RESEARCH LETTER

Xylella fastidiosa genotype differentiation by SYBR® Green-based QRT-PCR

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Abstract

Pathotype differentiation within *Xylella fastidiosa* (Wells) is necessary to accurately identify the causal agents of several leaf scorch diseases in agronomic crops and ornamentals. Three strain groups, Pierce's disease (PD), almond leaf scorch (ALS), and oleander leaf scorch (OLS), have been described in California and inhabit multiple hosts with varied severity. An SYBR® Green-based quantitative real-time PCR (QRT-PCR) protocol with a single primer set was developed for *X. fastidiosa* genotype differentiation utilizing melting temperature (T_m) analysis. The goal of this work was to develop a rapid diagnostic tool that could separate *X. fastidiosa* strains from one another similar to the placement in previous reports. Placement of eight PD, six ALS, and six OLS strains into respective strain groups was consistent with T_m curve analysis using a single primer set. These groupings were consistent with published data on the phylogenetic placement of the strains.

Introduction

Xylella fastidiosa (Wells) is a plant pathogenic bacterium that resides in the xylem tissue of plants, and induces leaf scorch disease in several economically impacted crops (Purcell, 1997). In transmission experiments, a strain of *X. fastidiosa* isolated from oleander was unable to cause disease in either grapes or almonds, although the bacterium was present in these plants (Almeida & Purcell, 2003). In further experiments, strains taken from grapes and almonds were unable to establish in oleander plants after mechanical inoculation of the bacteria (Almeida & Purcell, 2003). Interestingly, some strains of *X. fastidiosa* recovered from almond caused Pierce's disease (PD) symptoms in grapevines, while grapevine-isolated strains caused leaf scorch disease in almond plants (Almeida & Purcell, 2003).

Although all pathotypes have not been compared together in one study, classification of *X. fastidiosa* into distinct strain groups can be made through multiple lines of evidence. Those most clearly separated include the clades (1) citrus variegated chlorosis (CVC) and coffee leaf scorch (CLS) strains (possibly two separate groups), (2) PD strains, (3) oleander strains (OLS) (Marques *et al.*, 2002), and (4) Dixon-like almond leaf scorch (ALS) strains, and (5) hardwood strains including oak, maple, and mulberry (Hendson *et al.*, 2001; Chen *et al.*, 2002; Meinhardt *et al.*, 2003; Rodrigues *et al.*, 2003; Schaad *et al.*, 2004). Several techniques have been used in determining these different clades within *X. fastidiosa* species, including analysis of bacterial biofilm formation and morphology (Marques *et al.*, 2002), repetitive extragenic palindromic-PCR and randomly amplified polymorphic DNA-PCR (Pooler & Hartung, 1995; Hendson *et al.*, 2001; Chen *et al.*, 2002; Rodrigues *et al.*, 2003), CHEF (contour-clamped homogeneous electrophoresis) analysis (Hendson *et al.*, 2001), and enterobacterial repetitive intergenic consensus-PCR and restriction fragment length polymorphism (Hendson *et al.*, 2001). These studies agree with other phylogenetic studies based on a straight forward nucleotide sequence analysis that identifies differences within species; PD strains, OLS strains, ALS strains, mulberry (hardwood) strains, and CVC strains were grouped into different clades (Mehta & Rosato, 2001; Chen *et al.*, 2002; Almeida & Purcell, 2003; Rodrigues *et al.*, 2003). Most recently, *X. fastidiosa* was divided into three subspecies that are consistent with identified clades (Schaad *et al.*, 2004), but the ALS (Dixon-like) and OLS strains were not included. Schaad *et al.* (2004) named the PD strain 'ssp. *piercei*'; the hardwood group, peach, and plum groups were combined to become 'ssp. *multiplex*'; and the CVC strain group is 'ssp. *pauca*.'

In early genetic diversity studies of these different strains, the 16S–23S rRNA gene intergenic spacer region and 16S rRNA gene sequences were examined to determine the phylogenetic relationships (Hendson *et al.*, 2001; Mehta & Rosato, 2001). Another gene of interest, the *gyrase B* (*gyrB*) gene, encodes the β-subunit of DNA gyrase. This gene has evolved more rapidly than rRNA's due to base substitutions at the third position of several codons. Even with these substitutions, the *gyrB* gene is still conserved overall. This makes *gyrB* analysis suitable for strain identification because it is diverse enough to distinguish between strains, but also conserved enough to be used to have specific primers based on it (Yamamoto & Harayama, 1995). The *gyrB* gene of *X. fastidiosa* has a relatively low homology to the closely related species *Xanthomonas campestris* (Rodrigues *et al.*, 2003), which makes it a candidate for phylogenetic analysis.

DNA melting temperature analysis is a potentially vital tool to determine genetic variations as minute as single base pair alterations. The temperature at which DNA melts depends on several specific characteristics, making the analysis of the DNA melting temperature (T_m) curve a useful tool in distinguishing closely related DNA fragments (Szybalski, 1967; Gotoh, 1983; Li *et al.*, 2003; Papp *et al.*, 2003). T_m curve analysis using SYBR® Green technology provides qualitative measurements capable of distinguishing between even closely related DNA fragments (Karsai *et al.*, 2002; Ramos-Pyan *et al.*, 2003). The T_m is the point at which maximum fluorescence from the SYBR® Green molecule is lost due to amplicon denaturation (Papp *et al.*, 2003). A Poland algorithm has been used to determine the thermal stability of a piece of dsDNA. Using base stacking and denaturation behavior of dsDNA fragment, a predicted transition curve can be interpreted as a T_m curve (Poland, 1974; Steger, 1994). Divergent DNA fragments with single nucleotide changes can be identified by characteristic T_m profiles (Ririe *et al.*, 1997). *Xylella fastidiosa* field isolates naturally grouped based on the identity of *gyrB* amplicon T_m profiles.

The current methods used to detect the presence of *X. fastidiosa* and distinguishing among strains are time consuming. An efficient and early detection system that allows strain determination is an essential tool that aids in the prevention of pathogen spread to noninfected plants. In the studies presented here, primers specific for the *gyrB* gene in *X. fastidiosa* have been designed. Using quantitative real-time PCR (QRT-PCR) with SYBR® Green technology, followed by T_m curve analysis, *X. fastidiosa* strains were classified in < 2 h.

Materials and methods

DNA sequencing

Published *X. fastidiosa gyrB* sequences were collected from the National Center for Biotechnology Information (NCBI) including ALS-Dixon and OLS-ANN-1 from the NCBI Microbial Genomes Annotation Project, PD-Temecula1 (AF534960) (Van Sluys *et al.*, 2003), and PD-PCE-FG (AF534961) (Rodrigues *et al.*, 2003). To confirm homology within a strain group and to compare differences between groups, nearly complete *gyr*B sequences of three PD strains [STL (EF372240), I03 (EF372241), and Conn Creek (AY789017)], two ALS strains [#6 (AY789014) and 187 (AY789015)], and four OLS strains [TS5 (AY789016), Cathedral City (EF372237), and OLS Riverside (EF372238), and TR2 (EF372239)] were obtained from either the NCBI (for AY78901 4-AY789017) or by direct sequencing (for EF372237- EF372241) in the authors' laboratory. The *gyrB* gene was amplified using the degenerate primers Up1/Up2r (Yamamoto & Harayama, 1995) in a MyCycler (Bio-Rad Laboratories Inc., Hercules, CA) with the program 2 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, with a final extension of 7 min at 72 °C. The PCR products were then purified using a QiaQuick PCR product purification kit (Qiagen, Valencia, CA). The purified product was then sequenced in a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) following the manufacture's protocol. The resulting sequences were aligned and analyzed using the BIOEDIT sequence alignment editor (Hall, 1999).

PCR primers and Poland algorithm T_m values

Species-specific PCR primers were designed for the *X. fastidiosa gyrB* gene region following CLUSTALX (Thompson *et al.*, 1997) alignment of sequences. Regions of increased base variability between strains were also determined and exploited for this study (Fig. 1). Using PRIMER3 online oligo design software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), specific primers were designed to amplify 150–500-base pair (bp) fragments of the *gyrB* gene (Table 1).

Theoretical T_m values for sequences were entered into the Poland program, which is a service of the Biophysics Department at the Heinrich-Heine University, Dusseldorf, Germany (www.biophys.uni-dusseldorf.dc/local/POLAND/poland.html) (Steger, 1994). The program parameters were: temperature range of 72–92 °C, 0.5 °C step size, and no mismatched positions or strand concentration. The thermodynamic parameters were set at DNA (100 mM NaCl) with a dissociation constant (β) selected for a double strand at the default (β = 1.0 E+3/M). The program generated a T_m curve for each of the entered sequences. Peak T_m values for each strain were calculated using 42 primer sets.

Poland algorithm data were analyzed in three ways to determine the feasibility of genotype identification. Variations between the pathotypes of *X. fastidiosa* (PD, ALS, and OLS) were determined so that separation of local strains could be accomplished in a single-step reaction.

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PD (Temecula)  1   cagttagaggtgtcagcgaaacgcggtactacattacgttttaaaccggcaaaagagatcttcagtgatg 70
ALS (Dixon)    1   cagttagaggtgtcagcgaaacgcggtactacattacgttttaaaccggcaaaagagatcttcagtgatg 70
OLS (ANN)      1   cagttagaggtgtcagcgaaacgcggtactacattacgttttaaaccggcaaaagagatcttcagtgatg 70

PD (Temecula)  71  tggagtttcattacgaaaatcttgcaaagcgcctccgtgagttatccttcctcaattccggccttaaagt 140
ALS (Dixon)    71  tggagtttcattacgaaaatcttgcaaagcgcctccgtgagttatccttcctcaattccggccttaaagt 140
OLS (ANN)      71  tggagtttcattacgaaaatcttgcaaagcgcctccgtgagttatccttcctcaattccggccttaaagt 140

PD (Temecula)  141 tagtttgattgatgaacgtggtgagggtcggcgtgacgattttcactatgaaggtggtattcgtagcttt 210
ALS (Dixon)    141 tagtttgattgatgaacgtggtgagggtcggcgcgacgattttcactatgaaggtggtattcgtagcttt 210
OLS (ANN)      141 tagtttgattgatgaacgtggtgagggtcggcgtgacgattttcactatgaaggtggtattcgtagcttt 210

PD (Temecula)  211 gtagaacatttagctcaattgaagacccccattgcatccgaatgtaatttcggtcacaggggagcacaacg 280
ALS (Dixon)    211 gtagaacatttggctcaattgaagacccccgttgcattcgaatgtaatttcggtcaccggtgagcacaacg 280
OLS (ANN)      211 gtagaacatttggctcaattgaagacccccattgcatccgaatgtaatttcggtcaccggtgagcacaacg 280

PD (Temecula)  281 gcattgttgtggatgctgctttacagtggactgatgcctaccaagaaacaatgtattgttttacaaataa 350
ALS (Dixon)    281 gcattgttgtggatgttgctttacagtggactgatgcctaccaagaaacaatgtattgttttacaaataa 350
OLS (ANN)      281 gcattgttgtggatgttgctttacagtggactgatgcctaccaagaaacaatgtattgttttacaaataa 350

PD (Temecula)  351 cattccacaaaaagatggcggtacccaccttgctggcttccgtgctgcattgactcgtacgttgggtaat 420
ALS (Dixon)    351 cattccacaaaaagatggtggtacccaccttgctggcttccgtgctgcattgactcgtaccttgggtaat 420
OLS (ANN)      351 cattccacaaaaagatggcggtacccaccttgctggcttccgtgctgcattgactcgtaccttgggtaat 420

PD (Temecula)  421 tacattgagcagaatggggttgctaggcaagcgaagatcacttttctggtgatgatatgcgtgaagg 488
ALS (Dixon)    421 tacattgagcagaatgggattgctaggcaagcgaagatcacttttctggtgatgatatgcgtgaagg 488
OLS (ANN)      421 tacattgagcagaatggggttgctaggcaagcgaagatcacttttctggtgatgatatgcgtgaagg 488
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Fig. 1. Partial Gyrase B sequence (488 bp) of three *Xylella fastidiosa* pathotypes, highlighting 10 single base alterations among the strains (PD/ALS = 8, PD/OLS = 4, and ALS/OLS = 5).

Table 1. Primers designed to amplify regions of the Gyrase *B* gene that include several single nucleotide alterations

Primer	Sequence	Position*
INF1	GAAGGTGGTATTCGTAGCTTTGTAG	191–214
INF2	GTTTGATTGATGAACGTGGTGAG	143–165
INF3	TAGTTTGATTGATGAACGTGGTG	141–163
INR1	CATTGTTTCTTGGTAGGCATCAG	311–333
INR2	AAGCCAGCAAGGTGGGTAC	371–389
INR3	CATTCTGCTCAATGTAATTACCCA	413–435
OUTF1	AAGCGCCTCCGTGAGTTATC	097–116
OUTF2	CCTCCGTGAGTTATCCTTCC	102–121
OUTF3	GCCTCCGTGAGTTATCCTTC	101–120
OUTR1	CCTTCACGCATATCATCACC	469–488
OUTR3	TTCACGCATATCATCACCAG	467–486
F499†	CAGTTAGGGGTGTCAGCG	001–018
R907†	CTCAATGTAATTACCCAAGGT	409–429

*Position corresponds to Fig. 1.

†Primers F499 and R907 were published in Rodrigues *et al*. (2003).

The mean theoretical T_m for PD, ALS, and OLS strains for each of 42 primer sets were determined. Variations of more than 0.3 °C between each of the three strains denoted acceptable primer candidates. Primer pairs were analyzed to determine candidates for detecting T_m variations that separated the pathotypes from the South American strains including CVC strains (9a5c, SL1, and B14) and CLS strains (CM1 and P3) (Rodrigues *et al*., 2003). The feasibility of strain separation by T_m analysis was then determined.

Bacterial culture conditions and DNA extraction method

Cultures of *X. fastidiosa* were maintained on either PD3 agar medium (Hill & Purcell, 1995) or on PW (Davis *et al*., 1981) agar medium at 28 °C for 5–9 days. Cells were scraped from the medium with a sterile loop and suspended in sterile water, and DNA was extracted from cells using a Geneclean Spin Kit (Qbiogene, Carlsbad, CA) (Bextine *et al*., 2001).

Real-time PCR conditions

PCR was performed in a Rotor Gene 3000 (Corbett Research, Australia) using iQ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA) in 20 μL reactions with 2 μL of DNA template. Standardized conditions were used for all reactions beginning with an initial denaturing step of 3 min at 95 °C, followed by 40 cycles of the following parameters: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. At the end of the PCR, the temperature was increased from 72 to 99 °C at a rate of 0.5 °C/10 s, and the fluorescence was measured every 10 s. A T_m curve was created for each sample using DNA Sample Analysis System software Version 5 (Corbett Research, Australia).

All combinations of forward and reverse primers (42 sets) were tested for amplification of the *gyrB* fragment of the PD and ALS strains of *X. fastidiosa*. Empirical T_m variations between PD and ALS were determined and compared with theoretical T_m variations calculated using the Poland algorithm. In nine separate reactions with five replications of each, primer set 6 was tested for the ability to separate the

Table 2. Comparison of theoretical melting temperatures (T_m) for PD, ALS, or OLS genotype differentiation using 42 sets (only the most acceptable are shown)

	Combination		Theoretical T_m[†]			T_m difference (PD/ALS)[‡]		T_m difference (PD/OLS)[‡]		T_m difference (OLS/ALS)[‡]	
Set[*]	Forward	Reverse	PD	ALS	OLS	Theoretical	Actual	Theoretical	Actual	Theoretical	Actual
1	INF1	INR1	77.80	78.20	77.70	0.40	0.20	0.10	0.03	0.50	0.20
2	INF2	INR2	79.00	79.80	79.10	0.80	0.83	0.10	0.25	0.70	0.73
5	INF1	INR3	79.30	78.85	79.68	0.45	0.05	0.38	0.00	0.83	0.00
6	INF2	INR1	78.90	79.95	79.30	1.05	0.58	0.40	0.10	0.65	0.50
8	INF3	INR1	78.80	79.80	79.20	1.00	0.75	0.40	0.10	0.60	0.68
9	INF3	INR2	78.70	79.75	79.10	1.05	0.80	0.40	0.44	0.65	0.14
17	F499	INR1	79.13	79.85	79.93	0.72	0.53	0.80	0.08	0.08	0.30
18	F499	INR2	79.15	79.85	79.80	0.70	0.43	0.65	0.10	0.05	0.30
20	F499	OUTR1	79.85	80.50	79.90	0.65	0.20	0.05	0.02	0.60	0.21
22	INF1	R907	79.10	78.75	79.20	0.35	0.08	0.10	0.07	0.45	0.03
23	INF1	OUTR1	79.85	79.25	79.38	0.60	0.40	0.47	0.10	0.13	0.15
24	INF1	OUTR3	79.90	79.10	79.43	0.80	0.30	0.47	0.12	0.33	0.20
32	OUTF1	INR1	79.15	79.75	79.15	0.60	0.55	0.00	0.40	0.60	0.05
33	OUTF1	INR2	79.25	79.85	79.10	0.60	0.80	0.15	0.12	0.75	0.38
36	OUTF2	INR1	78.83	79.80	79.70	0.97	0.88	0.87	0.10	0.10	0.63
37	OUTF2	NR2	78.93	79.75	79.15	0.82	0.83	0.22	0.15	0.60	0.43
40	OUTF3	INR1	79.05	79.90	79.20	0.85	0.50	0.15	0.06	0.70	0.50
41	OUTF3	INR2	78.88	79.90	79.15	1.02	1.08	0.27	0.12	0.75	0.40

Also included are theoretical vs. actual T_m comparison between PD and ALS strains.

*Sets in bold separate PD, ALS, and OLS genotype from each other and were acceptable when tested.

[†]Theoretical T_m in °C calculated using Poland's algorithm, separation of all three genotype from one another was critical.

[‡]Difference between PD strains and ALS strains in °C, greater values indicate a more acceptable primer set.

PD (eight isolates), ALS (six isolates), and OLS (six isolates) genotypes.

Results

Primers were designed within a 1400-bp region of the *gyrB* gene that had several nucleotide differences between strain groups but not within a particular strain group. For example, all of the PD strains examined (Temecula1, PLE-FG, I03, Tulare, Conn Creek, and STL) were identical in sequence within the selected 488-bp region. All ALS strains (Dixon, 187, and #6) were identical to each other and all OLS strains (ANN-1, TS5, Cathedral City, Riverside, and TR2) were identical to each other. However, within this 488-bp region of the *gyrB* gene, there are a total of 10 single-base alterations among PD, ALS, and OLS genotypes (Fig. 1). PD and ALS strains had eight distinct nucleotide alterations, six of which resulted in AT to GC shifts. Five single-base differences were present between ALS and OLS, all of which were GC to AT shifts. PD and OLS strains had five alterations, four of which were AT to GC shifts. Because these shifts between AT and GC greatly affect dsDNA T_m, this section of the gene was chosen for strain identification. Several primer sets were designed to amplify nucleotide regions within the conserved but variable *gyrB* gene (Table 1), which resulted in products that ranged in size from 142 to 429 bp, with the average amplicon length being *c.* 317 bp.

Although the amplicons often had several base pairs in common, even the slightest difference in sequence caused a large change in T_m. For example, primer sets 36 and 40 had the same reverse primer (INR1) and forward primers that differed by only one nucleotide (OUTF2 and OUTF3); OUTF3 had one more base than OUTF2 (Table 1). Small differences in the nucleotide sequence of the two amplicons produced by primer sets 36 and 40 caused significant differences in T_m (Table 2). Set 36 produced an amplicon from the PD strain with a T_m of 78.83 °C, while the T_m for the same strain with set 40 was 79.05 °C. Similarly, the set 36 OLS amplicon had a T_m of 79.70 °C, while the set 40 OLS amplicon had a T_m of 79.20 °C. The ALS amplicon for set 36 had a T_m of 79.80 °C, compared with that for set 40 at 79.90 °C. In most cases, the theoretical values for T_m closely coincided with the experimental data collected from the T_m curves following QRT-PCR using SYBR®-Green technology. However, there were differences in the theoretical and actual values such as that seen for set 5. The theoretical values for T_m predicted a T_m variation of 0.45 °C between ALS and PD, while the actual difference was recorded to be only 0.05 °C.

The amplicon of primer set 6 produced amplicons with the largest difference in T_m for the different strains of *X. fastidiosa* with a theoretical difference of 1.05 °C between ALS and PD strains, 0.65 °C between ALS and OLS strains, and 0.40 °C between PD and OLS strains (Table 2). In all cases, the eight PD strain isolates tested were confirmed as

Table 3. Strains of *Xylella fastidiosa* used for strain group placement by SYBR® Green T_m separation

Strain	Collection host	Set 6 T_m placement	Referenced phylogenetic placement	References
I03	Grapevine	PD	PD	Costa *et al*. (2004), Meinhardt *et al*. (2003)
237*	Almond	PD	Not Placed	Costa *et al*. (2004)
PD-Conn Creek	Grapevine	PD	PD	Almeida & Purcell (2003), Hendson *et al*. (2001)
PD-STL	Grapevine	PD	PD	Almeida & Purcell (2003), Hendson *et al*. (2001)
PD-Traver	Grapevine	PD	PD	Almeida & Purcell (2003), Rodrigues *et al*. (2003)
ALS-Tulare*	Almond	PD	PD	Almeida & Purcell (2003), Hendson *et al*. (2001)
ALS-#1*	Almond	PD	PD	Hendson *et al*. (2001)
ALS-Fresno*	Almond	PD	PD	Almeida & Purcell (2003)
ALS-#3	Almond	ALS	ALS	Hendson *et al*. (2001)
ALS-#6	Almond	ALS	ALS	Almeida & Purcell (2003), Hendson *et al*. (2001)
ALS-#9	Almond	ALS	ALS	Hendson *et al*. (2001)
ALS-Dixon	Almond	ALS	ALS	Almeida & Purcell (2003), Hendson *et al*. (2001)
187	Almond	ALS	Not placed	Not referenced†
276	Almond	ALS	ALS	Costa *et al*. (2004)
OLS-TS5	Oleander	OLS	Not placed	Not referenced†
OLS-Texas	Oleander	OLS	Not placed	Not referenced†
OLS-Cathedral City	Oleander	OLS	OLS	Not referenced†
OLS-Riverside	Oleander	OLS	Not placed	Not referenced†
OLS-TR2	Oleander	OLS	Not placed	Not referenced†
OLS-ANN-1	Oleander	OLS	OLS	Almeida & Purcell (2003), Hendson *et al*. (2001), Mehta & Rosato (2001)

*Isolated from almond host plants, but phylogenetic analysis has consistently placed them within the PD pathotype.
†Strains provided by R. Hernandez-Martinez and Heather Costa, University of California, Riverside.

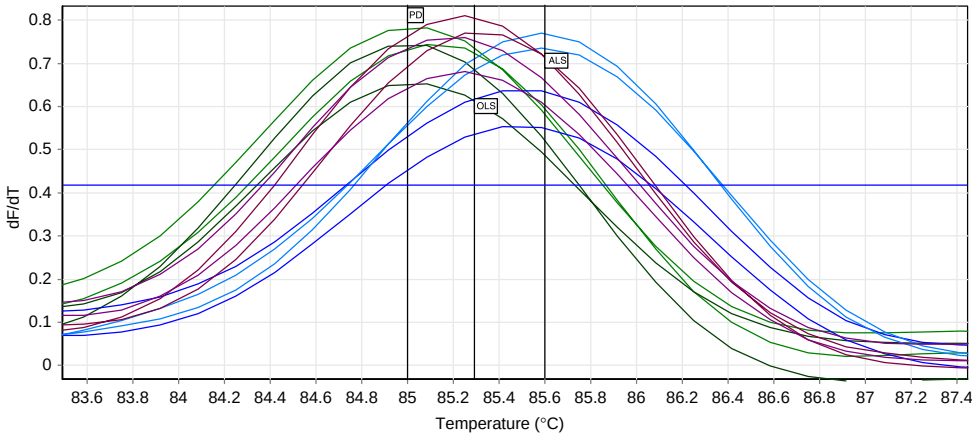

Fig. 2. Primer set 6 melting temperature curve for determination of PD (85.0 °C), OLS (85.3 °C), and ALS (85.6 °C) genotypes.

such through T_m curve analysis (Table 3), including ALS-Tulare, ALS #1, and ALS-Fresno. These strains were originally isolated from almond plants, but later identified as PD strains through sequencing and RFLP analysis (Almeida & Purcell, 2003). Six ALS isolates and six OLS isolates were consistently identified as ALS strains and OLS strains, respectively. PD strains were separated from OLS strains by 0.3 °C (Fig. 2). OLS and ALS strains were also separated by 0.3 °C, while PD and ALS strains had a difference of 0.6 °C T_m.

There were several primer sets that provided products with large T_m variations among the strains but did not adequately facilitate separation of two strains. For instance, primer set 36 had considerable variation between PD and OLS, but the melting temperatures of PD and ALS were both 79.15 °C. Another example was primer set 1, which was used to separate PD and OLS from ALS, but could not be used to adequately separate PD and OLS from each other (Table 2).

When DNA from PD and ALS strains were mixed in the same tube for PCR and T_m curve analysis, the strain with the higher concentration was identified by T_m analysis. Dual peaks did not appear and the T_m curve was not shifted.

Discussion

Xylella fastidiosa-associated disease symptoms in some host plants have been attributed to the presence of specific strains of the pathogen, which has been grouped into three subspecies, *piercei*, *multiplex*, and *pauca*, the former two occurring in the US (Schaad *et al.*, 2004). In the study by Schaad *et al.* (2004), no OLS strains were tested and analysis of strains of almond origin (none of which were Dixon-like strains) provided conflicting results, placing them in both ssp. *piercei* and *multiplex*. The Dixon-like ALS pathotype has been identified as being distinctly different from the PD pathotype (Hendson *et al.*, 2001; Almeida & Purcell, 2003); therefore, it is not classified as ssp. *piercei*. The OLS pathotype is distinctly different from both PD and ALS strain groups (Hendson *et al.*, 2001; Costa *et al.*, 2004). Although further analysis of the ALS and OLS strains could result in classification of two separate subspecies, this was not the aim of our work. Rather, a diagnostic technique for rapid selective detection of the PD, ALS, and OLS genotypes have been developed.

Currently, at least three major *X. fastidiosa* pathotypes that occur in the US are generally recognized: PD, ALS, and OLS. Interestingly, PD and ALS strains will not cause OLS and vice versa. However, the PD and ALS cause symptoms in other hosts. PD strains have been isolated from almonds expressing ALS symptoms; ALS strains have yet to be isolated from grapevine-expressing PD symptoms. This provides evidence of phenotypic difference (Almeida & Purcell, 2003). In addition to the pathotypes studied here, there is a group of strains that causes disease in hardwoods, classified as ssp. *multiplex* (Schaad *et al.*, 2004), which are distinctly different from other pathotypes and are found naturally in the eastern half of the US. There are two groups found only in Central and South America, CVC and CLS strains, classified as ssp. *pauca* (Schaad *et al.*, 2004). These strains are distantly related to the northern hemisphere strains.

The *gyrB* gene is conserved with only a few single nucleotide differences in sequence among *X. fastidiosa* genotypes. In a review of the sequences, the focus was on a gene region that contained 10 single base alterations, most of which were inversions (i.e. GC to AT changes that cause a decrease in the number of hydrogen bonds from three to two), that affect T_m. Using several of the primer sets, it was possible to take advantage of these properties. Primer set 6 could be used to amplify DNA from all *X. fastidiosa* strains that conserve some variations among genotypes, resulting in a 0.3 °C difference in T_m from one genotype to another. Theoretically, PD, ALS, and OLS pathotypes could all be clearly separated, because within the 192 bp region amplified by primer set 6, seven single nucleotide alterations were noted when strains the were compared (PD/ALS = 7, PD/OLS = 4, ALS/OLS = 3). This followed suit with the empirical differences, where the T_m of OLS was 0.3 °C higher than ALS, and ALS was 0.3 °C higher than PD (Fig. 2).

In most cases, the theoretical data for T_m obtained from the Poland data were similar to those obtained experimentally. However, experiments with primer sets ceased when empirical amplicon T_m values did not closely match the corresponding theoretical values. Because the goal of this work was to find a tool that could be used to differentiate strains for diagnostic purposes, work with any primer sets that returned inconsistent results was discontinued and the cause for variation was not determined. In all cases, the theoretical T_m values were lower than the experimental values because the PCR master mix components insulated the DNA in the PCR reaction, necessitating higher temperatures for DNA melting. However, strains were still separated efficiently because the PCR master mix was standard. In additional theoretical T_m analysis, we found several primer sets were found that could be used to clearly separate the California pathotypes (PD, ALS, and OLS) from ssp. *multiplex* and ssp. *pauca* from other groups.

SYBR® Green-based QRT-PCR should not be used as the sole means of strain identification or determining relatedness among strains. Certainly, the T_m of a short DNA fragment does not compare with direct sequencing in determining relatedness. Despite variation in the genome, several of the primer sets did not separate the *X. fastidiosa* pathotypes, probably because the sum total of the nucleotide alterations resulted in identical T_m. If a base inversion from GC to AT occurs at one position in a given amplicon, while another base inversion from AT to GC occurs at another position within the same amplicon, the overall effect is no change in T_m. The T_m separation protocol reported herein was designed to be used to rapidly categorize known or suspected strains of *X. fastidiosa* in a diagnostic setting.

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