Characterization of a *Pantoea stewartii* mutant affecting persistence in its flea beetle vector

Correa, Valdir R.[1], Majerczak, Doris R.[2], Ammar, El Desouky[3], Merighi, Massimo[2], Coplin, David. L.[2], Pratt, Richard C. , Redinbaugh, Margaret G. [2, 4] and Hogenhout, Saskia A.[3]

[1]Department of Horticulture and Crop Science, [2]Department of Plant Pathology, [3]Department of Entomology, OARDC/The Ohio State University. [4]USDA, ARS Corn and Soybean, Wooster-OH

Contact: Saskia.Hogenhout@bbsrc.ac.uk

Abstract

Stewart's wilt, a disease of corn characterized by wilting and leaf blight symptoms, is caused by *Pantoea stewartii*, a Gram-negative bacterium that is transmitted by the corn flea beetle, *Chaetocnema pulicaria*. Little is known about the interaction of *P. stewartii* with its flea beetle vector, including the mechanism(s) by which *P. stewartii* is transmitted. Recent work indicated that *P. stewartii* carries gene clusters for two separate type III secretion systems (TTSS). These TTSS gene clusters encode pili-like structures that enable these bacteria to inject virulence proteins into host cells. It has already been shown that one TTSS gene cluster is required for plant infection, whereas the other cluster is not (Coplin, et al. 1992, Frederick, et al. 2001). We hypothesize that the second TTSS is important for the transmission of *P. stewartii* by flea beetles. To detect *P. stewartii* in the beetles, two methods were used. The first method involved the detection of transformed *P. stewartii* that express *gfp* from a plasmid and in the second method *P. stewartii* was detected with specific antibodies linked to Alexa-fluor. The localization of the bacteria was visualized in dissected beetle guts with confocal laser scanning microscopy (CLSM). These experiments showed that the bacteria were mainly found in the lumen of the hindgut at up to 10 days after acquisition of the bacteria from corn plants. The localization of two *P. stewartii* mutants was also investigated. One mutant carried a mutation in the *ysaN* gene that is part of the second TTSS, and the other had a mutation in the *sapD* gene, which is part of an antibacterial peptide resistance ABC transporter. The results of colony-forming unit (CFU) counts and CLSM revealed that the *ysaN* mutants did not persist in the beetles, whereas wild type bacteria and the *sapD* mutant did. These results suggest that the second TTSS is involved in persistence of *P. stewartii* in the flea beetle vector. This is an important finding, because to our knowledge, it has never been shown that a single bacterium has two TTSS that are specifically involved in the infection of two diverse hosts (plants and insects).

Hypothesis

Our hypothesis is that the 2nd cluster of type III secretion system genes in the *Pantoea stewartii* genome is involved in the persistence of this bacterium in its flea beetle vector.

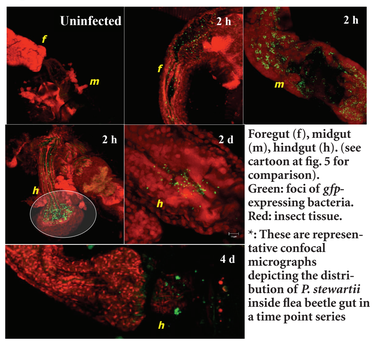
Objectives

- To determine the distribution of *P. stewartii* DC283 in its most important flea beetle vector, *Chaetocnema pulicaria*.
- To determine whether a DC283 mutant in which the *ysaN* gene of the second type III secretion system has been knocked out is less persistent in the flea beetle compared to wild type DC283 and another DC283 mutant.

Experimental Approaches

- Candidate genes for mutant analyses were obtained by random mutagenesis of the genome with mini-Tn5gus and then screened for genes that were down regulated by the HrpL alternate sigma factor.
- *P. stewartii* DC283 wild type and mutants containing plasmids expressing the *gfp* gene were localized in dissected flea beetle guts using CLSM.
- *P. stewartii* DC283 wild type and mutants were localized in dissected flea beetle guts by immunofluorescence CLSM (iCLSM) using specific antibodies to *P. stewartii* and a secondary antibody conjugated to Alexa fluor.
- Bacterial persistence of DC283 wild type and mutants in flea beetle guts was compared by counting the number of viable colonies per insect.

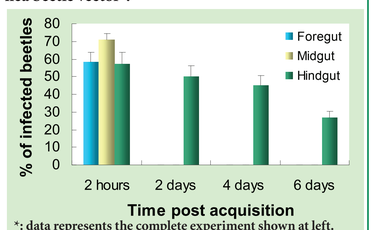
Figure 1. *P. stewartii* resides in the hindgut of its insect flea beetle vector



Foregut (f), midgut (m), hindgut (h). (see cartoon at fig. 5 for comparison). Green: foci of *gfp*-expressing bacteria. Red: insect tissue.

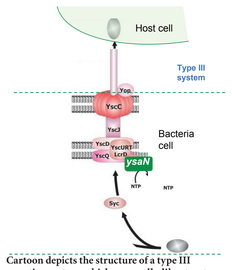
*: These are representative confocal micrographs depicting the distribution of *P. stewartii* inside flea beetle gut in a time point series

Summary of *Pantoea stewartii* localization inside flea beetle vector*.

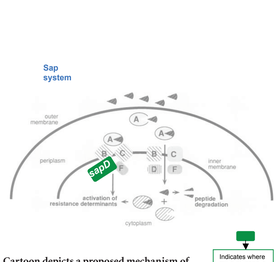


*: data represents the complete experiment shown at left.

Figure 2. Representation and proposed mechanism of action for a type III and an antibacterial peptide resistance ABC transporter candidate genes of *Pantoea stewartii*

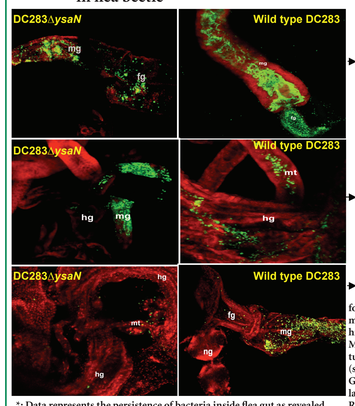


Cartoon depicts the structure of a type III secretion system, which are needle-like structure used by pathogenic bacteria to inject effector molecule (in gray bullet) into host cell. Adapted from: Henderson, et. al. 2004.



Cartoon depicts a proposed mechanism of action for the sap system. Briefly, bacteria with a defective system would be more sensitive to microbial anti-peptides, shown by the triangles. Adapted from: Parra-Lopez, et al. 1993.

Figure 4. Mutation in *ysaN* attenuates bacterial persistence in flea beetle



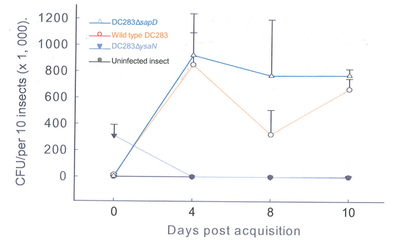
foregut (fg). midgut (mg). hindgut (hg). Malpighian tubules (mt) (see cartoon in fig. 5) Green: immuno-labeled bacteria Red: insect tissue.

*: Data represents the persistence of bacteria inside flea gut as revealed by immuno-labeling. Both wild type DC283 and DC283Δ*ysaN* persisted equally upon day four, however, persistence of DC283Δ*ysaN* severely decreased upon day 8.

Characteristics of the candidates genes

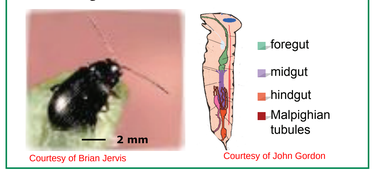
Mutants	Characteristics	Homology in animal pathogens and insect symbionts
ysaN	Structural protein of a type III secretion system (TTSS) apparatus (2nd gene cluster) from *P. stewartii.*	*Yersinia; Shigella and Sodalis*
sapD	Structural component of an antimicrobial peptide ABC transport system.	*Salmonella typhimurium*

Figure 3. Mutation in *ysaN* attenuates bacterial persistence in flea beetle*



*: data represents the number of colony forming unit per insect in a time series period following bacteria acquisition by insects. As seen, the numbers of DC283Δ*ysaN* cells did not persisted upon four days post infection.

Figure 5. Representation of the corn flea beetle and schematic drawing of parts composing its digestive tract



Courtesy of Brian Jervis

Courtesy of John Gordon

Summary/Conclusions

- Our results suggest that a gene in the 2nd cluster type III secretion system is important for bacterial persistence in flea beetles. As seen in fig. 3 and 4, beetles equally acquired both wild type and mutants; however, persistence of DC283Δ*ysaN* declined at four days upon bacterial uptake by insects as compared to wild type DC283 and DC283Δ*sapD*.
- The CLSM and iCLSM experiments suggest that *P. stewartii* remains in the gut lumen, particularly the hindgut lumen and did not seem to invade gut cells. However, it seems that *P. stewartii* can invade cells of the Malphigian tubules that protrude from the gut (see fig. 4, at day four).
- Because *P. stewartii* mainly colonizes the hindgut (see fig. 1), the most likely route for transmission is through defecation. This hypothesis is supported by our observation that flea beetles tend to cluster together in small groups on a maize leaves under growth chamber condition. Hence, they can easily facilitate the introduction of *P. stewartii* present in the defecated materials into plant by its feeding wounds.
- These results indicate that *Pantoea stewartii* might have evolved two distinct mechanisms for interacting with its plant and invertebrate host.

Ongoing/Future Experiments

- To determine whether *P. stewartii* can invade cells of the flea beetle gut and Malphigian tubules, we will conduct transmission electron microscopy experiments combined with immunogold labeling using our specific *P. stewartii* antibodies.
- To investigate whether or not *P. stewartii* can invade insect cells, we will conduct various cell invasion assays with Drosophila S2 cells.
- To assess whether the second TTSS is active in insects, we will examine the expression of genes in this cluster in flea beetles and maize.
- To confirm whether the 2nd TTSS is involved in flea beetle persistence, we will conduct complementation experiments in which we introduce the complete *ysaN* gene and other genes that may be affected by the transposon insertion mutagenesis into the DC283Δ*ysaN* strain. We expect that this (ese) complementation (s) will restore persistence of *P. stewartii* in the flea beetle gut.

Acknowledgements

Funding was provided by The OARDC Research Competitive Grants Program and the USDA, Cooperative State Research, Education & Extension Service (CSREES). We thank Kristen Willie, Mark Zimmerman, Angela D. Strock and Cameron Exner for their excellent technical assistance.

References

1- Henderson, I. R., Navarro-Garcia, F., Desvaux, M., Fernandez, R. C. and Aldeen S. A. Type V protein secretion pathway: the autotransporter story. Micr. and Mol. Biol. Rev. 68: 692.

2- Parra-Lopez, C.,Baer, M.T., and Groisman, E. A. genetic analysis of a locus required for resistance to antimicrobial peptides in *Salmonella typhimurium*. The EMBO Journal. 12: 4053.

3- Frederick, R. D., Ahmad, M., Majerczak, D. R., Arroyo-Rodríguez, A. S., Manulis, S. and Coplin, D. L. Genetic organization of the *Pantoea stewartii* subsp. *stewartii hrp* gene cluster and sequence analysis of the hrpA, hrpC, hrpN, and wtsE operons. Mol. Plant-Microbe Interact 14: 1213.

4- Coplin, D. L., Frederick, R. D., Majerczak, D. R., and Tuttle, L. D. 1992. Characterization of a gene cluster that specifies pathogenicity in *Erwinia stewartii*. Mol. Plant-Microbe Interact. 5: 81.