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Detection and Diversity Assessment of *Xylella fastidiosa* in Field-Collected Plant and Insect Samples by Using 16S rRNA and *gyrB* Sequences

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The causal agent of diseases in many economically important plants is attributed to the xylem-limited bacterium *Xylella fastidiosa*. The detection of this plant pathogen has been hampered due to its difficult isolation and slow growth on plates. Nearly complete nucleotide sequences of the 16S rRNA gene and partial sequences of the *gyrB* gene were determined for 18 strains of *X. fastidiosa* isolated from different plant hosts. A phylogenetic analysis, based on *gyrB*, grouped strains in three clusters; grape-isolated strains formed one cluster, citrus-coffee strains formed another cluster, and a third cluster resulted from all other strains. Primer pairs designed for the 16S rRNA and *gyrB* genes were extensively searched in databases to verify their in silico specificity. Primer pairs were certified with 30 target and 36 nontarget pure cultures of microorganisms, confirming 100% specificity. A multiplex PCR protocol was developed and its sensitivity tested. Sequencing of PCR products confirmed the validity of the multiplex PCR. *Xylella fastidiosa* was detected in field-collected plants, disease vector insects, and nonsymptomatic but infected plants. Specific detection of *X. fastidiosa* may facilitate the understanding of its ecological significance and prevention of spread of the disease.

The insect-transmitted bacterium *Xylella fastidiosa* (38) causes diseases in many economically important plants, including citrus, coffee, grapevine, peach, plum, almond, alfalfa, elm, oak, mulberry, maple, and oleander, by clogging the xylem and affecting water and nutrient transport (26). Control of these diseases, particularly citrus variegated chlorosis and Pierce's disease in grapevines, often relies on management of insect vectors and on inoculum reduction by elimination of diseased and surrounding plants or pruning of infected branches (26). Therefore, early detection and identification of the pathogen is a cornerstone in preventing the spread of disease.

Current methods of detecting the slow-growing microorganism *X. fastidiosa* rely mainly on isolation and cultivation to pure culture, followed by biochemical and serological analysis. These methods are labor- and time-consuming and are complicated by the presence of many contaminant bacteria associated with the plant host (19). Other methods include scanning electron (7, 11), phase-contrast (18), and fluorescence (14) microscopy. Primer sets based on an *Eco*RI restriction (23) and RAPD [random(ly) amplified polymorphic DNA] fragments (25) were reported previously. However, these primers do not target any coding sequence, and to date no coherent phylogenetic analysis of their amplicons has been performed.

Advances in molecular microbial ecology opened avenues for designing taxonomically meaningful highly specific PCR primers (3). The 16S rRNA gene has been widely used for this purpose, providing enough sequence information to allow the analysis of both close and distant phylogenetic relationships among microorganisms (30). However, when the resolution of the 16S rRNA gene-based analysis is too low to make inferences on the relatedness of very closely related microorganisms, an alternative taxonomic marker is needed. The gene encoding the β-subunit polypeptide of the DNA gyrase (*gyrB*) is estimated to evolve much faster than the 16S rRNA gene (35) while still maintaining a high correlation with the total genome homology analyzed by total DNA-DNA hybridization (36) and microarrays (24).

The present study addressed two issues associated with detection of the *X. fastidiosa*: (i) the assessment of the genetic diversity of strains isolated from different hosts through two conserved genes (16S rRNA and *gyrB*) and (ii) the design of primers and development of a multiplex PCR method that allowed specific and sensitive detection of this plant pathogen in small samples of plant and insect material.

Microorganisms included in the present study were purchased from culture collections or donated by other laboratories (Table 1). *X. fastidiosa* strains were grown in PW liquid medium (8), while others were grown in 523 medium (15) at 28°C. DNA isolation was performed with CTAB (cetyltrimethylammonium bromide) buffer according to the method of Doyle and Doyle (9). The DNA concentration was measured with the DyNA QUANT2000 Fluorometer (Amersham Pharmacia Biotech UK, Ltd., Buchinghamshire, United Kingdom).

The 16S rRNA gene was amplified by PCR with eubacterial primers (Table 2) (37). Amplifications were performed in a 50-μl reaction containing 0.2 μM concentrations of each primer, 200 μM concentrations of deoxynucleoside triphosphates, 1× *Taq* buffer, 1.5 mM $MgCl_2$, 2.0 U of *Taq* DNA polymerase (Invitrogen, Inc., São Paulo, Brazil), and 20 ng of DNA template. The PCR was initiated with a 3-min denaturation step at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, extension at 72°C for 2 min, and final extension for 7 min. For the *gyrB*

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TABLE 1. List of 66 bacterial strains used during specificity studies

Species	Strain	Biological origin	Geographic origin	Collection[a]
Xylella fastidiosa	9a5c[c]	*Citrus sinensis* (citrus)	São Paulo, Brazil	INRA
Xylella fastidiosa	CM1[c]	*Coffea arabica* (coffee)	São Paulo, Brazil	CCT 6737
Xylella fastidiosa	CRS1	*Coffea arabica*	São Paulo, Brazil	CCT 6738
Xylella fastidiosa	CRS2[c]	*Coffea arabica*	São Paulo, Brazil	CCT6739
Xylella fastidiosa	P3[c]	*Coffea arabica*	São Paulo, Brazil	CCT 6740
Xylella fastidiosa	6755	*Coffea arabica*	São Paulo, Brazil	CCT 6755
Xylella fastidiosa	MUL-1[c]	*Morus nigra* (mulberry)	Massachusetts, United States	ATCC 35868
Xylella fastidiosa	MUL-3[c]	*Morus nigra*	Massachusetts, United States	ATCC 35869
Xylella fastidiosa	ALS-BC[c]	*Prunus amygdalus* (almond)	California, United States	ATCC 35870
Xylella fastidiosa	PLM G83[c]	*Prunus salicina* (plum)	Georgia, United States	ATCC 35871
Xylella fastidiosa	ELM-1[c]	*Ulmus americana* (elm)	Washington, United States	ATCC 35873
Xylella fastidiosa	RGW-R[c]	*Ambrosia artimeisiifolia* (ragweed)	Florida, United States	ATCC 35876
Xylella fastidiosa	PCE-GG	*Vitis vinifera* (grape)	California, United States	ATCC 35877
Xylella fastidiosa	PWT-22[c]	*Vinca minor* (periwrinkle)	Florida, United States	ATCC 35878
Xylella fastidiosa	PCE-RR[c]	*Vitis vinifera*	Florida, United States	ATCC 35879[T]
Xylella fastidiosa	PCE-FG	*Vitis vinifera*	Florida, United States	ATCC 35881
Xylella fastidiosa	Temecula[c]	*Vitis vinifera*	California, United States	ATCC 700964
Xylella fastidiosa	Fetzer	*Vitis vinifera*	California, United States	B. C. Kirkpatrick
Xylella fastidiosa	Trever	*Vitis vinifera*	California, United States	B. C. Kirkpatrick
Xylella fastidiosa	SL1[c]	*Citrus sinensis*	Minas Gerais, Brazil	CCSM
Xylella fastidiosa	B14[c]	*Citrus sinensis*	São Paulo, Brazil	CCSM
Xylella fastidiosa	U175	*Citrus sinensis*	São Paulo, Brazil	CCSM
Xylella fastidiosa	3000	*Citrus sinensis*	São Paulo, Brazil	CCSM
Xylella fastidiosa	1725	*Citrus sinensis*	São Paulo, Brazil	CCSM
Xylella fastidiosa	11348	*Citrus sinensis*	Parana, Brazil	CCSM
Xylella fastidiosa	11066	*Citrus sinensis*	Parana, Brazil	CCSM
Xylella fastidiosa	11775	*Citrus sinensis*	Rio Grande do Sul, Brazil	CCSM
Xylella fastidiosa	Hib.5	*Hibiscus schizopetalus*	Brasilia, Brazil	CCSM
Xylella fastidiosa	12319	*Catharanthus* sp.	São Paulo, Brazil	IAPAR
Xylella fastidiosa	9746	*Prunus salicina*	Parana, Brazil	IAPAR
Xanthomonas albilineans	Xa13	*Saccharum officinarum*	São Paulo, Brazil	E. Gigliote
Xanthomonas axonopodis pv. *citri*	306	*Citrus sinensis*	São Paulo, Brazil	CCSM
Xanthomonas bromi		*Bromus carinatus*	France	LMG 947[T]
Xanthomonas campestris pv. *campestris*		*Brassica oleracea*	United Kingdom	ATCC 33913[T]
Xanthomonas campestris pv. *vesicatoria*		*Capsicum frutescens*	Brazil	IBSBF 251
Xanthomonas campestris pv. *cassavae*		*Manihot esculenta*	Brazil	IBSBF 270
Xanthomonas campestris pv. *manihotis*		*Manihot esculenta*	Brazil	IBSBF 278
Xanthomonas campestris pv. *citri*		*Citrus sinensis*	Brazil	IBSBF 338
Xanthomonas campestris pv. *malvacearum*		*Malva brasiliensis*	Brazil	IBSBF 555
Xanthomonas campestris pv. *citrumelo*		*Poncirus trifoliata*	Brazil	IBSBF 1011
Xanthomonas campestris pv. *pruni*		*Prunus salicina*	Brazil	IBSBF 1097
Xanthomonas campestris pv. *phaseoli*		*Phaseolus vulgaris*	Brazil	IBSBF 1394
Xanthomonas cordiae		*Codiaeum variegatum*	United States	LMG 8678[T]
Xanthomonas curcubitae		*Curcubita maxima*	New Zealand	LMG 690[T]
Xanthomonas hyacinthi		*Hyacinthus orientalis*	The Netherlands	LMG 739[T]
Xanthomonas melonis		*Cucumis melo*	Brazil	LMG 8670[T]
Xanthomonas translucens pv. *translucens*		*Hordeum vulgare*	United States	LMG 876[T]
Xanthomonas vasicola pv. *holcicola*		*Sorghum bicolor*	New Zealand	LMG 736[T]
Curtobacterium flaccunfaciens		*Citrus sinensis*	Brazil	W. Araújo
Enterobacter cloacae		*Citrus sinensis*	Brazil	W. Araújo
Methylobacterium extorquens		*Citrus sinensis*	Brazil	W. Araújo
Methylobacterium mesophilicum		*Citrus sinensis*	Brazil	W. Araújo
Methylobacterium radiotolerans		*Citrus sinensis*	Brazil	W. Araújo
Methylobacterium zatmanii		*Citrus sinensis*	Brazil	W. Araújo
Pantoea agglomerans		*Citrus sinensis*	Brazil	W. Araújo
Pseudomonas aeruginosa		Clinical material	United States	ATCC 27583
Pseudomonas aureofaciens		River	The Netherlands	ATCC 13985[T]
Pseudomonas chlororaphis		Plate	United States	ATCC 9447
Pseudomonas fluorescens		Rhizosphere	United States	ATCC 17467
Pseudomonas mendocina		Soil	Argentina	ATCC 25411
Pseudomonas putida		Soil	United States	ATCC 12633[T]
Pseudomonas syringae pv. *syringae*		*Triticum aestivum*	Brazil	IBSBF 1022
Agrobacterium tumefaciens		*Chrysanthemum morifolium*	Brazil	IBSBF 710
Candidatus Liberobacter africanus[b]		Citrus		INRA
Erwinia carotovora		*Primula obconica*	Brazil	IBSBF 913
Escherichia coli	DH10B		United States	Invitrogen, Inc.

[a] ATCC, American Type Culture Collection, Manassas, Va.; CCT, Coleção de Culturas Tropical, Fundação André Tosello, Campinas, Brazil; IBSBF, Instituto Biológico, Seção de Bacteriologia Fitopatologica, Campinas, Brazil; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; CCSM, Centro de Citricultura Silvio Moreira, Cordeirópolis, Brazil; E. Gigliote, Universidade Federal de São Carlos, Araras, São Paulo, Brazil; B. C. Kirkpatrick, Departament of Plant Pathology, University of California, Davis, Calif.; W. Araújo, Universidade de São Paulo, ESALQ, Departamento Genetica, Piracicaba, São Paulo, Brazil; INRA, Institut National de la Recherche Agronomique et Université Victor Ségale, Bordeaux, France.

[b] Only genomic DNA was used for analysis due to customs restrictions.

[c] Used for sequence analysis.

TABLE 2. Primer sequences, length, and target sites used in this study

Primer	Oligonucleotide sequence (5′-3′)	Length (no. of bases)	Position	Source or reference
FD1 eubacterial	AGA GTT TGA TCC TGG CTC AG	20	8–28[a]	37
RD1 eubacterial	AAG GAG GTG ATC CAG CC	17	1526–1542[a]	37
S-S-X.fas-0067-a-S-19	CGG CAG CAC ATT GGT AGT A	19	67–85[a]	This study
S-S-X.fas-0838-a-S-21	GCA AAT TGG CAC TCA GTA TCG	21	838–858[a]	This study
S-S-X.fas-0838-a-A-21	CGA TAC TGA GTG CCA ATT TGC	21	838–858[a]	This study
S-S-X.fas-1439-a-A-19	CTC CTC GCG GTT AAG CTA C	19	1439–1457[a]	This study
UP1S	GAA GTC ATC ATG ACC GTT CTG	21	307–329[b]	34
UP2RS	AGC AGG GTA CGG ATG TGC GAG CC	23	1519–1541[b]	34
FXYgyr499	CAG TTA GGG GTG TCA GCG	18	499–516[b]	This study
RXYgyr907	CTC AAT GTA ATT ACC CAA GGT	21	907–928[b]	This study

[a] Positions correspond to the *E. coli* 16S rRNA gene.
[b] Positions correspond to the *X. fastidiosa gyrB* gene.

gene, previously published degenerate PCR primers and thermal cycling conditions were used (Table 2) (34). Cloning and transformation were carried out according to the instructions provided with the pGEM-T Easy Vector System I (Promega Corp., Madison, Wis.). Cloned PCR products were bidirectionally sequenced on an ABI Prism 3100 automatic sequencer with BigDye chemistry (Applied Biosystems, Foster City, Calif.) at the Centro de Energia Nuclear na Agricultura, Universidade de São Paulo (Piracicaba, São Paulo, Brazil). Sequences were assembled and edited in the software Sequencher, version 4.0.5 (Gene Codes Corp., Ann Arbor, Mich.).

Sequences in public databases most similar to the determined *X. fastidiosa* 16S rRNA gene were identified by using the SEQUENCE MATCH and SEQUENCE ALIGN functions of the Ribosomal Database Project II (RDP; www.cme.msu.edu/RDP/html/index.html) (20). The sequences were then aligned against the most similar sequences in the ARB small subunit rRNA database by using the alignment algorithm in the ARB software package (www.mikro.biologie.tu-muenchen.de) (31), and the alignment was adjusted manually based upon elements of primary sequence and secondary structure. The *gyrB* gene sequences alignment was made by using the CLUSTALW program, version 1.7 (32) of the European Bioinformatics Institute (http://www.ebi.ac.uk/index.html). Alignment was visually inspected and manually corrected when necessary. All *X. fastidiosa* strains yielded nearly complete 16S rRNA gene sequences of 1,452 bp with levels of similarity among sequences ranging from 99.0 to 100%. All six coffee and citrus strains revealed only two nucleotide transversions along their 16S rRNA sequences, whereas strains isolated from grape showed five transitions and one transversion when strain PCE-RR was compared to Temecula and PD strains. The 16S rRNA gene, as the standard sequence for phylogenetic positioning in bacterial taxonomy, allowed clear distinction of *X. fastidiosa* from its nearest phylogenetically related species, *Xanthomonas campestris* (33). However, the high levels of sequence similarity among all *Xylella* strains did not allow an effective grouping according to their hosts. Attempts to analyze the genetic relatedness of *X. fastidiosa* strains through sequencing of the 16S rRNA gene (22) and 16S-23S intergenic space region (12, 22) have indicated limited variability that is not consistently clustered with the host. The second gene, the DNA gyrase–β-subunit polypeptide-coding gene (*gyrB*), evolves much faster than rRNAs, allowing synonymous substitutions at the third position of codons. The frequency of base substitution for the *gyrB* gene partial sequences (1,177 bp) was higher than that for the 16S rRNA gene. The similarity levels for *gyrB* sequences varied from 97.5 to 100%.

Phylogenetic trees from nucleotide sequences of both genes (Fig. 1) were constructed by using the neighbor-joining method (27) with genetic distances computed by using the Kimura two-parameter model (16). Phylogenetic trees had slightly different topologies for the 16S rRNA and *gyrB* genes, although three clusters were maintained in both sequence-derived trees. For the *gyrB* gene tree, the distinction was clear, with *Xylella* strains divided into a cluster of citrus and coffee strains with nearly identical sequences (one transition), a cluster of grape strains with three transversions and one transition, and a cluster with all other host-isolated strains (Fig. 1B), agreeing with those results obtained from DNA typing methods such as RAPD (4, 5, 17), Rep-PCR, and pulsed-field gel electrophoresis (12). The branching order of the *gyrB* nucleotide sequence-based tree was very similar to that based on the translated amino acid (GyrB)-based tree (data not shown). The only difference between these trees was the position of the strain Ann1 isolated from oleander. Multilocus sequence typing (21) of housekeeping genes under stabilizing selection, such as *gyrB*, is recommended as an alternative method to evaluate the robustness of clusters during a phylogenetic analysis (29). Whole-genome sequencing projects of other *Xylella* strains will soon become available and other functional genes could be selected for further strain classification at subspecies or pathovar level.

Once the genetic diversity of the plant pathogen *X. fastidiosa* from a diverse range of hosts was evaluated, primers for 16S rRNA gene of *X. fastidiosa* were designed by using the phylogeny of this species, close relatives were inferred by 16S rRNA gene alignment with the ARB software package, and their specificities were tested against over 32,000 16S rRNA sequences in the ARB database. The specificity of each primer was further tested against public databases by using the CHECK PROBE function of the RDP and the basic local alignment search tool (BLAST) program from the National Center for Biotechnology Information (2). The target regions for PCR primers were identified at three different locations: S-S-X.fas-0067-a-S-19 (positions 67 to 85), S-S-X.fas-0838-a-S-21 and S-S-X.fas-0838-a-A-21 (positions 838 to 858), and S-S-X.fas-1439-a-A-19 (positions 1439 to 1457) (Fig. 2A). Primers were named according to the Oligonucleotide Probe database (Table 2) (1). Theoretical hybridization targets of each primer were determined by extensively searching rRNA

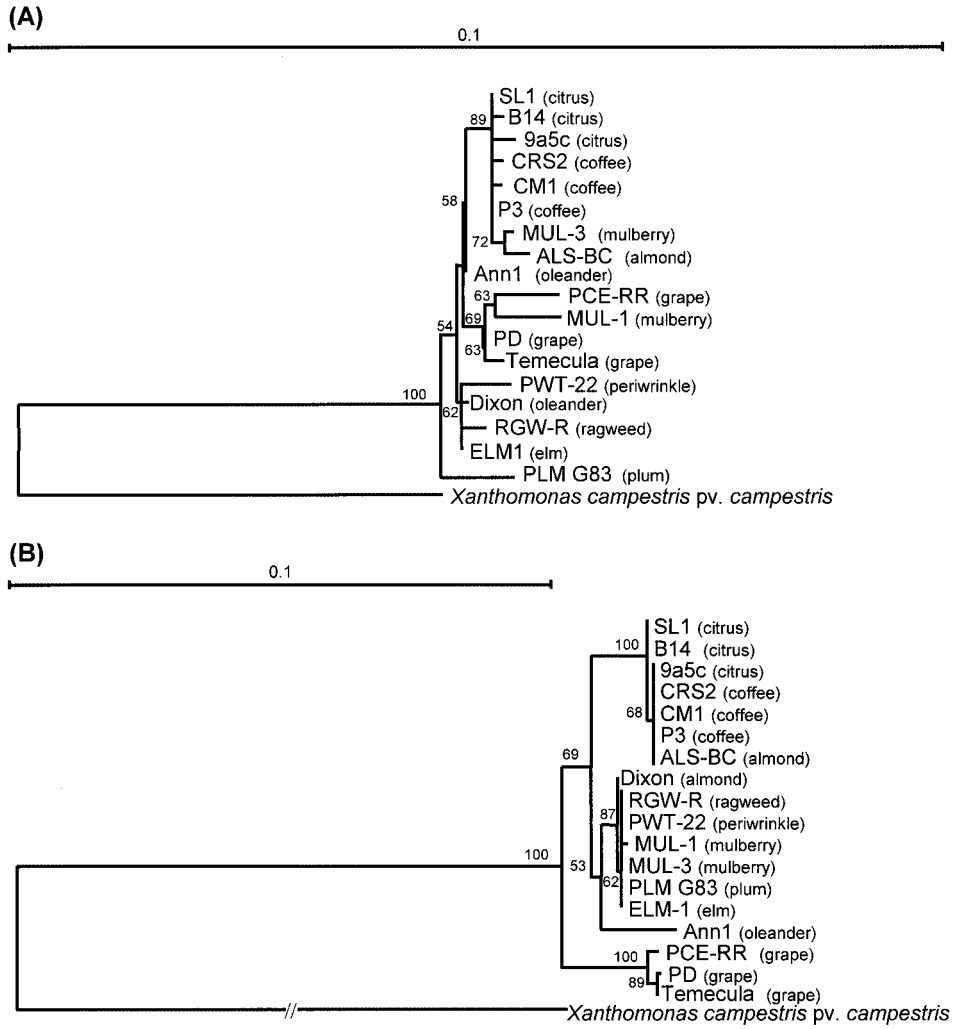

FIG. 1. Phylogenetic trees based on the nucleotide sequences of the 16S rRNA (A) and *gyrB* (B) genes of *X. fastidiosa* strains. Sequences of *gyrB* and 16S rRNA genes were submitted to GenBank under the following first and second accession numbers, respectively. When only one accession number is shown, it represents a *gyrB* sequence: Temecula (AF53460 and AF536760), PCE-RR (AF53461), PLM G83 (AF53462 and AF536761), RGW-R (AF53463 and AF536762), MUL-1 (AF53464), MUL-3 (AF53465 and AF536763), ELM-1 (AF53466 and AF536764), B14 (AF53468 and AF536765), SL1 (AF53469 and AF536766), CRS2 (AF534670 and AF536768), PWT-22 (AF53471), P3 (AF53472 and AF536769), ALS-BC (AF53473 and AF536770), and CM1 (AF53474 and AF536767). Unrooted trees were constructed by using the neighbor-joining method (27). The sequences for *Xanthomonas campestris* pv. *campestris* were treated as the outgroup. All sequences were generated during the present study, except for strains Ann1, PD, and Dixon. The scale is the expected number of substitutions per position. The numbers at the nodes represent percentage bootstrap values of 1,000 resamplings.

sequences (32,000 in all) available at the RDP. The PCR primer S-S-X.fas-0067-a-S-19 was designed by taking in consideration the presence of a stem-loop in the *X. fastidiosa* 16S rRNA secondary structure from positions 67 to 85 considering the *E. coli* numbering system (10). This primer yielded a perfect match for all 11 16S rRNA sequences of *X. fastidiosa* species present in the RDP. When one mismatch was allowed, no sequences were found to match this primer. The primer S-S-X.fas-0067-a-S-19 showed mismatches of two nucleotides for four xanthomonad sequences, *X. melonis*, *X. translucens*, *X. campestris* pv. *campestris*, and *X. albilineans.* Mismatches (positions 77 [T:G] and 84 [T:C]) were located at the central and 3′-end regions of the primer, allowing mismatch discrimination due to the stringency of the PCR conditions. The PCR primers S-S-X.fas-0838-a-S-21 and S-S-X.fas-0838-a-A-21 are reverse complements of each other, being located in the region from positions 838 to 858. Mismatches of one nucleotide were not found in this region. Mismatches of two nucleotides were found for sequences of 20 species belonging to the genus *Xanthomonas* and to *Stenotrophomonas maltophilia*. Mismatches were positioned at the central region of the primer (positions 842 [A:T] and 850 [T:G]). The third primer, S-S-X.fas-1439-a-A-19, is positioned at the end of a long stem-loop and also matches all ribosomal DNA sequences of *X. fastidiosa* species available at the RDP. One nucleotide mismatch (G:A) was found at position 1448 for sequences from *Burkholderia andropogonis*, *Janthinobacterium lividum*, *Pseudomonas lemoignei*, *Mycoplasma putrefasciens*, *Mycoplasma cottewii*, and *Mycoplasma yeatsii.* Specific primers targeting the *gyrB* gene were obtained by aligning 18 sequences from *Xylella* strains from

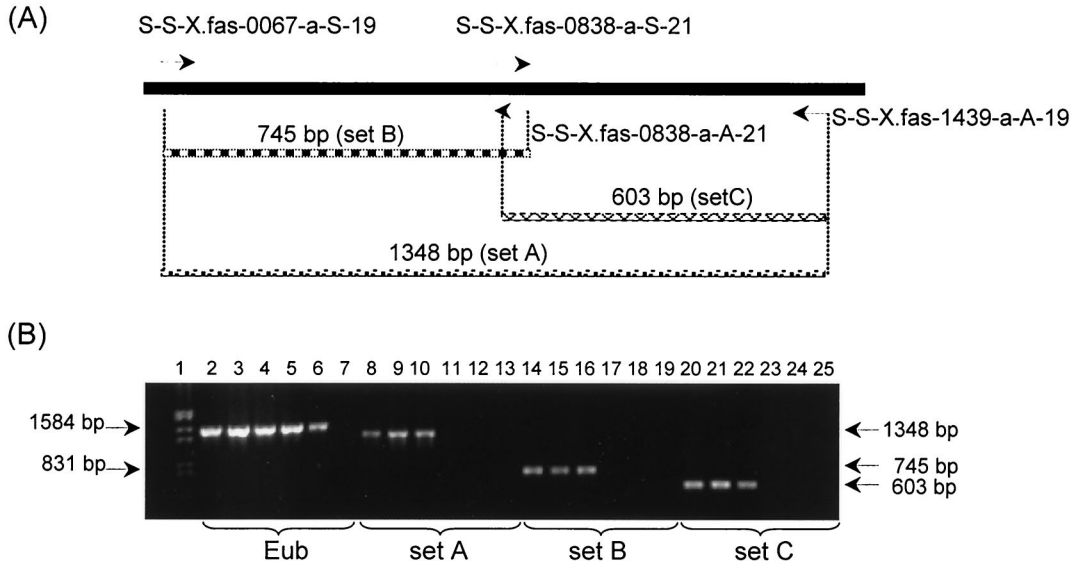

FIG. 2. (A) Scheme of relative position of the 16S rRNA gene-specific primers for *X. fastidiosa.* (B) Ethidium bromide-stained agarose gel of PCR products after amplification of the 16S rRNA gene with eubacterial (Eub) or *X. fastidiosa*-specific (sets A to C) primers. The expected sizes for specific primer sets A, B, and C are 1,348, 745, and 603 bp, respectively. Lanes: 1, molecular marker λ restricted with *Hin*dIII and *Eco*RI; 2, 8, 14, and 20, *X. fastidiosa* strain 9a5c; 3, 9, 15, and 21, strain Temecula; 4, 10, 16, and 22, strain MUL1; 5, 11, 17, and 23, *Xanthomonas campestris* pv. *campestris*; 6, 12, 18, and 24, *Xanthomonas axonopodis* pv. *citri*; and 7, 13, 19, and 25, negative control (H_2O).

different hosts and two downloaded sequences from the National Center for Biotechnology Information for *Xanthomonas campestris* pv. *campestris* and *Xanthomonas axonopodis* pv. *citri*. The forward primer FXYgyr499 with nucleotide positions from 499 to 516 and the antisense primer RXYgyr907 positioned at nucleotides 907 to 928 were synthesized (Table 2). Public database searches of these primers found no other target sequences than the *X. fastidiosa gyrB* gene, confirming their theoretical specificity.

The specificity of the *X. fastidiosa* 16S rRNA gene-targeted primers combined in pairs (sets A, B, and C) was experimentally tested with 30 *X. fastidiosa* isolates from different plant hosts and with 36 phylogenetically related microorganisms, endophytes, or plant pathogens (Table 2). When the PCR primer set A was used (Fig. 2A), a product of 1,348 bp was amplified only for *X. fastidiosa* DNA samples (Fig. 2B). In contrast, no PCR amplification was observed for any other species (Fig. 2B shows the results obtained with only two xanthomonads, but all 36 closely related isolates were tested). All isolates tested positive with 16S rRNA gene eubacterial primers, indicating that the DNA was suitable for amplification. The same results were observed with primer sets B and C, yielding PCR amplification products of 745 and 603 bp, respectively (Fig. 2B). PCR products from all primer combinations showed sizes corresponding to those expected from in silico analysis. The specificity of PCR amplification was also confirmed by subsequent partial sequencing of 10 randomly chosen PCR products, followed by RDP and GenBank database searching, yeielding 99% similarity matches to the *X. fastidiosa* 16S rRNA gene. Specific primers developed against the *gyrB* gene were also tested with DNA isolated from all strains (Table 2). Amplification products of the expected size (429 bp) were obtained only when *X. fastidiosa* DNA was used.

Experimental validation of different primer combinations with pure cultures of 66 strains (Table 1) attested to their specificity toward *X. fastidiosa*. One could use any selected combined primer pair for detecting this plant pathogen. Likewise, the partially sequenced *gyrB* gene allowed the design of a suitable PCR primer pair. The similarity of *gyrB* sequences of *X. fastidiosa* and its closest related species, *Xanthomonas campestris* pv. *campestris*, was low (73%); thus, the best primer pair was easily selected for differentiating these microorganisms. This pair was as specific as the 16S rRNA-targeting primers with the same pure cultures (data not shown).

Primers developed for the 16S rRNA and *gyrB* genes were combined for the development of a multiplex PCR protocol. Multiplex PCR conditions were maintained as described above with 0.2 and 0.4 μM concentrations of each 16S rRNA and *gyrB* primer, respectively. To test whether *X. fastidiosa* could be detected in a background of plant DNA, serial 10-fold dilutions of genomic DNA were mixed with citrus DNA and were PCR analyzed. The multiplex PCR with 16S rRNA and *gyrB*-specific primers yielded visible bands on ethidium bromide-stained agarose gels when as little as 2.9 pg from *Xylella* DNA was used (data not shown). This amount corresponds to 10^2 cells, calculated according to its genome size. When a nested multiplex PCR was used, the detection level had a 10-fold increase in sensitivity (10 cells), yielding visible PCR bands on an agarose gel. Increasing amounts of citrus DNA, up to 40 ng, did not interfere with detection, but a weak background smear was seen on the gel lane. The sensitivity of the multiplex PCR accords with that described for single PCR protocols developed for other plant pathogens, with a detection limit of 10^2 cells (23) or of 2.9 pg of target DNA (6). Because amplifications in a multiplex PCR compete for a limited amount of nucleotides and *Taq* polymerase, an efficient set of primers might influence the result of a less efficient one and thus the sensitivity of the protocol. Uneven amplifications were cor-

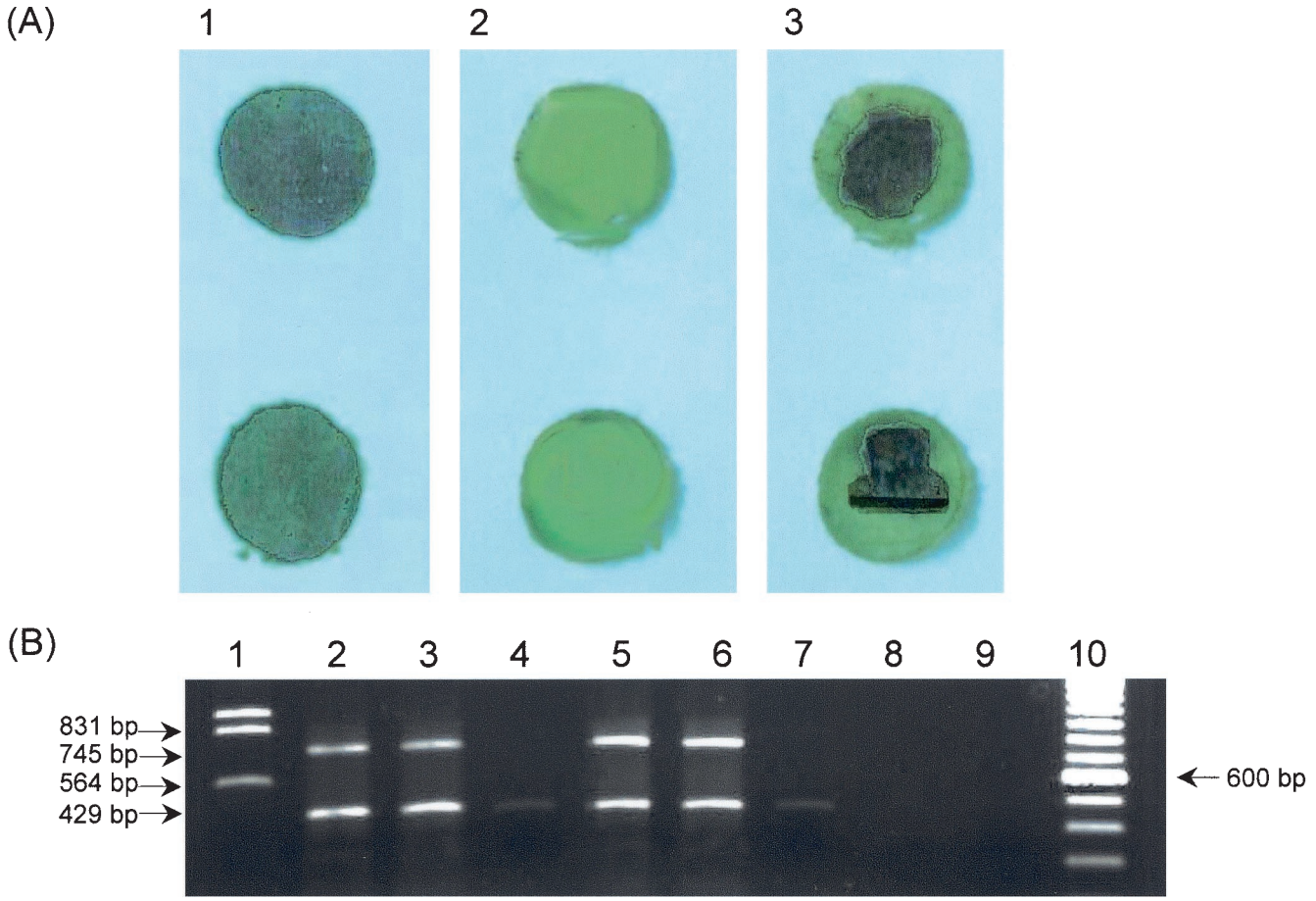

FIG. 3. Sensitivity of *X. fastidiosa* detection by multiplex PCR. (A) Leaf disks of a known diseased citrus plant used for DNA extraction. Subpanels: 1, nonsymptomatic area; 2, chlorotic area; 3, necrotic area. (B) Ethidium bromide-stained gel of coamplified PCR products from citrus leaf disks. The expected sizes are 745 and 429 bp for 16S rRNA (set B)- and *gyrB*-specific primers, respectively. Lanes: 1, molecular marker λ restricted with *Hin*dIII and *Eco*RI (947, 831, and 564 bp); 2 and 5, DNA extracted from nonsymptomatic area; 3 and 6, chlorotic areas; 4 and 7, necrotic area; 8, known healthy plant; 9, negative control (H_2O); 10, molecular marker (100-bp ladder).

rected by adding a twofold concentration of *gyrB*-specific primers. A direct explanation for this result is the presence of only one *gyrB* gene in the *X. fastidiosa* genome, whereas two copies of the 16S rRNA are found (28).

To test whether *X. fastidiosa* could also be detected from any part of a leaf tissue of a diseased citrus plant, leaf disks (7 mm in diameter) from nonsymptomatic, chlorotic, and necrotic areas were collected (Fig. 3A), and DNA was extracted and PCR tested. Multiplex detection with 16S rRNA primer set B and *gyrB* primers was obtained for nonsymptomatic and chlorotic leaf areas, but only a faint PCR product band for *gyrB*-targeted primers was observed with DNA extracted from necrotic leaf disks (Fig. 3B). These results suggest that phenolic compounds released during plant cell death inhibited the PCR or that target bacterial DNA recovery was insufficient for its detection.

In order to validate the specific primers in plant and vector insect samples, we tested known diseased citrus plants from four orchards. Leaves were randomly collected from plants with or without symptoms from four different citrus orchards (cities of Gavião Peixoto, Neves Paulista, Paraíso, and Santa Rita do Passa Quatro) and one plum orchard (city of Itapetininga) in the State of São Paulo, Brazil. The leaves were kept in labeled humid dark plastic bags and transported on ice to the laboratory (2 to 4 h until delivery time). Samples were kept in a cold room (10°C) for 1 day before DNA extraction. Plant DNA extraction was performed with CTAB buffer as described above. All citrus samples were determined to be positive in the multiplex PCR assay (data not shown). We also performed a blind experiment by asking a farmer to collect samples from a plum orchard thought to be contaminated with *X. fastidiosa*. PCR bands were present in 5 of 10 samples (data not shown). It was later confirmed that five of the positive samples were collected from trees with symptoms of the disease, whereas the others were sampled from nonsymptomatic plants. Sequencing of PCR products confirmed the specificity obtained by the molecular method, attesting to the utility of the protocol for detecting the pathogen in other economically important plants or reservoir hosts. Furthermore, insect DNA samples (L. P. Ciapina and E. G. M. Lemos, First *Xylella fastidiosa* Funct. Genomics Symp., poster abstr. 1, 2001) from four different species known as vectors of *X. fastidiosa*, namely, *Bucephalogonia xanthophis*, *Dilobopterus costalimai*, *Parathona gratiosa*, and *Acrogonia citrina*, that had been fed for 2 days in a citrus plant

infected with the pathogen yielded positive results only after a second round of PCR amplification. No PCR amplification occurred when DNA extracted from healthy laboratory-reared insects fed on noninfected plants was used as a template (data not shown). The need of a nested PCR for insect samples might be due to the low number of *X. fastidiosa* cells in the vector, as previously observed by culturing and CFU quantification (13). Nevertheless, a nested PCR assay with these primers would be very useful for epidemiological studies, as well as for finding new potential vectors in several crops in which *X. fastidiosa* causes diseases, since previously reported set of primers allowed detection through nested PCR only for citrus strains of *X. fastidiosa* (25).

Because the current disease control strategy is based mainly on the eradication of plants and branch pruning when symptoms have already manifested, a molecular method could greatly enhance orchard management by certifying nursery trees free of the pathogen, by chemical control of insect vectors above a significant number of positive results, and by random analysis of trees and plants avoiding the spread of disease.

The present study addressed the diversity, detection, and diagnosis of *Xylella fastidiosa*. Phylogenetic meaningful specific primer sets for the 16S rRNA or *gyrB* genes could be used as single or multiplex PCR for studying this plant pathogen and its ecological role in other host plants and still unknown vector insects.

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