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Sensitive and Specific Detection of *Xanthomonas campestris* pv. pelargonii with DNA Primers and Probes Identified by Random Amplified Polymorphic DNA Analysis†

SHULAMIT MANULIS,[1] LEA VALINSKY,[1] AMNON LICHTER,[1] AND DEAN W. GABRIEL[2]*

Department of Plant Pathology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel,[1] and Department of Plant Pathology, University of Florida, Gainesville, Florida 32611[2]

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The random amplified polymorphic DNA method was used to distinguish strains of *Xanthomonas campestris* pv. pelargonii from 21 other *Xanthomonas* species and/or pathovars. Among the 42 arbitrarily chosen primers evaluated, 3 were found to reveal diagnostic polymorphisms when purified DNAs from compared strains were amplified by the PCR. The three primers revealed DNA amplification patterns which were conserved among all 53 strains tested of *X. campestris* pv. pelargonii isolated from various locations worldwide. The distinctive *X. campestris* pv. pelargonii patterns were clearly different from those obtained with any of 46 other *Xanthomonas* strains tested. An amplified 1.2-kb DNA fragment, apparently unique to *X. campestris* pv. pelargonii by these random amplified polymorphic DNA tests, was cloned and evaluated as a diagnostic DNA probe. It hybridized with total DNA from all 53 *X. campestris* pv. pelargonii strains tested and not with any of the 46 other *Xanthomonas* strains tested. The DNA sequence of the terminal ends of this 1.2-kb fragment was obtained and used to design a pair of 18-mer oligonucleotide primers specific for *X. campestris* pv. pelargonii. The custom-synthesized primers amplified the same 1.2-kb DNA fragment from all 53 *X. campestris* pv. pelargonii strains tested and failed to amplify DNA from any of the 46 other *Xanthomonas* strains tested. DNA isolated from saprophytes associated with the geranium plant also did not produce amplified DNA with these primers. The sensitivity of the PCR assay using the custom-synthesized primers was between 10 and 50 cells. The techniques used could provide a general method to identify pathovar-specific DNA primers for rapid, sensitive, and specific detection and identification of plant-pathogenic bacteria.

The most important disease of the florist's geranium (*Pelargonium* x *hortorum*) is caused by *Xanthomonas campestris* pv. pelargonii (Brown) Dye (3, 14). Symptoms are characterized by wilting of the plant, localized water-soaked lesions, and stem rot (4, 5). This disease is widespread in various states of the United States and in Europe, Australia, and Israel and may cause heavy losses, particularly where geranium cuttings are propagated on a large scale. Under conditions unfavorable for disease development, the bacterium can survive on plant surfaces (epiphytically) or as latent infections within plants. The movement of infected, asymptomatic plants between greenhouses is the major means of dispersal of the pathogen (4). All commercial cultivars of geranium are susceptible to *X. campestris* pv. pelargonii. There is no effective chemical control, and therefore, the only practical method available today for controlling the disease is the use of disease-free planting material obtained after indexing the propagating stock. Geraniums are vegetatively propagated from these culture-indexed plants.

Currently, diagnosis of *X. campestris* pv. pelargonii is based on attempts to isolate the pathogen from plants in culture and serological assays using polyclonal antibodies (1, 15). Although the diagnostic procedures are generally satisfactory, false-positive results often occur. This can cause losses since growers are advised to discard all suspect plants. In addition, outbreaks of the disease sometimes occur in greenhouses in spite of the intensive culture indexing. These outbreaks may be the result of failure of the diagnosis methods to detect latent infections or low levels of epiphytic populations of the pathogen. Variation in the surface antigens of the pathogen may also lead to false-negative results with serological assays. Obviously, an additional reliable and more sensitive diagnostic procedure, based on a different principle, is necessary.

The objective of this research was to develop a sensitive, specific, and useful DNA-based method for identification of *X. campestris* pv. pelargonii. Since knowledge of the molecular biology of *X. campestris* pv. pelargonii is very scanty, the random amplified polymorphic DNA (RAPD) technique (18, 19) was chosen as a first step to evaluate variation among strains included within the pathovar. This technique uses the PCR to evaluate a number of different DNA primers of arbitrary sequence for the ability to amplify a unique pattern of DNA fragments. It does not require any prior information on the molecular biology of the organism, is technically easy and rapid, and requires only small amounts of DNA. We describe here the use of the RAPD technique to identify a unique, pathovar-specific DNA fragment. This DNA fragment was cloned, a partial DNA sequence was determined, and DNA primers that specifically amplified DNA only from strains of *X. campestris* pv. pelargonii were synthesized. The method used may have general application.

(A preliminary report on this study has been presented previously [10].)

* Corresponding author. Phone: (904) 392-7239. Fax: (904) 392-6532.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All strains of *X. campestris* pv. pelargonii

TABLE 1. *Xanthomonas* strains used

Xanthomonas strain and pathovar	Strain	Origin[a]
X. campestris pv.		
pelargonii	1/1, 27, 32, 35/23, 35/100, 129, 53/7, 54/16, 305, 479, 481, 1353, 1610, G, V/2, 7/4, 14/8, 28/1, 28/3, 28/4, 29/1, 29/2, CHSC	Israel
	068-877, 1360, 1362, 1366, 1369, 1370, 1750, 1786, 1792, 1799	Florida
	2007, 2008, 2009, 2303, 2304, 2527, 074-270, 073-2736	New York
	3105, 3106, 3108, 3112, 3114, 3116	Germany
	3138, 3139, 3140, 3141, 3142, 3143	Mexico
alfalfae	KX-1	Kansas
armoraciae	417	California
begoniae	077-3382	Florida
campestris	385, 418, 602, 528	Israel
citrumelo	3048	Florida
cyamopsidis	13D5	Arizona
	UCD	California
dieffenbachiae	X-326, 084-729	Florida
esculenti	084-1093	Florida
glycines	ATCC 17915	ATCC
	B-9-3	Brazil
holcicola	Xh66	Kansas
malvacearum	N, TX84	Texas
malvacearum-hibiscus	M84-11, X108, X10, X27	Florida
pisi	XP1	Japan
pruni	068-1008	Florida
translucens	82-1	Florida
vesicatoria	82-23	Florida
	592	Israel
vignicola	A81-331, CB5-1, XV19	Georgia
X. albilineans	Xalb	Florida
X. citri	3213	Florida
	635	New Zealand
X. fragariae	070-1277	Florida
X. phaseoli	LB-2, SC-3B, EK11, Xph25, Xpf11	Nebraska
	XP-JL	Kansas
	82-1, 82-2	Florida
	XP-JF	Missouri
	Bx-P118, Bx-P200, Bx-P203	Canada

[a] Strains were obtained from the collections of D. Zutra, J. Tammen, and D. W. Gabriel.

obtained in Israel were isolated from geranium plants by D. Zutra (ARO, Volcani Center, Israel). Other strains of *X. campestris* pv. pelargonii were from the collection of J. Tammen (The Pennsylvania State University, University Park). All strains were cultured routinely at 28°C on medium containing 5 g of peptone, 5 g of yeast extract, 11 g of glucose (sucrose for *Xanthomonas albilineans*), and 15 g of agar per liter. Liquid cultures for DNA extraction were grown on TY medium containing 5 g of tryptone, 3 g of yeast extract, and 0.9 g of $CaCl_2 \cdot 2H_2O$ per liter. Bacterial strains were stored at −80°C in 15% glycerol.

To isolate naturally occurring saprophytic bacteria associated with the geranium plant, small pieces of geranium cuttings were shaken overnight in a 0.9% NaCl solution at 4°C. The supernatant was centrifuged, and the pellet was resuspended in 0.5 ml of sterile water. A 10-liter sample was directly used for the PCR tests described below, and 50 μl was spread on the agar medium described above. The plates were incubated for 2 to 3 days at 28°C. The total bacteria growing on the plates was collected, and the DNA was extracted and used for PCR tests. These experiments were repeated five times. In addition, 20 individual colonies were isolated in pure culture and tested for pathogenicity on geranium plants and serologically as described below. The DNA was also extracted from each of these colonies and used for PCR tests.

Pathogenicity tests and ELISAs. Pathogenicity tests and enzyme-linked immunosorbent assays (ELISAs) of all *X. campestris* pv. pelargonii strains listed in Table 1 and on saprophytic bacteria isolated from geranium plants were carried out. *Pelargonium* x *hortorum* 'Palais' plants were used for pathogenicity assays. Inoculations were performed by puncturing the lower part of the stem with a needle dipped in an overnight culture of bacteria. The inoculated plants were examined for symptom development over a period of 3 weeks. All *X. campestris* pv. pelargonii strains produced typical disease symptoms, i.e., the stem turned brown to black, and in the final stage, the stem collapsed and rotted.

The competitive indirect ELISA was performed as described previously (9) with antiserum prepared against *X. campestris* pv. pelargonii strain 305.

DNA manipulations. Bacterial DNA was isolated as described by Lazo et al. (7). The procedures for agarose gel electrophoresis, staining, Southern blotting, restriction enzyme digestions, ligation, and transformation were as described by Sambrook et al. (12). Total DNA samples were electrophoresed in 0.8% agarose gels. DNA samples after PCR amplification were electrophoresed in 1.2% agarose gels. Hybridizations were performed at 42°C with DNA probes labelled with a digoxigenin-11-dUTP kit (Boehringer GmbH, Mannheim, Germany) as described in the manufacturer's instructions. PCR-amplified DNA fragments used as probes were recovered from agarose and purified by using the Geneclean kit (Bio 102, Inc., San Diego, Calif.). Purified, PCR-amplified DNA fragments were cloned by direct ligation into commercially prepared, linearized pGEM-T vector fragments exactly as described by the manufacturer (Promega Corp., Madison, Wis.). DNA sequencing was performed with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

Primers and PCR conditions. For RAPD analysis, PCR amplifications were carried out in 25-μl volumes and contained 50 ng of genomic DNA, 2 mM $MgCl_2$, 0.4 μM primer, 1.5 U of *Taq* DNA polymerase (Promega), 100 μM (each) deoxynucleoside triphosphate (dNTP) (Boehringer) in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% Triton X-100, under 2 drops of mineral oil. Amplification was performed in a thermal cycler (Hybaid, Teddington, Middlesex, United Kingdom) programmed for one cycle of 5 min at 94°C, 1 min at 35°C, and 2 min at 72°C; 40 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C; and a final extension for 5 min at 72°C. Ten-base oligonucleotide primers were purchased from Operon Technologies Inc., Alameda, Calif., 42 different arbitrary primers were tested from kits B, D, and F. The sequences of primers D5, D7, and D11 were 5′-TGAGCGGACA, 5′-TTGGCACGGG, and 5′-AGCGCCATTG, respectively.

For specific amplification of *X. campestris* pv. pelargonii, two 18-mer oligonucleotides from the borders of the 1.2-kb fragment were chosen for custom synthesis. DNA synthesis was performed by the ICBR DNA Sequencing Core Facility (University of Florida, Gainesville). The sequences of the primers were 5′-GAGTGTCCAGTGGCAAGC and 5′-GTTGCTGC CTCTTCCTGC. The 25-μl reaction mixture contained 1 mM $MgCl_2$, 0.2 μM each primer, 1 U of *Taq* DNA polymerase, and 50 μM each dNTP and template DNA and buffer as described above. Denaturation was done at 94°C for 5 min and then for

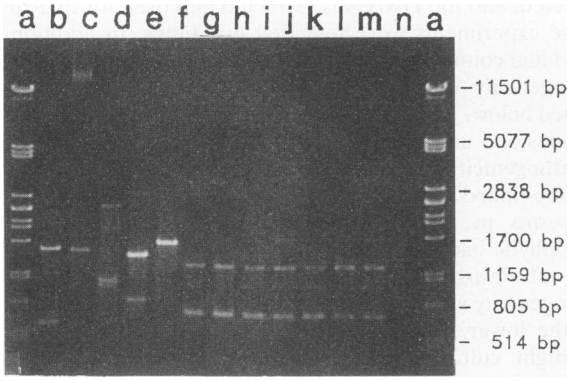

FIG. 1. RAPD patterns of different *X. campestris* pathovars generated with primer D5. Lanes: a, lambda DNA digested by *Pst*I; b to f, *X. campestris* pathovars campestris (strain 385; lane b), Vesicutoria (strain 592; lane c), citri (strain 3213; lane d), begoniae (strain 077-3382; lane e), and glycines (strain B-9-3; lane f); g to m, *X. campestris* pv. pelargonii strains 305 (lane g), 3114 (lane h), 3112 (lane i), 2009 (lane j), 129 (lane k), 1360 (lane l), and 068-877 (lane m); n, control reaction without DNA.

40 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C and for a final extension for 5 min at 72°C.

To determine the detection limits of these 18-mer primers, 10-fold serial dilutions of *X. campestris* pv. pelargonii strain 305 were made. In each of six experiments, 5-μl aliquots from each tube of the dilution series were added directly to the PCR mixture. The actual number of colonies in each series was determined by plating on solid agar medium.

RESULTS

RAPD analysis of *X. campestris* pv. pelargonii. To identify primers that generate RAPD patterns characteristic of *X. campestris* pv. pelargonii, we tested 42 different 10-mer primers with DNA extracted from five strains of the pathogen. Nine primers gave reproducible PCR banding patterns with strains of *X. campestris* pv. pelargonii and amplified from two to five DNA fragments of different sizes. The sizes of the products ranged from 0.5 to 2.5 kb. Three of these primers, D5, D7, and D11, were selected for comparative analyses by using DNA from other xanthomonads. All of the strains listed in Table 1 were subjected to PCR amplification with each of the three primers. Representative results are shown in Fig. 1 and 2. Amplification patterns obtained with primer D5 revealed two major products of 1.2 and 0.6 kb (Fig. 1). This pattern was common to all 53 *X. campestris* pv. pelargonii strains isolated from various geographical locations. This RAPD profile was also clearly distinguishable from those produced by all 46 other *Xanthomonas* strains tested (Table 1). Distinctive RAPD patterns were also obtained with primers D7 (not shown) and D11 (Fig. 2A).

Selection of a DNA fragment specific to *X. campestris* pv. pelargonii. The 1.2-kb DNA fragment amplified by primer D11 (Fig. 2A) was selected for further analyses. To test for homology among the similarly sized amplification products obtained with primer D11 and all *X. campestris* pv. pelargonii strains, the 1.2-kb DNA fragment was purified from an agarose gel, labelled with digoxigenin, and used as a probe against Southern blots and spot blots of DNA amplified by PCR from all strains in Table 1 by using the same primer. A Southern blot of the PCR products that hybridized with the 1.2-kb probe is illustrated in Fig. 2B. These results indicated that all 1.2-kb

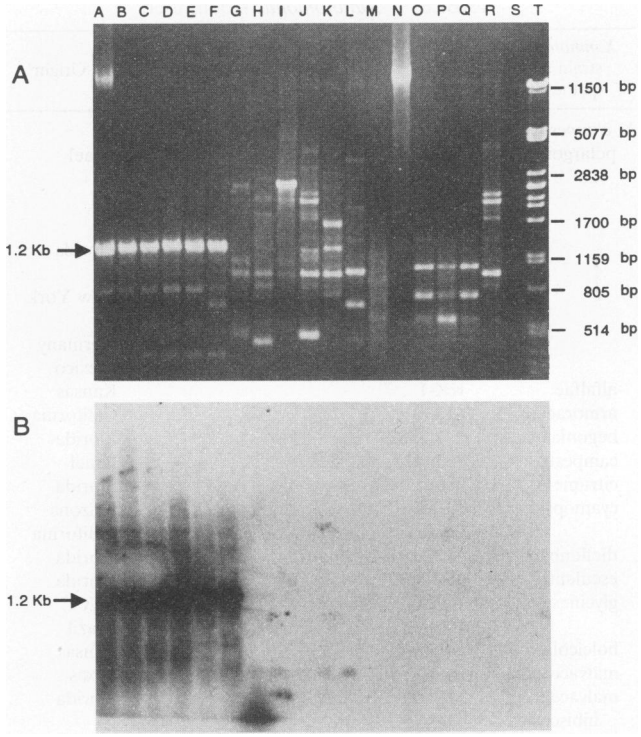

FIG. 2. (A) RAPD patterns of different *X. campestris* pathovars generated with primer D11. Lanes: A to F, *X. campestris* pv. pelargonii strains 305 (lane A), 479 (lane B), 1360 (lane C), 2008 (lane D), 3105 (lane E), and 3139 (lane F); G to R, *X. campestris* pathovars phaseoli (strain LB-2; lane G), citri (strain 3213; lane H), citrumelo (strain 3048; lane I), cyamopsidis (strain 13D5; lane J), malvacearum (strain N; lane K), dieffenbachiae (strain X-326; lane L), alfalfa (strain KX-1; lane M), glycines (strain B-9-3; lane N), and phaseoli (strains XP-JF [lane O], 82-2 [lane P], Xph25 [lane Q], and 82-1 [lane R]); S, control reaction without DNA; T, lambda DNA digested by *Pst*I. (B) Southern blot of the gel in panel A probed with a 1.2-kb fragment. Arrows indicate the size of the specific PCR product and the hybridized band.

DNA fragments which were generated with primer D11 from different *X. campestris* pv. pelargonii strains were homologous. No hybridization occurred with the PCR products of any other *X. campestris* strain that amplified with primer D11 (Fig. 2B). In dot blot experiments (results not shown), the 1.2-kb fragment hybridized with the PCR products of all *X. campestris* pv. pelargonii strains tested but not with those of any other *Xanthomonas* strain tested (Table 1).

To determine whether the entire 1.2-kb DNA fragment was unique to *X. campestris* pv. pelargonii, the 1.2-kb fragment was used as a hybridization probe against total DNA extracted from all strains shown in Table 1. The total DNA of 15 *X. campestris* pv. pelargonii strains and 10 strains representing different *Xanthomonas* pathogenicity groups was digested with *Sma*I, size fractionated on agarose gels, and subjected to Southern hybridizations. Representative results revealing that two bands with approximate sizes of 6.0 and 3.1 kb were present only in DNA of *X. campestris* pv. pelargonii strains are shown in Fig. 3. In dot blot experiments (results not shown), the 1.2-kb fragment hybridized with total DNA extracted from all *X. campestris* pv. pelargonii strains tested but not with that of any other *Xanthomonas* strain tested (Table 1).

Specific primers for detection of *X. campestris* pv. pelargonii by PCR. The 1.2-kb amplified fragment was cloned into the

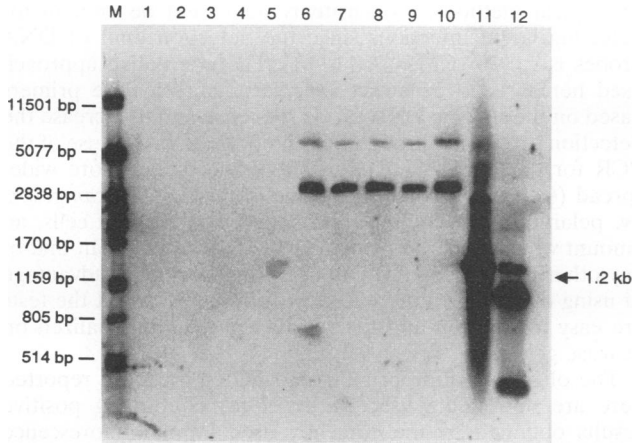

FIG. 3. Southern transfer of *Sma*I-restricted DNA of *X. campestris* strains probed with a 1.2-kb fragment. Lanes: M, lambda DNA digested by *Pst*I; 1 to 5, *X. campestris* pathovars dieffenbachiae (strain 084-729; lane 1), begoniae (strain 077-3382; lane 2), cyamopsidis (strain 13D5; lane 3), phaseoli (strain LB-2; lane 4), and campestris (strain 602; lane 5); 6 to 10, *X. campestris* pv. pelargonii strains 305 (lane 6), 35/23 (lane 7), 1366 (lane 8), 3106 (lane 9), and 3139 (lane 10); 11, RAPD products generated with primer D11; 12, 1.2-kb fragment partially digested by *Sma*I. An arrow indicates the approximate size of the probe.

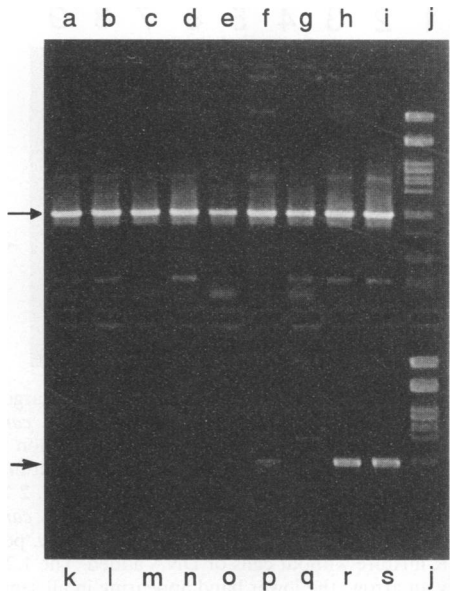

FIG. 4. Electrophoretic analysis of PCR-amplified DNA from *X. campestris* pathovars by using the specific primer pair for *X. campestris* pv. pelargonii. The upper arrow shows the size of the specific 1.2-kb PCR product. Lanes: a to i, p, r, and s, *X. campestris* pv. pelargonii strains 3139 (lane a), 1610 (lane b), 2527 (lane c), 1366 (lane d), 53/7 (lane e), 35/100 (lane f), 27 (lane g), 1/1 (lane h), 54/16 (lane i), 28/1 (lane p), 29/2 (lane r), and 479 (lane s); k to o and q, *X. campestris* pathovars dieffenbachiae (strain X-236; lane k), malvacearum (strain n; lane l), cyamopsidis (strain 13D5; lane m), citri (strain 3213; lane n), phaseoli (strain LB-2; lane o), and vesicatoria (strain 592; lane q); j (upper and lower lanes), molecular weight markers.

vector pGEM-T, and the DNA sequence of the first 300 bases from both terminal ends of the fragment was determined. Based on these DNA sequences, two 18-mer oligonucleotides were synthesized and used for subsequent PCR amplification tests. These 18-mer primers were expected to amplify a DNA fragment 180 bp shorter than the D11 RAPD primer since the DNA sequence upon which these primers were based was internal to the D11 primer sequence that formed the termini of the 1.2-kb fragment. To determine the specificity of these primers, PCRs were carried out with DNA of all of the strains listed in Table 1. Representative results are shown in Fig. 4. Amplification of *X. campestris* pv. pelargonii DNA with these primers produced one major band of the expected (1.2-kb) size. Occasionally, *X. campestris* pv. pelargonii samples produced only a weakly amplified 1.2-kb DNA fragment in some experiments (e.g., Fig. 4, lane p), but this was found to be due to sampling error and was not characteristic of any *X. campestris* pv. pelargonii strains tested. No amplification was observed with any *Xanthomonas* strain tested other than *X. campestris* pv. pelargonii.

The 1.2-kb DNA fragment amplified by RAPD primer D11 hybridized to the 1.2-kb fragment amplified by the specific 18-mer primers (blot not shown), indicating that the region amplified by the 18-mer primers is the same as the 1.2-kb region amplified by D11.

The 18-mer primers used in these PCR tests were evaluated comparatively against the *X. campestris* pv. pelargonii strain 305 antibody for accuracy in identification of *X. campestris* pv. pelargonii strains. All of the strains listed in Table 1 were subjected to ELISAs. Two *X. campestris* pv. pelargonii strains from Florida (068-777 and 1362) gave false-negative results, and one *X. campestris* pv. vignicola strain (CB5-1) gave a false-positive result. By contrast, the PCR tests using the specific 18-mer primers were entirely consistent with the pathogenicity tests. PCR tests and ELISAs were also compared by using saprophytic bacteria isolated from geranium plants. PCR amplifications were carried out with five pooled samples of saprophytic bacteria and with 25 purified colonies of saprophytic bacteria, all isolated from geranium plants. None of these bacteria exhibited an amplified PCR product, and none was pathogenic to geranium plants. However, 16 of the 25 purified colonies of saprophytic bacteria gave (false) positive ELISA results.

To determine the limit of detection of the PCR assay with these primers, serial dilutions of *X. campestris* pv. pelargonii culture were used as templates for PCR (Fig. 5). In dilution series tests repeated six times, the detection limit was found to be between 10 and 50 bacterial cells.

DISCUSSION

The economic importance of bacterial blight disease of geranium plants necessitates sensitive, reliable, and rapid detection methods. The serological methods developed for diagnosis of *X. campestris* pv. pelargonii (1, 2, 15), although considered reliable and rapid, are not sensitive enough to detect low levels of the pathogen. Furthermore, serological methods are not always reliable. In this study, two strains of *X. campestris* pv. pelargonii isolated in Florida did not react with the polyclonal antibodies in the ELISA, and 12 nonpathogenic strains isolated from geranium plants did react with the polyclonal antibodies in the ELISA. By contrast, the custom-synthesized primers, based on a pathovar-specific DNA sequence, were reliable. The 18-mer pathovar-specific primers correctly identified all *X. campestris* pv. pelargonii strains and distinguished these from all saprophytes isolated from geranium plants and all other xanthomonads tested.

DNA-based methods have provided powerful tools to iden-

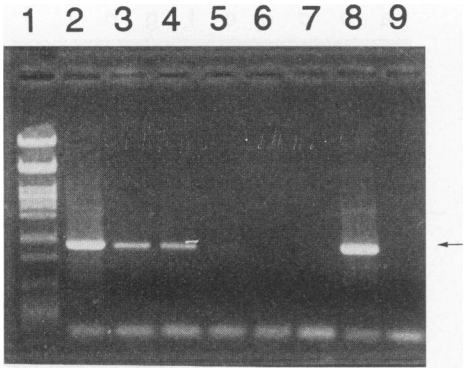

FIG. 5. Detection limit of the *X. campestris* pv. pelargonii-specific DNA primers and PCR. Tenfold serial dilutions of *X. campestris* pv. pelargonii strain 305 were used; 5 μl of each dilution was added directly to the PCR mixture. Lanes: 1, molecular weight markers; 2 to 7, 2×10^6 (lane 2), 2×10^5 (lane 3), 2×10^4 (lane 4), 2×10^3 (lane 5), 2×10^2 (lane 6), and 2×10^1 (lane 7) CFU of *X. campestris* pv. pelargonii per ml; 8, 10 ng of DNA of *X. campestris* pv. pelargonii; 9, control PCR mixture without cells or DNA added. The 1.2-kb band is indicated by an arrow; the lower band appearing in all samples is that of the 18-mer primers used.

tify microorganisms with high sensitivity and specificity. In this study, we have utilized the RAPD procedure to identify a specific DNA fragment unique to, and conserved in, all tested strains of *X. campestris* pv. pelargonii. This highly conserved DNA fragment appeared to be useful as a pathovar-specific DNA probe, and its DNA sequence provided the basis for synthesizing pathovar-specific oligonucleotide primers useful for sensitive detection of *X. campestris* pv. pelargonii. An alternative approach to that of using RAPD primers to identify a specific, highly conserved DNA fragment is to use subtraction hybridization for the same purpose (13). The disadvantages of subtraction hybridization are that they are much more difficult to perform and the identified fragments are likely to be strain specific, not pathovar or species specific.

The clonality of *X. campestris* pv. pelargonii was probably important to the ease of identification of a unique, highly conserved, and pathovar-specific DNA fragment. A number of different strains of *X. campestris* pv. pelargonii isolated from various geographical locations and from different geranium cultivars produced identical RAPD patterns with several arbitrary primers. These results support the clonal structure of the pelargonii pathovar which had previously been suggested on the basis of numerical analysis of protein electrophoresis (16, 17). For pathovars which consist of several different clonal groups, it may be necessary to identify several different DNA fragments, each unique and therefore specific for a different clonal group. For pathovars which exhibit little clonality, identifying unique, pathovar-specific DNA fragments may be more difficult. However, the large number of commercially available arbitrary primers and the ease with which each can be evaluated for polymorphisms may make the RAPD technique generally useful for the identification of pathovar- and species-specific DNA fragments.

DNA-based diagnostic methods appear to offer several advantages relative to serological methods. In this study, the DNA probe used was at least as sensitive as the serological methods ordinarily used for the detection of *X. campestris* pv. pelargonii in geranium plants (7a) but had the added advantage of being much more specific. In spite of the increased specificity of the DNA probe compared with that of serological methods, its sensitivity might not be enough for detecting latent infections since the detection limit of DNA probes is ca. 10^5 CFU (8, 11, 13). The alternative approach used here was to construct specific oligonucleotide primers based on the specific DNA probe sequence and to increase the detection sensitivity through PCR amplification. The use of the PCR for detecting plant pathogens is becoming more widespread (6). By constructing specific primers for *X. campestris* pv. pelargonii, we could detect between 10 and 50 cells, an amount which should provide a greater sensitivity than that of any other method for this pathogen. An additional advantage of using oligonucleotide probes with the PCR is that the tests are easy to perform and the results are readily visualized on agarose gels.

The oligonucleotide primers and PCR procedure reported here are now being used in Israel for confirming positive results obtained by the currently used immunofluorescence method. A further simplification of this procedure is desirable to apply it to the large-scale screening of geranium mother plants.

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