

ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted September 31, 2004 to October 1, 2005.

ABSTRACT

Xylella fastidiosa (Xf) 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. Further, these *tolC*⁻ mutant strains were unable to multiply in mechanically inoculated grape plants, indicating that strain survival in grape may be dependent on type I efflux pump activity. Both *in planta* growth and pathogenic symptoms were restored when the mutant was transformed with a broad host range vector expressing wild type *tolC*. This is the first report of a completely non-pathogenic mutant of Xf due to a single gene knockout and it is also the first report of complementation of a gene knockout using an autonomously replicating plasmid in Xf.

INTRODUCTION

Xylella fastidiosa (Xf) is a xylem-inhabiting Gram-negative bacterium that causes serious diseases in a wide range of plant species (Purcell and 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 used marker-interruption to generate site directed mutations in *tolC* in Xf PD strain Temecula and found that *tolC* was absolutely required for PD pathogenicity. This year we complemented the *tolC* mutation using an autonomously replicating shuttle vector.

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

Xf 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 *Bam*HI site by an *nptII* gene from pKLN18 (kindly provided by K. Newman and S. Lindow). One microgram of pJR6.3 DNA was used to transform electrocompetent cells (prepared by washing 10 ml of four day old PD3 broth culture of Xf 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 °C and were spread on PD3 plates amended with kanamycin (50 µg/ml). Plates were incubated at 28 °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 Xf.

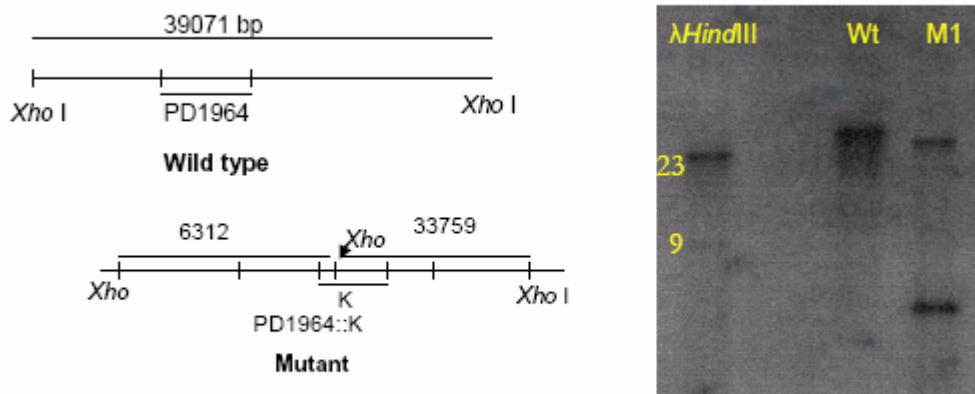


Fig. 1: Southern blot of *tolC::nptII* mutant (M1) and wild type total DNA cut with *Xho*I. *Xho* I is internal to the *nptII* gene. The probe was PD1964 (wild type *tolC* from Temecula, 1459 bp).

Plant inoculation assays were performed in collaboration with Don Hopkins, at the Mid-Florida Research and Education Center, Apopka, Florida. Grape plants (var. Carnignae) were inoculated with the wild-type *Xf* 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 *Xf tolC::nptII* mutants on grapes. All the three plants inoculated with the wild-type Temecula strain exhibited typical PD.



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

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 Carnignae) were inoculated in triplicates with wild-type *Xf* Temecula, mutant M1, M1/pJR13.2, and M1/pJR22.2. Both pJR13.2 and pJR22.2 complemented the mutant *tolC* strain M1, but symptoms were stronger with pJR22.2 in repeated experiments (Figure 3).



Figure 3. Leaves above the inoculation point on the stem of grape plants inoculated with wild type, *tolC*-mutant and *tolC*-mutant complemented using an autonomously replicating vector. A, wild-type PD strain Temecula, B, *tolC*-mutant strain M1 and C, M1 /pJR22.2. Photo taken 60 days after inoculation.

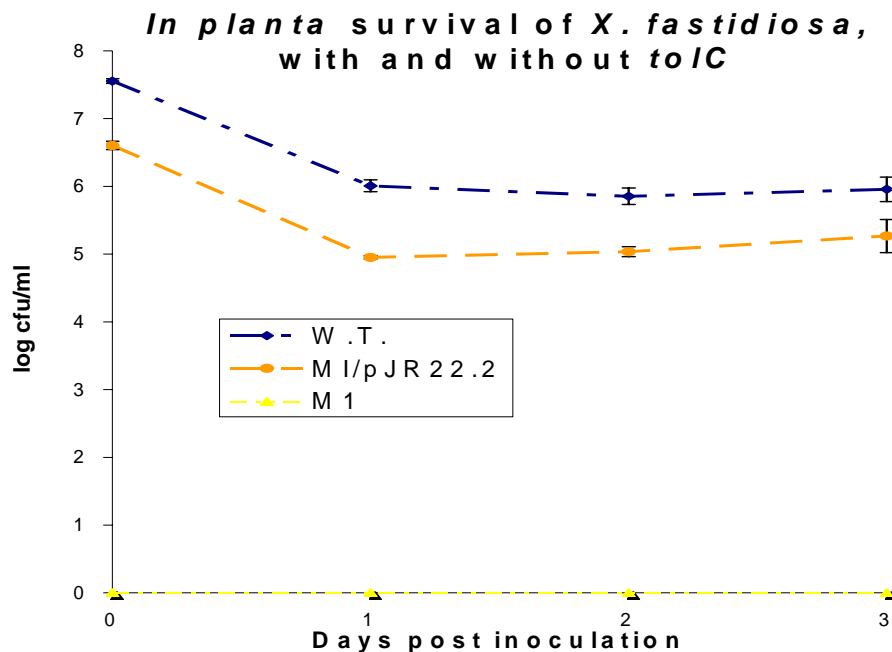


Figure 4. *In planta* survival of *Xf* Temecula wild-type (W.T.) and the complementation of *tolC*⁻ mutant strain M1 with pJR22.2 (*tolC*⁺).

***In planta* stability of pJR22.2.**

Perhaps surprisingly, mutant M1 could not be re-isolated from inoculated grape xylem, even 2 hrs after inoculation. It seems likely that grape xylem sap is toxic to M1. However, eight randomly isolated colonies from the sap of Carignane grape plants inoculated 60 days earlier with M1/pJR22.2 were positive for the presence of the vector by PCR analysis. pJR22.2 was isolated from two of the positive colonies and was confirmed by restriction digestion (data not shown). Stability studies *in planta* demonstrate that *tolC* is required for the survival of *Xf* in grape (Figure 4). Taken together with the re-isolation of the plasmid pJR22.2, these stability studies also demonstrate that the *Bordetella* replication origin on pBBR1MCS-5 is sufficiently stable as an autonomously replicating vector in *Xf* strain Temecula over a 60 day period to be useful for complementation (Figure 4).

CONCLUSIONS

Type I secretion gene *tolC* (PD1964) of *Xf* Temecula was disrupted by marker exchange mutagenesis. The mutant strains lost all pathogenicity and were rapidly killed in grape, indicating a critical role of *tolC* in both pathogenicity and survival of *Xf* in grape. Complementation assays using an autonomously replicating vector demonstrated: 1) that an autonomously replicating vector is available for complementation studies in *Xf* and 2) that *tolC* is required for pathogenicity, confirming a role of Type I secretion in both survival and pathogenicity of *Xf* in grape.

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FUNDING AGENCIES

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