# ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

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**Reporting period**: The results reported here are from work conducted from November 8, 2003 to October 31, 2004.

## ABSTRACT
*Xylella fastidiosa* Temecula sequence information reveals no type III, but two type I secretion systems, both dependent on a single *tolC* homologue. Marker exchange mutagenesis using pGEM-T as delivery vector and *nptII* as marker was employed to generate *tolC* disruptions. PCR and Southern blot analyses confirmed marker exchange at the *tolC* locus. Grape (var. Carignane) plants inoculated with mutant (*tolC*::*nptII*) strains exhibited no symptoms of PD, indicating that pathogenic ability of PD strains may be dependant on *tolC* and type I secretion. Complementation assays using *tolC* in the mutant strains are in progress to help confirm this hypothesis.

## INTRODUCTION
*Xylella fastidiosa* (*Xf*) is a xylem-inhabiting Gram-negative bacterium that causes serious diseases in a wide range of plant species (Purcell & Hopkins, 1996). Two of the most serious of these are Pierce's Disease (PD) of grape and Citrus Variegated Chlorosis (CVC). The entire genomes of both PD and CVC have been sequenced (Simpson et al., 2000). Availability of the complete genomic DNA sequence of both a PD and a CVC strain of *Xf* should allow rapid determination of the roles played by genes suspected of conditioning pathogenicity of CVC and/or PD. For example, analyses of the CVC and PD genomes showed that there was no type III secretion system, but there were at least two complete type I secretion systems present, together with multiple genes encoding type I effectors in the RTX (repeats in toxin) family of protein toxins, including bacteriocins and hemolysins. RTX proteins form pores in lipid bilayers of many prokaryotic and eukaryotic species and cell types; at least one is associated with pathogenicity in plants. However, lack of useful DNA cloning vectors and/or techniques for working with either CVC or PD strains have impeded progress in functional genomics analyses.
Last year we focused on attempts to perform marker-interruption in the PD strains using various suicide vectors and techniques. Although marker-interruption using suicide vectors is normally an efficient, single crossover event in many bacteria, repeated marker-interruption attempts with *X. fastidiosa* in our lab and in others have failed (Feil et al., 2003; Gaurivaud et al., 2001; Guilhabert et al., 2001). Since marker-exchange has now been reported to be successful with *X. fastidiosa* (Feil et al., 2003), we report here the utility of marker-exchange to generate *tolC* interruption in *X. fastidiosa* PD strain and the role of *tolC* in pathogenicity.

## OBJECTIVES
The primary objective of this work is to determine the effect of type I secretion gene knockouts on pathogenicity of a PD strain on grape.

## RESULTS
*X. fastidiosa* strain Temecula (Guilhabert, 2001), was grown in PD3 (Davis et al., 1981) and confirmed to be pathogenic on Madagascar periwinkle and Grape (var. Carnignane). Symptoms appeared after 2 months. Marker-exchange mutagenesis of *tolC* was performed using pJR6.3. This plasmid carries an internal fragment of PD1964 (*tolC* of Temecula) interrupted at an internal *BamH*I site by an *nptII* gene from pKLN18 (kindly provided by K. Newman and S. Lindow). One microgram of pJR6.3 DNA was use to transform electrocompetent cells (prepared by washing 10 ml of four day old PD3 broth culture of *X. fastidiosa* Temecula, serially with 10, 5, 2 ml of ice-cold deionized water and resuspending in 100 µl the same) by electroporation (1mm gap cuvettes; 1800 volts). Electroporated cells were allowed to recover in 1 ml of PD3 broth for 24 hours at 28 $^{o}$C and were spread on PD3 plates amended with kanamycin (50 µg/ml). Plates were incubated at 28 $^{o}$C for 10 days and single colonies were screened for interruption of *tolC* by PCR analysis and by Southern blot hybridization. The results (Figure 1) indicate that *tolC* gene can be disrupted and marker-exchange was efficient in generating gene-disruptions in *X. fastidiosa*.

Plant inoculation assays were performed in collaboration with Dr. Don Hopkins, at the Mid-Florida Research and Education Center, Apopka, Florida. Grape plants (var. Carnignae) were inoculated with the wild-type *X. fastidiosa* Temecula strain and the mutant (*tolC*::*nptII*) strain in triplicates. The plants were maintained under green-house conditions and were evaluated for Pierce's disease symptoms at 60 and 90 days after inoculation. The results (Figure 2) showed loss of pathogenicity of *X. fastidiosa tolC::nptII* mutants on grapes. All the three plants inoculated with the wild-type Temecula strain exhibited typical PD.

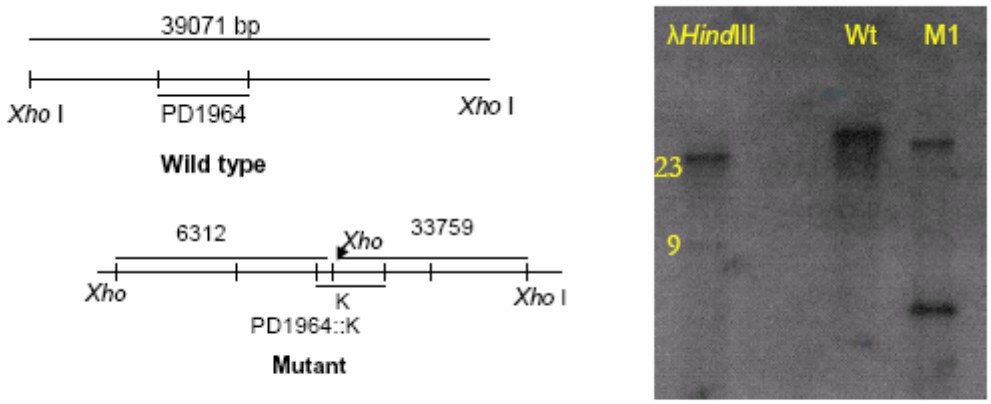


**Figure 1:** Southern blot of *tolC::aptII* mutant (MI) and wild type total DNA cut with *XhoI*. *XHO* I is internal to the *nptII* gene. The probe was PD1964 (wild type *tolC* from Temecula, 1459 bp).

For complementation assays, PD1964 was amplified by PCR, cloned into pGEM-T, verified by sequencing and sub-cloned into pUFR47, a wide host range replicon based on *repW* (DeFeyter et al., 1993) and pBBR1MCS-5, a wide host range replicon based on a *Bordatella* replication origin (Kovach et al., 1995). pUFR47 and pBBR1MCS-5 containing the entire *tolC* gene are referred as pJR13.2 and pJR22.2 respectively. Non-pathogenic Temecula mutant M1 was transformed with pJR13.2 and pJR22.2 independently by electroporation as described above. The cells were recovered in 1 ml of PD3 broth for 6 hours and were spread on PD3 plates amended with Gentamycin (5 µg/ml). The plates were incubated at 28 °C for 10 days and single colonies were screened for the presence of pJR13.2 /pJR22.2 and also for the integrity of *nptII* integration, by PCR assay. Grape plants (var Carnignane) were inoculated in triplicates with wild-type *X. fastidiosa* Temecula, mutant M1, M1/pJR13.2, and M1/pJR22.2 strains and are currently being monitored for Pierce's disease symptoms. Preliminary results indicate possible complementation using both vectors. These results need to be repeated and confirmed, and these tests are currently in progress.

**Figure 2:** Grape var. Camignane 90 days after inoculation with wild type Temecula (left) and *tolC::nptII* mutant M1 (right).

**CONCLUSIONS**

Type I secretion gene *tolC* (PD1964) of *X. fastidiosa* Temecula was disrupted by marker exchange mutagenesis. The mutant strains lost all pathogenicity, indicating a critical role of tolC in pathogenicity of *X. fastidiosa* on grape. Complementation assays are in progress and could result in a demonstration of a role of *tolC* in pathogenicity. If such a role can be confirmed, it would indicate several important molecular targets for potential PD control methods.

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**FUNDING AGENCIES**

Funding for this project was provided by the University of California Pierce's Disease Grant Program.