Journal of Microbiological Methods 89 (2012) 12–17

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Probe-based real-time PCR method for multilocus melt typing of *Xylella fastidiosa* strains

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ARTICLE INFO

Article history:
Received 15 December 2011
Received in revised form 30 January 2012
Accepted 2 February 2012
Available online 9 February 2012

Keywords:
Real-time PCR
Genotyping
Fluorophore
Quencher
Xylella fastidiosa
Pierce's disease

ABSTRACT

Epidemiological studies of Pierce's disease (PD) can be confounded by a lack of taxonomic detail on the bacterial causative agent, *Xylella fastidiosa* (*Xf*). PD in grape is caused by strains of *Xylella fastidiosa* subsp. *fastidiosa*, but is not caused by other subspecies of *Xf* that typically colonize plants other than grape. Detection assays using ELISA and qPCR are effective at detecting and quantifying *Xf* presence or absence, but offer no information on *Xf* subspecies or strain identity. Surveying insects or host plants for *Xf* by current ELISA or qPCR methods provides only presence/absence and quantity information for any and all *Xf* subspecies, potentially leading to false assessments of disease threat. This study uses a series of adjacent-hybridizing DNA melt analysis probes that are capable of efficiently discriminating *Xf* subspecies and strain relationships in rapid real-time PCR reactions.

1. Introduction

Xylella fastidiosa (*Xf*) as a species comprises a group of xylem-limited bacteria that cause leaf scorch diseases in a wide array of plant species. The bacteria are vectored between plant hosts by a number of generalist xylophagous insects. Several subspecies of the bacterium have been named, including *X. fastidiosa* subsp. *fastidiosa* (*Xff*), which causes Pierce's disease (PD) in grape; *X. fastidiosa* subspecies *sandyi* (*Xfs*), which causes oleander leaf scorch; *X. fastidiosa* subsp. *pauca* (*Xfp*), which causes citrus variegated chlorosis in South America; and a genetically diverse subspecies, *X. fastidiosa* subsp. *multiplex* (*Xfm*), which causes leaf scorch diseases in a large number of tree species (Schaad et al., 2004; Schuenzel et al., 2005). Many of the subspecies may occupy multiple plant hosts, but cause disease symptoms in only a select subset of potential hosts (Hopkins and Purcell, 2002). While methods for efficient detection of the bacterium exist, such as ELISA and qPCR, epidemiological studies can be hindered because the detection assays will detect all *Xf* subspecies, but do not provide individual subspecies or strain identification. Conventional PCR assays have been successfully used to identify *Xf* from environmental samples and provide strain differentiation (Chen et al., 2005; Hernandez-Martinez et al., 2007; Minsavage et al., 1994), but the methods are more laborious than real-time PCR, involving restriction digests and agarose gel electrophoresis of PCR amplicons in some cases, and provide a very limited amount of genetic information. Methods requiring culture of *Xf* for genotyping can be even more cumbersome. Isolating and culturing strains is a laborious and time-consuming process due to the fastidious nutritional requirements and slow growth habit of the bacterium. A multilocus sequence typing (MLST) system for *Xf* has been developed that is capable of generating sufficient genetic information to easily discriminate subspecies and strains (Scally et al., 2005), and although the method has been streamlined (Yuan et al., 2010), it remains a time-consuming and expensive process, and has only been applied to 146 *Xf* isolates in 7 years (http://pubmlst.org/fastidiosa/) (Jolley et al., 2004).

In order to complement more informative but more time-consuming assays such as MLST, we have developed several real-time PCR DNA probe sets capable of robust bacterial subspecies and strain differentiation that constitute a rapid multilocus melt typing (MLMT) system for *Xf*. The DNA probe sets are designed so that a fluorescently-labeled donor probe hybridizes in close proximity to a second acceptor probe that is coupled to a quencher, thus fluorescence is quenched when a DNA strand complementary to the probes is present (Cardullo et al., 1988). As the probes melt off of complementary DNA during a temperature ramp, the donor probe and acceptor probe are separated from one another, leading to an increase in fluorescence that is most rapid at the melting temperature (T_m) for the probes. For

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doi:10.1016/j.mimet.2012.02.002

a diagram of how the probes are positioned on template DNA, see Fig. 1C in (Wittwer et al., 1997). Single nucleotide polymorphisms (SNPs) within the sequence of one or both probes cause them to melt at different temperatures when hybridized to template DNA from different *Xf* subspecies. The probe sets designed here target many of the same genes utilized in the established MSLT assay (Scally et al., 2005; Yuan et al., 2010), so that a rapid preview of important strain differences can be generated. These probe sets are capable of identifying *Xf* DNA polymorphisms even when the *Xf* DNA is a small proportion of a mixed environmental isolation containing DNA from other species.

2. Materials and methods

Xf reference strains Dixon and Ann-1 were obtained from the American Type Culture Collection (Manassas, VA). Temecula DNA was kindly provided by Dr. Carlos Gonzalez, Texas A&M University. Bacterial cultures were grown in PD3 broth supplemented with 4 ml of 0.5% phenol red and 20 ml of 10% BSA per liter (Almeida and Purcell, 2003; Davis et al., 1981). Isolation of DNA from cultured bacteria was conducted using an alkaline lysis method as previously described (Sambrook et al., 1989). Insect samples were collected on yellow sticky traps outdoors and remained attached to the trap for a period of up to 2 weeks before the traps were collected and stored at 4 °C for a period of up to 3 years prior to DNA extraction. Xylophagous insects were removed from the sticky traps with orange oil before being subjected to a high-throughput silica-based DNA extraction method as previously described (Brady et al., 2011).

Insect DNA extractions subjected to melt analysis were from 93 insects collected in Dawson, Frio, Gillespie, Guadalupe, Hale, Hockley, Kimble, San Saba, Swisher, and Terry counties in Texas. The insect samples had been previously identified as *Xf* positive using an established qPCR assay (Schaad et al., 2002) and included 51 *Graphocephala hieroglyphica*, 20 *Homalodisca vitripennis*, 9 *Graphocephala versuta*, 8 *Oncometopia* sp., 2 *Clastoptera xanthocephala*, 2 *Xiphon flaviceps*, and 1 *Cuerna costalis*.

Genes were selected for genotyping based primarily on an existing MLST assay for *Xf* (Scally et al., 2005; Yuan et al., 2010). AlleleID software (Premier Biosoft International, Palo Alto, CA) was used to align sequences and indicate SNPs that could potentially discriminate between the completely sequenced reference strains Temecula (*Xff*), Dixon (*Xfm*), and Ann-1 (*Xfs*), as well as sequences deposited in GenBank. The FRET module in AlleleID was then used to select SNPs of interest and design adjacent-hybridizing probes with default settings for DNA melting assays to discriminate the SNP differences. Acceptor probes were manually designed to extend within 1 bp of the donor probe. An effort was made to select SNPs with high Simpson's *D* values (Hunter and Gaston, 1988; Simpson, 1949) in order to create individual probe sets with the highest possible index of discrimination. However, poor performance in trial melting assays led to placement of some probes over SNPs with a lower index of discrimination, but more easily interpreted probe-melting characteristics. Primers and probes designed for the project are listed in Table 1. Labeled probes were synthesized by Biosearch Technologies (Novato, CA), and primers were synthesized by Eurofins MWG Operon (Huntsville, AL). Acceptor probes were labeled with the inexpensive quencher dabcyl, regardless of the fluorophore used on the donor probe, because at the close distances involved, fluorophore energy is dissipated as heat by contact quenching instead of FRET (Marras et al., 2002). Real-time PCR was carried out in a 384-well LightCycler® 480 real-time PCR instrument (Roche Applied Science, Indianapolis, IN) using hard-shell® thin-wall PCR plates with microseal B adhesive seals (Bio-Rad, Hercules, CA). Immediately following amplification a DNA melt assay was conducted by allowing the probes and amplicon to anneal, then ramping up sample temperature while continuously collecting fluorescence data.

Asymmetric PCR reactions were conducted to preferentially amplify the DNA strand complementary to the probes. One exception was the *nuoN* reaction, which was easily scored with symmetrical PCR. Asymmetric PCR reactions contained 500 nM primer to amplify the complementary strand, 50 nM primer to amplify same strand as the probes, 500 nM acceptor probe, and 100 nM donor probe. Each PCR reaction contained 1 µl of 5X LightCycler® 480 genotyping PCR master mix (Roche Applied Science, Indianapolis, IN). PCR reactions contained 1 µl of template from a DNA extraction (Brady et al., 2011) in an overall reaction volume of 5 µl. The thermocycling protocol consisted of 95 °C for 10 min, followed by 45 amplification cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Following amplification, a melt program was conducted consisting of 95 °C for 2 min, 37 °C for 2 min, followed by a temperature ramp to 95 °C with acquisition of 10 fluorescence data points per degree Celsius. Primer and probe sequences used are listed in Table 1. PCR plates containing insect samples also contained 3 completely sequenced North American reference strains (Temecula-*Xff*, Dixon-*Xfm*, Ann-1-*Xfs*) as positive controls for T_m comparison purposes.

Multiple PCR amplifications were conducted on each of the 3 reference strains for all loci. Negative first derivative curves of fluorescence data were analyzed in the T_m module of the LightCycler® 480 software suite without any additional post-PCR normalization of the curves. Melting temperatures for each well were individually scored manually and T_m values were exported into SAS v9.2 (SAS Institute, Cary, NC) and submitted to analysis of variance to identify T_m differences between the three reference strains for each of the 9 loci using the GLM procedure. Student Newman–Keuls (SNK) post-hoc tests determined differences between means in the model.

Samples subjected to sequencing were first amplified by conventional PCR using PCR conditions previously described (Schuenzel et al., 2005) except that primers AY876798F 5′-GTGTACCTGGGGCTTGAGCTGTTG-3′, and AY876798R 5′-TTTCCGATGAACCATGGATCACATC-3′ were used to amplify a portion of the *nuoN* gene, because the MLST primers (Schuenzel et al., 2005) failed to amplify the *Xf* DNA from insect DNA extractions. The PCR amplicons were then gel purified in 1.0% agarose gel and cleaned using a Qiaex II purification kit (Qiagen, Valencia, CA). Gel purified PCR amplicons were sequenced using a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA) with the same primers used in PCR amplification.

3. Results

ANOVA of T_m values revealed highly significant differences ($p<0.0001$) between the reference strains within each of the 9 loci. Box and whisker plots (Fig. 1) reveal the narrow range of melt temperatures for each probe set used on the three *Xf* reference strains. Separation of adjacent-binding DNA probes leads to an increase in fluorescence, so that the negative first derivative melting curves form troughs instead of peaks (Fig. 2). This contrasts with some other types of probes and non-specific DNA binding dyes where melting curves form peaks. DNA probe sets designed for *Xf* loci showed melt differences of more than 15 °C between the reference strains for some loci. For instance, the *Xfm* strain (Fig. 2A, replicate curves in blue) produced melting troughs averaging 69.72 °C for the *cysG* locus, whereas the *Xfs* strain (replicate curves in green) produced melting troughs averaging 54.52 °C. A Student Newman–Keuls test of T_m differences within each locus separated all 3 reference strains except in the case of the *rfbD* locus, where the loci-spanning probe set (Pont-Kingdon et al., 2007) for the gene *rfbD* only distinguishes *Xff* from *Xfs* and *Xfm*, which both melt at the same temperature (Figs. 1, 2I). While all housekeeping genes used in the MLST assay had polymorphisms suitable for discriminating all three of the North American *Xf* reference strains, probe design constraints prevented the development of probes that could clearly distinguish all 3 reference strains from one another for the *rfbD* gene with a single