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

Kylie Weis, Abigail Conroy, Tyler Smith, Parker King, Neha Potnis

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 Bastion 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 liner 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. The BL21 cells were dilution plated on LB media with 0.2% glucose (inactive promotor with gene off), 0.2% arabinose (gene on with araC promoter), 0.002% arabinose, and 0.0002% arabinose. A negative control with a gene in the incorrect orientation was used. The yeast strains were dilution plated on SC-U media with 2% glucose (gene off) and 2% galactose (gene on with ga11 promoter). A negative control with a gene in the incorrect orientation was also used in addition to a positive control containing the gene *cidB* encoding a protein toxic to eukaryotes. The yeast was incubated at 28°C. If no toxic phenotype was seen, the assay was conducted again at 37°C, as heat stress on the yeast may exacerbate a toxic phenotype.

Results

The computational analysis showed a high degree of conservation of putative effectors between strains and species. Some variation was seen between *Xp* and *Xv*; however, these species are more evolutionarily distant. EasyFig figures showed the synteny of the T6SS clusters was also conserved between strains (data not shown). The phylogenetic tree revealed that a gene located in the cluster follows the evolutionary lineage of genomic core genes as the strains were grouped according to the those outlined in Newberry et al. (2019). Annotations and protein predictions for the putative effectors are shown in Table 1. BJD13_RS18380 and BJD13_RS18385 (yapH) are not part of the T6SS according to Bastion 6. Additionally, they are characterized as a two-part autotransporter for adhesin (Thieme et al., 2005).

An assay for toxicity of gene BJD13_RS18315 in prokaryotes (BL21 E. *coli*) is shown in Figure 1. No significant difference was seen between the negative control and the putative effector. Genes BJD13_RS18250 and BJD13_RS18310 also did not show a toxic phenotype or decrease in growth. Prokaryote toxin assays were also conducted on genes BJD13_RS18270, and BJD13_RS18340. These assays showed a slight decrease in colony diameter (1mm) compared to the negative controls. An assay for toxicity in eukaryotes (W303a/BY4741a *Saccharomy cescerevisiae*) 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 prokaryoteor eukaryote toxins.

The slight decrease in colony diameter in the prokaryotic toxin assay may be due to the stress of producing a large amount of protein with the promoter turned on in the presence of arabinose. While the eukaryotic toxin assay of gene BJD13_RS18340 shows a toxic phenotype, the mechanism by which it inhibits grow is unknown.

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.

Gene locus tag	Annotation/protein prediction	Gene locus tag	Annotation/protein prediction
BJD13_RS18245	Hypothetical Protein	BJD13_RS18325	Hypothetical protein
BJD13_RS18250	DUF4124 domain containing protein	BJD13_RS18330	Type VI secretion system associated FHA domain containing protein (TagH - NCBI) (ImpI -JGI/IMG)
BJD13_RS18255	CPBP family intramembrane metalloprotease	BJD13_RS18335	DNA-binding LysR family transcriptional regulator
BJD13_RS18260	Putative secreted protein	BJD13_RS18340	Aldo/keto reductase family oxidoreductase -NCBI dehydrogenase-like oxidoreductase - JGI/IMG
BJD13_RS18270	Jacalin-like lectin domain-containing protein	BJD13_RS18365	Serine/threonine phosphatase
BJD13_RS18305	Serine/threonine kinase	BJD13_RS18375	Hypothetical protein
BJD13_RS18310	Zn-binding PAAR domain containing type VI secreted protein	BJD13_18375	Histidine-type phosphatase -NCBI 4-phytase / acid phosphatase -JGI/IMG
BJD13_RS18315	Predicted methyltransferase	BJD13_RS18380	ShlB/FhaC/HecB family hemolysin secretion/activation protein
BJD13_RS18320	Type VI secretion system secreted protein VgrG	BJD13_RS18385	filamentous hemagglutinin N-terminal domain-containing protein, *yapH*

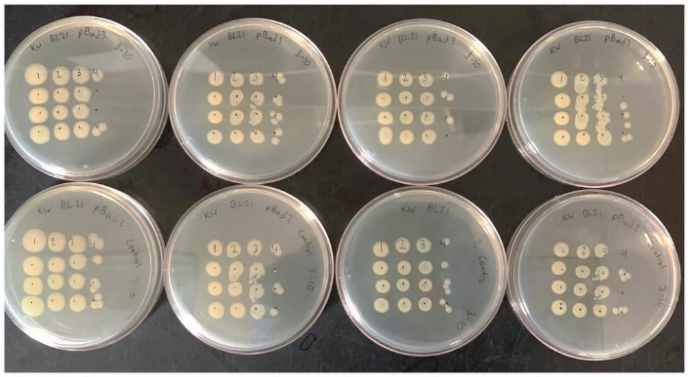

Figure 1. Shows aprokaryotic toxin assay. The top row of plates is BL21 E. coli cells with gene BJD13_RS18315. The bottom row is a negative control containing a plasmid with the gene in the reverse orientation. The first column of plates has 0.2% glucose (gene off), the second column has 0.2% arabinose (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.

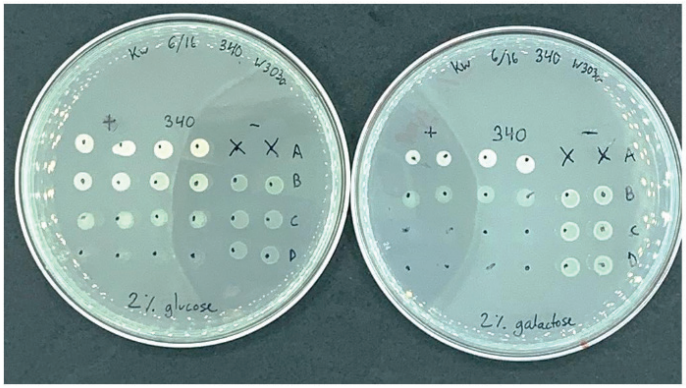

Figure 2. Shows the eukaryotic toxin assay in yeast. The plate on the left contains 2% glucose (gene off) and the plate on the right contains 2% galactose (gene on). There is a positive and negative control on each plate with the gene of interest, BJD13_RS18340 in the middle 2 columns. There are 2 replicates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

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