into the fruit mesocarp and nearby xylem vessels followed, once the fruit began to ripen. These results highlight the ability of Cmm to invade tomato fruit and seed through multiple entry routes.

The third objective was to assess correlation between disease severity and expression levels of Cmm virulence genes and tomato defense genes. The effect of plant age on expression of tomato defense related proteins during Cmm infection was analyzed by qRT-PCR. Five genes out of eleven showed high induction at early stages of infection of plants with 19/20 leaves compared to young plants bearing 7/8 leaves. Previous results showed that Cmm virulence genes were expressed at early stages of infection in young plants compared to older plants. Results of this study suggest that Cmm virulence genes may suppress expression of tomato defense-related genes in young plants allowing effective disease development. The possibility that *chpC* is involved in suppression of tomato defense genes is currently under investigation by measuring the transcript level of several PR proteins, detected previously in our proteomics study.

The fourth objective was to define genome location and stability of virulence genes in Cmm strains. New York isolates were compared to Israeli, Serbian, and NCPPB382 strains. The plasmid profiles of New York isolates were diverse and differed from both Israeli and Serbian strains. PCR analysis indicated that the presence of putative pathogenicity genes varied between isolates and highlighted the ephemeral nature of pathogenicity genes in field populations of Cmm.

Results of this project significantly contributed to the understanding of Cmm virulence, its movement within tomato xylem or externally into the seeds, the role of serine proteases in disease development and initiated research on global regulation of Cmm virulence. These results form a basis for developing new strategies to combat wilt and canker disease of tomato.

Achievements

Objective A: Elucidate the role of secreted serine proteases in Cmm virulence.

The genome of Cmm contains numerous genes encoding for extracellular serine proteases and cell wall degrading enzymes. To characterize their involvement in pathogenicity, mutants of these genes were tested for their ability to cause wilting, when tomato stems were puncture-inoculated, as compared to blisters formation on leaves, when plants were spray-inoculated. Tomato seedlings spray-inoculated with *Cmm382* (wild type) and *Cmm100* (lacking the plasmids pCM1 and pCM2) formed blisters on leaves eight days post inoculation, whereas *Cmm27* (lacking the PAI) did not.

These results suggest that plasmid-borne effectors that affect wilting are not required for blisters formation. GFP labeled *Cmm* strains showed that the wild type and *Cmm100* formed aggregates and penetrated mainly through trichomes, colonizing the intercellular space of the mesophyll tissue. In contrast, *Cmm27* was unable to penetrate and colonize the leaf. Mutants of nine genes encoding serine proteases from 3 different families (*chpC*, *chpG*, *chpE*, *chpF*, *ppaA*, *ppaC*, *sbtA*, *sbtB* and *sbtC*) showed reduction in wilting and blister formation compared with the wild type and *Cmm100*. The *chpC* mutant showed the highest reduction suggesting that it is a major factor in symptom development. Similarly, the *sbtA* mutant was impaired in pathogenicity but to a lesser extent than *chpC* mutant. Five mutants of cell wall degrading enzymes (*celB*, *xysA*, *xysB*, *pgaA* and *endx/y*) did not affect wilting, but the mutants in *pgaA* and *endx/y* were impaired in their ability to form blisters on leaves. Additional mutants, in perforin and sortase, were also unable to cause blisters but were not impaired in wilting. To further support the above results, expression levels of genes encoding virulence factors located on plasmids (*celA*, *pat-1*) and *chp/tomA* PAI (*chpC*, *ppaA*, *chpE*, *chpF*, *sbtA*) were measured by qRT PCR at various time points after spraying tomato leaves with *Cmm382*. Results showed that *celA* and *pat-1* were hardly expressed at the initial 48 h. In contrast, the serine proteases, *chpC* and *sbtA* were induced very early reaching the highest levels at 8-16 h after inoculation. Results of this part showed that Cmm differentially expressed virulence genes according to the site of penetration to tomato plant. A manuscript describing the results of this study is in preparation.

In addition, we isolated and characterized two Cmm transcriptional regulators, Vatr1 and Vatr2 that were identified based on proteomics experiments carried out previously on series of *in planta* and *in vitro* generated samples (Savidor et al. 2012, *J. Proteome Research* 11:736-750). Vatr1 and Vatr2 belong to TetR and MocR families of transcriptional regulators, respectively. Mutations in their corresponding genes caused attenuated virulence, with the *Δvatr2* mutant showing a more dramatic effect than *Δvatr1*. While both mutants grew well *in vitro* and reached a high titer *in planta*, they caused reduced wilting and canker development in infected plants compared to the wild-type

bacterium. They also led to a reduced expression of the ethylene-synthesizing tomato enzyme ACC-oxidase compared to wild-type Cmm and to reduced ethylene production in the plant. Transcriptomic analysis of wild-type Cmm and the two mutants under infection-mimicking conditions revealed that Vatr1 and Vatr2 regulate expression of virulence factors, membrane and secreted proteins, and signal transducing proteins. A 70% overlap between the sets of genes positively regulated by Vatr1 and Vatr2 suggests that these transcriptional regulators are on the same molecular pathway responsible for Cmm virulence. For further details see our publication Savidor et al. 2014.

Objective B: Determine the effect of bacterial virulence genes on movement of Cmm in tomato plants and identify the routes by which the pathogen contaminates seeds.

Colonization patterns and movement of Cmm during tomato infection was examined using a green fluorescent protein (GFP)-labeled strain. A plasmid expressing GFP in Cmm was constructed and found to be stable in planta for at least one month. The GFP provided a powerful tool to answer some fundamental questions that could not had been previously obtained for Cmm. The mode of Cmm colonization and movement in tomato xylem vessels had been thoroughly described and revealed various unique features including biofilm-like formation and a firm adherence to secondary cell wall of the xylem. Acropetal movement of the wild-type strain *Cmm* 382 in tomato resulted in an extensive systemic colonization of the whole plant reaching the apical region after 15 days, whereas *Cmm*100 or *Cmm*27 remained confined to the area surrounding the inoculation site. 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. For further details refer to our publication Chalupowicz et al. 2012.

To obtain stable GFP expression, which is required to follow Cmm movement to the seeds, we constructed a transposon plasmid that can be stably integrated into the Cmm chromosome and express GFP. Field strains of Cmm from New York were stably transformed with the eGFP gene and used to demonstrate that Cmm could not only access seeds systemically through the xylem, but also externally through tomato fruit lesions, which harbored high intra-and intercellular populations. Active movement and expansion of bacteria into the fruit mesocarp and nearby xylem vessels followed, once the fruit began to ripen. These results highlight the ability of Cmm to invade tomato fruit and seed through multiple entry routes. For further details refer to our publication Tancos et al. 2013.

Objective C: Assess correlation between disease severity and expression levels of *Cmm* virulence genes and tomato defense genes.

The proteomic study revealed that several PR proteins, protein phosphatases and kinases were differentially expressed in plants infected with *Cmm*. The effect of plant age on expression of tomato defense related proteins during *Cmm* infection was analyzed by qRT-PCR. Relative expression of eleven genes encoding tomato protein were selected from this analysis and measured in tomato plants bearing 7/8 leaves compared with 19/20. Five genes, cation binding protein, trypsin inhibitor, protein phosphatase, subtilisin activity and PR- STH-2, out of eleven showed high induction at early stages of infection of plants with 19/20 leaves compared to those bearing 7/8 leaves. Previous results showed that *Cmm* virulence genes are expressed at early stages of infection in young plants compared to older plants. Results of this study suggest that *Cmm* virulence genes may suppress expression of tomato defense-related genes in young plants allowing effective disease development. The possibility that *chpC* is involved in suppression of tomato defense genes is under investigation by measuring the transcript level of several PR proteins detected in our proteomics study when plants were inoculated with *chpC* mutant.

Objective D: Define genome location and stability of virulence genes in *Cmm* strains.

To better understand the genetic diversity among *Cmm* populations, New York isolates were compared to Israeli, Serbian, and NCPPB382 strains. New York *Cmm* isolates, collected from disparate bacterial canker of tomato outbreaks over the past eleven years, were characterized with a multilocus sequence analysis (MLSA) scheme that differentiated the 51 isolates into 21 haplotypes with a discriminatory power of 0.944. The MLSA scheme consisted of five housekeeping genes (*kdpA*, *sdhA*, *dnaA*, *ligA*, *gyrB*) and three putative pathogenicity genes (*celA*, *tomA*, *nagA*). Repetitive-PCR, with the BOX-A1R primer, confirmed the high diversity of *Cmm* isolates in New York by demonstrating all six PCR patterns (A, B, 13C, 65C, 81C, D) were present, with PCR patterns 'C' and 'A' being the most common. The MLSA scheme provided higher resolving power than the current repetitive-PCR approach. The plasmid profiles of New York isolates were diverse and differed from both Israeli and Serbian strains. PCR analysis indicated that the presence of putative pathogenicity genes varied between isolates and highlighted the ephemeral nature of pathogenicity genes in field populations of *Cmm*. Analysis of molecular variance between Serbian and New York *Cmm* isolates demonstrated that the two populations were not significantly different, with 98% genetic variation within each population and only 2% genetic variation between populations. For further details refer to our publication Tancos et al. 2015.

Description of cooperation:

The collaborating groups of Volcani Center, Cornell and Tel Aviv Universities were constantly in touch by E-mail and personal meetings. The American and the Israeli investigators (Barash and Chalupowicz) had met during the APS annual meetings in Providence, RI (2012) and Austin, Tx (2013) for results discussion, exchange of ideas and planning experiments. For instance, the plasmid for stable expression of GFP in Cmm was constructed by the Israeli team and supplied to the American team to visualize the routes by which tomato seeds are contaminated. Additionally, in order to investigate the behavior of Cmm under xylem-mimicking conditions, several mutants of Cmm (sortase, *chpC*) were sent to the American team for analysis by the microfluidic flow chamber technology which is available at Cornell University.

List of Publications:

Chalupowicz, L., Zellermann, 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.

Tancos, M.A., Chalupowicz, L., Barash, I., Manulis-Sasson, S. and Smart, C.D. (2013). Tomato fruit and seed colonization by *Clavibacter michiganensis* subsp. *michiganensis* through external and internal routes. *Applied and Environmental Microbiology* 79:6948-6957.

Savidor, A., Chalupowicz, L., Teper, D., Gartemann, K.-H., Eichenlaub, R., Manulis-Sasson, S., Barash, I. and Sessa, D. (2014). *Clavibacter michiganensis* subsp. *michiganensis* VatR1 and VatR2 transcriptional regulators are required for virulence in tomato. *Molecular Plant-Microbe Interactions* 27:1035-1047.

Tancos, M.A., Lange, H.W., and Smart, C.D. 2015. Characterizing the genetic diversity of the *Clavibacter michiganensis* subsp. *michiganensis* population in New York. *Phytopathology* 105:169-179.

Appendix

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Published papers

1. Chalupowicz, L., Zellermann, 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.
2. Tancos, M.A., Chalupowicz, L., Barash, I., Manulis-Sasson, S. and Smart, C.D. (2013). Tomato fruit and seed colonization by *Clavibacter michiganensis* subsp. *michiganensis* through external and internal routes. *Applied and Environmental Microbiology* 79:6948-6957.
3. Savidor, A., Chalupowicz, L., Teper, D., Gartemann, K.-H., Eichenlaub, R., Manulis-Sasson, S., Barash, I. and Sessa, D. (2014). *Clavibacter michiganensis* subsp. *michiganensis* VatR1 and VatR2 transcriptional regulators are required for virulence in tomato. *Molecular Plant-Microbe Interactions* 27:1035-1047.
4. Tancos, M.A., Lange, H.W., and Smart, C.D. 2015. Characterizing the genetic diversity of the *Clavibacter michiganensis* subsp. *michiganensis* population in New York. *Phytopathology* 105:169-179.

Manuscript in preparation

Chalupowicz, L., Reuven, M., Dror, O., Barash, I., Sessa, G., Gartemann, K.-H., Eichenlaub, R., Smart, C.D. and Manulis-Sasson, S. (2015). Serine proteases and cell-wall degrading enzymes are involved in blister formation on tomato leaves by *Clavibacter michiganensis* subsp. *michiganensis*.

Presentations in scientific meetings

- Chalupowicz, L., Dror, O., Zellermann, E., Eichenlaub, R., Gartemann, K., Sessa, G., Barash, I. and Manulis-Sasson, S. (2011). Colonization and movement of *Clavibacter michiganensis* subsp. *michiganensis* in the tomato plant. *Phytoparasitica* 39:247-248.
- Chalupowicz, L., Savidor, A., Eichenlaub, R., Gartemann, K.-H., Sessa, G., Barash, I. and Manulis-Sasson, S. (2012). An insight into the molecular interactions between *Clavibacter michiganensis* subsp. *michiganensis* and tomato. BARD Workshop on microbial virulence determinants and plant immunity. Tel Aviv University, Tel-Aviv, Israel.
- Savidor, A., Teper, D., Gartemann, K.-H., Eichenlaub, R., Chalupowicz, L., Manulis-Sasson, S., Barash, I., Tews, H., Mayer, K., Giannone, R.J., Hettich, R.L. and Sessa, G. (2012). The *Clavibacter michiganensis* subsp. *michiganensis*-tomato interactome reveals perception of pathogen by host and suggests mechanisms of infection. BARD Workshop on microbial virulence determinants and plant immunity. Tel Aviv University, Tel-Aviv, Israel.
- Savidor, A., Teper, D., Gartemann, K.-H., Eichenlaub, R., Chalupowicz, L., Manulis-Sasson, S., Barash, I., Tews, H., Mayer, K., Giannone, R., Hettich, R. and Sessa G. (2012). The *Clavibacter michiganensis* subsp. *michiganensis*-tomato interactome reveals perception of

pathogen by host and suggests mechanisms of infection. XV International Congress on Molecular Plant-Microbe Interactions, Kyoto, Japan.

Manulis-Sasson, S., Chalupowicz, L., Savidor, A., Sharabani, G., Eichenlaub, R., Gartemann, K-H., Sessa, G. and Barash, I. (2013). The molecular interactions between *Clavibacter michiganensis* subsp. *michiganensis* and tomato. The Annual Meeting of the Israeli Society for Microbiology, Bar Ilan University, Ramat Gan, Israel.

Tancos, M.A., Chalupowicz, L., Barash, I., Manulis-Sasson, S., and Smart, C.D. (2013). Tomato fruit colonization of *Clavibacter michiganensis* subsp. *michiganensis* via external and internal routes. *Phytopathology* 103:S2.143 (Abstract, presented at the American Phytopathological Society annual meeting, Austin, TX).

Manulis-Sasson, S., Chalupowicz, L., Savidor, A., Sharabani, G., Eichenlaub, R., Gartemann, K-H., Sessa, G. and Barash, I. (2013). The molecular interactions between *Clavibacter michiganensis* subsp. *michiganensis* and tomato. The Annual Meeting of the Israeli Society for Microbiology, Bar Ilan University, Ramat Gan, Israel.

Tancos, M.A., Chalupowicz, L., Barash, I., Manulis-Sasson, S., and Smart, C.D. (2014). Modes of seed infection by *Clavibacter michiganensis* subsp. *michiganensis* and population diversity in New York. Oral presentation at the American Phytopathological Society annual meeting, Minneapolis, MN.

Manulis-Sasson, S., Chalupowicz, L., Gartemann, K.-H., Sessa, G., and Barash, I. (2014). Insights into the molecular interactions between *Clavibacter michiganensis* subsp. *michiganensis* and tomato. Oral presentation at the 13th International Conference on Plant Pathogenic Bacteria, Shanghai, China.

Unpublished data

As part of objective A we suggested to identify tomato proteins interacting with Pat 1 and ChpC by a yeast two-hybrid system. However, although extensive efforts have been devoted, this part was not successful due to high background in the assay. The part concerning the effect of serine proteases on the expression of selected tomato defense proteins is under investigation using the mutants in *chpC* and *sbtA* which showed a major involvement in pathogenicity.

Based on the previous observations, Cmm appeared to be actively moving within the fruit pericarp (horizontally and basipetally), even though it's purported to be non-motile. Upon further *in planta* experimentation, Cmm was capable of both acropetal and basipetal motility at distances of 30 cm above the inoculation site and 20 cm below the inoculation site within 4 weeks. The calculated basipetal speeds were between 4.96-6.61 $\mu\text{m min}^{-1}$, which correlate well with the twitching speeds of *Xylella fastidiosa*. *In silico* analyses revealed putative pili (*tad* genes) potentially involved in biofilm formation, colonization, or movement. As a result, individual pili genes are being targeted for knockout.

To complement the *in planta* experiments, Cmm strains were analyzed *in vitro* with artificial xylem chambers, developed at Cornell to study movement of the xylem-limited bacterium *Xylella fastidiosa*. The microfluidic chambers mimic xylem vessels by providing a constant flow of media,

and bacterial movement can be recorded with digital cameras attached to inverted microscopes to give a time-lapse view of Cmm colonization. Preliminary observations revealed active bacterial movement against the flow of the media at speeds averaging $3.4\text{-}6.64\ \mu\text{m min}^{-1}$. Pili-mutants and mutants generated in Israel will be tested in the microfluidic chambers for biofilm formation and movement.

Wild-type Cmm strains have been analyzed within the microfluidic chambers using different medias (pure tomato sap, xylem mimicking media, or Luria-Bertani media) in order to explore differences in movement or aggregation patterns, thereby better representing an *in planta* environment. However due to unforeseen difficulties in the fabrication process of microfluidic chambers, the screening of Cmm strains/mutants is ongoing.