

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, including knockouts of associated with colicin V secretion and all three colicin effectors also resulted in significant loss of pathogenicity. This raised the possibilities that 1) colonization and pathogenicity of grapevines by *Xf* involves the exclusion of other bacteria from the xylem niche by way of these effectors, and/or 2) that these Type I effectors might directly affect plant cells. To test these ideas, PCR primers were designed to amplify all 8 hemolysins and all 3 colicins from *Xf* Temecula and used to amplify full length genes of all 11 effectors from Temecula. Surprisingly, these same primers were used to amplify nine equivalent homologs from EB92.1 and the predicted amino acid sequences of all 9 EB92.1 homologs were identical to the corresponding Temecula homologs. This result was confirmed by the results of the draft EB92.1 genomic DNA sequence obtained through funding of a separate project, and extended to include the remaining 2 effectors, which were 100% identical to Temecula. Furthermore, no additional hemolysins or colicins have been found to date in the draft EB92.1 genome that were not found in Temecula. These results strongly indicate that although these Type I effectors are important for plant colonization and subsequent development of PD, they are not directly responsible for the overt disease symptoms caused by Temecula (and not caused by biocontrol strain EB92.1, which has all 11 identical effectors). In addition, one hemolysin, one calcium binding hemolysin, all three colicins and *uidA* (GUS, as a control gene) were separately cloned into plant transient expression vectors and inoculated into tobacco and *Vitis vinifera* grape plants. GUS expression confirmed that the expression system worked, but no obvious plant responses were observed due to expression of the individual hemolysins or colicins inside the plant cell, further casting doubt on a direct role for these effectors in symptom elicitation.

LAYPERSON SUMMARY

Previously funded work has shown that the bacterial pathogen *Xylella fastidiosa* (*Xf*) relies upon a specific protein secretion device to both attack grape plants, causing Pierce's disease (PD), and to defend itself from natural grape plant defenses against bacterial pathogens. Previous work also demonstrated that the proteins secreted by *Xf* using the attack mechanism played a role in the development of PD symptoms. It is now shown that the proteins secreted by *Xf* do not directly elicit PD symptoms in grape, but instead play an indirect, and as yet unknown, role in allowing the pathogen to grow within grape plants. It is also shown that these proteins are widely conserved in *Xf* strains attacking almond, coffee and citrus, in addition to grape, making any technology that is effective in disabling these proteins in grape widely applicable to other crops as well.

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). 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 (**Figure 1**). 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. 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).

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 PD 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 PD 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 PD 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 additional factors aside from PCD 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.**

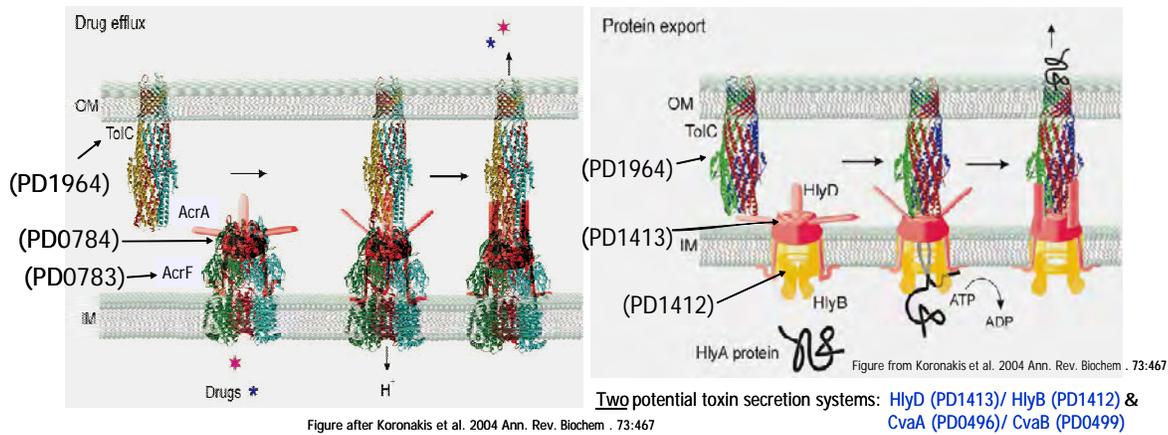


Figure 1. Type I machine for MDR (“Drug) efflux in *Xf* utilize *tolC* and *acrF/A* or *acrC/D* (left). Type I machine for protein export or secretion in *Xf* utilize *tolC* and *cvaA/B* or *hlyB/D* (right). Figures from Koronakis et. al. (2004).

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**).

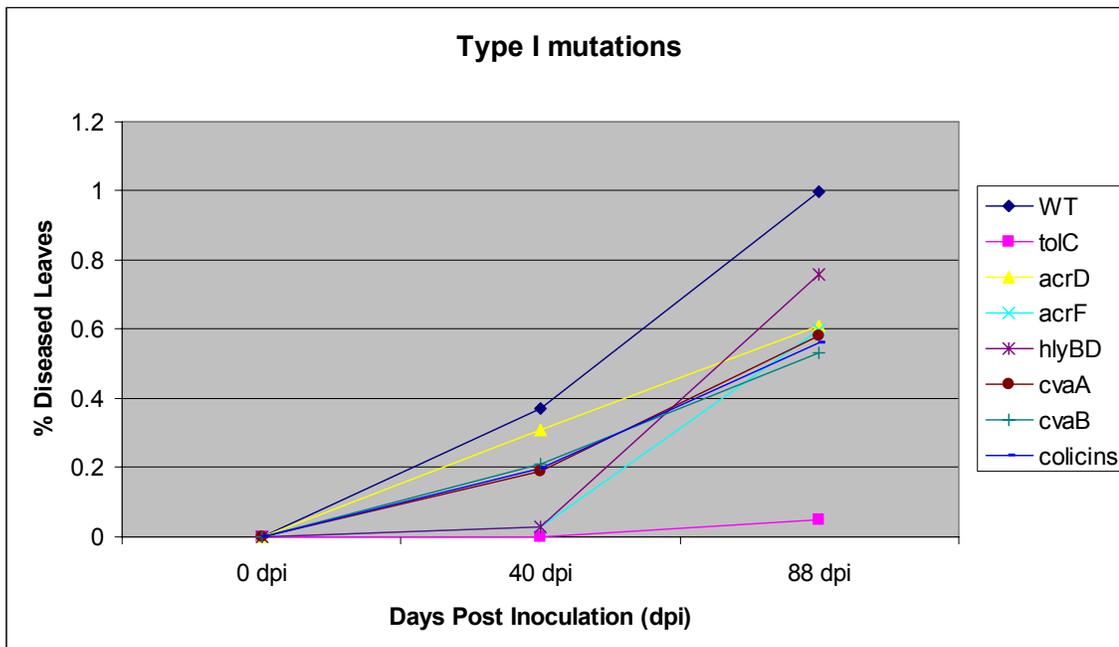


Figure 2. Grape var. Carignane inoculated with marker exchanged mutants of *acrF* (PD0783), *acrD* (PD1404), *hlyBD*; PD1412-1413), *cvaA* (PD0496) and *cvaB* (PD0499), and the three colicin V precursors PD0215, PD0216 and PD0217 (labeled “colicins”) and assessed for % diseased leaves at 40 and 88 days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

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 greenhouse 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**).

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 PD 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.

OBJECTIVES

1. Determine the specific effect of individual **Temecula hemolysins** PD0413, PD0282, PD0536, calcium binding hemolysin-type proteins PD1506, PD0305, PD2094, calcium binding protein PD2097 in eliciting PD symptoms of leaf scorch and PCD on *V. vinifera* plants and in enhancing growth in *V. vinifera* plants of *Xf* biological control strain EB92-1. Specifically:
 - a) Use our published pBBRMC5 vector system (Reddy et al., 2007) to move each of these genes, expressed by either their native or *lacZ* promoter into *Xf* biological control strain EB92-1 (Hopkins, 2005; described above), and inoculating *V. vinifera* plants. (Year 1).
 - b) If any hemolysins cause PD symptom expression, determine how much additional growth, if any, is provided by this ability to elicit PD symptoms (Year 1).
 - c) By transient expression assays of select hemolysin genes in grape leaves using a CaMV promoter and *A. tumefaciens* GV2260 delivery (Year 2).
2. Determine the effect of biocontrol strain **EB92-1 colicins** on multiple PD strains and in eliciting phytotoxic symptoms on *V. vinifera* plants. **A novel aspect of the use of EB92-1 as a donor strain is the expectation, based on our Type I colicin secretion component knockouts, that its colicin V or bacteriocin genes are determinative of its ability to perform as an effective biological control agent, and the speed with which this hypothesis can be tested.** Specifically:
 - a) PCR amplify, clone and sequence the homologs of Temecula colicins PD0215, PD0216, PD0217 and bacteriocin PD1427 from biocontrol strain EB92-1, overexpress the EB92-1 homologs individually in *E. coli* and determine the sensitivity of Temecula and at least 10 other PD strains and a variety of other pathogens to the three colicins using plate overlay assays (Year 1).
 - b) If any colicins or the bacteriocin are found from EB92-1 that kill or inhibit growth of all or most PD strains tested, at least one will be selected for transient expression assays in tobacco and grape leaves (Year 2).
 - c) If phytotoxicity is observed, confirmation of PCD elicitation by use of an Affymetrix *V. vinifera* chip will be performed (Not covered by this proposal).
 - d) If, as expected, no phytotoxicity is observed, transgenic tobacco plants will be made in Year 2 and subsequently tested by challenge inoculation for control of PD and/or elimination of inoculated *Xf* (late in Year 2).

RESULTS

All objectives of the proposal except (1.c.) were met by the rather surprising discovery that EB92.1 carries exactly the same repertoire of colicins, hemolysins, and calcium-dependent hemolysins as are found in Temecula, with 100% predicted amino acid identity for each homolog, and with no missing homologs in either strain (**Table 1**).

PCR primers were designed to amplify all 8 hemolysins and all 3 colicins from *Xf* Temecula. These were then used to amplify full-length genes of all 11 effectors from Temecula. These PCR products were cloned into pGEM-T and three random colonies selected and sequenced. Simultaneously, these same primers were then used to amplify equivalent homologs from EB92.1. Nine of the 11 reactions were successful; these PCR products were also cloned into pGEM-T and sequenced. Surprisingly, although PCR errors were detected, upon correction the predicted amino acid sequences of all nine EB92.1 homologs were identical to the corresponding Temecula homologs. This result was confirmed and extended to include all 11 homologs by finding each of the Temecula homologs by Blast using the results of the draft EB92.1 genome,

obtained through funding of a separate project. The draft EB92.1 currently contains 2,478,233 bps of genomic sequence in 210 contigs. The average contig length is currently 11,801 bps (Min: 106 bps, Max: 149,096 bps). By far the majority of the primary Blast hits are to Temecula; based on the size of the Temecula genome (2,519,802 nt), this draft EB92.1 genome is ca. 98% complete. No additional hemolysins or colicins were found to date in the draft EB92.1 genome that were not found in Temecula. More importantly, no additional hemolysins or colicins were present in Temecula that were not found in EB92.1. The fact that the complete repertoire of known Temecula Type I effectors were found, with 100% identity, in EB92.1 (which does not cause PD), means that PD symptoms per se cannot be caused by these effectors. Furthermore, since EB92.1 carries the identical colicins found in Temecula, with no additional colicins discovered and ca. 98% of the genome complete (and since a strain is resistant to its own colicins) means that it is unlikely that colicins from EB92.1 are responsible for the biocontrol properties of EB92.1.

Table 1. Predicted Type I secreted effectors in *Xf* Temecula and their homologs in other *Xf* pathovars.^a

Gene	Size in Kbp	Temecula (Grape)	Ann ^b (Oleander)	Dixon ^b (Almond)	M12 (Almond)	M23 (Almond)	9aC5 (Sweet orange)	EB92-1 ^b (Elderberry)
PD1427 Ca+ hemolysin	4.5	100%	-	-	-	95%	79%	100% (partial)
PD2094 Ca+ hemolysin	1.07	100%	96%	-	-	100%	-	100%
PD2097 Ca+ hemolysin	3.5	100%	88%	88%	88%	100%	81%	100%
PD1506 Ca+ hemolysin	5.1	100%	79%	93%	77%	94%	83%	100% (partial)
PD0305 Ca+ hemolysin	4.8	100%	72%	72%	71%	100%	77%	100% (partial)
PD0143 hemolysin	1.0	100%	99%	99%	99%	100%	99%	100%
PD0282 hemolysin	0.5	100%	100%	98%	98%	100%	92%	100%
PD0536 hemolysin	1.4	100%	99%	99%	99%	99%	96%	100%
PD0215 colicin	0.3	100%	96%	90%	89%	100%	90%	100%
PD0216 colicin	0.3	100%	93%	91%	-	100%	-	100%
PD0217 colicin	0.3	100%	-	98%	91%	100%	two homologs 88% and 79%	100%

^a Searched COG2931 in genomes to identify predicted Type I secreted proteins. Performed BLASTP to identify homologs.

^b Draft genome.

- Homolog not found.

CONCLUSIONS

Previous work demonstrated that not only is multidrug efflux critical to survival of *Xf* in grape, but also that Type I offensive protein secretion is needed, at least in part, to help condition host range and/or full pathogenicity to grape. Both multidrug efflux and Type I protein effector 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. This new work rules out a role for the identified hemolysins and colicins (a total of 11 identified Type I effectors) in directly conditioning pathogenicity, but preserves an indirect role for these Type I effectors in adaptation and perhaps growth in grape, which is then needed to allow full pathogenicity. These effectors **therefore represent promising and multiple additional targets for chemical and/or transgenic disease control strategies. Since these proteins are highly conserved in all *Xf* strains and are secreted, any technology developed with ability to attack these targets in grape would likely also have applications in citrus (against citrus variegated chlorosis), coffee (against leaf scorch), or almond (against leaf scorch).**

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