Plasmid-Based Hybridization Probes for Detection and Identification of *Xanthomonas campestris* pv. *citri*

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ABSTRACT

Hartung, J. S. 1992. Plasmid-based hybridization probes for detection and identification of *Xanthomonas campestris* pv. *citri*. Plant Dis. 76:889-893.

Two hybridization probes were developed from plasmid DNA of pathotype A strain XC62 of *Xanthomonas campestris* pv. *citri*. Plasmid pFL62.42 was constructed by cloning a 4.2-kb *Bam*HI fragment of an indigenous plasmid of strain XC62 in the vector pUC9. Plasmid pFL1 contains an internal 700-bp *Eco*RI fragment from pFL62.42; this fragment was also cloned in the vector pUC9. The plasmid probes were highly specific for *X. c. citri*. Both plasmids hybridized with DNA purified from 44 pathotype A strains of *X. c. citri*, which had been isolated from diseased *Citrus* spp. in 15 countries. A dot blot format with chemiluminescent detection by biotinylated probes was used for these experiments. Probe pFL62.42 was also used to detect 13 of 15 pathotype B, C, and D strains of *X. c. citri*, and probe pFL1 was used to detect 11 of 15 pathotype B, C, and D strains of *X. c. citri*. The limit of detection for pathotype A was between 2 and 7 ng of DNA per dot, and the limit of detection for pathotypes B, C, and D was about two- to fourfold higher. Neither plasmid hybridized with DNA from 56 strains of *X. campestris* associated with citrus bacterial spot disease. The probes also did not hybridize with DNA from four epiphytic strains of *X. campestris* isolated from *Citrus* foliage or with DNA from *Flavobacterium balustinum*, *Enterobacter cloacae*, *Escherichia coli*, *Erwinia carotovora*, *E. amylovora*, and *Pseudomonas putida*. When 16 other pathovars of *X. campestris* were screened by DNA dot blotting, limited cross reaction was observed with plasmid pFL62.42. Specificity of detection was improved with plasmid pFL1. Only DNA from *X. c. vignicola* and from a single strain of *X. c. bilvae* hybridized with plasmid pFL1. Probe pFL1 also specifically detected pathotype A of *X. c. citri* in extracts of leaf lesions. These probes will be useful in detecting *X. c. citri*.

Two diseases of *Citrus* spp. are caused by *Xanthomonas campestris*. Citrus bacterial canker (CBC), caused by *X. c.* pv. *citri* (Hasse) Dye, is a serious disease with a near worldwide distribution (4,29), recently including Florida. Citrus bacterial spot (CBS), caused by other strains of *X. campestris*, causes minor foliar damage primarily in citrus nurseries and is no longer considered a threat to the citrus industry. Because of the magnitude of the perceived threat to the citrus industry, detection of *X. c. citri* has been the objective of plant quarantines in both the United States and abroad (2), and the development of diagnostic methodologies has been a high priority. Because of the occurrence of strains of *X. campestris* that incite CBS on *Citrus* spp. and closely related plants, a high degree of specificity is now required. Monoclonal antibodies have potential as diagnostic probes (1,14). DNA-based probes are another promising approach. DNA probes derived from genomic DNA have been useful in restriction fragment length polymorphism (RFLP) studies (10,17). However, they are not useful as diagnostic probes, because the capacity to cross-react with other pathovars of *X. campestris*, which is useful in RFLP studies, compromises their specificity.

Pathotypes within *X. c. citri* have long been identified by host range, geographical origin, bacteriophage sensitivities, plasmid content, and serology (4–6,29). Pathotype A has the widest host range and a global distribution. Pathotypes B, C, and D have so far been restricted to lemon (*Citrus limon*) and lime (*Citrus aurantifolia*) in South America and Mexico (4,29). The existence of these pathotypes was confirmed at the DNA level by genomic fingerprinting (16) and RFLP analyses (10,17).

Strains of *X. campestris* that incite CBS disease can be distinguished from strains of *X. c. citri* by use of all of the methods mentioned above, as well as by symptomology (28) and DNA-DNA hybridization (9). The disease has been found in Florida citrus nurseries in spite of an eradication campaign.

RFLP analyses of plasmid DNA have been used to distinguish strains of *Pseudomonas syringae* pv. *pisi* (19) and also the several pathotypes of *X. c. citri* (24). In the course of the latter study, a 4.2-kb *Bam*HI fragment of plasmid DNA was cloned from strain XC62 (pathotype A) and shown to distinguish pathotypes of *X. c. citri* (24). The purpose of the present study was to evaluate the 4.2-kb *Bam*HI plasmid fragment and a subclone from it as hybridization probes for *X. c. citri*. The ability of the probes to detect *X. c. citri* was evaluated in tests with a unique, worldwide collection of strains of *X. c. citri*. The specificity of the probes was evaluated in tests with a wide variety of strains of other pathovars of *X. campestris*, especially strains of *X. campestris* associated with CBS, as well as bacteria from other genera.

MATERIALS AND METHODS

Bacterial strains. Fifty-nine strains of *X. c. citri* isolated from diseased *Citrus* spp. in 15 countries were used in this study (Table 1). Thirty-eight strains representing 17 pathovars of *X. campestris*, four nonpathogenic strains of *X. campestris* isolated from Mexican citrus, and six strains from other bacterial genera were also used in this study (Table 2). The pathovar designation for strains of *X. campestris* that cause CBS is controversial (11,32,33). Fifty-six of these strains from 18 locations were tested for sequences homologous to pFL62.42 and pFL1. These strains have all been described previously and include both aggressive and nonaggressive isolates (14,17,18). To confirm purity, all strains of *X. campestris* were stored as frozen glycerol stocks and were streaked on Wakimoto's potato semisynthetic medium (13). Strains from other genera were maintained on Luria-Bertani (LB) medium (21).

Hybridization probes. Plasmid pFL62.42 consists of a 4.2-kb *Bam*HI fragment cloned from an indigenous plasmid of the *X. c. citri* pathotype A strain XC62 in vector pUC9 (23,24). Plasmid pFL1 contains a 700-bp internal *Eco*RI fragment from the 4.2-kb *Bam*HI fragment cloned in the same vector by standard methods (21). Purified, intact plasmid DNA was labeled by nick translation using the Bio-Nick kit (GIBCO/BRL, Gaithersburg, MD) with biotinylated dATP for hybridizations.

Dot blot hybridizations. DNA was purified from cultures grown in LB broth as described (16). Dot blots were prepared as described (8), except 125 ng of genomic DNA per dot was applied, and the denaturation and dilution steps were performed in 96-well microtiter dishes. A twofold dilution series of homologous (XC62) DNA was included on each dot blot as a positive control and to estimate the sensitivity of the detection assay. Dot

Accepted for publication 24 February 1992.

Table 1. Strains of *Xanthomonas campestris* pv. *citri* used in this study[a]

Strain	Origin
Pathotype A[b]	
XC62, XC63	Japan
XC101, XC102	Guam
XC104, XC105	Thursday Island
XC106, XC107	Christmas Island
PH2, PH3, PH7	Philippines
Th76, Th7B, Th7C	Thailand
XC74, XC75, XC77	Reunion Island
MI1-1, MI2-2, MI3-1, MI4-7, MI7-4, MI14-10, MI15-7, MI16-3, MI18-2	Maldive Islands
MIJJ97A	Mauritius
XC100, XC297, XC298	Pakistan
XC269	Saudi Arabia
XC98	Yemen
XC118	New Zealand (type)[c]
XC91, XC92	Argentina
XC336, XC337	Uruguay
F598, F599, F600, XC308, XC312, XC320	Florida, USA
Pathotype B	
XC64, XC69, XC93, XC94, XC96, XC148, XC80, XC84	Argentina
Pathotype D	
XC90	Mexico
Pathotype C	
XC70, XC171, XC172, XC338, XC340, XC341	Brazil

[a] All strains are from the collection of phytopathogenic bacteria of the Fruit Laboratory, Beltsville Agricultural Research Center, Beltsville, MD.
[b] Pathotypes as described in references 4 and 24.
[c] National Collection of Plant Pathogenic Bacteria, NCPPB409.

Table 2. Additional bacterial strains used in this study

Strains	Pathover or host of isolation	Source or reference[a]
Xanthomonas campestris		
X60, X61	*alfalfae*	D. W. Gabriel
X3	*begoniae*	J. W. Miller
X32, X33	*bilvae*	IMI 8600, NCPBB
X6, X7, X8	*campestris*	J. W. Miller
X11, X12	*dieffenbachiae*	J. W. Miller
X151	*fici*	J. H. Graham, Gottwald et al 1991 (14)
X56	*holcicola*	E. L. Civerolo
X22	*macufoliigardeniae*	J. W. Miller
X203	*malvacearum*	E. L. Civerolo
X40	*manihotis*	W. Fry
X18, X20	*nigromaculans*	J. W. Miller
X25	*pelargonii*	J. W. Miller
X34, X35, X36, X45	*phaseoli*	NCPPB, NCPPB, D. W. Gabriel, ATCC
X69, X70	*pruni*	ICMP
X27, X37, X38	*vesicatoria*	J. W. Miller, R. E. Stall
X137, X143, X198	*Strelitzia reginae*	J. H. Graham, Gottwald et al 1991 (14)
G-55, 81-30, 82-38, 86-1	*vignicola*	R. E. Stall
T20, T22, T23, T24	*Citrus aurantifolia*; not pathogenic on *Citrus*	E. L. Civerolo
Other bacteria		
Flavobacterium balustinum 299		D. Roberts
Enterobacter cloacae E6		D. Roberts
Escherichia coli RR1		Boliver et al 1977 (3)
Erwinia carotovora subsp. carotovora EC14		D. Roberts
Pseudomonas putida 15819		D. Roberts
Erwinia amylovora 110R		Ritchie and Klos 1977 (25)

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, MD; IMI, Indian Mycological Institute; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpendon, England; ICMP, International Collection of Microbes from Plants, Auckland, New Zealand.

blots were made by using a Hybri-Dot manifold and nylon Photogene membranes (GIBCO/BRL). To fix the DNA, filters were baked for 90 min at 80 C. Hybridizations were done in 10-ml vol with roller bottles in a hybridization oven (Techne HB-1, Thomas Scientific, Swedesboro, NJ) at 68 C for 18 hr; a standard hybridization solution (6× SSC [1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0], 0.01 M EDTA, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate [SDS], and 100 μg/ml of denatured salmon sperm DNA) (21) and 400 ng of probe per filter were used. Posthybridization washes (50 milliliters per filter) (0.1× SSC, 0.5% SDS) were done twice at 68 C with roller bottles. After three washes with TBS-Tween (0.1 M Tris, 0.15 M NaCl, 0.05% Tween, pH 7.5), a Photogene chemiluminescent kit (GIBCO/BRL) was used, following the directions of the manufacturer, for detection of bacteria. After the addition of the chemiluminescent substrate, the filters were incubated in the dark for 150 min. Hyperfilm (Amersham Corp., Arlington Heights, IL) was exposed for 2–10 min. Southern blots of genomic DNA digested with *Bam*HI were prepared as described (16), but TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) (21) was used. Hybridization and detection were as described above. All blotting experiments were repeated at least once.

Detection of bacteria in citrus canker lesions. Strains XC312, XC320 (*X. c. citri* CBC pathotype A, Florida), F1, and F100 (*X. campestris* CBS, Florida) were grown from single colonies overnight in LPG (7 g of tryptone, 7 g of yeast extract, 7 g of glucose, pH 7.2) and adjusted to an OD_{590nm} of 0.1 with fresh LPG. Droplets (10 μl) were placed on the upper surfaces of newly expanded grapefruit leaves, and a 25-gauge needle was used to wound the leaf through the inoculum. After 42 days, lesions (6-mm disks) were removed with a paper punch. Disks from uninoculated leaves served as controls.

Four leaf disks per strain were ground in liquid nitrogen. The leaf powder was taken up in CTAB buffer (50 mM Tris-Cl, pH 8.6, 7 M NaCl, 10 mM EDTA, 1% CTAB, 2% sarkosyl, and 0.1% β-mercaptoethanol) (26), incubated at 60 C for 5 hr, extracted sequentially with phenol and chloroform, and then ethanol-precipitated. The DNA was resuspended in 25 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and diluted serially for dot blot assays as described above.

RESULTS

Probe pFL62.42 hybridized with DNA from all *X. c. citri* pathotype A strains from 13 countries (44 of 44), as well as 13 of 15 strains of pathotypes B, C, and D from South America (Fig. 1A). Strong signals were obtained from strains XC98

and XC100 (Fig. 1A; c-6 and c-10, respectively), which were previously shown to be distinguishable from other strains of *X. c. citri* in genomic fingerprinting and RFLP analyses (16,17). DNA from the pathotype B strain XC64 and pathotype C strain XC70 failed to hybridize with the probe. All other B, C, and D strains hybridized with the probe; however, scanning laser densitometry of these blots, with a dilution series of strain XC62 DNA as an internal standard, showed that these (pathotypes B, C, D) signals were much weaker than the signals from pathotype A strains (Fig. 1A, row e; data not shown). In contrast to the strains of *X. c. citri*, the probe did not hybridize to any of the 56 CBS strains tested or to DNA from four epiphytic strains of *X. campestris* isolated from lime in Mexico (Fig. 1C). DNA from 16 other pathovars of *X. campestris* was also tested for sequences homologous to pFL62.42. Most strains did not have sequences homologous to pFL62.42. However, hybridization signals were detected from single strains of *X. c. alfalfae*, *X. c. bilvae*, *X. c. campestris*, and from four tested strains of *X. c. vignicola*. Very faint signals, approximately equal in intensity to the signal produced by 1 ng of DNA from strain XC62, were detected for DNA from several other pathovars (Fig. 1B). In contrast to the results from pathotypes B, C, and D of *X. c. citri*, scanning laser densitometry showed that the signals from *X. c. bilvae* and *X. c. vignicola* were similar in intensity to those of pathotype A strains of *X. c. citri* (Fig. 1B, row e; data not shown).

Duplicate filters processed with those in Figure 1A–C were probed with plasmid pFL1. Plasmid pFL1 retained the homology of pFL62.42 for *X. c. citri* (Fig. 1D) and also did not hybridize to DNA from CBS strains of *X. campestris* (Fig. 1F). The specificity of pFL1 for *X. c. citri* compared to other pathovars of *X. campestris* was better than that of pFL62.42. Only *X. c. bilvae* and *X. c. vignicola* produced significant hybridization signals with this probe (Fig. 1E). However, detection of pathotype C strains was somewhat less successful with pFL1 than with pFL62.42 (Fig. 1A,D, row f).

No hybridization signal was detected with DNA isolated from strains belonging to other bacterial genera when the gel-purified insert from pFL62.42 was used as a hybridization probe (Fig. 2A). DNA from *Escherichia coli* RR1 hybridized to vector sequences when intact pFL62.42 was used as the probe (Fig. 2B).

The sizes of the DNA fragments that hybridized to pFL1 were compared after *Bam*HI digestion and Southern blotting. In the *X. c. citri* pathotype A strains tested, the homologous fragment was at 4.2 kb as expected (Fig. 3A). In contrast, the homologous fragment(s) in strains of heterologous pathovars were larger than 20 kb as was true for strains of *X. c. citri* with pathotypes B and D (Fig. 3B). Analogous results were observed when pFL62.42 was used as the hybridization probe (24). Pathotype C strains of *X. c. citri* were not detected in this experiment, which was consistent with the dot blot results with this probe (Fig. 1D).

Probe pFL1 also detected homologous DNA sequences when present in leaf disks that contained CBC-A lesions (Fig. 4). For both strains tested, pFL1 produced distinctly positive signals from each tested canker, even after the lesion extract had been diluted 1:32 or more. No signal was observed from the leaf disks taken from uninoculated control leaves or from leaf disks that contained CBS lesions, even after prolonged exposure of the chemilumigrams. Another DNA isolation method, which combined the boiling "mini-prep" procedure (21) with glassmilk (Bio-101, La Jolla, CA) purification, produced similar results with lesion extracts ground in liquid nitrogen (not shown).

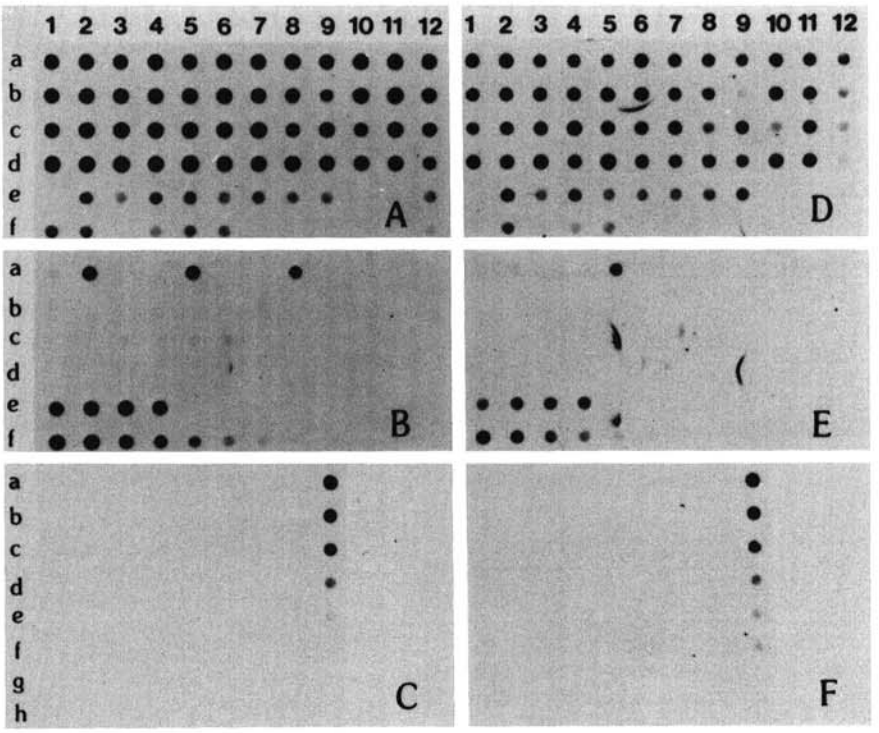

Fig. 1. Chemiluminescent detection of *Xanthomonas campestris* pv. *citri* with biotinylated hybridization probes in duplicate blots. **(A–C)** Probe used was pFL62.42; **(D–F)** probe used was pFL1. **(A)** and **(D)** Blots of DNA from 44 pathotype A strains of *X. c. citri* in rows a–d, columns 1–11; strains are listed in Table 1. Row e contains DNA from the nine pathotype B and D strains (listed in Table 1). Row f contains DNA from the six pathotype C strains (Table 1). Column 12 contains a twofold dilution series of XC62 (homologous) DNA, beginning with 125 ng in dot a-12. **(B)** and **(E)** Blots of DNA from 34 strains representing 16 pathovars of *X. campestris* (Table 1) in rows a–c, columns 1–8; row d, columns 1–6; and row e, columns 1–4. Row f contains a twofold dilution series of XC62 DNA, beginning with 125 ng in dot f-1. **(C)** and **(F)** Blots of DNA from 56 strains of *X. campestris* associated with citrus bacterial spot disease in rows a–g, columns 1–8. Row h, columns 1–4 contain DNA from epiphytic strains of *X. campestris* isolated in Mexico. Column 9 contains a twofold dilution series of XC62 DNA, beginning with 125 ng in dot a-9.

DISCUSSION

Diagnostic probes have been developed for various phytopathogenic bacteria in recent years, including *P. s. tomato* (8), *Clavibacter michiganense* subsp. *michiganense* (31), and *X. c. phas-*

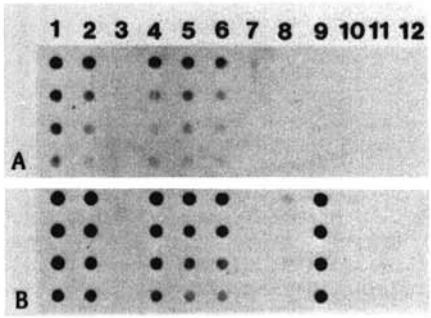

Fig. 2. Chemiluminescent dot blot detection assay for DNA from strains of *Xanthomonas campestris* pv. *citri* and strains of other bacterial genera in duplicate blots. **(A)** Probe used was the 4.2-kb *Bam*HI fragment from pFL62.42; **(B)** probe used was pFL62.42. DNA from strains XC62, XC69, XC70, and XC90 (*X. c. citri*), X33 (*X. c. bilvae*), 86-1 (*X. c. vignicola*), 299 (*Flavobacter balustinum*), E6 (*Enterobacter cloacae*), RR1 (*Escherichia coli*), EC14 (*Erwinia carotovora* subsp. *carotovora*), 15819 (*Pseudomonas putida*), and 110R (*Erwinia amylovora*) are in rows 1–12.

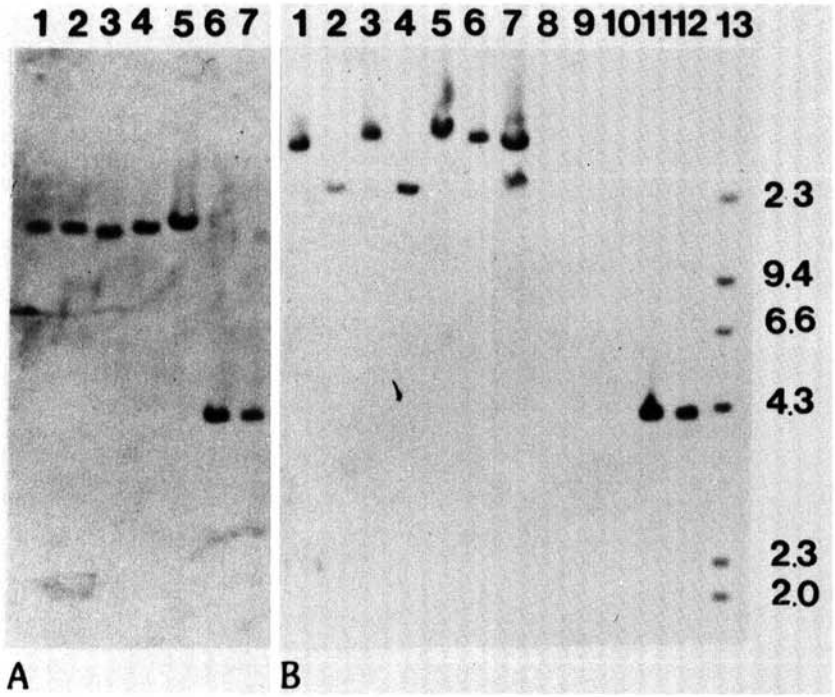

Fig. 3. Southern blot of *Xanthomonas campestris* genomic DNA after digestion with *Bam*HI. The probe for both blots was pFL1. **(A)** *X. c. vignicola* strains G55, 81-30, 82-38, 86-1 in lanes 1–4; *X. c. bilvae* strain X33 in lane 5; *X. c. citri* strains XC62 and XC100 (pathotype A) in lanes 6 and 7. **(B)** *X. c. citri* strains XC69, XC93, XC94, XC96, XC80, XC84, and XC90 (pathotypes B and D) in lanes 1–7; strains XC70, XC171, and XC172 (pathotype C) in lanes 8–10; strains XC62 and XC100 (pathotype A) in lanes 11 and 12. Sizes of biotinylated λ/*Hin*dIII markers (lane 13) are shown in the margin.

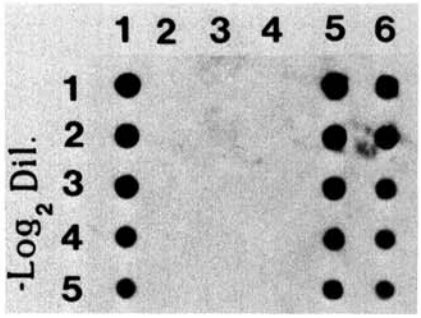

Fig. 4. Specific detection of pathotype A of *Xanthomonas campestris* pv. *citri* in leaf lesions by biotinylated probe pFL1. Column 1 contains purified DNA from strain XC62 (positive control) beginning with 125 ng. Column 2 contains DNA from control inoculation sites (negative control). Columns 3 and 4 contain DNA purified from lesions incited by citrus bacterial spot strains F1 and F100, respectively. Columns 5 and 6 contain DNA purified from lesions incited by citrus bacterial canker-A strains XC312 and XC320, respectively.

eoli (12). In at least two cases, the biological function encoded by the DNA probe was known. A portion of the phaseolotoxin gene of *P. s. phaseolicola* was used to specifically detect that pathogen (27). A plasmid fragment that cross-hybridized with genes in the indole-3-acetic acid biosynthetic pathway of *P. s. savastanoi* was used as a diagnostic probe for *E. herbicola* (22).

Previous work based on genomic DNA fingerprinting or RFLP analyses showed a distinct clonal population structure within *X. campestris* and within *X. c. citri* (10,16,17,20). Recently, in the analysis of RFLP data from plasmid DNA, the pathotypes of *X. c. citri* were also separated in a manner entirely consistent with genomic DNA analyses (24) as predicted by the hypothesis of the evolution of a "balanced genome" (7). Because plasmids are generally present in multiple copies per cell, good sensitivity could be expected. Thus, the idea of a plasmid-based diagnostic probe became attractive in this system. A 4.2-kb *Bam*HI fragment from a plasmid of strain XC62 and a 700-bp fragment derived from it were chosen for this purpose because of the widespread occurrence of the 4.2-kb *Bam*HI fragment in strains of *X. c. citri* with pathotype A (24).

The functions (if any) encoded by pFL62.42 and pFL1 are unknown, although the conservation of the sequence within pathotype A strains and the presence of related sequences in pathotype B, C, and D strains of *X. c. citri* may suggest a role in host selection or some other biologically important function. It is significant that none of the strains of *X. campestris* that cause CBS disease had any sequences homologous to either pFL62.42 or pFL1. This is consistent with their limited relatedness to *X. c. citri* (9,11,17) and with their possible incidental association with citrus (15,18). The presence of homologous sequences in the single available strain of *X. c. bilvae* is interesting, because the strain was originally isolated from *Feronia elephantiacum*, a member of the Rutaceae.

A pathogenicity locus, which included a gene designated *pthA*, was isolated recently from a genomic library of a pathotype A strain of *X. c. citri* (30). *pthA* was included on a 3.7-kb *Sst*I-*Sal*I fragment, and no hybridization signal was observed in the region of 4.2 kb in *Bam*HI digests of genomic DNA of *X. c. citri* (30). When pFL62.42 and pFL1 were used to probe similar digests, a band at 4.2 kb was observed (Fig. 3) (24). Thus, the hybridization probes described in this paper are unrelated to *pthA*.

The ability of these probes to hybridize to homologous sequences present in lesion extracts make them particularly valuable as diagnostic tools. DNA isolation was required for this purpose. Spurious hybridization signals were detected from F1 and F100 lesions (CBS) when leaf squashes or similar unpurified extracts were probed (not shown).

The probes described in this paper provided excellent specificity within *X. campestris* for strains of *X. c. citri*, especially those belonging to pathotype A, the most widespread and virulent pathotype. The sensitivity of detection was in the range of 2–7 ng of DNA per dot (Fig. 1) or the DNA present in a single canker lesion (Fig. 4). Interference from other xanthomonads associated with *Citrus* spp. was not observed. The demonstrated effectiveness of these probes with a nonradioactive label and the effect of quarantine regulations directed against *X. c. citri* on commerce may make these probes particularly useful in regulatory situations and in complementing current serological (6), phagetyping, and pathological methods (4).

ACKNOWLEDGMENTS

I thank A. S. O'Connor and J. H. Spisso for their skilled technical assistance, Susan Koehler for the CTAB extractions, and Olivier P. Pruvost for initiating this research when he was a visiting scientist in this laboratory.

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