Detection of *Xanthomonas campestris* pv. *vesicatoria* Associated with Pepper and Tomato Seed by DNA Amplification

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ABSTRACT

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Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed was achieved by amplification of a DNA fragment of the bacterium by the polymerase chain reaction. Oligonucleotide primers specific for the hypersensitive reaction and pathogenicity (*hrp*) gene cluster were used in the reaction. The method includes extraction of total DNA from buffered seed washings to which sodium ascorbate and insoluble polyvinylpolypyrrolidone were added. The *hrp* fragments were amplified from the DNA preparations if cells of *X. c.* pv. *vesicatoria* were added to seed washes. Identification of an *hrp* fragment as that from *X. c.* pv. *vesicatoria* was attained by restriction enzyme analyses of the amplified fragment. The minimum number of cells that could be detected in washes from pepper or tomato seed was from 10^2 to 10^3 cfu per ml. This was about 1,000 times fewer than the minimum number of cells detected with an enzyme-linked immunosorbent assay. The pathogen was also detected in washes obtained from several lots of naturally contaminated pepper and tomato seed, one of which contained background bacterial microflora greater than 10^7 cfu per g of seed.

Additional keyword: detection of bacteria

Bacterial spot, caused by *Xanthomonas campestris* pv. *vesicatoria* is one of the most important diseases of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) plants (16,45). The disease is characterized by necrotic lesions on leaves, stems, and fruits. In warm and rainy weather, bacterial spot may cause severe defoliation of the plants that results in reduced yield (34). Furthermore, diseased fruits may not be suitable for fresh-market sale (6,33). Bacterial spot is also of major concern in the certification program in the pepper and tomato transplant industry in the southern U.S., where measures are enforced for the production of disease-free transplants (10,12).

Xanthomonas campestris pv. *vesicatoria* has been reported to be associated with tomato and pepper seed (2,8,9,18,43), and symptoms of bacterial spot have been observed in seedlings from contaminated seed lots (9,14). Consequently, the use of pathogen-free seeds and transplants has become an important part of the strategy for control of bacterial spot (10,16). However, the detection and identification of *X. c.* pv. *vesicatoria* associated with seed is still difficult with the methods available. Semiselective media developed specifically for *X. c.* pv. *vesicatoria* (11,25,42) may support the growth of strains of other pathovars of *X. campestris* as well as nonpathogenic strains of *Xanthomonas*. Because these bacteria may have cultural characteristics similar to *X. c.* pv. *vesicatoria* (11,12,24,25,42), additional physiological and/or pathogenicity tests are required to determine with certainty the identity of the xanthomonads recovered from seed (11,24).

The use of polyclonal and monoclonal antibodies in serological tests has also been examined for specific identification of *X. c.* pv. *vesicatoria* (3,4,29,30,38). However, the heterogeneous nature of antigenic determinants in immunogens of the xanthomonads has been a major constraint on the use of polyclonal antibodies in the specific identification of these plant pathogens. For instance, polyclonal antibodies developed for strains of *X. c.* pv. *vesicatoria* cross-reacted with other pathovars of *X. campestris* (30). Also, strains of *X. c.* pv. *vesicatoria* are serologically diverse (4,38), and no single monoclonal or polyclonal antibody has been obtained that reacted to all strains of this pathogen (3,17, 46). Nevertheless, enzyme-linked immunosorbent assays (ELISA) may be useful under certain conditions for detection of *X. c.* pv. *vesicatoria* (44).

Recently, *X. c.* pv. *vesicatoria* has been identified by nucleic acid-based techniques (7,17,20,23). Restriction fragment length polymorphism (RFLP) analyses of genomic and plasmid DNA have been used to differentiate several groups of xanthomonads, including some strains of *X. c.* pv. *vesicatoria* (20,21). Furthermore, DNA probes were selected for the purpose of identification of specific strains of *X. c.* pv. *vesicatoria* (7,23). The banding profiles resulting from endonuclease digestion of amplified hypersensitive reaction and pathogenicity (*hrp*)-related DNA fragments were used to identify different groups of strains within *X. c.* pv. *vesicatoria* and to distinguish them from other plant-pathogenic xanthomonads (22,23). The amplification of specific regions of the bacterial genome by the polymerase chain reaction (PCR) and restriction enzyme analysis (REA) of the region may be a sensitive technique for identification of plant-pathogenic xanthomonads associated with different plant parts.

The objective of this study was to determine if plant-pathogenic xanthomonads associated with pepper or tomato seed could be detected by a procedure based on the amplification of DNA fragments related to the *hrp* gene cluster of *X. c.* pv. *vesicatoria* (23). Furthermore, the presumptive identification of *X. c.* pv. *vesicatoria* in washings from pepper and tomato seed was determined from band profiles obtained by electrophoresis of endonuclease-digested amplified *hrp* fragments was tested. Detection of *X. c.* pv. *vesicatoria* associated with naturally contaminated seed by the PCR and REA procedures was compared with seed transmission tests and with culture on selective media. The sensitivities of the PCR procedure and ELISA for detection of *X. c.* pv. *vesicatoria* in seed washes were compared.

MATERIALS AND METHODS

Seed sources. Eleven pepper seed lots and four tomato seed lots were examined for the presence of *X. c.* pv. *vesicatoria.* Tomato seed lot 51-1b from M. Meadows, Rogers Seed Company, Naples, Fla., was

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thought to be free of *X. c.* pv. *vesicatoria* and was used as a control. The Marglobe and Manalucie seed lots were obtained through the Florida Department of Agriculture and Consumer Services, Tallahassee, in 1983 and were found previously to be free of the pathogen by seed transmission tests (R. E. Stall, *unpublished*). The seed lot DF was extracted without fermentation from diseased tomato fruits in 1992. The pepper seed lots SP2.92, SP66.92, SP124.92, SP133.92, SP135.92, and SP306.92 were obtained from Grace O'Keefe of the Georgia Department of Agriculture, Tifton, who detected the presence of viable plant-pathogenic xanthomonads by plating a portion of extracts from 30,000 seeds of each seed lot onto semiselective media (Grace O'Keefe, *personal communication*). Seed lots PK-1-PF, PK-2-PF, and PL-3-P were obtained from diseased pepper fruit by J.-F. Wang, AVRDC, Shanhua, Taiwan, in 1993. Seed lot GH was obtained from symptomless pepper fruit harvested from plants that had been inoculated with *X. c.* pv. *vesicatoria* in a greenhouse in Gainesville, Fla., and Jupiter was a commercial lot of seed of that cultivar that was obtained in 1985. Whether the latter two seed lots were contaminated with *X. c.* pv. *vesicatoria* was not known.

The presence of *X. c.* pv. *vesicatoria* on each seed lot was determined by seed transmission tests, recovery on selective media, and by the PCR-REA procedure. Each lot was tested by the above methods in two tests with three replications of each treatment in each test. Some lots were in numerous tests to determine the appropriate conditions for preparation of the samples for PCR detection.

Recovery of bacteria from seeds and seedlings. Bacteria were recovered from seeds or seedlings by an ultrasonication technique (13,28). Two and one-half grams of seed, or 10 g of the aerial part of seedlings were added to 20 ml or 150 ml, respectively, of phosphate buffer (8.5 mM K_2HPO_4, 7.5 mM KH_2PO_4, 0.02% Tween 20, pH 7.0) in a beaker and sonicated for 20 min in a model B-22-4 ultrasonic cleaner (Branson Cleaning Equipment Co., Shelton, Conn.). The buffer (seed wash) was separated from the seeds or aerial parts of seedlings by filtration through sterile cheesecloth in sterile glass funnels. Portions of the seed washes were used for detection of *X. c.* pv. *vesicatoria* by PCR or by growth of the bacterium on a selective medium. The seeds collected in the cheesecloth were used for seed transmission tests.

Growth on semiselective medium. Bacterial populations in the washes were determined by the dilution plate count method (48). Ten-fold dilutions were made in sterile tap water, and 0.1 ml of appropriate dilutions was plated onto yeast-extract nutrient agar (41) for total bacterial numbers and on Tween Medium B (24,25) for populations of *X. c.* pv. *vesicatoria.* Circular, raised, and yellow colonies surrounded by precipitate on Tween Medium B were presumed to be xanthomonads (24,25).

Transmission tests. Transmission of *X. c.* pv. *vesicatoria* from seed to seedlings was tested with all 15 pepper and tomato seed lots included in this study. Two and one-half grams of seed (ca. 1,000 seeds) were planted in a sterilized soil mixture in a plastic pot, 15 cm in diameter. The pots were placed in a shaded greenhouse with ambient temperatures of 25 to 35°C. A plastic bag, tightened to each pot with a rubber band, was placed over each pot for 3 days beginning at emergence to maintain high humidity. The seedlings were examined for lesions when in the first true-leaf stage of growth. The seedlings were then cut at the soil line for recovery of bacteria.

Extraction of DNA from washes. DNA extracts were obtained from seed or seedling washes with a modification of the method described by Ausubel et al. (1). Samples of 600 µl of the seed or seedling washes were transferred to 1.5-ml microfuge tubes, and sodium ascorbate (Sigma Chemical Co., St. Louis, Mo.) and insoluble polyvinylpolypyrrolidone (PVPP) (Sigma) were added for a final concentration of 0.2 M and 0.1%, respectively. Prior to use, the PVPP was acid washed by the procedure described by Holben et al. (15). The samples were homogenized by vortexing. The homogenate was pelleted by centrifugation in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, N.Y.) for 2 min at 16,000 × *g*. The pellet was resuspended in 567 µl of TE buffer (10 mM Tris·Cl, pH 8.0; 1 mM EDTA, pH 8.0). Proteinase K (Boehringer Mannheim, Indianapolis, Ind.) and sodium dodecyl sulfate (SDS) (Sigma) were added for a final concentration of 100 µg/ml and 0.5%, respectively. After incubation for 1 h at 37°C, sodium chloride and hexadecyltrimethyl ammonium bromide (Sigma) were added to each preparation for a final concentration of 0.7 M and 1%, respectively. The preparations were incubated for 10 min at 65°C. DNA was purified by treatment with chloroform/isoamyl alcohol (24:1). The samples were vortexed and centrifuged for 5 min at 16,000 × *g*. A

Table 1. Sensitivity of enzyme-linked immunosorbent assay (ELISA) and DNA amplification procedures for detection of *Xanthomonas campestris* pv. *vesicatoria* from tomato seed supernatants

Sample	Total population of bacteria on yeast-extract nutrient agar (cfu/ml)	*X. campestris* recovered on Tween B medium (cfu/ml)	ELISA (A_{nm492})[a]	Amplification of *hrp* fragment[b]	
				355 bp	840 bp
1	2.9×10^8	2.0×10^8	2.79 ± 0.01	+[c]	+
2	7.3×10^7	6.7×10^7	2.09 ± 0.24	+	+
3	5.4×10^6	4.6×10^6	0.51 ± 0.01	+	+
4	5.6×10^5	5.5×10^5	0.21 ± 0.01	+	+
5	6.8×10^4	6.8×10^4	0.16 ± 0.02	+	+
6	5.8×10^3	5.2×10^3	0.16 ± 0.01	+	+
7	1.7×10^2	1.4×10^2	0.17 ± 0.01	+	–
8	2.6×10^2	7.0×10^1	0.17 ± 0.01	–	–
9	1.5×10^2	Not detected	0.16 ± 0.01	–	–

[a] Values are the average (±SD) of the spectrophotometer readings of three wells.
[b] Aliquots of 300 µl and 600 µl of the seed washings were used in the ELISA assay and for extraction of DNA, respectively. Aliquots of 10 µl of DNA template were used in the DNA amplification assay.
[c] – = no *hrp* fragment amplified; + = *hrp* fragment amplified.

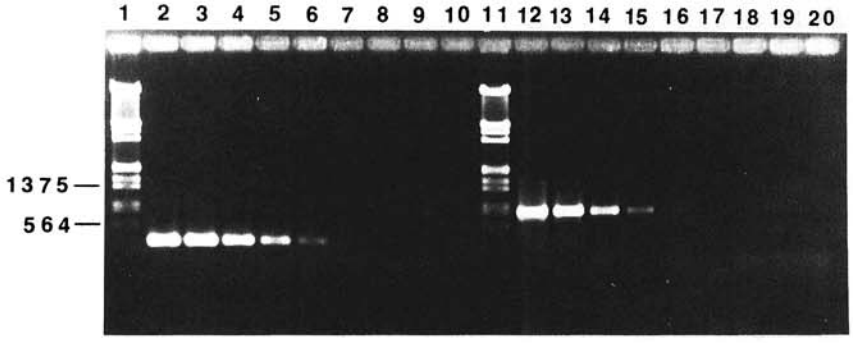

Fig. 1. Amplification of the 355-bp (lanes 2 to 10) and the 840-bp (lanes 12 to 20) *hrp* fragments from washings of tomato seeds containing different concentrations of *Xanthomonas campestris* pv. *vesicatoria* 75-3. Lanes: 1 and 11, Phage λ restricted with *Eco*RI and *Hin*dIII; 2 and 12, 2.6×10^8 cfu per ml; 3 and 13, 2.9×10^7 cfu per ml; 4 and 14, 0.9×10^6 cfu per ml; 5 and 15, 3.4×10^5 cfu per ml; 6 and 16, 1.0×10^4 cfu per ml; 7 and 17, 3.3×10^3 cfu per ml; 8 and 18. 3.0×10^2 cfu per ml; 9 and 19, 4.0×10^1 cfu per ml; and 10 and 20, no bacteria added. The number of cells of *X. c. vesicatoria* was determined by plating on Tween Medium B. Molecular sizes of bands are in base pairs.

second purification was accomplished by adding phenol/chloroform/ isoamyl alcohol (25:24:1) and centrifuging as described above. DNA was precipitated by adding 0.6 volumes of isopropanol and incubating for 30 min at –20°C. The samples were then centrifuged for 20 min at 16,000 × *g*. The DNA pellet obtained was resuspended in 70% ethanol and centrifuged again. After drying, the pellet was dissolved in 50 µl of TE buffer and stored at 4°C.

DNA amplification and REA. Two sets of oligonucleotide primers selected from the nucleotide sequence of the *hrp* gene cluster of *X. c.* pv. *vesicatoria* were used in this study (23). Primers RST2 plus RST3 delineated a 840-bp fragment, and RST9 plus RST10 delineated a 355-bp fragment. Both sets of primers map to the complementation group *hrpB* of the *hrp* gene cluster of *X. c.* pv. *vesicatoria* (23). Oligonucleotide primers were synthesized with a model 394 DNA Synthesizer (Applied Biosystems, Foster City, Calif.) by the DNA Synthesis Laboratory, University of Florida, Gainesville.

DNA fragments were amplified in a reaction mixture of 50 µl containing 5 µl of 10X buffer (500 mM KCl, 100 mM Tris·Cl [pH 9.0 at 25°C], 1% Triton X-100), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Boehringer Mannheim), 25 pmol of each primer, and 1.25 units of *Taq* polymerase (Promega, Madison, Wis.). A volume of 5 to 10 µl of the bulk DNA preparation obtained from the seed or seedling samples was added to the reaction mixture, unless otherwise stated. The mixture was overlaid with 50 µl of light mineral oil. Thirty amplification cycles were performed in an automated thermocycler PT-100-60 (MJ Research, Watertown, Mass.) according to the following programs: 30 s of denaturation at 95°C, 30 s of annealing at 62°C, and 45 s of extension at 72°C for the primers RST2 plus RST3; and 30 s of denaturation at 95°C, 30 s of annealing at 52°C, and 45 s of extension at 72°C for the primers RST9 plus RST10. For both programs, the initial denaturation step was 5 min at 95°C, and the last extension step was extended to 5 min. The PCR products were extracted once with 25 µl of chloroform/isoamyl alcohol (24:1) before electrophoresis in 0.9% agarose gels in TAE buffer (40 mM Tris·acetate, 1 mM EDTA, pH 8.2) according to standard procedures (37).

The extracted PCR products were digested with the frequent-cutting endonucleases *Cfo*I, *Hae*III, or *Taq*I, according to conditions specified by the manufacturer (Promega). The digested fragments were separated by electrophoresis in 4% agarose gels (3% NuSieve and 1% Seakem GTG [FMC BioProducts, Rockland, Maine]) in TAE buffer at 8 V/cm. Phage λ *Pst*I-digested DNA fragments were used as molecular standards. The gels were stained with 0.5 µg of ethidium bromide per ml for 40 min and then destained in 1 mM $MgSO_4$ for 1 h and photographed over a UV transilluminator with type 55 Polaroid film. The restriction pattern obtained was compared with the standard banding profiles established for the different groups of plant-pathogenic xanthomonads (22).

ELISA assay. A suspension of strain 75-3 (tomato race 1) of *X. c.* pv. *vesicatoria* was added to the washes of seed lot 51-1b at appropriate concentrations before ultrasonication for detection by ELISA. Monoclonal antibodies were available for detection of this strain (17). A procedure that was developed for detection of low populations of *X. c.* pv. *vesicatoria* was used (44). Aliquots of 300 µl of the seed washes containing different concentrations of cells of *X. c.* pv. *vesicatoria* were transferred to 1.5-ml microfuge tubes and an equal amount of EDTA/lysozyme lysis

Table 2. Detection of plant-pathogenic xanthomonads in naturally contaminated pepper and tomato seed

Seed sample	Total population of bacteria on YNA (cfu/g seed)[a]	Recovery of xanthomonads		Amplification of *hrp* fragment[c]		Source of the seed[d]
		Tween[a]	Transmission[b]	355 bp	840 bp	
Pepper						
SP2.92	3.2×10^2	–[e]	–	+	+	GOK
SP66.92	1.9×10^3	–	–	(+)	+	GOK
SP124.92	3.2×10^2	–	+	+	+	GOK
SP133.92	$>2.4 \times 10^5$	–	+	+	+	GOK
SP135.92	1.6×10^2	–	–	–	+	GOK
SP306.92	0.8×10^2	–	+	+	+	GOK
Jupiter	4.0×10^2	–	–	–	–	RST
GH	5.4×10^2	–	–	(+)	(+)	RST
PK-1-PF	$>4.0 \times 10^3$	–	–	–	+	JFW
PK-2-PF	$>4.0 \times 10^3$	–	+	(+)	+	JFW
PL-3-P	$>4.0 \times 10^3$	–	–	+	+	JFW
Tomato						
51-1b	4.0×10^3	–	–	–	–	MM
DF	1.1×10^7	–	+	+	+	RST
Marglobe	4.6×10^3	–	–	–	ND	RST
Manalucie	1.0×10^3	–	–	–	ND	RST

[a] Yeast-extract nutrient agar (YNA) is a general medium for isolation of bacteria (40) and Tween Medium B (Tween) (25) is selective for *Xanthomonas*.

[b] Presence of plant-pathogenic xanthomonads was determined by detection by amplification of *hrp*-related fragment.

[c] Amplification of fragments related to the *hrpB* of *Xanthomonas campestris* pv. *vesicatoria* by 30 cycles of polymerase chain reaction.

[d] GOK, G. O'Keefe, Georgia Department of Agriculture, Tifton, Ga.; JFW, J. F. Wang, The Asian Vegetable Research and Development Center Tainan, Taiwan; MM, M. Meadows, Rogers NK, Naples, Fla.; RST, R. E. Stall, University of Florida, Gainesville.

[e] – = negative result; + = positive result; (+) = weak positive result; ND = not determined.

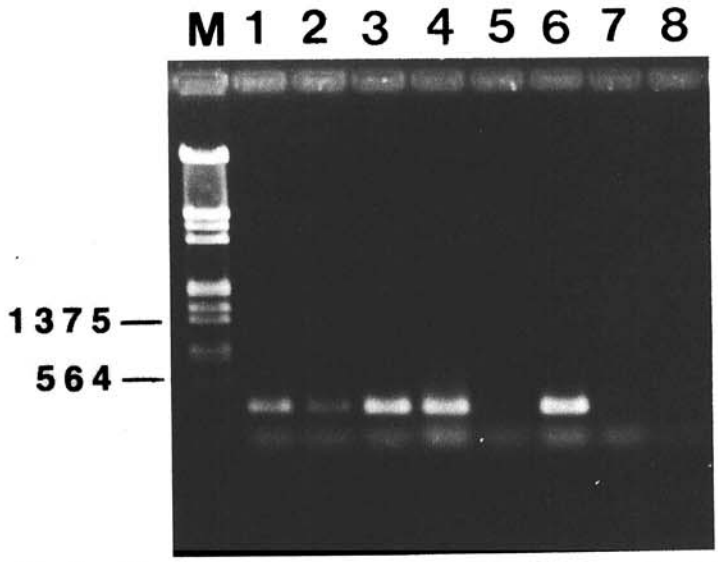

Fig 2. Amplification of the 355-bp *hrp* fragment from DNA extracted from washings of different pepper seed lots. Lanes: M, phage λ restricted with *Eco*RI and *Hin*dIII; 1, SP2.92; 2, SP66.92; 3, SP124.92; 4, SP133.92; 5, SP135.92; 6, SP306.92; 7, GH; 8, Jupiter.

buffer (2.0 g of KH_2PO_4, 11.5 g of Na_2PO_4, 0.14 g of EDTA disodium, 0.02 g of thimerosal, 0.2 g of lysozyme (Sigma), 1 liter of deionized water) was added. The samples were homogenized by vortexing and incubated for at least 16 h at room temperature.

Immulon 2, flat-bottom, 96-well microtiter plates (Dynatech Laboratories, Chantilly, Va.) were coated with a polyclonal antibody raised against *X. c.* pv. *vesicatoria* 75-3 (44). The plates were then incubated at 4°C overnight. The coating buffer and phosphate-buffered saline (PBS) used were described by Clark and Adams (5). All further incubations were for 2 h at 37°C. The microtiter plates were washed with a solution containing 0.8% NaCl and 0.1% Tween 20 in deionized water (NT rinse) (G. C. Somodi, *unpublished data*). Plates were then blocked with bovine albumin (Sigma) and shaken to remove excess liquid. Aliquots of 100 µl of each sample in EDTA/lysozyme lysis buffer were added to three wells on the microtiter plate and incubated. The microtiter plates were rinsed with NT rinse and a monoclonal antibody, designated 2H10, was added to all wells and the plates were incubated. The microtiter plates were rinsed again with NT rinse, and alkaline phosphatase conjugated goat antimouse (Sigma) was applied to the plate. The microtiter plates were incubated and then rinsed four times with the final washing buffer of Tris-buffered saline (1.5 g of Tris·base, 2.2 g of NaCl, final volume 250 ml with deionized water, pH 7.5). Substrate and amplifier were added according to the instructions specified by the manufacturer of the ELISA Amplification System (Gibco BRL, Gaithersburg, Md.). Readings were made at A_{492} 15 min after addition of the amplifier with an EAR 400 AT plate reader (SLT-Labinstruments, Grödig, Austria).

The sensitivity of the PCR procedure and ELISA for detection of *X. c.* pv. *vesicatoria* was compared in two experiments with three replications of each treatment.

RESULTS

Detection of *X. c.* pv. *vesicatoria* in seed washes. An 840-bp DNA fragment was amplified with primers RST2 plus RST3 from DNA preparations obtained from the washes of tomato seed lot 51-1b to which 10^6 cells per ml of *X. c.* pv. *vesicatoria* 75-3 were added. The fragment was not amplified if cells of *X. c.* pv. *vesicatoria* were not added. A 355-bp fragment was also amplified with primers RST9 plus RST10, if the bacterial cells were added, but not if the cells were not added (data not shown). Those fragment sizes were expected for each set of primers and the DNA bands obtained by digestion of each fragment with the three endonucleases were as expected for the A group of *X. c.* pv. *vesicatoria* (23).

In preliminary studies, amplification of the *hrp* fragment occurred from DNA preparations only if DNA was extracted from contaminated washes that contained sodium ascorbate and PVPP. However, in subsequent experiments amplification of the *hrp* fragment occurred from DNA preparations if the contaminated washes were processed without those two reagents. No inhibition of the amplification process by the two reagents was observed when a series of 5 µl, 10 µl, and 15 µl of the DNA preparation from contaminated washes of seed lot 51-1b were each added to buffer containing 0.2 M sodium ascorbate and 0.1% PVPP, and to buffer without the reagents. Thus, sodium ascorbate and PVPP were added to all other preparations for PCR detection of *X. c.* pv. *vesicatoria* in this study.

Sensitivity of DNA amplification. When 10-fold dilutions of *X. c.* pv. *vesicatoria* 75-3 were added to washes of seedlot 51-1b and DNA was extracted from them, the minimum number of cells detected was about 10^2 cfu per ml with the primers RST9 plus RST10 (Table 1, Fig. 1). In contrast, the minimum number of cells of *X. c.* pv. *vesicatoria* detected was at least 10 times higher when the primers RST2 plus RST3 were used for amplification of the 840-bp fragment (Table 1, Fig. 1). The lower sensitivity of the primers RST2 plus RST3 for detection of *X. c.* pv. *vesicatoria* was also observed with washes from pepper seed lots (data not shown). In comparison, the minimum number of cells of *X. c.* pv. *vesicatoria* detected in the seed washes by ELISA was greater than 10^5 cfu per ml (Table 1).

Detection of plant-pathogenic xanthomonads in washes from naturally contaminated seed lots. Amplification of the expected fragments occurred from DNA extracted from washes of 11 of 15 seed lots (Table 2). The results were the same in both experiments, but the intensity of the bands of DNA in agarose gels after electrophoresis of the amplified product sometimes differed. The expected DNA fragments were amplified in spite of background bacterial microflora of up to 10^7 cfu per g of seed in one lot (Table 2). Even though primers RST9 plus RST10 detected the lowest levels of bacteria, xanthomonads were detected on more lots of seed with the primers RST2 plus RST3. The 840-bp fragment was amplified with the latter primers from all seed lots known to be contaminated with *X. c.* pv. *vesicatoria* and not amplified from all seed lots suspected to be not contaminated. The primers RST9 plus RST10 failed to amplify the 355-bp fragment from the pepper seed lots SP135.92 (Fig. 2) and PK-1-PF even though amplification of the 840-bp fragment with primers RST 2 plus RST 3 indicated the presence of plant-pathogenic xanthomonads in these seed lots (Table 2). Both sets of primers amplified expected fragments in low yield from DNA extracted from washes from the GH seed lot and did not from the washes from the Jupiter seed lot.

The band profiles resulting from digestion of each of the amplified 840-bp fragments with the endonucleases *Taq*I and *Cfo*I were identical to the *hrp* band profile of group A strains of *X. c.* pv. *vesicatoria*, except for two. Interestingly, the 840-bp fragment amplified from DNA extracted from washings of seed lots SP66.92 and SP133.92 produced a complex banding profile (data not shown) compared with the profiles established for different groups of plant-pathogenic xanthomonads (22). The profile of group A strains (with SP133.92) and of group B strains (with SP66.92) of *X. c.* pv. *vesicatoria* could be distinguished in the complex profile, however. REA of the 840-bp fragment obtained with the GH seed lot was not successful because of low yield of the fragment. The band profiles obtained for each of the 355-bp fragments after digestion with either endonuclease *Cfo*I or *Hae*III were identical to the *hrp* profile of group A strains of *X. c.* pv. *vesicatoria* (46; R. P. Leite, *unpublished data*). Low yields of the 355-bp fragment from DNA extracted from washes of seed lots SP66.92, GH, and PK-2-PF precluded REA.

The attempts to recover plant-pathogenic xanthomonads from washes of the naturally contaminated seed lots on both yeast-extract nutrient agar and Tween B media were unsuccessful (Table 2). With some seedlots a large background bacterial microflora in washings prevented the identification of any possible xanthomonads that may have grown. Similarly, bacterial spot was not positively identified in seedlings in the seed transmission test. Although lesions on cotyledons were sometimes found, bacterial spot could not be presumed. However, the presence of viable cells of *X. c.* pv. *vesicatoria* on the seedlings from five seed lots was confirmed by detection of the bacterium by amplification of *hrp* fragments from bulked DNA extracted from washes of the seedlings (Table 2).

DISCUSSION

Specific and sensitive methods for detection of plant pathogenic bacteria associated with seeds and other plant propagative materials are needed (26,36,39). The amplification of *hrp* sequences by PCR and REAs of the amplified DNA sequences can be a useful tool for the detection and identification of plant-pathogenic xanthomonads on plant material. Saprophytic bacteria and opportunistic xanthomonads are not detected because they do not have *hrp* genes. Furthermore, sequence variation within the *hrpB* operon among plant-pathogenic xanthomonads allows selection of primers that have different specificities, as was illustrated by the dif-

ferent specificities of the two sets of primers used in this work and in work published previously (23). The primer set RST9 plus RST10 apparently did not amplify a *hrp* fragment from extracts of some seeds that were amplified with the primer set RST2 plus RST3. On the other hand, the latter primers probably amplified a *hrp* fragment of another plant pathogenic xanthomonad on two lots of seed in addition to that of *X. c.* pv. *vesicatoria*. The complex band profiles after endonuclease digestion of the amplified fragment would support that hypothesis. This is not surprising since several xanthomonads that produce hypersensitivity on pepper and tomato plants have been isolated from pepper and tomato seed (22). Since other plant-pathogenic xanthomonads may be detected with RST2 and RST3, these primers may possibly be used to detect plant-pathogenic xanthomonads on other types of seed. We are continuing to evaluate other primer sets from the *hrpB* operon for increased specificity for *X. c.* pv. *vesicatoria*, however.

The REAs of amplified DNA can detect amplification of nonspecific DNA in addition to amplification of DNA from nontarget xanthomonads that might be on the plant material. Thus, the analyses are advantageous as a control for false-positive detection. Occasionally, low yields of the *hrp* fragments occurred that precluded REA. A second round of amplification with a set of primers internal to the primers used for the initial amplification can overcome the low yield problem. Thus, a "nested" set of primers will be needed for characterization of the amplified fragments in some cases.

The sensitivity of the DNA amplification method ranged from about 10^2 to 10^3 cfu per ml of seed washings when cells were added. This level of detection is at least 1,000 times more sensitive than the level obtained with ELISA and is comparable to the levels obtained with the most sensitive techniques available for detection of bacteria (32,36). However, this level of sensitivity of detection may be improved further by including a step for concentration of the cells in the sample, or to enrich target bacteria by plating washes on a selective medium (35,39,40). These steps will concomitantly concentrate unwanted saprophytic microflora. Although the presence of a large background microflora is a major limitation in the detection of plant-pathogenic xanthomonads in pepper and tomato seed by plating on general or even semiselective media (11,25), the specificity of the DNA amplification procedure is likely to overcome this kind of problem. The specificity of the method allowed for the detection of plant-pathogenic xanthomonads against background bacterial microflora larger than 10^7 cfu per g of seed in our tests.

The reagents sodium ascorbate and PVPP prevent the action of potential inhibitors of PCR. The presence of inhibitors of PCR has been a major concern in the extraction of high quality DNA from environmental and plant samples (15,27, 47). The reagents were necessary for the detection of *Xylella fastidiosa* in extracts of plant tissue (27). The addition of the reagents to the seed washes before nucleic acid extraction was sometimes needed and they may be more important in cases in which large quantities of seed or plant material are sampled for detection of xanthomonads. The addition of sodium ascorbate and PVPP to the washes of seeds of the tomato lot 51-1b did not interfere with the recovery of DNA, nor the amplification of the DNA fragments by PCR.

A basic feature of our recovery of bacterial DNA was the initial separation of intact bacterial cells from the seed washes by centrifugation. This was followed by lysis of the cells and purification of the recovered DNA. This DNA extraction step may require more work than the direct lysis method that involves the release and extraction of DNA without prior separation of the cells from the original matrix (31,47). However, a DNA extraction procedure may produce DNA of higher quality for PCR amplification (15,47). In fact, inconsistent results (22) in the detection of *X. c.* pv. *vesicatoria* in seed washes were obtained with a quick approach (19) that employs direct lysis of the bacterial cells without DNA extraction.

Transmission of *X. c.* pv. *vesicatoria* to seedlings was detected with only five of 11 seed lots that tested positively for the bacterium in the DNA amplification procedure. Detection of the bacterial spot pathogen by the DNA amplification procedure may be at levels below that which will result in seed transmission, or nonliving cells of the pathogen may contribute to the DNA amplification procedure, but not to seed transmission. Research is needed for optimizing the conditions for seed transmission of *X. c.* pv. *vesicatoria* and to determine the efficiency of the bacterium in seed transmission to pepper and tomato seedlings under optimum conditions (2, 8,9).

In conclusion, the procedure based on amplification and analysis of *hrp*-related fragments from bacteria was deemed to be sensitive and specific for detection of plant-pathogenic xanthomonads associated with pepper and tomato seed. Although different xanthomonads cannot be distinguished by the size of the amplified DNA fragment with a given set of primers from the *hrpB* operon (23), the sequence variation present in *hrp* genes from different strains of *Xanthomonas* was valuable for differentiation of these plant-pathogenic xanthomonads. Further, comparison of the restriction enzyme profiles to predetermined profiles of *X. c.* pv. *vesicatoria* (22) allowed detection of the pathogen on seed.

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