# GENETIC ANALYSIS OF *ZONULA OCCLUDENS TOXIN* (*ZOT*) GENE IN TEXAS ISOLATES OF *XYLELLA FASTIDIOSA*

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## ABSTRACT
Multiple subspecies of the phytopathogenic bacterium *Xylella fastidiosa (Xf)* exist which are pathogenic to distinct plant hosts, such as grapes, oleander, almonds, and citrus. Previously, DNA sequence analysis of the *mopB* and *gyr*B genes has been used to separate *Xf* strains into their subspecies groups. In this study, DNA sequence analysis of the *Zot* gene was used to corroborate the genetic variation found between three Texas strains of *Xf*, a grape strain, a weed strain, and an oleander strain (*BAN POL 055*, *GIL BEC 628A*, and *MED PRI 025* respectively). This approach provided variable gene sequences that allow for categorization of *Xf* at the population level. The *Xf* gene that encodes the z*onula occludens toxin* (*Zot*) is homologous to the *Zot* found in *Vibrio cholerae*, which is involved in tight junction modulation and disruption between host cells. The results of the analysis of this gene were consistent with the phylogeny found using the more conserved *mop*B and *gyr*B genes at the subspecies level and can be used to differentiate populations within subspecies. The analysis of these variable genes and gene regions provide additional opportunities for new diagnostic and disease management techniques.

## LAYPERSON SUMMARY
In this study, we sequenced several regions of the *Xylella fastidiosa (Xf)* genome. *Xf* has been implicated as the cause of several plant diseases that cause plant death and crop loss, including PD, almond leaf scorch, and citrus variegated chlorosis. By identifying and comparing the sequences of *Zot*, we are able to determine the relationship of the different *Xf* subspecies, and may also be able to identify different populations of *Xf*, such as those from California versus those from Texas. This will allow researchers to track the spread of Pierce's disease (PD) among others, and may be useful in detailing the mechanisms by which *Xf* causes disease.

## INTRODUCTION
*Xylella fastidiosa (Xf)* is a Gram-negative, xylem-limited, fastidious, insect-transmitted, gammaproteobacteria (26). Multiple subspecies have been described, including *Xf piercei* which causes Pierce's disease (PD)*, Xf sandyi* which cases oleander leaf scorch (OLS)*, Xf multiplex* which causes almond leaf scorch (ALS), *Xf 9a5c* which causes citrus variegated chlorosis (CVC) and others (19) (21) (23). *Xf* has distinctly different host ranges; though some strains of *Xf* are only pathogenic in a single host species, others cause disease in a variety of hosts (1) (10).

The *Zonula occludens toxin* (*Zot*) in *Xf* strains has been suggested as a new potential virulence factor in CVC caused by *Xf* strain *9a5c* (23). A homologous protein of the *Zot* family is found in many *Vibrio cholerae* strains and has been linked to disruption of tight junctions (11), and diarrheagenicity in *V. cholerae* that lack the cholerae toxin (5). Recently, a *Zot*-like protein was found in *Stenotrophomonas maltophilia* strains, which can cause severe health problems such as endocarditis and bacteremia (7). A *Zot* gene can also be found in strains of phytopathogens that are closely related to *Xf*, namely *Xanthomonas campestris,* which causes lesions and loss of water in plant tissue (2), and *Ralstonia solanacearum*, which causes bacterial wilt in a variety of plants (9).

Previous studies have shown that most of the sequence variation in *Xf* subspecies occurs in coding regions derived from bacteriophages (15). These regions are responsible for alterations to the chromosomes, including sequence rearrangements and deletions, of *Xf* subspecies, and therefore have an impact on the evolution of the genome of *Xf* (16) (24) (25). Several studies have shown that bacterial *Zot* genes have originated from bacteriophages (3) (7) (11) (13). The *Zot* gene found in *V. cholerae* has great sequence similarity to the protein product I (pI) of the filamentous phage Pf1, and is most likely derived from a Pf1-like phage (13). The pI protein, which shares similarity to many *Zot* proteins in *Xf*, has both an extracellular and intracellular region, and is necessary for phage packing and transport across the cell membrane in many filamentous phages (13) (22). The *Zot* gene discovered in *S. maltophilia* is reported to originate from the phage φSMA9, which is similar to the phage φLf which infects *Xanthomonas* species (3) (7). *Zot* genes in *Xf* share 55% identity with orthologs in the filamentous phage φLf of *Xanthomonas campestris pv. vesicatoria* and phage φSMA9 of *Stenotrophomonas maltophilia* , and with less than 30% identity to orthologues of *X. campestris pv. campestris* and RSM1 phage of *Ralstonia solanacearum* (15).

The *Zot* gene and respective protein are useful in comparing *Xf* because of its prophage origins, link to pathogenicity, cosmopolitan nature, and its variability in both nucleotide and amino acid sequences. In this work, the *Zot* genes of three Texas *Xf* strains, a grape strain (*BAN POL 055*), an oleander strain (*MED PRI 025*), and a multiplex strain (*GIL BEC 628A*)

were compared. These sequences were then translated and modeled *in silico* to compare the effects of the substitutions on protein structure. Finally, phylogenetic analysis was used to show the utility of the *Zot* gene in indentifying subspecies as well as populations within clades.

**OBJECTIVES**

1. Identify taxonomic differences between *Xf* strains that allow *Xf* population dynamics to be studied below the subspecies level.
2. Investigate the role of the ZOT protein in grape pathogenicity.

**RESULTS AND DISCUSSION**

**G**enbank accession numbers for *Xf BAN POL 055, MED PRI 025, GIL BEC 628A* were **GQ429146**, **GQ429147**, and **GQ891884**, respectively. These samples are labeled according to their subspecies (i.e. *Xf BAN POL 055* is labeled *Xf piercei* for ease of use). Sample *Xf BAN POL 055* had nucleotide sequences that align to the *Xf Temecula1* and *M23* with 99% identity while sample *Xf MED PRI 025* aligned with 99% identity the unfinished nucleotide sequence of *Xf Ann-1,* and *Xf GIL BEC 628A* aligned with to *Xf M23, Temecula1* and *Ann-1* with greater than 87% identity (**Table 1**).

**Table 1**. Nucleotides, Protein, and Homologous Structure Alignment Scores of *Zot1*.

| *Xf* | Nucelotide | | | | | | | | Protein | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Temecula1*** | | ***M23*** | | ***Ann-1*** | | | | e-value | | samples | |
| | e-value | Identity | e-value | Identity | e-value | Identity | ***BAN POL 055*** | | **d1ixza** | **c2r2aB** | ***BAN POL 055*** | |
| ***BAN POL 055*** | 0 | 99% | 0 | 99% | 0 | 93% | Identity | ***MED PRI 025*** | 9.7 E-16 | NA | Identity | ***MED PRI 025*** |
| ***MED PRI 025*** | 0 | 92% | 0 | 92% | 0 | 99% | 92.50% | Identity | 2.1 E-20 | 1.80 E-27 | 92.50% | Identity |
| ***GIL BEC 628A*** | 0 | 91% | 0 | 91% | 0 | 90% | 89.70% | 89.10% | 0.012 | 8.10 E-17 | 88.30% | 89.90% |

These results reveal homology between *Xf BAN POL 055* and *Xf M23 and Temecula1* and homology between *Xf MED PRI 025* and *Xf Ann-1*. Sequence variation between sample strains was also found. *Xf BAN POL 055* and *Xf MED PRI 025* shared 92.5% identity, *Xf BAN POL 055* and *Xf GIL BEC 628A* shared 87.9% identity, and *Xf MED PRI 025* and *Xf GIL BEC 628A* shared 87.1% identity. A hypervariable region was found between base pairs 40 to 160 in all three subspecies. Additionally, *Xf GIL BEC 628A* contained a large insertion at basepairs 344 to 387 not found in either *Xf BAN POL 055* or *Xf MED PRI 025* (**Figure 1**).

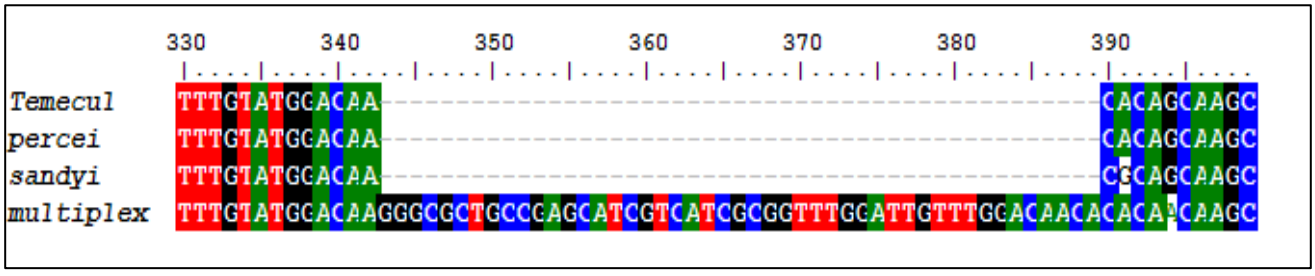


**Figure 1.** The insertion sequence appeared in multiple samples of *Xf GIL BEC 628A*, all collected in central Texas.

*In silico* translation of the *Zot* gene in all three subspecies yielded several amino acid changes and as well as changes in the predicted tertiary structure (data not shown). Sequence variation exists between members of *Xf* subspecies particularly in a hypervariable region found at the N-terminus of the *Zot1* protein (data not shown). Protein sequences submitted to Pfam returned significant results from the *Zot* family (PF05707) with e-values of at least $2.8e^{-11}$. In a homologous structure search,

the *Xf* subspecies were placed in two different groupings. *Xf GIL BEC 628A* and *MED PRI 025* matched the crystal structure of the N-terminus domain of the *Zot* protein of the *Neisseria meningitidis* (SCOP code c2r2aB) with an e-value of $8.1e^{-17}$ and $1.8e^{-27}$ respectively (**Table 1**). While *Xf BAN POL 055* matched a protein in the P-loop containing nucleoside triphosphate hydrolases superfamily (SCOP code d1ixza) with an e-value of $9.7e^{-16}$ (**Table 1**). As a reference, *Xf Temecula1* was also submitted to PHYRE and also matched the protein d1ixza in the triphosphate superfamily. *Xf GIL BEC 628A* and *MED PRI 025* also aligned to the protein d1ixza, but with a higher e-value than their respective alignments to c2r2aB (**Table 1**).

Phylogenetic comparison shows three distinct groupings of *Xf Zot* proteins (data not shown). These groupings were not consistent with current organization of *Xf* clades. Closer analysis of the individual *Zot* proteins revealed that three distinct homologues of *Zot* proteins exist in *Xf*. Each form was placed in the *Zot* superfamily according to HMM searches performed at Pfam. Additionally, these homologues of *Zot* are found across many taxa, including *Xanthomonas campestris* and *Stenotrophomonas maltophilia*. Finally, protein sequence comparisons between the four copies of the *Zot* gene found in *Xf Ann-1* show that only two copies share a high degree similarity (an error value of 0.0) while all other comparisons show positive error value scores, thus indicating high sequence variation. The *Zot* proteins thus form three distinct homologues hereby named *Zot1*, *Zot2,* and *Zot3*. *Zot1* includes the three sequences from *Xf BAN POL 055, MED PRI 025,* and *GIL BEC 628A* as well as two additional sequences from *Xf Temecula1* (**gi|28198830|** and **gi|28198817|**), two additional sequences from *Xf M23* (**gi|182681531|** and **gi|182681517|**), and one additional sequence from *Xf Ann-1* (**gi|71902114|**). *Zot2* includes one sequence from *Xf Temecula1* (**gi|28198835|**), one sequence from *Xf M23* (**gi|182681534|**), two sequences from *Xf Ann-1* (**gi|71901575|** and **gi|71728661**), one from *Xf Dixon* (**gi|71164684|**) and two sequences from *Xf M12* (**gi|170730250**| and **gi|170730245**|). *Zot3* includes one sequence from *Xf Ann-1* (**gi|71728117**) and two sequences from *Xf 9a5c* (**gi|15838473**| and **gi|15838468**|).

Phylogenetic analysis comparison of *Zot1* proteins corroborated analysis of the *mopB* and *gyrB* proteins. The *Xf BAN POL 055* sample grouped with *Xf Temecula1* and *M23* sequences, and the *Xf MED PRI 025* sequence grouped with the sequence from *Xf Ann-1*, while *the Xf GIL BEC 628A* sequence branched separately from the other sequences (**Figure 2**).

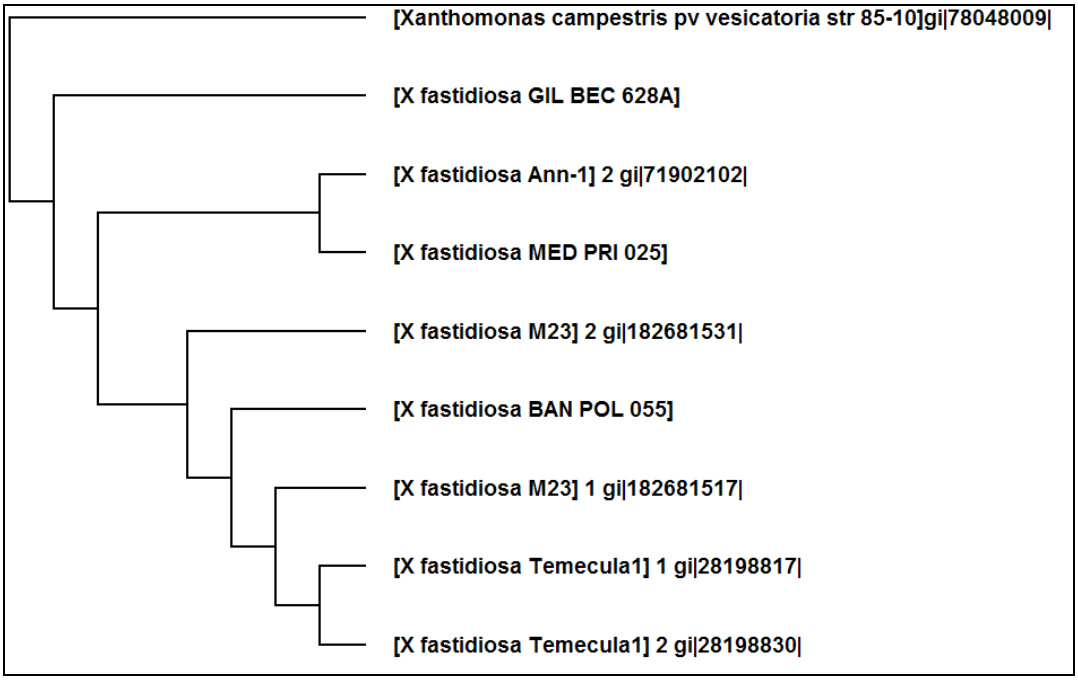


**Figure 2.** Cladogram of *Zot1* using sequences found in NCBI. Alignment performed using ClustalW and visualized using Treeview v1.6.6. Genbank accession numbers follow organism name. Sequences labeled according to source organism and genomic location. Sequence from *Xanthomonas campestris pv vesicatoria str 85-10* is a filamentous phage Cf1c related protein belonging to the *Zot* family.

The sequence variation in *Zot1* found in Texas subspecies of *Xf* is evident and can be exploited for classification and identification. All tested isolates contained *Zot1* representing all three clades of *Xf* present in Texas. *In silico* protein analysis revealed changes in amino acid sequence, as well as predicted changes in tertiary structure. Phylogenetic analysis using the *Zot1* protein accurately grouped the sample *Xf* strains to their respective clades. Finally, sequence variation within the *Zot1* gene was great enough to show differentiation between populations of *Xf piercei* populations in California and Texas, thus allowing a greater degree of classification of *Xf*.

*Zot1* was chosen for this study as the *Zot1* gene is the most common form of *Zot* in *Xf Temecula1* and *M23*. Future study will elucidate the relationship between *Zot1*, *Zot2*, and *Zot3* and determine if they are paralogous, xenologous, or a combination of the two. Additionally, future study will describe which Texas isolates, if any, contain the *Zot2* or *Zot3* gene. Alignment analysis performed using BLAST*n* and BLAST*p* shows that several *Xf* genomes, including *Xf M12,* and *9a5c* do not contain *Zot1* based on searches conducted in the EST, Genome, Chromosome, and Nucleotide libraries of NCBI (data not shown). Additionally, *Zot1* does not appear in *Xf Dixon,* though this may be a result of an incomplete *Dixon* genome.

The exact origin of *Zot1* is not certain; however, by examining other, closely related *Zot* genes, we may make inferences. *Zot2* appears to have evolved from filamentous bacteriophages in *Vibrio cholerae* and *Stenotrophomonas maltophilia* (7) (13). The *Zot2* protein found in *Xf* has significant homology to another *Zot*-like protein found in strains of *S. maltophilia* (7). This *Zot* protein is the first virulence factor in clinical isolates of *S. maltophilia* strains, and shows a similarity to the *Zot2* protein in *Xf Dixon* with an error value of $1e^{-128}$, and appears to have arisen from infection from phage φSMA9 (7). Though, *Zot1* and *Zot2* share little identity (less than 5%), it can be deduced that *Zot1* arose in *Xf* through phage integration as in *Stenotrophomonas maltophilia* and *Vibrio cholerae*. In a BLAST*x* search at NCBI, *Xf BAN POL 055* matched *Xanthomonas* phage Cf1c protein labeled Cf1cp4, which contains a putative *Zot* protein. Additionally, several of the phage related proteins found in *S. maltophilia* strain c5 match phage related protein sequences surrounding a *Zot1* gene found in *Xf Temecula1*. Finally, the direction of the open reading frame of the *Zot1* gene in *Xf BAN POL 055* and *GIL BEC 628A* differs from *Xf MED PRI 025*, and the genomic context of the *Zot1* gene differs between each strain. Genomic rearrangements of *Xf* prophage regions have been described before (16). Initial analysis of the alignment of the *Xf Ann-1* partial genome to both *Xf Temecula1* and *M23* shows that the *Zot1* gene and its associated phage integrases are found in the opposite direction and in different locations in the genome as can be inferred from the necessary use of different primer sets at the beginning and end of the *Zot1* gene, as well as a study of the genomic context of *Xf M23, Temecula1,* and *Ann-1* found at NCBI. Reorganizations such as these have previously been attributed to large prophage regions that act as recombination sites (16). Thus, it is possible that some *Xf* ancestors received the *Zot1* gene after branching. Another possibility is that phage-related recombination removed the *Zot1* gene from some *Xf* subspecies after a branching event

Little is known about the *Zot* protein's structure due to the complexity of isolating *Zot* for analysis as a result of its transmembrane region (7) (13) (22). *In silico* translations and structure predictions offer great insights into protein function and classification and has been found to be accurate and sensitive. By using *in silico* analysis, the sequence variation found in *Zot* genes between subspecies has been shown to yield differences in protein structure. These differences appear near the N-terminus of the *Zot1* protein, which is extracellular in other species of bacteria (7) (13) (22). It is possible to infer that changes made in the *Zot* protein are driven by host-pathogen interactions. Further study regarding the location and expression of the *Zot1* protein is necessary to fully elucidate the relationship.

The first step in determining host range in differentially pathogenic bacteria is placing the bacteria into clades (17). Many techniques for identification and classification exist; however, the complexity of *Xf* pathogen makes categorization based on morphology or pathogenicity difficult (1). Additionally, *Xf* has been shown to have limited genomic variability within clades and region, and that the majority of strain specific genes occur in prophage regions, though they contain genomic islands which enable rapid evolution (25). Techniques that focus on classification based on well conserved regions shared by all *Xf* subspecies might then miss putative evolutionary growth and adaptation. The *Zot1* gene insertion is an excellent target for QRT-PCR, and the small sequence differences can be targeted by restriction enzyme digestion analysis for quick and accurate identification and classification of *Xf* subspecies and populations.

**CONCLUSIONS**

Comparative analysis *Zot1*genes and proteins provide accurate, population level differentiation therefore allows researchers greater ability to track the spread of economically important phytopathogens. Additionally, *in silico* translation and analysis of *Zot1* describes in greater detail differences between strains, and describes possible conformation changes that result from sequence changes between strains. Taken together, these results show that *Zot1* is a useful target for differential sequence analysis and can be used to elucidate the phylogenetic history of *Xf*, and its spread through the U.S.

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