Detection of *Xanthomonas campestris* pv. Citri by the Polymerase Chain Reaction Method

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pFL1 is a pUC9 derivitive that contains a 572-bp *Eco*RI insert cloned from plasmid DNA of *Xanthomonas campestris* pv. citri XC62. The nucleotide sequence of pFL1 was determined, and the sequence information was used to design primers for application of the polymerase chain reaction (PCR) to the detection of *X. campestris* pv. citri, the causal agent of citrus bacterial canker disease. Seven 18-bp oligonucleotide primers were designed and tested with DNA from *X. campestris* pv. citri strains and other strains of *X. campestris* associated with *Citrus* spp. as templates in the PCR. Four primer pairs directed the amplification of target DNA from *X. campestris* pv. citri strains but not from strains of *X. campestris* associated with a different disease, citrus bacterial spot. Primer pair 2-3 directed the specific amplification of target DNA from pathotype A but not other pathotypes of *X. campestris* pv. citri. A pH 9.0 buffer that contained 1% Triton X-100 and 0.1% gelatin was absolutely required for the successful amplification of the target DNA, which was 61% G+C. Limits of detection after amplification and gel electrophoresis were 25 pg of purified target DNA and about 10 cells when Southern blots were made after gel electrophoresis and probed with biotinylated pFL1. This level of detection represents an increase in sensitivity of about 100-fold over that of dot blotting with the same hybridization probe. PCR products of the expected sizes were amplified from DNA extracted from 7-month-old lesions from which viable bacteria could not be isolated. These products were confirmed to be specific for *X. campestris* pv. citri by Southern blotting. This PCR-based detection protocol will be a useful addition to current methods of detection of this pathogen, which is currently the target of international quarantine measures.

Citrus bacterial canker (CBC) occurs in many citrus-producing tropical and subtropical countries around the world. It is a major problem in all areas in which high temperatures and rainfall occur at the same time of year (5). Recent outbreaks of CBC in Florida have stimulated a great deal of research into the biology of the causal pathogen, *Xanthomonas campestris* pv. citri (Hasse) Dye (reviewed in reference 23). Because the pathogen is the target of international quarantine efforts (2), the development of rapid and reliable procedures for the diagnosis of this pathogen has been a priority. This task has been complicated by the presence of another distinct disease in Florida citrus nurseries, citrus bacterial spot (CBS), caused by other strains of *X. campestris* (21). Although the taxonomic position of *X. campestris* pv. citri has been controversial (8, 25, 26), there is widespread agreement that the strains of *X. campestris* that cause CBS (*X. campestris* pv. citrumelo [8]) are quite distinct from and should not be confused with any strains of the pathotypes (A to C) (4) that cause CBC and that CBC pathotype A [*X. citri* (ex Hasse, 1915) (8)] is readily distinguished from CBC pathotypes B and C (*X. campestris* pv. aurantifolii [8]).

We and others have shown that restriction fragment length polymorphism analysis of the genomic DNA of these strains can separate the strains into groups that are entirely consistent with groups independently derived from other data (7, 11). This work was recently extended by a restriction fragment length polymorphism analysis of plasmid DNA from strains of *X. campestris* pv. citri (15). During the course of

that study, a 4.2-kb *Bam*HI fragment was found in 85% of the most pathogenic strains (pathotype A) of *X. campestris* pv. citri. After molecular cloning from strain XC62 as pFL62.42, this fragment distinguished subtypes of *X. campestris* pv. citri in Southern blots and, importantly, did not cross-react with 54 strains of *X. campestris* that cause CBS. An internal 572-bp *Eco*RI fragment from pFL62.42 was cloned as pFL1, and the two fragments were used to develop a rapid and sensitive dot blot assay for *X. campestris* pv. citri (9). Even strains of *X. campestris* pv. citri, such as XC100 (pathotype A, Pakistan), which lacked the 4.2-kb *Bam*HI fragment (15) still produced a strong hybridization signal in this assay (9). Thus, although some strains display *Bam*HI polymorphisms, the homologous region is nonetheless conserved.

The polymerase chain reaction (PCR) (18) allows the rapid, specific, and sensitive detection of DNA sequences and thus is ideally suited to the detection of plant pathogens. We report the development of a PCR-based assay for *X. campestris* pv. citri based on the DNA sequence of the *Eco*RI insert in pFL1 (9).

(A preliminary report of this work has been presented [12].)

MATERIALS AND METHODS

DNA sequence determination and primer design. The *Eco*RI insert from pFL1 (9) was cloned into sequencing vectors M13mp18 and M13mp19 by standard methods (13). The complete nucleotide sequence of both strands of the insert was determined by dideoxy sequencing with the Sequenase system (United States Biochemical, Cleveland,

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Ohio). Oligonucleotides 18 bp in length were designed by use of the computer program Nuc-it (Compu-Right, Gaithersburg, Md.). Paired primers that showed low homology to other sequences in the target fragment and that had closely matched calculated thermal melting points were selected.

PCR assays and molecular methods. PCR assays were performed with a DNA thermal cycler (Perkin-Elmer Cetus) and 50-μl reaction mixtures that typically contained 50 ng of genomic DNA, deoxynucleoside triphosphates at 200 μM each, and primers at 1 μM each. Three reaction buffers were used: 10 mM Tris-Cl (pH 8.3)–50 mM KCl–3 mM Mg^{2+} (buffer I) (Perkin-Elmer Cetus), buffer I with 3% formamide and 7% glycerol (buffer II), and 50 mM Tris-HCl (pH 9.0)–20 mM NaCl–1% Triton X-100–0.1% gelatin–3 mM Mg^{2+} (buffer III) (3). Denaturation was done at 95°C for 70 s, annealing was done at 58°C (unless noted otherwise) for 60 s, and extension was done at 72°C for 60 s plus an additional 2 s per cycle for 30 cycles (program I). Subsequently, we found that sensitivity was improved by running amplification program I for only 2 cycles and linking it to a program with a 30-s denaturation step for 33 cycles; the other reaction parameters were unchanged (program II). Aliquots containing 25% of the reaction mixture were removed and subjected to agarose gel electrophoresis in 3% composite agarose gels (3 parts Nusieve:1 part standard LE agarose [FMC, Rockland, Maine]) or 1.5% LE agarose gels. The oligonucleotide primers were obtained commercially (Genosys, The Woodlands, Tex.). Southern blotting, DNA labeling and hybridization, and chemiluminescence detection (Photogene; GIBCO BRL, Gaithersburg, Md.) were done as described previously (9, 15). The bacterial strains used were described previously (9–11).

Detection of X. campestris pv. citri in lesions. Immature leaves of greenhouse-grown grapefruit (*Citrus paradisi*), were inoculated by placing 10-μl droplets of freshly grown *X. campestris* pv. citri XC320 (pathotype A, Florida) (optical density at 600 nm, 0.1) on the leaf surface and stabbing through the inoculum droplet with a sterile needle. After 7 months in the greenhouse, the lesions were old and dry at the time of the two assays described below. The first assay used purified DNA as the source of template DNA; the second used a crude water extract as the source of template DNA.

(i) **Assay 1.** For each sample, two lesions (or controls; 7.5 mg, fresh weight) were removed with a paper punch and ground together in liquid nitrogen, and DNA was extracted by a hexadecyltrimethylammonium bromide (CTAB) protocol (17). The powdered leaf disks were dispersed in 150 μl of extraction buffer (50 mM Tris [pH 8.0], 0.7 M NaCl, 10 mM EDTA, 1% CTAB, 0.1% 2-mercaptoethanol) and incubated at 60°C for 1 h. The mixture was extracted twice with chloroform-isoamyl alcohol (24:1), and the supernatant was precipitated with isopropanol and washed with 70% ethanol. The precipitate was dissolved in 150 μl of TE (10 mM Tris, 1 mM EDTA) buffer (pH 8.0) prior to the assay. The PCR assay was performed as described above in quadruplicate by use of primer pairs 2-3 and 1-5 and 5 μl of the extracts as the source of template DNA.

(ii) **Assay 2.** Individual lesions (or controls; 7.5 mg, fresh weight) were removed with a paper punch and minced with a razor blade in 100 μl of distilled H_2O (dH_2O). Five microliters of the resulting water extracts was used as the source of template DNA. Prior to amplification, the prepared reaction mixtures were incubated at 95°C for 10 min to lyse any bacteria and then at 55°C for 12 min after the addition of proteinase K to 10 μg/ml. Proteinase K was inactivated by a second 10-min incubation at 95°C. Tubes were then incu-

bated at 20°C for 3 min for the addition of Amplitaq DNA polymerase (Perkin-Elmer Cetus) (3). At this point, PCR amplification program I described above was carried out. Dilution series from these extracts were also prepared and plated on LPG_{CX} agar (peptone, 7 g; yeast extract, 7 g; glucose, 7 g; agar, 15 g; dH_2O, 1 liter; cycloheximide, 100 mg; pH 7.2), and the colonies that developed were enumerated. This assay was also performed with 3-week-old lesions incited by strain XC63 (pathotype A, Japan).

Estimates of limits of detection. Assay 2 described above was also used to estimate the limit of detection for bacterial strain XC63 after preparation of a simple dilution series. Aliquots of 10 μl from each dilution were used as template DNA. Aliquots of the dilution series were plated to enumerate the number of CFU per reaction. A dilution series of purified DNA from strain XC62 (pathotype A, Japan) was prepared and assayed as described above to estimate the limit of detection for purified homologous DNA by these methods. PCR amplification program II was used for these experiments, which were repeated once.

Nucleotide sequence accession numbers. The nucleotide sequence data presented in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession numbers X70682 and XCSEQ.

RESULTS

The DNA sequence of the 572-bp target fragment was 60.6% G+C (Fig. 1). Primer pairs 2-3, 4-5, 6-7, and 1-5 were expected to prime the amplification of products of 222, 462, 478, and 261 bp, respectively, with homologous (XC62) DNA as the target template (Fig. 1).

No amplification of homologous target DNA was achieved with buffer I or II at any annealing temperature from 45 to 65°C. The only exception was with primer pair 1-5, which successfully primed the amplification of its target sequence when annealed at 65°C (data not shown). In contrast, specific amplification products were produced in buffer III at all annealing temperatures from 45 to 65°C and with all four primer pairs. The results from the 55°C annealing reaction were typical (Fig. 2A). Nonspecific products were eliminated when the annealing temperature was 60°C (Fig. 2B).

Primer pair 2-3 was used to amplify target sequences in genomic DNA from 12 CBC pathotype A strains originally isolated in 12 countries (10, 11) (Fig. 3A). This primer pair did not find target sequences in DNA from five strains of pathotypes B and C of *X. campestris* pv. citri or in DNA from four strains of *X. campestris* associated with CBS (Fig. 3B). The other primer pairs detected all 12 pathotype A strains tested but yielded variable results with strains of pathotypes B and C (data not shown). A product was also found when DNA from single strains of *X. campestris* pv. vignicola and *X. campestris* pv. bilvae were tested with primer pair 2-3 (Fig. 3B) as well as with other primer pairs.

A dilution series of genomic DNA of strain XC62 was prepared, and aliquots were used as templates for PCR amplification. Aliquots that contained only 25 pg of genomic DNA were successfully detected after amplification (primer pair 2-3) (Fig. 4A). A dilution series of cultured cells of strain XC63 yielded a limit of detection of about 10 CFU per reaction after amplification (primer pair 2-3) and Southern blotting (Fig. 4B and C).

Specific amplification of target DNA was observed after CTAB extraction of 7-month-old, dry lesions incited by strain XC320 in four of four assays when two different primer pairs (1-5 and 2-3) were used (Fig. 5). Water extracts

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                    6 ─────────────────►
                       4 ───────────────────►
                    3 ──────────────────────►
5'CGTTGCACCT CCCGCTGCAT GGCGTTGGTG TCGTCGCTTG TATGGCCTAT AGTCGATACT GGAAATCATT ATATATATAC ATAGATAAGT
3'GCAACGTGGA GGGCGACGTA CCGCAACCAC AGCAGCGAAC ATACCGGATA TCAGCTATGA CCTTTAGTAA TATATATATG TATCTATTCA


  ATATATATAC CTATCAACCC TTGAGGAAGG GCTTCCAGTG CCTCGATCAC GATGTCCTTC TCCGTGACCC TGCCGCCTTC TCTTCCGAGG
  TATATATATG GATAGTTGGG AACTCCTTCC CGAAGGTCAC GGAGCTAGTG CTACAGGAAG AGGCACTGGG ACGGCGGAAG AGAAGGCTCC

                                                          1 ───────────────────►
  CCTGAGGCCT GCGCGCGCAG GTGATCAGCC AGTTCCTCGG GGAAGTTGAA GATTTTTTGC ACCCGTGCGC GGCCGTAGCG CTGCGTTTCC
  GGACTCCGGA CGCGCGCGTC CACTAGTCGG TCAAGGAGCC CCTTCAACTT CTAAAAAACG TGGGCACGCG CCGGCATCGC GACGCAAAGG
                                                       ◄─────────── 2

  TCAGCAGCGG GCGGCGTGGC TGCCGGTGTC GTGGTCACGG CAGCAGGTGC CACCAGCGGC TTCTCGGCCT TGTCCGCCTC CGCCTTCGAG
  AGTCGTCGCC CGCCGCACCG ACGGCCACAG CACCAGTGCC GTCGTCCACG GTGGTCGCCG AAGAGCCGGA ACAGGCGGAG GCGGAAGCTC


  GAAGCGTCGG TCTTGGCGGC TTGAACTGCG CCGTGTTGGG CTTGCCTTCA TGCTCATGCC ATCCACCTCC TTGAAGAAGG CTTCCATCTC
  CTTCGCAGCC AGAACCGCCG AACTTGACGC GGCACAACCC GAACGGAAGT ACGAGTACGG TAGGTGGAGG AACTTCTTCC GAAGGTAGAG


  GGCGATGGCG GCTTGATCGC GGCCCAACTC CTGAACGGTC GCACCCTCGC CAATGGCGCG GCGGAAGGCC ACGCGCTCGC AGACCTTGGT
  CCGCTACCGC CGAACTAGCG CCGGGTTGAG GACTTGCCAG CGTGGGAGCG GTTACCGCGC CGCCTTCCGG TGCGCGAGCG TCTGGAACCA
                                                      ───── 7
                            ◄────────────── 5
  GGGCAGCACG GTCAGCTTCT GCCTCGGCCA AG 3'
  CCCGTCGTGC CAGTCGAAGA CGGAGCCGGT TC 5'
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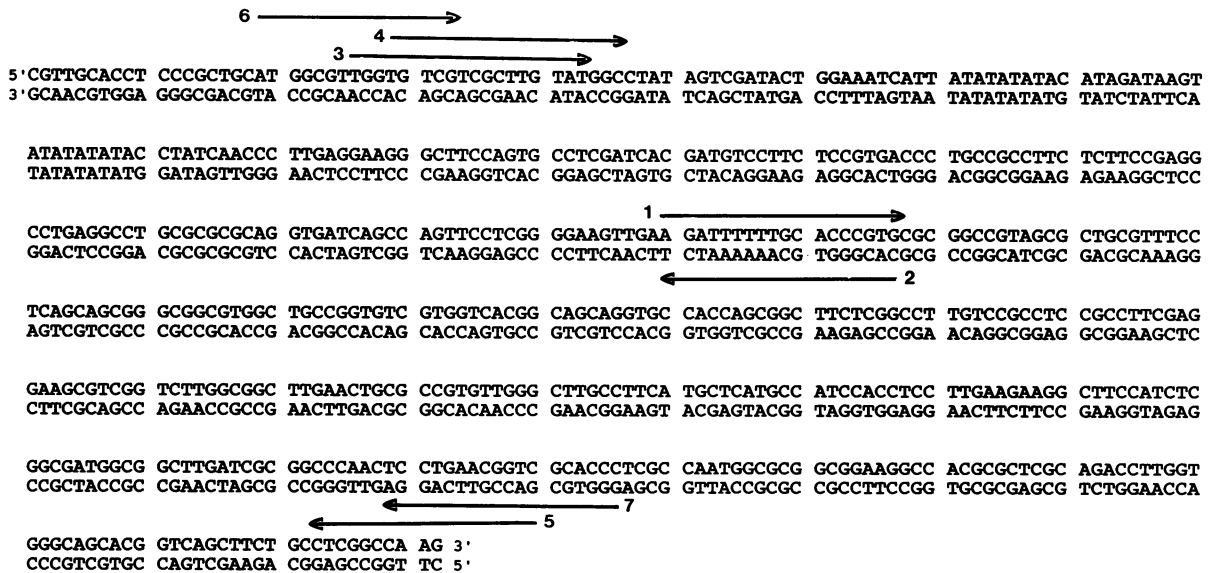
FIG. 1. DNA sequence of the 572-bp insert of pFL1. Arrows indicate the relative positions and direction of priming of the primers used in this study.

from this same set of lesions also produced specific products after amplification (Fig. 6A). These products were detectable even when the extracts were diluted 100-fold prior to amplification if the amplification products were then subjected to Southern blotting and hybridization with probe pFL1 (Fig. 6B). No products were observed from healthy noninoculated leaf disks assayed as controls, and no viable bacteria were recovered from the necrotic lesions used in these assays. The assays were repeated with succulent 3-week-old lesions incited by strain XC63, with similar results, except that specific detection in triplicate assays was achieved after 1,000-fold dilution, corresponding to 100 to 800 CFU per assay. Viable bacteria were recovered from these lesions (data not shown).

DISCUSSION

Because of the high G + C content and the fact that the target sequence was part of a plasmid, difficulty in achieving amplification was anticipated. Since buffer composition can affect PCR results, the standard PCR buffer recommended by Perkin-Elmer Cetus was compared with the same buffer supplemented with the cosolvents glycerol and formamide (19, 22) and with a third buffer (3). The composition of the reaction buffer was critical for the successful amplification of *X. campestris* pv. citri DNA. Buffer III differs from the standard PCR buffer in that it has a higher pH and contains 1% Triton X-100 and 0.1% gelatin. We do not know which of these ingredients is most responsible for the success of the amplification.
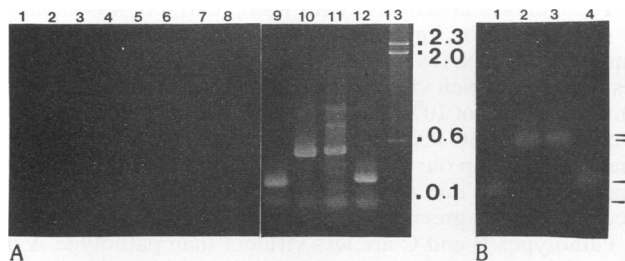

FIG. 2. (A) Successful amplification of target DNA is dependent on the reaction buffer. Lanes: 1 to 4, buffer I; 5 to 8, buffer II; 9 to 12, buffer III. Primer pair 2-3 was used for samples 1, 5, and 9; primer pair 4-5 was used for samples 2, 6, and 10; primer pair 6-7 was used for samples 3, 7, and 11; and primer pair 1-5 was used for samples 4, 8, and 12. The annealing temperature was 55°C. (B) As for panel A, lanes 9 to 12, except that the annealing temperature was 60°C. The sizes (in kilobases) of lambda *Hind*III standards (lane 13) are given between the panels. The template DNA was 50 ng of *Eco*RI-digested pathotype A (XC62) DNA for all reactions. Arrowheads in the right margin denote the expected positions of specific amplification products.
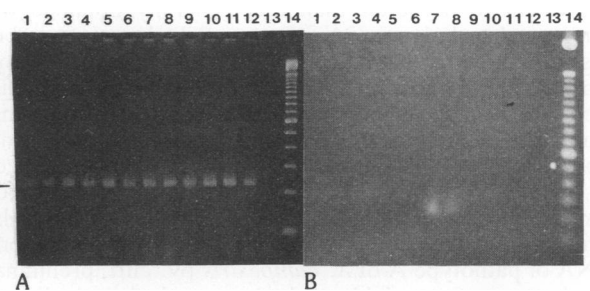

FIG. 3. Specific amplification of target DNA from pathotype A of *X. campestris* pv. citri by the PCR. (A) Template DNA was from pathotype A strains of *X. campestris* pv. citri from 12 countries. (B) Template DNA was from pathotype B and C strains of *X. campestris* pv. citri (lanes 1 to 5), *X. campestris* pv. alfalfae, *X. campestris* pv. bilvae, and *X. campestris* pv. vignicola (lanes 6 to 8), and CBS strains of *X. campestris* (lanes 9 to 12). The no-DNA control reaction was in lanes 13. Primer pair 2-3 was used, and annealing was done at 58°C. The GIBCO BRL 100-bp ladder was run in lanes 14, with the lowest band of 100 bp. The position of the predicted 222-bp product is marked in the margin.
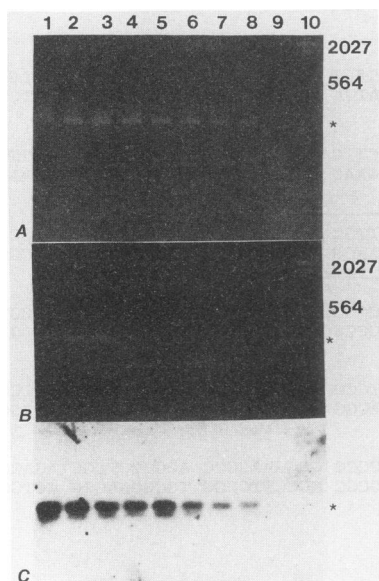
FIG. 4. Limits of detection of *X. campestris* pv. citri, starting with purified DNA and cultured cells. (A) Detection of 25 pg of strain XC62 DNA. Lanes: 1 to 8, 50 ng, 25 ng, 5 ng, 2.5 ng, 500 pg, 250 pg, 50 pg, and 25 pg of genomic DNA as the template, respectively. Primer pair 2-3 was used. (B) Dilution endpoint assay for the detection of strain XC63. Lanes 1 to 4 contained 1.6×10^5 to 1.6×10^2 CFU per reaction; lanes 5 to 8 contained 80, 40, 20, and 10 CFU/10 μl; lane 9 contained the dH$_2$O control. Primer pair 2-3 was used to prime amplification. The positions (*) and sizes (in kilobases) of lambda *Hind*III markers are given on the right. (C) Southern blot of the gel in panel B with biotinylated pFL1 as a probe. The position of the expected product is indicated by an asterisk.
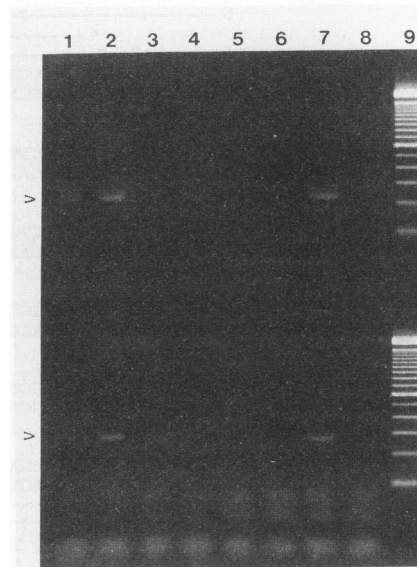

FIG. 5. Detection of strain XC320 DNA after CTAB extraction of necrotic leaf lesions. Lanes: 1 to 4, DNA extracted from four lesion pairs; 5 and 6, DNA extracted from healthy leaf controls; 7, positive control; 8, negative control (dH$_2$O); 9, 100-bp ladder. PCR assays with primer pairs 2-3 and 1-5 are shown in the top and bottom halves of the gels, respectively. Arrowheads denote the positions of the predicted products.

In this study, the PCR method used (amplification program II) allowed us to detect 10 CFU/10 μl or the equivalent of 1,000 CFU/ml. The simple reduction of the denaturation step (95°C) from 70 s (program I) to 30 s (program II) increased sensitivity about 20-fold (data not shown). Although this sensitivity does not represent the detection of a single cell, which is theoretically possible with the PCR, it is equivalent to or better than that generally obtained with serological techniques (i.e., enzyme-linked immunosorbent assay, indirect immunofluorescence) (20) by use of polyclonal (6) or monoclonal (1) antibodies. Plating on semiselective agar media (16) also has the potential to detect single viable cells but is more time-consuming. The application of "booster PCR" may well allow the detection of single cells of *X. campestris* pv. citri without blotting, as has been shown recently for *Agrobacterium tumefaciens* (14). Also, while amplification was always successful with genomic DNA of pathotype A of *X. campestris* pv. citri, preliminary results suggest that yield may be improved when the DNA is digested with *Bam*HI or *Eco*RI prior to amplification (data not shown). This possibility is probably due to the supercoiled (plasmid) state of the target DNA, which would allow it to reanneal more quickly after heat denaturation than linear DNA. The specificity of the reaction products was confirmed in all cases by the absence of a product in the negative controls, by the predicted product size, and by Southern blotting. Therefore, this PCR assay, whose sensitivity can doubtless be improved, will be a useful addition to previous detection methods because of the demonstrated

combination of speed, sensitivity, and specificity, which are critical parameters of any detection assay for bacteria.

Specific amplification of target DNA was successful when we started with intact bacteria (Fig. 4B and C). In previous work with probe pFL1 in a dot blot format, specific detection required purified DNA and was not possible with lysed bacteria because of the production of a nonspecific signal with the strepavidin-alkaline phosphatase-conjugated reagent used for chemiluminescence detection. Thus, the PCR-based assay presented here represents an increase in speed and flexibility as well as an increase in sensitivity of at least 100-fold over that of the previously used dot blot method of detection.

The increase in sensitivity for lesion extracts was similar. It should also be emphasized that *X. campestris* pv. citri pathotype A DNA was specifically detected in dry necrotic lesions from which viable bacteria were not recovered (Fig. 5). Populations of 10^6 CFU per lesion have been reported for 9-month-old CBC lesions on grapefruit (24). The absence of viable bacteria in our 7-month-old CBC lesions on grapefruit may have been due to the frequent high temperatures that occurred in our greenhouse during this period.

Pathotypes B and C are less virulent than pathotype A of *X. campestris* pv. citri and are much less widely distributed (4); therefore, they pose less of a threat to the citrus industry. The clear detection of pathotype A and the lack of detection of pathotypes B and C represent a useful complement to the previously used serological and dot blot assays (1, 6, 9). The results of these PCR assays are consistent with the results of a hybridization analysis of these same strains, in which pathotype B and C strains yielded consistently weaker results than pathotype A strains in dot blot assays (9) and produced homologous bands of different sizes in Southern blots (15). DNAs from other bacterial species and from other pathovars of *X. campestris* were not detected in dot
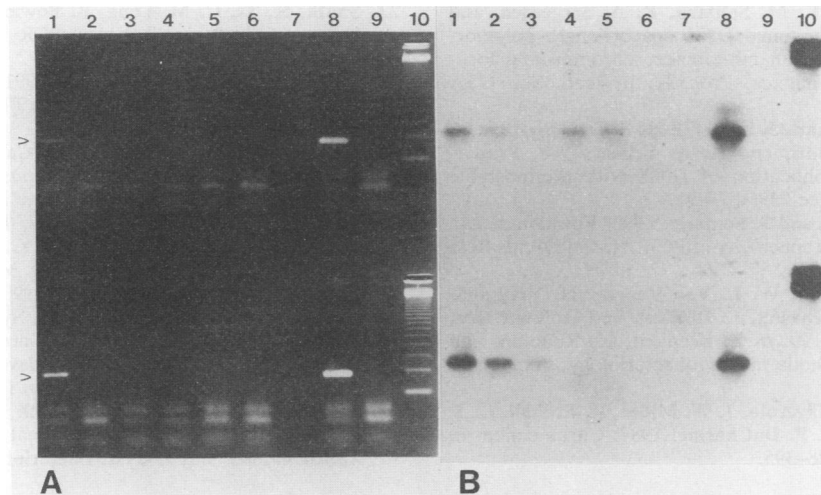
FIG. 6. (A) Detection of strain XC320 directly in exudates from single cankers. (Upper half of the gel) Lanes: 1 to 3 and 4 to 6, 10-fold dilution series from lesions 1 and 2; 7, empty; 8, positive control (XC104 DNA); 9, negative control (dH₂O); 10, 123-bp ladder. (Lower half of the gel) Lanes: 1 to 3 and 4 to 6, 10-fold dilution series from lesion 3 and from the healthy control; 7 to 10, as in the upper half of the gel. Amplification was directed by primer pair 2-3. (B) Southern blot of the gel in panel A probed with pFL1. Arrowheads denote the positions of the predicted products.

blot assays with pFL1 as a probe, except for *X. campestris* pv. vignicola and *X. campestris* pv. bilvae (9), analogous to the PCR results reported here. These strains are not known to occur on citrus and are unlikely to cause problems in the practical application of these methods, although caution is warranted.

Significantly, none of the primer pairs found targets in the limited number of CBS strains tested (Fig. 3B), consistent with the results of dot blot assays with pFL1 as a probe for 54 CBS strains (9). It is, however, conceivable that nonspecific amplification products could be produced by untested bacterial strains with the primers described here. However, if observed, these could be readily distinguished from specific amplification products on the basis of size and hybridization analyses. The excellent specificity of detection demonstrated for pFL1 in the dot blot experiments was retained by the PCR assay. PCR-based detection will be a useful addition to detection methods used for pathotype A of *X. campestris* pv. citri, which is the target of international quarantine regulations (2).

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