ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

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ABSTRACT
In previous work, we discovered that: 1) *tolC* was absolutely required not only for pathogenicity, but also for survival of *Xylella fastidiosa* (*Xf*) strain Temecula in *Vitis vinifera* grapevines; 2) that the loss of multi-drug resistance (MDR) efflux through the Type I secretion system was the primary reason that *tolC*⁻ Temecula could not survive in grapevines; and 3) that gene knockouts of Type I system components associated with offensive Type I effector secretion also resulted in significant loss of pathogenicity. Both hemolysins and colicin V effectors are found in the Temecula genome. Surprisingly, knockout mutations of both 1) the Type I components associated with colicin secretion and 2) the three colicin V effectors in Temecula resulted in a significant loss of pathogenicity of the mutants, raising the possibilities that colonization and pathogenicity of grapevines by *Xf* involves the exclusion of other bacteria from the xylem niche and/or that these colicins might directly affect plant cells.

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 (**Figure 1**).

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 (*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). 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.

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 effector secretion (offensive, to either promote pathogenicity or secrete antimicrobial peptides). The outer membrane protein TolC has been shown to be essential for MDR efflux and pathogenicity in *Erwinia chrysanthemi* (Barabote et al., 2003) and in *Xf* (Reddy et al., 2007). Our general working hypothesis has been that *Xf* is a highly opportunistic species and that (at least) many of its strains have a very wide host range that is limited by at least two factors of unknown weight: the host range of its insect vectors, and its *intrinsic host range factors that may or may not elicit obvious pathogenic symptoms.* This working hypothesis was based on several observations. First, Freitag (1951) identified 75 asymptomatic host species for PD out of 100 plant species tested. In support of these older published test results, it is now clear that at least some PD, CVC and coffee leaf scorch strains of *Xf* can grow well in coffee, tobacco and periwinkle. These results all strongly indicate that some strains are capable of using the xylem sap of many plant species as growth medium, and may be restricted primarily by the lack of vectors to take them to other plant species.

Second, PD strains inoculated on grape both grow and elicit leaf scorch symptoms, but on tobacco cultivar Samsun, PD strains grow, but elicit no symptoms (Harakava Ph.D. thesis); on citrus, PD strains neither grow nor elicit symptoms (Hopkins, 1977). Similarly, the coffee strain does not grow or cause symptoms in citrus, but the CVC strain causes limited symptoms in coffee and mainly chlorosis in tobacco (Harakava, personal communication). These results indicate: 1) that

symptoms are host specific and induced only in a subset of host species; and 2) that the host specific symptom induction depends on the *Xf* strain as well as the plant species infected.

PD strains produce a host-specific elicitor of PD involving programmed cell death (PCD). Symptoms of leaf scorch in PD are not expected of a pathogen that merely blocks xylem vessels. Vascular wilts, such as periwinkle wilt, are more typical of xylem vessel blockage. Leaf scorch must be caused by another factor, long ago proposed to be a toxin (Raju and Wells, 1986). However, the limited evidence provided to support the toxin theory at the time was found to be an artifact caused by components of the culture medium (Goodwin et al., 1988) and the matter seemed settled (Hopkins, 1989) until very recently. At the 2007 Pierce's Disease Research Symposium, Gilchrist and colleagues reported evidence that *Xf* PD strains elicited PD and programmed cell death (PCD) or apoptosis in *V. vinifera*, but not *V. californica* grapes, and that anti-apoptotic genes cloned from grape variety Chardonnay and retransformed into plants using a strong (CaMV) promoter strongly suppressed symptoms of PD and PCD (Gilchrist & Lincoln, 2007 Pierce's Disease Research Symposium Proceedings, pp 252-5). In recent years, a large and growing number of bacterial protein toxins have been discovered that behave as virulence factors, and many of these bacterial toxins induce apoptosis (Schiavo,G.; van der Goot, F.G. 2001). One emerging theme from studies of these bacterial toxins is that they frequently interfere with host pathways, thereby eliciting programmed cell death (for a review see Weinrauch and Zychlinsky, 1999). Since symptoms of PD are suppressed by anti-apoptotic gene expression, it becomes likely that the pathogen is producing a PCD elicitor, or "toxin". This "toxin" or elicitor has yet to be identified.

Elicitation of symptoms of PD and PCD enhances Xf growth in hosts. A major question has always been whether or not the symptoms of leaf scorch on grape contribute to pathogen growth or spread on grape or are merely gratuitous. Gilchrist's lab discovered that the anti-apoptosis genes both strongly suppressed symptoms of PD and in addition, limited the bacterial titer (at six months post inoculation) to that which is usually seen on the asymptomatic host, *V. californica* (ie., to ca. 10^4 cfu/gram stem tissue instead of 10^8 cfu/gram stem tissue observed at point of death of *V. vinifera* ; refer Table 2 of the PowerPoint presentation by Gilchrist, Lincoln, Ward and Cook, 2007 Pierce's Disease Research Symposium, available online; confirmed by Dave Gilchrist in personal communication). This data indicates that elicitation of programmed cell death (PCD) can contribute to additional *Xf* growth in hosts, but is not required for opportunistic (parasitic) growth of *Xf*, at least not in some hosts.

Of course, the early work of Freitag (1951) mentioned above demonstrated that PD symptom elicitation is not required for growth of PD strains in a variety of hosts. The converse is also true; several non-PD *Xf* strains are known to be capable of asymptomatic growth in *V. vinifera* (Hopkins, 2005). Indeed, *Xf* strain EB92-1, isolated from elderberry, has been found to be highly effective as a biological control agent against PD in the field for 12-18 months, and "only strains that were able to multiply and systemically colonize without producing significant symptoms were able to protect against virulent strains" (Hopkins, 2005). An important series of questions regarding host specific symptoms and host range now may be quantitative in nature: how much additional growth is provided by ability to elicit PCD and/or PD symptoms? Are multiple elicitors involved? How host-specific are these elicitors? Is some low level of PCD, below the level required to elicit symptoms, required for host range? The anti-apoptosis genes in Gilchrist's study suppressed, but presumably did not eliminate, programmed cell death (PCD) in the host, thereby resulting in suppression of symptoms and limiting bacterial growth. What if PCD were eliminated? Would all or most *Xf* growth also then be eliminated, and the plant be a nonhost?

A related question is whether or not ability to elicit PCD ultimately restricts ability to infect plants that might otherwise be hosts, such as PD strains inoculated on citrus. In other words, since PD strains do not grow in all plants, is (are) the PCD elicitors (all) host specific? Are there additional factors, aside from insect transmission and symptom elicitation that may limit host range? As described in some detail below, recent work from our lab indicates that the answer to the host range question indicates that there are likely dditional factors aside from elicitors that may limit host range. These factors may involve colicins used for competitive exclusion of other bacteria that may colonize the same ecological niche. Our general working hypothesis regarding the very wide host range of the entire, highly opportunistic *Xf* species but more limited range of individual strains has been expanded to include three factors affecting host range: 1) the host range of its insect vectors (not examined by our methods); 2) the ability of Xf to elicit PCD with or without leaf scorch symptoms on *V. vinifera;* and 3) ability of *Xf* to competitively exclude other bacteria from its xylem vessel niche. If the primary factor(s) that determine host range can be identified, then additional targets for chemical, biological and/or transgenic controls would be made available.

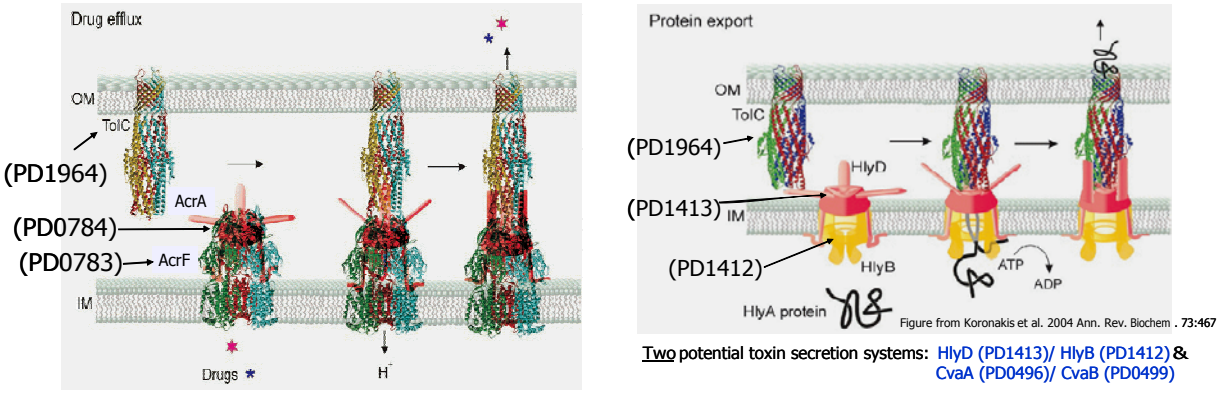

Figure 1: Type I machine for MDR ("Drug) efflux in *X. fastidiosa* utilize *tolC* and *acrF/A* or *acrC/D* (left). Type I machine for protein export or secretion in *X. fastidiosa* utilize *tolC* and *cvaA/B* or *hlyB/D* (right). Figures from Koronakis et. al. (2004).

OBJECTIVES

Two specific Type I toxin secretion systems are found in both CVC strain 9a5c and PD strain Temecula. These are an alpha-hemolysin-like system [in Temecula, it utilizes TolC (outer membrane, PD1964) and HlyB/D (inner membrane, PD1412/periplasm, PD1413)], and a colicin V-like system [in Temecula, it utilizes TolC (outer membrane, PD1964) and CvaA/B (inner membrane, PD0496/periplasm, PD0499)]. These Type I systems are also found together with multiple genes encoding type I effectors of the RTX (repeats in toxin) family of protein toxins (Lally et al., 1999).

Critically, all sequenced Xf strains carry only a single *tolC* gene; therefore, a knockout of this single gene in Temecula eliminated all Type I secretion, both offensive and defensive (Reddy et al. 2007). Unfortunately, the loss of MDR efflux in Temecula resulted in the strain being undetectable even a few minutes after inoculation of the *tolC*$^{-}$ mutation; therefore, the loss of pathogenicity reflected the loss of the strain itself and any offensive role of Type I secretion in pathogenicity, due to loss of secretion of either hemolysin and/or colicins could not be tested.

Type I secreted effectors found in the sequenced CVC and PD strains were a bacteriocin (XF2407 in CVC and ortholog PD1427 in PD) that resembles a *Rhizobium* host range factor (Oresnik et al., 1999), three hemolysins (XF0175, XF0984 and XF1280) in CVC and orthologs PD0413, PD0282 and PD0536, respectively, in PD), calcium binding hemolysin-type proteins (XF0668, XF1011, XF2759 in CVC and orthologs PD1506, PD0305, PD2094, respectively, plus an additional calcium binding protein PD2097), and three colicin V precursors (XF0262, XF0263 in CVC and orthologs PD0215, PD0216 and PD0217 in PD). The discovery of such a large group of RTX toxins is likely significant because both genomes carry representatives of both major RTX toxin types: the alpha-hemolysin group that are toxic to a very wide range of eukaryotic cell types (Lally et al., 1999), and the colicin V group, which is not known to us to affect eukaryotic cells. Among the symptoms elicited by CVC strains on citrus are brown, necrotic and slightly gummy lesions on the undersides of leaves that are suggestive of toxin activity. The earliest symptom caused by PD strains on grape is leaf scorch, which is also strongly suggestive of toxin activity.

RESULTS

Rather than attempt knockouts of multiple and potentially redundant effectors, initial experiments focused on knockouts of three apparently separate components of Type I secretion: 1) MDR efflux only: *acrD* (PD1404) and *acrF* (PD0783); 2) Type I hemolysin secretion only: the periplasmic component *hlyB* (PD1412) and the inner membrane component *hlyD* (PD1413); and 3) colicin V secretion only: the inner membrane component *cvaA* (PD0496) and the periplasmic component *cvaB* (PD0499). Surprisingly, knockouts of any of the three Type I system strongly reduced pathogenicity (**Figure 2).**

The colicin V precursors (PD0215, PD0216 and PD0217) are clustered in the Temecula genome, allowing the simultaneous knockouts of all three colicins in a single recombination event by marker exchange, which was accomplished and documented as described (Reddy et al. 2006). Plant inoculation assays using the colicin V knockout mutant 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 Δ (PD0215, PD0216 and PD0217)::nptII strain (labeled "colicins" in **Figure 2**). The plants were maintained under green-house conditions and were evaluated for PD symptoms at 60 and 90 days after inoculation. All plants inoculated with the wild-type Temecula strain exhibited typical PD (not shown).

Again to our surprise, pathogenicity was strongly reduced by eliminating just the colicin effectors (**Figure 2**).

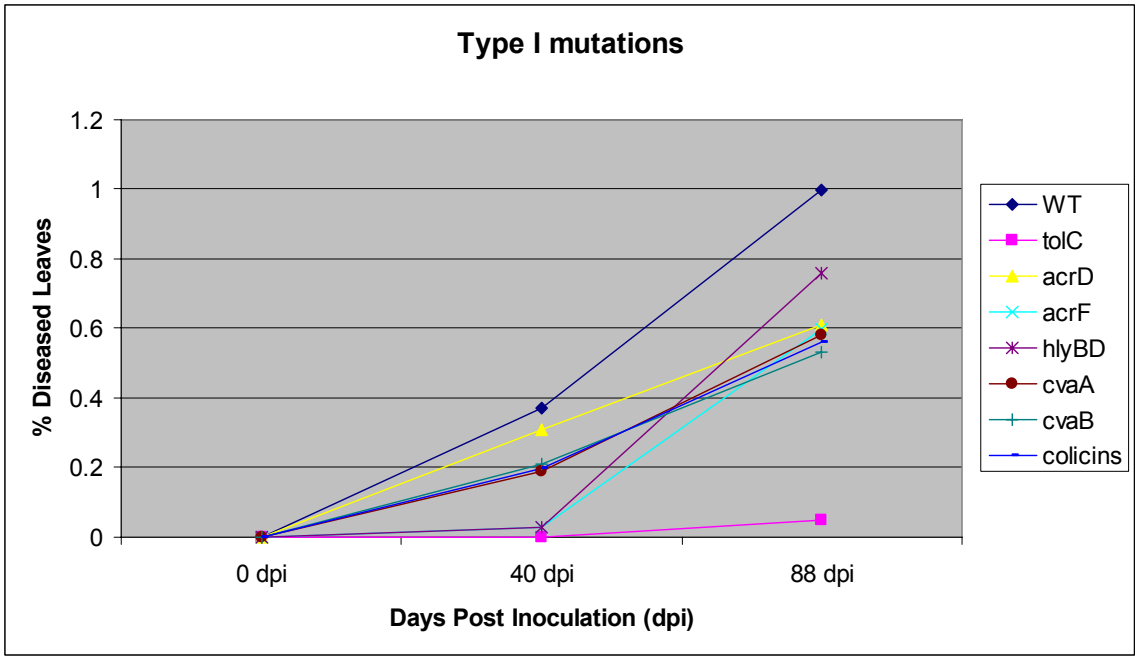

Figure 2. Grape var. Carignane inoculated with marker exchanged mutants of *acrF* (PD0783), *acrD* (PD1404), *hlyBD;* PD1412-1413*), cvaA (*PD0496*) and cvaB* (PD0499), and the three colicin V precursors PD0215, PD0216 and PD0217 (labeled "colicins") and assessed for % diseased leaves at 40 and 88 days post inoculation.

Note that genes considered to be dedicated to both hemolysin (*hlyBD*) and colicin (*cvaAB*) secretion exhibited greatly reduced symptoms, and not just delayed symptoms. The *acrF* (MDR efflux) mutant, but not *hlyBD* (hemolysin secretion) was sensitive to berberine chloride (Gabriel, 2007 Pierce's Disease Research Symposium Proceedings, p190-3), as expected. These results strongly support a role of both colicins and hemolysins in pathogenicity. In this regard, it is important to note that hemolysins are known to behave as apoptotic toxins in insects and animal pathogens (Vigneux et al., 2007). Therefore hemolysins may have a direct role in PD pathogenicity. Some colicins have a structural domain similar to Bcl-2 like proteins that are involved in apoptosis of animal cells (Boya et al. 2001), and can inhibit proliferation of cancer cells. Therefore the colicins may have a direct role in PD pathogenicity as well; alternatively or additionally, they may have a role in suppressing growth of bacteria that may compete for colonization of the xylem niche. These potential roles are currently under investigation.

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, including the putative Type I effectors annotated as "colicin V precursors". 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 PD. Additionally, since colicin-like effectors appear to be important in conditioning pathogenicity, they represent additional targets for chemical, biological and/or transgenic disease control strategies.

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