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Review

Genome-wide study of *Xylella fastidiosa:* Effects of detection and strain relationships

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The xylem limited plant pathogenic bacterium *Xylella fastidiosa* causes economically important diseases on agronomic, horticultural and landscape plants. This review includes the current status of polymerase chain reaction (PCR) based systems for detection and characterization of *X. fastidiosa*, and presents a genome-wide analysis of strain differentiation. The use of genomics data for strain comparisons will improve the understanding of the genetic determinants of strain specific pathogenicity and virulence. The genome-level analysis can be applied to design new strategies for management and control of *Xylella fastidiosa* associated diseases in a wide range of crops.

Key words: Comparative genomics, PCR, diagnosis, database, phylogeny, evolution

INTRODUCTION

Xylella fastidiosa causes economically-important diseases of food crops including Pierce's disease (PD) of grapevine (*Vitis* spp.), citrus variegated chlorosis (CVC) of *Citrus* spp., almond leaf scorch (*Prunus amygdalus*), and coffee leaf scorch (*Coffea arabica*) (Purcell and Hopkins, 1996; Hopkins and Purcell, 2002 Almeida and Purcell, 2003). Xf also causes leaf scorch disease in a wide range of landscape trees and ornamental plants, such as elm, maple, mulberry, oak, sycamore, and oleander (Gould and Lashomb, 2005).

The bacterium proliferates in the xylem of infected hosts (Hopkins, 1989) and is transmitted by several species of xylem-sap feeding insect vectors (*Homoptera, Cidcadellidae* and *Cercopidae*). Transmission efficiency varies widely among vector species (Purcell and Hopkins, 1996). Infection by the bacterial pathogens may result in reduced plant growth, wilting, leaf scorch, twig dieback, decline and death of some plants (e.g., grapevines and plums).

X. fastidiosa was described as the type species of a new genus based on analyses of 16S rRNA signature sequences, (Wells et al., 1987). The current classification includes all strains of *X. fastidiosa* as one species with several sub-species that differ in plant host range and pathogenicity (Schaad et al., 2004; Schuenzel et al., 2005). Strain identification and differentiation was previously based on pathogenicity, nutritional requirements in culture, and by using several molecular biomarkers. In the recent years, the genomes of several strains of *X. fastidiosa* have been sequenced, facilitating improved and high-throughput molecular tools for pathogen detection and epidemiological studies. Here, we review the available DNA based methods for *X. fastidiosa* diagnosis and strain differentiation and genome-wide variation among strains. We also comment on the significance of genomic variation studies as applied to *X. fastidiosa* biology.

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PCR TOOLS FOR DETECTION AND STRAIN CHARACTERIZATION OF *XYLELLA FASTIDIOSA*

PCR is a valuable tool for detection, diagnosis and characterization of phytopathogens (Alvarez, 2004). This technology is especially useful in the case of fastidious pathogens that are difficult to isolate and characterize

based on traditional laboratory tests for pathogen identify-cation. Rapid development of genomic techniques for characterization of bacteria over the past decade has greatly improved the quality of plant pathogen detection and has advanced our knowledge in understanding genetic relationships among pathogens.

For most of the pre-genomic era, *X. fastidiosa* PCR protocols were based on partially characterized DNA (Chen et al., 1992, Minsavage et al., 1994; Pooler and Hartung, 1995a; 1995b; Costa et al., 2000; Chen et al., 2000; Hendson et al., 2001). With the availability of whole-genome sequence information for several *X. fastidiosa* strains, PCR protocols were developed for *X. fastidiosa* detection (Rodrigues et al., 2003; Chen et al., 2005a; 2005b; Chen and Civerolo, 2005; Olson et al, 2006; Francis et al., 2006) and for characterization of strain diversity (Coletta- Filho et al., 2001; Schaad et al., 2002; Lin et al., 2005b; Schuenzel et al., 2005; Scally et al., 2005).

PCR-based protocols using single pair of primers have contributed significantly to pathogen detection and strain differentiation. Primers RST31/RST33, which generate a 733 base pair (bp) PCR product, have been the most widely used primers for *X. fastidiosa* detection (Minsavage et al., 1994). Primers CVC-1 and 272-2 -int (Pooler and Hartung, 1995b), which generate a 500 bp PCR product, were used for the specific detection of CVC strains. A sequenced characterized amplified region (SCAR) marker was developed for detection and differentiation of PD strains of *X. fastidiosa*, using the primer pair Xf-1/REP-2 that amplifies a 350 bp fragment. (Travensolo et al, 2005). A summary of current information on primers for *X. fastidiosa* detection and strain differentiation is presented in Table 1.

Real-time PCR protocols are sensitive, reliable and have the added advantage of quantifying bacterial population levels in a sample. Several such protocols have been developed for *X. fastidiosa*. PCR quantification of CVC strains in Brazil was achieved with primers based on a target sequence identified by random amplification of total genomic DNA (Oliveira et al., 2000; Pooler and Hartung, 1995b). A PD specific diagnostic protocol was developed using conserved sequences of the 16S rRNA and 16S-23S internal transcriber spacers (ITS) (Schaad, et al., 2002). In this system, the 16S RNA primers were more sensitive than the ITS primers due to the high number of template DNA copies per *X. fastidiosa* cell. However, as the 16S rRNA region is highly conserved among related taxa, it is recommended that both primer sets be employed to achieve both increased sensitivity and high specificity. Furthermore, the 16S rRNA amplicon was smaller than 80bp, making it difficult to resolve the PCR product from primer dimers by electrophoresis in agarose gels. Detection of *X. fastidiosa* in the glassy-winged sharpshooter vector (GWSS) using real time PCR with SYBR Green (Bextine, et al., 2005) is less reliable than the TaqMan system because SYBR Green cannot discriminate the specific target DNA amplicon from nonspecific PCR products or primer dimers (Giulietti et al., 2001). Recently, a TaqMan-based real-time quantitative PCR system (Francis et al., 2006) using HL5/HL6 primers based on whole genome sequence information was developed to reliably detect and identify *X. fastidiosa* in grapevines and almond trees (early in the spring), as well as in insect vectors, without the need for nested PCR.

The presence of PCR inhibitors, especially in samples collected late in the season when symptoms are fully expressed has been an inherent problem with *X. fastidiosa* detection. Few of the above protocols have specifically addressed this problem to mitigate the effect of inhibitors in plant and insect extracts that interfere with PCR (Minsavage et al., 1994; Bextine and Miller, 2004; Francis et al, 2006) . Immunocapture PCR (Pooler et al., 1997) and addition of Chelex 100 (Ciapina et al., 2004) have been used to avoid inhibition of PCR for sample extracted from insect vectors. A Bio-PCR protocol has been developed (Fatmi et al., 2005) to avoid the inhibitory effect of sap for detection of *X. fastidiosa* in samples from grape and citrus.

Insect vectors generally harbor low concentrations of the *X. fastidiosa*. Hill and Purcell (1995) estimated that less than 100 viable bacterial cells within an insect vector were sufficient for the transmission of *X. fastidiosa* to grape plants. These low level populations are below the detection limit for isolation or ELISA, and explain the reported requirement for nested PCR to detect *X. fastidiosa* in insect vectors (Pooler et al., 1997, Ciapina et al., 2004 and Rodrigues et al., 2003). Genome-based PCR primes are highly specific and sensitive for detection of *X. fastidiosa* in insect vectors by standard and real-time PCR (TaqMan) with a detection threshold of 10 bacterial cells per insect, (Francis et al, 2006).

Most of the current PCR protocols were developed and tested using DNA template samples extracted from axenic cultures of *X. fastidiosa*. Application of these diagnostic procedures to clinical samples is affected by the specificity and efficiency of the primers. This is particularly problematic for plant or insect vector template DNA preparations that often also contain endophytic or endosymbiotic bacteria. Despite these limitations, PCR based techniques remain the method of choice for rapid detection of *X. fastidiosa* and are particularly suited for processing large numbers of samples in surveys and epidemiological studies of *X. fastidiosa* associated diseases.

WHOLE GENOME SEQUENCING EFFORTS AND GENERAL GENOME FEATURES

X. fastidiosa was the first plant pathogenic bacterium whose genome was completely sequenced. There are several online resources that provide information that covers various aspects of *X. fastidiosa* genomics and bio-

Table 1. PCR protocols currently available for *X. fastidiosa* detection/strain differentiation.

CONVENTIONAL PCR				
Primer name[a]	**Sequence 5'------3'[b]**	**Size[c]**	**Strains**	**References**
RST31	5'-GCG TTA ATT TTC GAA GTG ATT CGAT TGC-3	733	General	Minsavage
RST33	5'-CAC CAT TCG TAT CCC GGT G-3		detection	et al., 1994
272-1-int	5'-CTG CAC TTA CCC AAT GCA TCG-3'	600	General	Pooler & Hartung
272-2-int	5'-GCC GCT TCG GAG AGC ATT CCT-3'		detection	1995b
CVC-1	5 -AGATGAAAACAATCATGCAAA-3	500	CVC	Pooler & Hartung
272-2-int	5 -GCC GCT TCG GAG AGC ATT CCT-3		Specific	1995b
SSXfsa0067aS19	5'-CGG CAG CAC ATT GGT AGT A-3'	Set A	General	Rodriguez, et al.,
SSXfas1439aA19	5'-CTC CTC GCG GTT AAG CTA-3	1348	detection	2003
SSXfsa0067aS19	5'_CGG CAG CAC ATT GGT AGT A-3	Set B	General	Rodriguez, et al.,
SSXfa0838aA21	5'-CGA TAC TGA GTG CCA ATT TGC-3'	745	detection	2003
SSXfas0838aAS1	5'-CGA TAC TGA GTG CCA ATT TGC-3'	Set C	General	Rodriguez, et al.,
SSXfas1439aA19	CTC CTC GCG GTT AAG CTA	603	detection	2003
FXYgyr499	5-CAG TTA GGG GTG TCA GCG-3'	429	General	Rodriguez, et al.,
RXYgyr907	5'-CTC AAT GTA ATT ACC CAA GGT-3'		detection	2003
Xf-1	5'-CGGGGGTGTAGGAGGGGTTGT-3'	350	PD Specific	Travensolo
REP 2	5'-ICGICTTATCI GGCCTAC-3'			et al, 2005
Teme150fc	5 TCT ACC TTA TCG TGG GGG AC 3	348	PD	Chen et al
Teme454rg	5 AAC AAC TAG GTA TTA ACC AAT TGC C 3 ,		Specific	2005b
Teme150fc	5 TCT ACC TTA TCG TGG GGG AC 3	700	PD	Chen et al
Xf16s1031r	5 AAG GCA CCA ATC CAT CTC TG 3		Specific	2005b
Dixon454fa	5 CCT TTT GTT GGG GAA GAA AA 3	847	ALSD	Chen et al
Dixon1261rg	5 TAG CTC ACC CTC GCG AGA TC 3		Specific	2005b
BBXFOUTF1	5'-AAG CGC CTC CGT GAG TTA TC-3'	381	General	Olson et al,
BBXFOUTR1	5'-CCT TCA CGC ATA TCA TCA CC-3			2006
HL5	5'-AAG GCA ATA AAC GCG CAC TA-'3	221	General	Francis
HL6	5'-GGT TTT GCT GAC TGG CAA CA-3'			et al., 2006
REAL TIME PCR- TaqMan				
ITS-Oligos			General	Schaad
XfF1	5' AAA AAT CGC CAA CAT AAA CCC A 3'			et al., 2002
XfR1	5' CCA GGC GTC CTC ACA AGT TAC 3'	n/a		
XfP1 (Probe)	5' (6FAM) ACC TAT GCC AAC ATC AAA CCC TGA ATG CA (TAMRA) 3'			

Table 1. Contd

16 S Oligos			General*	Schaad
XfF2	5’ CTC GCC ACC CAT GGT ATT ACT AC 3’	n/a		et al., 2002
XfR2	5’ CTG GCG GCA GGC CTA AC 3’			
XfP2 (Probe)	5’ (6 FAM) ATG TGC TGC CGT CCG ACT TGC ATG TAMRA 3’			
CVC-1 F	5’-AGA TGA AAA CAA TCA TGC AAA-3’		CVC	Oliveira,
CCSM-1-R	5’-GCG CAT GCC AAG TCC ATA TTT-3’	n/a	SPECIFIC	et al., 2002
TAQCVCV (PROBE)	5’-FAM AAC CGC AGC AGA AGC CGC TCA TC TAMRA p-3’			
HL5	5’-AAG GCA ATA AAC GCG CAC TA-’3		General	Francis
HL6	5’-GGT TTT GCT GAC TGG CAA CA-3’	n/a		et al., 2006
p5/6FAM (Probe)	FAM 5’-TGG CAG GCA GCA ACG ATA CGG CT 3’BHQ-1			
	* = non specific also reacts with other plant pathogenic bacteria (Schaad, 2002) n/a = non applicable			

Table 2. Internet data resources for *X. fastidiosa* research.

	Organization	Features	Web address
1	University of California at Berkeley	A scientific and community internet resource on plant diseases caused by the bacterium *X. fastidiosa.*	http://www.cnr.berkeley.edu/xylella/
2	Organization for Nucleotide Sequencing and Analysis (ONSA)	First website of *X. fastidiosa* strain 9a5c genome database	http://aeg.lbi.ic.unicamp.br/xf/
3	National Center for Biotechnology Information (NCBI)	Central warehouse for scientific information	http://www.ncbi.nlm.nih.gov/
4	Integrated genomics Inc.	Genome and gene sequences of three strains (9a5c, Dixon, Ann1) of *Xylella fastidiosa*	http://www.integratedgenomics.com/genomereleases.html
5	Joint Genomic Institute (JGI)	Contains information on gene pathway database of the two draft strains, Dixon and Ann1.	http://genome.jgi-psf.org/mic_cur1.html
6	The Institute for Genome Research	Reannotated database for (9a5c, 418 more genes) and Temecula-1(861 more genes).	http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi
7	European Bioinformatics Institute	Integr8 database	http://www.ebi.ac.uk/integr8/OrganismHomeAction.do?orgProteomeID=47
8	Citrus Research Board, CA; USDA-ARS, Parlier, CA	Comparative database of 9a5c, Ann1, Dixon and Temecula1 strains of *X. fastidiosa*	http://cropdisease.ars.usda.gov/CVC_index.htm
9	EMBL-EBI	Genes of strain 9a5c are categorized according to different criteria such as 3D structure, homologues and functional class etc	http://jura.ebi.ac.uk:8765/
10	Dna data bank of Japan (DDBJ)	Genome of strain 9a5c presented in a graphic view with additional search functions	http://gib.genes.nig.ac.jp/single/index.php?spid=Xfas_9A5C
11	Munich Information Center for Protein Sequences (MIPS)	Protein structure analysis for the strain 9a5c	http://pedant.gsf.de/

informatics (Table 2).

The *X. fastidiosa* CVC strain has a genome size of 2.67 Mb and carries two plasmids, pXF51 (51.1 Kb) and pXF1.3 (1.3 Kb) (Simpson et al., 2000). A total of 2,249 open reading frames (ORFs) were predicted of which 58.4% were assigned putative functions based on homology with known genes. A cosmid map using 1,056 clones was generated, which was later used to assist in contig assembly, determine colinearity and fill in seq-uence gaps (Frohme et al., 2000) . A second round of annotation predicted an additional 131 potential ORFs (Bhattacharyya et al., 2002). The PD-causing *X. fastidiosa* Temecula-1 strain has a genome size of 2.51 Mb (chromosome) and a single plasmid, pXFPD1.3 (1.34 kb) (Van Sluys et al., 2003). A total of 2,066 ORFs were originally predicted 68.1% of which, were assigned a putative function. The genomes of two additional *X. fastidiosa* strains from oleander and almond have been mostly sequenced (~95%), but several gaps remain to be resolved (Bhattacharyya et al., 2002). For the Ann-1 strain associated with oleander leaf scorch, 93 contigs (2.6 Mb) have been assembled and predicted to encode 2,870 ORFs. The partial sequence of the Dixon strain associated with almond leaf scorch was assembled from 121 contigs (2.4 Mb) and encoded 2,681 ORFs. For both strains, 62% of the predicted ORFs were assigned putative functions. The GC content of these four strains ranges between 51.8 and 52.7%.

WHOLE GENOME COMPARATIVE STUDIES AND STRAIN SPECIFIC FEATURES

To date, there are few bacterial species for which genomic information of multiple strains is available. Comparative whole genome sequence analyses of the four sequenced *X. fastidiosa* strains have provided increased understanding of genome-wide variations that presumably are critically important in strain divergence, host specificity and pathogenicity (see below).

In silico comparative analyses among *X. fastidiosa* strains and with sequenced *Xanthomonas* strains have been done (Bhattacharyya et al., 2002; Van Sluys et al., 2002; 2003; Moreira et al., 2005; Doddapaneni et al., 2006). Highlights of these identified strain specific gene variations that have direct applicability in new biomarker development are discussed below.

Bhattacharyya et al. (2002) identified 1,705 ORF clusters conserved (minimum average similarity of 82%) among *X. fastidiosa* strains. These authors concluded that a putative prophage insertion located next to the hypothetical replication terminus may account for strain specificity to citrus. In addition, 133 ORFs were identified as specific to the Dixon strain. Of these ORFs specific to the Dixon strain, 78 are hypothetical (e.g., no known function) and 55 showed similarity to other bacterial genome sequences involved in conjugal transfer or type II restriction and modification system components. Similarly, the Ann-1 strain genome has 180 strain specific ORFs that are mainly composed of phage-related proteins and plasmid maintenance coding genes. Strain 9a5c has the highest number (375) of specific ORFs.

Van Sluys et al. (2002) compared the genomes of eight plant- associated bacteria, including *X. fastidiosa* strain 9a5c, and *Xanthomonas axonopodis* pathovar *citri* (XAC). Comparative analysis of the 9a5c and Temecula-1 geno-mes (Van Sluys et al., 2003) identified a small (1.3 Kb) PD strain specific plasmid. Further, they concluded that the chromosome of Temecula- 1 strain is 150 kb smaller than that of 9a5c. Nearly, 98% of the Temecula-1 genes also are present in 9a5c strain, with a high average identity of (98%); these genes may constitute the core genome of the *X. fastidiosa*. ORFs that exhibited a higher degree of divergence may be related to plant and insect host inter-actions, and include those encoding fimbrillins, hemag-glutinins, colicins, hemolysins, toxins, drug resistance proteins, and DNA restriction modification enzymes.

More extensive analysis of differences in the genomes of 9a5c and Temecula 1 was done by Monteiro et al. (2005), who described some previously unreported ORFs. Strain 9a5c has five large unique chromosomal regions with more than eight genes and nearly all these are flanked by ORFs encoding putative phage proteins. In 9a5c, there are 20 conjugal transfer protein encoding ORFs. Temecula-1 carries four unique genomic regions and has the smallest number of unique ORFs (Bhattacharyya et al., 2002; Van Sluys et al., 2003). The first region has three ORFs, including a site-specific DNA-methyltransferase. The second region, which is the largest of the four (PD0906–PD0951), has 30 unique coding sequences (CDSs), encoding 16 phage-related proteins and 14 hypothetical proteins. The third region has four genes, including ORFs for a proteic killer suppression and a virulence- associated protein (*vapI*). The fourth region codes for a phage-related endolysin, a type II restriction enzyme (*nspV*), and its cognate methylase.

Moreira et al. (2005) concluded that the Temecula-1 strain has 51 strain-specific ORFs (2.47%) and the 9a5c strain has 152 (6.78%) strain-specific ORFs. Nearly, 50% of these ORFS in both the strains are hypothetical, and many reside within mobile genetic elements. They also identified three major genomic rearrangements with a putative phage-integrase ORF at one end. If prophage regions are excluded from the sequences and the rearrangements reoriented *in silico*, the two genomes are nearly collinear. Thus, phage appears to be actively involved in rearrangement of the *X. fastidiosa* genome, with structural divergence between the two genomes mediated by lateral gene transfer via transduction. In addition to these rearrangements, each genome has a specific genomic island, designated as giCVC in strain 9a5c and giPD in the PD strain. The giCVC island was initially thought to be citrus-strain specific (Bhattacharya et al., 2002). However, giCVC was subsequently determined to be present in different strains of *X. fastidiosa* from South America (Van Sluys et al., 2003).

Comparative genomic analysis of the four strains (two complete and two drafts) suggests that there are 1,579 homologous genes representing 87 families and 194 non-coding sequences, which accounts for 72.6% and 3.6%, respectively, of the total sequenced genome size (Doddapaneni et al., 2006). Based on the BLAST search analysis, 108 (6.8%) of these were unique to *X. fastidiosa* with no known homology to the sequenced bacterial genomes. There were 10 to 241 strain-specific unique genes and 68 to 147 non-coding (intergenic) sequences in each strain. The highest number of strain-specific genes occur in the strain CVC-9a5c (241 genes) followed by Ann1 (145 genes), Dixon (96 genes) and Temecula-1 (10 genes). This, and previous results suggest that the Temecula-1 strain most closely represents the ancestral genome of *X. fastidiosa*. A portion of these strain specific genes, when BLAST searched against the NCBI database did not show homology to any of the previously sequenced bacterial genomes, suggesting that these are also unique to that strain. There are nine such genes in strain Temecula- 1, 54 in strain Dixon, 83 in strain Ann-1 and 60 in strain 9a5c. Further, a small portion of these strain- specific genes showed significant differences in terms of their codon usage and GC composition from other *X. fastidiosa* genes suggesting their xenologous origin. There are 27 such genes in the strain 9a5c, 25 in strain Ann1, and 7 in strain Dixon, respectively. The above studies identified macro level differences among the genomes of different *X. fastidiosa* strains that can readily serve as loci specific for detection, strain diversity and population genetic studies.

Using suppression subtractive hybridization (SSH) method, Harakava and Gabriel (2003) identified 18 genes that are specific to the CVC strain and absent in a PD-causing strain. Seven of these genes were from the 51-kb mega plasmid of the CVC strain and the other 11 were located on the chromosome. Loss of virulence after several passages in axenic culture has been reported for a number of pathogenic bacteria (Behr et al., 1999). The main reason for attenuation was attributed to the loss of the ability of bacterial cell attachment to specific sites in the host (Masuzawa et al., 1994). Similarly, virulent strains of *X. fastidiosa* causing PD may partially or totally lose virulence after successive transfers in culture medi-

lose their ability to aggregate, suggesting a connection between aggregation and pathogenicity of *X. fastidiosa*. Monteiro et al. (2001) showed that inoculation of strain 9a5c into periwinkle (*Catharanthus roseus*), showed more severe symptoms when plants were inoculated with cells at the eighth transfer in culture medium compared to symptoms produced by inoculation of bacterial cells at the 58th passage. To confirm this, De Souza et al. (2003) used whole genome DNA micro-arrays spotted with approximately 2,200 ORFs from strain 9a5c. Total RNA obtained from cells at the first passage and cells after several passages were labeled and used in a competitive hybridization assay. Seven ORFs encoding pathogenicity-related proteins were expressed mainly in the FP condition. Three of these ORFs are related to adhesion functions characterized in related bacteria.

An alternative approach was based on comparative microarray hybridizations (Koide et al., 2004) using the nonpathogenic *X. fastidiosa* strain J1a12 associated with CVC and the highly pathogenic CVC strain 9a5c. Based on this analysis, 14 coding sequences of strain 9a5c were absent or highly divergent in strain J1a12. Of these, arginase (responsible for multiplication of the pathogen in multiple hosts due to its ability to down-regulated host nitric oxide production) and a fimbrial adhesion precursor of type III pilus (responsible for cell aggregation) were absent in J1a12 strain (Koide et al., 2004). Using strain 9a5c as a reference, 12 *X*. *fastidiosa* isolates were compared on a microarray platform (Nunes et al., 2003). The results suggested that *X. fastidiosa* has a flexible gene pool with up to 18% of the total genome comprised of horizontally acquired elements, (prophages, plasmids, and GIs). A majority of these mobile elements were transcriptionally active and displayed coordinated responses to environmental stimuli. Based on real time PCR analy-sis, De Souza et al. (2005) showed that adhesion is important at the beginning of biofilm formation, whereas genes related to adaptation are essential for maintenance of the bacteria in planta. Nucleotide sequences of selec-ted RAPDs from *X. fastidiosa* strains were explored to identify previously unreported single nucleotide polymerphism (SNPs), loci associated with horizontal transfer events, and a cryptic plasmid (Chen et al., 2005a). In conclusion, the above comparative studies have shown that difference in pathogenicity among *X. fastidiosa* strains and presumably host specificity is genetically controlled. Identifying the key elements involved in such processes would aid in the design of better diagnostic tools.

NEW OR UNDEREXPLORED MOLECULAR TOOLS

Availability of whole genome sequence information can be used to design high-throughput marker systems such as SNPs and insertion/deletion (INDEL) . To date, there are few reports on the utilization of SNPs for *X. fastidiosa* strain differentiation (Chen et al., 2005a; Chen and Civerolo, 2005). A related study has used minor sequence variations in multilocus sequence typing (MLST) design to examine phylogenetic relationships among *X. fastidiosa* strains (Scally et al., 2005). In a more recent study, multiple alignments of the 1,579 conserved ORFs in the four sequenced genomes identified 12,754 SNPs and 14,449 INDELs in the common ORFs and 20,779 SNPs and 10,075 INDELs in the 194 non-coding sequences. The average mutation frequency for conserved ORFs among the six compared groups was 3.93%, which equals one mutation per 25 to 26 nucleotides (Doddapaneni et al., 2006). The average SNP frequency was 1.08×10^{-2} per base pair, which translates to approximately one SNP for every 93 bp of the DNA. The average INDEL frequency was 2.06×10^{-2} per base pair, which equates to approximately one INDEL per 30 bp of DNA. Thus, INDELs occurred within 53.1% (812) of all genes, and SNPs occurred in every gene. Studies in other bacterial systems suggest that SNPs can be used for strain diversity assessment with gene-based locus-specific SNPs useful for functional genotyping (Pearson et al., 2004; Levy et al., 2004). SNPs can be used as a routinely screening tool to identify disease outbreaks and appearance of new variants. Information from such SNPs can help develop genome-wide linkage disequilibrium (LD) haplotypes for phenotype association studies. INDELs play a major role in sequence divergence between closely related DNA sequences in animals, plants, insects and bacteria (Britten et al., 2003). Understanding the functional significance of the existing INDELs, especially those causing frame shift mutations (internal INDELs) and altered transcripts (external INDELs), will aid in identification of underlying causes of host preferences shown by distinct Xf strains, particularly in cases where macro level genomic variation is lacking. As both SNPs and INDEL markers are adaptable to multiplexing and high-through-put screening platforms, these procedures are amenable to high through-put sampling. Multi- locus simple sequence repeat (SSR) DNA markers were combined with fluorescent-labeled primers to generate a high-throughput multiplex genetic analysis system for *X. fastidiosa* (Lin et al., 2005a). Using this system, pathogen diagnostic and genetic analysis data can be automated. The ability to detect polymerphism in a population of *X. fastidiosa* within a plant or insect hosts makes this system an ideal tool for studies of *X. fastidiosa* population genetics and epidemiological studies focusing on risk assessment. Similarly, strain specific ORFs and non-coding sequences can be used in a microarray chip format (e.g., PCR arrays), which are rapid and suitable for on site pathogen detection. Development of more robust multiplex real-time PCR protocols will improve detection and monitoring distribution and seasonal variation in *X. fastidiosa* populations, particularly in cases when more than one strain is present in the same host plant.

MOLECULAR PHYLOGENY AND *XYLELLA FASTIDIOSA* STRAIN RELATIONSHIPS

Originally, 25 strains of *X. fastidiosa* isolated from 10 different plant species were grouped into a single bacterial species. These strains showed similar phenotypic and genotypic characteristics, with 51 to 53% GC content and DNA sequence identity of 85% or more (Wells et al., 1987).

Based on the DNA homology studies, PD-associated *X. fastidiosa* strains could be differentiated from the strains causing phony disease of peach, plum leaf scald, and periwinkle wilt (Kamper et al., 1985). In a more recent study, Schaad et al. (2004) proposed that different pathovars of *X. fastidiosa* formed a single species. North American strains were further sub- divided into two subspecies, *X. fastidiosa* subsp. *piercei* (PRC) and *X. fastidiosa* subsp. *multiplex* (MULT), with each subspecies retaining at least 84% internal sequence similarity.

Pulse field electrophoresis was used in RFLP-based fingerprinting of *X. fastidiosa* strains isolated from grape, sycamore, plum, goldenrod, and oak to determine phylogenetic relationships (Leite, et al., 1993). Chen et al. (1992) used RFLP fingerprinting of 24 *X. fastidiosa* strains to confirm that strains associated with Pierce's disease, alfalfa dwarf, and almond leaf scorch form a closely related taxonomic group.

Using RAPDs, Chen et al. (1995) demonstrated that strains isolated from oak were closely related to strains isolated from grapevine and more distantly related to strains isolated from periwinkle and plum. Using the same approach, PD-associated strains were shown to be more similar to a ragweed strain than to an almond strain and less similar to CVC strains (Pooler and Hartung, 1995a). These were the first studies indicating the exis-tence of several host specific subgroups of *X. fastidiosa*: the oak group, the grape-alfalfa-almond-ragweed group, the mulberry group, the plum-elm group, and the citrus group. Subsequently, RAPD similarity indices further sup-ported the existence of groupings for strains collected from the same host plant species compared to the strains from different host plants (Hendson et al., 2001). RAPD analysis also revealed that the genetic similarity was greater than 70% between CVC-associated strains and coffee strains compared to less than 50% similarity between CVC -associated strains and grapevine strains (Lacava et al., 2001). However, contradictory conclusions were noted with respect to the extent of intra-host diversity in the study of Lacava et al. (2001) and those using citrus strains, in which only limited diversity was observed (Coletta-Filho and Machado, 2002).

There are several reports on strain diversity using single locus markers (gene and non- coding) such as 16S rRNA (Mehta et al., 2001; Mehta and Rosato, 2001; Rodrigues et al., 2003), 16S-23S intergenic spacer sequences (Kamper et al., 1985; Hendson et al., 2001; Schaad et al., 2004), *gyr*B (Rodrigues et al., 2003). However, the above studies did not result in a consensus phylogenetic pattern and tree structure, probably due to lack of informative sites and phylogenetic signals (Schuenzel et al., 2005) . Using single nucleotide polymephisms (SNPs) in the 16S rRNA gene (16S rDNA), genotypically distinct types of *X. fastidiosa* strains, G-type and A-type with distinct bacterial colony morphologies were shown to coexist simultaneously in the same almond leaf scorch affected orchard (Chen et al., 2005b).

Using genomic information of 9a5c, Coletta-Filho et al. (2001) identified simple sequence repeats (SSRs) with potential variable numbers of tandem repeat (VNTR) loci in CVC strains. Using the sequence information for all four sequenced strains, thirty-four repeat DNA loci were selected for SSR primer design and were PCR validated using 43 *X. fastidiosa* strains isolated from four crops (22 from grape, 10 from citrus, 6 from almond, and 5 from oleander) (Lin et al., 2005b). Thus, these multilocus SSR primers, distributed throughout the *X. fastidiosa* genome, can differentiate and cluster *X. fastidiosa* strains from grape, almond, citrus, and oleander to their host of origin. *X. fastidiosa* lacks mono- and di- repeat regions in its genome but is rich in 7- and 8-nucleotide repeats. These results are in contrast to other Gram -negative bacteria such as *E. coli*. For example in *E. coli* (strain K12) there are 19,200 mono- and 7,575 di-nucleotide repeats. Repeat units equal or greater than 6 nucleotides were rare in the *E. coli* genome. The evolutionary and adaptive implications of the various classes of repeat motifs among bacteria are not known (Lin et al., 2005b). Markers based on SSRs or VNTRs offer greater power of discrimination compared to RAPDs and single locus markers for strain differentiation and diversity studies. These markers offer another potential tool for *X. fastidiosa* strain differentiation and for studying epidemiology and population diversity. Phylogenetic analysis based on DNA sequence information of seven chromosomal genes (*holC*, *rfbD*, *gltT*, *cysG* , *petC*, *pilU*, and *leuA*) with a total of 9,288 bp was used to construct a Maximum-likelihood tree (Schuenzel et al., 2005). Sequence information was derived from 25 North American *X. fastidiosa* isolates; a single South American CVC strain was used as the outgroup. Three major clades were identified: *X. fastidiosa* subspecies *piercei* (PD and some ALS isolates) and *X. fastidiosa* subsp. *multiplex* (oak strains (OAK), peach strains (PP), plum leaf scald (PLS), and some ALS strains) and the third clade consisting of all of the oleander leaf scorch (OLS) strains, named *X. fastidiosa* subsp. *sandyi*.

The biogeographical history of *X. fastidiosa,* especially that of the more thoroughly studied North American strains, is confounded. For example, data obtained using RAPDs (Hendson et al., 2001) conflict with the results of sequence based comparisons (Schuenzel et al., 2005). In the first report of genetic variation among PD strains of *X. fastidiosa* from California, the north coastal strains (fr-om Alameda, Napa, Santa Cruz, and Sonoma counties)

were more similar to each other than to Southern California strains (from Ventura, San Luis Obispo, and Los Angeles counties) or to the central California strains (of Fresno, Tulare, and Contra Costa counties). The authors reasoned that either these differences among PD strains evolved over the past 130-plus years or that genetically different strains were introduced into the state or that some PD strains are indigenous to western North America. However, some of these differences could have been due to the extrachromosomal plasmid DNA, which varied in grape strains from the east and west coasts of the United States that retain similar chromosomal DNA (Hendson et al., 2001). In contrast, geographical substructuring, especially between the northern and southern California strains within the *X. fastidiosa* subsp. *piercei* (PRC) clade was not observed (Schuenzel et al., 2005; Doddapaneni et al., 2006). Minor differences (0.14% at synonymous sites *vs* 3% strain level difference) were observed within MULT between the western ALS strains and the eastern OAK and PP strains, but this identified causation is not clear with respect to geography and plant hosts (Schuenzel et al., 2005) . It is possible that ALS strains do not occur in the east and that OAK and PP do not occur in California. If so, biogeographic structure for these closely related strains may be derived from a series of intermediates across the United States (Schuenzel et al., 2005). In this regard, it is noteworthy to mention that oleander leaf scorch has been reported in Texas (Huang et al., 2004) and *X. fastidiosa* of elm in Oklahoma has been reported (Olson et al., 2006).

In contrast to the situation in North America, there is less confusion regarding distribution of CVC and coffee strains in South America (DeLima et al., 1998, Qin et al., 1998; Chen et al., 2002; Picchi et al., 2006). Kitajima et al (1975) identified rickettsia-like bacteria associated with plum leaf scald disease in Brazil. These rickettsia-like bacteria are now known to be *X. fastidiosa*. It is also noted in this paper that plum leaf scald was first reported in the delta region of the Parana River in Argentina circa 1935. Moreover, Kitajima et al (1975) noted that this disease also affected several other *Prunus* species. CVC disease may have been preceded in Brazil by the recently described coffee leaf scorch, also known as ‘requiema do cafe´. Further, there are reports of CVC vectors feeding on coffee plants (Paradela-Filho et al., 1997). Because the citrus has largely replaced coffee in Sao Paulo state after the coffee industry had experienced a prolonged decline, it is likely that the CVC strains are derived from pre-existing coffee strains (Li et al., 2001; Chen et al., 2002). Further, based on conjunct analysis of PCR-RFLP in the four loci of the restriction-modification system, 13 haplotypes were detected among different *X. fastidiosa* strains for which geographic distribution patterns and host associations were noted (Picchi et al., 2006). Similarly, for CVC strains, genetic differences were correlated with different geographic regions corresponding with host origin (Coletta-Filho et al., 2003). However, the authors did not find a correlation between genetic distance and the geographic regions of the origin-nation of populations.

Distinct origins of *X. fastidiosa* in the Southern and Southeastern States was shown using RFLP analysis of the 16S rDNA and 16S-23S intergenic spacer and by rep-PCR fingerprinting (Mehta et al., 2001). Strains isolated from coffee, grapevine, plum and pear were used in this study. The results showed separate geographic grouping of Citrus strains from States of São Paulo and Sergipe from the strains isolated from Southern States.

EVOLUTIONARY PERSPECTIVES OF *X. FASTIDIOSA* STRAINS

Diseases caused by *X. fastidiosa* have been predominantly reported from the Americas except for three reports from Taiwan (Leu and Su, 1993), Cosavo (Berisha et al., 1998) and from Costa Rica (Aguilar et al., 2005) suggesting the endemic nature of this organism in Americas. Phylogenetic analyses (Schuenzel et al., 2005) based on synonymous-site divergence (~ 3%) suggests that the ancestral *X. fastidiosa* was a clonal organism which diverged more than 15,000 years ago into three clades (*X. fastidiosa* subsp. *pierce*i, *multiplex* and *sandyi*). This inference suggests the long existence of *X. fastidiosa* strains in the Americas before the introduction of non-native crop plants such as grape and citrus that exhibit distinct symptoms upon infection. This hypothesis is in general agreement, at least with respect to PD and CVC, since there are no reported cases of CVC in Asia (where the citrus originated) and PD in Europe (where susceptible *Vitis* spps. originated) (Chen et al., 2002). The PD strain is believed to have evolved in the south-eastern US, where *V. vinifera* grapes were never succes-sfully established because of PD. Similarly, the CVC strain is believed to be endemic to Brazil. Schuenzel et al. (2005) observed differences in the synonymous-site evolutionary rates between these clades with *X. fasti-diosa* subsp. *multiplex* evolving ~ 3 times slower than the other two clades. These authors concluded that a low level (~0.1%) of genetic differentiation which represents a recent divergence event of ALS isolates from the PP, PLS, and OAK strains presumably was due to host plant adap-tation within *X. fastidiosa* subsp. *multiplex*. These conclu-sions assume that the estimation of divergence time based on the *E. coli* turnover rates represent those of bacterial mutation rates in nature. Doddapaneni et al. (2006) used pair-wise comparison methods to show that Temecula-1 has the least unpaired nucleotide percentage deviation from the other three sequenced strains suggesting that Temecula-1 strain had diversified at a slower rate. Similarities between the strains were 9a5c+Temecula-1 > Ann1+Temecula-1 >> Ann1+Dixon > Dixon+Temecula-1 >> 9a5c+Ann1 > 9a5c+Dixon, with “>>” identifying the degree of observed differences that

were supported by ANOVA analysis with Duncan grouping. However, these pair-wise comparisons based on SNPs alone showed a different trend, with the three North American strains showing least strain variability. The strain relationship based on their SNP analysis alone can be concluded as: Ann1+ Temecula-1 > Ann1+ Dixon > Dixon+Temecula-1 > 9a5c + Dixon > 9a5c + Ann1 > 9a5c + Temecula-1. As the above analysis and the four-way comparison results showing that the Temecula-1 strain has the lowest number of strain specific genes, it appears that the Temecula-1 strain has undergone the fewest genetic changes among the four strains and may most closely represent the ancestral *X. fastidiosa* geno-me. Furthermore, high similarity between the genomes of 9a5c and Temecula-1 could indicate the recent origination of 9a5c from Temecula-1. Due to a higher number of strain specific genes, we also may conclude that 9a5c is evolving at a faster rate compared to the other three strains.

Phylogenetic trees of *X. fastidiosa* strains are typified by long internal branch lengths with groupings of short terminal branches that largely correspond to host-specific groupings. This type of tree topology is indicative of limited intra- host (strain) variation and may have resulted from strong selection pressures imposed by different hosts over an extended period of time.

CONCLUSIONS AND FUTURE DIRECTIONS

Significant improvements in the past few years in technologies for detection and strain differentiation have permitted assessment of genetic diversity among *X. fastidiosa* strains. Availability of whole genome information for strains isolated from different hosts and geographical regions has made it possible to characterize strain specific differences that may aid in the design of disease management strategies. Further studies of *X. fastidiosa* will likely include the elucidation of strain-specific pathogenicity mechanisms and virulence determinants, as micro-variation among *X. fastidiosa* strains are associated with specific biological characteristics. New information gathered on genetic variation can be used to develop the next generation of technologies for rapid and high-throughput detection of *X. fastidiosa*, disease monitoring, early disease forecast, improved quarantine measures, and development of new strategies for introduction of genetic resistance traits in crops susceptible to *X. fastidiosa*.

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