

# BIOLOGICAL CONTROL OF PIERCE'S DISEASE WITH NON-PATHOGENIC STRAINS OF *XYLELLA FASTIDIOSA*

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## ABSTRACT

This project is to construct and test nonpathogenic strains of *X. fastidiosa* derived from a pathogenic Pierce's disease strain for competitive exclusion of the pathogen in grapevines. Potential virulence genes were selected from comparative genome sequence analyses as well as DNA macroarray studies of differential gene expression. A more comprehensive analysis of differential gene expression with a DNA microarray approach is an outcome of this project and is funded under a new grant. Disruption of xanthan gum production by mutations in gumD and gumH resulted in less of a slime layer and fewer adhering cells than the wild-type strain on plastic and wood surfaces. However, when biofilm formation was quantitatively measured in polystyrene microtiter plates, both gumD and gumH mutants formed significantly more biofilm than the wild-type *X. fastidiosa*. In addition, the disruption of rsmA resulted in significantly more biofilm than the parent strain. Virulence assays in grapevine are still in progress, but gum mutants showed fewer symptoms than the parent strain in an alternative host assay. The analysis of potential virulence genes has also had side benefits for epidemiological work on *Xylella* by providing new primer sets for differentiate certain host strains of *X. fastidiosa*.

## INTRODUCTION

A general approach that is being considered to manage Pierce's disease is biological control of the bacterial pathogen. Specific biological control approaches include the use of antagonistic endophytic bacteria isolated from the xylem of grape, bacteriophages, and interference with bacterial intercellular signaling. Another approach is the possible use of a nonpathogenic strain of *X. fastidiosa* derived from a pathogenic Pierce's disease strain for competitive exclusion of the pathogen in grapevines. This concept has certain advantages over other biological control approaches and considerable precedent in bacterial, fungal, and viral systems, including the biological control of xylem-inhabiting bacterial pathogens.

Colonization and protection of plants with less virulent or completely nonpathogenic strains of plant pathogens has been demonstrated in a number of bacterial (Wilson and Lindow, 1993), fungal (Sneh, 1998), and viral systems (Fulton, 1986). Some studies relied on naturally occurring avirulent strains, while other researchers have developed defined nonpathogenic mutants of pathogenic strains for this purpose, following the expectation that they would have the same ecological requirements for growth and are therefore ideal competitors (Wilson and Lindow, 1993). Another advantage of this approach is the specificity of the interaction, which reduces or eliminates possible deleterious effects on non-target organisms (Cook et al., 1996). This concept has also been demonstrated for the xylem-inhabiting vascular wilt pathogen, *Ralstonia* (*Pseudomonas*) *solanacearum* (Frey et al., 1994). Nonpathogenic mutants of this pathogen still colonized vascular tissues and resulted in high protection rates against the pathogenic strain. Our goal is to test this concept of competitive exclusion with nonpathogenic, or reduced virulence mutants, of the xylem-inhabiting *Xylella fastidiosa* for biological control of Pierce's disease.

## OBJECTIVES

1. Construct deletion mutations in putative virulence genes of
2. Test mutant strains for virulence in grapevines.
3. Test mutant strains for biological control of pathogenic strains in grapevines.

## RESULTS AND CONCLUSIONS

### Results

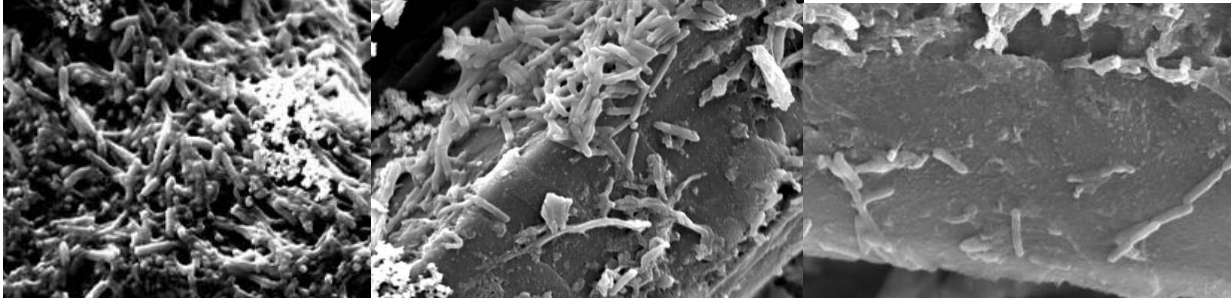
#### *Selection of candidate virulence genes*

We have utilized the full genome sequences of *Xylella fastidiosa* strains (Bhattacharyya, A., et al. 2002; Simpson et al., 2000; Van Sluys et al., 2003) to select open reading frames specifying putative pathogenicity and virulence factors. In addition, we constructed a DNA macroarray with about 100 of these genes to analyze their expression in different *Xylella* strains *in planta* and *in vitro* (Hernandez-Martinez et al., 2002). This work follows the hypothesis that many genes important in virulence and symptom will be differentially expressed in the bacterium grown in culture vs. during infection of plants. We have shown that these genes are expressed to varying degrees ranging from none to very high. However, since over 50% of the *Xylella fastidiosa* genome consists of genes with no known function, a more comprehensive approach toward the identification of virulence genes is necessary. We have recently obtained funding from the CDFR in a separate project to continue this work through the use of full genome microarrays.

### **Mutational analysis of virulence genes**

Construction of several virulence gene mutants has been done using the EZTN transposon or by insertional cloning of antibiotic resistance cassettes to create disruptive insertions into cloned genes that were amplified by PCR based on genomic sequence. The mutated clones have been subcloned into pUC129 and gene knockouts were constructed through homologous recombination in *Xylella fastidiosa*. Among virulence genes included in mutational studies are those of the *gum* operon, for which we have recently constructed successful knockout mutations in *Xylella*. Other genes we are manipulating include a number of regulatory genes that likely control other virulence factors, such as RsmA.

Disruption of xanthan gum production by mutations in *gumD* and *gumH* resulted in less of a slime layer and fewer adhering cells than the wild-type strain on plastic and wood surfaces.



Wild-type on wood surface

*gumD* mutant on wood

*gumH* mutant on

However, when biofilm formation was quantitatively measured in polystyrene microtiter plates (Espinosa-Urgel et al., 2000; O'Toole and Kolter, 1998), both *gumD* and *gumH* mutants formed significantly more biofilm than the wild-type *X. fastidiosa*. In addition, the disruption of *rsmA* resulted in significantly more biofilm than the parent strain.

Virulence assays on grapevine have not been consistent in our laboratory, so assessment of the effects of these mutations on virulence of *Xylella* is still underway. We are also working to develop more rapid virulence assays with other hosts. Chorotic and necrotic symptoms were obtained by infiltration of Chenopodium leaves with wild-type Pierce's disease strains of *X. fastidiosa*, but the gum mutants appeared to cause fewer or no symptoms on this host. The *rsmA* mutant caused similar symptoms to the wild-type strain. Since RsmA is a global regulator, the effects of the *rsmA* mutation are being studied at a broader level using macroarray/microarrays to determine the expression profiles of other genes in *Xylella* in the *rsmA* mutant vs. wild type.

**Additional findings from the project.** The analysis of potential virulence genes has also had side benefits for epidemiological work on *Xylella*. We previously reported that while the *gumB* gene was conserved in all *Xylella* strains tested, we could digest the PCR product of this gene with frequent-cutting restriction endonucleases and differentiate certain host strains of *X. fastidiosa*. This was particularly important in our project on assessing inoculum sources of the grape strain in southern California, where we needed to differentiate grape strains from the oleander strain that is also present but not a threat to vineyards. We subsequently found that a number of our primer sets that we had designed for the macroarray project above were able to differentially amplify DNA from different host strains. We now have the ability to rapidly differentiate strains by PCR amplification with differential primers from cultures or often directly from infected tissues. We also used these primers to detect whether individual glassy-winged sharpshooters can carry and transmit multiple strains of *X. fastidiosa*.

### **CONCLUSIONS**

We have not yet achieved the original goal of this project, to produce nonpathogenic mutants and test them for biological control of Pierce's disease. However, we have made progress in identifying potential virulence genes and in genetically modifying this difficult bacterium. Xanthan gum genes appear to play a role in colonization of abiotic surfaces and affected virulence in at least one plant assay. Our new project that involves a much comprehensive approach toward the identification of virulence genes using a DNA microarray approach will greatly enhance our ability to achieve the goals of this biological control project. Mutants generated in that study will continue to be assessed for virulence and tested for the ability to reduce Pierce's disease symptoms through competitive exclusion.

### **REFERENCES**

- Bhattacharyya, A., et al. 2002. Whole-genome comparative analysis of three phytopathogenic *Xylella fastidiosa* strains. Proc. Natl. Acad. Sci. USA 99:12403-12408.
- Cook, J., W.L., Bruckart, J.R. Coulson, M.S. Goettel, R.A. Humber, R.D. Lumsden, J.V. Maddox, M.L. McManus, L. Moore, S.F. Meyer, P.C. Quimby, J.P. Stack, and J.L. Vaughn. 1996. Safety of microorganisms intended for pest and plant disease control: A framework for scientific evaluation. Biological Control. 7:333-351.
- Espinosa-Urgel, M., A. Salido, and J. Ramos. 2000. Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. J. Bacteriol. 182:2363-2369.

- Frey, P., P. Prior, C. Marie, A. Kotoujansky, D. Trigalet-Demery, and A. Trigalet. 1994. Hrp- mutants of *Pseudomonas solanacearum* as potential biocontrol agents of tomato bacterial wilt. *Appl. Environ. Microbiol.* 60:3175-3181.
- Fulton, R.W. 1986. Practice and precautions in the use of cross protection for plant viruses. *Annu. Rev. Phytopathol.* 24:67-81.
- Hernandez-Martinez, R., C. K. Dumenyo, and D. A. Cooksey. 2002. Gene expression profile of pathogenicity factors in *Xylella fastidiosa*. *Phytopathology (Abstr.)* 92:S36.
- O'Toole, G.A., and R. Kolter. 1998. Initiation of biofilm in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathway: a genetic analysis. *Mol. Microbiol.* 28:449-461.
- Simpson, A.J. G., F.C. Reinach, P. Arruda, F.A. abreu, M. Acencio, R. Alvarenga, L.M.C. Alves, J.E. Araya, G.S. Baia, C. S. Baptista, et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa* *Nature* 406:151-159.
- Sneh, B. 1998. Use of non-pathogenic or hypovirulent fungal strains to protect plants against closely related fungal pathogens. *Biotechnology Advances* 16:1-32.
- Van Sluys, M.A., et al. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *J. Bacteriol.* 185:1018-26.
- Wilson, M., and S.E. Lindow. 1993. Release of recombinant microorganisms. *Annu. Rev. Microbiol.* 47:913-944.

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