GENOMIC SEQUENCING OF BIOCONTROL STRAIN EB92-1 AND IDENTIFICATION OF ELICITOR(S) OF EFFECTIVE DEFENSE IN *VITIS VINIFERA* AGAINST PIERCE'S DISEASE

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ABSTRACT

A draft genome of infectious but asymptomatic *Xylella fastidiosa* (*Xf*) strain EB92.1 that currently contains 2,478,730 bps of genomic sequence in 191 contigs has been created. By far the majority of the primary Blast hits were to *Xf* strain Temecula; based on the size of the Temecula genome (2,519,802 nt); based on the size of the Temecula genome (2,519,802 nt), this draft EB92.1 genome is ca. 98% complete. Comparative analyses of Temecula vs. EB92.1 revealed that EB92.1 is highly similar to Temecula in both gene order and synteny.

Type I, II and V secretion system effectors are all found in Temecula and were comparatively examined in EB92.1. No additional Type I effectors (hemolysins or colicins) were found to date in the draft EB92.1 genome that were not found in Temecula and the complete repertoire of known Temecula Type I effectors were found. This might indicate that Pierce's disease (PD) symptoms *per se* are not likely caused by these effectors, although an essential role in host colonization or adaptation is still indicated for these effectors. However, two EB92.1 type V subtilisin-like serine protease autotransporters were found to be only 71% and 69% identical, respectively, to their Temecula homologs, PD0218 and PD0313. It is possible that these variant serine proteases affect maturation of one or more Type I effectors. This idea is under experimental investigation.

A type II secreted Temecula lipase, PD1703, appeared to be completely missing from EB92.1. An *in vitro* lipase assay confirmed that EB92.1 showed no secreted lipase activity, while Temecula exhibited strong secreted lipase activity in the same assay. This lipase is an apparent *lipA* homolog of *Xanthomonas oryzae*; *lipA* is known to directly contribute to pathogenic symptoms of *X. oryzae*. A DNA clone carrying PD1703 conferred strong secreted lipase activity to *Xanthomonas*; similar tests are underway to transform EB92.1 with this lipase.

LAYPERSON SUMMARY

Xylella fastidiosa strain EB92.1 is infectious to grapevines but causes no symptoms and has been used for biological control of Pierce's disease (PD). We have determined the genomic DNA sequence of EB92.1 to 98% completion, allowing comparisons of most genes of this strain to strain Temecula, which causes PD. What are the PD-determining genes in Temecula that are not found in EB92.1? Most of the EB92.1 genes are nearly identical in gene order and protein coding with those found in Temecula. However, notable exceptions were found in three genes that are suspected to be involved either in creating the symptoms of PD or in combating competitive bacterial strains in the same xylem niche. If any of these three genes can be proven to contribute to the disease, it will become a new molecular target with potential to control the disease.

INTRODUCTION

Type I multidrug resistance (MDR) efflux system of *Xylella fastidiosa* (*Xf*) is absolutely required for both pathogenicity, and more importantly, survival of the Pierce's disease (PD) pathogen in grape (Reddy et al., 2007). Knockout mutations of the single *tolC* gene in strain Temecula are extremely sensitive to compounds produced by susceptible *Vitis vinifera* grapes, including phytoalexins, and mutants such as strain M1 were not recovered from *Vitis vinifera* grapes after inoculation (Reddy et al., 2007). As a part of the work involving defense compound and phytoalexin sensitivity tests, we discovered that the wild type strain Temecula, with its lone multidrug resistance (MDR) efflux system, is much more sensitive to plant-derived antimicrobial chemicals than most other bacterial plant pathogens, which all carry multiple MDR efflux systems. This may mean that the opportunistic *Xf* is living at the quantitative edge of survival, and ***Xf* may only be capable of surviving in the xylem vessels of hosts in which a defense response is not triggered.**

Hopkins (2005) discovered an effective PD biocontrol strain, *Xf* EB92-1, which infects grapevine and survives for many years. EB92-1 can be inoculated in a single location in a *V. vinifera* grapevine and the entire plant is protected from PD for years (Hopkins, 2005). How does this strain infect grape, and yet not cause disease? What factors are present in Temecula that may be triggers of host defense? What factors are different?

OBJECTIVES

This is the first year of a two year project. The first year's objectives were:

1. Obtain nearly the complete EB92-1 genome DNA sequence.
2. Compare EB92-1 with Temecula and identify all unique ORFs and differences, ranking the top 40 candidate ORFs for evaluation as elicitors.

RESULTS AND DISCUSSION

A draft genome of infectious but asymptomatic *Xf* strain EB92.1 that currently contains 2,478,730 bps of genomic sequence in 191 contigs has been created. The average contig length is currently 12,977 bps (Min: 100 bps, Max: 149,098 bps). By far the majority of the primary Blast hits were to *Xf* strain Temecula; based on the size of the Temecula genome (2,519,802 nt); based on the size of the Temecula genome (2,519,802 nt), this draft EB92.1 genome is ca. 98% complete. Comparative analyses of Temecula vs. EB92.1 revealed that EB92.1 is highly similar to Temecula and the gene order exhibits a very high level of synteny. The average GC Content is 53% (Min: 30%, Max: 70%). The gene coding percentage is 81%.

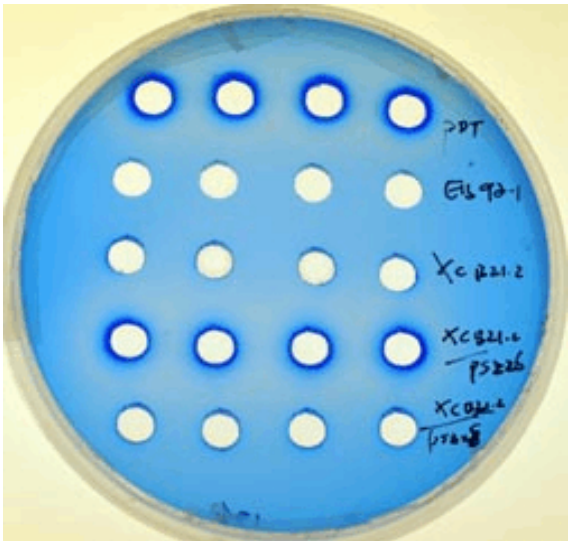
Predicted gene elements:

* 2387 genes coding for mRNA (protein coding ORFs)
* 48 genes coding for tRNA
* 3 genes coding for rRNA
* 0 repetitive elements

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 repertoir 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 are not likely caused by these effectors, although an essential role in host colonization or adaptation is still indicated (Flores-Cruz et al. 2009).

Type I, II and V secretion system effectors are all found in Temecula and were comparatively examined in EB92.1. No additional Type I effectors (hemolysins or colicins) were found to date in the draft EB92.1 genome that were not found in Temecula and the complete repertoire of known Temecula Type I effectors were found, with 100% identity, in EB92.1. This might indicate that PD symptoms *per se* are not likely caused by these effectors, although an essential role in host colonization or adaptation is still indicated for these effectors. However, two EB92.1 type V subtilisin-like serine protease autotransporters were found to be only 71% and 69% identical, respectively, to their Temecula homologs, PD0218 and PD0313. Guilhabert and Kirkpatrick (2005) reported a Tn*5* mutation in PD0218 as mildly reduced in pathogenicity. Since a mutation in PD0218 causes failure of Temecula to secrete a Type I bacteriocin, PD1427 (Igo, 2009 PD Symposium Proceedings, p97-101), it is possible that these variant serine proteases affect maturation of one or more Type I effectors. A modified, but functional PD0218 may secrete a modified bacteriocin that is toxic to Temecula and help explain the biological control properties of EB92.1. This idea is under experimental investigation.

A potentially significant discovery is that a type II secreted Temecula lipase, PD1703, was completely missing within an assembled contig of EB92.1, evidently as a result of a genomic deletion. This result was confirmed by multiple PCR analyses of the evidently deleted region. An *in vitro* lipase assay was conducted using Tween 20 as the substrate and 0.01% Victoria Blue B as indicator, exactly as described by Samad et al. (1989)



In the figure at left, each row of four wells was filled with 50 ul of cell culture supernatant in the following order (top row to bottom row):

<--Temecula (labeled PDT)

<--EB92.1 (labeled EB92-1)

<--*Xanthomonas citri* B21.2 (labeled Xc B21.2)

<--*X. citri* B21.2 transconjugant plus cloned PD1703, driven by native promoter and cloned in pBBR1-MCS5 (downstream from *lacZ* promoter).

<--*X. citri* B21.2 transconjugant plus cloned PD1702, driven by native promoter and cloned in pBBR1-MCS5 (downstream from *lacZ* promoter).

The top row (Temecula supernatant) and fourth row (*X. citri* B21.2 transconjugant plus cloned PD1703 supernatant), clearly demonstrate strong amounts of secreted lipase in the culture supernatants (not concentrated or purified). This level of lipase activity is not present in the supernatants of EB92.1, *X. citri* B21.2, or *X. citri* B21.2 transconjugant with another lipase (PD1702) cloned from Temecula, but also present (with one amino acid substitution) in EB92.1. These assays were repeated twice, and including as a control *X. citri* / pBBR1-MCS5 (not shown).

The PD1703 lipase is a homolog of *lipA* from Xanthomonas oryzae (GenBank Accession XOO0526). The *X. oryzae lipA* is known to be a cell wall degrading esterase/lipase that elicits host defenses, including programmed cell death, in rice (Jha et al. 2007; Aparna et al. 2009). Mutations of *lipA* in *X. oryzae* lead to a partial loss of pathogenicity on rice (Rajeshwari et al. 2005; Jha et al. 2007).

Finally, a unique hemagglutin-like protein in Temecula, PD0986, appeared to be missing in the EB92.1 genome. Since mutations of hemagluttinins in *Xf* show increased virulence are thought to be important in impairing *Xf* movement in the plant xylem (Guilhabert & Kirkpatrick, 2005), loss of PD0986 may well assist the asymptomatic EB92.1 in increasing its movement in grapevine and make it more effective as a biocontrol agent. Although the EB92.1 genome is incomplete, it deserves mention that the Temecula region in which this locus is found appears to be missing from the middle of a large EB92.1 contig. There were no good hits of PD0986 to any small contigs, making it appear unlikely that further sequencing will uncover this locus in EB92.1.

CONCLUSIONS

The identification of two genes that encode comparatively significantly different modified serine protease autotransporters that in turn may affect Type I effectors provides potential additional support for the idea that Type I effectors, such as colicins and hemolysins, can affect pathogenicity. However, even knockout mutations of these autotransporters do not explain the major differences between the biocontrol strain EB92.1 and PD-causing Temecula. The discovery of a *lipA* homolog in Temecula that is missing in EB92.1, and which is known in the literature as a pathogenicity factor and defense elicitor may help explain, at least in part, why EB92.1 fails to elicit PD symptoms. This must be confirmed, but this potential lead would not have been made without the genomic sequence of both Temecula and EB92.1. If pathogenicity genes are found in Temecula that are not found in biological control strain EB92-1, and if these genes can be demonstrated to be primarily responsible for generating PD symptoms, then methods to interfere with the mechanism of action of such genes may be found that would not be otherwise considered.

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