Specific Detection of *Pseudomonas syringae* pv. *phaseolicola* DNA in Bean Seed by Polymerase Chain Reaction-Based Amplification of a Phaseolotoxin Gene Region

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ABSTRACT

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The polymerase chain reaction (PCR) is described for the specific detection of the bean halo blight pathogen *Pseudomonas syringae* pv. *phaseolicola* in bean seed. The method involves the amplification of a segment of the *tox* (phaseolotoxin) gene cluster (23). Two oligonucleotide primers, designed according to the sequence of this segment, allowed the exclusive amplification of a 1.9-kb fragment from total DNA extracted from pure cultures of the pathogen and from water extracts of soaked bean seed. The amplified segment was visually detected by ethidium bromide staining when the original samples contained approximately 10^3 colony-forming units (cfu) per milliliter and by Southern blot hybridization in DNA extracts from pure cultures of the pathogen that contained as few as 10 cfu/ml. DNAs from 57 strains of other bacteria, including the bean pathogens *P. s.* pv. *syringae* and *Xanthomonas campestris* pv. *phaseoli,* and presumptive saprophytes associated with bean seed did not contain sequences homologous to the 1.9-kb fragment and did not produce this or other discrete bands upon amplification. The detection threshold in water extracts from soaked bean seeds was 10–20 cfu/ml. Presumptive epiphytes present at concentrations $\geq 10^4$ cfu/ml did not interfere with detection even when the pathogen was present at concentrations of ≤ 30 cfu/ml. In control experiments, heterologous DNA increased the intensity of the 1.9-kb band. The method was used to detect the pathogen in a commercial seed lot that failed to yield the pathogen by conventional methods.

Pseudomonas syringae pv. *phaseolicola* (5) is a seedborne pathogen that causes halo blight of beans and is found worldwide (7,33–35,42). The only practical control for this serious disease is the use of pathogen-free seeds. Many states and countries have strict quarantine laws regarding *P. s. phaseolicola* (7,9,33,35,42). In Idaho, where 80% of the bean seeds in the United States are grown, regulations require that seeds be assayed by the state department of agriculture for freedom from contamination, and the seed must be grown in isolation for one season. Furthermore, all seed crops are field-inspected, and if halo blight is found, the crop must be destroyed. A seed soak-plant inoculation technique is used by some seed companies in the United States for the detection of *P. s. phaseolicola* (17,42). Other laboratory assays are based on immunological methods (10,38–40) or plating on the fluorescence-promoting King's B (KB) agar (16,39) or on the semiselective modified sucrose peptone (MSP) agar (22). Serological tests have not been widely accepted for routine testing because of their relatively low sensitivity and frequent false positive results. The use of the MSP agar facilitates the differentiation of the pathogen from other saprophytic fluorescent pseudomonads in most seed lots. Nevertheless, differentiation of *P. s. phaseolicola* from saprophytes can be difficult in seed lots that carry a large population of saprophytic bacteria. Furthermore, this medium does not select against the brown spot pathogen *P. s.* pv. *syringae.* Consequently, time-consuming and expensive biochemical and pathogenicity tests are needed to differentiate between the two pathogens. Thus, there exists a pressing need for a rapid diagnostic assay that is highly sensitive and specific for the halo blight pathogen. A combination of plating on MSP agar with subsequent testing of suspect colonies for phaseolotoxin production (13), with a slight modification of the microbiological assay developed earlier (31), is a significant improvement over other methods. However, this method may not be suitable when the ratio of epiphytes and saprophytes to pathogenic bacteria in the seed lot is high.

Nucleic acid hybridization probes consisting of genes encoding virulence factors, ribosomal RNA, or DNA segments that are unique to particular pathogens often provide the sensitivity and the specificity needed for the detection of pathogens in natural samples. Dot-blot hybridization with a 2.6-kb DNA probe derived from the phaseolotoxin gene cluster (*tox;* 23) has been used to specifically detect and identify suspected colonies of *P. s. phaseolicola* isolated on MSP or KB agar (29). This method worked well for field diagnosis of suspected halo blight lesions on mature pods where large numbers of *P. s. phaseolicola* are present (N. W. Schaad, *unpublished*). However, DNA hybridization alone lacked the sensitivity needed for direct detection of low populations of the pathogen or to detect it when high populations of saprophytic bacteria were present.

Amplification of specific DNA sequences by means of the polymerase chain reaction (PCR; 6,12) allows for the detection of small numbers of the target organism (2,3,32,36,41). An ideal situation for the application of PCR in phytosanitary practice and disease epidemiology is that in which DNA sequences exist that are unique to a given pathogen. The phaseolotoxin gene cluster constitutes a good candidate region as a potential PCR target for the detection of *P. s. phaseolicola* for two reasons. First, production of this toxin is a characteristic trait of this pathogen (20,21). We are unaware of any proven exception to this, although there is indirect evidence that some strains of the related pathogen *P. s.* pv. *glycinea* produce a phaseolotoxinlike substance(s) (8). Second, the 2.6-kb DNA segment derived from this cluster hybridizes with *P. s. phaseolicola* strains but not with other plant-pathogenic or saprophytic bacteria (23,29, this study). We report the use of a PCR-mediated amplification of a region contained within this DNA segment as a highly sensitive method for the specific detection of *P. s. phaseolicola* in contaminated bean seeds. Preliminary reports of this work have been published (24,25).

MATERIALS AND METHODS

Media, chemicals, molecular and other reagents. KB agar (16) and yeast-dextrose-$CaCO_3$ agar (YDC) (43) were used as general plating media. The selective MSP agar (22) and the medium for *Xanthomonas campestris* pv. *phaseoli* (MXP) (4) were used for *Pseudomonas* and *Xanthomonas* strains, respectively. Liquid cultures of these bacteria were grown in 523 broth (15) at 21–24 C. *Escherichia coli* strains carrying recombinant plasmids were grown in Luria broth or Luria agar (19) supplemented with 25 μg/ml of tetracycline or 50 μg/ml of ampicillin (Sigma Chemical Company, St. Louis, MO) at 37 C. Restriction endonucleases, T4 DNA ligase, and bacterial alkaline phosphatase were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Proteinase K, RNase A, random priming kit, and the Genius nonradioactive labeling kit were obtained from Boehringer-Mannheim Biochemicals. *Taq* DNA polymerase was purchased from Perkin Elmer-Cetus (Norwalk, CT) or from Promega Biotec (Madison, WI). The sources of other chemicals and materials were as follows: α-[^{32}P]dCTP, New England Nuclear Research Products, Du Pont Co., Boston, MA; deoxyribonucleotide triphosphates, United States Biochemical Corporation, Cleveland, OH; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 5 Prime-3 Prime, Inc., West Chester, PA; plasmid pT7/T3α-18, Bethesda Research Laboratories; Nytran nylon filters, Schleicher & Schuell, Inc., Keene, NH.

Bacterial strains, plasmids, DNA sequencing, and primers. The bacterial strains used are listed in Table 1. Plasmid pRCP2 is a pUC8 derivative carrying an 8.3-kb *Eco*RI insert derived from the Tn*5* insertion mutant NPS4336 of *P. s. phaseolicola* NPS3121, which is deficient in the production of phaseolotoxin (23). The 8.3-kb insert consists of a 2.6-kb *Eco*RI genomic fragment plus a copy of the transposon Tn*5* (5.7 kb) inserted in the position indicated in Figure 1. The regions to the right and left of the Tn*5* insertion were separately cloned as an *Eco*RI-*Xho*I and an *Eco*RI-*Hpa*I fragment in the plasmid vector pT7/T3α-18 that had been digested with either *Eco*RI plus *Sal*I or *Eco*RI plus *Hin*cII, respectively. The resulting plasmids, pPT18A and pPT18B, were used as templates to establish the nucleotide sequence near the *Eco*RI ends of the 2.6-kb *tox* segment with commercially available sequencing primers, according to the dideoxy chain termination method (28). The entire 2.6-kb *Eco*RI segment was subsequently sequenced in both directions with defined deletions and internal sequencing primers (Hatziloukas et al, *unpublished*). These primers, and those used for PCR, were synthesized in a commercial DNA synthesizer (PCR-Mate, Applied Biosystems, Inc., Foster City, CA).

TABLE 1. Bacteria tested and results obtained with the polymerase chain reaction method

Bacterium Strain	Source	1.9-kb band
Pseudomonas syringae pv. *phaseolicola*		
NPS3121	Peet et al (23)	+
PC-32, PC-72	Commercial seed, this study	+
C-199, C-200, C-206, C-209, C-223A, C-233, C-235, C-237, C-252A, C-254, C-447, C-267	N. W. Schaad et al (29)	+
P. s. pv. *syringae*		
C-203, C-204, C-205, C-227, C-239, C-273, C-303, C-318, C-332, C-383, C-448, C-454, C-475, C-492, PC-20[a]	N. W. Schaad	−
P. s. pv. *tomato*		
C-171, C-441, C-300	R. Gitaitis, Tifton, GA	−
P. s. pv. *pisi*		
C-302	K. Kimble, Davis, CA	−
Pseudomonas putida	R. L. Stahl, Gainesville, FL	−
Xanthomonas campestris pv. *vesicatoria*	N. W. Schaad	−
X. c. pv. *malvacearum*	N. W. Schaad	−
X. c. pv. *alfalfae*	N. W. Schaad	−
X. c. pv. *campestris*	N. W. Schaad	−
X. c. pv. *phaseoli*	N. W. Schaad	−
Escherichia coli DH5α	Bethesda Research Laboratories	−
Pseudomonas saprophytes 27 strains[b]	N. W. Schaad, this study	−
Other saprophytes 5 strains[b]	N. W. Schaad, this study	−

[a] Commercial seed, this study.
[b] Obtained from bean-seed washes plated on King's B agar.

Origin and processing of seed samples. The following bean seed materials were used: 1) seed of *Phaseolus vulgaris* cv. Hystyle (Harris Moran Seed Co., San Juan Bautista, CA) that had been found to be free of the halo blight pathogen by both PCR analysis and plating on MSP agar; 2) field-thrashed cull bean seed from Idaho; 3) seed from plants grown in the greenhouse (Harris Moran Seed Co.) and infected with *P. s. phaseolicola* C-199; and 4) five commercial seed lots grown in Tanzania that were suspected of being contaminated with *P. s. phaseolicola.*

Seeds were soaked in sterile phosphate buffer, 0.05 M, pH 7.1, containing 0.85% NaCl and 0.01% Tween-20 (phosphate-buffered saline [PBS]), either individually (1 ml per seed) or in batches of 800–2,000 seeds (0.5 ml per seed), for 15–18 h at 4 C (22). Aliquots of seed extract were centrifuged for 10 min in a Sorvall GSA rotor at 10,000 rpm and resuspended in PBS to obtain 10-fold (10×) and 100-fold (100×) concentrated samples. Four 100-μl aliquots of the original (1×) and the 10× and 100× samples were plated directly onto MSP and KB agar to determine the bacterial population, and the remaining sample was stored at −85 C in 1.5-ml microcentrifuge tubes for later DNA extraction.

DNA was extracted from the seed extract by the cetyltrimethylammonium bromide (CTAB) method (1). To facilitate precipitation of DNA during extraction from samples with low bacterial populations, 5 μg of yeast t-RNA (Sigma) was added at the isopropanol precipitation step. The final pellets were dissolved in 40 μl of sterile double distilled water for 15–18 h at 5 C before

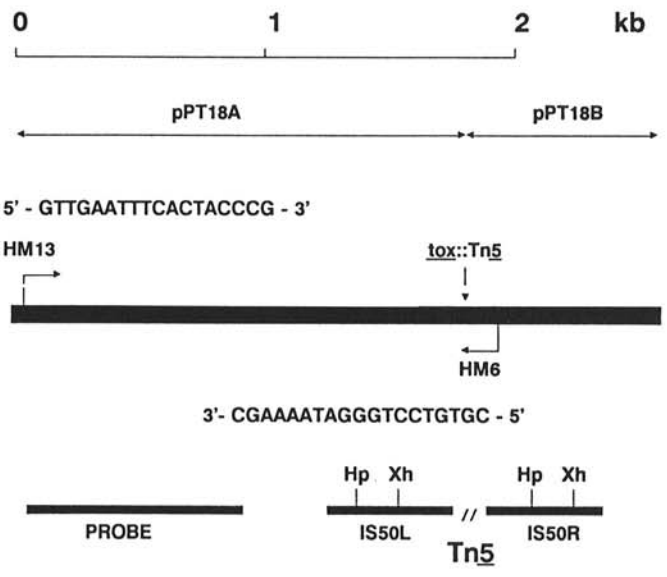

Fig. 1. Schematic diagram of the 2.6-kb DNA fragment. The scale (in kb) and the segments contained in the pTP18A and pTP18B are depicted above the physical map of the fragment. The location of the Tn*5* insertion that inactivates phaseolotoxin production in strain NPS4336 (23) is marked by the arrow pointing downward. Arrows pointing to the right and left indicate the location and orientation of the HM6 and HM13 primers, whose sequence is also given. The thinner bar on the lower left depicts the 0.9-kb fragment used as the hybridization probe; the one on the lower right depicts the terminal portions of the inverted repeats of Tn*5* (IS50L and IS50R, not drawn to scale) that carry the restriction sites used for the subcloning of the pTP18A and pTP18B inserts. Hp and Xh stand for *Hpa*I and *Xho*I endonuclease sites, respectively.

amplification.

DNA amplification, agarose gel electrophoresis, and Southern blot hybridizations. The following conditions were used for routine amplification reactions: primers HM6 and HM13 (Fig. 1), 0.4 μM each; 1.25 units of *Taq* polymerase; deoxynucleotide triphosphates and buffers according to the suppliers' recommendations; final reaction volume of 50 μl. Amplification reactions were routinely done in quadruplicate with the following thermal profile: 1 min incubation at 94 C (denaturation), 2 min at 60 C (annealing), and 1 min at 72 C (elongation). After 30 such cycles, a 10-μl aliquot from each reaction tube was treated with 2 μl of RNase A stock for 10 min and run in a 1% agarose gel in TAE buffer (0.04 M Tris[hydroxylmethyl]-aminomethane-acetate, 0.002 M EDTA, pH 8.0). The amplified products were visualized by ethidium bromide staining. The purpose of the RNase A treatment was to allow visualization of potential low molecular weight PCR products that might comigrate with RNA in the agarose gel. For positive identification of the PCR products the gels were then blotted onto Nytran membranes, hybridized with a 0.9-kb DNA probe fragment (Fig. 1) that was internal to the amplified segment, and washed twice in 2× saline sodium citrate (2× SSC; 0.3 M NaCl, 0.03 M Na-citrate)-0.1% sodium dodecyl sulfate (SDS) at 65 C and twice in 0.1× SSC-0.1% SDS at 65 C, as described (1,19).

Sensitivity thresholds. The sensitivity thresholds for detection by PCR of *P. s. phaseolicola* in pure culture and in bean seeds were determined as follows: 1) Cultures of *P. s. phaseolicola* were grown overnight in 523 broth, adjusted to a turbidity of 0.1 (OD_{600}) (numbers of colony-forming units are given in Results and Discussion), and serially diluted in sterile distilled water by 10^{-5}, 10^{-6}, 5×10^{-7}, 2×10^{-7}, 10^{-7}, and 10^{-8}. Five 100-μl aliquots from each dilution were plated in triplicate on KB agar plates, and another five were used to extract DNA with the CTAB protocol and the addition of yeast t-RNA as described. The entire DNA extract was used in the amplification reactions, and the PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. Duplicate gels were blotted onto Nytran filters and analyzed by Southern hybridization. 2) Bean pods at the mature green stage were inoculated with a 26-gauge needle by injection of a suspension containing approximately 10^8 cfu/ml of *P. s. phaseolicola*. Pods on another group of plants were merely wounded with a needle and kept in a separate area to serve as controls. Once the pods reached full maturity, they were hand-harvested and dried. The seeds were then divided into two groups, those that had halo blight lesions and those that did not. Each group contained approximately 70 seeds. Ten seeds from each group were taken randomly and soaked individually in 10 ml of PBS for 15 h at 4 C. The extracts were serially diluted and plated in triplicate onto MSP agar to determine the number of colony-forming units of *P. s. phaseolicola* per seed or were frozen for later DNA extraction. None of the seeds from the control group contained the pathogen, determined on the basis of nutritional properties (see below). The 10 seeds from inoculated pods contained 2.0×10^7, 1.0×10^7, 1.0×10^7, 2.0×10^6, 1.7×10^6, 1.0×10^6, 7.5×10^5, 6.0×10^4, 3.2×10^4, and 2.5×10^3 (mean of 4.6×10^6) colony-forming units per seed and tested positive for *P. s. phaseolicola*. Six other seeds randomly chosen from the inoculated group were mixed individually with an equal number of separate batches of 2,000 seeds from a lot that was found to be free of the pathogen by plating on MSP agar and PCR analysis. Similar batches without added contaminated seeds were used as controls. Each batch was soaked in 1,000 ml of PBS, and 100-μl aliquots were removed for DNA extraction and amplification, as described above.

Pathogenicity and nutritional tests. For taxonomic and pathogenic identification, colonies growing on KB or MSP agar that were suspected of being *P. s. phaseolicola* were purified by streaking and tested for nutritional properties that are diagnostic for this pathovar (ability to utilize mannitol, inability to utilize *myo*-inositol and *i*-erythritol as sole carbon sources; 27) and for pathogenicity on *P. vulgaris* cv. Hystyle by standard methods (27).

RESULTS AND DISCUSSION

Selection of primers and specificity of the amplification reaction. The nucleotide sequence was determined for various segments of the 2.6-kb DNA fragment derived from the *tox* gene cluster. The Tn*5* insertion in mutant NPS4336 interrupts a gene required for toxin production (23; Hatziloukas et al, *unpublished*); however, the biochemical defect responsible for the Tox^- phenotype of this mutant has not been established. To find a suitable combination of primers, several pairs of 17- to 21-mer oligonucleotides were initially tested for the ability to amplify DNA fragments from total DNA extracts of *P. s. phaseolicola* C-199, with *E. coli* DH5α and *P. s. syringae* C-227 as negative controls. The optimal concentration of primers, the thermal profile, amount of input DNA, and other parameters were examined in preliminary experiments to establish the reproducibility of the PCR amplification, maximize its yield, and insure amplification specificity. The oligonucleotides HM6 and HM13 (Fig. 1) produced a band of the expected size (1.9 kb, Fig. 2) after 20–30 cycles. The number of cycles depended on the DNA concentration at the start of the experiment. This band hybridized to the internal 0.9-kb probe fragment. A weak secondary band (approximately 4.5 kb) and several faint bands (Fig. 2), seen only when DNA from pure cultures of *P. s. phaseolicola* was used for amplification, did not hybridize to the internal probe fragment (data not shown) and thus represent nonspecific amplification products. The same was true of the smear of products with predominantly high molecular weights seen with *P. s. syringae* strains (Fig. 2). Since the 1.9-kb fragment was by far the predominant and verifiable PCR product, no attempt was made to further optimize the specificity of amplification with the HM6 and HM13 primers.

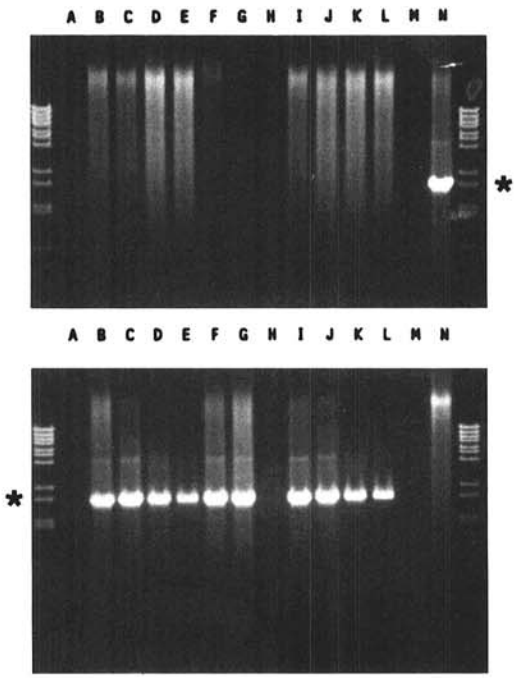

Fig. 2. Polymerase chain reaction amplification products separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The left and right lanes (unmarked) in each panel contain molecular weight standards (0.6-μg phage lambda DNA digested with *Bst*EII), and lanes A and M are empty. *Pseudomonas syringae* pv. *phaseolicola* strains are represented in lane N of the upper panel (strain C-199) and B–G and I–L in the bottom panel (strains C-200, C-206, C-209, C-223A, C-235, C-237, C-251A, C-254, C-247, and PC-72, respectively). Lanes B–L in the upper panel and H and N in the bottom panel represent *P. s. syringae* strains C-204, C-205, C-273, C-303, C-318, C-332, C-383, C-448, C-454, C-475, C-492, C-239, and C-203, respectively. The asterisks mark the diagnostic 1.9-kb band seen with all *P. s. phaseolicola* strains. The starting samples for the amplification reactions contained 1 μg of DNA (estimated to be approximately 1.33×10^5 genome equivalents [11]) per reaction tube.

Several other primer pairs that were expected to amplify shorter fragments produced multiple bands under the amplification conditions used in this study.

The 13 strains of *P. s. phaseolicola* tested with the HM6-HM13 primer pair were scored positive (Table 1). In contrast, 57 strains of other bacteria representing three pathovars of *P. syringae,* seven pathovars of *X. campestris, P. putida, E. coli,* and 32 presumably saprophytic isolates from bean-seed washes were negative (Table 1, Fig. 2). Thus, the HM6-HM13 primer pair displayed the expected specificity of amplification under our conditions.

Sensitivity of detection in pure cultures and bean-seed extract. When pure cultures of *P. s. phaseolicola* were used for DNA extraction and amplification, the minimum starting cell concentration needed to detect the characteristic PCR product (1.9-kb DNA band) by ethidium bromide staining after a 30-cycle amplification was approximately 10^4 cfu/ml (Fig. 2). The sensitivity was greatly improved when Southern hybridization was used to detect the PCR product. In one representative experiment, an average of 3.6 cfu per plate (36 cfu/ml of pure culture suspension) was determined, and the 1.9-kb band was detected by Southern hybridization in all four replicate amplifications. When the starting cell suspensions contained an average of 10 cfu/ml (actual colony-forming units per plate on KB agar were 0, 0, 1, 1, and 3), the 1.9-kb band was detected in only some of the replicate amplifications. Thus, the sensitivity of our procedure with pure cultures of the pathogen approached the theoretical detection threshold of PCR.

To establish the detection threshold in seed lots and to compare the relative efficiency of PCR versus the standard MSP agar plating method, we tested water extracts from seed mixtures containing single infected seeds from pod-inoculated plants mixed with 2,000 "clean" seeds. All seed mixtures were provisionally scored positive for *P. s. phaseolicola* by plating of the extracts on MSP agar (Table 2). However, the presence of large numbers of saprophytes made the identification of the pathogen exceedingly difficult. When individual colonies from the MSP agar plates were individually examined by standard nutritional and pathogenicity tests, the pathogen was confirmed in only two of the six extracts (Table 2). These results are typical for MSP agar plating because the medium is only semiselective and allows the growth of other *Pseudomonas* strains associated with bean seed (22). When these extracts were tested by PCR, four of them were scored positive on the basis of the amplification of a 1.9-kb band (Table 2). The number of positive PCR assays in replicate amplification reactions generally was correlated with the apparent concentration of the pathogen in the seed extract. Thus, extracts 1 and 2, in which the presence of the pathogen was confirmed by the combined agar plating-nutritional and pathogenicity tests, were scored positive in all four replicate amplifications. In contrast, with extracts 3 and 5, which were scored negative in the above tests, only some of the replicate amplifications produced the 1.9-kb band. Finally, two extracts (4 and 6) tested negative by both methods. It is possible that the infected seeds that had been added to these batches had low pathogen populations. Assuming that pathogen multiplication was minimal during seed soaking at 4C, such seeds may not have released the pathogen in sufficient numbers of colony-forming units per milliliter of extract to permit detection. Although the number of extracts tested from individual seeds and seed mixtures is not large and the efficiency of pathogen release from the infected seeds during soaking was not investigated in this study, it is interesting to note that the proportion of PCR-negative seed extracts (1/3) was the same as the proportion of individual infected seeds (3/10) that produced extracts containing the pathogen at $<10^5$ cfu/ml (<1 cfu/100 μl). The above results showed that our method detected the halo blight pathogen in water extracts from batches that contained one infected seed in 2,000 seeds. This threshold would only apply to seeds that contain a sufficiently high pathogen population to yield at least 10 cfu/ml of extract.

The tolerance levels for bean halo blight in commercial seed beans vary from zero (42) to one in 30,000 seeds (35). Soaking this number of seeds requires a larger volume of water or buffer than that used in this study, and this concomitantly reduces the concentration of the pathogen's colony-forming units per unit volume of extract. Since this problem can be remedied by concentrating the extract, we applied the same procedures to 10× and 100× concentrated aliquots of the seed extracts described above (Table 2). The two PCR-negative extracts (4 and 6) remained negative at both the 10× and the 100× concentrations. With two of the PCR-positive extracts (1 and 2), the 10× concentration gave the same results as the nonconcentrated extract, but the 100× concentration gave less reproducible amplification (extract 1) or no amplification (extract 2). In the two other positive extracts (3 and 5), the 100× concentration slightly improved detection, relative to the 10× concentration. These results suggest that by concentrating the extracts from large seed batches, the limit of detection can potentially be improved to one infected seed in at least 20,000 healthy seeds. However, excessive concen-

TABLE 2. Comparison of plating and polymerase chain reaction (PCR) methods for detection of *Pseudomonas syringae* pv. *phaseolicola* in the water extracts from seed batches that contained single infected seeds[a]

Seed batch no.	Seed-extract concentration	Mean cfu/plate			Number of PCR-positive aliquots/ number of aliquots tested
		Total bacteria (KB)[b]	*P. s. phaseolicola*		
			Suspected (MSP)[c]	Number confirmed/ number tested[d]	
1	1×	≈1,000	25	3/3	4/4
	10×	>1,000	14	0/3	4/4
	100×	C[e]	0	NA[f]	1/4
2	1×	80	5	3/5	4/4
	10×	>1,000	6	0/2	4/4
	100×	C	0	NA	0/4
3	10×	C	0.25	0/1	2/4
	100×	C	0.50	0/2	3/4
4	10×	C	0.75	0/1	0/4
	100×	C	7	0/7	0/4
5	10×	C	0.25	0/1	1/4
	100×	C	0.25	0/1	3/4
6	10×	C	4	0/1	0/4
	100×	C	1	0/3	0/4

[a] Individual seeds that were known to be infected with *P. s. phaseolicola* were added to 2,000 noninfected seeds and soaked for 15 h as described (22).
[b] KB = King's B agar. Values are averages of quadruplicate platings.
[c] MSP = Modified sucrose peptone agar. Values are averages of quadruplicate platings.
[d] The number of colonies confirmed or not confirmed as being *P. s. phaseolicola* after being individually tested for nutritional properties and pathogenicity.
[e] Confluent growth on plates.
[f] Not applicable.

tration of the extracts has a variable effect and can in fact lower the efficiency of detection. A likely reason for this is the presence of inhibitors that are not eliminated in the course of DNA extraction. Other investigators (26) have noted the presence of inhibitors in seed extracts, particularly after prolonged soaking. Since we tested seeds from only one cultivar, we do not know whether seeds from other cultivars present the same problem.

With seed extracts that contained low concentrations of *P. s. phaseolicola* (≤10 cfu/ml), the frequency of positives in replicate amplification reactions and the intensity of hybridization between the 1.9-kb PCR band and the 0.9-kb probe fragment were often greater than with pure cultures containing the pathogen at the same concentration (data not shown). This suggested that either DNA from nonviable cells of *P. s. phaseolicola* in the seed was amplified or that nontarget DNA in the bean-seed extract enhanced the efficiency of amplification. Since the first possibility could not be tested easily, we examined the effect of nontarget DNA on PCR efficiency. *P. s. phaseolicola* DNA (0.1 pg, approximately 13 genome equivalents per aliquot, assuming a genome size equal to *P. aeruginosa* [5.85 Mb; 11]) was amplified alone as well as in the presence of 50-, 5×10^3-, 5×10^5-, and 5×10^7-fold excess of DNA from *P. s. syringae* C-227 and *P. putida*. In the presence of 5×10^3-, 5×10^5-, and 5×10^7-fold excess nontarget DNA, the intensity of the 1.9-kb band was greater than when the target DNA was amplified alone. The nontarget DNA alone, present at the same concentrations, did not yield a 1.9-kb PCR product when analyzed by Southern blot hybridization. Although the basis for the observed enhancement was not investigated, it is possible that nontarget DNA sequesters potential inhibitors of *Taq* polymerase, such as those originating from the seeds (26), or protects the target (initial product) from metal ion-induced DNA cleavage (30), from the 5′→3′ exonuclease activity of *Taq* polymerase, or from other nucleases. Alternatively, a large excess of nontarget DNA favors the kinetics of amplification by reducing effective diffusion volume in the sample.

Assays of commercial seeds. We assayed five commercial seed lots for the presence of *P. s. phaseolicola* by PCR as well as by plating on KB and MSP agar. These lots were chosen because they had been independently tested for the presence of the halo blight pathogen by the French government's Regional Plant Protection Service in Angers, France, with the KB agar plating method and by the Idaho Department of Agriculture with their standard agar plating-serological method (J. F. Chauveau and G. L. West, respectively; *personal communication*). All five lots had been found free of the halo blight pathogen, but lots I and IV were reported positive for *X. c. phaseoli* by the Idaho and French laboratories, respectively.

We did not detect *P. s. phaseolicola* in lots II, III, IV, and V, either by PCR or by our agar plating methods. These findings agree with those of the two laboratories mentioned. However, we obtained from lot I the characteristic 1.9-kb band in two and three of the four replicate amplifications of the 10× and 100× extracts, respectively. When aliquots from the 1× extract of this seed lot were plated on MSP agar, several colonies that were morphologically similar to *P. s. phaseolicola* were found. However, all were positive for *myo*-inositol and *i*-erythritol utilization (unlike *P. s. phaseolicola*) and nonpathogenic on the bean cultivar Hystyle. The extracts from lots IV and V gave 300–400 colonies on MSP agar that were suspected of being *P. s. phaseolicola*. Several of these colonies were tested for the nutritional properties mentioned above, but none were confirmed to be *P. s. phaseolicola*. These results demonstrate the superiority of the PCR-based detection method in a commercial seed lot. Lots I and IV, which had been found to carry *X. c. phaseoli* by the Idaho and French laboratories, respectively, were examined for *X. c. phaseoli* by plating on MXP agar. This pathogen was found in lot IV but not in lot I.

The specificity of detection of *P. s. phaseolicola* by the method described here is due in large part to the fact that the DNA region serving as a PCR target is absent from other bean pathogens, saprophytes, or epiphytes that may be associated with bean seeds. The 2.6-kb DNA fragment does not hybridize to DNA from a large number of bacterial strains that have been tested in this and in previous studies (23,29), which include representatives of all Gram-negative pathogens of bean and other plants as well as nonpathogenic bacteria. In a preliminary report (37), other workers showed that primers designed from a different region of the *tox* gene cluster amplified the target fragment from all 27 strains of *P. s. phaseolicola* but not from 21 other unspecified bacterial strains that were genetically close to this pathogen or had been isolated from bean. *P. s. phaseolicola* is the only proven phaseolotoxin producer among plant pathogens (21), although one report (8) indicates that the related pathogen *P. s. glycinea* may produce phaseolotoxinlike compounds.

Important advantages of PCR-based screening of commercial bean seed for halo blight are sensitivity of detection and the short time needed to perform the assays (2–3 days). A method that combines plating on the semiselective MSP agar and screening of individual colonies for phaseolotoxin production (with the *E. coli* bioassay [31]) apparently has sensitivity for the detection of the bean halo blight pathogen in seed (13) comparable to the PCR method described here. This method is technically simple but requires 6 days to perform, and its sensitivity depends strongly on the efficiency of isolation on MSP agar, which in our experience is good only with seed lots carrying low populations of saprophytes or epiphytes. Furthermore, the *E. coli* bioassay would fail with all potential Tox^- strains. One the other hand, PCR would fail with such strains if they lack the PCR target region or suitable primer binding sites. There is a reported tendency in some strains to change from Tox^+ to Tox^- in the laboratory (21), and Tox^- strains are occasionally found in nature (14,21). However, the frequency and epidemiological significance of Tox^- strains of the bean halo blight pathogen are unknown, and such strains have not been observed in Idaho (N. W. Schaad, *unpublished*), where the majority of bean seeds in the United States are grown. It is commonly believed that in plants there is a natural selection for the toxigenic trait (21).

The PCR-based method described here has the potential to detect both live and dead cells. Because the latter would indicate prior infection and because of the low tolerance levels for halo blight infection in commercial bean seed, it would be prudent not to use such lots as seed. Nevertheless, if knowledge of pathogen viability is desirable in bean seed certification, seed lots could be initially screened by PCR, and those shown to be positive could be retested by methods based on viable cells. Alternatively, it should be possible to employ cell-extraction methods (3) or reverse transcription PCR (6,12,18) to amplify the mRNA encoded by the *tox* region. Although the stability of various mRNAs in *P. s. phaseolicola* is unknown, most mRNAs of bacteria are short-lived and are rapidly degraded in dead cells. Thus, reverse transcription PCR of mRNAs should detect only cells that are viable at the time the seed lot is tested. The main problems in implementing this approach would be the added time and cost.

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