Rapid and Sensitive Colorimetric Detection of *Xanthomonas axonopodis* pv. *citri* by Immunocapture and a Nested-Polymerase Chain Reaction Assay

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ABSTRACT

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We have developed a sensitive and specific assay for *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus bacterial canker. The assay is based on sequential nested amplification by polymerase chain reaction (PCR) of a region of plasmid DNA that is very highly conserved in *X. axonopodis* pv. *citri*. Specific amplification products were observed in reactions containing three or fewer target molecules, an improvement of 50- to 100-fold over single-stage PCR, and similar results were observed when beginning with purified DNA or living bacterial cells. Colorimetric detection of amplification products was performed with the DIANA (detection of immobilized amplified nucleic acids) method, which uses labeled primers to allow amplification product capture and detection in a microtiter plate. Predicted amplification products were produced from all strains of *X. axonopodis* pv. *citri* and from four of six strains of *X. axonopodis* pv. *aurantifolii* but not from other xanthomonads, including citrus epiphytes, except for *X. axonopodis* pv. *vignicola* and one strain isolated from *Feronia elephantiacum*, consistent with previous hybridization results. No amplification products were observed from strains of *X. axonopodis* pv. *citrumelo* that incite citrus bacterial spot disease in Florida citrus nurseries. Amplification was completely inhibited by copper hydroxide when present in the reaction mix at 13.6 μg/ml. Concentrated leaf extracts from tangelo and mandarin orange, but not similar extracts from other citrus varieties, also inhibited amplification. Immunomagnetic separation of target bacteria prior to amplification was used to concentrate and recover *X. axonopodis* pv. *citri* from samples containing compounds that inhibit amplification (i.e., copper and concentrated citrus extracts). Immunocapture, by concentrating target bacteria from dilute plant extracts, improved the sensitivity of the assay by 100-fold over nested-PCR alone. The combination of sensitivity, specificity, and speed of the assay could make this a widely used assay both in plant quarantine and in areas where *X. axonopodis* pv. *citri* is endemic and clean planting stock programs are to be initiated.

Additional keywords: citrus canker, *Xanthomonas campestris* pv. *citri*

There are three distinctive diseases of citrus caused by readily distinguishable xanthomonads. These strains were recently reclassified, as were all xanthomonads that cause plant disease (36). The most important of these diseases, citrus bacterial canker (CBC), caused by *Xanthomonas axonopodis* pv. *citri* (synonym *X. campestris* pv. *citri* pathotype A) (11) occurs in many subtropical and tropical areas in which high temperatures and rainfall occur at the same time of year (8,34). This pathogen is a target of international phytosanitary quarantine efforts. A second group of strains, limited to lemon trees (*Citrus limon* (L.) N. L. Burm.) in South America, cause a milder form of CBC. These strains are now referred to as *X. axonopodis* pv. *aurantifolii* (36) (synonym *X. campestris* pv. *aurantifolii*).

In the countries of Southeast Asia, the Indian subcontinent, and South America where *X. axonopodis* pv. *citri* is endemic (8), long-distance movement of the pathogen occurs through infected plants produced in nurseries. In such countries, infected nursery plants may be the most important primary inoculum for subsequent epidemics (8). Thus, production of healthy plant material in infested areas is important and can be achieved only if specific and sensitive detection techniques are available. Recent outbreaks of CBC in Florida, where it is not endemic, have prompted a great deal of research on the pathogen (33) and has prompted the development of rapid and sensitive methods for detection. Correct identification of *X. axonopodis* pv. *citri* in Florida is particularly important because of the occurrence in citrus nurseries of a third distinct disease, citrus bacterial spot (CBS), caused by strains referred to as *X. axonopodis* pv. *citrumelo* (synonym *X. campestris* pv. *citrumelo*). There is general agreement based on symptomatology, epidemiology, and host range data that strains of *X. axonopodis* pv. *citrumelo* that cause CBS are not closely related to other xanthomonads that cause diseases of citrus (33). Thus, strains of *X. axonopodis* pv. *citrumelo* are not targets of international phytosanitary efforts and should not be detected by an assay for *X. axonopodis* pv. *citri*, as is true for other xanthomonads.

We previously described a DNA hybridization probe assay for *X. axonopodis* pv. *citri*, which satisfied this criterion (9), based on the observation that a 4.2-kb *Bam*HI fragment was present in plasmid DNA from 85% of strains of *X. axonopodis* pv. *citri* but was absent from *X. axonopodis* pvs. *aurantifolii* and *citrumelo* (29). The limit of detection of the DNA probe was about 2 ng of ge-

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nomic DNA or 5 × 10^5 genome equivalents. Sensitivity was increased to about 160 cells per reaction by a standard polymerase chain reaction (PCR) assay. Detection of as few as 10 cells in an assay tube was observed, but this required Southern hybridization analysis of the PCR products (14).

PCR-based detection methods have been described for several other important plant pathogens (17). Several analytical methods for detection of PCR products that have sensitivities comparable to detection of products after agarose gel electrophoresis have been described recently (6,16,18). These colorimetric methods may be amenable to automation and also can be used to quantify the initial number of target molecules in a sample using a competitive PCR approach (20,21). Immunomagnetic capture (IC) has been widely used to concentrate and purify target bacteria from water (7,25) and from food and clinical samples (16,18,26,27,31,38).

We report on specific detection of *X. axonopodis* pv. *citri* in the range of single cells or genome equivalents per assay tube by nested-PCR (N-PCR) and either gel electrophoresis or a colorimetric assay performed in microtiter dishes. We also have used magnetic beads coated with the well-characterized mouse monoclonal antibody (MAb) A1 (1) to concentrate *X. axonopodis* pv. *citri* from plant samples prior to PCR amplification. In addition to concentrating the target bacteria, IC purified target cells away from inhibitors of PCR.

MATERIALS AND METHODS

PCR reaction conditions. Primers for the PCR were designed with the MacVector computer program (IBI Kodak, New Haven, CT) based on the sequence of the 572-nucleotide *X. campestris* pv. *citri* insert in pFL1 (14) and were obtained commercially (Cruachem, Dulles, VA). Plasmid DNA was purified after alkaline lysis of overnight cultures using either centrifugation through CsCl density gradients (24) or the Insta-Prep spin column procedure (5-Prime 3-Prime, Boulder, CO). Genomic DNA was purified as described previously (10). DNA concentrations were estimated by fluorometry (TKO 100, Hoefer Scientific, San Francisco) and by spectrophotometry at 260 and 280 nm. PCR reactions contained reaction buffer (3) with 3 mM $MgCl_2$, 0.5 µM each primer, 125 µM nucleotides, and 1.25 units of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN, or Life Technologies, Grand Island, NY) per 25 µl of reaction. The first round of PCR was with primers 4 and 7 (14) and consisted of 35 cycles of 95°C for 70 s, 58°C for 60 s, and 72°C for 60 s. For N-PCR, 1 µl from the first reaction was used as template with primers 94-3 bio (5′-biotin-CTCGATCACGATGTCCTTCTCC) and 94-4 lac (5′-<u>AATTGTTATCCGCTCACAATT</u>GTGGATGGCATGAGCATGAAG). The first 21 nucleotides of this primer (underlined) were derived from the sequence of the *lac* operator of *Escherichia coli* (22) and were added at the 5′ end of a 21-nucleotide sequence specific for the product amplified by primers 4 and 7. These primers directed the amplification of a 315-nucleotide product from DNA or cells of *X. axonopodis* pv. *citri*. Twenty cycles at the same temperature profile as above were used for the second round of PCR.

When purified plasmid DNA was used as template for PCR, known amounts of DNA were added to tubes containing the complete reaction mix. *X. axonopodis* pv. *citri* cells also were used directly for PCR as described previously (14). For experiments to determine detection sensitivity beginning with cultured cells, bacteria from an overnight culture on sucrose peptone agar (SPA) plates (15) were suspended and serially diluted in deionized water. One microliter of serially diluted bacterial suspensions was added to PCR reactions, and aliquots from these dilutions were immediately plated in duplicate on SPA plates with a spiral plater (InterScience, St. Nom la Breteche, France) to obtain direct estimates of the number of viable bacteria added to each PCR reaction. Negative control reactions (no DNA or bacteria) were run in all experiments.

Detection of PCR products. PCR reaction products were detected by electrophoresis through 2% NuSieve (FMC Bioproducts, Rockland, ME) agarose (3:1) gels and staining with ethidium bromide or by using the detection of immobilized amplified nucleic acids (DIANA) (Dynal International, Oslo, Norway) colorimetric procedure (18). In this procedure, PCR products from the second round of PCR were captured via the 5′ biotin moiety of primer 94-3 bio using streptavidin-coated super paramagnetic polystyrene beads (Dynal) (100 µg per reaction) and a magnetic separation stand. The bound products were washed once and resuspended in 100 µl of DIANA buffer (25 mM Tris HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 10 mM dithioreitol, and 0.05% Tween 20) and transferred to microtiter trays. The samples were incubated for 20 min with 50 µl of *Lac* I repressor::β-galactosidase fusion protein (2 units/ml) per well. After four washes with 150 µl of DIANA buffer, the substrate ONPG (*O*-nitrophenyl-β-D-galactoside) was added (3 mg/100 µl of DIANA buffer), and the microtiter plates were incubated at room temperature for 15 min. Color development was stopped by the addition of 75 µl of stop solution (1M Na_2CO_3, pH 12), and the A_{405} was determined. Assays were conducted with three or four replicates. *X. axonopodis* pv. *citri* strain JA159-1, a typical strain originally isolated in Reunion, France, from mandarin orange (*C. reticulata* 'Clementine') in 1981 was used for these experiments.

Specificity of N-PCR for *X. axonopodis.* pv. *citri*. Aliquots (1 µl) from bacterial suspensions made in deionized water from freshly streaked SPA plates were used as template in N-PCR assays. The 19 strains of *X. axonopodis* pv. *citri* and their origins were as follows: XC59, Brazil; XC62 and XC63, Japan; XC74, XC75, and XC77, Reunion Island, France; XC78, the Philippines; XC80 and XC84, Thailand; XC105 and XC106, Thursday and Christmas Islands, Australia; XC118, New Zealand; XC98, South Yemen; XC100 and XC156 Pakistan; and XC269, XC323, XC328, and XC331, Saudi Arabia. The six strains of *X. axonopodis* pv. *aurantifolii* and their origins were as follows: XC64, XC69, and XC93, Argentina; XC90 and Mex 14B, Mexico; and XC70, Brazil. Strains representing *X. axonopodis* pvs. *alfalfae, malvacearum, manihotis, phaseoli, vesicatoria,* and *vignicola*; *X. vasicola* pv. *holcicola*; *X. arboricola* pv. *pruni*; and *X. hortorum* pv. *pelargonii* were tested in the same manner. In addition, strains formerly classified as *X. campestris* pvs. *nigromaculans, fici, and bilvae,* but presently unclassified, as well undesignated strains from *Strelitzia reginae* (9) were tested in the same manner. Eleven strains (13) of *X. axonopodis* pv. *citrumelo* also were assayed by N-PCR.

Bacteria living as epiphytes on citrus foliage were recovered from freshly collected leaves of sweet orange and grapefruit (provided by R. H. Brlansky, Citrus Research and Education Center, Lake Alfred, FL). Groups of three leaves were ground in deionized water. The extracts were filtered through cheesecloth and plated on SPA plates that contained cycloheximide (100 µg/ml). The plates were incubated at 27°C for 3 days, and the confluent growth was suspended in deionized water, diluted to contain approximately 100 CFU/µl, and assayed by N-PCR. Eight separate pools from sweet orange and grapefruit were assayed. Epiphytic xanthomonads previously isolated from citrus (9) also were grown on SPA plates and assayed as described above. *X. axonopodis* pv. *citri* strain XC62 was used for a positive control in these experiments.

Because of the extreme sensitivity of the N-PCR, amplified products were initially observed in some of the negative control reactions as well as in some reactions with DNA from pathovars of *X. campestris* that do not have DNA homologous to pFL1 (9). To eliminate contaminating DNA from the reactions, a new set of pipettes that never dispensed DNA template or PCR products was used exclusively to make up the PCR reaction mixes (19). In addition to autoclaving, water, buffer solutions, and mineral oil for overlays were aliquotted and treated with short-wave (254 nm) UV light (30 min) in a Stratalinker 1800 (Stratagene, La Jolla, CA) (28). All pipetting was done with filtered pipette tips.

Test of citrus extracts for PCR inhibition. Healthy plants of seven citrus clones were grown in a screened greenhouse. The clones used were Mexican lime SRA619 (*C. aurantifolia*), Tahiti lime SRA 58 (*C. latifolia*), combava (*C. hystrix*), mandarin orange 'Clementine' SRA63 (*C. reticulata*), sweet orange 'Valencia' SRA360 (*C. sinensis*), grapefruit 'Henderson' SRA336 (*C. paradisi*), and tangelo 'Orlando' SRA 46 (*C. paradisi* × *C. reticulata*). Five grams of citrus leaves was homogenized in 50 ml of Tris HCl buffer (pH 7.1) using a Stomacher model 400 blender (Seward Medical, London). *X. axonopodis* pv. *citri* strain JA159-1 cells were added to citrus extracts to obtain a final concentration of 10 or 1,000 CFU/µl. One microliter of this mixture was added in a total of 25 µl for the first round of PCR, and 1 µl of the products of these reactions was used in the second amplification cycle. Four positive control reactions also were done (10 or 1,000 bacteria without citrus extracts). Three reactions with neither bacteria nor extracts served as negative controls for the PCR reagents, and two reactions per citrus variety containing citrus extracts only (no added bacteria) were used to verify that the citrus tissue was not naturally infested with *X. axonopodis* pv. *citri*. A second experiment was performed with 10 µl of each citrus extract added per 25 µl of reaction. A third experiment was done with mandarin orange and tangelo extracts diluted 1:5 and 1:25. For the IC experiments, 500 µl of leaf extracts from grapefruit 'Henderson' SRA336, tangelo 'Orlando' SRA 46, and mandarin orange 'Clementine' SRA63 were adjusted to contain 10 and 1,000 CFU of strain JA159-1 per µl. IC was performed as described below.

Effect of copper hydroxide on amplification. Citrus leaves (Mandarin orange 'Clementine') were collected from a nursery sprayed with copper hydroxide (250 g/hl) 4 h previously. The copper concentration in 20 samples, each containing 20 leaves, was deter-

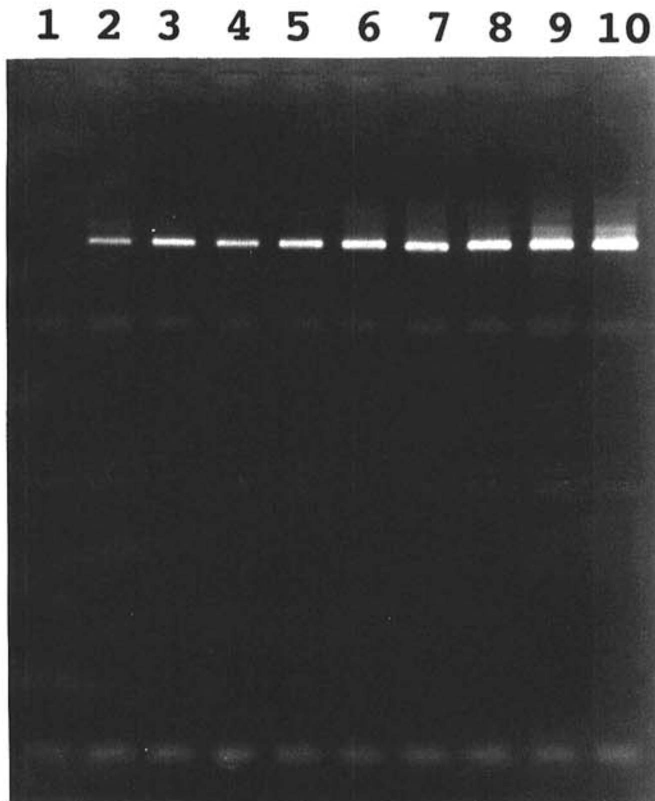

Fig. 1. Detection of nested polymerase chain reaction-amplified products of *Xanthomonas axonopodis* pv. *citri* by agarose gel electrophoresis with pFL1 as a model template. Upper half of gel, lanes 1–10: the estimated number of copies of pFL1 as starting template were 1.5, 2.9, 15, 29, 150, 290, 1,500, 2,900, 15,300, and 29,000, respectively. Colorimetric data from a similar experiment are presented in Table 1. Reactions in the lower 10 lanes did not receive template DNA (negative controls).

TABLE 1. Amplification and detection of model template pFL1 by nested polymerase chain reaction and gel electrophoresis or colorimetry

Copies of pFL1/ 25 µl of reaction[a]	Electrophoresis[b]	Colorimetry[c]
0	–	0.028 ± 0.005
0.5	–	0.038 ± 0.014
1	–	0.165 ± 0.014
12	+	ND[d]
48	+	0.292 ± 0.028
96	+	0.466 ± 0.012
190	+	0.385 ± 0.035
380	+	0.307 ± 0.035
3,800	+	0.313 ± 0.056
19,000	+	0.255 ± 0.025
48,000	+	0.784 ± 0.037

[a] Estimated by spectrofluorometry, the molecular weight of the plasmid, and Avogadro's number. Each assay tube contained 1 µl of DNA solution. Multiply by 1,000 to determine number of target molecules per ml of solution assayed.

[b] Presence (+) or absence (–) of unique band of predicted size after agarose gel electrophoresis.

[c] Absorbance at 405 nm after cleavage of the substrate *o*-nitrophenyl-β-D-galactoside by the *lac* repressor::β-galactosidase fusion protein using the DIANA (detection of immobilized amplified nucleic acids) protocol described in text. Mean ± standard deviation of three replicates

[d] Not done.

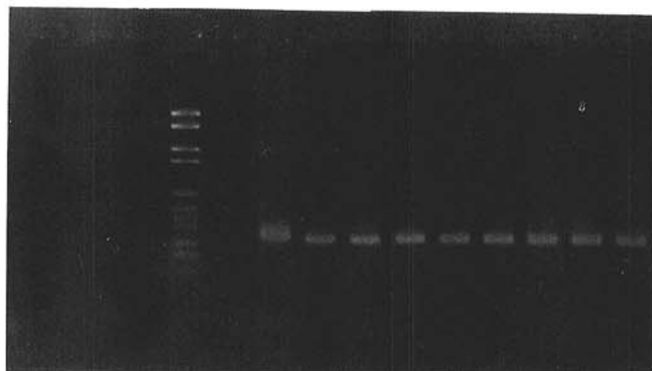

Fig. 2. Detection of nested polymerase chain reaction-amplified products by agarose gel electrophoresis with *Xanthomonas axonopodis* pv. *citri* strain JA159-1 cells as template. Lanes 1–3, dH_20; lane 4, Boehringer Mannheim marker VI; lane 5, empty; and lanes 6–14, reactions contained approximately 1, 4, 11, 88, 231, 386, 3,500, 15,000, and 29,000 CFU prior to amplification, respectively. Colorimetric assay data from this experiment are presented in Table 2.

TABLE 2. Colorimetric detection of cultured *Xanthomonas axonopodis* pv. *citri* strain JM 159-1 after nested polymerase chain reaction (PCR) amplification

Viable bacteria/ 25 µl of reaction[a]	Mean A_{405}[b]
0 (negative control)	0.057 ± 0.006
1	0.460 ± 0.026
4	0.490 ± 0.029
11	0.415 ± 0.010
89	0.438 ± 0.010
231	0.468 ± 0.023
386	0.505 ± 0.029
3,500	0.509 ± 0.060
15,300	0.468 ± 0.029
29,000	0.573 ± 0.029

[a] Estimated by plating (spiral system) aliquots of dilutions in duplicate on sucrose peptone agar after aliquots were removed for PCR assay. Multiply by 1,000 to determine viable CFU/ml in the solution assayed.

[b] Mean ± standard deviation of mean for three replicates.

mined by flame atomic absorption spectrometry. For the first amplification experiment, copper hydroxide (Champion Flow, Calliope, France) was added to standard PCR reagents to give 340 µg, 34 µg, 3.4 µg, 340 ng, 34 ng, and 3.4 ng of copper hydroxide per 25 µl of PCR reaction (13.6 mg/ml to 136 ng/ml). Two sets of reactions, which contained 10 or 1,000 CFU of JA 159-1, were subjected to N-PCR using our standard method. For the IC experiments, copper hydroxide was added to similar cell suspensions of strain JA159-1 to final concentrations of 1,360 and 13.6 µg/ml. These concentrations are equivalent to the concentrations of 34 µg and 340 ng of copper hydroxide per 25 µl of reaction described above. IC was performed as described below.

Magnetic IC. The MAb A1 (1) was obtained as diluted ascites fluid and was purified by protein G affinity chromatography (NYGene, Hawthorne, NY). The purified MAb was quantified spectrophotometrically at 280 nm and incubated with paramagnetic Dynabeads M-280 precoated with sheep anti-mouse immunoglobulin G (IgG) (Dynal) overnight at 4°C with shaking (125 rpm). Forty microliters (0.4 mg) of beads was coated with 4 or 0.04 µg of MAb (10 or 0.1 µg of MAb per 10^6 beads), depending on the experiment. The bead-MAb complex was separated from the solution by applying a magnet to the wall of the tubes. The excess of unbound MAb was eliminated by four washes (30 min each) with 2 volumes of phosphate buffered saline (PBS) with 0.1% bovine serum albumin. The complex was incubated with 500 µl of serially diluted bacterial suspensions (from 10 to 10^8 cells of strain JA159-1 per ml of Tris-HCl buffer, pH 7.2) for at least 1 h at 4°C with gentle shaking. Immunocaptured bacteria were separated magnetically from the solution, washed three times with PBS buffer, and suspended in 10 µl of sterile water. The beads were boiled for 5 min and briefly centrifuged at 13,000 × *g*. The N-PCR was done using 1 µl of the supernatant.

RESULTS

Sensitivity of N-PCR with model templates and *X. axonopodis* pv. *citri*. N-PCR provided extremely sensitive detection of model template pFL1, which contains a single copy of the target sequence. Amplification products of the expected size were observed with agarose gel electrophoresis (~3 target molecules; Fig. 1) and detected colorimetrically (~1 target molecule; Table 1). Because of the low number of template molecules used in these experiments, no products were visible after agarose gel electrophoresis of the first-round PCR products, as expected (14; data not shown). No amplification products were observed in an equal number of negative control reactions. Very low numbers of bacteria, in the range of single cells, were detected easily after agarose gel electrophoresis of N-PCR products (Fig. 2) and also were detected colorimetrically (Table 2). The colorimetric assay was uniform over five orders of magnitude of bacterial numbers per sample.

Specificity of the N-PCR. A product of the expected size was produced by N-PCR when DNA from strains of *X. axonopodis* pv. *citri* was used as template for N-PCR (Fig. 3). All tested strains produced the expected product, including four strains from Saudi Arabia (Fig. 3, bottom) that do not react with MAb A1 used for IC in other experiments reported here. Four additional strains of *X. axonopodis* pv. *citri* from India, Iran, and Oman also produced a product of the expected size after N-PCR (data not shown). The assay also detected four of the six strains of *X. axonopodis* pv. *aurantifolii* tested (Fig. 3).

Twenty-three additional xanthomonads, including strains of at least three other *Xanthomonas* species and seven other pathovars of *X. axonopodis*, also were tested by N-PCR. No amplification

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

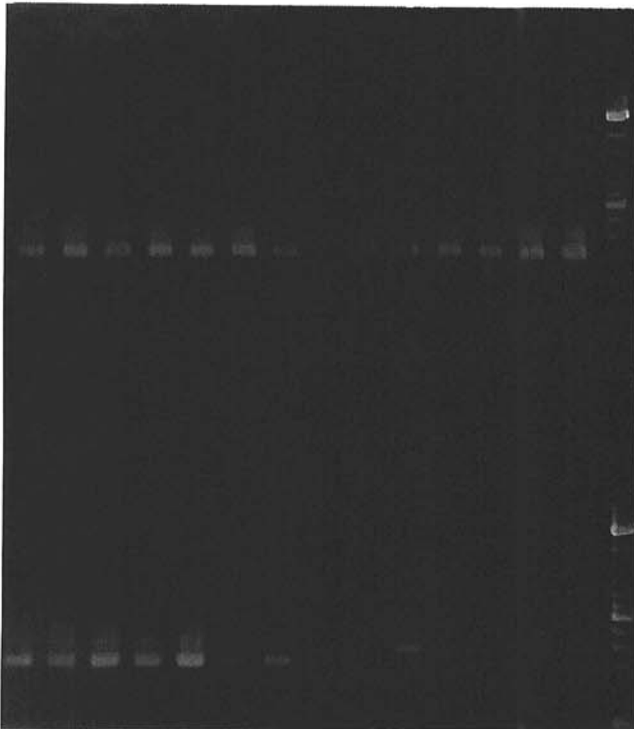

Fig. 3. Strains of *Xanthomonas axonopodis* pvs. *citri* and *aurantifolii* subjected to nested polymerase chain reaction. Upper half of gel, lanes 1–14: *X. axonopodis* pv. *citri* strains XC59, XC62, XC63, XC74, XC75, XC77, XC78, XC80, XC84, XC105, XC106, XC118, XC98, and XC100, respectively (11); and lane 15, the 100-bp ladder. Lower half of gel, lanes 1–5: *X. axonopodis* pv. *citri* strains XC156, XC269, XC323, XC328, and XC331 from Saudi Arabia, respectively (37); lanes 6–11: *X. axonopodis* pv. *aurantifolii* strains XC64, XC69, Mex 14B, XC93, XC90, and XC70, respectively (11,29); lanes 12–14, negative controls; and lane 15, 100-bp ladder.

1 2 3 4 5 6 7 8 9 10 11 12 M

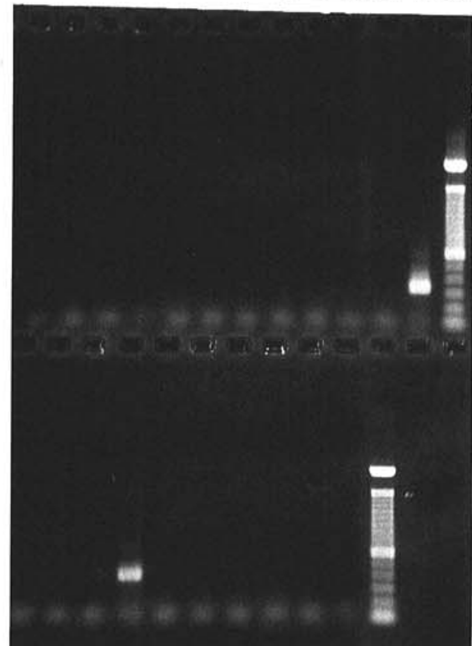

Fig. 4. Cells of various xanthomonads subjected to nested polymerase chain reaction. Upper half of gel: amplification products from strain/*Xanthomonas axonopodis* pathovar, lane 1: X37/*vesicatoria*; lane 2: X40/*manihotis*; lane 3: X45/*phaseoli*; lane 4: X56/*holcicola*; lanes 5 and 6: X69 and X70/*pruni*; lanes 7, 8, and 10: isolates from *Strelitzia reginae*; lane 9: X151/*fici*; lane 11: X203/*malvacearum*; lane 12: XC118/*citri*; and lane M: 100-bp ladder. Lower half of gel: lanes 1–10: amplification products from 10 negative control (no template DNA) reactions; and lane 11: 100-bp ladder. Strains have been described previously by Hartung (9).

product was observed for these strains, except for a strain formerly classified as *X. campestris* pv. *bilvae* (rutaceous host) and *X. axonopodis* pv. *vignicola*, consistent with previous hybridization assays (Fig. 4; some data not shown). Strains of *X. axonopodis* pv. *citrumelo* were tested separately and did not produce product bands after N-PCR (Fig. 5). None of the epiphytic bacteria tested in pooled extracts obtained from citrus leaves produced product bands after N-PCR. This also was true for epiphytic xanthomonads originally isolated from citrus in Mexico (Fig. 6)

Effect of citrus extracts and copper on amplification. The addition of 10 µl of any of the seven citrus extracts per 25 µl of reaction inhibited amplification (data not shown). Detection of *X. campestris* pv. *citri* by N-PCR was not inhibited by 1 µl of extracts of Tahiti lime, combava, 'Valencia' sweet orange, 'Henderson' grapefruit, and Mexican lime. As few as 10 bacteria per µl of extract were detected with agarose gel electrophoresis in these experiments. This is equal to approximately 10^5 CFU/g of citrus leaves. Some inhibition of amplification was observed using 1 µl of extracts from mandarin orange and tangelo and was overcome by diluting the extract 1:5 prior to adding it to the PCR reactions. Amplification also was completely inhibited by the presence of copper hydroxide at a concentration of 13.6 µg/ml in the reaction mixtures, whereas only faint amplification products were observed when copper hydroxide was present at 1.36 µg/ml or 136 ng/ml in the reaction mixtures (Table 3; some data not shown). The copper concentration in 20 samples of 20 leaves each was 1,115 ± 98 µg/g of leaf tissue when assayed 4 h after spraying. After preparation of citrus extracts and PCR reactions, this produced a concentration of copper hydroxide of 4.46 µg/ml in the reaction mixture, a concentration inhibitory to amplification.

Sensitivity of IC-N-PCR. When using beads coated with 10 µg of MAb per 10^6 beads (4 µg of MAb/0.4 mg of beads) for IC, specific amplification was obtained for target concentrations from 100 bacteria per ml of sample to 1×10^8 bacteria per ml of sample (Table 4). N-PCR without IC detected 1,000 or more bacteria per ml, consistent with other data presented in this paper. Decreasing the concentration of capture antibody to 0.04 µg/0.4 mg of beads during the coating procedure allowed us to increase the sensitivity by 10-fold (i.e., 10 bacteria per ml of sample) (Table 4). Compared to N-PCR, which detects 1,000 cells per ml of sample (1 cell per µl assayed), the sensitivity of IC-N-PCR was 100 times greater.

Elimination of inhibitors of PCR by IC. Amplification products of the expected size were obtained after IC-N-PCR of samples that contained 20 or 2,000 *X. axonopodis* pv. *citri* cells per ml and copper hydroxide at 1,360 or 13.6 µg/ml (Table 3). Specific amplification products also were observed after IC-N-PCR when 20 or 2,000 *X. axonopodis* pv. *citri* cells were added per ml of citrus extracts (i.e., 'Henderson' grapefruit, 'Orlando' tangelo, and 'Clementine' mandarin). This sensitivity represents about 200 CFU/g of citrus leaf tissue assayed.

DISCUSSION

We have developed a N-PCR–based assay for *X. axonopodis* pv. *citri*, an important pathogen of citrus that is subject to international phytosanitary quarantine. This assay improves the sensitivity of our previous single-stage PCR assay (14) approximately 50- 160-fold to the range of a single template molecule or bacterium per assay tube based on detection of amplified DNA products by either agarose gel electrophoresis (Figs. 1 and 2) or a colorimetric procedure (Tables 1 and 2).

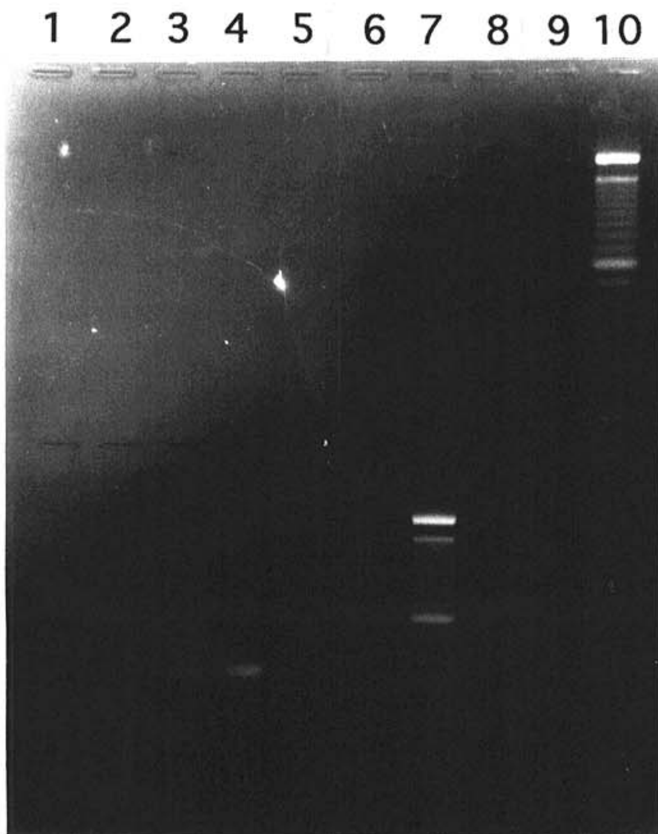

Fig. 5. Strains of *Xanthomonas axonopodis* pv. *citrumelo* subjected to nested polymerase chain reaction. Upper half of gel, lanes 1–9: amplification products from strains F1, F6, F49, F54, F100, F228, F230, F269, and F270, respectively (13); and lane 10: 100-bp ladder. Lower half of gel, lanes 1 and 2: amplification products from strains F300 and F359; lanes 3 and 4: *X. axonopodis* pv. *citri* strains XC62 and XC63; lanes 5 and 6: negative controls (no template DNA); and lane 7: 100-bp ladder.

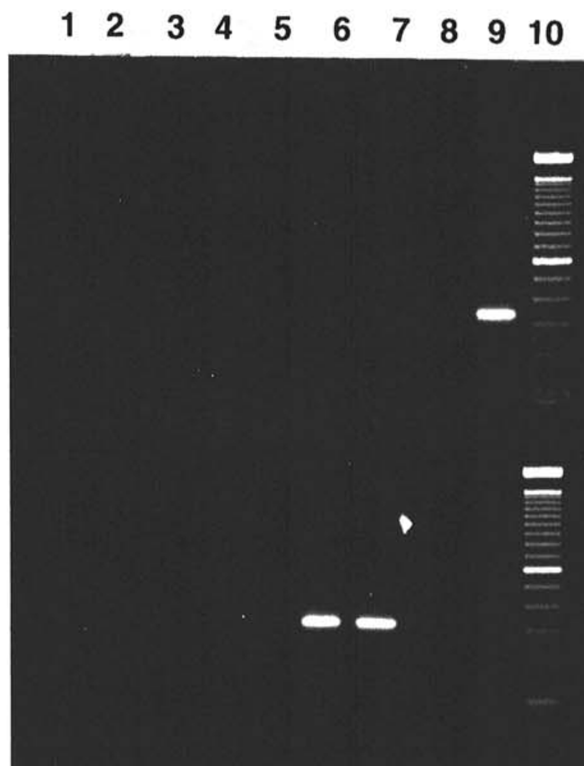

Fig. 6. Epiphytic bacteria from citrus foliage subjected to nested polymerase chain reaction. Upper half of gel, lanes 1–3 and 8: pooled *Xanthomonas axonopodis* strains from grapefruit leaves; lanes 4–7: pooled strains from sweet orange leaves; lane 9: *X. axonopodis* pv. *citri* strain XC62 as positive control; and lane 10: 100-bp ladder. Lower half of gel, lanes 1–5: *Xanthomonas* strains T20–T24 from Mexican citrus (9); lanes 6 and 7: *X. axonopodis* pv. *citri* strain XC62 as positive control; lanes 8 and 9: negative controls (no bacteria); and lane 10: 100-bp ladder.

For these assays, strain JA159-1 was suspended and diluted in sterile distilled H_2O prior to assay by N-PCR. Aliquots were plated within 30 min to determine viable cell counts. Substantial cell lysis prior to plating would have caused our sensitivity to be overestimated. However, under these conditions log-transformed viable cell counts of JA159-1 decreased only 0.08 to 0.10 log per h, so this effect is not significant (O. Pruvost, *unpublished data*).

The assay retains the excellent specificity of the previous dot blot and PCR assays (9,14). The expected DNA fragment was amplified only from strains of *X. axonopodis* pvs. *citri* and *vignicola* and a single strain originally isolated from *F. elephantiacum*, a member of the Rutaceae (Figs. 3 and 4), consistent with our previous dot blot results (9). N-PCR reactions from all strains of *X. axonopodis* pv. *citri* tested (23/23), representing 15 widely separated geographic origins, contained an amplification product of the expected size after gel electrophoresis. The assay also readily detected most strains of *X. axonopodis* pv. *aurantifolii* tested (4/6) (Fig. 3). The nondetection of some of these strains is consistent with previous hybridization assays (9,29) and is not a cause of concern since *X. axonopodis* pv. *aurantifolii* strains are restricted geographically and by host range (34) and are taxonomically distinct (36). Products were not observed from *X. axonopodis* pv. *citrumelo,* the causal agent of CBS disease (33) (Fig. 5) or from epiphytic bacteria, including xanthomonads isolated from citrus (Fig. 6).

The colorimetric assay may be adaptable to automation because no electrophoresis of products is required. Products can be detected in a standard 96-well microtiter dish by a two-step process: streptavidin capture of the PCR products via a 5′ biotin "tag" on primer 94-3 bio and detection based on a *lac* operator sequence added to primer 94-4 lac. This *lac* operator "tag" is the target of a *Lac* I repressor::β-galactosidase fusion protein. The fusion protein cleaves ONPG substrate, releasing a yellow product that is readily detected visually or with an automated plate reader.

Extracts of citrus inhibited PCR reactions, initially limiting the sensitivity of the assay when used to detect bacteria in plant tissue. Copper hydroxide, commonly used as an agricultural spray on citrus leaves, also inhibited PCR (Table 3). Removal of bacteria from samples by IC reduced inhibitors of PCR, including copper and unknown components of concentrated citrus extracts and, therefore, is a great improvement of the N-PCR assay described in this paper. However, inhibition of amplification was occasionally observed with samples that contained concentrated citrus extracts of 'Orlando' tangelo and 'Clementine' mandarin, even after IC (data not shown).

IC prior to amplification of *X. axonopodis* pv. *citri* increased the sensitivity of the assay 100-fold compared to N-PCR alone. The ratio of 10 μg of purified IgG per 10^6 beads used in initial experiments was chosen based on the manufacturer's procedure. However, using less than 0.1 μg of IgG per 10^6 beads was reported to improve the binding of *Listeria monocytogenes* (32). Our data are consistent with this report. A prerequisite for IC is to have antibodies that recognize the target bacteria. The well-characterized MAb A1 is specific to strains of *X. axonopodis* pv. *citri* (1). Therefore, the IC step prior to N-PCR may enhance the specificity of the assay as well as greatly increase its sensitivity. However, recently, some strains of *X. axonopodis* pv. *citri* from Saudi Arabia were reported not to react with MAb A1 (37). A different capture antibody will be necessary if IC is to be used to remove these strains from plant extracts. However, our N-PCR assay produced an amplification product of the expected size when applied to these bacteria from culture (Fig. 3).

Plasmid-based PCR assays for *X. axonopodis* pv. *phaseoli* (2) and *Erwinia amylovora* (5) with sensitivity and specificity similar to our own have been described. Any PCR-based assay for a pathogenic bacterium that relies on a plasmid DNA target could be misleading if the plasmid is transmissible. However, the cryptic plasmids of *X. axonopodis* pv. *citri* are not known to be transmissible, as are, for example, plasmids encoding copper resistance in *X. campestris* pv. *vesicatoria* (4,35) or virulence in *Pseudomonas syringae* pv. *atropurpurea* (30). Also, dendrograms that summarize restriction fragment length polymorphism data based on genomic (12) or plasmid DNA of *X. axonopodis*. pv. *citri* (29) are strikingly similar. This data is consistent with the plasmids being stably maintained in *X. axonopodis* pv. *citri* and is inconsistent with the plasmids being recently transferred into *X. axonopodis* pv. *citri.* Because the target sequence is plasmid-borne the sensitivity of detection assays based on the sequence may be enhanced since it may be present in multiple copies per cell. Improved sensitivity and specificity were observed using a plasmid-based PCR assay compared to a genomic-based PCR assay with *Chlamydia trachomatis* (23).

TABLE 3. Inhibition of nested polymerase chain reaction (N-PCR) by copper hydroxide relieved by immunocapture (IC) of target bacteria (*Xanthomonas axonopodis* pv. *citri*) prior to amplification

Experiment 1: N-PCR[a]			Experiment 2: IC-N-PCR[b]		
μg $Cu(OH)_2$/ml	CFU/ reaction	Product[c]	μg $Cu(OH)_2$/ml	CFU/ml	Product
0	0	–	0	0	–
0	0	–	0	0	–
0	1,000	+	0	2,000	+
0	1,000	+	0	2,000	+
0	10	+	0	20	+
0	10	+	0	20	+
1,360	1,000	–	1,360	2,000	+
1,360	1,000	–	1,360	2,000	+
13.6	1,000	–	13.6	2,000	+
13.6	1,000	–	13.6	2,000	+
1,360	10	–	1,360	20	+
1,360	10	–	1,360	20	+
13.6	10	–	13.6	20	+
13.6	10	–	13.6	20	+

[a] Suspensions of *X. axonopodis* pv. *citri* strain JA 159-1 were added to give the indicated final number of viable bacteria per 25 μl of reaction (estimated turbidimetrically). Multiply by 40 to determine the estimated number of CFU/ml of reaction mixture. The reaction mixtures contained the indicated concentrations of copper hydroxide.

[b] IC followed by N-PCR. The suspensions (500 μl) were prepared with the indicated concentrations of copper hydroxide and bacteria and were incubated for 1 h with magnetic beads coated with monoclonal antibody A1 (*X. axonopodis* pv. *citri* specific). Bacteria bound to the beads were collected and subjected to N-PCR.

[c] Presence (+) or absence (–) of the predicted amplification product after agarose gel electrophoresis of 50% of the amplification reaction mixture.

TABLE 4. Comparison of sensitivities of nested polymerase chain reaction (N-PCR) and immunocapture followed by N-PCR (IC-N-PCR) for the detection of *Xanthomonas axonopodis* pv. *citri*

CFU/ ml of test solution[a]	N-PCR[b]	IC-N-PCR[c] Experiment 1	IC-N-PCR[c] Experiment 2
0	–	–	–
10	ND[d]	–	+
10^2	–	+	ND
10^3	+	+	+
10^4	+	+	ND
10^5	+	+	+
10^6	+	+	ND
10^7	+	+	ND
10^8	+	+	ND

[a] Strain JA159-1 in deionized water. CFU/ml estimated based on serial dilutions of a suspension of cells from a 24-h culture on sucrose peptone agar adjusted turbidimetrically to 10^8 CFU/ml.

[b] One microliter of each suspension was added to 24 μl of PCR amplification reaction mix and subjected to N-PCR amplification as described in text.

[c] Five hundred microliters of each suspension was subjected to IC and N-PCR amplification as described in text. In experiment 1, the capture antibody was used at a rate of 10 μg/10^6 beads, and in experiment 2, the capture antibody was used at a rate of 0.1 μg/10^6 beads.

[d] ND = not done; presence (+) or absence (–) of an amplification product of the expected size after agarose gel electrophoresis.

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