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, 2002 to October 31, 2003.

ABSTRACT
In an effort to make knockout mutations of a TolC homologue, marker interruption was attempted using two different vectors, one of them newly constructed for the purpose. Although these vectors worked well in *Xanthomonas*, repeated efforts to obtain such mutants in *X. fastidiosa* failed. In an attempt to utilize splice-overlap PCR to obtain marker eviction and generate marker-free exconjugants, a marker-eviction vector was constructed. Again, although the vector and method worked well in *Xanthomonas,* the method failed in *X. fastidiosa.* Marker exchange experiments are currently underway.

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 described the transformation of two *X.f* PD strains using the small, stable, broad host range shuttle vector, pUFR047 (De Feyter et al., 1993). Both the vector and the transformed PD strains are available upon request. The vector is relatively stable in both PD strains.

This year we have 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; Monteiro et al., 2001; Guilhabert et al., 2001). We report here that attempts to use three different suicide plasmid constructs that we have successfully used for marker interruption or marker-eviction in *Xanthomonas* have all failed, despite repeated attempts. Since marker-exchange has now been reported to be successful with *X. fastidiosa* (Feil et al., 2003), we recently turned to this method.

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).
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, 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. Because of reports that others had problems with electroporation of pUFR047 into PD strains, we again confirmed transfer from *E. coli* DH5∀ to the spontaneous Rif resistant PD-1R strain by electroporation.

We attempted knockout mutagenesis on TolC, indicated below from CVC as XF2586, and also found in the PD strain as PD1964. We constructed marker-interruption plasmids using an internal fragment of PD1964 cloned by PCR in pUFR012,

which we had used for exactly the same purposes in *Xanthomonas* (Kingsley et al., 1993). Initial experiments failed. We then tried to knockout *fimA* using the same method. Marker-exchange had been reported for *fimA* at last year's symposium (Feil et al., 2003). These experiments also failed. We thought it might have to do with our level of kanamycin resistance, so we obtained the same *nptII* gene as had been used by Feil et al (2003) and created pAC3 (see Figures 1-3 below). pAC3 differs from pUFR012 principally by having the *nptII* gene transcribed in the same direction as *lacZ* (thus giving somewhat higher levels of resistance to kanamycin).

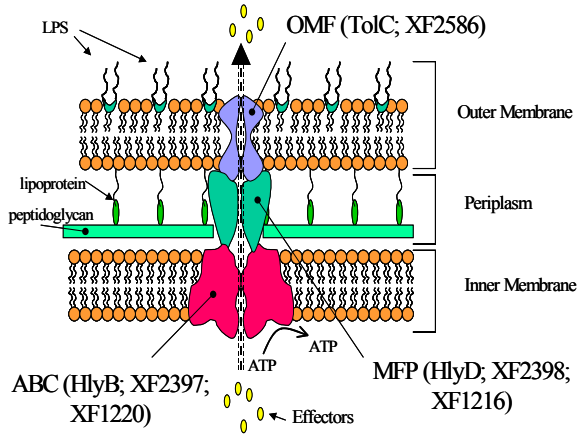

Figure 1. Type I Secretion system, including ABC (ATP binding cassette), MFP (membrane fusion protein) and OMF (outer membrane factor).

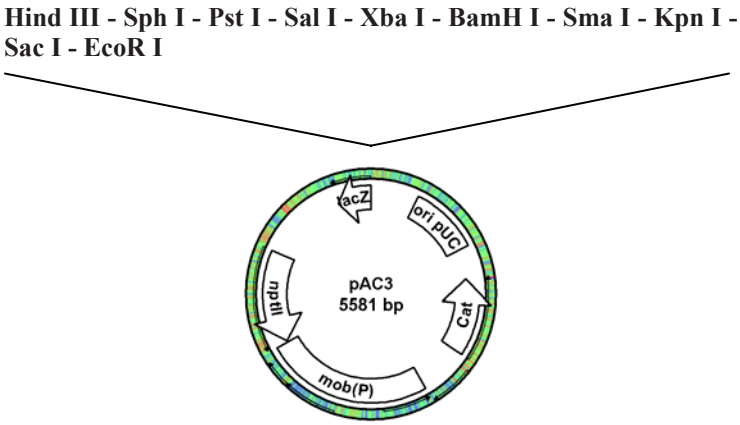

Figure 2. Suicide vector pAC3, with enhanced kanamycin resistance.

Again, we could not obtain marker-interruption mutants of either gene target. Simultaneously, we created another vector in order to attempt splice-overlap PCR deletions (marker eviction) of the target. This method involves constructing a gene deletion *in vitro* using splice-overlap extension PCR to generate and then fusing two ~500 bp flanking DNA fragments (Horton, 1995), and then site-specifically inserting the deletion into the genome in place of the wild-type gene using a two-step, SacB-assisted, marker-eviction mutagenesis (Hoang et al., 1998; Reyrat, 1998). We constructed pUFR080 for the purpose. Again, although the method works well in our hands in several different xanthomonads (unpublished), the method requires initial formation of a cis-merodiploid, and it failed in the PD strains we used.

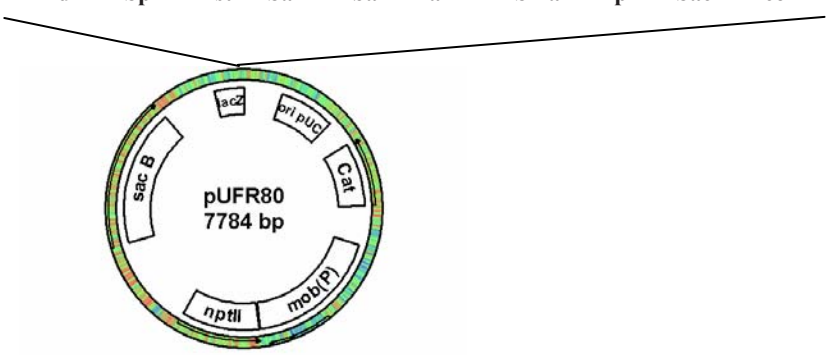

Figure 3. Suicide vector pUFR080, with enhanced kanamycin resistance and levan sucrase gene, used for marker eviction mutagenesis.

The reasons for the failure of marker-interruption are unknown, but speculation centers on the possibility that the origin of replication (pUC) of the suicide vectors may interfere with chromosomal replication (Feil et al, 2003). We have since turned to marker-exchange mutagenesis, and have obtained a no cost extension on the project to complete the objectives.

CONCLUSIONS

Despite our expectations to the contrary, our repeated efforts to obtain marker interruption and marker eviction mutants failed. Both methods required the formation of cis-mero diploids using a pUC based vector, and confirm recently published observations of others. We have repeated electroporation of our repW based vector, pUFR047, and have a relatively stable vector that should useful for complementation.

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FUNDING AGENCIES

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