Parameters for Specific Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in Potato Stems and Tubers By Multiplexed PCR-ELISA

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ABSTRACT

A multiplex PCR-ELISA protocol for detection of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) was developed that is based on primers for amplification of three single-copy, unique DNA sequences, Cms50, Cms72, and Cms85. The three sequences were simultaneously amplified from the genomes of all 42 strains of *Cms* that were tested including variant mucoid forms, but not from strains representing five related subspecies, and *Rathayibacter rathayi* and *Rhodococcus faciens*. The lowest limit of detection by gel electrophoresis was estimated to be approximately 300 CFU per mL when cells were spiked into potato core fluid, but sensitivity increases approximately 10-fold using PCR-ELISA. Inclusion of a sea anemone DNA fragment engineered so it could be amplified from the Cms72 primer set provided the simultaneous signal that the system functioned properly when any sample was free of the pathogen. The addition of hydrolyzed casein to the reaction mix was demonstrated to markedly reduce or eliminate inhibition of PCR by plant cell components or contaminants. Multiplex PCR-ELISA detection of *Cms* was determined to be verifiable for analysis of both stems and tubers based on the amplification of multiple sites in its genome, it provides absolute specificity, and it was more sensitive than detection based on gel electrophoresis of PCR products and serological approaches.

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ADDITIONAL KEY WORDS: Bacterial ring rot, *Cms*-specific DNA, multiple-site PCR amplification.

INTRODUCTION

A worldwide policy of zero tolerance for the presence of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) in seed stock is presently in effect to reduce the spread of bacterial ring rot disease of potato. Proper implementation of this policy requires methods of detection that are both highly sensitive and absolutely specific for *Cms*. Detection of *Cms* has been difficult because it is frequently present in potato tubers and stems in low numbers and variant morphological forms can compromise certain serological methods of detection. The indirect fluorescent antibody staining assay (IFAS) (De Boer and Copeman 1980; De Boer and Hall 1988; Slack et al. 1979) and an enzyme-linked immunosorbent assay (ELISA) (De Boer et al. 1988) are typically used for detection. The MAb 9A1 antibody used in IFAS assays is highly specific (De Boer and Wieczorek 1984) and the detection sensitivity is estimated to no less than 10^4 colony-forming units/mL (cfu/mL) (Baer and Gudmestad 1993). However, detection by ELISA has shown cross-reactivity with other unidentified microbes (Crowley and De Boer 1982) and an inability to detect nonmucoid *Cms* strains (Baer and Gudmestad 1993).

DNA-based methods of *Cms* detection have recently been examined in an effort to increase sensitivity and retain high specificity. PCR primers have been developed to amplify sequences of an indigenous plasmid of *Cms* (Schneider et al., 1993; Firrao and Locci 1994) Rademaker and Janse 1994), as well as sequences within the RNA intergenic transcribed spacer region (Li and De Boer 1995) and single-copy DNA sequences (Mills et al. 1997). PCR primers for amplification of three single-copy *Cms* DNA sequences, designated Cms50, Cms72 and Cms85 (Mills et al. 1997) were previously shown by Southern hybridization to have no homology with DNA from other species of bacteria tested, including *Clavibacter* subsp.

insidiosus, *michiganensis* and *tessellarius*, *Pseudomonas syringae*, pv. *syringae*, *Erwinia carotovora* subsp. *carotovora*, and *Agrobacterium tumefaciens*, nor with DNA from other microbes that have been shown to cross-react with antisera made to *Cms*. Moreover, PCR detection of *Cms* in potato core fluid was also highly sensitive and approximately 100 cfu of potato core fluid were detected with the Cms85 primers.

Amplification of single-copy, unique DNA sequences provides an opportunity to verify the presence or absence of the pathogen with molecular probes. DNA probes constructed to have homology with one of the DNA strands of the PCR product will not form hybrids with spuriously amplified DNA from other species. Using the primer sets for amplification of Cms50 and Cms72, Baer et al. (2001) applied previously reported protocols (Mills and Russell 1997) to compare the efficacy of PCR-ELISA (enzyme-linked oligonucleosorbent assay) with serological approaches. They showed that this system has greater sensitivity than serological methods of testing, and that its specificity was uncompromised when challenged with strains from other subspecies of *Clavibacter* and strains of other species that are problematic in serological tests. However, their results also revealed certain inconsistencies in the ability of the system to detect *Cms* that could be attributed to problems with PCR inhibition rather than the limits of detection by this system. In certain cases where a dilution series was examined, cells were detected at both high and low dilution, but not at intermediate levels. These results are characteristic of a system that is lacking a component for the identification of samples where the PCR has failed for a variety of reasons.

The culmination of the development of a complete system for multiplexed PCR detection of *Cms* is reported here. This system has been optimized to enable the multiplexed PCR amplification of three *Cms*-specific fragments in approximately equal amounts, and the simultaneous amplification of a heterologous DNA molecule from one of the *Cms* primer sets. Biotinylated capture probes for each of the three *Cms*-specific fragments and the heterologous DNA fragment provide a convenient method of verifying that samples contain *Cms*, and evidence that the system is functioning properly when samples are free of the pathogen. This system also incorporates acid hydrolyzed casein to suppress frequently encountered inhibition of PCR amplification by components in plant cell extracts, debris or soil, thereby eliminating or greatly reducing the possibility of false-negative results.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

Cms strains and other bacteria used in this study are presented in Table 1. All strains were grown at ambient temperature on solidified and liquid NBY (Vidaver 1967) and PDA amended with 20 g $CaCO_3$/L.

Plant Material

Infected stems and tubers were generously provided by Oscar Gutbrod (Oregon State University) from field plots. Stems were received in ziplock bags and crushed directly in them using pliers with Velcro-lined jaws. A 100-µL aliquot of the cell sap was transferred into each of two 0.5-mL microfuge tubes. One tube was frozen (–20 C) and the other was centrifuged at 14,000 x g for 5 min. The supernatant was removed, InstaGene matrix (Bio-Rad Laboratories, Hercules, CA, USA) was added in an amount equal to the volume of the cell pellet, and the sample was treated according to the manufacturer's instructions. Tubers were washed free of dirt and dried. After removal of a thin slice of tissue from the stolon end, three plugs approximately 3 mm^2 were removed from the exposed tissue and transferred to a 1.7-mL microfuge tube containing 50 µL of filter-sterilized phosphate buffered saline (PBS) (Sambrook et al. 1989). The sample was macerated using the procedure described by Bertoni and Mills (1987), an additional 150 µL of PBS was added, and the suspension was incubated for 30 min at room temperature and centrifuged as before. A 100-µL volume of InstaGene Matrix was added to the sample pellet, which then was treated as previously described.

DNA Manipulation, Oligonucleotide Design, Southern Blot Analysis and PCR Conditions

Total DNA was extracted from bacterial strains using the CTAB procedure (Ausubel et al. 1992) and amplified by multiplex PCR using an Ericomp TwinBlock EasyCycler (Ericomp, San Diego, CA, USA). Conditions were optimized to produce approximately equal amounts of Cms50, Cms72, and Cms85 amplicons from internal primer sets and varying concentrations of genomic template DNA. The forward and reverse primers were as previously described (Mills et al. 1997) except that the reverse primer for Cms50 had the following sequence: 5'CCTGAGCAACGACAAGAAAAATATG3'. The multiplex PCR conditions were an initial incubation at 94 C for 5 min, followed by 10 cycles of 45 s at 94 C, 60 s at 63 C, and 10 s at 72 C, fol-

TABLE 1—*Reaction of* Clavibacter michiganensis *subsp.* sepedonicus (CMS) *and other bacterial strains to molecular probing, IFAS analysis and multiplex PCR with primers that amplify Cms 50, Cms72, and Cms85.*

Species and strain number	Colony Morphology[a]	IFAS Reaction[b]	^{32}P-Labeled Probes[c] Cms50	Cms72	Cms85	Multiplex PCR[d]	Source[e]
C. m.* subsp. *sepedonicus							
CS3	mucoid	+	+	+	+	+	1
CS5	mucoid	+	+	+	+	+	1
CS12	mucoid	+	+	+	+	+	1
CS13	mucoid	+	+	+	+	+	1
CS14	mucoid	+	+	+	+	+	1
CS15	mucoid	+	+	+	+	+	1
CS16	mucoid	+	+	+	+	+	1
CS17	mucoid	+	+	+	+	+	1
CS20	mucoid	+	+	+	+	+	1
CS57	mucoid	+	+	+	+	+	1
CS106	mucoid	+	+	+	+	+	1
CS118	mucoid	+	+	+	+	+	1
CS50/100	mucoid	+	+	+	+	+	5
CS100/250	mucoid	+	+	+	+	+	5
CSCA	mucoid	+	+	+	+	+	5
CSCAw	mucoid	+	+	+	+	+	5
cms3	mucoid	+	+	+	+	+	5
cms5	mucoid	+	+	+	+	+	5
cms6	mucoid	+	+	+	+	+	5
cms7	mucoid	+	+	+	+	+	5
cms8	mucoid	+	+	+	+	+	5
cms9	mucoid	+	+	+	+	+	5
As-1	mucoid	+	+	+	+	+	5
As-1-R	mucoid	+	+	+	+	+	5
Beta 1230	mucoid	+	+	+	+	+	5
BJ19	mucoid	+	+	+	+	+	5
BRR7	mucoid	+	+	+	+	+	5
33111 ATCC	mucoid	+	+	+	+	+	5
33113 ATCC	mucoid	+	+	+	+	+	5
4850 ATCC	mucoid	+	+	+	+	+	5
P45 ATCC	mucoid	+	+	+	+	+	5
299	mucoid	+	+	+	+	+	3
378 NCPPB	mucoid	+	+	+	+	+	3
379 NCPPB	mucoid	+	+	+	+	+	3
2140 NCPPB	mucoid	+	+	+	+	+	3
2884 NCPPB	mucoid	+	+	+	+	+	3
3467 NCPPB	mucoid	+	+	+	+	+	3
3833 NCPPB	mucoid	+	+	+	+	+	3
3898 NCPPB	mucoid	+	+	+	+	+	3
3917 NCPPB	mucoid	+	+	+	+	+	3
wi2	intermediate	+	+	+	+	+	5
Csep-1	intermediate	+	+	+	+	+	5
087-10	intermediate	ND	+	+	+	+	4
151-3	nonmucoid	ND	+	+	+	+	4
SD1#2	nonmucoid	+	+	+	+	+	5
C. m.* subsp. *insidiosus							
10253 ATCC		-	-	-	-	-	4
33114 ATCC		-	-	-	-	-	4
C. m.* subsp. *michiganensis							
4450 ATCC		-	-	-	-	-	4
C. m.* subsp. *tessallarius							
33566 ATCC		-	-	-	-	-	4

TABLE 1—*Continued.*

Species and strain number	Colony Morphology[a]	IFAS Reaction[b]	^{32}P-Labeled Probes[c] Cms50	Cms72	Cms85	Multiplex PCR[d]	Source[e]
C. m.* subsp. *nebraskensis							
27794 ATCC		-	-	-	-	-	4
C. m.* subsp. *inanicus							
NZ23496		-	-	-	-	-	4
Rathayibacter rathayi							
2980		-	-	-	-	-	2
Rhodococcus facians							
3263		-	-	-	-	-	2
Agrobacterium tumefaciens							
K24		ND	-	-	-	-	7
K84		ND	-	-	-	ND	7
96 field isolates		ND	-	-	-	ND	7
Enterobacter cloacae							
JL1157		ND	-	-	-	[f]	6
Alcaligenes xylosoxidans* subsp. *denitrificans							
JL3095		ND	-	-	-	ND	6
Unidentified Strains							
G		-	-	-	-	ND	1
N		-	-	-	-	[g]	1
02		-	-	-	-	[g]	1

[a]In reference only to *Cms* strains.
[b]Reaction with 9A1 anti-*Cms* monoclonal antibody.
[c]32P-labeled probes (Sambrook et al. 1989) made of Cms50, Cms72 and Cms85 and hybridized with Southern-blotted, *Sma*I-digested genomic DNA.
[d]PCR performed with Cms50, Cms72 and Cms85 internal primer sets unless otherwise noted. + = Predicted band size detected. - = Not detected.
[e]Number denotes person from whom strain was obtained.
1. S. De Boer, Agriculture Canada Research Station, Centre for Animal & Plant Health, Charlottetown, Prince Edward, Canada.
2. M. Romantschuk, University of Helsinki, Helsinki, Finland
3. David Stead, Central Science Laboratory, Sand Hutton, U. K.
4. D. Opgenorth, California Department of Food and Agriculture, Sacramento, CA USA
5. N. Gudmestad, Department of Plant Pathology, North Dakota State University, Fargo, ND USA
6. J. Loper, USDA ARS, Horticultural Crops Laboratory, Corvallis, OR USA
7. L. Moore, Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR USA
[f]PCR performed only with Cms85 primer set.
[g]PCR performed only with Cms85 and Cms72 primer sets.
Symbols/acronyms: IFAS = Immunofluorescent antibody stain; ND= not determined; ATCC, American Type Culture Collection; CDFA, California Dept. Food and Agriculture

lowed by 40 cycles of 30 s at 92 C, 30 s at 63 C, and 15 s at 72 C. The amplification reaction mix consisted of a 10-fold dilution of 10x Amplitaq Gold enzyme buffer (PE Applied Biosystems, Foster City, CA, USA), 2.75 mM $MgCl_2$, 200 µM dNTPs, 1µM of the primer sets that amplify Cms50 and Cms85, and 0.5 µM of the Cms72 primer set, 1.0 unit of Amplitaq Gold polymerase (PE Applied Biosystems) and 1 to 10 µL of sample in a total reaction volume of 25 µL. The relative concentrations of amplified Cms50, Cms72, and Cms85 PCR products following multiplex PCR were determined from bands excised from an agarose gel. The agarose plugs were soaked in water, the DNA was purified by passage through a QIAquick™ column (QIAGEN, Inc., Chartworth, CA, USA) and the concentration of each product was determined using a GeneQuant RNA/DNA Calculator (Pharmacia LKB Biotechnology, Pisctaway, NJ, USA). Acid hydrolyzed casein (Schleicher and Schuell, Keene, NH,

USA) was added to the reaction mix (final conc., 0.05%, wt/vol) to suppress inhibition of the PCR by plant cell components.

Following multiplexed PCR with *Cms*-specific primers and total DNA from various bacterial species, 20 µL of each reaction were analyzed by gel electrophoresis and Southern-blot hybridization with radioactive probes made of Cms50, Cms72, and Cms85 as previously described (Mills et al. 1997) to ascertain whether any bands had homology. To assure that template DNA of each species was suitable for amplification, forward and reverse primers (F 5'GAGGCAGCAGTGGGAATATT3' and R 5'CCCTACTAAAAGAGGTTTACAACCC 3') were used to prime the amplification of a conserved 100 bp sequence from the bacterial 16s ribosomal subunit rDNA gene.

Multiplex PCR ELISA Detection

When multiplex PCR ELISA was performed, 8 µM digoxigenin (DIG-ll-dUTP) (Boehringer and Mannheim, Indianapolis, IN, USA) was present in the reaction mix to label the PCR products. Five-prime biotinylated oligonucleotide capture probes designated Cp50 5'CTTTTGCCAGATTCAGGTCACCACG3', Cp72 5'CTTAGACGAACTGCTCCTGAACGG3', and Cp85, 5' AGGCATCCTGTTCCGTCTGGG3' were designed to be complementary to one strand of Cms50, Cms72 and Cms85, respectively, and purchased from Oligos Etc., (Wilsonville, OR, USA). Following multiplex PCR, 5-µL aliquots of the reaction were delivered to three microfuge tubes, each of which contained 10 ng of either Cp50, Cp72 or Cp85 oligonucleotide capture probe in 45 µL of PCR buffer. These tubes were placed in the thermocycler and initially incubated at 94 C for 5 min to denature the PCR products, and then at 70 C for 5 min to allow each capture probe to anneal to the complementary strand of the PCR product. The tubes were quickly chilled on ice, 100 µL of filter-sterilized PBS buffer was added, and 150 µL of this solution was transferred to the well of a streptavidin-coated microtiter plate (Boehringer Mannheim, Indianapolis, IN, USA). DIG-11-dUTP labeled DNA was detected using instructions included with a DIG detection ELISA (ABTS®) reagent kit (Cat. No. 1 531 434 Boehringer Mannheim). Intensity of the colorimetric reaction was determined at 405 nm with a reference wavelength of approximately 490 nm using a microtiter plate reader.

Sensitivity of Detection of Cms in Potato Core Fluid by PCR

Potato core fluid was "spiked" with predetermined concentrations of *Cms* cells of strain Cs3 as previously described (Russell and Mills 1997), to simulate natural levels of infection. The Cs3 culture was grown in NBY broth medium to an $OD_{600} = 0.5$, which corresponded to approximately 10^8 colony forming units (CFU/mL) and serially diluted by 10-fold increments into water. At each dilution, 5 replicas of 10 µL were immediately delivered onto solidified NBY medium to obtain an estimate of the CFU at each dilution and to establish a dilution end point. Twenty microliters of each dilution were also added to 180 µL of the potato core fluid and 100 µL were immediately frozen; the remainder was treated with InstaGene Matrix (Bio-Rad Laboratories) according to the manufacturer's instructions. At each dilution, 10 µL of either the frozen or the InstaGene™ Matrix-treated potato core fluid were then used to estimate the sensitivity of detection of *Cms* by multiplex PCR. The products of each PCR reaction were electrophoresed through a 4% agarose gel and stained with ethidium bromide to determine the minimum CFU that yield a detectable PCR product.

Development of an Internal Control to Specifically Monitor Cms72 Primer Function and PCR Inhibition

In three separate reactions, sea anemone DNA (a generous gift from Virginia Weiss, Oregon State University) was used as the template to generate randomly amplified fragments by PCR from the forward and reverse primers that amplify Cms50, Cms72, and Cms85. To ensure that the primers would anneal to random fragments, less stringent conditions were initially used. The conditions were 50 cycles 30 s at 92 C, 30 s at 37 C, and 60s at 72 C. Fragments were separated by gel electrophoresis and those of approximately 100-250 bp in size were excised from the gel by a toothpick and transferred to water to dissolve the DNA. The fragments were amplified again using the standard PCR conditions for amplification of Cms50, Cms72, and Cms85 fragments. Specific fragments were gel-purified and cloned into the Xcm1-derived vector (Borovkov and Rivkin 1997) and one designated SA72 was used in this study to specifically monitor Cms72 primer function with tuber-derived samples.

Immunodetection of Cms *Strains*

Pure cultures of bacteria and putative infected plant material were analyzed by an indirect fluorescent antibody staining (IFAS) procedure (De Boer and Wieczorek 1984) using a kit (Agdia, Inc., Elkart, IN, USA) that employs the 9A1 anti-*Cms* monoclonal antibody.

RESULTS

Development of Conditions for Multiplex PCR

The forward and reverse primer sets used for PCR amplification of Cms50, Cms72, and Cms85 sequences in this study produce single PCR products of 192 bp, 161 bp, and 205 bp, respectively, from *Cms* genomic DNA. The reaction conditions were established for multiplex PCR that included optimizing the annealing temperature for the three primer sets, establishing primer concentrations, and designing a new reverse primer for the amplification of Cms50 (see **Materials and Methods**) to obtain primers with similar annealing temperatures for near uniform amplification of all three fragments. Using these new PCR conditions, all three PCR products were synthesized in approximately equal amounts when *Cms* genomic DNA was not limiting (Figure 1). When the sample was diluted to 3 CFU per 10 µL in the potato core fluid approximately equal amounts of the Cms50 and Cms72 products were observed in the gel, but the Cms85 product was barely visible (Figure 1). No additional PCR products were visualized indicating that members of different primer sets were not priming the amplification of DNA from other sites within the genome.

The specificity of this multiplex PCR system was then examined with representative strains of *Cms*, as well as closely related *Clavibacter* subspecies and other species of bacteria (Figure 2 and Table 1). The multiplex PCR conditions primed the amplification of the three fragments from all 45 *Cms* strains analyzed regardless of their colony morphology and origin, and the typical gel pattern for the three fragments is shown in Figure 2 lane 11. Non-specific amplicons were occasionally detected from DNA templates of the eight heterologous strains examined (Figure 2, lanes 3-10), and a fragment of approximately 148 bp was amplified from genomic DNA of *C. m.* subsp. *insidiosum* (Figure 2A, lane 3). However, probes made of Cms50, Cms72 and Cms85 did not hybridize to Southern blots with PCR products made from the 8 heterologous strains (data not shown). Fragments of 50 to 75 bp that were observed in all lanes, including the tube that had no template DNA (lane 2), were assumed to be primer dimers. To demonstrate that the DNA templates of the eight species were suitable for amplification, a reaction was carried out using forward and reverse primers that amplify a conserved 100 bp sequence from the 16S ribosomal subunit rDNA repeat. For each DNA sample, a robust PCR product of the appropriate size was obtained (data not shown).

Negating Plant Cell Inhibitors of the PCR

Occasionally *Cms* cells were visible by Gram staining in some infected stem cell extracts, but their presence was only weakly, or sometimes undetected by multiplex PCR. This observation suggested that inhibitors of the PCR were present, and acid hydrolyzed casein was determined to be an effective product of numerous compounds examined to suppress PCR inhibition. At concentrations of 0.05%, 0.1%, and 0.2% (wt/vol) casein suppressed total PCR inhibition observed in stem extracts (Figure 3). The inhibition was most effectively suppressed at 0.05% casein but, interestingly, in the absence of plant cell extracts, amplification of genomic Cms72 DNA isolated from cultured Cs3 cells was completely inhibited at 0.2% casein.

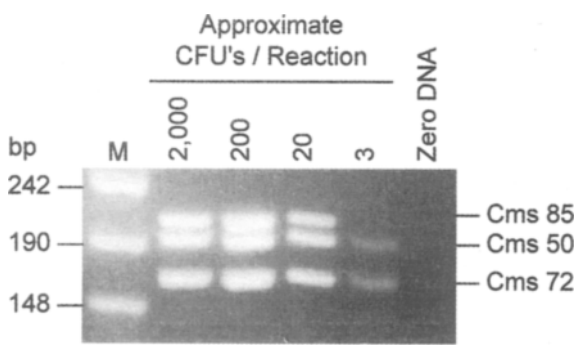

FIGURE 1.
The sensitivity of multiplex PCR using potato tuber core fluid spiked with varying concentrations of *Cms* cells.

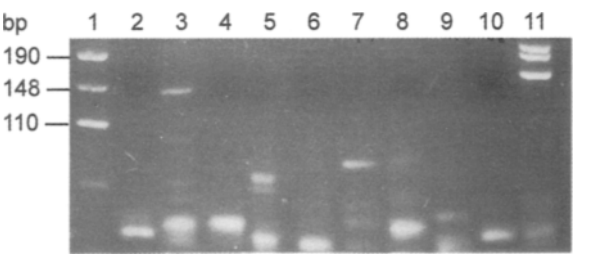

FIGURE 2.
Specificity of *Cms*-specific primers in multiplex PCR. Primers that amplify Cms50, Cms72, and Cms85 DNA were challenged with genomic DNA from eight bacterial species and an intermediate mucoid strain of C. *m.* subsp. *sepedonicus*. Lane 1, marker; lane 2, zero DNA; lane 3, C. *m.* subsp. *insidiosus* 33114 ATCC; lane 4, C. *m. insidiosus* 10253 ATCC; 5, C. *m.* subsp. *tessalarius* 33566 ATCC; lane 6, C. *m.* subsp. *michiganensis* 4450 ATCC; lane 7, C. *m.* subsp. *iranicus* NZ3496; lane 8, *Rhodococcus facians* 3263; 9, *Rathayibacter rathayi* 2980; 10, C. *m.* subsp. *nebraskensis* ATCC 27794; 11, C. *m.* subsp. *sepedonicus* Wi2.

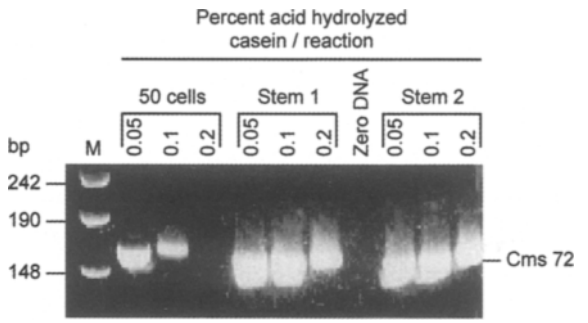

FIGURE 3.
Efficacy of hydrolyzed casein as an agent for relieving PCR inhibition in potato stem cell extracts. The DNA templates were obtained from cultured Cs3 cells (approx. 50 cells per reaction) and two stem samples that initially scored negative using primers for amplification of Cms72, but were known to contain *Cms* from Gram staining.

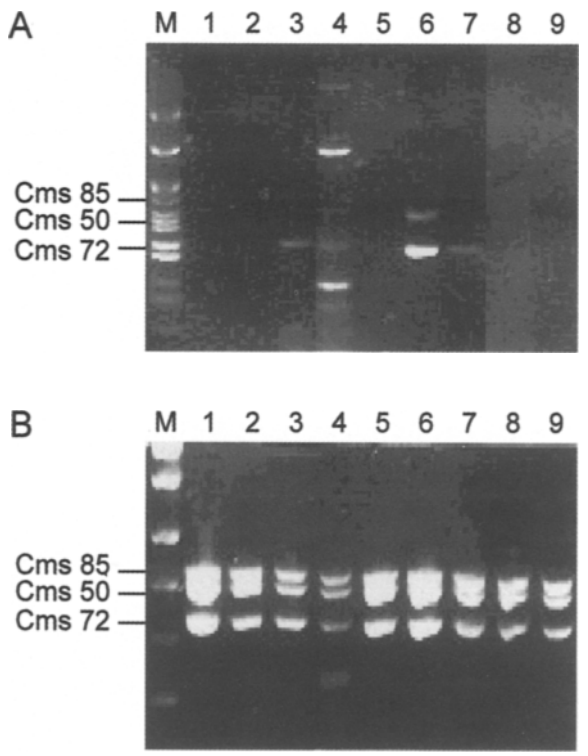

FIGURE 4.
The effect of using acid hydrolyzed casein to relieve the inhibition of multiplex PCR in potato stem samples infected with *Cms*. A. Multiplex PCR analysis in the absence of hydrolyzed casein. Lane 1, molecular weight standards and the relative positions of the expected PCR products, Cms85, Cms50 and Cms72; lanes 2-10, potato stem samples from infected plants. B. Multiplex PCR carried out with hydrolyzed casein (0.05%, final conc.) in the reaction mix. Samples are as indicated in panel A.

Various stem samples that initially scored negative by multiplex PCR were then analyzed with a reaction mix containing hydrolyzed casein added to a final concentration of 0.05% (wt/vol). In the absence of casein, PCR products were either not produced, or were of various sizes (Figure 4A, lane 5) that occasionally were of the expected size (Figure 4A, lane 6). However, in no case were three products of the expected size amplified. When these same samples were analyzed in the presence of 0.05% casein, PCR inhibition was avoided, and three very robust PCR products of the expected sizes were detected in each sample (Figure 4B).

Verification of Primer Function in Each Sample

An internal control was developed to demonstrate that the multiplex system was uninhibited and fully operational for any sample in which *Cms* was not detected. A 253 bp fragment was isolated from the sea anemone genome and its ends were engineered so it could be amplified from the forward and reverse primers that amplify Cms72. This fragment, designated SA72, was added to the multiplex reaction mix at various concentrations to establish conditions that do not compete for primer annealing with Cms72 template, but allow for its detection in the absence of Cs3 DNA template. In the absence of *Cms* template DNA, as little as 5 to 9 fg of SA72 DNA template was sufficient to observe a PCR product (data not shown). However, competition for primer annealing was apparent as *Cms* genomic DNA was increased in the reaction mix. In the experiment shown in Figure 5, the SA72 PCR product was produced when the reaction mix contained 90 fg of SA72 template DNA, but not when it contained 9 fg. In other experiments where the concentration of *Cms* genomic DNA was lower, the SA72 PCR product was occasionally detectable when the template concentration was 5 or 9 fg. Competition for primer annealing to Cms72 template was not apparent when SA72 template DNA was at 90 fg, and robust synthesis of Cms72 product was readily detected by gel electrophoresis (Figure 5).

Sensitivity of Detection of PCR Products by Gel Electrophoresis and ELISA

The unique sequences of Cms50, Cms72, and Cms85 provided the opportunity to determine whether all three fragments were amplified in approximately equal amounts, and to make a comparison of the sensitivity of detection by PCR-ELISA and by gel electrophoresis alone. Following multiplex

PCR, total DIG-11-dUTP-labeled Cms50, Cms72, and Cms85 DNA was quantified and diluted to obtain the end-point dilution for detection of each fragment by gel electrophoresis (Figure 6A). First, it can be seen that amplification of Cms72 is greater than amplification of Cms50 and Cms85, but that all three products are detected when 15 ng of total PCR product DNA is fractionated by gel electrophoresis (Figure 6A). In the lane containing 3 ng DNA, only the Cms72 product was barely visible. To determine the level of sensitivity conferred by PCR-ELISA, biotinylated capture probes, Cp50, Cp72 and Cp85 were used in separate tubes to capture one of the DNA strands of the respective PCR products at each dilution (Figure 6B). The colorimetric reaction was visually detectable above the control (zero DNA) for each fragment when approximately 0.3 ng of total PCR product was analyzed, which is approximately 10-fold more sensitive than visualization of the faintest band by gel electrophoresis. Measurement of the absorbance at 405 nm (Table 2) corroborated the results of visual inspection of the colorimetric changes at each dilution, and it was determined that the concentration of Cms72 product at the limit of detection was approximately twofold and fourfold greater than Cms85 and Cms50 amplicons, respectively.

Analysis of Tuber Samples by Multiplex PCR ELISA with Hydrolyzed Casein and SA72 in the Reaction Mix

The efficacy of detection of DIG-labeled PCR products by multiplex PCR-ELISA was examined using tuber samples and typical results are presented in Figure 7. When the samples apparently contained abundant *Cms* template DNA, robust

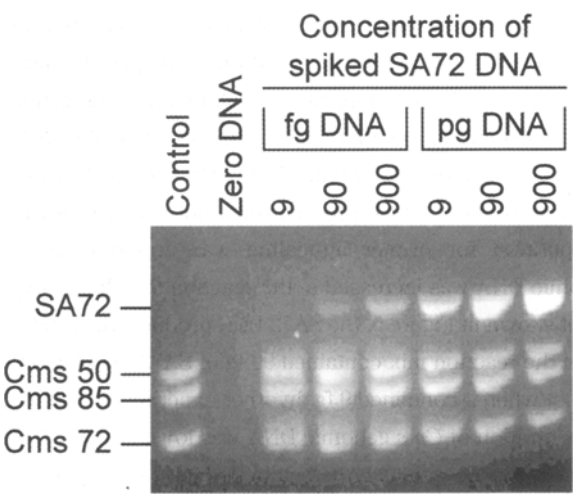

FIGURE 5.
Multiplex PCR of three regions of the *Cms* genome and various concentrations of an engineered sea anemone fragment (SA72) from the Cms72 primer set.

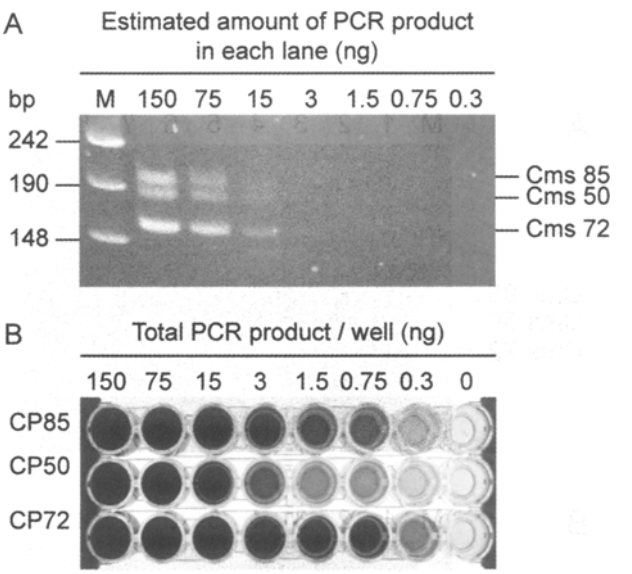

FIGURE 6.
Comparison of the sensitivity of multiplexed PCR detection of *Cms* by gel electrophoresis of PCR products and by PCR-ELISA. The concentration of DNA following multiplexed PCR was determined and diluted for analysis as indicated for each lane. A. Gel electrophoresis. Lane M, molecular weight standards. B. PCR-ELISA analysis of the amount of DNA shown in each lane of panel A.

TABLE 2—*Measurements of absorbance in multiplexed PCR-ELISA assays with ABTS® and defined concentrations of DNA.*

	Approximate concentration of DNA (μg)						
Probe	50	25	5	1	0.5	0.2	0.1
Cp85	3.707[a]	3.175	1.962	0.945	0.507	0.422	0.098
Cp50	3.556	3.075	1.445	0.488	0.227	0.200	0.057
Cp72	3.770	3.593	3.329	1.811	1.032	0.852	0.256

[a]For all entries, absorbance measured at 405 nm with background subtracted.

bands of Cms50, Cms72, and Cms85 were detected by gel electrophoresis and the SA72 control fragment was not amplified (Figure 7A, lanes 7, 9, and 10). In these samples the Cms72 template DNA appears to be in vast excess relative to the SA72 template and primer annealing to Cms72 out-competed primer annealing to, and amplification of, SA72 DNA. In samples with apparently low levels of *Cms* DNA template, the SA72 fragment was typically amplified, as were at least two of the three *Cms*-specific sequences (Figure 7A, lanes 4 and 6). The robust amplification of SA72 in those samples indicated that the PCR was not inhibited by plant cell components. When samples were uninfected, the SA72 product was typically robust (lanes 2, 3, 5, and 8), verifying that the system was functional.

Specific demonstration that *Cms* was present or absent in the tuber samples was accomplished using capture probes Cp50, Cp72, Cp85 and CpSA72 (Figure 7B). The control capture probe CpSA72 detected SA72 in all samples except those that contained robust bands of Cms50, Cms72, and Cms85 (lanes 7 and 9). The samples that were presumed uninfected by gel electrophoresis (Figure 7A, lanes 2, 3, 5, and 8) were confirmed uninfected by PCR-ELISA (Figure 7B). PCR-ELISA also confirmed that Cms72 and Cms85 were amplified, but not Cms50, in the sample shown in lane 6. This result demonstrates the necessity of using multiple primer sets to verify the presence of *Cms* because some samples contain low cell numbers. Moreover, comparison of the intensity of the ELISA reaction for Cms72 and Cms85 in this sample clearly demonstrates the increased level of sensitivity and ease of detection by PCR-ELISA relative to visual detection of bands by gel electrophoresis.

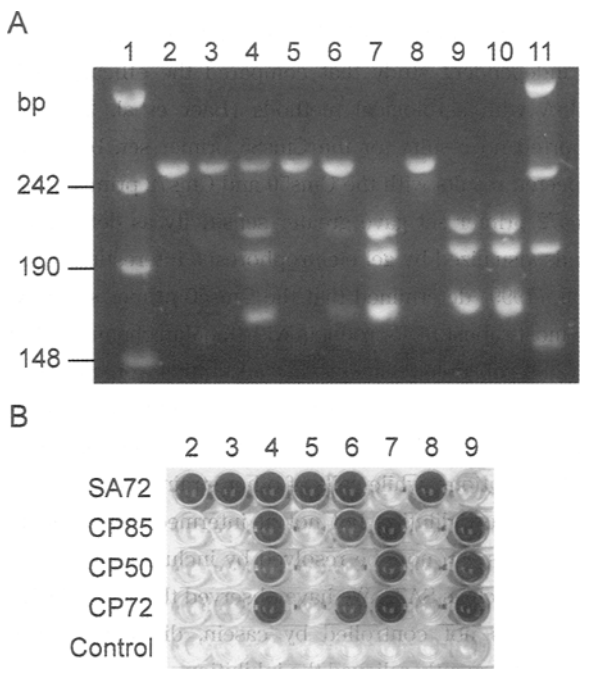

FIGURE 7.
Multiplexed PCR detection of *Cms* in infected potato tubers. A. Detection of PCR products by gel electrophoresis. B. PCR-ELISA detection of an equivalent amount of sample as in the lanes 2-9 of panel A. Lanes 1 and 11, molecular weight standards; lanes 2-10, tuber samples.

DISCUSSION

We have developed and evaluated a multiplex PCR-ELISA approach for the detection of *Cms* in both potato stems and tubers. This system is based on primer sets that amplify three separate, single-copy DNA sequences, Cms50, Cms72, and Cms85 (Mills et al. 1997). Because these three sequences are unique to *Cms*, the system offers the opportunity for absolute specificity and verification of the presence or absence of this pathogen at three genomic loci. The three loci from all 45 of the *Cms* strains examined were amplified with this multiplex system. When the analysis was expanded to include other strains (Mills et al. 1997), all of approximately 70 strains were detected by amplification of Cms50, Cms72, and Cms85. Among these were strains that produce the nonmucoid colony morphology. The variant nonmucoid isolates occasionally escape detection by ELISA tests (Baer and Gudmestad 1993). Random priming and amplification of DNA occasionally occurred with template DNA from closely related subspecies of *C. michiganensis* and other bacterial species. However, the spurious PCR products of various sizes have no homology with Cms50, Cms72, and Cms85 and would not be detected using DNA-based capture probes. False positive readings have never been observed with template DNA from another species.

Combining the three primer sets in a single reaction tube did not result in primer combinations that foster the priming and amplification of other fragments that could have limited the amplification of the expected PCR products. Although various annealing conditions and primer concentrations were used together with a new reverse primer for Cms50 to establish an optimal condition for multiplex PCR, the end-point dilutions of the PCR products indicated that the amplification of Cms72 was twofold and fourfold greater than Cms85 and Cms50, respectively (Table 2). At the dilution end point of 300 cfu/mL of potato tuber core fluid, amplification of Cms72 and

Cms50 DNA was detected (Figure 1). This level of sensitivity represents 3 cfu in 10 µL of potato core fluid used for PCR analysis, which approximates the sensitivity previously observed by PCR of single fragments (1 X 10^2 cfu/mL) (Mills et al. 1997; Baer et al. 2001), and is approximately 30-fold more sensitive than detection by immunodetection tests (Li and De Boer 1995). However, it should be noted that with the conditions defined here, amplification of either Cms50 or Cms85 may not always occur at the limits of detection (see Figures 1 and 7). The ability to detect the DIG-11-dUTP labeled PCR product by ELISA provided a 10-fold greater level of sensitivity than by gel electrophoresis and should provide a similar increased level of sensitivity with other DNA-based methods such as TaqMan probes and molecular beacons.

This multiplex PCR detection system has two additional features that can eliminate the possibility of false negative readings. First, the inclusion of SA72 template DNA in the reaction mix, which is amplified from primers that are used to amplify Cms72, ensures that primers that specifically amplify *Cms* template DNA function properly for each sample. Second, the addition of 0.05% (wt/v) casein hydrolysate to the reaction mix proved to be effective for relieving the occasional inhibition of PCR presumed to be caused by plant cell components, soil, and debris. Previously De Boer et al. (1995) had shown that BLOTTO, a non-fat milk cocktail typically used to reduce non-specific binding of proteins and nucleic acids to Southern and Western blots, also relieved PCR inhibition at concentrations of 1-5 % (v/v), and the precise concentration was adjusted with each polymerase batch. They also determined that similar concentrations of hydrolyzed casein were not effective. As shown in Figure 4, concentrations of casein above 0.20% (wt/v) inhibited PCR amplification of purified *Cms* template DNA, indicating that it is effective at only very low concentrations. Moreover, 0.05% casein did not inhibit different brands and batches of DNA polymerase. For uninfected plants, the amplification of SA72 template DNA was an absolute requirement for assurance that PCR inhibition was not a factor. In other multiplex PCR detection systems, different forward and reverse primers were used to amplify plant DNA (Weller et al. 2000) to monitor for PCR inhibition. Amplification of a heterologous fragment (SA72) with primers that amplify a pathogen target sequence (Cms72) guarantees that the reaction conditions are appropriate when the target PCR product is not detected in a sample.

In two previous studies, the primer sets that amplify Cms50, Cms72, and Cms85 (Baer et al. 2001; Schaad et al. 1999) were shown to be specific to *Cms* strains among approximately 30 strains that were screened. Schaad et al. (1999) used a fluorescent TaqMan probe homologous to Cms50 and found that BIO-PCR (Schaad et al. 1995) provided 10-fold higher sensitivity than conventional PCR for detection of *Cms* in potato tuber extracts. The core tissue extraction shaker incubation procedure of Dinesen and DeBoer (1995) was used by Schaad et al. (1999) and also in this study to estimate the sensitivity of detection. The differences in sensitivity reported here (300 cfu/mL) and by Schaad et al. (1999) (ca. 1000 cfu/mL) using classical PCR may be attributed our use of InstaGene™ Matrix to extract DNA and inclusion of casein to control inhibitors of the PCR. Subsequently, we discovered that 30-fold smaller cores macerated to a pulp directly in a microfuge tube was likely to release more bacteria than would be expected to be released from 1-cm cores that are shaken in liquid. The recent automated approach to sampling potato tubers for pathogens described by Souza-Dias et al. (1999) may easily be adopted for this detection system.

The primer sets and some of the reaction conditions developed for multiplex PCR reported here were provided for an independent study that compared the efficacy of PCR-ELISA with serological methods (Baer et al. 2001). They reported no results for the Cms85 primer set, but obtained expected results with the Cms50 and Cms72 primer sets. The Cms72 primer set gave greater sensitivity as determined by bands visualized by gel electrophoresis. Interestingly, Schaad et al. (1999) determined that the Cms50 primer set provided the most robust PCR products with TaqMan chemistry. Baer et al. (2001) observed that certain *Cms* strains were not detected at 240 cfu/rxn, but were detected at 0.4 cfu/rxn. Conversely, cells of some strains were detected at 240 cfu/rxn, but not at further dilutions, while cells of other strains were detected at high and low dilution but not at intermediate levels. These ambiguities can now be resolved by inclusion of an internal control, such as SA72. We have observed that when PCR inhibition was not controlled by casein, diluting the sample twofold frequently relieved the inhibition.

In developing this detection system, we initially observed interactions between combinations of different primer sets that affected sensitivity. By altering all of the components of the reaction mix, as well as the annealing temperatures, and

carrying out PCR with a RoboCycler (La Jolla, CA, USA), the optimum conditions for multiplex PCR amplification of the three PCR products and the SA72 fragment were established. To amplify approximately equal amounts of the three PCR products, the concentration of the Cms72 primer set was reduced to one-half that of the other two primer sets, and a new reverse primer for Cms50 was used to optimize the annealing of this primer set.

The major advantages of the multiplex PCR-based detection system described here are the elimination of erroneous positive reactions by amplification of unique *Cms* DNA sequences; the verification of the presence or absence of the pathogen at three loci; the inclusion of an internal control fragment that is amplified by primers specific for pathogen target DNA to eliminate false negative results; the inclusion of a component that relieves the inhibition of PCR that is common when samples contain soil, plant cell components, or other debris; and the demonstration that the PCR products are suitable for detection by ELISA, TaqMan probes and molecular beacons.

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