Detection of the Bean Common Blight Bacteria, *Xanthomonas campestris* pv. *phaseoli* and *X. c. phaseoli* var. *fuscans*, Using the Polymerase Chain Reaction

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ABSTRACT

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A 3.4-kb plasmid DNA fragment (p7) of *Xanthomonas campestris* pv. *phaseoli* that hybridizes specifically to *X. c. phaseoli* was subcloned and partially sequenced to design primers for a polymerase chain reaction (PCR) assay for the detection of bacteria causing common blight in bean. In Southern hybridization experiments, p7X3 and p7X4 subfragments showed high specificity for total genomic DNA of pathogenic *X. c. phaseoli*, whereas the p7X2 segment also weakly hybridized to DNA extracted from some strains belonging to other *X. campestris* pathovars. For each of these p7 fragments, a set of G+C-rich oligonucleotide primers was devised and tested under high-stringency conditions in PCR assays. The p7X4-derived primers specifically directed the amplification of a 730-bp fragment from DNA of 27 pathogenic *X. c. phaseoli* isolates from various geographic regions, whereas DNA from 61 other strains of *X. campestris, Pseudomonas syringae* pv. *phaseolicola* (the cause of bean halo blight), and plant-pathogenic species of *Agrobacterium, Clavibacter*, and *Erwinia* did not produce any discrete bands upon amplification on ethidium bromide-stained agarose gel. An additional fragment of 550 bp was occasionally amplified from the DNA of *fuscans* strains of *X. c. phaseoli*. The p7X4 primers successfully mediated the amplification of PCR products of the expected size from DNA extracted from common blight lesions on bean leaves. The high melting temperature of these primers allowed us to shorten the PCR assay time considerably by using a two-step repetitive cycle—95 C for denaturation and 72 C for annealing and extension. As little as 100 fg of *X. c. phaseoli* DNA (approximately 10 cfu genomic equivalent) could be detected on ethidium bromide-stained agarose gel after a 35-cycle PCR-amplification assay using p7X4-derived primers, and the threshold of detection could be lowered to 10 fg of DNA (approximately 1 cfu genomic equivalent) with two successive amplifications of 20 and 35 cycles.

Additional keywords: diagnosis, *Phaseolus vulgaris*.

Common bacterial blight of bean (*Phaseolus vulgaris* L.), caused by *Xanthomonas campestris* pv. *phaseoli* and its variant *X. c. phaseoli* var. *fuscans*, which produces a melaninlike compound, is a major disease in many bean production areas throughout the world (10,35). Yield reductions of up to 40% directly attributable to common blight infection have been reported in North and South America (36,39). Seeds, contaminated either internally or externally, constitute the primary source of inoculum (26), and epidemiological studies have shown that one infected seed in 10,000 is sufficient to result in an outbreak of the disease in the field (33).

The development of resistant cultivars derived from *X. c. phaseoli*-resistant *P. acutifolius* germ plasm and the use of pathogen-free seeds are the only practical methods to control bean common blight (25). The success of seed certification and breeding programs for common blight depends primarily on the availability of reliable methods for detecting the pathogen in bean tissues. Diagnosis of the disease in the field on the basis of visual symptoms has limitations, especially since *X. c. phaseoli* can survive in tissues of resistant plants without causing symptoms (5). Current assays to identify and quantify *X. c. phaseoli* in bean tissues by plating on selective media, phage typing, immunoassay, and host inoculation (31) are valuable but too labor-intensive and not sufficiently precise for routine use. Moreover, nonpathogenic *X. campestris* strains can colonize bean plants and debris (13), further complicating identification of *X. c. phaseoli*. Therefore, there is a need

for rapid, specific, and highly sensitive diagnostic methods for the bean common blight pathogen.

Since the early 1980s, nucleic acid hybridization probes have been developed and used for disease diagnosis and identification of plant pathogens (21). The direct targeting of the genetic information of a pathogenic agent allows for a high degree of specificity and circumvents many of the difficulties associated with serological detection methods. However, there are limitations to the sensitivity of the hybridization probes, particularly when a large excess of heterologous DNA or RNA is present with

TABLE 1. Bacterial strains and pathogenicity on bean

Strain	Location	Source	Pathogenicity[a]
Xanthomonas campestris pv. *phaseoli*			
EK11, Xp816	Nebraska	M. Schuster	+
Bxp98, XpF	Ontario	Unknown	−
ATCC 9563, ATCC 10199		ATCC	+
ATCC 10198		ATCC	−
Xcp9, Xcp50, Xcp51, Xcp53, Xcp72	Wisconsin	R. L. Gilbertson	+
Xcp47, Xcp56	Brazil	J. C. Faria	+
Xcp93	Columbia	E. L. Civerolo	+
Xcp98	Uganda	E. L. Civerolo	+
Xcp99	Florida	E. L. Civerolo	+
XcpN	Nebraska	A. K. Vidaver	+
XcpG2	Guatemala	R. L. Gilbertson	+
X. c. phaseoli var. *fuscans*			
Bxp12, Xp fusc wallen	Ontario	B. N. Dhanvantari	+
ATCC 13464[b]		ATCC	−
ATCC 19315		ATCC	+
Xcp59	Nebraska	A. K. Vidaver	+
Xcp60	Wisconsin	R. L. Gilbertson	+
XcpG3	Guatemala	R. L. Gilbertson	+
ES1	Brazil	J. C. Faria	+
Mal1, Mal6, Mal9, Mal10	Malawi	R. L. Gilbertson	+
X. c. alfalfae			
XA1	Kansas	D. L. Stuteville	nt
X. c. armoraciae			
275	Wisconsin	D. K. Willis	nt
X. c. campestris			
Cp670, Cp800, Cp907	Quebec	M. Lacroix	−
Xcc295	Wisconsin	R. L. Gilbertson	nt
X. c. carotae			
Xcarl-Xcar6	California	R. L. Gilbertson	nt
X86-X90	Wisconsin	D. K. Willis	nt
X. c. holcicola			
XCH429, XCH474	Kansas	J. L. Leach	nt
X. c. juglandis			
252	California	R. L. Gilbertson	nt
X. c. pelargonii			
ATCC 8721		ATCC	−
Pg730, Pg913	Quebec	M. Lacroix	−
X. c. pruni			
ATCC 10016		ATCC	−
X. c. vesicatoria			
ATCC 11633		ATCC	−
Vs715, Vs834	Quebec	M. Lacroix	−
325	Wisconsin	D. K. Willis	nt
X. c. vitians			
93-00, 93-10	California	R. L. Gilbertson	nt
Vt840	Quebec	M. Lacroix	−
X. fragariae			
Fg38	Quebec	M. Lacroix	−
X. oryzae pv. *oryzae*			
X061, X086	Philippines	J. L. Leach	nt
Xanthomonas saprophytes (bean)			
5, 23L, D11, D21, D10B, NTL, P3L3, 232	Wisconsin	R. L. Gilbertson	−
Xb1, Xb2, Xb4, Xb5	Alberta	P. Audy	−
Agrobacterium tumefaciens			
LBA 4404	The Netherlands	R. A. Schilperoort	−
Clavibacter michiganense subsp. *sepedonicus*			
RR2	Alberta	H. C. Huang	−
Erwinia carotovora subsp. *atroseptica*			
BL3	Alberta	H. C. Huang	−
E. herbicola			
EH-11	Wisconsin	R. L. Gilbertson	nt
Pseudomonas syringae pv. *phaseolicola*			
HB9, HB33, HB93, PS90SC-5	Alberta	G. Saindon	+[d]
ATCC 11355[c], ATCC 11365[c], ATCC 19304		ATCC	+[d]

[a] + = Pathogenic, − = nonpathogenic, nt = not tested.
[b] Does not produce melaninlike pigment on media.
[c] Documented as nonpathogenic (1).
[d] Halo blight disease.

a low population of target molecules. The polymerase chain reaction (PCR) (27) overcomes those constraints by allowing exponential enzymatic amplication of minute amounts of target DNA using thermostable DNA polymerases. This approach offers the rapidity, specificity, and sensitivity required for diagnostic procedures. PCR-based detection and identification protocols have been recently described for diverse groups of plant pathogens (for review see Henson and French [15]). The development of a PCR assay specific for pathogenic *X. c. phaseoli* would significantly improve the accuracy and efficiency of diagnosis of bean common blight, enable the establishment of higher seed certification standards, and accelerate the selection of *X. c. phaseoli*-resistant bean cultivars in breeding programs.

Gilbertson et al (11) identified a 3.4-kb plasmid DNA probe (p7) harboring a repetitive sequence present in both the plasmid and the chromosome of pathogenic *X. c. phaseoli*. That probe hybridized extensively with common blight bacteria DNA in colony, squash, and dot blot hybridization assays but not with nonpathogenic xanthomonads from bean debris. With the dot blot procedure, 10^3 cfu of *X. c. phaseoli* (approximately 10 pg of DNA) could be detected on a 3-day exposed autoradiogram using ^{32}P-labeled p7 probe (11). In the present study, we describe the use of G+C-rich primers, designed from selected sequences of the p7 *X. c. phaseoli*-plasmid fragment, in a highly sensitive and rapid PCR-based assay for detection of bean common blight bacteria in bean leaf lesions.

MATERIALS AND METHODS

Bacterial strains and media. All bacterial cultures (Table 1) were freshly prepared from stocks stored in 7% (v/v) dimethyl sulfoxide (DMSO) at −80 C (3). *X. c. phaseoli* strains were maintained on yeast-dextrose-$CaCO_3$ (YDC) (38) and semiselective MXP (6) and YSSM-XP (7) agar. Strains of *Pseudomonas syringae* pv. *phaseolicola* (causal agent of bean halo blight) were cultured on KB (17) and semiselective MSP (22) media. All other bacteria were cultured on nutrient agar (Difco) containing 0.25% glucose (NGA).

Pathogenicity tests. *X. c. phaseoli* strains were tested for pathogenicity on bean cv. Othello, US1140, or Topcrop. *P. s. phaseolicola* strains were tested on bean cv. Centralia, and all other bacterial strains were tested on US1140 or Topcrop. Bean plants were grown in 15-cm-diameter pots (two plants per pot) in a controlled growth cabinet (25 C, 16-h photoperiod) and inoculated 7 days after emergence. Bacterial strains were grown in shake culture at 26 C for 18 h in nutrient broth containing 0.25% glucose. Bacterial cells were pelleted at 10,000 *g* for 30 s and resuspended into sterile 10 mM $MgSO_4$ just prior to inoculation (0.2 A_{600nm}, approximately 2×10^8 cfu/ml). Unless specified otherwise, inoculation was onto the adaxial surface of half-expanded unifoliate leaves using a multiple-needle method (2). Control plants were mock-inoculated with a sterile 10 mM $MgSO_4$ solution. The leaves were thoroughly misted with water, and each pot was caged with a plastic bag. Care was taken to avoid any contact between the leaves and the bag. After 3 days of incubation in the growth cabinet, the bags were removed and the plants watered to avoid any foliage desiccation. Blight symptoms were assessed 5 and 10 days after inoculation. Three pathogenicity tests were independently conducted for common and halo blight strains using two plants in each test. For all other bacterial strains the pathogenicity tests were conducted once. A few *X. c. phaseoli* strains were also tested using stem puncture (31) or razor blade (23) inoculation methods.

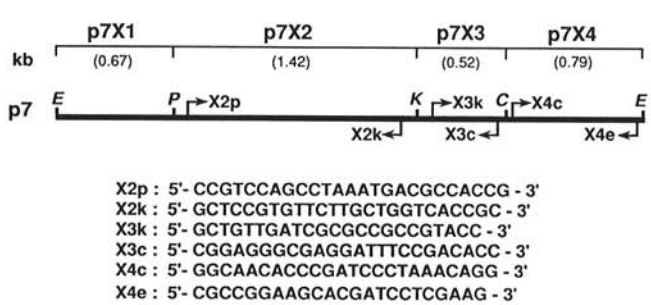

Fig. 1. Schematic diagram of p7 DNA subfragments. The upper scale indicates the relative position, the name (in boldface), and the size in kilobases (in parentheses) of each p7 DNA subfragment. The lower scale (in boldface) describes the p7 restriction map for *Eco*RI (*E*), *Pst*I (*P*), *Kpn*I (*K*), and *Cla*I (*C*). Primer name and sequence and relative position of priming are shown. Arrows indicate the direction of DNA extension for each primer.

Subcloning, sequencing, and primer design. Plasmid p7 is a pBS(+) derivative harboring a 3.4-kb *Eco*RI fragment that contains repetitive sequence(s) found in *X. c. phaseoli* genomic and plasmid DNA (11). Partial restriction analysis of the 3.4-kb DNA insert was performed using *Cla*I, *Hin*dIII, *Kpn*I, *Sal*I, *Pst*I, *Xpa*I, and *Xho*I (New England Biolabs, Beverly, MA). The selected fragments generated from p7 by restriction enzyme digestion (Fig. 1)—*Eco*RI-*Pst*I (p7X1), *Pst*I-*Kpn*I (p7X2), *Kpn*I-*Cla*I (p7X3), and *Cla*I-*Eco*RI (p7X4)—were eluted from a 0.8% low-melting-temperature agarose gel and ligated into similarly digested pBluescript II KS (Stratagene, La Jolla, CA) vector (3). Recombinant plasmids were transformed into *Escherichia coli* strain DH5α using a $CaCl_2$ transformation procedure (28). Subclones p7X2, p7X3, and p7X4 (Fig. 1) were partially sequenced by the dideoxynucleotide chain termination method (29) using modified T7 DNA polymerase (Sequenase, U.S. Biochemicals, Cleveland, OH) or by a dye fluorescent sequencing method (Dyedeoxy terminator, Applied Biosystems, Foster City, CA) using *Taq* polymerase (Ampli*Taq*, Perkin-Elmer Cetus, Norwalk, CT). Paired primers having similar thermal melting points, high G+C content, and low homology to other p7 available DNA sequences were designed for each of the sequenced subfragments (Fig. 1).

Extraction of bacterial genomic and bean leaf tissue DNA. Freshly grown single colonies on agar media were used to start nutrient broth cultures. Bacterial cultures were grown at 26 C for 18 h, and cells from 1 ml of broth were used for DNA extraction. Total genomic DNA was isolated following two small-scale procedures. In the first extraction protocol (9), broths were centrifuged for 2 min at 10,000 *g* and bacterial cells were washed with 1 ml of 150 mM NaCl, 50 mM EDTA, pH 8.0 (NE buffer). Cells were pelleted for 30 s at 10,000 *g* and resuspended in 600 μl of NE buffer containing 90 μg of proteinase K (BDH, Vancouver, BC) and 1% (w/v) sodium dodecyl sulfate (SDS). The bacterial suspensions were mixed thoroughly by vortexing and incubated at 50 C for 1 h. The lysates were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and the DNA was precipitated by adding 0.1 volume of 3.0 M sodium acetate, pH 5.2, and one volume of isopropanol to the aqueous supernatant. The DNA was spooled out with a freshly flame-sealed Pasteur pipette, rinsed in 70% ethanol, and dissolved overnight at 4 C in 500 μl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing 5 μg of DNAse-free RNAse A (Sigma Chemical Company, St. Louis, MO). The solution was extracted with an equal volume of chloroform/isoamyl alcohol (24:1), and the DNA was reprecipitated by adding 0.1 volume of 3.0 M sodium acetate, pH 5.2, and two volumes of 95% ethanol. The DNA was spooled out, rinsed in 70% ethanol, dissolved in 100 μl of TE (10 mM Tris[hydroxylmethyl]-aminomethane, 1 mM EDTA) buffer, and used in Southern hybridization experiments. For the PCR assays, the DNA was extracted by a quick alkaline DNA extraction method for plant material adapted from Wang et al (37). Bacterial broths were centrifuged (10,000 *g*, 2 min), cells were resuspended in 500 μl of 0.5 N NaOH, and 5 μl of the lysate was transferred to a new tube containing 495 μl of 20 mM Tris-HCl, pH 8.0. Yield of DNA for both protocols was measured by the Hoechst dye assay method with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Franscisco, CA) according to the manufacturer's instructions.

DNA was extracted from bean leaf tissue by the same alkaline procedure (37) described above. Leaf tissue (40 mg) was ground

in 400 μl of 0.5 N NaOH in a 1.5-ml tube, and 5 μl of the extract was transferred to a new tube containing 495 μl of 20 mM Tris-HCl, pH 8.0.

All DNA samples (from leaves and bacteria) were aliquoted and kept at −20 C until use.

Southern hybridization. DNA samples were digested with restriction endonuclease *Eco*RI (Bethesda Research Laboratories, Gaithersburg, MD) for 16 h at 37 C, and nucleic acid fragments were electrophoresed in 0.8% agarose gel using Tris-borate-EDTA buffer (28). DNA fragments were denatured and blotted onto Nytran nylon membranes (Schleicher & Schuell, Keene, NH) by capillary transfer (32). Prehybridization was carried out at 25 C for 4 h in 1% (w/v) Sarkosyl, 200 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and hybridization was performed at 65 C for 16 h in 1% (w/v) Sarkosyl, 1 M NaCl, 50 mM Tris-HCl, pH 7.5, and 10% (w/v) dextran sulfate (Sigma). Gel-purified p7X2, p7X3, and p7X4 insert DNA were ^{32}P-radiolabeled by random priming (8) and used as probes. Filters were washed three times at 25 C for 20 min in prehybridization buffer and once at 65 C for 1 h in 0.1× SSPE buffer (15 mM NaCl, 1 mM NaH_2PO_4, 0.1 mM EDTA, pH 7.4) and 0.1% (w/v) Sarkosyl. Membranes were exposed at −80 C to Kodak X-Omat AR film with Dupont Cronex Lightning (Sigma) intensifying screens.

PCR assays. PCR-amplification assays were routinely performed in a 25-μl reaction mixture containing 10–50 ng of bacterial genomic DNA; 200 μM each of dATP, dCTP, dGTP, and dTTP; 0.5 μM each of upstream and downstream primers; 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [w/v] gelatin); and 1.25 units of *Taq* DNA polymerase (Perkin-Elmer Cetus). Mineral oil (molecular biology grade, Sigma) was added (25 μl) to prevent evaporation. PCR amplifications were performed in a TwinBlock System (Ericomp, Inc., San Diego, CA) or a TC480 (Perkin-Elmer Cetus) thermocycler and, unless otherwise indicated in the text, with the following thermal profile: initial denaturation at 95 C for 1 min followed by 35 repeated cycles of melting, annealing, and DNA extension at 95 C for 1 min, 65 C for 1 min, and 72 C for 2 min, respectively. For the last cycle, the extension time was increased to 10 min. In some experiments, annealing and DNA extension were performed simultaneously at 72 C for 2 min. The amplified DNA fragments were electrophoresed in 1.0% agarose gels in Tris-acetate-EDTA buffer (28) and visualized with ultraviolet light following ethidium bromide staining.

Assessment of sensitivity thresholds. To ascertain the concentration of *X. c. phaseoli* cells in relation to turbidity, 8-h nutrient broth cultures of pathogenic *X. c. phaseoli* var. *fuscans* strains Bxp12 and Xcp60 and non-*fuscans* strains EK11, Xp816, and Xcp50 were adjusted spectrophotometrically to 0.1 A_{600nm} (approximately 1×10^8 cfu/ml), serially diluted in sterile water, and plated on NGA media. To assess the concentration of DNA extracted in relation to the number of colony-forming units, bacteria from 1 ml of 16-h broth cultures adjusted to 0.5 A_{600nm} (approximately 5×10^8 cfu/ml) were pelleted and processed through the alkaline lysis DNA extraction method described above. The samples were RNAse-treated and twice phenol-chloroform-extracted, and the DNA was ethanol-precipitated. Recovered DNA was estimated by fluorometry and serially diluted for PCR sensitivity experiments. Three independent repetitions were conducted to assess the sensitivity thresholds. PCR products were analyzed as described earlier.

RESULTS

Pathogenicity of bacterial strains on bean. All but four of 31 *X. c. phaseoli* strains induced common blight symptoms on bean cv. Othello, US1140, or Topcrop 5–7 days after inoculation (Table 1). The three non-*fuscans* strains, Bxp98, XpF, and ATCC 10198, and *fuscans* strain ATCC 13464 were rated nonpathogenic, as the plants were symptomless 4 wk after inoculation by multiple-needle, stem puncture, and razor blade methods. These strains carried cryptic plasmids, as do all pathogenic *X. c. phaseoli* strains (19), but did not display the typical starch hydrolysis activity

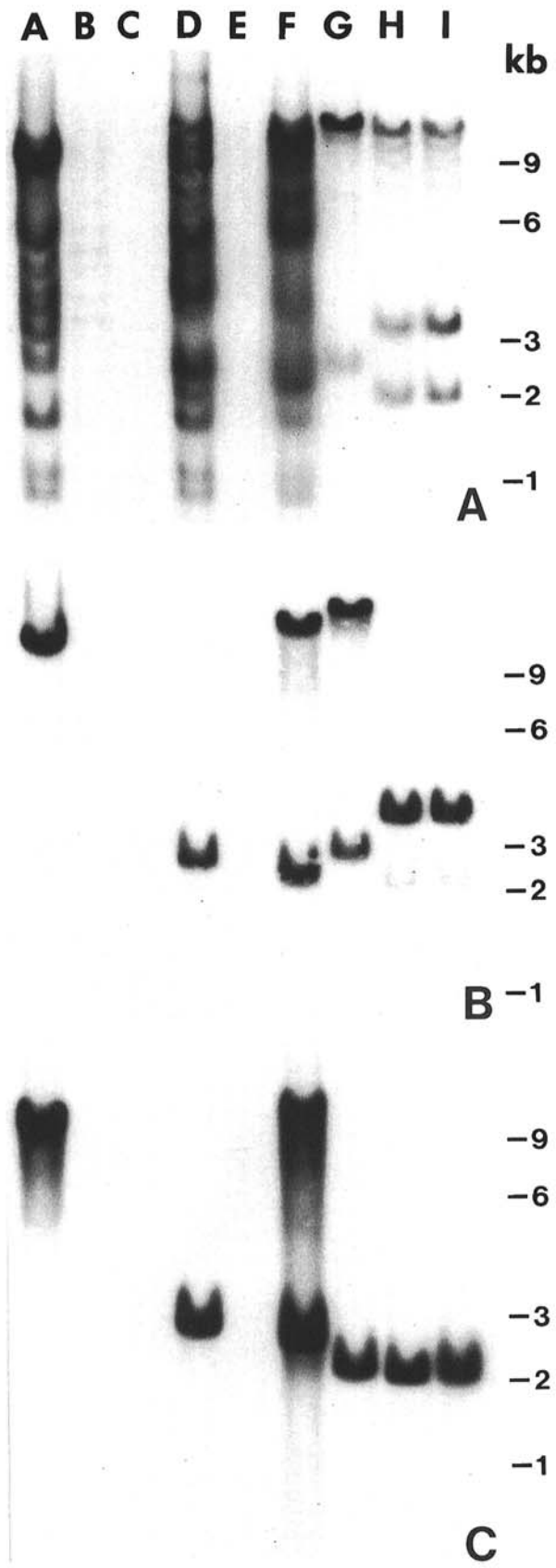

Fig. 2. Southern hybridization analysis of total genomic DNA of *Xanthomonas campestris*. DNA was digested with *Eco*RI, fractionated on a 0.8% agarose gel, transferred to Nytran nylon membrane, and probed with ^{32}P-labeled **A,** p7X2, **B,** p7X3, and **C,** p7X4 DNA. *X. c. vesicatoria* ATCC 11633 (lane B); non-*fuscans X. c. phaseoli* EK11 (lane A) and Bxp98, ATCC 9563, ATCC 10198, and ATCC 10199 (lanes C–F); and *X. c. phaseoli* var. *fuscans* ATCC 19315, Bxp12, and Xp fusc wallen (lanes G–I). Numbers on the right indicate the sizes in kilobases.

on semiselective starch-bearing medium for *X. c. phaseoli*. These strains may be *X. c. phaseoli* variants with altered pathogenicity and are hereafter referred to as nonpathogenic *X. c. phaseoli*.

All *P. s. phaseolicola* strains induced typical halo blight symptoms on Centralia beans 7 days after inoculation (Table 1), including strains ATCC 11355 and ATCC 11365, documented as not pathogenic on bean (1). No symptoms were observed on beans inoculated with bacteria of other pathovars of *X. campestris* or any other pathogenic genera tested (Table 1).

Specificity of p7 subfragments. Partial restriction analysis of the p7 plasmid DNA generated four relevant subfragments (Fig. 1): *Eco*RI-*Pst*I (p7X1), *Pst*I-*Kpn*I (p7X2), *Kpn*I-*Cla*I (p7X3), and *Cla*I-*Eco*RI (p7X4). The fragments p7X2, p7X3, and p7X4 hybridized strongly to Southern blots of *Eco*RI-restricted total genomic DNA of pathogenic *X. c. phaseoli* strains but not to the nonpathogenic ones (Fig. 2). Fewer bands were probed in blots of *Eco*RI-restricted plasmid DNA, confirming that, as p7 itself (11), the p7 subfragments target plasmid and chromosome sequences (data not shown). The *fuscans* strains Bxp12 (lane H) and Xp fusc wallen (lane I) exhibited the same pattern of hybridization against the three probes, but this pattern differed from that of *fuscans* strain ATCC 19315 (lane G) with the p7X2 and p7X3 DNA (Fig. 2). Variable patterns of hybridization were detected among the non-*fuscans* strains of *X. c. phaseoli*, with the p7X2 fragment displaying a much higher level of polymorphism for non-*fuscans* than for *fuscans* strains (Fig. 2A). The three p7 fragments did not hybridize to the DNA extracted from *X. fragariae* and the bean halo blight agent, *P. s. phaseolicola*, or that from *Erwinia, Clavibacter*, and *Agrobacterium* strains (Table 2). The p7X3 and p7X4 DNA exhibited complete specificity for pathogenic *X. c. phaseoli* strains, even after a 7-day exposure of the filters, whereas p7X2 hybridized slightly to the genomic DNA of other *X. campestris* pathovars, particularly to that of *X. c. vesicatoria* strains (Table 2; Fig. 2A, lane B).

Primer specificity. Sets of 23–25 oligonucleotide primers encoding G+C-rich sequences (60–70%) were devised for p7X2, p7X3, and p7X4 fragments (Fig. 1) and used under stringent conditions (annealing at 65 C) in PCR assays. The primers derived from p7X2 and p7X3 sequences did not display suitable specificity for pathogenic *X. c. phaseoli* strains because DNA fragments of comparable sizes (0.35 and 1.26 kb, respectively) were amplified from genomic DNA of other *X. campestris* pathovars (data not shown). Attempts to generate larger and more specific PCR products using upstream p7X2 primer (X2p), with either of the downstream primers X3c or X4e, were unsuccessful. However, the upstream p7X3 primer (X3k) paired with the downstream p7X4 primer (X4e) directed the amplification of a specific 1.15-kb DNA fragment from only pathogenic *X. c. phaseoli* strains (Fig. 3). The p7X4 primers also exhibited a complete specificity by directing the amplification of a distinctive 0.73-kb DNA product from pathogenic *X. c. phaseoli* templates (Fig. 4). Furthermore, the pathogenic *fuscans* strains occasionally presented an additional band at 0.55 kb with the p7X4-derived primers (Fig. 4). This second PCR product usually appeared when less than 10 ng of template DNA was used in the reaction. With the downstream X4e primer paired with the upstream X4c or X3k primers, a two-step thermal profile of DNA melting at 95 C for 1 min and simultaneous annealing and extension at 72 C for 2 min resulted in similar amplification of PCR-products in significantly less time (30%) than required to complete a 35-cycle PCR program.

Detection of *X. c. phaseoli* in infected bean leaf tissue. *X. c. phaseoli* var. *fuscans* and non-*fuscans* were successfully detected after amplification of DNA extracted from common blight lesions on bean leaves 7 days after inoculation (Fig. 5). No products were observed from control, mock-inoculated leaf tissue (Fig. 5, lane J), indicating that the p7X4 primers did not anneal to bean nucleic acid under the stringent conditions used in our assay.

Sensitivity threshold. As little as 100 fg of *X. c. phaseoli* DNA (approximately 10 cfu genomic equivalent) from EK11 non-*fuscans* and Xcp60 *fuscans* strains was detected on ethidium bromide-stained agarose gel after a 35-cycle PCR assay primed by X4c and X4e oligonucleotides (Fig. 6). Comparable sensitivity was obtained with the two other non-*fuscans* strains assayed (Xp816 and Xcp50), but the threshold of detection was 1 pg DNA with the *fuscans* strain Bxp12. The sensitivity threshold was lowered to the genomic equivalent of a single cell (approximately 10 fg of DNA) by two successive PCR amplifications in which a 5-μl aliquot from a 20-cycle PCR program was transferred to a fresh reagent cocktail for another 35 cycles (Fig. 6).

TABLE 2. Southern hybridization of p7 subfragments[a]

Strain	p7X2	p7X3	p7X4
Xanthomonas campestris pv. *phaseoli*			
EK11, ATCC 9598, Xp816, ATCC 10199	+++	+++	+++
Nonpathogenic Bx98, XpF, ATCC 10198	−	−	−
X. c. phaseoli var. *fuscans*			
Bxp12, Xp fusc wallen, ATCC 19315	+++	+++	+++
Nonpathogenic ATCC 13464	−	−	−
X. c. campestris			
Cp800	−/+	−	−
Cp670, Cp907	−	−	−
X. c. pelargonii			
ATCC 8721, Pg730, Pg913	−/+	−	−
X. c. pruni			
ATCC 10016	−/+	−	−
X. c. vesicatoria			
ATCC 11633, Vs715, Vs834	+	−	−
X. c. vitians			
Vt840	−/+	−	−
X. fragariae			
Fg38	−	−	−
Pseudomonas syringae pv. *phaseolicola*			
HB9, HB33, HB93, PS90SC-5, ATCC 11355, ATCC 11365, ATCC 19304	−	−	−
Clavibacter michiganense subsp. *sepedonicus*			
RR2	−	−	−
Erwinia carotovora subsp. *atroseptica*			
BL3	−	−	−
Agrobacterium tumefaciens			
LBA 4404	−	−	−

[a]+++ = Strong hybridization, + = weak hybridization, −/+ = very faint hybridization, − = no hybridization.

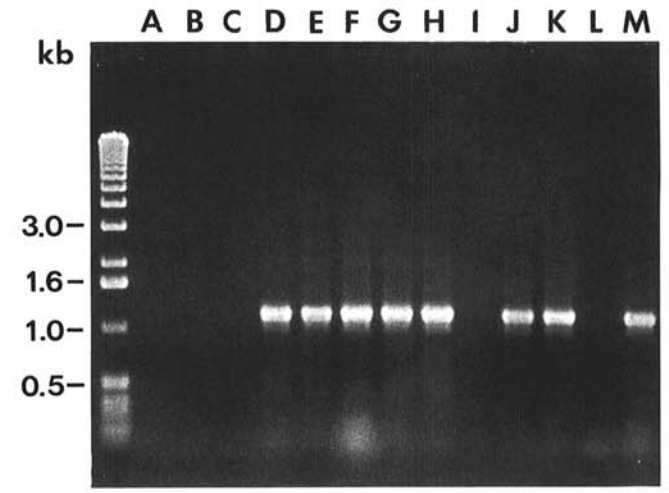

Fig. 3. Ethidium-bromide stained gel of PCR-amplification products directed by X3k and X4e primers. Template DNAs (20 ng) were from *Xanthomonas campestris* pv. *vesicatoria* ATCC 11633 (lane A); *Pseudomonas syringae* pv. *phaseolicola* HB9 (lane B); non-*fuscans X. c. phaseoli* Bxp98, Xp816, EK11, ATCC 9563, and ATCC 10199 (lanes C–G); *X. c. phaseoli* var. *fuscans* ATCC 19315, ATCC 13464, Xp fusc wallen, and Bxp12 (lanes H–K); no-DNA control reaction (lane L); and p7 DNA control reaction (lane M). Annealing was done at 65 C. Molecular weight standard (1.0-kb ladder, BRL) was run in the left lane (unmarked), and numbers on the left indicate sizes in kilobases.

DISCUSSION

In this study, we established a restriction map for the 3.4-kb plasmid DNA fragment (p7) of *X. c. phaseoli*, subcloned selected fragments, and determined partial nucleotide sequences of the fragments in order to select sets of G+C-rich primers suitable for PCR-mediated detection of the bean common blight bacteria. We identified two p7 subfragments (p7X3 and p7X4) that hybridized exclusively to total genomic DNA of pathogenic *X. c. phaseoli*, and we used certain combinations of primers derived from those sequences to direct the amplification of specific DNA fragments from nucleic acid extracted from common blight bacteria or common blight lesions on bean leaves. With two successive amplification runs, we could detect PCR products from template nucleic acid equivalent to that from a single cell of *X. c. phaseoli*.

The species *X. campestris* represents a complex group of plant-pathogenic bacteria that are similar in morphology but different in host range. These host range differences are recognized by the subspecies designation "pathovar," but this has caused some confusion in the taxonomy of these bacteria (34). Accurate identification of plant-pathogenic *X. campestris* can be difficult and time-consuming. Therefore, our initial objective in this study was to ascertain the pathogenicity of putative *X. c. phaseoli* strains and other *X. campestris* bacteria on bean. All but four putative *X. c. phaseoli* strains induced typical common blight symptoms, whereas none of the other tested *X. campestris* strains or bacteria of other genera were pathogenic. The four strains that did not induce common blight symptoms, which included two ATCC strains, carried cryptic plasmids but lacked the usual starch hydrolysis activity (6,7), and their genomic DNA did not hybridize to the p7 subfragments. Because the p7 fragment has been shown to hybridize to *X. c. phaseoli* plasmid and genomic DNA from 50 geographically representative strains (11), it is unlikely that the four nonpathogenic *Xanthomonas* are variants of formerly pathogenic *X. c. phaseoli*; the DNA fragment(s) lost from these strains must have included all the p7 repetitive DNA sequences and encoded the genetic determinant(s) of pathogenicity and/or host range and starch hydrolysis. Alternatively, these strains are not, and never were, *X. c. phaseoli* and may belong to other *X. campestris* pathovars or nonpathogenic *X. campestris* forms such as those previously described as associated with beans (13). This situation emphasizes the possibility that xanthomonads associated with common blight disease are not necessarily pathogenic and the need for a rapid, reliable, and specific diagnostic test for pathogenic *X. c. phaseoli*.

The p7X3 and p7X4 fragments exhibited complete specificity for pathogenic *X. c. phaseoli*, whereas, like p7 itself (11), p7X2 DNA weakly hybridized to the genomic DNA of other *X. campestris* bacteria. The p7X2 probe hybridizes to numerous restriction fragments of DNA from non-*fuscans* strains of *X. c. phaseoli*, suggesting that this region contains one or more repetitive elements that probably account for some of the incomplete specificity of the p7 plasmid DNA. Repetitive sequences have been previously reported in *X. c. vesicatoria* (16) and *X. oryzae* pv. *oryzae* (20), two taxa that hybridized slightly with p7 DNA, both in this study and that by Gilbertson et al (11).

Our Southern hybridization analysis of *Eco*RI-restricted *X. c. phaseoli* DNA with probes from p7 revealed substantial genetic diversity among common blight strains. In a previous report (12), restriction fragment length polymorphism analysis demonstrated that *fuscans* and non-*fuscans* strains shared enough similarities to be considered common blight pathogens but could be classified into two distinct groups based on significant genetic differences (12). In this study, we show that some of the repetitive sequence(s) responsible for the polymorphisms are located on the p7X2 fragment. The p7X3 and p7X4 fragments revealed some polymorphisms among *X. c. phaseoli* strains even though they did not contain repetitive elements.

While we were able to identify *X. c. phaseoli*-specific fragments from p7 (p7X3 and p7X4), the specificity of detection was a critical consideration in the development of a PCR-based diagnostic assay. Because of its extreme level of sensitivity, false-positive reactions caused by mispriming or lack of primer specificity can compromise the credibility of the PCR approach as a routine diagnostic method. To reduce the possibility of primer annealing to alternative sequences, G+C-rich primers with high thermal melting points were designed from p7X2, p7X3, and p7X4 DNA sequences. Despite the use of a stringent thermal profile (annealing

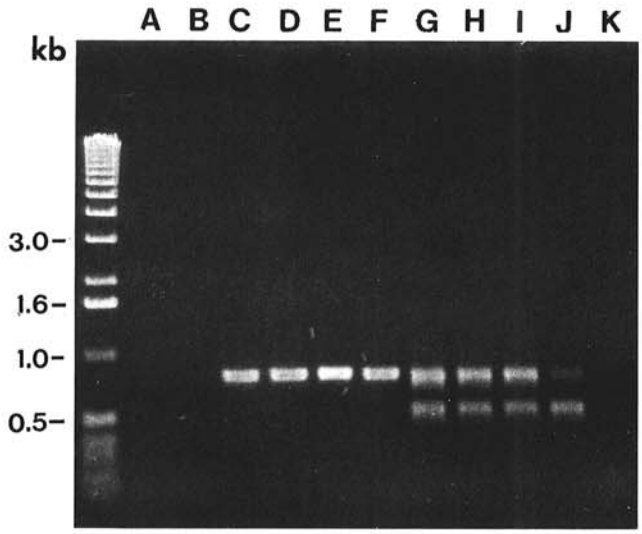

Fig. 4. Ethidium-bromide stained gel of PCR-amplification products directed by X4c and X4e primers. Template DNAs (5 ng) were from *Xanthomonas campestris* pv. *vesicatoria* ATCC 11633 (lane A); *Pseudomonas syringae* pv. *phaseolicola* HB9 (lane B); non-*fuscans X. c. phaseoli* Xp816, EK11, ATCC 9563, and ATCC 10199 (lanes C–F); *X. c. phaseoli* var. *fuscans* ATCC 19315, Xp fusc wallen, Bxp12, and Xcp59 (lanes G–J); and no-DNA control reaction (lane K). Annealing was done at 65 C. Molecular weight standard (1.0-kb ladder, BRL) was run in the left lane (unmarked), and numbers on the left indicate sizes in kilobases.

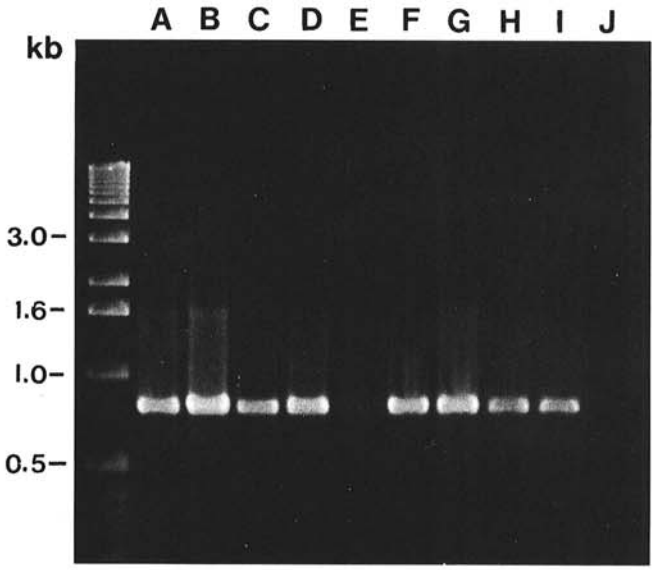

Fig. 5. Ethidium-bromide stained gel of PCR-amplification products for detection of *Xanthomonas campestris* pv. *phaseoli* in infected bean leaf tissue. Template DNAs were extracted from bacterial cells in pure culture (lanes A–D) and from 7-day-old common blight leaf lesions (lanes F–I) for non-*fuscans X. c. phaseoli* Xcp50 (lanes A and F) and Xcp51 (lanes B and G); *X. c. phaseoli* var. *fuscans* Xcp60 (lanes C and H) and Mal1 (lanes D and I); no-DNA control reaction (lane E); and mock-inoculated leaf DNA control reaction (lane J). Primer pair X4c and X4e were used at an annealing temperature of 72 C (two-step PCR). The samples from bacterial cells initially contained 50 ng of DNA, and no determination was performed for the DNA extracted from bean leaf tissues. Molecular weight standard (1.0-kb ladder, BRL) was run in the left lane (unmarked), and numbers on the left indicate sizes in kilobases.

at 65 C), only the downstream X4e primer paired with the upstream X4c or X3k oligonucleotides directed PCR amplification exclusively from the strains causing common blight.

No polymorphisms were usually observed in the PCR products generated by specific priming of *fuscans* and non-*fuscans* common blight strains. Occasionally, the p7X4 primers directed the amplification of an additional band at 0.55 kb from *fuscans* DNA, particularly when the priming occurred at 65 C with less than 10 ng of template DNA. The sporadic amplification of this smaller fragment suggests the presence of an alternative target sequence for one of the p7X4 primers in the *fuscans* DNA. This putative sequence is probably sufficiently degenerate to be primed only rarely by the X4c or X4e oligonucleotides, but other primers could likely be devised to differentiate between *fuscans* and non-*fuscans* common blight strains.

PCR detection of *X. c. phaseoli* from common blight lesions on bean leaves was efficiently performed using a quick alkaline extraction protocol. In practice, preparation of DNA suitable for PCR required less than 2 min per sample. The grinding step was the most tedious part of sample processing, and we attempted to dispense with it by simply boiling the samples in a sodium hydroxide solution (18), but the results were less reliable.

Gilbertson et al (11) reported the detection of 10 pg of *X. c. phaseoli* DNA (approximately 10^3 cfu) by dot blot hybridization using radioactively labeled p7 DNA, following a 3-day exposure of the filter. That level of sensitivity was about 100–1,000 times superior to the threshold of detection reported for an enzyme immunoassay method for bean blight bacteria (31). The methodology we present in this paper provides a very rapid and sensitive assay for *X. c. phaseoli*. A 35-cycle PCR run using a three-temperature program required less than 4 h and allowed detection of as little as 100 fg (approximately 10 cfu genomic equivalent) of *X. c. phaseoli* DNA within a regular 8-h day. Using a 37-cycle PCR program, Bereswill et al (4) could detect as few as 50 cells of *Erwinia amylovora*. Comparable limits of detection were also reported for *X. c. citri* (14) and *P. s. phaseolicola* (24), but only after Southern blot hybridization of their PCR products. With one of the *fuscans* strains (Bxp12) used in our sensitivity assay, the threshold of detection was 1 pg (approximately 100 cfu genomic equivalent) of DNA, 10-fold more than that of the four other strains tested, suggesting that the copy number of target sequences carried on the plasmid and genomic DNA might be variable within *X. c. phaseoli* strains. A similar strain-dependent sensitivity was reported with a PCR assay for *Pseudomonas solanacearum* in which thresholds of detection varied from 5 to 100 cells (30). We did not investigate the sensitivity of the PCR assay in the presence of bean tissue or other bacterial DNA, but Seal et al (30) reported that the presence of 20- and 200-fold excess of other bacteria did not reduce the sensitivity of their assay for *P. solanacearum*.

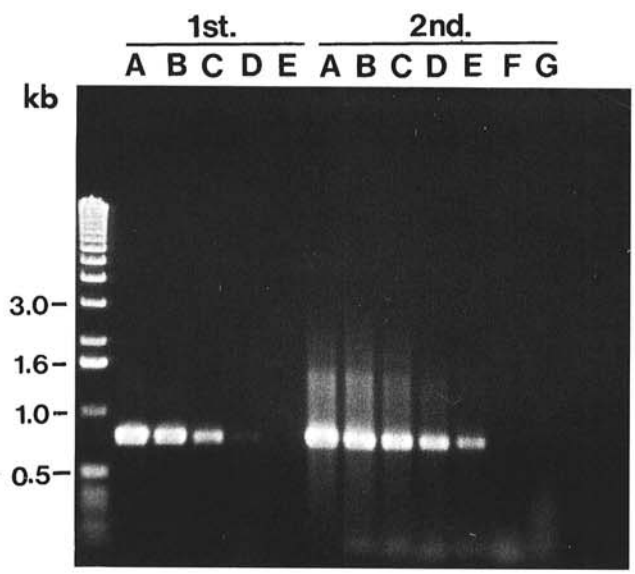

Fig. 6. Ethidium-bromide stained gel of PCR-amplification products to assess the sensitivity of the PCR assay for *Xanthomonas campestris* pv. *phaseoli* after one amplification run or two successive amplification runs. Primer pair X4c and X4e were used at an annealing temperature of 72 C (two-step PCR). PCR products were from one 35-cycle amplification run or two successive amplification runs (20-cycle run followed by 35-cycle run) initiated with 100 pg (lane A), 10 pg (lane B), 1 pg (lane C), 100 fg (lane D), 10 fg (lane E), and 1 fg (lane F) of DNA from non-*fuscans X. c. phaseoli* EK11; the no-DNA control reaction is in lane G. Molecular weight standard (1.0-kb ladder, BRL) was run in the left lane (unmarked), and numbers on the left indicate sizes in kilobases.

The rationale for designing high-melting-temperature primers was to prevent nonspecific amplification and shorten our PCR assay from the traditional three-step to a two-step temperature profile in which annealing and extension were combined. A 35-cycle, two-step PCR program took 1 h less than a three-step program to complete and gave the same level of sensitivity. With both programs, it was possible to achieve the genomic equivalent of a single-cell limit of detection with two successive PCR amplifications of 20 and 35 cycles. With its specificity and sensitivity, the simple and short PCR assay described here has great potential as a reliable procedure for the detection and identification of the bean common blight pathogen in culture and in infected plant tissue. Preliminary data show promise for detection of *X. c. phaseoli* in seed.

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