Development of PCR-Based Assays for Detecting *Xanthomonas campestris* pv. *carotae*, the Carrot Bacterial Leaf Blight Pathogen, from Different Substrates

X. Q. Meng, K. C. Umesh, R. M. Davis, and R. L. Gilbertson, Department of Plant Pathology, University of California, Davis 95616

ABSTRACT

Meng, X. Q., Umesh, K. C., Davis, R. M., and Gilbertson, R. L. 2004. Development of PCR-based assays for detecting *Xanthomonas campestris* pv. *carotae*, the carrot bacterial leaf blight pathogen, from different substrates. *Plant Dis.* 88:1226-1234.

Detection of the carrot bacterial leaf blight pathogen, *Xanthomonas campestris* pv. *carotae*, was achieved using polymerase chain reaction (PCR) along with primer pairs developed from sequences of cloned random amplified polymorphic DNA (RAPD) fragments. Primer pairs 3S and 9B directed the amplification of ~350-bp and ~900-bp (or ~2 kb) DNA fragments, respectively, from genomic DNA of all known *X. campestris* pv. *carotae* strains tested, but not from that of 13 other *X. campestris* pathovars or other bacterial species, including yellow non-xanthomonad bacteria isolated from carrot tissues and seeds. In tests conducted with an extensive collection of *X. campestris* pv. *carotae*-like strains isolated from different substrates from California, Idaho, Oregon, Washington, and Canada, the 3S primer pair directed the amplification of the ~350-bp target fragment from all strains. These results indicated that the 3S primer pair is highly specific for *X. campestris* pv. *carotae* detection. Using the 3S primer pair, PCR assays were developed for detection of *X. campestris* pv. *carotae* from colonies on agar media, carrot leaf and stem tissues, and seeds. These tests could be performed in a single day. The PCR-based seed assay detected *X. campestris* pv. *carotae* from lots with contamination rates ranging from 2×10^2 to 2.3×10^8 CFU per gram of seed. This assay gave results similar to a seed-wash dilution plating assay and proved more sensitive than an enzyme-linked immunosorbent assay (ELISA)-based assay.

Additional keywords: seedborne pathogen

Bacterial leaf blight of carrot (*Daucus carota* subsp. *sativus*), caused by *Xanthomonas campestris* pv. *carotae*, is an important seedborne disease with a worldwide distribution. Under favorable conditions, this disease can cause significant yield losses (8,14,15,17,23,25). Foliar symptoms of bacterial leaf blight can be easily confused with other diseases, such as Alternaria leaf blight, particularly early in the season. This complicates disease diagnosis and selection of appropriate disease management strategies.

Contaminated carrot seeds are an important primary source of inoculum for bacterial leaf blight. Therefore, planting seed free of *X. campestris* pv. *carotae* contamination is an important disease management strategy. However, detection of this pathogen on carrot seeds can be difficult and time-consuming. The most commonly used

method is a seed-wash dilution plating assay, which involves washing seeds in buffer and plating serial dilutions of concentrated or unconcentrated washate on the semi-selective medium, XCS agar (19). After incubation at 28°C for 5 days, *X. campestris* pv. *carotae* colonies are identified based on colony morphology (light yellow, convex, and mucoid). As XCS agar is semi-selective, growth of saprophytic bacteria (including yellow bacteria that are not pathogenic on carrots) can complicate identification. Therefore, pathogenicity tests must be completed to positively identify representative *X. campestris* pv. *carotae*-like colonies. These tests are laborious, require greenhouse or growth chamber facilities, and take approximately 3 to 4 weeks to complete. A rapid and sensitive method for detection and identification of *X. campestris* pv. *carotae* colonies on XCS agar medium and/or direct detection from plants or seeds could improve bacterial leaf blight management.

Polymerase chain reaction (PCR) has become a useful tool for detection of plant pathogenic bacteria, including *X. campestris* pathovars (1,4,7,11,12,20–22). In this report, the development and application of a PCR-based method for detection of *X. campestris* pv. *carotae* is presented, together with methods for detecting the

pathogen from agar media, carrot leaf and stem tissues, and seeds.

MATERIALS AND METHODS

Isolation and characterization of *X. campestris* pv. *carotae* strains and pathogenicity tests. Five to 10 carrot leaves or umbels with blight symptoms from each carrot field were surface-disinfested in 5% sodium hypochlorite for 1 min, rinsed three times in sterilized distilled water, blotted dry, and cut into small pieces with a razor blade. Two or three drops of sterile distilled water were added, and the suspension was streaked onto plates of XCS agar medium. Plates were incubated at 28°C for 5 days. *X. campestris* pv. *carotae*-like colonies (light yellow, convex, and mucoid) were subcultured onto plates of 523 medium (13) and incubated at 28°C for 3 to 4 days.

One to three *X. campestris* pv. *carotae*-like strains from each carrot field or seed lot were selected for pathogenicity tests using a method modified from that of Umesh et al. (23). Cells from a single colony of the strain to be tested were inoculated into 4 ml of 1× yeast tryptone (YT) broth in a 10-ml culture tube (Fisher Scientific Co., Pittsburg, PA). Tubes were placed on a rotary shaker (250 rpm) and incubated at 28°C for 18 to 24 h. These cell suspensions were diluted 20-fold with sterile water, which generated suspensions of $\sim 1 \times 10^6$ CFU/ml (based on dilution plating on 523 medium). Carrot seedlings (cv. Caropak) at the 3- to 4-leaf stage (~35 days old) were sprayed with bacterial suspensions until runoff with a hand-held air pump sprayer. At least 10 carrot seedlings were inoculated with each strain in three independent experiments. Control plants were sprayed with diluted YT broth. Following inoculation, seedlings were covered with a plastic bag and placed in a greenhouse. After 48 h, bags were removed, plants were placed in a mist chamber, and symptom development was assessed 3 to 4 weeks later.

Carrot seed lots and seed-wash dilution plating assay for *X. campestris* pv. *carotae*. A total of 64 carrot seed lots were obtained from California, Oregon, and Washington during the 2001 to 2003 growing seasons. Seeds were assayed for *X. campestris* pv. *carotae* using the method of Kuan et al. (14) with minor modifications (23). Briefly, 10 g of carrot seeds were incubated in 100 ml of 0.85% NaCl at 4°C

Corresponding author: R. L. Gilbertson
E-mail: rlgilbertson@ucdavis.edu

Present address of K. C. Umesh: California Department of Food and Agriculture, 1220 N Street, Room A-372, Sacramento, CA 95814

Accepted for publication 18 June 2004.

Publication no. D-2004-0830-01R
© 2004 The American Phytopathological Society

for 16 to 18 h in a 250-ml flask. Two drops of Tween 20 were added, and then flasks were placed on a rotary shaker (250 rpm) for 2 min. The seed wash was filtered through four layers of sterilized cheesecloth and centrifuged at $10,000 \times g$ for 10 min. The resulting pellet was resuspended in 10 ml of 0.85% NaCl, serial dilutions (10^0 to 10^{-5}) were prepared in 0.85% NaCl, and 100- μ l aliquots of each dilution were spread onto each of three plates of XCS agar medium. Plates were incubated at 28°C for 5 days, and *X. campestris* pv. *carotae*-like colonies were counted.

The following tests were done to assess the efficacy of the seed-wash dilution plating assay. The recovery efficiency on XCS agar medium was compared with that on 523 medium by plating dilutions of a strain (Xcc12) on both media in three independent experiments. Also, two seed-wash methods: (i) the standard 16- to 18-h wash at 4°C and (ii) a 2-h wash at room temperature, were compared in three independent experiments with five seed lots from California and one from Washington, all having different levels of contamination.

Isolation of total genomic DNA from bacterial cells. Total genomic DNA was isolated from cells of *X. campestris* pv. *carotae*, 13 other known *X. campestris* pathovars, and four other species of bacteria using the method of Audy et al. (4) with minor modifications. Cells from single colonies were inoculated into 8 ml of 1× YT broth in a 25-ml polypropylene tube (Fisher Scientific Co.). Tubes were placed on a rotary shaker (250 rpm) and incubated at 28°C for 24 h. Cell suspensions were transferred into 15-ml polypropylene tubes, and tubes were centrifuged at $9,200 \times g$ for 10 min. Cell pellets were resuspended in 1 ml of NE buffer (150 mM NaCl, 50 mM EDTA [pH 8.0]), and suspensions were transferred into 1.5-ml Eppendorf tubes. Tubes were centrifuged at $10,000 \times g$ for 5 min, and cells were washed with 1 ml of NE buffer and resuspended in 600 μ l of NE buffer containing proteinase K at 150 μ g/ml (BDH, Vancouver, BC, Canada) and 1% sodium dodecyl sulfate (SDS). This suspension was vortexed for 1 min and incubated at 50°C for 1 h. Cell lysates were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and DNA was recovered by precipitation with isopropanol. The DNA was spooled out with a flame-sealed Pasteur pipette, rinsed in 70% ethanol, and resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Random amplified polymorphic DNA (RAPD) analysis and cloning and sequencing of RAPD fragments. RAPD analysis of total genomic DNA of selected *X. campestris* pv. *carotae* strains was performed as previously described for *X. campestris* pv. *vitians* (6,7) using Operon primer set AB. RAPD products were frac-

tionated in 1% agarose gels in 1× Tris Borate EDTA (TBE) buffer. Selected RAPD fragments were gel-purified using silica matrix (GeneClean, BIO 101, La Jolla, CA).

RAPD fragments were cloned with the TA cloning system (Invitrogen, San Diego, CA), and recombinant plasmids were identified by restriction enzyme digestion with *Eco*RI (18). Sequences of cloned fragments were determined using the dideoxy nucleotide chain-termination method and Sequenase (USB, Cleveland, OH). Primers were designed from DNA sequences using the software Primer Designer (Version 2.0, Scientific & Educational Software, Durham, NC).

PCR with *X. campestris* pv. *carotae* primer pairs and gel electrophoresis. The PCR was carried out in a 50- μ l reaction mixture containing 1× PCR buffer (20 mM Tris-HCl [pH 9.0], 37.5 mM KCl, and 7.5 mM Triton X-100); 250 μ M each of dATP, dCTP, dGTP, and dTTP; 1 μ M each of forward and reverse primers; 2 units of *Taq* DNA polymerase (Perkin Elmer Cetus, Emeryville, CA); 4 ng of bacterial total genomic DNA in a DNA Thermal Cycler 480 (Perkin Elmer Cetus), an Eppendorf Mastercycler gradient (Eppendorf Scientific Inc., Westbury, NY), or a Primus 96 plus PCR system (MWG Biotech Inc., High Point, NC). The PCR profile was 30 cycles of 94°C for 1 min, 65°C for 45 s, and 72°C for 2 min.

To determine the threshold level of DNA detectable with PCR and various primer pairs, serial dilutions of genomic DNA of *X. campestris* pv. *carotae* (strain 108-2L2-1) were prepared and used in the PCR. PCR products were fractionated in 1% agarose gels in 0.5× TBE, stained with ethidium bromide, and visualized with an ultraviolet transilluminator (Fisher Scientific Co.) and an IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA).

To test the specificity of the designed primer pair for *X. campestris* pv. *carotae*, PCR was conducted using total genomic DNA of one strain of 13 other known *X. campestris* pathovars (*X. campestris* pv. *alfalfae*, *X. campestris* pv. *cucurbitae*, *X. campestris* pv. *incanae*, *X. campestris* pv. *malvacearum*, *X. campestris* pv. *manihotis*, *X. campestris* pv. *oryzae*, *X. campestris* pv. *pelargonii*, *X. campestris* pv. *phaseoli*, *X. campestris* pv. *pruni*, *X. campestris* pv. *raphani*, *X. campestris* pv. *translucens*, *X. campestris* pv. *vesicatoria*, and *X. campestris* pv. *vitians*) and four other species of bacteria (*Agrobacterium tumefaciens*, *Clavibacter michiganensis* subsp. *michiganensis*, *Erwinia carotovora* subsp. *carotovora*, and *Pseudomonas syringae* pv. *phaseolicola*). The 13 strains of these other *X. campestris* pathovars were kindly provided by L. du Toit (Mount Vernon Research and Extension Unit and Department of Plant Pathology, Washington State Uni-

versity). Strains of the four other species of bacteria were from our laboratory.

PCR detection of colonies from agar plates. For detection of colonies, boiled cell extracts of bacterial colonies growing on plates of 523 or XCS agar medium were used as previously described for *X. campestris* pv. *vitians* (7). Cells were resuspended in 100 μ l of sterile water prior to boiling.

PCR detection from carrot plant tissues. For detection from carrot plant tissues, plants with bacterial leaf blight symptoms collected from the field or carrot seedlings sprayed with a known strain (01C5L31) in a greenhouse were used. Tissue from seedlings inoculated with sterile distilled water was used as a negative control. Four methods of DNA extraction were tested: (i) The NaOH + PVP method of Wang et al. (24), in which 10 mg of leaf tissue (four or five ~2 mm² pieces of leaf tissue from borders of diseased and healthy tissue) was placed in an Eppendorf tube, thoroughly ground in 100 μ l of 0.5 N NaOH + 0.5% PVP (wt/vol) with a Kontes pestle, and 5 μ l of this extract was transferred to another tube with 495 μ l of 20 mM Tris-HCl (pH 8.0). Two microliters of this extract was used in a 50- μ l PCR reaction. (ii) The Dellaporta method (10), in which DNA was extracted from 10 to 15 mg of leaf tissue, resuspended in 500 μ l of sterile water, and 2 μ l of this DNA extract was used in a 50- μ l PCR reaction. (iii) The CTAB method (26), in which DNA was extracted from 10 to 15 mg of leaf tissue with cetyltrimethylammonium bromide (CTAB) buffer (prewarmed to 60°C), resuspended in 500 μ l of sterile water, and 2 μ l used in a 50- μ l PCR reaction. (iv) The DNeasy Plant Minikit method (Qiagen Inc., Valencia, CA), in which DNA was extracted from 0.5 to 1.0 mg of leaf tissue. Tissue was ground into a fine powder in liquid nitrogen with a mortar and pestle, transferred to a 1.5-ml Eppendorf tube, and DNA extracted following manufacturer's instructions. Four microliters of this DNA extract was used in a 25 μ l PCR reaction. For all four methods, serial dilutions of DNA extracts were also prepared and used in the PCR.

PCR detection from carrot seeds. For detection from carrot seeds by PCR, 10 g (~10,000 seeds) of seeds were washed as described for the seed-wash dilution plating assay, and the pellet was resuspended in 10 ml of 0.85% NaCl. From this suspension, 0.5 or 1 ml was added to a 1.5-ml Eppendorf tube, the tube was centrifuged at $10,000 \times g$ for 10 min, and the resulting pellet was used for DNA extraction by four methods: (i) The NaOH + PVP method (24), in which the pellet was resuspended in 100 μ l of 0.5 N NaOH + 0.5% PVP (wt/vol); 5 μ l of this suspension was added to 495 μ l of 20 mM Tris-HCl (pH 8.0), and 2 μ l of this extract was used in a 50- μ l PCR reaction. (ii) A modified Dellaporta

method (10), in which the pellet was resuspended in 600 µl of Dellaporta buffer and 1% SDS, and 2 µl of this DNA extract was used in a 50-µl PCR reaction. (iii) The CTAB method (26), in which the pellet was resuspended in 800 µl of 2% CTAB buffer, and 2 µl of this extract was used in a 50-µl PCR reaction. (iv) The Qiagen DNeasy Tissue Kit method (Qiagen Inc.), in which the pellet was resuspended in 180 µl of ATL buffer (as described in the proto-

col for isolation of genomic DNA from gram-negative bacteria), and 4 µl of this DNA extract was used in a 25-µl PCR reaction. For all four methods, serial dilutions of DNA extracts were also prepared and used in the PCR.

To test whether *X. campestris* pv. *carotae* could be detected from carrot seed with the PCR-based seed assay following a hot water treatment, two seed lots with contamination levels of 1.1×10^6 and $2.3 \times$

10^7 CFU/g were treated with hot water (52°C for 25 min), a treatment known to eradicate *X. campestris* pv. *carotae* (3,25). To detect the pathogen from these seed lots by PCR, the 2-h seed wash method followed by the Qiagen DNeasy Tissue Kit extraction and PCR was used as described above.

Threshold of detection for the PCR assays. To determine the threshold of detection of *X. campestris* pv. *carotae* from carrot seed for the PCR-based seed assay, DNA extracts were prepared from seed-wash pellets using the NaOH + PVP, Dellaporta, and CTAB extraction methods from five naturally contaminated seed lots from California. Contamination levels of these seed lots ranged from 2×10^2 to 5.7×10^7 (determined with the seed-wash dilution plating assay). To assess the threshold in terms of the minimum number of contaminated seeds detectable in 10,000, one, two, or 10 artificially infested seeds were added to 10 g of seed previously tested free of *X. campestris* pv. *carotae*. These lots were washed for 16 to 18 h at 4°C, seed-wash pellets were obtained, DNA extracts were prepared from the pellets by the CTAB method, and PCR analysis was performed with the 3S primer pair. These assays were performed in three independent experiments, except for the minimum number of contaminated seed, which was conducted twice.

Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). Polyclonal antiserum against *X. campestris* pv. *carotae* (strain Xcc12) was made by Antibodies Inc. (Davis, CA) by injecting Giant New Zealand rabbits with heat-killed cells. Immunoglobulin G (IgG) was purified from crude antisera using the E-Z-Sep kit (Pharmacia Biotech, Alameda, CA) following the manufacturer's instructions. The IgG concentration was adjusted to 1.0 mg/ml in 0.01 M phosphate buffered saline (PBS), pH 7.4. An enzyme (alkaline phosphatase)-immunoglobulin conjugate was prepared by the one-step glutaraldehyde method, and the optimum concentration for ELISA was determined as described by Avrameas (5). DAS-ELISA was performed as described by Alvarez and Lou (2) in 96-well flat-bottom polystyrene microtiter plates (Immulon-4, Dynatech Laboratories, Alexandria, VA) with 200 µl each of IgG (1:1,000 dilution), seed-wash suspension (prepared as described for the seed-wash dilution plating assay), enzyme conjugate (1:2,000 dilution), and substrate (*p*-nitrophenyl phosphate, 10 mg/ml) per well. The plates were incubated for coating, sample reaction, and enzyme conjugate at 37°C for 2 h or at room temperature for 4 h. The plates were washed between incubation steps with 0.01 M PBS containing 0.5% Tween 20 using a vacuum/pressure plate washer (Dynatech Ultra Wash II, Dynatech Laboratories, Chantilly, VA). The substrate was allowed

Table 1. Geographic location, origin, year of collection, designation, total number, and pathogenicity of *Xanthomonas campestris* pv. *carotae*-like strains tested by polymerase chain reaction (PCR) with the 3S primer pair in this study

Geographic location	Origin	Year of collection	Designation ^a (field or previous name)	Strains tested by PCR	Pathogenic strains/total strains tested
Arvin, CA	Plants	2001	AV6	15	1/1
			AV7	10	1/1
		2002	108-5	15	2/2
Bakersfield, CA Cuyama, CA	Plants Unknown Plants	2001	108-10	10	2/2
			BK620	4	1/1
		1990	Car001	1	1/1
		1999	XCC9927-36, XCC9942, 43	12	
		2001	01C4	20	2/2
			01C5	10	1/1
		2002	01C6	21	1/1
			02C1	16	2/2
			02C2	16	2/2
			02C3	16	2/2
			02C4	16	2/2
			02C5	10	2/2
			02C6	10	2/2
			02C7	10	2/2
Lancaster, CA	Plants	1999	XCC9924-26, XCC9937-41, XCC9958-61	12	
Rosedale, CA	Plants	2002	108-1	14	2/2
			108-2	11	2/2
			108-3	12	2/2
			GF11	18	2/2
			GW201A	15	2/2
			GWA2J	15	2/2
Idaho Madras, OR	Unknown Plants	1990	Car003	1	1/1
		2001	126-6	12	1/1
			CF	16	1/1
			GLY	4	
			HHL	11	1/1
			H+T	14	1/1
			MS	14	1/1
			NMS	16	1/1
		2002	F1	10	2/2
			F2	12	2/2
			F3	12	2/2
			F4	12	2/2
			F5	14	2/2
			F6	12	2/2
			F7	12	2/2
			F8	12	2/2
Washington	Plants	1994	Car004	1	
			Car005	1	
		2000	Car006	1	1/1
			Car007	1	
		2001	WA1-12	12	3/3
			WA13-15	3	1/1
			WA21-24	4	1/1
			WA25, 26	2	1/1
Canada	Unknown	1990	CAR	29	29/29
			Car002	1	1/1
			Total	548	98/98

^a *X. campestris* pv. *carotae* strains (Car001-007 and 29 strains from Washington in 2002) were kindly provided by L. du Toit (Mount Vernon Research and Extension Unit and Department of Plant Pathology, Washington State University).

to react at room temperature for 4 h before reading color development. The plates were read at 405 nm on a microplate reader (Molecular Devices, Sunnyvale, CA). Suspensions prepared from carrot seed tested free of *X. campestris* pv. *carotae* were added to at least six wells per plate as negative controls. Reactions were considered positive if the absorbance values were greater than the mean absorbance plus three times the standard deviation of the negative controls.

RESULTS

Isolation of strains from carrot plants and seed lots. From 1993 to 1996, 23 *X. campestris* pv. *carotae*-like strains were isolated from carrot seed lots or diseased carrot leaves or stems from five locations in California (Cuyama Valley, Davis, Kern County, Madera, and Owens Valley). The identity of these 23 strains as *X. campestris* pv. *carotae* was confirmed by pathogenicity tests.

Carrot disease surveys were conducted in central Oregon (around Madras) carrot seed production fields on 28 and 29 June 2001 and 8 July 2002. In 2001, 87 *X. campestris* pv. *carotae*-like strains were collected from carrot plants from seven fields (Table 1). In 2002, 96 *X. campestris* pv. *carotae*-like strains were collected from carrot plants from eight fields (Table 1). A carrot disease survey was conducted in Washington (around Moses Lake) on 20 July 2001, and 21 *X. campestris* pv. *carotae*-like strains were collected from carrot plants from four of seven fields (Table 1).

X. campestris pv. *carotae*-like strains also were recovered from carrot seed lots. They were recovered from two of four and two of eight selected commercial seed lots planted in California in 2001 and 2002, respectively. Contamination levels ranged from 1.1×10^2 to 6.8×10^7 CFU/g, and 63 strains of *X. campestris* pv. *carotae* were collected (Table 2). In 2001, *X. campestris* pv. *carotae*-like strains were recovered from 10 seed lots from Oregon. Contamination levels ranged from 6.6×10^5 to 4.0×10^8 CFU/g, and 71 strains of *X. campestris* pv. *carotae* were collected (Table 2). *X. campestris* pv. *carotae*-like colonies were recovered from 2 of 2 and 8 of 10 seed lots from Washington in 2001 and 2002, respectively. Contamination levels ranged from 3.2×10^3 to 5.7×10^7 CFU/g, and 134 strains of *X. campestris* pv. *carotae* were collected (Table 2).

Of the 548 strains isolated from carrot plants (545 strains) or unknown substrates (3 strains) from California, Oregon, and Washington, all 98 strains tested were pathogenic on carrot (Table 1). Of the 268 strains isolated from carrot seed lots, all 16 strains tested were pathogenic on carrot (Table 2).

Generation and testing of primer pairs. RAPD analysis of *X. campestris* pv. *carotae* genomic DNA with the Operon

AB primers revealed that primer AB9 (5'-GGGCGACTAC-3') directed the amplification of DNA fragments common to all 23 strains collected from California from 1993 to 1996 (data not shown). Two of these fragments (~400 bp and ~950 bp) were cloned, and the nucleotide sequences of the ends of each fragment were determined (data not shown). Based on these sequences, the following PCR primer pairs were designed: 3S (3SF: 5'-CATTCC-AAGAAGCAGCCA-3' and 3SR: 5'-TCGCTCTTAACACCGTCA-3') and 9B (9BF: 5'-TGCCTGGCTACGGAATTA-3' and 9BR: 5'-ATCCACATCCGCAACCAT-3'). When tested with total genomic DNA of the 23 strains collected from California from 1993 to 1996, the 3S primer pair directed the amplification of the ~350-bp target DNA fragment from all strains, whereas the 9B primer pair directed the amplification of the ~900-bp target DNA fragment from 18 strains and an ~2 kb fragment from five strains (data not shown). Representative results for 17 strains from California, Oregon, and Washington are shown in Figure 1A (3S primer pair) and Figure 1B (9B primer pair).

The 3S and 9B primer pairs were then evaluated for sensitivity based on the capacity to direct the amplification of the target fragment from serial dilutions of total genomic DNA of *X. campestris* pv. *carotae* (strain 108-2L2-1). In these experiments, the limit of detection (i.e., the smallest amount of DNA from which the target fragment was amplified) for the 3S

primer pair was ~122 fg of total genomic DNA (Fig. 2A) and ~2 pg for the 9B primer pair (Fig. 2B). Thus, the limit of detection of the 3S primer pair was ~20-fold lower than that of the 9B primer pair. The 3S primer pair was selected for subsequent experiments because it: (i) directed the amplification of the same-sized target fragment (~350 bp) from all *X. campestris* pv. *carotae* strains and (ii) provided greater sensitivity in the PCR.

The 3S primer pair did not direct the amplification of DNA fragments from total genomic DNA of strains of 13 other known *X. campestris* pathovars and the four other species of bacteria.

PCR detection from colonies on agar media. In preliminary tests, the 3S primer pair directed the amplification of the ~350-bp target fragment from boiled bacterial cell extracts prepared from single colonies of known strains of *X. campestris* pv. *carotae* grown on plates of 523 medium (data not shown). Therefore, this method was used to test *X. campestris* pv. *carotae*-like colonies recovered from different substrates from California, Idaho, Oregon, Washington, and Canada. The 3S primer pair directed the amplification of the ~350-bp target fragment from all 785 *X. campestris* pv. *carotae*-like strains collected from carrot plants and seeds from California, Oregon, and Washington in 2001 and 2002, including 704 strains not tested for pathogenicity (Tables 1 and 2). The 3S primer pair also directed the amplification of the target fragment from 24 strains col-

Table 2. Geographic location, year of collection, seed lot designation, level of contamination with *Xanthomonas campestris* pv. *carotae*, and number and pathogenicity of *X. campestris* pv. *carotae*-like strains tested by polymerase chain reaction (PCR) with the 3S primer pair in this study

Geographic location	Year of collection	Seed lot designation	Level of contamination (CFU/g of seed) ^a	Strains tested by PCR	Pathogenic strains/total strains tested
Cuyama, CA	2001	GP6	6.8×10^7	15	3/3
		HRR	1.5×10^8	18	3/3
	2002	SK	1.1×10^4	20	2/2
	2002	PS1202	1.0×10^2	10	2/2
Rosedale, CA Madras, OR	2001	Xcc1	2.3×10^8	8	
		Xcc2	1.0×10^7	6	
		Xcc3	4.0×10^8	7	
		Xcc4	3.7×10^6	5	
		1L	9.8×10^5	7	1/1
		2L	6.6×10^5	4	1/1
		1M	1.4×10^6	9	1/1
		2M	4.7×10^6	6	1/1
		1H	2.7×10^7	9	1/1
		2H	2.8×10^6	10	1/1
		WASD2	3.2×10^3	20	
Washington	2002	WASD3	2.4×10^4	20	
		WB	3.2×10^6	12	
		WC	6.3×10^5	12	
		WD	3.6×10^7	12	
		WE	3.5×10^6	10	
		WF	1.0×10^6	12	
		WG	1.1×10^6	12	
		WK	2.3×10^7	12	
		WL	5.7×10^7	12	
		Total		268	16/16

^a Level of contamination presented as CFU per gram of seed as determined by the seed-wash dilution plating assay.

lected from California in 1999 (Table 1) and the seven *X. campestris* pv. *carotae* strains from California, Idaho, Washington, and Canada provided by L. du Toit (Table 1). These results confirmed that these strains were *X. campestris* pv. *carotae*, and that the 3S primer pair has the capacity to direct the amplification of the target fragment from a diversity of *X. campestris* pv. *carotae* strains.

During the course of isolating *X. campestris* pv. *carotae* from carrot plants and seeds, bacteria with different colony morphologies (e.g., yellow nonmucoid, white mucoid, and white flat and nonmucoid) were isolated. When two to five colonies representing these types were tested by PCR with the 3S primer pair, no DNA fragments were amplified. These results were consistent with the specificity of the 3S primer pair for detection of *X. campestris* pv. *carotae*.

PCR detection from carrot tissues. The target fragment was amplified with the

3S or 9B primer pairs from extracts prepared from diseased leaves using all four methods (i.e., NaOH + PVP, Dellaporta, CTAB, and Qiagen DNeasy Plant Minikit). No DNA fragment was amplified from equivalent extracts prepared from symptomless control leaves. In many cases, the target fragment was amplified from dilutions of DNA extracts rather than from undiluted extracts, indicating the presence of PCR inhibitors (Fig. 3). Using the Qiagen DNeasy Plant Minikit, *X. campestris* pv. *carotae* was consistently detected from dilutions of DNA extracts prepared from diseased carrot leaves and umbels collected from the seed-producing fields of Oregon (data not shown) or from leaves of seedlings inoculated with *X. campestris* pv. *carotae* strain 01C5L31 in greenhouse experiments (Fig. 3).

Recovery from carrot seed and development of a PCR-based seed assay. The recovery efficiency of *X. campestris* pv. *carotae* on 523 medium was considerably

higher than that on XCS agar medium. In dilution plating experiments, an average of 6.5×10^5 CFU/g was recovered on 523 medium compared with 1.5×10^5 CFU on XCS agar medium; this represents an ~77% reduction in plating efficiency on XCS agar medium.

There was no difference in recovery from seed with the standard 16- to 18-h wash at 4°C compared with a 2-h wash at room temperature for five contaminated seed lots from California in three independent experiments (data not shown). In similar experiments performed with a contaminated seed lot from Washington, an average of 2.3×10^7 CFU/g (three independent experiments) was recovered using both seed wash methods. These results indicate that the 2-h wash is sufficient for recovering *X. campestris* pv. *carotae* from carrot seed.

For PCR detection of *X. campestris* pv. *carotae* from seeds, DNA extracts were prepared from seed wash pellets from seed lots with contamination levels ranging from 2.3×10^2 to 5.7×10^7 CFU/g using the NaOH + PVP, Dellaporta, and CTAB methods. The target fragment was amplified with the 3S or 9B primer pair from extracts prepared with all three methods (data not shown); however, amplification of the target fragment was usually from diluted rather than undiluted extracts, indicating the presence of PCR inhibitors. The detection threshold was 5.8×10^3 CFU/g with the NaOH + PVP method, and 2.3×10^2 CFU/g with the CTAB and Dellaporta methods. Using the CTAB method, a single infected seed was detected in 10 g of seed (~10,000 seeds). Based on the seed-wash dilution plating assay, the contamination level of this lot was 6.2×10^2 CFU/g.

Because it became commercially available during the course of this study, the Qiagen DNeasy Tissue Kit was investigated as a method to prepare DNA extracts from seed-wash pellets. In three independent experiments, the target fragment was amplified with the 3S primer pair from extracts prepared from pellets from seed lots with contamination levels of 2.7×10^7 , 2.8×10^6 , and 2.0×10^2 CFU/g (Fig. 4). As with the other DNA extracts prepared from leaves or seed, amplification of the target fragment was usually from diluted rather than undiluted extracts.

The two seed-wash methods (16 to 18 h at 4°C and 2 h at room temperature) were compared with the Qiagen DNeasy Tissue Kit DNA extraction method for PCR detection of *X. campestris* pv. *carotae* from two seed lots with contamination levels of 1.1×10^6 and 2.3×10^7 CFU/g. In three independent experiments, the target fragment was amplified from extracts prepared from both seed lots using each seed-wash method (Fig. 5). Thus, there was no difference between these seed-wash methods for PCR detection of *X. campestris* pv. *carotae* from seed.

Fig. 1. Agarose gels showing polymerase chain reaction (PCR) with total genomic DNA of *Xanthomonas campestris* pv. *carotae* strains and the **A**, 3S and **B**, 9B primer pairs. Lane M contains DNA size markers (1-kb DNA ladder, Gibco BRL). Lane 1 is a positive control (PCR with genomic DNA of known strain XCC9940) showing amplification of the ~350-bp target fragment with the 3S primer pair (**A**), or the ~900-bp (or ~2 kb) target fragment with the 9B primer pair (**B**). Lane 2 is a negative control (PCR with sterile H₂O). Lanes 3 to 5 are strains 108-2L2-1, C4L1-1, and GWA2J-1 from California carrot plants; lanes 6 to 8 are strains GP6-2, GP6-6, and HRR4 from carrot seed lots planted in California; lanes 9 to 11 are strains NMSL1-1, NMSL2-2, and HHLU2-3 from Oregon carrot plants; lanes 12 to 14 are strains 2L-1, 2M-1, and 1H-14 from carrot seed lots produced in Oregon; lanes 15 to 17 are strains WA1, WA10, and WA21 from Washington carrot plants, and lanes 18 to 19 are strains WASD2-6 and WASD3-9 from carrot seed lots produced in Washington.

When the two hot water treated seed lots were assayed with the seed-wash dilution plating method, no *X. campestris* pv. *carotae* colonies were recovered. However, when assayed with the PCR-based seed assay (the 2-h seed wash method followed by the Qiagen DNeasy Tissue Kit extraction), the target fragment was amplified from diluted extracts (10^{-1} to 10^{-5}) prepared from both seed lots treated with hot water. Therefore, *X. campestris* pv. *carotae* was detected by PCR from hot water treated seed from which viable bacteria were no longer recovered with the seed-wash dilution plating assay.

Finally, the seed-wash dilution plating and PCR-based assays were each used to assay 17 carrot seed lots intended for planting in California during the 2001 to 2003 growing seasons. With the seed-wash dilution plating assay, *X. campestris* pv. *carotae* was recovered from four of the 17 seed lots (contamination levels ranging from 2×10^2 to 1.5×10^8 CFU/g). These same four seed lots also were positive for *X. campestris* pv. *carotae* by the PCR-based seed assay. The pathogen was not recovered/detected from the other 13 seed lots with the seed-wash dilution plating assay/PCR-based seed assay. Thus, the two tests gave similar results for these seed lots.

Detection from carrot seed with a DAS-ELISA assay. Color development (positive reaction for *X. campestris* pv. *carotae*) was observed from seed wash suspensions prepared from seed lots with contamination levels $>10^5$ CFU/g. The absorbance values of seed wash suspensions from seed lots having contamination levels $<10^4$ CFU/g did not differ from those of seed wash suspensions prepared from lots tested free of *X. campestris* pv. *carotae* contamination (data not shown).

DISCUSSION

Effective management of bacterial leaf blight begins with the planting of seed lots tested free of contamination. Our disease survey results in central Oregon, which showed a high incidence of bacterial leaf blight and seed contamination by *X. campestris* pv. *carotae* in this major carrot seed production area, demonstrate the need for effective methods for detection of contamination of carrot seeds by *X. campestris* pv. *carotae*. The seed-wash dilution plating assay, together with pathogenicity testing of representative *X. campestris* pv. *carotae*-like colonies, is currently the standard method of detection (9,14). While generally effective, this approach is laborious and time-consuming, often taking as long as 1 month to complete. Thus, development of more rapid PCR-based methods for detection and identification of *X. campestris* pv. *carotae* and of seed contaminated by this pathogen should improve the capacity to detect and manage bacterial leaf blight.

Fig. 2. Agarose gels showing the limits of polymerase chain reaction (PCR) detection for the A, 3S and B, 9B primer pairs. The primer pairs were used in the PCR with serial dilutions of total genomic DNA of *Xanthomonas campestris* pv. *carotae* (strain 108-2L2-1). Lanes Mp and M contain the DNA size markers, 1-kb Plus and the 1-kb DNA ladder, respectively (Gibco BRL). Lanes 1 to 11 contain 0 ng, 10 ng, 1 ng, 500 pg, 125 pg, 31 pg, 9 pg, 2 pg, 488 fg, 122 fg, and 31 fg of total genomic DNA, respectively.

Fig. 3. Agarose gel showing polymerase chain reaction (PCR) detection of *Xanthomonas campestris* pv. *carotae* from leaves of carrot seedlings with bacterial leaf blight symptoms (artificially inoculated with strain 01C5L31). DNA extracts were prepared with the Qiagen DNeasy Plant Minikit, and PCR was performed with 3S primer pair. Lane M contains the DNA size markers (1-kb DNA ladder, Gibco BRL). Lane N is a negative control (PCR with a DNA extract from a symptomless control plant). P is a positive control (PCR with genomic DNA of *X. campestris* pv. *carotae*). Lanes 10^0 to 10^{-6} represent PCRs with the indicated serial dilutions of the DNA extract.

The 3S and 9B primer pairs were designed based on sequences of cloned RAPD fragments, an approach previously used to develop specific primers for detection of *X. campestris* pv. *vitiensis* (7). The nature of these RAPD fragments, in terms of the *X. campestris* pv. *carotae* genome, is unknown. However, the specificity of the primer pairs was established by the fact that the target DNA fragment was amplified from known *X. campestris* pv. *carotae* strains, but not from 13 other known *X. campestris* pathovars or four other species of bacteria. The 9B primer pair directed

the amplification of the ~900-bp target fragment from most *X. campestris* pv. *carotae* strains; however, an ~2.0-kb fragment was amplified from some strains, indicating some level of genetic diversity in this region of the genome. On the other hand, the 3S primer pair directed the amplification of the ~350-bp target fragment from all *X. campestris* pv. *carotae* strains tested, indicating less genetic diversity in this region of the genome. Together with the finding that the 3S primer pair was more sensitive than the 9B primer pair, the 3S primer pair was selected as the *X.*

campestris pv. *carotae*-specific primer pair for this study.

The 3S primer pair directed the amplification of the target fragment (i.e., the ~350-bp fragment) from 816 *X. campestris* pv. *carotae* strains, including those from various geographical locations (California, Idaho, Oregon, Washington, and Canada) and substrates (infected plants and contaminated seeds). This included 785 *X. campestris* pv. *carotae*-like colonies recovered from carrot plants with bacterial leaf blight symptoms and seed from California, Oregon, and Washington in 2001 and 2002. The 3S primer pair also directed the amplification of the target fragment from a large number of *X. campestris* pv. *carotae* strains in Europe (M. Asma, *personal communication*). Together, these results indicate that the 3S primer pair is a reliable tool for detection of *X. campestris* pv. *carotae* strains in most carrot-producing areas.

During the course of these experiments, the 3S primer pair did not direct the amplification of the target DNA fragment from non-*X. campestris* pv. *carotae*-like bacteria (i.e., having different colony morphologies) isolated from carrot leaves and seeds. However, the 3S primer pair directed the amplification of an ~350-bp fragment from an *X. campestris* pv. *carotae*-like bacterium isolated from a coriander seed lot produced in Idaho in 2002. Preliminary results indicated that this bacterium is a xanthomonad that is pathogenic on coriander and weakly pathogenic on carrot; however, it is genetically distinct from known *X. campestris* pv. *carotae* strains (A. Poplowsky, R. L. Gilbertson, and L. du Toit, *unpublished results*). This xanthomonad could be a distinct strain of *X. campestris* pv. *carotae* adapted to coriander or a different *X. campestris* pathovar that infects umbelliferous crops. This finding indicates the importance of continued testing of the 3S primer pair with xanthomonads from other related crops and locations, as well as other bacteria to further define the specificity of this primer pair. Taken together, the results of PCR tests with *X. campestris* pv. *carotae* and other bacteria indicate that the 3S primer pair is, for the most part, highly specific for *X. campestris* pv. *carotae* detection.

PCR with the 3S primer pair could greatly reduce the time and facilities required for routine detection and identification of *X. campestris* pv. *carotae* and could be used in ecological and epidemiological studies. Thus, protocols were developed for the use of the 3S primer pair and PCR for detection of *X. campestris* pv. *carotae* from various substrates. Using the boiled-cell method to release the genomic DNA template, a large number of colonies can be tested in a short period of time (~6 h). This method was used to confirm that 785 strains collected from carrot plants and seeds in 2001 and 2002 were *X. campestris*

Fig. 4. Agarose gels showing polymerase chain reaction (PCR) detection of *Xanthomonas campestris* pv. *carotae* from contaminated carrot seed lots (seed lots 1H and 2H were produced in Oregon in 2001 and had contamination levels of 2.7×10^7 and 2.8×10^6 CFU/g of seed, respectively; and seed lot PS1202 was planted in Rosedale, CA, in 2002 and had a contamination level of 1.0×10^2 CFU/g of seed) with the PCR-based seed assay. DNA extracts were prepared from concentrated seed washate (pellets) with the Qiagen DNeasy Tissue Kit, and PCR was performed with the 3S primer pair. Lanes Mp and M contain DNA size markers, 1-kb Plus and the 1-kb DNA ladder, respectively (Gibco BRL). Lane N is a negative control (PCR with sterile H₂O). Lanes 10⁰ to 10⁻⁷ represent PCRs with the indicated serial dilutions of the DNA extract.

pv. *carotae*. PCR detection of *X. campestris* pv. *carotae* colonies provides many advantages, including rapid detection and differentiation of these colonies from non-pathogenic yellow bacteria sometimes isolated from carrot plants and seeds.

Because of the ubiquitous nature of *X. campestris* pv. *carotae* as a contaminant of carrot seed, it is important that all carrot seed lots be assayed for this pathogen, preferably before any type of treatment (e.g., hot water treatment or seed coating). However, the time-consuming and laborious nature of the seed-wash dilution plating assay makes this goal impractical. In our attempt to develop a more rapid seed assay, we found DAS-ELISA to be much less sensitive than the seed-wash dilution plating assay. Therefore, our efforts focused on development of a reliable PCR-based seed assay. In the development of this assay, it was first established that equivalent amounts of *X. campestris* pv. *carotae* were recovered from seed after 2-h and 16- to 18-h washes. Thus, the washing time for both the standard seed wash and PCR-based seed assays can be reduced. From concentrated seed washate, all four

DNA extraction methods tested produced a DNA extract (template) that allowed for PCR amplification of the target fragment with the 3S primer pair. However, the DNA extracts generated with all four methods had to be diluted for reliable PCR amplification of the target fragment, indicating that PCR inhibitors were present in these carrot seed extracts. The presence of PCR inhibitors was previously reported in the development of a PCR assay for detection of *Alternaria radicina* from carrot seed (16). Because the commercially available Qiagen DNeasy Tissue Kit provided a DNA extract that allowed for consistent amplification of the target fragment, it was selected as the standard method to prepare the DNA template for the PCR-based seed assay. Using this assay, *X. campestris* pv. *carotae* was detected from seed lots with contamination levels ranging from 2×10^2 to 2.3×10^8 CFU/g, which is in the range of detection of the seed-wash dilution plating assay. It is significant that there was good correlation between results obtained for the PCR-based seed assay and the seed-wash dilution plating assay. Thus, all seed lots that tested positive by the seed-

wash dilution plating assay were also positive by the PCR-based assay, but no lots tested positive by PCR and negative by the seed-wash dilution plating assay or vice versa. Thus, this PCR-based seed assay shows promise for routine detection of *X. campestris* pv. *carotae* from carrot seed.

There are also disadvantages to the PCR-based seed assay, including the inability to differentiate viable versus nonviable cells, the presence of PCR inhibitors, and the lack of a quantitative component of the test. Because the PCR test detects pathogen DNA, it is possible that free DNA from nonviable bacteria can be detected, thereby giving a false positive result. Indeed, this was demonstrated with the PCR detection of *X. campestris* pv. *carotae* (presumably DNA) from hot water treated seed from which no viable bacteria were recovered. Nevertheless, detection of the DNA from a seed lot demonstrates a history of contamination, which would indicate the need for further assay and/or treatment. The presence of PCR inhibitors was solved by using a series of dilutions of the seed pellet DNA extracts. Quantification is important because bacterial leaf blight is not a zero tolerance disease, and lower levels of contamination (i.e., $<10^4$ CFU/g) may not give rise to significant disease in the field (23). The development of a quantitative PCR-based assay (e.g., real-time PCR) could address this issue. Taking these issues into consideration, we envision an approach where, prior to treatment, carrot seed lots would be tested by PCR. Positive lots would be assayed additionally with the seed-wash dilution plating assay. Lots confirmed to be contaminated would be subjected to treatment (e.g., hot water) and follow-up confirmatory seed wash assays.

In summary, PCR-based assays with the 3S primer pair were developed for rapid detection of *X. campestris* pv. *carotae* from different substrates, including carrot tissues and seeds. The 3S primer pair is highly specific and detected all known strains. These PCR-based assays have the potential to improve routine detection of this important carrot pathogen.

ACKNOWLEDGMENTS

This study was partially supported by grants from the California Carrot Advisory Board. We thank F. Crowe, L. du Toit, M. Derie, and J. J. Nunez for assistance in collecting carrot plant and seed samples.

LITERATURE CITED

1. Adachi, N., and Oku, T. 2000. PCR-mediated detection of *Xanthomonas oryzae* pv. *oryzae* by amplification of the 16S-23S rDNA spacer region sequence. J. Gen. Plant Pathol. 66:303-309.
2. Alvarez, A. M., and Lou, K. 1985. Rapid identification of *Xanthomonas campestris* pv. *campestris* by ELISA. Plant Dis. 69:1082-1086.
3. Ark, P. A., and Gardner, M. W. 1944. Carrot bacterial leaf blight as it affects roots. Phytopathology 34:416-420.

Fig. 5. Agarose gels showing polymerase chain reaction (PCR) detection of *Xanthomonas campestris* pv. *carotae* from two contaminated carrot seed lots (seed lots WG and WK were produced in Washington and had contamination levels of 1.1×10^6 and 2.3×10^7 CFU/g of seed, respectively). The PCR-based seed assays involved either a 16- to 18-h at 4°C wash (upper gel) or a 2-h at room temperature wash (lower gel). DNA extracts were prepared from concentrated seed washate (pellets) with the Qiagen DNeasy Tissue Kit, and PCR was performed with the 3S primer pair. Lane M contains the DNA size markers (1-kb DNA ladder, Gibco BRL). Lane N is a negative control (PCR with sterile H₂O). Lane P is a positive control (PCR with genomic DNA of *X. campestris* pv. *carotae*). Lanes 10⁰ to 10⁻⁵ represent PCRs with the indicated serial dilutions of the DNA extracts.

4. Audy, P., Laroche, A., Saindon, G., Huang, H. C., and Gilbertson, R. L. 1994. Detection of the bean common blight bacteria, *Xanthomonas campestris* pv. *phaseoli* and *X. c. phaseoli* var. *fuscans*, using the polymerase chain reaction. *Phytopathology* 84:1185-1192.
5. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry* 6:43-52.
6. Barak, J. D., and Gilbertson, R. L. 2003. Genetic diversity of *Xanthomonas campestris* pv. *vitians*, the causal agent of bacterial leafspot of lettuce. *Phytopathology* 93:596-603.
7. Barak, J. D., Koike, S. T., and Gilbertson, R. L. 2001. Role of crop debris and weeds in the epidemiology of bacterial leaf spot of lettuce in California. *Plant Dis.* 86:169-178.
8. Blodgett, E. C. 1944. Carrot disease in Idaho in 1943. *Plant Dis. Rep.* 28:764-769.
9. Davis, R. M., and Raid, R. N. 2002. Compendium of Umbelliferous Crop Diseases. American Phytopathological Society, St. Paul, MN.
10. Dellaporta, S. L., Woods, J., and Hicks, J. B. 1983. A plant miniprep, Version II. *Plant Mol. Biol. Rep.* 1:19-21.
11. Glick, D. L., Coffey, C. M., and Sulzinski, M. A. 2002. Simultaneous PCR detection of the two major bacterial pathogens of geranium. *J. Phytopathol.* 150:54-59.
12. Hartung, J. S., Daniel, J. F., and Pruvost, O. P. 1993. Detection of *Xanthomonas campestris* pv. *citri* by the polymerase chain reaction method. *Appl. Environ. Microbiol.* 59:1143-1148.
13. Kado, C. I., and Heskett, M. G. 1970. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. *Phytopathology* 60:969-976.
14. Kuan, T.-L., Minsavage, G. V., and Gabrielson, R. L. 1985. Detection of *Xanthomonas campestris* pv. *carotae* in carrot seed. *Plant Dis.* 69:758-760.
15. Pflieger, F. L., Harman, G. E., and Max, G. A. 1974. Bacterial leaf blight of carrots: Interaction of temperature, light and inoculum procedures on disease development of various carrot cultivars. *Phytopathology* 64:746-749.
16. Pryor, B. M., and Gilbertson, R. L. 2001. A PCR-based assay for detection of *Alternaria radicina* on carrot seed. *Plant Dis.* 85:18-23.
17. Saad, S. M., and Wade, E. K. 1972. Bacterial blight of carrot in Wisconsin. *Plant Dis. Rep.* 56:744-746.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Schaad, N. W., and Stall, R. E. 1988. *Xanthomonas*. Pages 81-94 in: *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN.
20. Sulzinski, M. A., Moorman, G. W., Schlaghauser, B., and Romaine, C. P. 1997. A simple DNA extraction method for PCR-based detection of *Xanthomonas campestris* pv. *pelargonii* in geraniums. *J. Phytopathol.* 145:213-215.
21. Sulzinski, M. A., Moorman, G. W., Schlaghauser, B., and Romaine, C. P. 1998. PCR-based detection of artificial latent infections of geranium by *Xanthomonas campestris* pv. *pelargonii*. *J. Phytopathol.* 146:111-147.
22. Toth, I. K., Hyman, L. J., Taylor, R., and Birch, P. R. J. 1998. PCR-based detection of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* in plant material and its differentiation from *X. c.* pv. *phaseoli*. *J. Appl. Microbiol.* 85:337-336.
23. Umesh, K. C., Davis, R. M., and Gilbertson, R. L. 1998. Seed contamination thresholds for development of bacterial leaf blight of carrots caused by *Xanthomonas campestris* pv. *carotae*. *Plant Dis.* 82:1271-1275.
24. Wang, H., Qi, M., and Cutler, A. J. 1993. A simple method for preparing plant samples for PCR. *Nucleic Acids Res.* 21:4153-4154.
25. Watson, R. D. 1948. Carrot bacterial blight control in Idaho. *Plant Dis. Rep.* 32:238-239.
26. Zhang, Y. P. 1996. Etiological study of cherry stem pitting disease and molecular characterization of sour cherry green ring mottle virus. Ph.D. diss. University of California, Davis.