Investigation of the Function of the Putative Type VI Secretion System Effectors of the Plant Pathogen Xanthomonas

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Abstract
An analysis of the putative effectors of the type VI secretion system of Xanthomonas perforans, Xanthomonas euvesicatoria, and Xanthomonas vesicatoria was conducted to elucidate the conservation and function of these effectors. The type VI secretion system's function is currently not well defined in Xanthomonads but is inferred to play a role in epiphytic fitness. Computational methods, literature reviews, and in-lab toxin assays in both prokaryotic and eukaryotic organisms were used to access the putative effectors of this secretion system. Initial findings suggest these effectors are conserved and serve diverse functions including virulence, pathogenicity, prokaryotic competition, predation defense, and nutrient acquisition.

Introduction
Xanthomonas perforans (Xp), Xanthomonas euvesicatoria (Xe), and Xanthomonas vesicatoria (Xv) cause bacterial leaf spot disease on tomato and pepper plants, both economically important crops. These pathogens use a type III secretion system to inject virulence and immune suppression effectors into the host plant (Üstün & Börnke, 2014). The type IV secretion system delivers toxins to kill neighboring bacteria to decrease competition (Sgro et al., 2019). The function of the type VI secretion system (T6SS) in Xp, Xe, and Xv is not as well characterized. While many proteobacteria pathogens have a T6SS, the function of the secretion system and its effectors may differ between species. In Pseudomonas aeruginosa, the T6SS shows anti-bacterial capabilities, injecting toxin into other bacteria (Hood et al., 2011). Vibrio cholerae's T6SS targets both prokaryotes and eukaryotes (Crisan, 2020). A serine/threonine kinase of the T6SS of Xanthomonas citri decreases predation by amoebas (Bayer-Santos et al., 2018).

The type VI secretion system consists of core genes encoding for proteins that form a complex similar to a syringe with a spike-tipped rod in a contractile sheath (Zouedet al., 2014). Within the cluster of core genes are additional genes believed to be effectors due to their proximity to the core genes. Effectors are proteins that are secreted by the system, usually by attaching to the tip or spike protein VgrG. Knowing the function of these effectors is essential to ascertaining the purpose of the T6SS in Xanthomonas species affecting tomato and pepper.

Methods
The computational analysis utilized the Alabama Supercomputer to run BLAST (basic local alignment search tool) on all strains of Xp, Xe, and Xv available in the National Center for Biotechnology Information (NCBI) database to assess conservation of the putative effectors across strains and between species. The program Basction 6, a T6SS effector predictor based on genetic markers from a database of known effectors (Wang et al., 2018), was used to gauge the likelihood of the secretion of the putative effectors. A phylogenetic tree based on the gene BJD13_RS18385 in the T6SS cluster was made using MEGAX: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al., 2018). EasyFig was used to construct figures showing the conservation of the T6SS clusters. Annotations and protein predictions for the putative effectors were found by running BLASTx (Altschul et al., 1990) through NCBI (https://www.ncbi.nlm.nih.gov/), the Joint Genomic Institute's Integrated Microbial Genomes & Microbiomes system (JGI/IMG) (Chen et al., 2021), UniProt (Bateman et al., 2021), and PaperBLAST (Price et al., 2017). Annotations of genes with high identity and coverage percentage in other species were also considered.

Toxin assays were conducted by first designing primers spanning the open reading frame of each putative effector. These putative effector genes from Xp strain AL65 were then amplified through polymerase chain reaction (PCR) using Taq polymerase and confirmed.
by length in an agarose gel. Taq polymerase leaves a single nucleotide (A) on the 3’ end of the PCR product. The PCR products were cloned into pBAD TOPO and pYES TOPO vector plasmids by matching the overhanging nucleotide on the PCR product with an overhanging (T) nucleotide on the linear vector plasmid. These plasmids were then transformed into *Escherichia coli* and plated on selective media. Growth on the selective plates indicated presence of the plasmid. PCR with the gene specific primers and gel electrophoresis was used to confirm presence of the gene of interest in the plasmid. A miniprep was done and the extracted plasmids sent for Sanger sequencing to confirm correct orientation of the gene within the plasmid. pBAD plasmids with genes in the correct orientation were then transformed into BL21 cells. pYES plasmids with genes in the correct orientation were transformed into yeast strains W303a and BY4741a. These assays showed a slight decrease in colony diameter (1mm) compared to the negative controls. An assay for toxicity in eukaryotes (W303a/BY4741a *Saccharomyces cerevisiae*) is shown in Figure 2. This figure shows the putative effector BJD13_RS18340 having a toxic phenotype with decreased growth on the 2% galactose plate.

**Discussion**

The conservation of the putative effectors suggests they impart an essential function to the pathogen, or they would tend to acquire mutations. Annotations and protein predictions reveal the diverse functions of these effectors. The jacalin-lectin domain containing protein likely prevents recognition by the plant’s immune response. The Zn-binding protein has the potential to have a variety of functions (Sharma et al., 2019). In addition to showing a toxic phenotype in yeast, BJD13_RS18340 or oxidoreductase may be used to degrade toxins from competing microorganisms (Taylor et al., 2006). The serine/threonine kinase likely protects against amoeba predation as seen in *X.citri* (Bayer-Santos et al., 2018). Additionally, the hypothetical proteins, DUF4124 domain containing protein, intramembrane metalloprotease, and predicted methyltransferase are possibly prokaryote or eukaryote toxins.

While toxin assays still need to be conducted on the remaining putative effectors, initial findings suggest that the effectors and the T6SS may have a diverse array of functions important to the survival of the pathogen. Further understanding of the T6SS in *Xanthomonas* is pertinent to mitigating the devastation caused by bacterial leaf spot disease on tomato and pepper.
Table 1. Shows the gene locus tag of the putative T6SS effectors and the annotation or protein prediction from NCBI, JGI/IMG, UniProt, or PaperBLAST.

<table>
<thead>
<tr>
<th>Gene locus tag</th>
<th>Annotation/protein prediction</th>
<th>Gene locus tag</th>
<th>Annotation/protein prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJD13_RS18245</td>
<td>Hypothetical Protein</td>
<td>BJD13_RS18325</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>BJD13_RS18250</td>
<td>DUF4124 domain containing protein</td>
<td>BJD13_RS18330</td>
<td>Type VI secretion system associated FHA domain containing protein (TagH - NCBI) (Impal - JGI/IMG)</td>
</tr>
<tr>
<td>BJD13_RS18255</td>
<td>CPBP family intramembrane metalloprotease</td>
<td>BJD13_RS18335</td>
<td>DNA-binding LysR family transcriptional regulator</td>
</tr>
<tr>
<td>BJD13_RS18260</td>
<td>Putative secreted protein</td>
<td>BJD13_RS18340</td>
<td>Aldo/keto reductase family oxidoreductase -NCBI dehydrogenase-like oxidoreductase - JGI/IMG</td>
</tr>
<tr>
<td>BJD13_RS18270</td>
<td>Jacalin-like lectin domain-containing protein</td>
<td>BJD13_RS18365</td>
<td>Serine/threonine phosphatase</td>
</tr>
<tr>
<td>BJD13_RS18305</td>
<td>Serine/threonine kinase</td>
<td>BJD13_RS18375</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>BJD13_RS18310</td>
<td>Zn-binding PAAR domain containing type VI secreted protein</td>
<td>BJD13_RS18375</td>
<td>Histidine-type phosphatase -NCBI 4-phytase / acid phosphatase -JGI/IMG</td>
</tr>
<tr>
<td>BJD13_RS18315</td>
<td>Predicted methyltransferase</td>
<td>BJD13_RS18380</td>
<td>SHB/FhaC/HecB family hemolysin secretion/activation protein</td>
</tr>
<tr>
<td>BJD13_RS18320</td>
<td>Type VI secretion system secreted protein VgrG</td>
<td>BJD13_RS18385</td>
<td>filamentous hemagglutinin N-terminal domain-containing protein, yapH</td>
</tr>
</tbody>
</table>

Figure 1. Shows apokaryotic toxin assay. The top row of plates is BL21 E. coli cells with gene BJD13_RS18315. The bottom row is a negative control containing a plasmid with the gene in the reverse orientation. The first column of plates has 0.2% glucose (gene off), the second column has 0.2% arabinose (gene on), the third column has 0.002% arabinose, and the fourth column has 0.0002% arabinose. There are four replicates on each plate and four 1/100 dilutions from left to right. No toxic phenotype was seen.

Figure 2. Shows the eukaryotic toxin assay in yeast. The plate on the left contains 2% glucose (gene off) and the plate on the right contains 2% galactose (gene on). There is a positive and negative control on each plate with the gene of interest, BJD13_RS18340 in the middle 2 columns. There are 2 replicates of each group on both plates with four 1/10 dilutions from top to bottom. A toxic phenotype is seen.
Acknowledgments
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References


Üstün, S., & Börnke, F. (2014). Interactions of Xanthomonas type-III effector proteins with the plant
