A Polymerase Chain Reaction Protocol for the Detection of *Clavibacter xyli* subsp. *xyli*, the Causal Bacterium of Sugarcane Ratoon Stunting Disease

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ABSTRACT

Pan, Y.-B., Grisham, M. P., Burner, D. M., Damann, K. E., Jr., and Wei, Q. 1998. A polymerase chain reaction protocol for the detection of *Clavibacter xyli* subsp. *xyli*, the causal bacterium of sugarcane ratoon stunting disease. 82:285-290.

A polymerase chain reaction (PCR) protocol was developed that specifically detected *Clavibacter xyli* subsp. *xyli*, the causal agent of sugarcane ratoon stunting disease. Generic PCR products from the intergenic transcribed spacer (ITS) region of 16S-23S ribosomal DNA of *C. xyli* subsp. *xyli* and *C. xyli* subsp. *cynodontis* were cloned and sequenced. Based on a multiple sequence alignment among these two sequences and other nonredundant highly homologous sequences from the database, two *C. xyli* subsp. *xyli*-specific PCR primers were designed, Cxx1 (5' CCGAAGTGAGCAGATTGACC) and Cxx2 (5' ACCCTGTGTTGTTTCAACG). These two 20-mer oligonucleotides primed the specific amplification of a 438-bp DNA product from genomic DNA samples of 21 *C. xyli* subsp. *xyli* strains. Amplification was not observed with genomic DNA of one *C. xyli* subsp. *cynodontis* strain, five strains of four other *Clavibacter* species, and two strains of two *Rathayibacter* species. The 438-bp PCR product also was amplified directly from cultured *C. xyli* subsp. *xyli* cells and from *C. xyli* subsp. *xyli*-infected sugarcane vascular sap with a unique reaction buffer containing polyvinylpyrrolidone and ficoll. Extraction of genomic DNA was not necessary prior to PCR assay.

Ratoon stunting disease (RSD) of sugarcane (*Saccharum* interspecific hybrids) is caused by *Clavibacter xyli* subsp. *xyli*, a small, fastidious, gram-positive, xylem-limited coryneform bacterium (12,13,22,38). The disease occurs in all sugarcane production areas of the world and can cause up to 50% yield loss in susceptible cultivars under drought conditions (6). Diagnosis of RSD is difficult because *C. xyli* subsp. *xyli*-infected plants show no external symptoms. Yield loss of infected plants is due to reduced vigor and tillering, especially in ratoon crops. Internal symptoms, including a salmon pink discoloration just below the growing point of young cane and an orange-red discoloration of the nodal

vascular bundles in mature cane stalks, are unreliable because these symptoms vary within and among cultivars. Isolation of the bacterium is difficult due to its fastidious nature and the lack of an optimized medium. Microscopic and immunological procedures (12,20,23,24,39) are effective in detecting *C. xyli* subsp. *xyli* later in the growing season when the bacterium is at a high titer; however, detection can be unreliable with these methods in early to mid-season, when a management decision must be made about which stalks are to be used as the seed cane for the next crop cycle. Immunological methods also are limited by the availability of anti-*C. xyli* subsp. *xyli* antibodies.

Recently, various DNA probes have been developed to identify and detect microorganisms (19,25). For example, DNA probes derived from the ribosomal intergenic transcribed spacer (ITS) region of 16S-23S ribosomal DNA proved useful in the identification of eubacteria (5), and plasmid-borne DNA probes detected *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes (16,35,37). PCR protocols were developed to detect *Xanthomonas albilineans*, another xylem-limited bacterial pathogen causing the sugarcane leaf scald disease (26,34). DNA-based diagnostic tools for *C. xyli* subsp. *xyli* detection that used either *C. xyli* subsp. *xyli*-specific DNA probes from unknown genomic region (7) or *C. xyli* subsp. *xyli*-

specific PCR primers within the 16S-23S ribosomal DNA (3,17) have been reported; however, none of these reports provided DNA sequence information. In a recent study, a 566-bp L1/G1-primed DNA product from the ITS region of the 16S-23S ribosomal DNA of *C. xyli* subsp. *xyli* was found to be *C. xyli*-specific and was used as a DNA probe to detect *C. xyli* subsp. *xyli* in tissue blot hybridizations (*unpublished*). The objective of this study was to develop specific primers and a PCR protocol for the detection and identification of *C. xyli* subsp. *xyli*.

MATERIALS AND METHODS

Bacterial strains. Two local *C. xyli* subsp. *xyli* strains, *16296* and *CP53-1*, and a collection of 19 *C. xyli* subsp. *xyli* strains isolated from sugarcane grown in different geographic areas (provided by S. M. Brumby, Bureau of Sugar Experiment Stations, Australia) were included in the study. The collection included *C. xyli* subsp. *xyli* strains *UQ2271* (isolated in Australia), *UQ2272* (United States), *QPF83S* (Australia), *QPF110A* (Australia), *QPF110B* (Australia), *QPF115* (Australia), *QPF122* (Australia), *QPF124* (Australia), *QPF130* (Australia), *QPF138* (Australia), *QPF153* (Australia), *QPFUNK* (Australia), *BBERGROY1* (Australia), *BIB* (Brazil), *B2NA56-79* (Brazil), *B3SP70-3370* (Brazil), *LIA* (United States), *JI* (Japan), and *SIA* (South Africa). Genomic DNA samples of *Clavibacter michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *nebraskensis*, *Rathayibacter iranica*, and *R. tritici* were provided by X. Li (Terragen, Vancouver, Canada). *X. albilineans*, seven unidentified sugarcane saprophytic bacterial isolates (34), and a genomic DNA sample of strain *Cxc7* of *C. xyli* subsp. *cynodontis*, the causal agent of Bermuda-grass stunting disease (13,14), from Mary Metzler (33) were also included in the study.

Amplification, cloning, and sequencing of PCR products. PCR reaction mix, thermal cycling program, agarose gel electrophoresis, radioactive labeling of probe, Southern hybridization, and gel purification of PCR products were according to Pan et al. (34). L1/G1-primed PCR products from the local *C. xyli* subsp. *xyli* strain

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Accepted for publication 16 December 1997.

Publication no. D-1998-0116-02R

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16296 and the *C. xyli* subsp. *cynodontis* strain Cxc7 were gel purified and cloned into pCR2.1 vector using an Original TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's recommendations. Recombinant clones were selected by colony hybridization. Plasmid DNAs were extracted from 3-ml overnight TOP10F⁺ cultures by the alkali lysis method (36), and DNA con-

centrations were determined on GeneQuant II spectrophotometer (Pharmacia Biotech, Piscataway, NJ). Six recombinant plasmid clones per construct were sequenced on an automatic DNA Sequencer ABI200, Model 377 (Applied Biosystems, Foster City, CA) at the DNA Sequencing Facility, Iowa State University, Ames.

Computer-assisted DNA sequence analysis and primer design. Individual

DNA sequences were visually checked and edited against corresponding electropherograms to obtain the final nucleotide sequences, 566 bp for *C. xyli* subsp. *xyli* and 546 bp for *C. xyli* subsp. *cynodontis*. The 566-bp sequence was used to search for its nonredundant, highly homologous sequences in GenBank+EMBL+DDBJ+PDB database using the BLASTN program of NCBI (2). A total of four GenBank se-

Primer G1			
Cxx	<u>GAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCT</u>	50	
Cxc	<u>GAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCCGGATCACCTCCT</u>	50	
Primer Cxx2			
Cxx	TTCTAAGGAGCATCTGGCACCCTGTGTTGTTTTCACGCAGAAATTGTCC	100	
Cxc	TTCTAAGGAGCATCTGGCACCT-----CGGG---TGTCC	82	
.			
Cxx	AGGCGCCGGATCTGAGACAGTACTTATCACATCGGTACGACTGGGTCTCA	150	
Cxc	AGGCGCCGGATCTGAGACAGTACTTATCACTTCGGTAAGACTTGGTCTCA	132	
.			
Cxx	GCCGGTCAGCTCATGGGTGGAACATTGACATTGGTGCGGAGCCGAACGGC	200	
Cxc	GACGGT-AGCTCATGGGTGGAACATTGACATTGGTGCGGAGCCGAACGGC	181	
.			
Cxx	TCGAACTTAGTACGCCTGCTTGACAGGAAGGAACAGTTCGGACCGGGGAGC	250	
Cxc	TCGAACTCAGTACGTCTCTTCGGAGAAAGGAACGGTTCGATCCTGGAAGC	231	
.			
Cxx	CTCGCACATGCACGCTGTTGGGTCTGAGGGACCGGACCTCATCGCTGTG	300	
Cxc	CCCGCACATGCACGCTGTTGGGTCTGAGGGACCGGGCCTCATCG-----	276	
.			
Cxx	TCTTCAAGACGCTGAGATGAGAACCGAATCCTCTGGACCTTTTTTGGTGC	350	
Cxc	-----ATCG-----GATGAGAACTGGA-CCTC-GGACCTTTTCTTGTG	313	
.			
Cxx	GCCATAACTGGGCCGGCGGAAAAG-TACCGCCCGTACTTTGAGAACTACA	399	
Cxc	GTCATAATTTCGGCTGACGGAAAAGGTACCGCCCGTACTTTGAGAACTACA	363	
.			
Cxx	CAGTGGACGCGAGCATCTTAGATTCGGACCTTCGGGTTCGGATCACAAAA	449	
Cxc	CAGTGGACGCGAGCATCTTAGATTCGAACCTTCGGGTTCGGATCACAAAA	413	
.			
Primer Cxx1			
Cxx	GATGATCATAGTACTTTTCGAGTACTGATTAGATCATTTGGTCAATCTGC-T	498	
Cxc	GATGATCACAGTACTTTTCGAGTACTGATTAGATCATTTGGTCAATCTGCGT	463	
.			
Cxx	C-----ACTTCGGTGA-----GCCGATCAATTCAA-CTCATGTGATT	534	
Cxc	CGAATCTACTTCGATAGAGGAGACGCCGATCGATTCAAATCATGTGATT	513	
.			
Primer L1			
Cxx	-CAAGTTCTAAGAAGCAAACGGTGGATGCCTTG	566	
Cxc	TCAAGTTTCTAAGAGCAAACGGTGGATGCCTTG	546	
.			

Fig. 1. Aligned nucleotide sequences of the L1/G1-primed polymerase chain reaction (PCR) amplification products from the intergenic transcribed spacer region of 16S-23S ribosomal DNA of *Clavibacter xyli* subsp. *xyli* (Cxx) and *C. xyli* subsp. *cynodontis* (Cxc). The sequence of primer G1 (27) and the inverted complimentary sequence of primer L1 (27) are underlined. The sequence of primer Cxx2 and the inverted complimentary sequence of primer Cxx1 are boxed. Base transitions are indicated by a dot underneath the bases, and base transversions are indicated by an asterisk.

quences representing four subspecies of *C. michiganensis* (GenBank Accession No. U09378, U09379 or U09380, U09381, and U09382) (29) and the two *C. xyli* subspecies sequences from this study were aligned using the PC/GENE Program (IntelliGenetics, Mountain View, CA). PCR primers Cxx1 and Cxx2 were designed with the Primer Detective software program (CLONTECH Laboratories, Palo Alto, CA).

Specificity and sensitivity of PCR protocol. Genomic DNA samples from all the bacterial species listed were tested for the specificity of primers Cxx1 and Cxx2. PCR reactions were assembled on ice and started in a preheated (95°C) PTC-100 Programmable Thermal Controller (Model 60, MJ Research, Watertown, MA) with a PCR program of 95°C for 5 min, 40 cycles of 95°C for 1 s, 57°C for 1 s, and 72°C for 30 s, and final extension at 72°C for 5 min. Each 25- μ l volume reaction mixture contained 50 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 0.4 μ M each primer, 0.625 units of *Taq* DNA polymerase (Promega Corp., Madison, WI), and 1 μ l of sample. For sensitivity tests, the concentration of a genomic DNA sample from the local *C. xyli* subsp. *xyli* strain 16296 was determined on a DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA). Tenfold serial dilutions of this DNA sample were prepared in sterile water. A range between 10 fg and 10 ng was tested by PCR.

Detection of *C. xyli* subsp. *xyli* in sugarcane vascular sap. The efficacy of the PCR protocol to detect *C. xyli* subsp. *xyli* in sugarcane vascular sap was compared with the tissue blot immunobinding assay (TB-IBA) (24). Both a tissue and a vascular sap sample were collected from the basal internode of each of 120 sugarcane stalks. The stalks were from plants in a nursery grown from cane that was either inoculated with *C. xyli* subsp. *xyli* (60

stalks) or hot-water treated (60 stalks). The tissue samples for TB-IBA were prepared according to the methods of Harrison and Davis (24), and the sap samples were extracted by positive pressure. Following extraction, the sap samples were stored at -20°C for 2 months. Sap samples were thawed at 37°C and vortexed to resuspend. A 500- μ l aliquot of each sap sample was transferred to a new microfuge tube and spun at 13,450 $\times$ g. The supernatant was decanted, and the pellet was resuspended in the remaining supernatant by vortexing immediately before a 1- μ l aliquot was taken for PCR assay using the same reaction mixture, except that 0.8% (wt/vol) polyvinylpyrrolidone (MW = 360,000) and 0.8% (wt/vol) ficoll (MW = 400,000) also were included.

RESULTS

DNA sequence of the L1/G1-primed PCR products from *C. xyli* subsp. *xyli* and *C. xyli* subsp. *cynodontis*. The nucleotide sequence of one strand of the L1/G1-primed PCR products from *C. xyli*

subsp. *xyli* and the sequence from *C. xyli* subsp. *cynodontis* are shown in Figure 1. The total number of bases for the product from *C. xyli* subsp. *xyli* is 566, of which 134 are adenosine, 139 cytosine, 158 guanosine, and 135 thymine, with a GC content of 52.5%. The total number of bases for the product from *C. xyli* subsp. *cynodontis* is 546, of which 132 are adenosine, 132 cytosine, 152 guanosine, and 130 thymine, with a GC content of 52%. Alignment of the two sequences revealed the following sequence variations: (i) 31 transitions and 12 transversions; (ii) three 1-base, one 3-base, one 4-base, one 12-base, and one 15-base deletion in *C. xyli* subsp. *cynodontis*-derived PCR product; and (iii) four 1-base, one 6-base, and one 7-base deletion in *C. xyli* subsp. *xyli*-derived PCR product (Fig. 1). Overall, the two sequences shared 89.9% sequence identity.

Selection of *C. xyli* subsp. *xyli*-specific PCR primers. From the 566-bp *C. xyli* subsp. *xyli* sequence, Primer Detective software program identified nine 20-mer

Fig. 3. (A) Ethidium bromide-stained polymerase chain reaction (PCR) products following amplification with primers Cxx1 and Cxx2 from 10-fold dilution series of *Clavibacter xyli* subsp. *xyli* genomic DNA. Lane designation: 1 and 11, 100-bp size markers (600, 500, 400, and 300 bp, respectively) (MBI Fermentas, Amherst, NY); 2, positive control using *C. xyli* subsp. *xyli* cells; 3, negative control using sterile water; 4, 10 ng of DNA template; 5, 1 ng; 6, 100 pg; 7, 10 pg; 8, 1 pg; 9, 100 fg; 10, 10 fg. (B) Autoradiograph of Southern blot of gel in panel A probed with a 566-bp ³²P-labeled L1/G1-primed PCR product from *C. xyli* subsp. *xyli* genomic DNA.

Primer Cxx2			
Cxx	51	TTCTAAGGAGCATCTGGC	ACC-CTGTGTTGTTTCAACG
Cxc	51	TTCTAAGGAGCATCTGGC	ACC-CT-----CGGG---TGTC-CAGGCGCCGGATC
Cmi	45	TTNNAAGGAGCATGTG	CACCTCTCCTCTGTAT-----ACAGGGTAGATCAOGGGTGCCAAGTC
Cmn	45	TTCTAAGGAGCATGTG	CACCTCTCCTCTGTAT-----ACAGGG-AGATCACGGNTGCCAAGTC
Cms	29	TTCTAAGGAGCATGTG	CACCTCTCCTCTGTAT-----ACAGGG-AGATCACGGGTGCCAAGTC
Cmm	25	TTCTAAGGAGCATGTG	CACCTCTCCTCTGTAT-----GCAGGG-AGATCAAGGGGTGCCAAGTC

Primer Cxx1			
Cxx	481	ATCATTGGTCAAT-CTG-----CTCACTTCGGT	GAGCCG-----AT
Cxc	445	ATCATTGGTCAAT-CTGCGTCAATCTACTCGATAGAGGAGACGCCGATCGATTCAAATC	505
Cmi	420	ATCATTGGTCAATCTCTG-----TCTCCTCTAGGGGAGGCG-----AA	469
Cmn	418	ATCATTGGTCAATCTCTG-----TCTCCTCTAGGGGAGGCG-----AA	467
Cms	403	ATCATTGGTCAATCTCTG-----TCTCCTCTAGGGGAGGCG-----AA	452
Cmm	398	ATCATTGGTCAATCTCTG-----TCTCCTCTAGGGGAGGCG-----AA	438

Fig. 2. Aligned nucleotide sequences among *Clavibacter xyli* subsp. *xyli* (Cxx), *C. xyli* subsp. *cynodontis* (Cxc), *C. michiganensis* subsp. *insidiosus* (Cmi) (GenBank Accession No. U09378), *C. michiganensis* subsp. *nebraskensis* (Cmn) (GenBank Accession No. U09381), *C. michiganensis* subsp. *sepedonicus* (Cms) (GenBank Accession No. U09382), and *C. michiganensis* subsp. *michiganensis* (Cmm) (GenBank No. U09379 and U09380) around the regions of primers Cxx1 and Cxx2. The inverted complimentary sequence of Cxx1 and the sequence of Cxx2 are in the boxes. Base position numbers shown on the left begin with primer G1 for *C. xyli* subsp. *xyli* and *C. xyli* subsp. *cynodontis*, others were defined by Li and De Boer (29).

primers toward the G1 primer end (data not shown) and one reverse 20-mer primer toward the L1 primer end (Figs. 1 and 2, position 487 to 506 in *C. xyli* subsp. *xyli* strand) for consideration. Upon multiple sequence alignment, the sequences of six of the nine primers toward the G1 end were highly homologous to those of the four subspecies of *C. michiganensis* included in this study (data not shown). Of the remaining three primers (69 to 88, 81 to 100, and 84 to 103), the one from base 69 to base 88 was selected because it spanned the most variable spacer region among these bacterial species (Fig. 2). In this region, a 5-base deletion and an additional thymine were found from the four subspecies of the *C. michiganensis*. Also in this region, there was a 15-base deletion in *C. xyli* subsp. *cynodontis*. In addition, other forms of sequence variation existed among these bacterial species. This primer was designated as Cxx2 with a sequence of 5'

ACC CTG TGT TGT TTT CAA CG. The other reverse primer toward the L1 primer end (487 to 506) was designated as Cxx1 with a sequence of 5' CCG AAG TGA GCA GAT TGA CC.

Specificity of the Cxx1 and Cxx2 primers. As expected, primers Cxx1 and Cxx2 primed the amplification of a 438-bp DNA product from genomic DNA samples of all the 21 *C. xyli* subsp. *xyli* strains. No amplification product was observed from genomic DNA samples of *C. xyli* subsp. *cynodontis*, *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *nebraskensis*, *C. michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *insidiosus*, *R. iranicus*, *R. tritici*, *X. albilineans*, or saprophytic bacteria from sugarcane. In parallel control PCR experiments using universal primers L1 and G1, all the DNA samples produced a PCR product (data not shown).

Sensitivity of the PCR protocol. Using the PCR protocol, the 438-bp PCR product was observable in agarose gels with ethidium bromide staining (0.5 µg/ml) from PCR reactions in which as little as 1 pg of *C. xyli* subsp. *xyli* genomic DNA template was present (Fig. 3A). The detection limit was not improved by Southern blot hybridization with a ³²P-labeled 566-bp L1/G1-primed PCR probe (Fig. 3B).

Detection of *C. xyli* subsp. *xyli* in sugarcane vascular sap. Primers Cxx1 and Cxx2 primed the amplification of the 438-bp DNA product directly from cultured *C. xyli* subsp. *xyli* cells and from 37 of 60 vascular sap samples from stalks grown from *C. xyli* subsp. *xyli*-infected stalks using the PVP- and ficoll-containing PCR reaction mixture (Table 1, examples are shown in Fig. 4). In the comparison of the

PCR protocol with TB-IBA, 34 samples tested positive both by PCR and by TB-IBA, three tested positive by PCR but not by TB-IBA, and seven tested positive by TB-IBA but not by PCR. The RSD bacterium was not detected by either method in 76 samples, including all of the 60 sap samples from stalks of plants grown from hot-water treated stalks.

DISCUSSION

Specific primers and a PCR protocol were developed for the detection of *C. xyli* subsp. *xyli*. Current diagnostic methods for *C. xyli* subsp. *xyli* (4,8–11,21,28,39) are effective late in the growing season when titers of the bacterium are high. In general, the detection limit is 10⁶ CFU/ml by enzyme immunoassay (9), 10^{4–5} by the fluorescent-antibody-direct-count-on-filters technique (11), and 10⁶ by TB-IBA (24). In a related study, about 10⁵ CFU of *X. albilineans* per ml was detected by TB-IBA in 50% of the assays (8). The detection limit for *C. xyli* subsp. *xyli* by our PCR protocol was 1 pg of genomic DNA, either by ethidium bromide staining or by Southern blot hybridization. This was comparable to our PCR protocol for *X. albilineans* (34). Due to the difficulty in titrating *C. xyli* subsp. *xyli* cells because of the bacterium's fastidious nature, sensitivity tests on cells were not successful.

PCR-based detection methods for *Clavibacter* species using primers derived from plasmid-borne genes or genomic DNA have been reported in recent years (3,15,17,18,29,30,35). Dreier et al. (15) developed a PCR procedure using primers originated from a plasmid-borne gene *pat-1* sequence to detect virulent *C. michi-*

Table 1. Detection of *Clavibacter xyli* subsp. *xyli* by polymerase chain reaction (PCR) and tissue blot immunobinding assay (TB-IBA)

Samples	PCR ^a	TB-IBA ^b
34	+	+
7	–	+
3	+	–
76	–	–
Subtotal 120		
Total positive	37	41

^a Sap samples used for PCR were collected from the same stalks from which tissue blots were prepared. + = *C. xyli* subsp. *xyli* detected, – = not detected.

^b Anti-*C. xyli* subsp. *xyli* polyclonal antibodies were used as the primary antibody (Cocalico Biologicals, Reamstown, PA 17567).

Fig. 4. (A) Ethidium bromide-stained polymerase chain reaction (PCR) products following amplification with primers Cxx1 and Cxx2 from sugarcane vascular sap samples (lanes 4 to 23), from cultured *Clavibacter xyli* subsp. *xyli* cells (lane 2), and from sterile water (lane 3). DNA size markers (lanes 1 and 24) are 1,000, 750, 500, and 300 bp, respectively. (B) Autoradiograph of Southern blot of gel in panel A probed with a 566-bp ³²P-labeled L1/G1-primed PCR product from *C. xyli* subsp. *xyli* genomic DNA.

ganensis subsp. *michiganensis* strains in infected tomato tissue. Firrao and Locci (18) employed PCR to identify *C. michiganensis* subsp. *sepedonicus*. Li and De Boer (29) sequenced the 16S-23S ribosomal DNA regions of five subspecies of *Clavibacter* and designed a pair of primers within the ITS region that were highly specific to the subspecies *C. michiganensis* subsp. *sepedonicus*. PCR protocols for the detection of *C. xyli* subsp. *xyli* have been reported by Lopes and Damann (30), Astua-Monge et al. (3), and Fegan et al. (17); all used primers derived from the ITS region of 16S-23S ribosomal DNA. However, Lopes and Damann (30) only used the universal primers L1 and G1, which also prime amplification products from other bacterial species (27). No primer sequence information was reported by Astua-Monge et al. (3) and Fegan et al. (17). While the L1/G1-derived 566-bp DNA probe was specific at the species level, it did not differentiate the two subspecies *C. xyli* subsp. *xyli* and *C. xyli* subsp. *cynodontis*. In contrast, the PCR protocol developed in this study was highly specific to *C. xyli* subsp. *xyli* because primers Cxx1 and Cxx2 were selected from the most variable ITS regions, where *C. xyli* subsp. *cynodontis* and other closely related *Clavibacter* species had either deletions or nonhomologous sequences. These two primers primed the amplification of the expected 438-bp DNA product only from the 21 *C. xyli* subsp. *xyli* strains, including a worldwide collection of 19 strains and two locally isolated strains. The PCR protocol also can be used to differentiate *C. xyli* subsp. *xyli* from *C. xyli* subsp. *cynodontis*, a task that was not possible using serological, biochemical, and DNA hybridization methods (13,14; unpublished). The 89.9% sequence identity between the two L1/G1-primed PCR products from these two subspecies may account for the fact that the 566-bp L1/G1-primed PCR product from *C. xyli* subsp. *xyli* hybridized to the 546-bp PCR product from *C. xyli* subsp. *cynodontis* (unpublished).

Unlike previous reports (3,15,17,29), we found that PCR could be performed directly on *C. xyli* subsp. *xyli* cells from culture and from *C. xyli* subsp. *xyli*-infected sugarcane vascular sap, although a PVP- and ficoll-containing reaction mixture was used when dealing with sap sample (unpublished). A limiting factor in applying the PCR protocol to detect *C. xyli* subsp. *xyli* from sugarcane vascular sap is PCR inhibitors (25,29,30,34). Various reagents have been added to nucleic acid extraction buffer or PCR reaction buffer by different researchers to circumvent the inhibition problem, including PVP (31), polyethylene glycol (PEG) (31), blotto (10% skim milk) (29), BSA (32), and H₂O₂ (1). Diluting (30) or washing (34) field samples before PCR assay may also reduce

inhibitors' effects. In this study, inclusion of 0.8% (wt/vol) PVP (MW = 360,000) and 0.8% (wt/vol) of ficoll (MW = 400,000) in reaction buffer, which are used regularly in DNA hybridization studies, improved the efficiency of PCR assay on vascular sap samples (unpublished). We do not know the exact mechanism, but these high-molecular-weight polymeric matrices may irreversibly adsorb inhibitor compounds (31). However, we feel that even with the addition of these compounds in the reaction buffer, the presence of inhibitors in sugarcane vascular sap may still affect the efficiency of PCR detection. This could be one reason why *C. xyli* subsp. *xyli* was not detected in the seven sap samples tested positive by TB-IBA but not by PCR. The development of a highly specific PCR protocol offers some distinct advantages over other diagnostic methods. Sap samples are more easily transported than stalk tissue samples and can be stored for longer periods of time before processing. The PCR protocol can also have other applications where tissue blots may be inadequate, e.g., other sugarcane tissues, other host plants, soil samples. Experiments are currently being conducted to increase the level of sensitivity of PCR performed directly on sap samples.

In conclusion, a PCR protocol was developed that specifically amplified a 438-bp DNA product from the ITS region of 16S-23S rDNA from *C. xyli* subsp. *xyli*, the causal agent of the sugarcane ratoon stunting disease (12,22,38). In addition, the efficiency of PCR detection in sugarcane vascular sap was improved by the inclusion of PVP and ficoll, two high-molecular-weight polymeric matrices, into the PCR reaction buffer. The PCR protocol can be immediately applicable in many sugarcane diagnostic laboratories to help detect and control one of the most serious sugarcane diseases.

ACKNOWLEDGMENT

We thank Steve M. Brumbly (Bureau of Sugar Experiment Stations, Australia) for providing us with a diverse collection of 19 *C. xyli* subsp. *xyli* strains, Xiang Li (Terragene, Vancouver, Canada) for genomic DNA of five strains of *C. michiganensis* and two strains of *Rathayibacter*, Mary Metzler (University of Turku, Turku, Finland) for genomic DNA of *C. xyli* subsp. *cynodontis* strain Cxc7, Chris A. Clark (Louisiana State University, Baton Rouge) for various *X. albilineans* and other *Xanthomonas* strains, Jeffrey W. Cary, Ken C. Ehrlich, and Barbara A. Triplett (USDA, ARS, Southern Regional Research Center, New Orleans, LA) for DNA sequence analysis, and Kathryn Z. Warnke for technical assistance. This research was partially supported by a competitive grant from the American Sugar Cane League of the U.S.A., Inc., Thibodaux, LA.

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