Development of a qPCR Protocol to Detect the Cotton Bacterial Blight Pathogen, *Xanthomonas citri* pv. *malvacearum*, from Cotton Leaves and Seeds

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Abstract

Bacterial blight, historically a seed-borne disease of cotton (*Gossypium hirsutum*) is caused by *Xanthomonas citri* pv. *malvacearum*, resulted in significant economic losses prior to development of resistant varieties and implementation of acid-delinting of planting seed. Periodic outbreaks have been associated with seed since the early twentieth century; of note, the disease has experienced a resurgence since 2011. Effective management of bacterial blight is dependent on accurate diagnosis and detection of the pathogen. Currently, detection of *X. citri* pv. *malvacearum* is performed by time-consuming microbiological methods. In this study, a novel and sensitive TaqMan-based qPCR protocol was developed to test for *X. citri* pv. *malvacearum* in cotton plant tissue. The primers developed are specific

to five races of *X. citri* pv. *malvacearum*, but not to other *Xanthomonas* species or cotton-associated nonpathogenic bacteria. The efficiency of this assay was evaluated on artificially inoculated cotton leaves and seed, on naturally infected cotton leaves, and on bolls and seed originating from bacterial blight symptomatic bolls. The protocol's efficiency from artificially inoculated plant tissue was 10^2 copies g^{-1} and 37 copies from 1 g seed for leaves and seed, respectively. In addition, *X. citri* pv. *malvacearum* was detected from 94% of the seed samples originating from blight symptomatic bolls. The qPCR protocol provides a rapid and accurate method for diagnosis and detection of bacterial blight and offers a tool for monitoring *X. citri* pv. *malvacearum* and potentially reducing its spread in seed.

The genus *Xanthomonas* is notable in its phytopathogenic diversity and phenotypic uniformity (Starr 1981; Vauterin et al. 2000). Due to its economic importance, *Xanthomonas* has been the subject of several large-scale taxonomic (Ah-You et al. 2009; Vauterin et al. 2000; Yang et al. 1993) and pathogenesis studies (De Feyter et al. 1993; Lee et al. 2005). In addition, over the past several years, careful attention has been directed to the identification and nomenclature of species within the genus (Vauterin et al. 1995; Young et al. 2008). Numerous important pathogens are classified within the genus *Xanthomonas*. In fact, most economic crops are affected by at least one species of *Xanthomonas*.

Xanthomonas citri is the most heterogeneous species within the genus (Brunings and Gabriel 2003; Graham et al. 2004). Names within the *citri* group generally include a post-species epithet indicating the pathovar (pv.) designation that indicates the strain causing disease in a particular host plant. Several important diseases are caused by members of the *citri* group, including bacterial canker of citrus (*Citrus* spp.; caused by *X. citri* pv. *citri*), bacterial pustule of soybean (*Glycine max* (L.) Merr.; caused by *X. citri* pv. *glycines*), and bacterial blight of cotton (*Gossypium hirsutum* L.; caused by *X. citri* pv. *malvacearum*) (Ah-You et al. 2009; Kemerait et al. 2017; Phillips et al. 2017). Bacterial blight of cotton, also referred to as seedling blight, angular leaf spot, vein blight, black arm, and

bacterial boll rot, was first reported in Alabama in 1891 and initially referred to as black rust (Atkinson 1891). Bacterial blight of cotton can result in the production of characteristic, angular, water-soaked lesions on leaves (Innes 1983; Thaxton and El-Zik 2001; Verma 1986). Bacterial blight can also be responsible for the production of lesions on all cotton plant parts from cotyledon-stage cotton to adult plants including the fruit or boll that encloses the seed. Severe bacterial blight infection can result in premature defoliation and boll shed, resulting in yield losses (Jalloul et al. 2015; Schaad et al. 2006). Historically, bacterial blight of cotton has been a major cotton disease producing substantial yield losses, ranging from 10 to 77% in susceptible cultivars during periods of severe epidemics (Hillocks 1992; Razaghi et al. 2012; Thaxton and El-Zik 2001; Umeha et al. 2010). In the United States, bacterial blight tends to appear in fairly geographically narrow regions. The disease does occur in parts of Oklahoma and Texas, and more recently epidemics have occurred in the mid-southern and southeastern regions of the United States (Kemerait et al. 2017; Phillips et al. 2017; Rothrock et al. 2012). When growing cotton is not in the field, *X. citri* pv. *malvacearum* has the ability to overwinter on cotton plant residue for short periods (months) and in seed cotton and lint for more extended periods (years) (Davis and Sandidge 1977; Verma 1986). However, the reported cases of long-term survival have all been in environmentally protected, indoor locations, for example, in the laboratory and at cotton gins (Davis and Sandidge 1977; Innes 1983; Mehta et al. 2005; Thaxton and El-Zik 2001; Verma 1986). Moreover, *X. citri* pv. *malvacearum* survives poorly in soil without the presence of infected plant residue (Massey 1930), and has been reported to have a shorter survival period in moist than in dry soil (Brinkerhoff and Fink 1964). Tillage is one form of management, since *X. citri* pv. *malvacearum* is a poor competitor with other soil-borne organisms (Thaxton and El-Zik 2001; Verma 1986). Even though the bacterium can survive between seasons in dried field debris, the primary means of transmission remains infected/infested seed (Innes 1983; Thaxton and El-Zik 2001; Verma 1986).

Seed is widely reported to be the primary source of inoculum for the initiation of bacterial blight in cotton fields (Brinkerhoff and

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Funding: This research was supported by a cooperative research agreement from Cotton Incorporated (grant no. 13-763) and by the U.S. Department of Agriculture (USDA) National Institute of Food and Agriculture (grant no. MIS-401170).

*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures are published online.

Accepted for publication 27 August 2018.

Hunter 1963; Innes 1983; Schnathorst 1964; Thaxton and El-Zik 2001; Verma 1986). The infection rates of cottonseed with *X. citri* pv. *malvacearum* has previously been reported to range from <1% to between 10 and 20% (Archibald 1927; Bain 1939; Chester 1938; Rolfs 1915; Schnathorst 1968). Bacterial blight can be destructive even where only a single infected plant per 6,000 plants is observed (Hillocks 1992; Tarr 1961). One symptomatic plant can serve as a source for the pathogen to move throughout a field by local dissemination under favorable environmental conditions and via mechanisms that include wind-driven rain and agricultural equipment (Brown 1942; Davis and Sandidge 1977; King and Brinkerhoff 1949; Verma 1986). Long-range movement of the bacterium (>3.2 km) has not been reported (Brown 1942; Massey 1929).

Bacterial blight was largely eliminated in the United States following the general adoption of acid-delinting of cotton seed in the 1970s. Acid-delinting removes the cotton lint from the seed coat, thus eliminating a prime tissue that supports bacterial survival. Acid-delinting also tends to remove less dense seed from seed lots; such seeds may be harboring the bacterium within the embryo (Cherry and Leffler 1984; Delouche 1986; Innes 1983). Nonetheless, acid-delinting does not completely eradicate the organism from seed since internal seed infection does occur (Brinkerhoff and Hunter 1963; Schnathorst 1964, 1968). Reports of detection of *X. citri* pv. *malvacearum* following acid-delinting indicate the organism may still be present at low frequencies (≈ 0.1 to 1%) (Alexander et al. 2012; Bain 1939; Rothrock et al. 2012; Schnathorst 1968).

Planting pathogen-free seed is an important first step to reduce the incidence and impact of bacterial blight. However, current methods of detecting *X. citri* pv. *malvacearum* from cotton planting seed and for screening seed are extremely laborious and outdated (Heintz 1997). In the past, efforts to develop a method to detect specific species within the genus *Xanthomonas* from seed was unsuccessful due to the high degree of specificity required to detect certain genotypes among the pathovars. To date, detection of *X. citri* pv. *malvacearum* from cottonseed is based on time-consuming microbiological methods to recover the microorganism. Such methods rely on nonspecific microbiological media and identification of colony morphology followed by, in some cases, plant inoculation to verify pathogenicity of the bacteria isolated (Brinkerhoff and Hunter 1963; Hunter and Brinkerhoff 1964; Rothrock et al. 2012; Schnathorst 1968). Specific serological techniques to detect bacteria from seed have been developed in other plant systems, but not for cotton. The presently available enzyme-linked immuno-sorbent assays (ELISA) that use antibodies against species of *Xanthomonas* lack the ability to distinguish between different pathovars within distinct subgroups. One non-ELISA method was developed to detect the carrot (*Daucus carota* subsp. *sativus* (Hoffm.) Schübl. & G. Martens) bacterial leaf blight bacterium (*X. citri* pv. *carotae*) from seed using PCR primers that were developed based on random amplified polymorphic DNA (RAPD) analysis (Meng et al. 2004). The reproducibility and time-efficiency of quantitative, real-time PCR presents the possibility of assaying seed-borne pathogens rapidly and accurately (Gitaitis and Walcott 2008). Following the widespread outbreaks of bacterial blight of cotton in the mid-southern United States in 2011, multiple microbiological methods were serially combined to detect *X. citri* pv. *malvacearum* from 34 potentially infected cotton seed lots (Rothrock et al. 2012). The combined methods, while being able to positively identify *X. citri* pv. *malvacearum*, required substantial time and included several labor-intensive seed washings for each seed lot followed by morphological identification of *X. citri* pv. *malvacearum* in vitro and confirmation of pathogenicity in planta.

A sensitive method to detect *X. citri* pv. *malvacearum* in seed requires the specificity to discriminate among the races. Races of *X. citri* pv. *malvacearum* tend to be geographically distinct and incite disease in specific cotton producing areas (AbdelRahim et al. 2013; Abdo-Hasan et al. 2008). Twenty-two races of *X. citri* pv. *malvacearum* have been described (Hillocks 1992; Jalloul et al. 2015; Phillips et al. 2017). Race 18 is the one most frequently encountered in the United States. To date races 20, 21, and 22 have been reported

only from Central Africa (Abdo-Hasan et al. 2008; Allen and West 1991; Baldwin and Wrather 1973; Hunter et al. 1968; Hussain 1984; Jalloul et al. 2015; Thaxton and El-Zik 2001; Verma et al. 1975). To avoid false positives, a method to detect *X. citri* pv. *malvacearum* in cotton needs to discriminate it from multiple species of *Xanthomonas* (Heintz 1997; Heintz and Akinwunmi 1997).

Development of a rapid and accurate procedure for detection of *X. citri* pv. *malvacearum* from cottonseed, as well as field-infected cotton plant tissue, can potentially be helpful to mitigate the damage and limit the spread of the disease. In the field, a light infestation of bacterial blight may go unnoticed. The current procedures used to scout fields for seed production are based on the presence of disease symptoms on plant material, oftentimes at the end of the growing season when disease symptoms can be obscured by multiple, late-season foliar diseases, weathering, and insect damage. Bacterium-carrying seed could be harvested, commercially released, and subsequently spread the pathogen over a large geographic area. The specific objective of this research was to develop a rapid and accurate detection method for *X. citri* pv. *malvacearum* from cotton plant material.

Materials and Methods

Bacterial strains and DNA samples. Several strains of the bacterial blight pathogen, *X. citri* pv. *malvacearum*, representing five known races (race 1, 2, 3, 12, and 18), as well as two additional strains of *Xanthomonas* were used for DNA extraction (Table 1). The *X. citri* pv. *malvacearum* strain MSCT1, with an unknown race designation, was isolated from infected cotton leaves collected in Yazoo County, MS in 2011. Pathogenicity of the five races and strain MSCT1 was confirmed by vacuum inoculation of cotton seedlings.

Each bacterium was streaked onto nutrient yeast broth (NBY) agar medium and grown for 2 days at 28°C (Gross and DeVay 1977). A single colony of each bacterium was recovered and added to liquid NBY medium with continuous shaking (200 rpm) at 28°C overnight (approximately 8 h). Bacterial cells were harvested from 5 ml of the liquid medium by centrifugation. The resulting bacterial cell pellet was used for DNA extraction. Genomic DNA was extracted with Wizard Genomic DNA Purification Kit (Promega, WI, USA) according to the manufacturer's protocol for gram-negative bacteria. DNA quantity and quality were verified with a Qubit Fluorometer (Invitrogen, NY, USA).

Primer design and PCR modification. A genome comparison method was used to identify specific gene sequences from 19 *Xanthomonas* strains (Table 2) for the PCR detection of pathogenic *Xanthomonas* strains using DNASTAR Lasergene software package v12.2 (Wisconsin) (Burland 2000; Zhu et al. 2009). The noncoding region (692300-692504) of the MSCT1 chromosome (GenBank

Table 1. List of *Xanthomonas* strains or genomic DNA used in the standard and qPCR development

Bacterium	Isolate number	Source ^a
<i>Xanthomonas citri</i> pv. <i>malvacearum</i> MSCT1	MSCT1	Showmaker et al. 2017
<i>Xanthomonas citri</i> pv. <i>malvacearum</i> Race 1	Race 1	P. M. Thaxton
<i>Xanthomonas citri</i> pv. <i>malvacearum</i> Race 2	Race 2	P. M. Thaxton
<i>Xanthomonas citri</i> pv. <i>malvacearum</i> Race 3	Race 3	P. M. Thaxton
<i>Xanthomonas citri</i> pv. <i>malvacearum</i> Race 12	Race 12	P. M. Thaxton
<i>Xanthomonas citri</i> pv. <i>malvacearum</i> Race 18	Race 18	P. M. Thaxton
<i>Xanthomonas citri</i> spp. <i>citri</i> 306	XAC306	Nian Wang
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> 85	XCV85	Zhaohui Chu
<i>Xanthomonas campestris</i> pv. <i>campestris</i> ATCC 33913	XCC33913	Zhaohui Chu

^a Refers to the acknowledgment for providers' affiliations.

Accession number: CP017020), which is located downstream of the gene BGK55-3070 encoding a homolog of pyruvate dehydrogenase (Showmaker et al. 2017), was identified as a unique DNA segment via Blastn analysis and used for primer and probe design. One pair of pathovar specific primers (MSCT1-P2F and MSCT1-P2R; Table 3) was designed based on the selected gene sequence. To determine the optimized annealing temperature of the primer pairs, a temperature gradient PCR was carried out using a range of annealing temperatures from 50 to 60°C.

Strains MSCT1, *X. citri* pv. *malvacearum* race 1 to 3, 12, and 18, and the noncotton pathogen strains *X. citri* spp. *citri* 306, *X. campestris* pv. *vesicatoria* 85, and *X. campestris* pv. *campestris* ATCC 33913 were used for the initial PCR comparison. PCR reactions were carried out in a 25- μ l reaction volume containing 5.0 μ l PCR buffer (5 $\times$), 2.5 μ l MgCl₂ (25 mM), 0.75 μ l dNTPs (10 mM each), 0.75 μ l each forward and reverse primers (10 μ M), 0.25 μ l Taq polymerase (5 U μ l⁻¹, Promega Corporation), and 1 μ l (50 ng μ l⁻¹) DNA. The PCR reaction was conducted with the following parameters: an initial heating step at 95°C for 3 min; followed by 40 cycles of denaturation at 94°C for 30 s, annealing at temperature gradient of 50 to 60°C for 20 s, and extension at 72°C for 20 s; and final extension at 72°C for 5 min. Universal 16S rDNA primers (27F and 1492R; Eden et al. 1991) were used for DNA quality control. The PCR products were run on 1% (w/v) agarose gels in Tris-Acetate-EDTA buffer with ethidium bromide, and photographed using ChemiDoc MP System (BioRad Inc., CA, USA). Xan-MSCT-P1 (Table 3) was synthesized commercially (Eurofins MWG Operon LLC, Louisville, KY) and labeled at the 5'-terminal nucleotide with 6-carboxy-fluorescein (FAM) reporter dye and at the 3'-terminal nucleotide with Black Hole Quencher (BHQ)-1.

DNA templates for standard curves. Strain MSCT1 was cultured in liquid NBY medium and genomic DNA was extracted as described above. A Promega pGEM-T Easy Vector PCR clone system was used to clone the PCR amplicon (the PCR assay was described above with primers MSCT1-P2F and MSCT1-P2R) of MSCT1 to generate the plasmid pMSCT1-p2. Plasmid DNA was isolated using the Qiagen plasmid Miniprep kit. Both plasmid and genomic DNA were used to generate the standard curves to test the sensitivity of the TaqMan Probe. Plasmid and genomic DNA samples were quantified with a Qubit Fluorometer and stored at -80°C. PCR was performed based on the plasmid and genomic DNA of MSCT1 as described above. The PCR amplicon was sequenced to confirm the sequence was derived from MSCT1.

Sensitivity and specificity of the qPCR assay. To optimize the qPCR protocol, plasmid pMSCT1-p2 was serially diluted in 10-fold

increments over a 7-log scale. The serial dilution with DNA concentrations from 10 ng μ l⁻¹ to 10⁻⁶ ng μ l⁻¹ was used to generate the standard curve to quantify the sensitivity of the primers in triplicate. Primers and reaction conditions were initially screened using SYBR green-based qPCR (Agilent Technologies, Inc.) on an Mx3005P Q-PCR Systems (Agilent Technologies, Inc.). All qPCRs were completed with duplicate technical replicates and no-template negative controls. Following successful reactions for duplicate qPCR runs with no evidence of nonspecific amplification by agarose gel analysis, reaction conditions were optimized for TaqMan based qPCR (TaqMan Master Mixes; Agilent Technologies, Inc.) results.

Final reaction mixtures included 0.5 μ l (10 μ M) of each forward and reverse primer, 0.3 μ l (10 μ M) TaqMan Black Hole Quencher probe, 10 μ l (2 $\times$) TaqMan master mix, 1 μ l of sample DNA, and PCR-grade water to a total reaction volume of 20 μ l. The two-step qPCR protocol was performed according to the manual provided within the TaqMan master mix kit (10 min for DNA polymerase activation at 95°C followed by 40 cycles of 95°C for 30 s and 60°C for 1 min). A standard curve was drawn by plotting the threshold cycle (Ct) against the natural log of the copy number of molecules. A copy number of DNA was calculated with the following equation: copies μ l⁻¹ = [6.022 $\times$ 10²³ (copy mol⁻¹) $\times$ amount (g)] $\times$ [length (bp) $\times$ 660 (g mol⁻¹ bp⁻¹)]⁻¹ (Cho et al. 2011).

Validation of qPCR methodology with artificially inoculated and naturally infected plant samples. Cotton seedlings (cv. Phyto-gen 499WRF; Dow AgroSciences, Indianapolis, IN) were grown in an open growth chamber under an 8:16 h light:dark cycle at approximately 23°C. Seedlings were produced by planting three seeds in one-gallon pots with topsoil in a greenhouse and raised until they were 4 weeks old. Seedlings were subsequently sprayed with an MSCT1 bacterial suspension (washed cells in phosphate buffer, 0.1 M, pH 7.0) originating from overnight cultures grown on NBY medium at a concentration of approximately 1,000 CFU ml⁻¹ based on estimates obtained using a spectrophotometer and a cell density

Table 3. Primers, optimized annealing temperature, and size of product

Primer name	Primer sequence	Tm (°C)	Size of product
Primer			
MSCT1-P2F	TATTTATTTATCCCACCAGAGG	47.6	205 bp
MSCT1-P2R	TCAGAGTATTCAGAGTAAGTGCC	47.6	
TaqMan Probe			
Xan-MSCT-P1	TTGGATCCATCCCCGCCTGAAG	63.6	

Table 2. Bacterial strains, specific disease caused by each strain, and additional molecular information regarding the strains used for genome comparison

Bacterial strain ^a	Disease	Sequence method	Source (GenBank)
<i>X. albilineans</i> GPE PC73	Leaf scald of sugarcane	Complete genome	NC013722.1
<i>X. axonopodis</i> pv. <i>citri</i> strain 306	Citrus canker	Complete genome	NC003919.1
<i>X. axonopodis</i> pv. <i>glycines</i> CFBP 2526	Bacterial pustule of soybean	Whole genome shotgun	NZCM002268.1
<i>X. axonopodis</i> pv. <i>glycines</i> CFBP 7119	Bacterial pustule of soybean	Whole genome shotgun	NZCM002264.1
<i>X. axonopodis</i> Xac29-1	Citrus canker	Complete genome	NC020800.1
<i>X. campestris</i> pv. <i>campestris</i> 8004	Black rot of cabbage	Complete genome	NC007086.1
<i>X. campestris</i> pv. <i>campestris</i> ATCC 33913	Black rot of cabbage	Complete genome	NC003902.1
<i>X. campestris</i> pv. <i>campestris</i> CN14	Black rot of crucifers	Whole genome shotgun	CM001871.1
<i>X. campestris</i> pv. <i>campestris</i> CN15	Black rot of crucifers	Whole genome shotgun	CM001890.1
<i>X. campestris</i> pv. <i>campestris</i> CN16	Black rot of crucifers	Whole genome shotgun	CM001897.1
<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10	Bacterial spot of pepper	Complete genome	NC007508.1
<i>X. citri</i> pv. <i>malvacearum</i> X18	Cotton bacterial blight	Whole genome shotgun	CM002136.1
<i>X. citri</i> pv. <i>malvacearum</i> X20	Cotton bacterial blight	Whole genome shotgun	CM002029.1
<i>X. citri</i> subsp. <i>citri</i> Aw12879	Citrus canker	Complete genome	NC020815.1
<i>X. oryzae</i> pv. <i>oryzae</i> KACC 10331	Rice bacterial blight	Complete genome	NC006834.1
<i>X. oryzae</i> pv. <i>oryzae</i> MAFF 311018	Rice bacterial blight	Complete genome	NC007705.1
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99A	Rice bacterial blight	Complete genome	NC010717.1
<i>X. oryzae</i> pv. <i>oryzicola</i> BLS256	Rice bacterial streak	Complete genome	NC017267.1
<i>X. citri</i> pv. <i>malvacearum</i> MSCT1 ^b	Cotton bacterial blight	Complete genome	GCA001719145.1

^a *Xanthomonas* strains available from the NCBI genome database were used for genome comparison, the strains selected were pathogenic to different hosts.

^b A complete genome sequence of *X. citri* pv. *malvacearum* MSCT1 was used in this study for sequence comparison and primer design.

measurement at OD₄₂₀. Leaves were collected 48 h postinoculation and DNA was extracted from 1 g leaf tissue, representing approximately one true leaf, using Wizard Genomic DNA Purification Kit (Promega, WI, USA) according to the manufacturer's protocol for Gram-negative bacteria. The bacterial cell population present on the leaf tissue surface was estimated using a routine serial dilution procedure. Briefly, inoculated leaves were collected and washed with sterile distilled water to remove bacteria. DNA from noninoculated leaves was used as a negative control. The extracted DNA was dissolved in 50 µl elution buffer and maintained in a -80°C freezer. Experiments were conducted three times.

For verification of the developed qPCR method on cottonseed, a bacterial cell suspension (2 ml containing approximately 1,000 cells) was mixed with 10 g cottonseed (cv. PhytoGen 499WRF). Infested seeds were subsequently dried in a laminar flow hood for 1 h prior to further experimentation, and three replicates were conducted. To recover bacteria from artificially infested seed, the infested seed lot was soaked in 50 ml sterile distilled water for 30 min. Seed without addition of MSCT1 cells was used as a negative control. The liquid supernatant was collected for DNA extraction as described above. The DNA was dissolved in 50-µl elution buffer and maintained in a -80°C freezer or used for the qPCR assay. Real-time, quantitative PCR assays were conducted in duplicate for each extract as described above. The qPCR experiment was repeated three times independently.

For the purposes of validating the qPCR protocol with naturally infected cotton plant tissue, 15 cotton leaf and/or boll samples representing geographically distinct locations and exhibiting characteristic symptoms associated with bacterial blight (e.g., water-soaked lesions on leaves and bolls) were collected from Georgia ($n = 9$), Mississippi ($n = 2$), Tennessee ($n = 1$), and Texas ($n = 3$) during 2016. In addition, healthy asymptomatic plant tissues (three leaf samples and three boll samples) were collected from a cotton field located in Starkville, MS at the R. R. Foil Plant Science Research Center and served as negative controls. Initially, the bacteria were isolated from the plant samples and the bacterial isolates suspected to be *X. citri* pv. *malvacearum* were used for genomic DNA extraction. Suspected *X. citri* pv. *malvacearum* was recovered from all infected plant material by culturing the infected plant material on NBY medium, incubating the plates for 2 days under ambient laboratory conditions, and recovering single isolates exhibiting the proper colony morphology and coloration. The *X. citri* pv. *malvacearum* culture cells were used as a positive control. Secondly, detection of the pathogen using qPCR directly from cotton samples was conducted. Briefly, the symptomatic lesions were cut from the plant samples for total DNA extraction using Wizard Genomic DNA Purification Kit. The samples (≈ 0.1 g each) were macerated in a 1.5 ml microcentrifuge tube containing 100 µl of sterile water. After brief centrifugation (2,000 rpm for 30 s), the supernatants were collected for total DNA extraction as described above. The qPCR was conducted as described above.

In 2017, bacterial blight-infected bolls exhibiting water-soaked lesions were collected from geographically distinct fields. Boll samples were collected from Georgia ($n = 3$), Mississippi ($n = 2$), and Texas ($n = 5$). Each sample, in this case consisting of a location, was broken down into 10-boll replicates. Replicate samples were ginned separately on a table-top 10-saw gin (Continental Type 10, Continental Gin Company, Lubbock, TX) to separate lint and seed. Fuzzy seed were subsequently delinted using concentrated sulfuric acid (H₂SO₄; 95%), neutralized with sodium carbonate (soda ash; Na₂CO₃) as a buffering agent, and dried in a forced air heater (40°C) for approximately 30 min or until seeds were dry. Prior to laboratory-based analyses, immature cottonseeds were removed from each replicate sample based on the coloration (all nonblack seed) and size of seed deemed "immature." Therefore, only seeds that were the color and size to be candidates for commercial sale were used in the subsequent analysis. Two different methods were used to confirm the presence of *X. citri* pv. *malvacearum* from cottonseed: 1) the qPCR methodology, and 2) a culture plate method. To complete the two methods, aliquots of delinted seeds (2 to 10 g) were

ground using a sterilized blender (Waring Blender 7011S, Torrington, CT, USA) for 20 s. One commercial seed lot (cv. PhytoGen 499WRF) from which *X. citri* pv. *malvacearum* was not detected by using the culture-based method was included as a control. Sterile distilled water (3 ml g⁻¹ seed) was added to a 50-ml sterile Falcon tube containing the ground seed and vortexed for 5 s (setting no. 9, Vortex Genie; Scientific Industries, Bohemia, NY). The samples were placed on the lab bench for 20 min and the supernatant was collected after centrifugation (300 × g for 5 min) at 25°C to remove large seed tissue pieces. An aliquot (0.5 ml for each sample) of the supernatant was used for bacterial isolation using a culture-based method on NBY agar plates. Bacterial colonies suspected to be *X. citri* pv. *malvacearum* based on representative colony morphology and color were subsequently purified after 2 days on fresh NBY agar plates and genomic DNA was extracted using Wizard Genomic DNA Purification Kit for sequencing confirmation. The remainder of the supernatant (≈ 5 to 10 ml) was centrifuged at 4,750 × g for 10 min to collect bacterial cells, and the resulting pellets were used for genomic DNA extraction. After efficacy comparisons, Powersoil DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) was chosen for extraction of genomic DNA as described by the manual with the following modification. A bead beater homogenizer (Mini Beadbeater-8, Bio-Spec Products, Inc., Bartlesville, OK, USA) was used for breaking cells (2 min at the maximum speed). DNA samples were measured by a NanoDrop spectrophotometer (ThermoFisher Scientific, USA) and qPCR was conducted on the StepOnePlus Real-Time PCR System with the Maxima Probe qPCR Master Mix (ThermoFisher Scientific). The qPCR program (50°C 2 min and then 95°C 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) was used based on the recommendations of the manufacturer. Genomic DNA of MSCT1 was used as a positive control, and two negative controls (sterile distilled water and the genomic DNA from the non-*X. citri* pv. *malvacearum* infested seed) were included. Three replicates were conducted for each sample. The samples with significantly lower Ct values than that of the healthy seeds were considered as *X. citri* pv. *malvacearum* positive.

Results

Primer design and PCR modification. Using a genome comparison method, the genome sequences of 19 strains of cotton pathogenic and nonpathogenic *Xanthomonas* strains were compared to identify *X. citri* pv. *malvacearum*-specific sequences. Sequences that were only identified in cotton pathogenic (i.e., *X. citri* pv. *malvacearum*) strains were selected and used for primer design. Blast analysis of the candidate gene sequences indicated that the selected sequence was a noncoding region located downstream of the pyruvate dehydrogenase gene (GenBank Accession number: CP017020, Nucleotide sequence: 692300-692504). The PCR primers designed, MSCT1-P2F and MSCT1-P2R, were based on the selected sequence (Table 3).

Gradient PCR analysis showed that the primers MSCT1-P2F and MSCT1-P2R produced the expected amplicon at the annealing temperature 56°C without nonspecific amplification from the genomic DNA of strain MSCT1. With the annealing temperature (56°C), the primers MSCT1-P2F and MSCT1-P2R exclusively amplified the gene region of pathogenic races 1, 2, 3, 12, 18, and MSCT1 (Supplementary Figs. S1 and S2). Using the primer pairs, no cross amplification was observed with the other three non pathovar *malvacearum* strains XAC306, XCV85, and XCC33913.

Sensitivity and specificity of the qPCR protocol. Standard curves were generated using both the cloned DNA fragment in plasmid pMSCT1-p2 (Fig. 1) and the MSCT1 genomic DNA (Fig. 2). The cut-off (Ct) value for all assays was 35. The slope of the curves (k) was used to determine the average amplification efficiency (E) with the equation $E = 10^{[-1/k]}$, where $E = 2$ corresponds to 100% efficiency (Ramakers et al. 2003). The amplification efficiency for the standard curve generated by plasmid pMSCT1-p2 (Fig. 1) was 100%. Melting curve analysis using the SRYBR Green method determined that all PCR products shared the same melting temperature (Supplementary Figs. S3 and S4). When genomic DNA of MSCT1

was used for amplification, the efficiency for the standard curve (Fig. 2 and Supplementary Fig. S5) was 104%. The assay exhibited a strong linear response ($R^2 = 0.998$ and 0.996 , respectively). The detection limit of the reaction was one cloned copy of the *X. citri* pv. *malvacearum* target gene (Table 4).

The DNA samples of the three nonpathogenic strains XAC306, XCV85, and XCC33913 were used as negative controls to test qPCR specificity. The DNA templates used in the assay were 1 ng in a 20- μ l reaction mixture. For the particular primer set, no cross amplifications were noted for any of the five distinct cotton-pathogenic *X. citri* pv. *malvacearum* races in the presence of the target pathogen, and no signals were obtained in reactions using DNA of the three nonpathogenic strains.

Validation of qPCR methodology with artificially inoculated and naturally infected plant samples. Using the DNA extracted from the cotton leaf and seed samples, TaqMan based qPCR successfully detected *X. citri* pv. *malvacearum* and no PCR amplicon was observed from negative controls. The detection limit was determined to be 10^2 copies from 1 g of fresh cotton leaf material and 37 copies from 1 g of seed.

The qPCR method validated successfully the initial diagnosis of the plant samples collected in 2016 (Table 5). All bacteria isolated and suspected to be *X. citri* pv. *malvacearum* from the naturally infected cotton tissue samples were positive for *X. citri* pv. *malvacearum* in the qPCR analysis, while the qPCR detections from the healthy leaf and boll samples were *X. citri* pv. *malvacearum*-negative. In addition, 31 of the 33 seed lots collected from bolls exhibiting symptoms of bacterial blight tested positive for *X. citri* pv. *malvacearum* using the qPCR analysis (Table 5). In contrast, only 15 of the qPCR-positive seed samples (31 seed lots) were confirmed to carry *X. citri* pv. *malvacearum* using microbiological media-based isolation methods (Table 5). Based on the morphology of the colonies on NYB and 16S rDNA sequencing, *X. citri* pv. *malvacearum* was not found on the agar plates from 16 qPCR-positive seed lots or from the two qPCR-negative seed samples.

Fig. 1. A standard curve generated using plasmid pMSCT1-p2. A range of DNA concentrations of the plasmid from 1 ng to 10^{-6} ng, which were converted to copy numbers as described by Cho et al. (2011). Three replicates were conducted for each concentration.

Fig. 2. A standard curve generated using genomic DNA of MSCT1. The standard curve was generated using genomic DNA of MSCT1 in a range from 50 ng to 5×10^{-4} ng, which were converted to copy numbers as described by Cho et al. (2011). Three replicates were conducted for each concentration.

Discussion

Since 2011, a resurgence of bacterial blight throughout the U.S. cotton producing regions has resulted in concern regarding the potential presence of *X. citri* pv. *malvacearum*-infected commercial cotton planting seed (Phillips et al. 2017; Rothrock et al. 2012). As a result of the 2011 outbreak in the U.S. mid-South, a report of *X. citri* pv. *malvacearum*-infected seed representing multiple cultivars from multiple seed companies was made by Rothrock et al. (2012). Early detection is important for assessing the health of cotton plants, as well as for reducing the likelihood of *X. citri* pv. *malvacearum* becoming an issue in subsequent seasons as a result of its initial introduction from infected cotton planting seed. Since the early twentieth century, cotton planting seed has been reported as a primary source of bacterial blight in cotton fields (Brinkerhoff and Hunter 1963; Hibbard 1910; Innes 1983; Jenkins 1919; Schnathorst 1964; Thaxton and El-Zik 2001; Verma 1986). Bacterial blight can be destructive even if only one *X. citri* pv. *malvacearum*-containing seed is planted in a field, as the pathogen can be easily disseminated short distances within a field during favorable environmental conditions, both by natural means, such as blowing rain, and as a result of the agricultural practices involved in farming cotton (Brown 1942; Davis and Sandidge 1977; Tarr 1961). Therefore, having the ability to rapidly detect *X. citri* pv. *malvacearum* from infected plant material as well as from cotton planting seed is an important tool for limiting the incidence and impact of bacterial blight. Visual detection of bacterial blight on young cotton plants shortly after emergence is difficult. Young cotton plants may exhibit damage from other foliar leaf spot pathogens, or from the widespread use of herbicides that can result in injury to seedling cotton and the foliage of young cotton plants following either pre- or postemergent herbicide applications (Kendig et al. 2007; Kirkpatrick and Rothrock 2001). In addition, where bacterial blight occurs on cotyledon cotton, injury to developing cotton plants can accelerate the spread of bacterial blight from infected plants since *X. citri* pv. *malvacearum* requires natural openings or wounds for infection (Brinkerhoff 1970; Brinkerhoff and Hunter 1963; Hillocks 1992; Thaxton and El-Zik 2001; Verma 1986).

Development of specific primers and DNA probes from the 16S to 23S rDNA spacer region (Sakthivel et al. 2001; Siguier et al. 2006), repeated elements, and siderophore receptor (Zhao et al. 2007) for the purposes of identification and detection have been previously reported for *X. oryzae* pv. *oryzae*, the causal agent of bacterial blight in rice. A similar strategy was applied for the detection of *X. arboricola* pv. *pruni* in *Prunus* sp. (Palacio-Bielsa et al. 2011). However, with regard to the rice qPCR test, it has become evident that the genetic coverage was insufficient to discriminate among the different pathogens of *X. oryzae*. The insertion sequence may undergo periodic extinction in prokaryotic genomes (Cho et al. 2011; Raaijmakers et al. 1994; Siguier et al. 2006). In the current study, we identified some unique regions in the *X. citri* pv. *malvacearum* genomes using a comparative genomic analysis, which ensured specificity of the selected qPCR primers and probe. Specificity of the primers and probe were important since *X. citri* pv. *malvacearum* races other than race 18 have previously been reported from the U.S. cotton producing regions (Brinkerhoff 1970; Phillips et al. 2017; Thaxton et al. 1992).

Table 4. Mean cycle threshold (Ct) of 10-fold serial dilutions of MSCT1 cloned DNA and genomic DNA as determined by the TaqMan qPCR

Plasmid	Plasmid DNA		Genomic DNA	
	Log copies μ l ⁻¹	Ct values	Log Weight μ l ⁻¹	Ct values
2.85 $\times 10^8$	13.58	8.454	50 ng	17.46
2.85 $\times 10^7$	16.43	7.454	5 ng	20.16
2.85 $\times 10^6$	19.54	6.454	500 pg	22.98
2.85 $\times 10^5$	23.46	5.454	50 pg	26.73
2.85 $\times 10^4$	26.42	4.454	5 pg	30.12
2.85 $\times 10^3$	29.87	3.454	500 fg	33.82
2.85 $\times 10^2$	33.35	2.454	50 fg	36.13

Previously, Umesha et al. (2010) reported the detection of *X. citri* pv. *malvacearum* from cottonseed using morphological, biochemical, and molecular tests developed with ITS primers. However, the specificity of the primers was limited due to limited information regarding the sequences of the pathogenic strains. Recently our research team completed genome sequencing of the local *X. citri* pv. *malvacearum* strain MSCT1 (Showmaker et al. 2017), which made it possible to identify pathovar-specific genetic loci based on genomewide sequence comparison (Burland 2000). In the current study, TaqMan qPCR primers and probe were designed from a non-coding region and the primer set only produced an amplicon under the conditions described above from the *X. citri* pv. *malvacearum* strains pathogenic to cotton, but not from nonpathogenic *Xanthomonas* isolates on cotton. Moreover, the primers based on the selected sequence were specific to the strains of cotton-pathogenic *X. citri* pv. *malvacearum* included in the analysis (races 1, 2, 3, 12, 18, and MSCT1). In addition, when the primers were applied to strains of the bacterium not reported to be pathogenic to cotton (XAC306, XCV85, and XCC33913) no PCR amplicons were obtained.

The sensitivity threshold of 10^2 copies from 1 g fresh cotton leaf material; and 37 copies from 1 g of artificially infected cotton seed samples for the *Xanthomonas* TaqMan qPCR assay was equal to or better than those obtained from previously reported assays for other phytopathogenic bacteria (Dreo et al. 2007; Palacio-Bielsa et al. 2011; Sakthivel et al. 2001; Zhao et al. 2007). The assay we developed surpasses the sensitivity levels reported previously for the conventional PCR method (Hartung et al. 1993). The differences in detection limit between the *X. citri* pv. *malvacearum* DNA isolated from pure bacterial cultures and DNA isolated from plant tissue

may be due to inhibitors present within cotton plant tissues, especially interference from cotton DNA. Extraction of high-quality genomic DNA from cotton plant tissue is difficult because of high concentrations of polysaccharides, phenolic compounds, oxidizable quinones, and additional interfering substances (Demeke and Jenkins 2010; Paterson et al. 1993). In addition, some of the compounds in cotton plant tissues can serve as PCR inhibitors, especially to qPCR (Baeumler et al. 2006). The presence of inhibitors in genomic cotton DNA could interfere with the PCR amplification efficiency and result in reduced detection efficiencies compared with those achieved from DNA isolated from bacterial cultures. In the current study, bacterial isolates from naturally infected field samples exhibiting classical bacterial blight symptoms were confirmed to be *X. citri* pv. *malvacearum* using the qPCR protocol. The isolation-based microbiological method did not recover the pathogen from the qPCR positive samples, which may result from rich bacterial endophytes and lack of selective media for *X. citri* pv. *malvacearum*. Diverse bacterial endophytes have previously been recovered from crops including cotton (McInroy and Kloepper 1995). We also noted that cotton seeds grown in different geographical regions exhibited different endophytic bacterial compositions (S. Lu and T. W. Allen, unpublished). On the other hand, the qPCR approach may potentially result in false positive responses due to the numerous uncharacterized bacteria present in cottonseed and other tissues. Therefore, additional research may be necessary to reduce the likelihood of false positives occurring by increasing our understanding and awareness of the known and unknown bacteria that occur on and within cotton plant tissues. However, avoiding false positives may be difficult since the bacterial communities contained within individual seed lots collected from

Table 5. Field collected cotton plant tissue samples from 2016 originating in Georgia, Mississippi, Tennessee, and Texas and 2017 bacterial blight infected bolls originating from Georgia, Mississippi, and Texas used to validate the qPCR methodology and compare with the previous microbiological media methods employed for the detection of the bacterial blight pathogen, *Xanthomonas citri* pv. *malvacearum*, from infected plant samples

State	Sample ID	County	Cultivar ^a	Bacterial blight response ^b	Bacterial blight symptoms	Plant material ^c	qPCR result	Result of microbiological media ^d
Georgia	16-GA-1	Ben Hill	DPL 1555 B2RF (1)	Susceptible	Y	Leaf	Positive (1)	Positive (1)
Georgia	16-GA-2	Ben Hill	DPL 1555 B2RF (1)	Susceptible	Y	Boll	Positive (1)	Positive (1)
Georgia	16-GA-3	Ben Hill	DPL 1555 B2RF (1)	Susceptible	Y	Leaf	Positive (1)	Positive (1)
Georgia	16-GA-4	Ben Hill	DPL 1555 B2RF (1)	Susceptible	Y	Boll	Positive (1)	Positive (1)
Georgia	16-GA-5	Ben Hill	PHY 333 WRF (1)	Susceptible	Y	Leaf	Positive (1)	Positive (1)
Georgia	16-GA-6	Ben Hill	PHY 333 WRF (1)	Susceptible	Y	Boll	Positive (1)	Positive (1)
Georgia	16-GA-7	Seminole	DPL 1538 B2XF (1)	Susceptible	Y	Leaf/boll	Positive (1)	Positive (1)
Georgia	16-GA-8	Bainbridge	PHY 333 WRF (1)	Susceptible	Y	Leaf/boll	Positive (1)	Positive (1)
Georgia	16-GA-9	Bainbridge	PHY 333 WRF (1)	Susceptible	Y	Leaf/boll	Positive (1)	Positive (1)
Mississippi	16-MS-1	Washington	PHY 499 WRF (1)	Susceptible	Y	Leaf	Positive (1)	Positive (1)
Mississippi	16-MS-2	Washington	PHY 333 WRF (1)	Susceptible	Y	Leaf	Positive (1)	Positive (1)
Tennessee	16-TN-1	Carroll	NG 3406 B2XF (1)	Susceptible	Y	Boll	Positive (1)	Positive (1)
Texas	16-TX-1	Dawson	PHY 499 WRF (1)	Susceptible	Y	Leaf	Positive (1)	Positive (1)
Texas	16-TX-2	Hale	NG 3406 B2XF (1)	Susceptible	Y	Leaf	Positive (1)	Positive (1)
Texas	16-TX-3	Hale	NG 3517 B2XF (1)	Mostly susceptible	Y	Leaf	Positive (1)	Positive (1)
Georgia	17-GA-1	Appling	DPL 1747 NRB2XF (7)	Susceptible	Y	Black seed	Positive (7)	Positive (4)
Georgia	17-GA-2	Irwin	DPL 1646 B2XF (3)	Partially resistant	Y	Black seed	Positive (2)	Positive (0)
Georgia	17-GA-3	Tift	DPL 1646 B2XF (4)	Partially resistant	Y	Black seed	Positive (3)	Positive (2)
Mississippi	17-MS-1	Washington	PHY 499 WRF (4)	Susceptible	Y	Black seed	Positive (4)	Positive (1)
Mississippi	17-MS-2	Washington	PHY 499 WRF (5)	Susceptible	Y	Black seed	Positive (5)	Positive (0)
Texas	17-TX-1-T	Dawson	NG 3406 B2XF (3)	Susceptible	Y	Black seed	Positive (3)	Positive (3)
Texas	17-TX-2-T	Dawson	NG 3406 B2XF (3)	Susceptible	Y	Black seed	Positive (3)	Positive (3)
Texas	17-TX-3-J	Dawson	PHY 499 WRF (1)	Susceptible	Y	Black seed	Positive (1)	Positive (1)
Texas	17-TX-4-J	Dawson	NG 3406 B2XF (1)	Susceptible	Y	Black seed	Positive (1)	Positive (0)
Texas	17-TX-5-J	Dawson	NG 3406 B2XF (1)	Susceptible	Y	Black seed	Positive (1)	Positive (0)
Texas	17-TX-6-J	Dawson	NG 3406 B2XF (1)	Susceptible	Y	Black seed	Positive (1)	Positive (1)

^a Abbreviations for specific cotton cultivars are as follows: DPL = DeltaPine, PHY = Phytogen, and NG = NexGen. Numbers after cultivars indicate the number of replicate seed samples obtained from each location based on a 10 boll sample exhibiting water-soaked lesions associated with bacterial blight.

^b Response of cotton varieties to bacterial blight (race 18) are based on information as provided in Kemerait et al. (2017). A susceptible response is based on >90% incidence in inoculated field trials, mostly susceptible is based on 60 to 80% incidence, partially resistant is based on 10 to 25% incidence, and a resistant response is based on <10% incidence in inoculated field trials.

^c In instances where black seed was the main variable considered for testing, bolls attributed to the particular variety exhibited symptoms associated with bacterial blight (e.g., water-soaked lesions on bolls that penetrated the carpal wall).

^d Observations following the microbiological media methods were based on the colony morphology and resulting molecular analysis of those isolates believed to be *X. citri* pv. *malvacearum*.

different geographic locations may in fact be quite diverse (*unpublished data*). While differences in the fungal endophytic community in both space and time have previously been reported (Ek-Ramos et al. 2013), this has not been a topic considered in the scientific literature for bacterial communities in cultivated cotton.

Even though bacterial blight has been well documented to arise as a result of seedborne *X. citri* pv. *malvacearum*, there is still some conjecture as to whether or not the organism survives the acid-delinting process on the outside of the seed coat or if the organism survives within embryonic seed tissues (Alexander et al. 2012; Archibald 1927; Bain 1939; Chester 1938; Rolfs 1915; Schnathorst 1968). Previous reports suggest that the greatest percentage of the bacterium is present on seed lint, and this remains one of the main reasons for commercial acid-delinting as a seedborne disease management practice (Gregory et al. 1999). In addition, acid-delinting is performed so the seed coat can be more evenly coated with seed treatment chemicals and to increase the flowability of seed through modern planting equipment that rely on vacuum systems (Alexander et al. 2012). However, acid-delinting, although one of the preferred forms of bacterial blight management, is not fool proof. In the current study, lint was removed from seed with sulfuric acid and “immature” seed was excluded for bacterial isolation or qPCR detection to provide a situation similar to commercial seed production practices. However, *X. citri* pv. *malvacearum*–positive detection rates were still high (>45% for microbiological media isolation techniques and >93% for qPCR) and suggest that even if *X. citri* pv. *malvacearum* does not survive on the outside of the seed coat, that it more likely survives within the embryo, which is not affected by acid-delinting.

Previous research results suggested that *X. citri* pv. *malvacearum* can live on cotton plants that do not exhibit bacterial blight symptoms (Davis and Sandidge 1977; Stoughton 1930). Lack of symptom expression could result from a prolonged nonconductive environment following germination of infected seed. Where bacterial blight-susceptible cultivars are produced for cotton planting seed, and certification relies on field scouting to determine the presence of the disease, such determinations are difficult, and the bacterium could enter the seed production pipeline and increase the likelihood of bacterial blight being introduced during the subsequent season. Moreover, the disease can occur in cotton plants that do not exhibit symptoms on bolls, but still harbor *X. citri* pv. *malvacearum* in infected seed due to the systemic nature of the infection within the plant (Davis and Sandidge 1977; Stoughton 1930). All infected bolls collected during 2017 were observed to exhibit a moderate level of bacterial blight infection as evidenced by the presence of multiple water-soaked lesions on the boll and direct penetration of the pathogen into the carpal wall. Boll and subsequent seed infection was even documented in one variety that has been previously reported to be partially resistant. Therefore, it is strongly suggested that strict scouting practices should be used for detecting the bacterium in field situations where bacterial blight-susceptible cultivars are produced for the purpose of seed production. In addition, it is our professional opinion that fields containing bolls exhibiting bacterial blight symptoms on cotton bolls regardless of the published reaction of the cultivar should be excluded from seed production to limit the spread of the disease. Even though the current research considered the role of cotton exhibiting bacterial blight symptoms, the potential for cottonseed originating from bacterial blight-resistant varieties serving as a source of inoculum should be investigated.

In conclusion, this study demonstrates that the primers and probe set developed are highly specific for some of the more commonly observed *X. citri* pv. *malvacearum* races from the United States previously reported to be pathogenic to cotton. The main objective of this research was to create a rapid detection method and reduce the time required to positively confirm the presence of *X. citri* pv. *malvacearum* from cotton plant material. As compared with time-consuming culture-based methods, the TaqMan qPCR assay is reliable and efficient, and it has the potential to be used both as a diagnostic tool to confirm the presence of bacterial blight from cotton plant tissue as well as be used for quality control in cotton seed production systems.

Acknowledgments

We thank N. Wang (Univ. of Florida) and Z. H. Chu (Shandong Agric. Univ.) for providing genomic DNA samples of the *Xanthomonas* bacteria. The authors also thank W. Briggs, H. Y. Kelly (Univ. of Tennessee), B. Phillips, J. Story, J. Woodward (Texas A&M Univ.), and T. Wheeler (Texas A&M Univ.) for supplying infected cotton plant material to validate methods. Additional thanks are extended to S. M. Baird (Miss. State Univ.) for technical assistance and W. J. Mansour (Miss. State Univ.) and T. H. Wilkerson (Miss. State Univ.) for field sample collection and ginning cotton boll samples. We thank P. M. Thaxton for providing strains of *X. citri* pv. *malvacearum* for comparison. Lastly, we thank K. Jordan and S. Stetina (USDA-ARS, Stoneville, MS) for help with acid-delinting cottonseed samples.

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