ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

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ABSTRACT
In previous work, marker exchange mutagenesis of the single *tolC* gene in Pierce's Disease (PD) strain Temecula (PD1964) was shown to result in a total loss of pathogenicity on grape. The tolC mutant strains were not recovered after inoculation into grape xylem, strongly indicating that drug efflux is critical to survival of this fastidious pathogen. The multidrug efflux role of TolC was investigated, and the *tolC* mutant strain M1 was found to be much more sensitive to antimicrobial compounds than the wild type Temecula strain. TolC in *Xyllela fastidiosa* (*Xf*) is the common outer membrane component of two drug efflux systems (AcrF/A and AcrC/D) and two "toxin" secretion systems (CvaA/B and HlyB/D). Knockout mutations of *acrD* and *acrF* resulted in reduced symptoms of pathogenicity, in keeping with a redundant role in drug efflux. Importantly, knockout mutations of *cvaA, cvaB* and *hlyB/D* also elicited reduced symptoms of pathogenicity, indicating a potentially offensive role for Type I secretion in conditioning *Xf* pathogenicity.

INTRODUCTION
In Gram-negative bacteria, multidrug resistance (MDR) efflux pumps are composed of three protein components, two of which are localized in the inner membrane, and one, TolC, that traverses both the periplasm and outer membrane (Koronakis et al. 2004). The process of MDR efflux is energy dependant and utilizes either ATP or the transmembrane electrochemical gradient. At least five characterized families of MDR efflux pumps exist in bacteria: the ATP-binding cassette (ABC) family (Davidson and Chen 2004), the major facilitator (MF) family (Pao et al. 1998), the small multidrug resistance (SMR) family (Paulsen et al 1997), the resistance-nodulation-cell division (RND) family (Tseng et al. 1999), and the multidrug and toxic compound extrusion (MATE) family (Brown et al. 1999). All utilize TolC as a common periplasm/outer membrane protein component.

In addition to (defensive) MDR efflux, TolC is also essential for type-I dependent secretion of a variety of degradative enzymes and offensive effectors, some of which are antibiotic and others involved in plant or animal pathogenicity. These include a variety of hydrolases (proteases, phosphatases, esterases, nucleases and glucanases) and protein toxins, including hemolysins and bacteriocins (Koronakis et al. 2004). Orthologs of *tolC* are highly conserved among diverse Gram-negative pathogenic bacteria, and strains typically carry multiple homologues per strain (Sharff et al. 2001), including all sequenced strains of *Xanthomonas, Pseudomonas* and *Ralstonia.*

Xylella fastidiosa 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). Analyses of the CVC and PD published genomes showed that there was no type III secretion (*hrp*) 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.

OBJECTIVES
There are two main purposes for Type I secretion (refer Figure 1) : multi-drug resistance or MDR efflux (in this case, defense against presumably anti-microbial chemicals in the xylem sap of grape), and toxin secretion (offensive, to promote pathogenicity). The outer membrane protein TolC has been shown to be essential for MDR efflux and pathogenicity in *Erwinia chrysanthemi* (Barabote et al., 2003) and more recently in *Xf* (Reddy et al., 2007). The purpose of this study was to further investigate the MDR efflux role and to determine a potential role for Type I secretion of (offensive) toxins by *Xf*.

1. Pathogenicity tests following disruption of four Temecula genes, in addition to *tolC*. The additional genes encode substrate-specific, periplasmic portions of at least four Type I Secretion Systems found in the Temecula genome:
   a. multi-drug resistance (MDR) efflux pumps, specifically, *mexC* (PD0202), *acrF* (PD0783) and *acrA* (PD0784), and
   b. toxin secretion, specifically, hemolysin (*hlyD*; PD1413) and colicin (*cvaA*; PD0496).
2. Assay the minimum inhibitory concentration of agents known to have an effect on bacteria that have been compromised in MDR efflux capability, including a) the detergent SDS, b) the hydrophobic chemical DOC, c) the antimicrobial agent from Rhubarb, Rhein, d) the isoflavonoid genistein, e) the alkaloid berberine and f) the grape phytoalexin resveratrol. If any of these assays are successful in inhibition of mutants, they may be very useful to help confirm complementation, since they would be more rapid assays than pathogenicity tests. However, they would not be a substitute for the

pathogenicity tests needed to confirm potential offensive and/or defensive roles of the Type I secretion systems in PD strain Temecula.

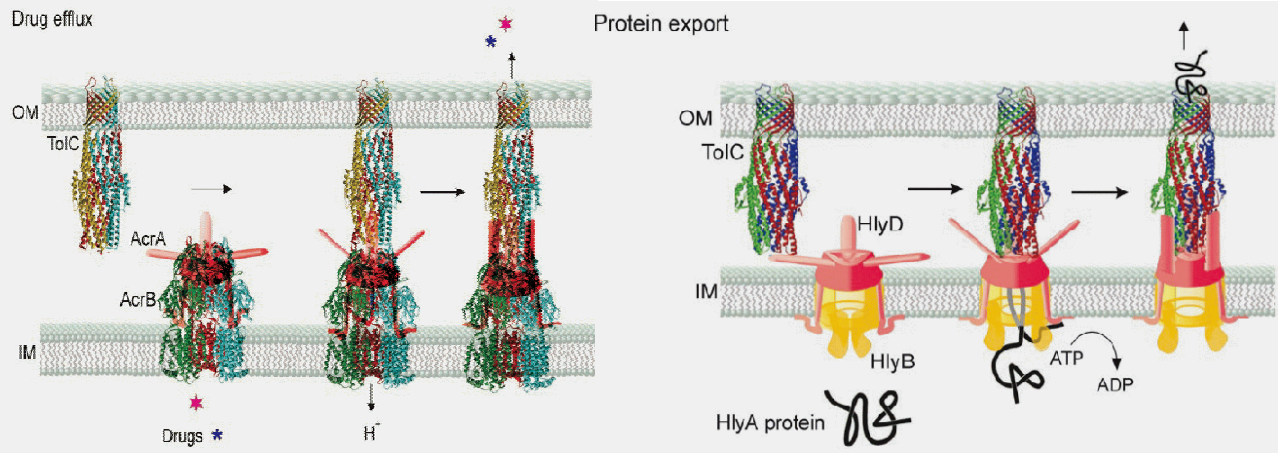

Figure 1. Type I machine for MDR ("Drug) efflux in *Xf* utilize *tolC* and *acrF/A* or *acrC/D* (left). Type I machine for protein export or secretion in *Xf* utilize *tolC* and *cvaA/B* or *hlyB/D* (right). Figures from Koronakis et. al. (2004).

RESULTS

1. **Pathogenicity tests following disruption of:**

a. MDR efflux pump genes *mexC* (PD0202), *acrF* (PD0783) and *acrA* (PD0784). The MDR efflux system genes *mexC* (PD0202) and *acrF* (PD0783) were successfully disrupted by marker exchange as described (Reddy et al., 2006) and used in assays described below. Despite many repeated attempts, we failed to disrupt *acrA* (PD0784); it is possible that *acrA* knockouts are lethal on the growth media used for selection. Since we have confirmed a critical role for MDR efflux in Reddy et al. (2007) and using *acrF*, we have dropped further attempts to disrupt *acrA*.

Plant inoculation assays using *mexC* and *acrF* 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 *Xf* Temecula strain and the mutant (*mexC*::*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. Not surprisingly, the *mexC::nptII* and *acrF::nptII* mutants on grapes had lost pathogenicity. All plants inoculated with the wild-type Temecula strain exhibited typical PD (not shown).

b. Protein export mutants *acrF* (PD0783), *acrD* (PD1404), *hlyBD;* PD1412-1413*), cvaA (*PD0496*) and cvaB* (PD0499). Mutations at these loci were generated using *nptII* as the marker and pGEM-T as the delivery vector. The mutants were verified Southern blot analysis and by using PCR analysis as described (Reddy et al., 2007; not shown).. Plant inoculation assays were performed as above. **All mutants listed were less pathogenic than the wild type, even after 90 days (refer Figure 2).**

2. Both *tolC*- and *acrF*- mutants were much more sensitive to antimicrobial chemicals berberine (an alkaloid DNA intercalating agent), genistein (an isoflavone phytoalexin precursor), rhein (an anthraquinone), and also to the surfactant Silwet L-77 than the wild type Temecula strain**,** confirming Reddy et al. (2007) that MDR efflux is required by *Xf* for pathogenicity (Table I).

3. Neither hlyBD, cvaB nor cvaA were sensitive to the antimicrobial chemical berberine, in keeping with their presumed role in Type I protein export, rather than drug efflux. The colicin Type I system secretion protein *cvaA* (PD0496) was successfully disrupted by marker exchange as described (Reddy et al., 2006) and used in assays as described above. However, in one experiment to date (to be repeated two additional times), no reduction of pathogenicity was observed in comparisons with wild type Temecula. These results provide a very preliminary indication that colicins may not be strongly involved in elicitation of PD symptoms.

Despite repeated efforts, we have failed to date to disrupt *hlyD* (PD1413), predicted to be involved in hemolysin secretion. We do not yet understand the reasons for this, but are working to obtain this mutation, which is important to test the hypothesis that Type I secretion of hemolysin results in some of the symptoms of PD.

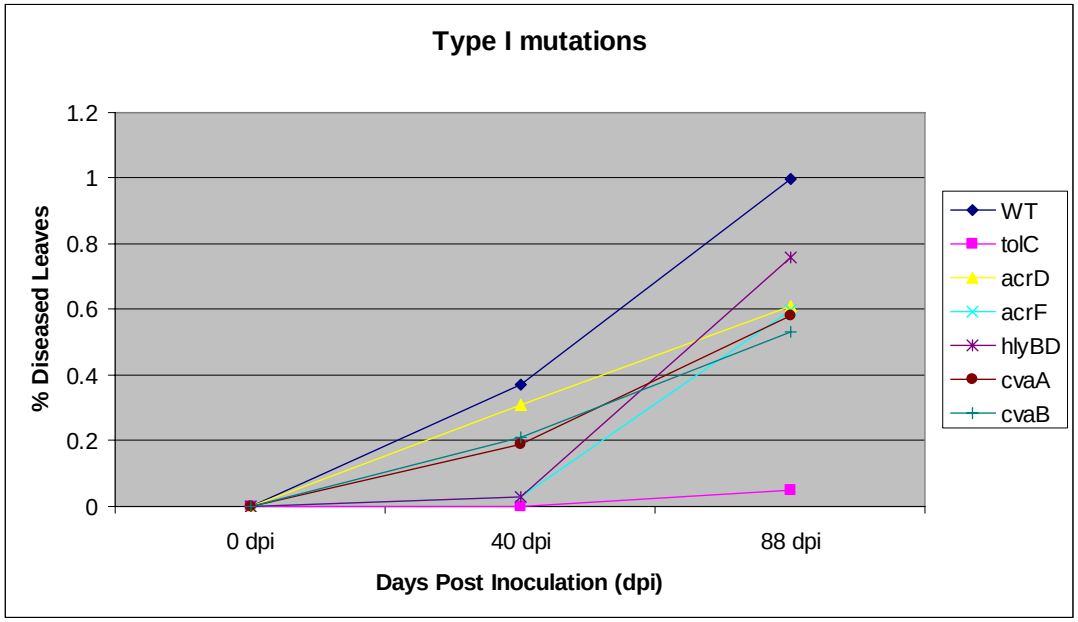

Figure 2. Grape var. Carignane inoculated with marker exchanged mutants of *acrF* (PD0783), *acrD* (PD1404), *hlyBD;* PD1412-1413*), cvaA (*PD0496*) and cvaB* (PD0499) and assessed for % diseased leaves at 40 and 88 days post inoculation. Complementation of tolC has been achieved, and complementation of the other mutations is in progress.

Table 1. Minimum inhibitory concentrations (MICs) of four phytochemicals and Silwet L-77 on Temecula, *tolC*– and *acrF*- Temecula mutants.

Chemical	MIC (µg/ml)			Fold difference
	Temecula	*tolC*⁻	*acrF*⁻	
Berberine	25	.02	.02	1000X
Genistein	5	0.5	NT	10X
Resveratrol	12.5	12.5	NT	1X
Rhein	50	.05	NT	1000X
Silwet L-77	>2000 ppm	20	20	>100X

CONCLUSIONS

This work demonstrates that not only is multidrug efflux critical to survival of *Xf* in grape, but also that Type I secretion is needed for full pathogenicity. Both multidrug efflux and Type I secretion depend upon a single tolC gene present in the *Xf* genome. Since TolC is exposed to the outer surfaces of bacteria, these combined results make TolC a vulnerable and specific target for both chemical and transgenic approaches to control Pierce's Disease.

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