ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

Project Leader:
Dean W. Gabriel
Plant Pathology Department
University of Florida
Gainesville, FL 32611

Cooperator:
Richard Lee
Citrus Research and Education Center
University of Florida
Lake Alfred, FL

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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.

There have been only three reports of transformation of *Xf*; one with a commercial transposase-transposon complex (Guilhabert et al. 2002) and two others with narrow host range plasmids that utilize origins of replication derived from *Xf*. One plasmid is an integrative vector that carries the CVC chromosomal origin of replication that provides a brief period of unstable replication in *Xf* (Monteiro et al. 2001). The second is a replicative shuttle vector that carries the pUC origin for replication in *Escherichia coli* and a rolling circle replicon derived from a cryptic CVC plasmid (Quin and Hartung 2001). However, this plasmid proved unstable in the absence of antibiotic selection.

We describe here the transformation of two *Xf* /PD strains using the small, stable, broad host range shuttle vector, pUFR047 (De Feyter et al. 1993). This vector is one of a series of well characterized conjugational shuttle vectors based on repW and is widely used to shuttle DNA fragments from *E. coli* to various species and strains of *Xanthomonas*, where the vector is stabilized in the absence of antibiotic selection by the parA locus (De Feyter et al.1990). This is the first report of stable transformation of any *Xf* strain using a broad host range cloning vector.

OBJECTIVES

This is a two year proposal with three objectives: 1) develop an effective functional genomics tool kit for efficient transformation and gene knock-out experiments in a PD strain (Year 1); 2) determine culture conditions for activation of type I secretion (Year 2), and 3) determine the effect of type I secretion gene knockout experiments on pathogenicity of a PD strain on grape (Year 2).

RESULTS AND CONCLUSIONS

PD strains of *X. fastidiosa*, PD-A (Hopkins 1985) and Temecula, (Guilhabert et al. 2001), were grown in PD3 (Davis et al. 1981)medium supplemented with MOPS (3-4[morphomino] propane sulfonic acid), (Gabriel et al. 1989) Both strains were confirmed to be pathogenic on Madagascar periwinkle. Symptoms appeared after 3 months. pUFR047 was transferred from *E. coli* DH5 to the spontaneous Rif resistant PD-1R strain by triparental conjugation. Selection was on PW-H containing gentamycin (Gm), 1.5 mg/L, and Rif, 75 mg/L. Transfer by conjugation was very inefficient and difficult to reproduce due to overgrowth of *E. coli* donor and/or helper colonies resistant to rifamycin; only a few PD-1 exconjugants were rescued from the selection plates. Presence of pUFR047 in the transformants was confirmed by agarose gel electrophoresis of alkaline lysis minipreps of the PD-1 exconjugants, transformation of *E. coli* with these minipreps followed by detection of pUFR047 in the transformants, and PCR using IncW repA-specific primers and miniprep DNA. In Figure 1A is shown the results of an alkaline lysis miniprep of a PD-1 transformant. By contrast with conjugal transfer, pUFR047, with and without a 3 kb DNA insert, was readily transferred by electroporation into both the PD-1 and Temecula at a frequency of ca. 50 transformants/ microgram DNA. PD cells were harvested by centrifugation, washed twice and resuspended in 0.3 ml of 10% glycerol. The cells were electroporated with 0.5-1 g of plasmid DNA at 1.8 kV to generate a pulse of 5.8 to 6.0 ms. Cells were then incubated at 28^{o}C for 24 h with constant shaking at 100 rpm and then selected by plating on PD3 agar medium supplemented with 2 mg/L Gm.

Maintenance of pUFR047 was measured in the absence of antibiotic selection for 30 generations. Cultures sampled at the beginning of each cycle were plated on both selective and nonselective medium by serial 10-fold dilutions in MOPS buffer, pH 6.2, containing 0.001% Silwet L-77 to disperse clumps. Use of Silwet L-77 greatly facilitated reproducibility in the cell counts, and did not appear to be toxic to either PD strain at concentrations used (data not shown). After 30 generations of growth, 48% of the cells retained the plasmid in the absence of antibiotic selection (Figure 2).

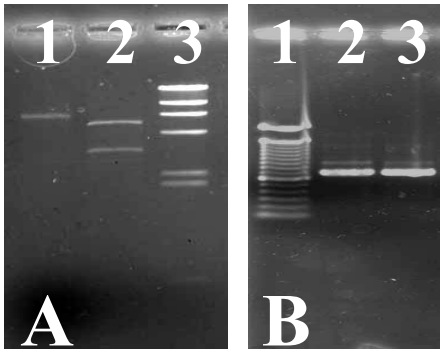

Figure 1. Transformation of PD-1using pUFR047. A. Plasmid DNA extracted from a single colony of PD-1 after transformation with pUFR047. Lanes: 1) undigested; 2) digested with *Bgl*II, 3) Lambda digested with *Hin*dIII. B. PCR product amplified by *X. fastidiosa*-specific primers RST31 and RST33 1) 100 bp DNA ladder; 2) PD-A; 3) PD-A /pUFR047.

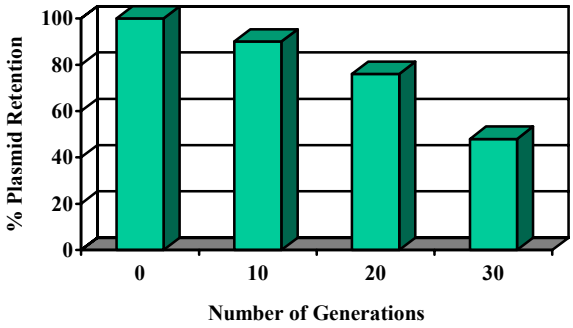

Figure 2. Plasmid maintenance in PD-1 in broth culture. Cultures were grown with antibiotic to late-exponential-growth phase, and diluted 1/1000 into fresh broth in the absence of antibiotic. Growth was continued to late-exponential phase, and the dilution growth procedure repeated for three cycles.

REFERENCES

Davis, M.J., W.J. French and N.W. Schaad. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr Microbiol. 6:309-314.

De Feyter, R., C.I. Kado, and D.W. Gabriel. 1990. Small, stable shuttle vectors for use in *Xanthomonas*. Gene 88:65-72.

De Feyter, R., Y. Yang and D.W. Gabriel. 1993. Gene-for-genes interactions between cotton *R* genes and *Xanthomonas campestris* pv. malvacearum *avr* genes. Mole Plant-Microbe Inter. 6:225-237.

Gabriel, D.W., M.T. Kingsley, J.E. Hunter, and T.R. Gottwald. 1989. Reinstatement of *Xanthomonas citri* (*ex* Hasse) and *X. phaseoli* (*ex* Smith) and reclassification of all *X. campestris* pv. citri strains. Intern. J. System. Bacteriol. 39:14-22.

Guilhabert, M.R., L.M. Hoffman, D.A. Mills and B.C. Kirkpatrick. 2001. Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. Mole Plant-Microbe Inter. 14:701-706.

Hopkins, D.L. 1985. Physiological and pathological characteristics of virulent and avirulent strains of the bacterium that causes Pierce's Disease of grapevine. Phytopathology 75:713-717.

Monteiro, P.B., D.C. Teixeira, R.R. Palma, M. Garnier, J-M. Bove and J. Renaudin. 2001. Stable transformation of the *Xylella fastidiosa* Citrus Variegated Chlorosis strain with oriC plasmids. Appl. Environ. Microbiol. 67:2263-2269.

Purcell, A. H. and D.L. Hopkins. 1996. Fastidious, xylem limited plant pathogens. Annu. Rev. Phytopath. 34:131-151.

Qin, X. and J.S. Hartung. 2001. Construction of a shuttle vector and transformation of *Xylella fastidiosa* with plasmid DNA. Curr. Microbiol. 43:158-162.

Simpson, A. J. G. et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151-159.

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