

## Development of a Polymerase Chain Reaction Protocol for Detection of *Xylella fastidiosa* in Plant Tissue

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### ABSTRACT

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A 7.4-kb *EcoRI* fragment of genomic DNA of *Xylella fastidiosa* strain PCE-RR (ATCC 35879) was used as a probe and was conserved in 18 strains of *Xylella*. The nucleotide sequence of a 1.0-kb internal *EcoRV* portion of the fragment was determined, and oligonucleotides were selected for primers that amplified genomic DNA specific to *X. fastidiosa* in 33 strains tested by the polymerase chain reaction (PCR). Plant extracts for PCR and enzyme-linked immunosorbent assay (ELISA) were obtained by maceration of grape petioles and by vacuum extraction of citrus stems.

Known cell numbers of *X. fastidiosa* were added to the plant extracts contained in a succinate-citrate-phosphate buffer prior to assay. Amplification of DNA by PCR was inhibited in the presence of plant extracts unless sodium ascorbate and acid-washed polyvinylpyrrolidone were added to the extraction buffer. Detection of *Xylella* by PCR was 100-fold more sensitive than by ELISA; the limits of detection were  $1 \times 10^2$  cfu/ml for PCR and  $2 \times 10^4$  cfu/ml for ELISA. Restriction endonuclease digestion of PCR amplification products with *RsaI* differentiated two pathotypes of *X. fastidiosa*.

*Additional keywords:* citrus variegated chlorosis, Pierce's disease of grapevine, plum leaf scald.

*Xylella fastidiosa* Wells et al (21), a gram negative, xylem-limited bacterium, is responsible for economic losses in many agriculturally important plants, including grapevine, peach, plum, and citrus (9). Pierce's disease of grapevine, for example, limits the commercial production of bunch grapes in the southeastern United States (7). *X. fastidiosa* also causes leaf scorch and declines in many urban shade trees, including elm, sycamore, oak, and maple (18). It also could be involved in some of the current forest decline problems that are of unknown etiology.

Certain strains of *X. fastidiosa* appear to have very wide host ranges; for example, strains that cause Pierce's disease of grapevine were shown to infect at least 28 families of monocotyledonous and dicotyledonous plants (3). However, many of the hosts are symptomless, and in some of these hosts, the populations of *X. fastidiosa* were low and difficult to detect. Strains of *X. fastidiosa* can be grouped into two or more pathotypes (2,12). Strains that cause Pierce's disease are genetically uniform, whereas those from various tree hosts are diverse. Sensitive detection methods and techniques to distinguish the pathotypes are needed for epidemiological and ecological studies of this bacterium.

Current methods of detection used in surveys for *X. fastidiosa* include electron, phase contrast, and fluorescence microscopy; culture of the bacterium; and enzyme-linked immunosorbent assay (ELISA) (4,10,18). However, microscopy is slow and inefficient and limited with respect to the amounts of host tissue that can be observed; culturing is very slow and inconsistent with some hosts and strains of the bacterium; and the limit of sensitivity of ELISA prohibits the detection of low numbers of cells. Recently, amplification of pathogen-specific DNA sequences by the polymerase chain reaction (PCR) has been used in highly sensitive methods for detection of various pathogens in their plant hosts (5).

Our objectives were to develop a sensitive and specific detection protocol for *X. fastidiosa* by using the PCR amplification of

specific DNA sequences and to compare the sensitivity and utility of this detection system with ELISA. Restriction endonuclease digestion of the PCR products was also evaluated for use in the differentiation of pathotypes of the bacterium.

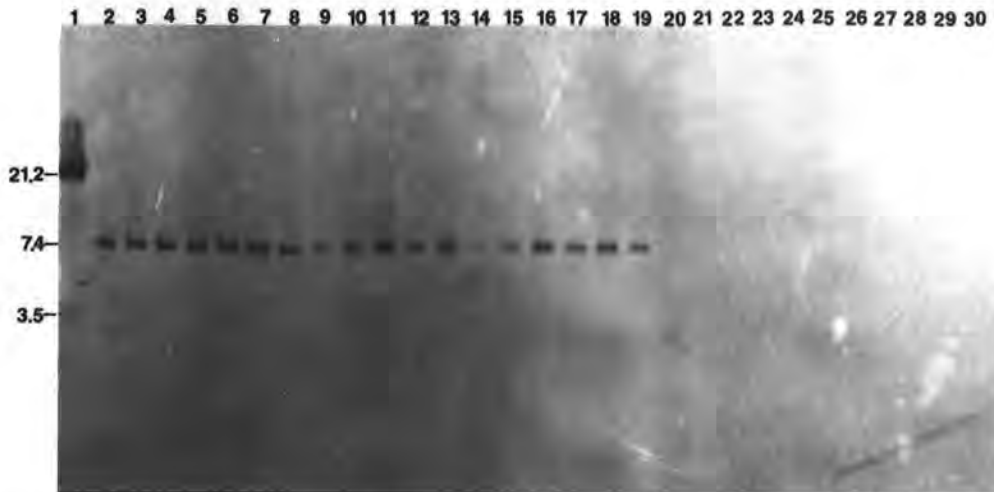
### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Strains of *X. fastidiosa* belonging to the Pierce's disease (PD) group, PD-1r, PD-1FD, PD-4, PD88-1M, PD91-1, PD92-4, PCE-FG (ATCC 35881), Grape H, PD92-3, PD92-9, PD88-5A, PCE-RR (ATCC 35879), and CB-9 (isolated from citrus), were maintained in PD3 or buffered charcoal-yeast extract (BCYE) medium (8) at 28 C. Other strains, PLM-G83 (ATCC 35871, plum), 83-G1 (goldenrod), O88-9 (oak), OAK (ATCC 35874, red oak), SYC86-1 (sycamore), and SYC89-A (sycamore), were maintained in BCYE or periwinkle wilt (PW) medium (8) at 28 C. An additional group of Brazilian strains that had been isolated from citrus trees with citrus variegated chlorosis (CVC) (IAPAR [Instituto Agronômico do Paraná, Londrina, PR, Brazil] 9712, IAPAR 9713, IAPAR 9714, IAPAR 9766, IAPAR 10437, and IAPAR 10438), from plums with leaf scald (IAPAR 9746, IAPAR 9748, IAPAR 9765, IAPAR Coeur de Lion, IAPAR Santa Rita, IAPAR Santa Rosa, and IAPAR Rosada de Camelati), and from grapevine with Pierce's disease (Instituto Biológico Seção Bactérias Fitopatogênicas [IBSBF] 755) were maintained in BCYE at IAPAR. The DNA of these strains was extracted in Brazil and used in tests in Florida. Strains were routinely subcultured every 14 days. Long-term storage was at -70 C in PD3 broth plus 30% glycerol for PD strains and in PW broth plus 30% glycerol for all other strains. Strains of *X. fastidiosa* used for DNA extractions were grown in PW broth for 7 days on a rotary shaker at 28 C. Various bacteria used in probe and primer specificity tests (Figs. 1 and 2) were grown in nutrient broth for 12-16 h on a rotary shaker at 30 C for DNA extraction.

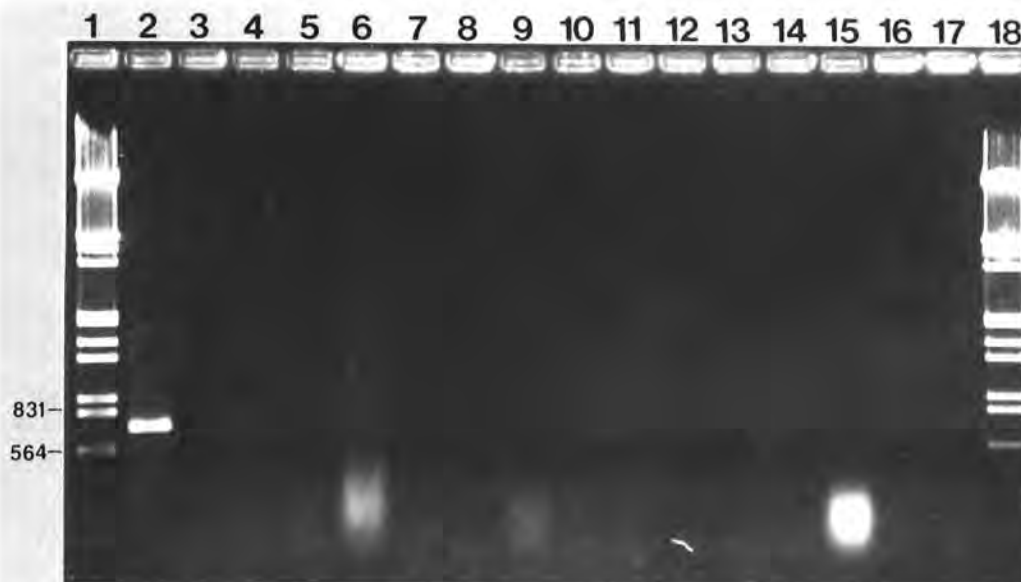
**Cloning, Southern hybridization, and DNA sequencing.** Genomic DNA was obtained from broth cultures by the CTAB (cetyltrimethylammonium bromide) extraction method (1). Restriction endonuclease digestions, agarose gel electrophoresis, and cloning of DNA fragments were performed by standard procedures (14). DNA fragments that were conserved in strains of *X. fastidiosa* were purified from agarose gels by the freeze-squeeze method (20). These fragments were cloned into pLAFR3 (19) or pBluescript 11 KS +/- (Stratagene, La Jolla, CA) for labeling or sequencing experiments. Plasmid clones were trans-

formed into *Escherichia coli* strain DH5 $\alpha$  (Bethesda Research Laboratories, Gaithersburg, MD) for routine maintenance.

Southern hybridizations (14) were performed by labeling linearized plasmid DNA or PCR product DNA with the Genius non-radioactive DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and probing against target DNA immobilized on Nytran membranes (Schleicher and Schuell, Keene, NH). DNA fragments cloned into the polylinker of pBluescript were sequenced by the dideoxy method with Sequenase T7 DNA polymerase (United States Biochemicals, Cleveland,



**Fig. 1.** Southern hybridization assay in which labeled DNA of plasmid pX10-2 was used as a probe against *Eco*RI-digested genomic DNA from various strains of *Xylella fastidiosa* and other bacteria. Lane 1, *Eco*RI + *Hind*III-digested  $\lambda$  DNA; lanes 2-19, *X. fastidiosa* strains PD-4, PD-1r, PD-1FD, PD88-1M, PD91-1, PD92-4, PCE-FG (ATCC 35881), Grape H, PD92-3, PD88-5A, PCE-RR (ATCC 35879), CB-9, PLM-G83 (ATCC 35871), 83-G1, 088-9, OAK (ATCC 35874), SYC86-1, and SYC89-A, respectively; lane 20, *Xanthomonas campestris* pv. *vesicatoria* strain XV56; lane 21, *X. c. pruni* strain FLA-1; lane 22, *X. c. phaseoli* strain 85-6; lane 23, *X. fragariae* strain GC6265; lane 24, *X. maltophilia* (= *Stenotrophomonas maltophilia* [16]) strain ASM-1; lane 25, *Xanthomonas* sp. (saprophyte) strain T-55; lane 26, *Pseudomonas syringae* pv. *syringae* strain INB; lane 27, *P. s. tomato* strain 987; lane 28, *P. solanacearum* strain GMI 1000; lane 29, *Erwinia stewartii* strain SW2; and lane 30, *Agrobacterium tumefaciens* strain I050. Molecular weights of markers are given in kilobase pairs.



**Fig. 2.** Agarose gel electrophoresis of the products from the polymerase chain reaction amplification (primers RST31/RST33) of DNA from various bacteria (R. E. Stall collection). Target DNA (100 ng) sources: lane 2, *Xylella fastidiosa* strain PCE-RR (ATCC 35879); lane 3, *Xanthomonas campestris* pv. *vesicatoria* strain 75-3; lane 4, *X. c. campestris* strain ATCC 33913; lane 5, *X. maltophilia* (= *Stenotrophomonas maltophilia* [16]) strain ASM-1; lane 6, *Xanthomonas* sp. (saprophyte) strain T-55; lane 7, *Acidovorax avenae* subsp. *citruilli* strain W3; lane 8, *Pseudomonas pseudoalcaligenes* strain ATCC 29625; lane 9, *P. syringae* pv. *syringae* strain INB; lane 10, *P. solanacearum* strain K60; lane 11, *P. putida* strain PR300; lane 12, *Erwinia stewartii* strain SW2; lane 13, *E. carotovora* strain KSR347; lane 14, *E. herbicola* strain NF33; lane 15, *Escherichia coli* strain HB101; lane 16, *Agrobacterium tumefaciens* strain I050; and lane 17, *Clavibacter michiganense* strain 75-1. Lanes 1 and 18 contain 0.5  $\mu$ g of *Eco*RI + *Hind*III-digested  $\lambda$  DNA. Molecular weights of markers are given in base pairs.

OH). Oligonucleotide primers used in PCR amplification tests were synthesized with a 394 DNA synthesizer (Applied Biosystems, Foster City, CA) at the University of Florida, ICBR Facility, Gainesville. Primers synthesized were RST29 (5'-GC-AACAAACAACGAACAAAGGCAATGC-3'), RST30 (5'-GTG-TAAGTTGAACACCGTCGTGG-3'), RST31 (5'-GCGTTAA-TTTTCGAAGTGATTCGATTGC-3'), RST32 (5'-CGAAC-AAAGCAATGCCGC-3'), and RST33 (5'-CACCATTTCG-TATCCCGGTG-3').

**Preparation of plant samples for ELISA and PCR.** Rooted cuttings of grapevine, *Vitis vinifera* 'Carignane,' were grown in the greenhouse at 28–33 C during the day and 20–25 C at night. Plants with symptoms of leaf marginal necrosis were produced by needle inoculation of stems (8) with a PD strain of *X. fastidiosa*. Petioles and leaf veins (1.0 g) from PD-inoculated, naturally infected, or noninfected plants were ground with a mortar and pestle in 5 ml of sterile distilled water or sample buffers and filtered through cheesecloth to remove plant debris. Sample buffers were either succinate-citrate-phosphate (SCP) (disodium succinate, 1.0 g/L; trisodium citrate, 1.0 g/L; K<sub>2</sub>HPO<sub>4</sub>, 1.5 g/L; and KH<sub>2</sub>PO<sub>4</sub>, 1.0 g/L; pH 7.0) or SCPAP (SCP, 0.02 M sodium ascorbate, and 5% acid-washed insoluble polyvinylpyrrolidone [PVPP; Sigma Chemical Company, St. Louis, MO]) (6,17).

In some experiments, known concentrations of *X. fastidiosa* were added to the sample buffer at an early stage in the extraction procedure. Suspensions of bacteria in buffer from 4- to 6-day-old cultures were standardized to an optical density of 0.25 (10<sup>7</sup>–10<sup>8</sup> cfu/ml) at A<sub>600</sub> nm with a spectrophotometer. Suspensions were added to the mortar containing petioles and leaf veins from noninfected plants and then ground with a pestle.

Xylem fluid samples were extracted from naturally infected or noninfected rough lemon (*Citrus jambhiri* Lush.) citrus rootstock seedlings. Stem segments (4–12 mm in diameter and 2–3 cm long) were vacuum extracted (4) with SCP buffer or SCP buffer containing sodium ascorbate. In some instances, standardized bacterial suspensions were added to the extract before the samples were assayed by ELISA or PCR.

For ELISA, the plant sample extracts in SCP or SCPAP were diluted serially (10-fold) in the respective buffers containing noninfected plant tissue prior to dilution (1:1) in a 2× concentration of ELISA extraction buffer (15). For PCR, DNA was extracted from aliquots of the samples diluted for ELISA. The actual concentrations of *X. fastidiosa* in the samples were determined by dilution plating on PD3 medium.

**ELISA.** The ELISA procedure was similar to that previously described (10,15) with slight modifications. Flat-bottom microtiter plates were coated with gamma globulin by incubation for 4 h at room temperature (25 C). The plant extracts were added to the wells, and the plates were incubated overnight at 6 C. The wells were washed, alkaline phosphatase conjugated antibody was added, and the plates were then incubated at room temperature for 6 h. The enzyme substrate (*p*-nitrophenyl phosphate, 1 mg/ml) was allowed to react for 30 min, and then the reactions were terminated by adding 50 µl of 3 M NaOH per well. Plates were read at A<sub>405 nm</sub> in a microplate auto reader (Bio-Tek Instruments Inc., Winooski, VT). A suspension of 10<sup>5</sup> cfu/ml of a PD strain of *X. fastidiosa* in ELISA extraction buffer was used as a positive control. A mean absorbance greater than the mean of the negative control wells plus four times the standard deviation was determined to be a positive reaction.

**PCR amplification.** PCR amplification of DNA was done in a DNA thermocycler (M. J. Research, Watertown, MA) with *Taq* DNA polymerase (Promega, Madison, WI). Individual PCR samples (50 µl) contained 1× amplification buffer (supplied by the manufacturer with the enzyme), 100 µM of each dNTP, 50 µM of each primer, 1.25 U *Taq* DNA polymerase, and either 100 ng of purified *X. fastidiosa* genomic DNA in 4 µl of TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) or 4 µl of plant sample extract. Oligonucleotide primers, dNTPs, 10× amplification buffer, and enzyme were diluted in sterile distilled water on ice to prepare a mastermix solution for each PCR experiment. Individual reactions were assembled on ice by the addition of

46 µl of mastermix solution to 4 µl of sample in sterile 500-µl microcentrifuge tubes. Each reaction was overlaid with 50 µl of molecular biology-grade mineral oil (Sigma) before PCR.

In preliminary experiments, attempts were made to extract DNA suitable for PCR amplification from plant samples by direct boiling in sterile water, by lyse and boil methods (11,13), and by standard DNA isolation procedures (1). Subsequently, DNA was extracted from aliquots of the plant samples prepared for ELISA by the CTAB method with slight modification. Briefly, 400 µl of lysing solution (TAE buffer containing 0.75% sodium dodecyl sulfate and 0.15 mg of freshly dissolved Proteinase K per milliliter) was gently mixed into 200 µl of ELISA sample in 1.5-ml microcentrifuge tubes. The tubes were incubated at 37 C for 1.5 h. Next, 100 µl of 5 M NaCl and 80 µl of CTAB-NaCl solution (4.1% NaCl and 10% CTAB) prewarmed to 65 C were added. The tubes were inverted several times and incubated at 65 C for 10 min. Samples were extracted once with an equal volume of chloroform-isoamyl alcohol (24:1) followed by one extraction with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous solution by the addition of 0.6 volume of isopropanol and incubation of the tubes at –70 C for 15 min. The tubes were centrifuged for 20 min at room temperature, and the pellets were washed in 70% ethanol and recentrifuged for 15 min. The DNA pellets were redissolved in 50 µl of 1× PCR buffer containing 0.5% Tween 20 (Sigma), and the solutions were boiled for 10 min before samples were removed for PCR. DNA preparations were stable when stored at 4 C.

For PCR amplification experiments, a 733-bp region of *X. fastidiosa* genome was amplified with the primer set RST31/RST33. Template DNA was initially denatured in the thermocycler at 95 C for 1 min. This was followed by 40 PCR cycles of denaturation at 95 C for 30 s, primer annealing at 55 C for 30 s, and DNA extension at 72 C for 45 s. A final cycle included the denaturation and annealing steps followed by extension at 72 C for 5 min before cooling to 4 C. The PCR products were extracted once with 25 µl of chloroform-isoamyl alcohol before aliquots (typically 15 µl) containing gel tracking dye were added to wells of 1% agarose gels in TAE buffer (14). The gels were run at 5 V/cm for 1.5 h (5 cm) and then stained with ethidium bromide (10 mg/ml) for 20–30 min. The PCR products were photographed in gels on a UV transilluminator with Polaroid type 55 film (Polaroid Corp., Cambridge, MA).

**Restriction endonuclease digestion of PCR products.** Aliquots (5 µl) of individual PCR products were digested in 10 µl total volume reactions containing 3 µl of TAE buffer, 1 µl of enzyme (8–12 U), and 1 µl of 10× restriction buffer (supplied by the manufacturer, Promega or Sigma). Digestions were incubated at 25 or 37 C, dependent on the enzyme, for 2–15 h. The digestions were terminated by the addition of 1 µl each of 250 mM EDTA, pH 8.0, and gel tracking dye. Samples were heated to 65 C for 5 min and placed in ice before being loaded into wells in 3:1% NuSieve-GTG agarose gels (FMC BioProducts, Rockland, ME). Gels were run in TAE buffer at 5 V/cm for 2 h. The gels were stained for 30 min in ethidium bromide, destained in 1 mM MgSO<sub>4</sub> for 30 min, and then photographed. Enzymes used were *AluI*, *AlwI*, *CfoI*, *DraI*, *EcoRI*, *EcoRV*, *HaeIII*, *HindIII*, *HinfI*, *HpaII*, *PstI*, *RsaI*, *Sau3AI*, *SpeI*, *TaqI*, *Tru9I*, and *XbaI*.

## RESULTS

***X. fastidiosa*-specific probes.** Individual random *Bam*HI, *Eco*RI, and *Hind*III DNA digestion fragments were visually assessed as being present in genomic digests of 18 strains of *X. fastidiosa*. Several conserved fragments were cloned from Pierce's disease strain PCE-RR (ATCC 35879) and individually labeled for use as probes. A 7.4-kb *Eco*RI fragment (clone pX10-2) hybridized to all strains tested and was selected for further evaluation (Fig. 1). This fragment did not hybridize to genomic digests of *Agrobacterium tumefaciens*, *Erwinia stewartii*, *Pseudomonas solanacearum*, several *P. syringae* pathovars, saprophytic *Xanthomonas* sp., *X. maltophilia* (= *Stenotrophomonas maltophilia* [16]), *X. fragariae*, or various *X. campestris* pathovars.



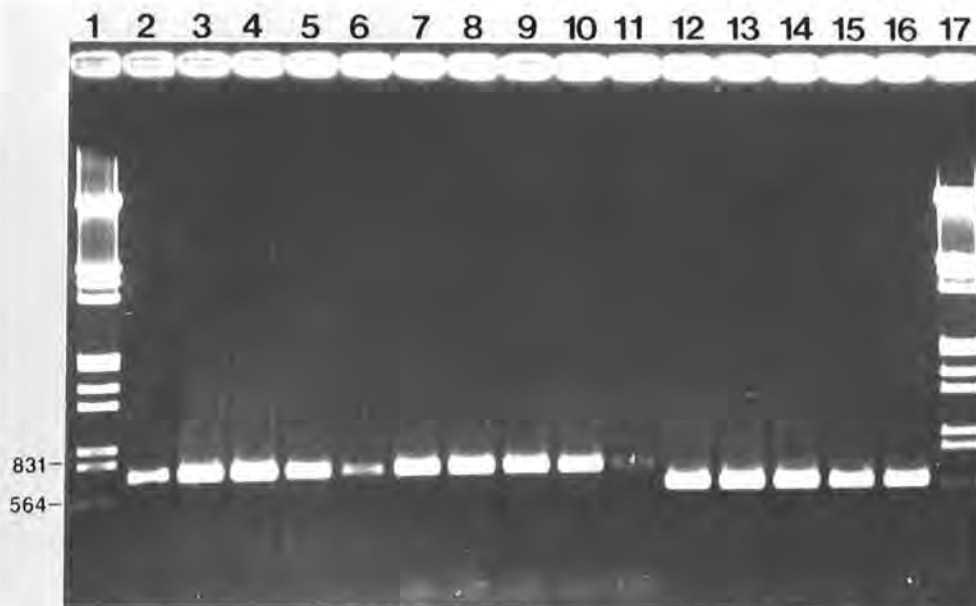
Digestion of pX10-2 with *EcoRV* generated an internal 1-kb DNA fragment, which when labeled and used as a probe against *EcoRV* genomic digests of all strains of *X. fastidiosa*, hybridized without polymorphisms. On the basis of the conserved nature and suitable size for PCR amplification, this 1-kb fragment was sequenced to obtain potential primer oligonucleotide sequences.

**Selection of PCR primers specific for *X. fastidiosa*.** Initially, five oligonucleotide primers were synthesized, which would, in pair combinations, amplify three DNA regions of 600, 720, or 733 bp from the genome of *X. fastidiosa* (Fig. 3). The expected fragment sizes for each primer set were successfully amplified by PCR from 33 diverse strains of *X. fastidiosa*.

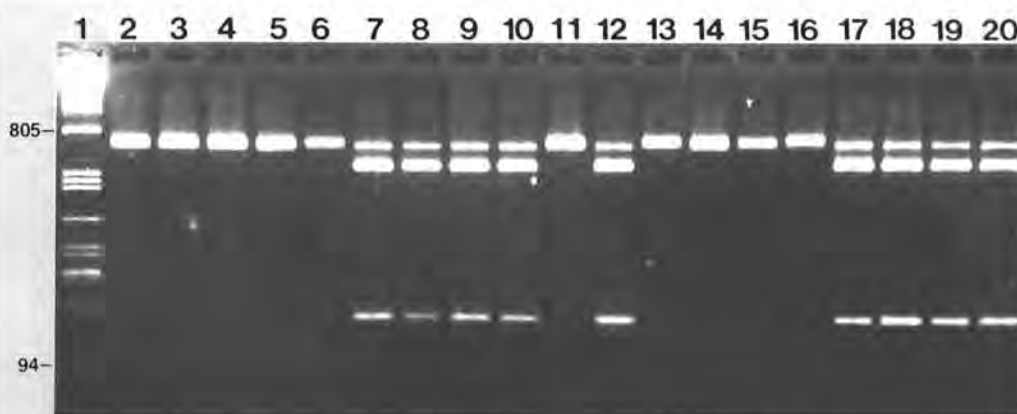
In analyses of the PCR products by restriction endonuclease digestion with 17 enzymes, only primer set RST31/RST33 (733 bp) generated products that allowed for the differentiation of the two pathotypes of *X. fastidiosa*. Digestion of the 733-bp PCR

products with *RsaI* followed by electrophoresis through NuSieve agarose gel differentiated strains of the Pierce's disease and CVC groups from strains of *X. fastidiosa* from other hosts (Fig. 4). Because of the possible usefulness of this differentiation in future epidemiological studies, primer set RST31/RST33 was used in plant sample detection experiments. No apparent PCR amplification of DNAs from a collection of several plant-pathogenic or saprophytic bacteria, as well as from noninfected plant extracts, occurred with these primers (Fig. 2).

**PCR detection of *X. fastidiosa* added to plant extracts.** In preliminary tests, known concentrations of viable *X. fastidiosa* cells were added to plant tissues, and several simple methods for preparing plant extracts for PCR amplification were evaluated. Direct boiling in water, lyse and boil methods, and standard DNA extraction methods were not successful. Dilution of the samples to concentrations of 1:100 or 1:1,000 in buffer or water prior



**Fig. 3.** Agarose gel electrophoresis of the products of the polymerase chain reaction amplification of DNA from various strains of *Xylella fastidiosa* with three primer sets. Lanes 2–6, primers RST29/RST30; lanes 7–11, primers RST31/RST33; and lanes 12–16, primers RST32/RST33. Target DNA (100 ng) sources: lanes 2, 7, and 12, Pierce's disease strain PCE-RR (ATCC 35879); lanes 3, 8, and 13, plum leaf scald strain PLM-G83 (ATCC 35871); lanes 4, 9, and 14, citrus variegated chlorosis strain IAPAR 10437; lanes 5, 10, and 15, oak leaf scorch strain OAK (ATCC 35874); and lanes 6, 11, and 16, sycamore leaf scorch strain SYC86-1. Lanes 1 and 17 contain 0.5  $\mu$ g of *EcoRI* + *HindIII*-digested  $\lambda$  DNA. Molecular weights of markers are given in base pairs.



**Fig. 4.** NuSieve agarose gel electrophoresis of *RsaI* restriction digestion fragments of the polymerase chain reaction products (primers RST31/RST33) from the DNA of strains of *Xylella fastidiosa* representing pathotype groups. Target DNA (100 ng) sources: lanes 2–6, Pierce's disease strains PCE-RR (ATCC 35879), PCE-FG (ATCC 35881), PD-4, PD92-4, and IBSBF 755; lanes 7 and 8, oak strains OAK (ATCC 35874) and 088-9; lanes 9 and 10, sycamore strains SYC86-1 and SYC89-A; lane 11, citrus blight strain CB-9; lane 12, goldenrod strain 83-G1; lanes 13–16, citrus variegated chlorosis strains IAPAR 9712, IAPAR 9713, IAPAR 10437, and IAPAR 10438; and lanes 17–20, plum strains PLM-G83 (ATCC 35871), IAPAR 9746, IAPAR 9748, and IAPAR Coeur de Lion. Lane 1 contains 3  $\mu$ g of *PstI*-digested  $\lambda$  DNA. Molecular weights of markers are given in base pairs.

to the DNA extraction procedure allowed for detection of *X. fastidiosa* by PCR.

To avoid the necessity for sample dilution, inhibitors of PCR were inactivated by the addition of acid-washed PVPP and sodium ascorbate to the SCP buffer (SCPAP) in which the tissue was ground as previously described (6,17). PCR amplification was achieved in the presence of undiluted plant tissue extracts (Fig. 5). This method was adopted in all further experiments.

In the preparation of vacuum extracts from trees, the most consistent PCR results were obtained when sodium ascorbate was added to the SCP buffer passed through the xylem vessels. PVPP was added to the extracts after passage of the buffer through the plant.

**Sensitivity of PCR compared with ELISA for detection of *X. fastidiosa*.** Replicated dilutions of suspensions of *X. fastidiosa* added to grape petiole extracts or citrus xylem extracts were tested for detection of the pathogen by ELISA and PCR amplification of DNA (Table 1 and Fig. 6). PCR amplification of DNA consistently detected populations of *X. fastidiosa* 100-fold lower than those detected by ELISA. The limit of detection with ELISA ranged from  $2 \times 10^4$  to  $1 \times 10^5$  cfu/ml, whereas  $2 \times 10^2$  to  $1 \times 10^3$  cfu/ml were detected by PCR amplification. The PCR method consistently detected *X. fastidiosa* in extracts from three naturally infected grapevine tissue sources as well as from artificially contaminated samples. In addition, the PCR method detected *X. fastidiosa* in seven of seven ELISA-positive, naturally

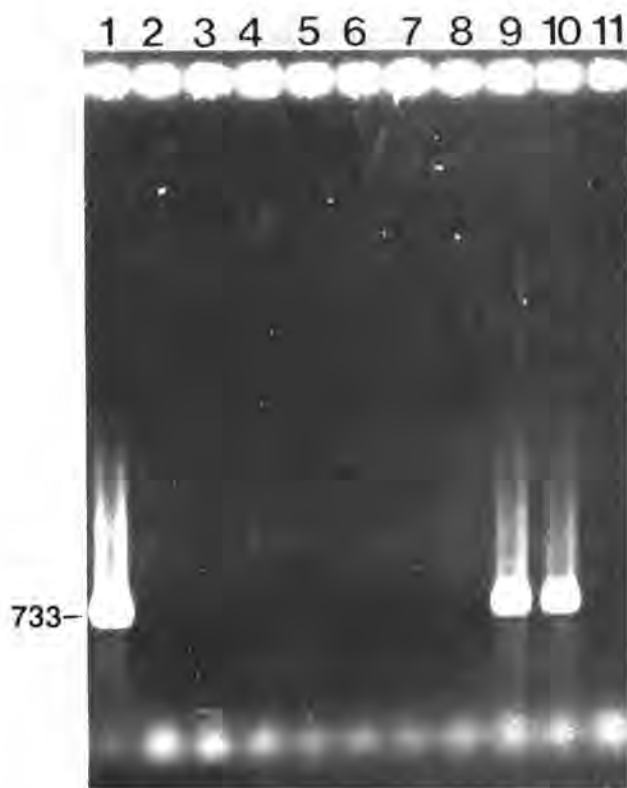
infected citrus rootstocks sampled. Noninfected tissue samples used as negative controls during PCR experiments revealed no false positive results in our tests.

## DISCUSSION

Oligonucleotide primers specific for *X. fastidiosa* and conserved in 33 strains isolated from various hosts were used in a PCR detection procedure that was more sensitive than ELISA. The procedure detected as few as 200 cfu/ml of sample. However, since DNA was extracted from only 200  $\mu$ l of sample containing approximately 40 cfu and less than one-tenth of the extracted DNA was added to the PCR reaction mixture, the procedure actually allowed for the detection of 3–4 cfu. By comparison, a minimum of 20,000 cfu/ml of sample was required for a positive ELISA test with 200  $\mu$ l of sample per well, which is equivalent to 4,000 cfu.

DNA from *X. fastidiosa* was not amplified from grape petiole extracts unless the plant extract was diluted 100-fold or more with buffer or water, evidence that phenolic compounds or polysaccharides from the grapevine tissue inhibited the PCR reaction (17). Since diluting the plant samples with water or buffer decreased sensitivity of the method, a way to inactivate or remove PCR inhibitors was needed. The addition of acid-washed PVPP and sodium ascorbate to the plant sample buffer was compatible with both methods of tissue extraction (tissue maceration and vacuum extraction of xylem vessels) commonly used for detection of *X. fastidiosa*. Insoluble PVPP present in the sample extracts was centrifuged from the aqueous phase to the interface layer at the chloroform extraction step during DNA isolation or was removed from microtiter plate wells in washing steps during ELISA and, therefore, did not pose any special handling problems during PCR and ELISA assays.

Restriction endonuclease digestion of the PCR amplification products with *Rsa*I allowed for the differentiation of two pathotypes of *X. fastidiosa*. This correlates with other studies that have shown that there are at least two pathotype groups of *X. fastidiosa*, the Pierce's disease group and the group causing other diseases, often referred to as the phony peach group (2,12). The phony peach group may consist of several pathotypes, although research has not conclusively differentiated them. The ability to distinguish the Pierce's disease strains by restriction enzyme digestion of PCR products will be a valuable tool in epidemiological studies of Pierce's disease of grapevine. Since the Pierce's disease strain is reported to have a very wide natural host range (3), the PCR procedure will be a convenient and rapid method of determining whether a strain of *X. fastidiosa* discovered in a new host is a Pierce's disease strain or possibly a new strain. Further research is needed in the evaluation of new primers that will allow for finer differentiation within and between the pathotypes of *X.*

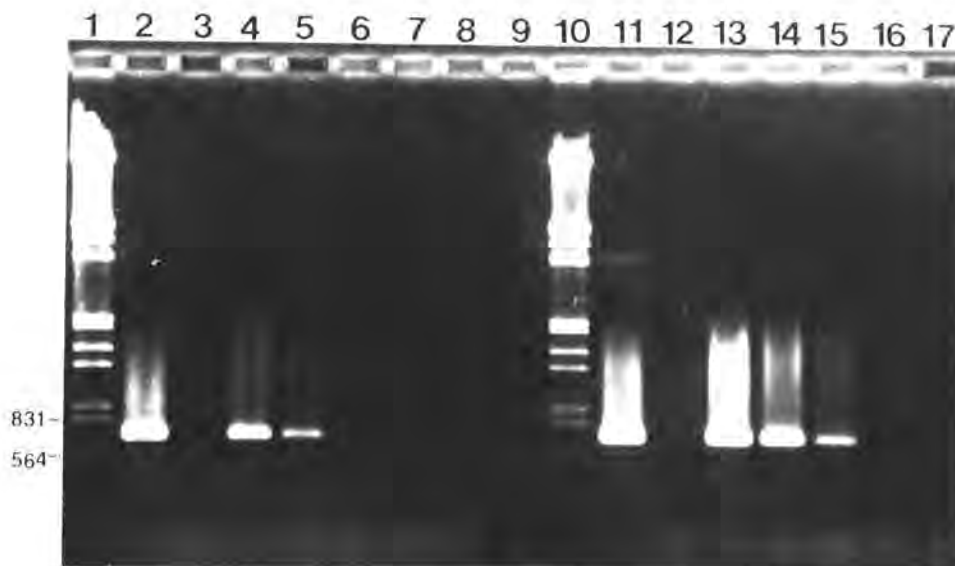


**Fig. 5.** Agarose gel electrophoresis of the products of the polymerase chain reaction (PCR) amplification (primers RST31/RST33) of DNA extracted from dilutions of *Xylella fastidiosa* present in undiluted non-infected grape tissue extracts. Target DNA sources: lane 1, 100 ng of genomic DNA from *X. fastidiosa* strain PCE-RR (ATCC 35879); lanes 2, 3, 7, and 8, noninfected plant extract without (lanes 2 and 3) or with (lanes 7 and 8) polyvinylpyrrolidone (PVPP) and sodium ascorbate; lanes 4–6, 10-fold dilutions of *X. fastidiosa* added to plant extract in succinate-citrate-phosphate (SCP) buffer without PVPP and sodium ascorbate; and lanes 9–11, 10-fold dilutions of *X. fastidiosa* added to plant extract in SCP buffer containing PVPP and sodium ascorbate. Lanes 4 and 9 correspond to a concentration of  $1.8 \times 10^4$  cfu/ml as determined by dilution plating on PD3 medium. Molecular weight of PCR products is given in base pairs.

**TABLE 1.** Comparison of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) amplification of DNA for the detection of *Xylella fastidiosa* in plant extracts

Sample	Minimum concentration detected (cfu/ml) <sup>a</sup>	
	ELISA	PCR
<b>Experiment 1</b>		
Noninfected grape petiole extract		
+ <i>X. fastidiosa</i>	$2.8 \times 10^4$	$2.8 \times 10^2$
Infected grape petiole extract	$6.1 \times 10^4$	$6.1 \times 10^2$
<b>Experiment 2</b>		
Noninfected grape petiole extract		
+ <i>X. fastidiosa</i>	$1.8 \times 10^5$	$1.8 \times 10^3$
Noninfected citrus xylem extract		
+ <i>X. fastidiosa</i>	$1.3 \times 10^5$	$1.3 \times 10^3$

<sup>a</sup> Actual numbers of live bacteria in the samples were determined by dilution plating on PD3 medium. Data represent the lowest concentrations of bacteria in the 10-fold dilutions that were positive by the respective assays in replicated experiments.



**Fig. 6.** Agarose gel electrophoresis of the products of the polymerase chain reaction amplification (primers RST31/RST33) of DNA extracted from dilutions of *Xylella fastidiosa* added to undiluted noninfected grape petiole extracts and citrus xylem vacuum extracts in succinate-citrate-phosphate buffer containing polyvinylpyrrolidone and sodium ascorbate. Target DNA sources: lanes 2 and 11, 100 ng of genomic DNA from *X. fastidiosa* strain PCE-RR (ATCC 35879); lane 3, noninfected grape petiole extract; lanes 4–8, 10-fold dilutions of *X. fastidiosa* added to grape petiole extract; lane 12, noninfected citrus xylem extract; and lanes 13–17, 10-fold dilutions of *X. fastidiosa* added to citrus xylem extracts. Lanes 4 and 13 correspond to  $1.8 \times 10^6$  and  $1.3 \times 10^3$  cfu/ml, respectively, as determined by dilution plating on PD3 medium. Lane 9 is empty. Lanes 1 and 10 contain  $0.5 \mu\text{g}$  of *EcoRI* + *HindIII*-digested  $\lambda$  DNA. Molecular weights of markers are given in base pairs.

*fastidiosa* by PCR amplification or by restriction digestion of PCR amplification products.

Currently, there are no rapid, accurate diagnostic techniques available to determine whether or not a strain of the bacterium in a newly discovered host is a Pierce's disease strain, one of the other host strains, or an entirely new strain. The sensitivity of the PCR method for detecting *X. fastidiosa* in host tissue should make it useful for both research and disease diagnostic programs. The current method of choice for surveys or epidemiological studies involving large numbers of samples is ELISA. The concentration of bacteria in many hosts may be very low and at the limit, or below the limit, of detection with ELISA. Since PCR is at least 100-fold more sensitive than ELISA, the PCR protocol could be useful in research to identify alternate hosts of the Pierce's disease pathogen. In addition, the PCR protocol may be adapted for detection of *X. fastidiosa* in insect vectors.

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