Selection of Polymerase Chain Reaction Primers from an RNA Intergenic Spacer Region for Specific Detection of *Clavibacter michiganensis* subsp. *sepedonicus*

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ABSTRACT

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Specific polymerase chain reaction (PCR) primers targeting genomic DNA were selected for sensitive detection of *Clavibacter michiganensis* subsp. *sepedonicus*, causal agent of bacterial ring rot disease of potato. The intergenic spacer region, approximately 500 bp, between the 16S and 23S rRNA genes of *Clavibacter michiganensis* subsp. *sepedonicus*, *michiganensis*, *insidiosus*, *nebraskensis*, and *tessellarius* were initially amplified and sequenced. Subsequently, a pair of PCR primers (Sp1f and Sp5r) was selected on the basis of the sequence data. The primers specifically amplified a 215-bp fragment when *Clavibacter michiganensis* subsp. *sepedonicus* genomic DNA was used as template but did not amplify DNA from phenotypically related bacteria, including species of *Rathayibacter* (formerly *Clavibacter*) and other subspecies of *C. michiganensis*; serologically related bacteria isolated from potato stems; or other unknown saprophytic bacteria isolated from potato tubers. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by PCR using these primers was more sensitive than enzyme-linked immunosorbent assay (ELISA) and immunofluorescence tests based on monoclonal antibodies. Amplification products were obtained by PCR for all potato tuber samples that were positive for *Clavibacter michiganensis* subsp. *sepedonicus* by ELISA and immunofluorescence. In addition, tubers from ring rot-infected plants, which tested negative in ELISA and immunofluorescence, were positive in PCR. However, 12 tubers from healthy plants were all negative in both the serological tests and PCR.

Additional keywords: DNA sequencing.

Bacterial ring rot disease of potato is caused by a gram-positive coryneform bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*. Bacteria from infected seed tubers enter plant stems through vascular tissue, invade stolons, and infect progeny tubers (7). Both internal and external symptoms are found commonly in infected potato tubers toward the end of the storage period because the disease continues to develop within stored tubers (16). However, the pathogen survives well on potato tubers in storage with or without symptoms. Since infected seed potatoes are the major source of initial inoculum each growing season (7), detection of low levels of *Clavibacter michiganensis* subsp. *sepedonicus* in seed potatoes plays an important role in disease control and eradication. Studies have shown that the bacterial population density that develops in the plant and progeny tubers depends on the population density in the seed tuber at planting (7,8). Quarantine and certification regulations have been adopted by many countries to avoid or minimize the chances of introducing *Clavibacter michiganensis* subsp. *sepedonicus* through commercial seed potatoes. In Canada, zero tolerance in the potato certification program has been applied for both import and export of seed potatoes.

The most widely used approaches for detection of bacterial ring rot are enzyme-linked immunosorbent assay (ELISA) and immunofluorescence; eggplant bioassay is sometimes used to complement these tests. ELISA and immunofluorescence based on monoclonal antibodies (MAbs) have proven to be very useful as sensitive potato indexing tests for bacterial detection in Canada's potato certification program. Immunofluorescence, in particular, has been widely used in Canada and several European countries for detection of bacterial ring rot in commercial seed potatoes. However, questions remain regarding determination of the positive/negative threshold for the serological tests. Occasionally, cross-reacting bacteria are also a problem (11).

Some attempts have been made to evaluate DNA-based techniques for detecting the bacterial ring rot pathogen (13,15,18,20, 23). In an earlier study, a DNA probe was developed on the basis of a plasmid found in most *Clavibacter michiganensis* subsp. *sepedonicus* strains (12). Polymerase chain reaction (PCR) primers were based on the sequences of DNA plasmid probes (13,20,23). Although the primers worked well for specific amplification, *Clavibacter michiganensis* subsp. *sepedonicus* strains without the plasmid, such as strain P45 (19), which is virulent on potato, could not be amplified by some of the primers (20,23). Another problem with these DNA-based techniques is cross-hybridization of probes and primers with other subspecies of *C. michiganensis*, such as *Clavibacter michiganensis* subsp. *insidiosus* (20). In addition, no data are available for practical application of these primers for detecting *Clavibacter michiganensis* subsp. *sepedonicus* in potato tissue samples.

The objective of this study was to select and apply PCR primers, targeting the intergenic spacer region between 16S and 23S rRNA genes of *Clavibacter michiganensis* subsp. *sepedonicus*, for specific amplification and detection of the bacterial ring rot

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pathogen in potato tuber samples. Another objective was to compare the sensitivity of PCR detection with that of ELISA and immunofluorescence based on MAbs.

MATERIALS AND METHODS

Bacterial strains. Bacterial cultures used for DNA sequencing include type strains of *C. michiganensis* subsp. *michiganensis* (LMG 7333), *sepedonicus* (LMG 2889), *insidiosus* (LMG 3663), and *nebraskensis* (LMG 5627) obtained from the Culture Collection of the Laboratorium Voor Microbiologie Rijksuniversiteit Gent, Belgium (LGM), and *Clavibacter michiganensis* subsp. *tessellarius* (ATCC 33566) obtained from the American Type Culture Collection, Rockville, MD (ATCC). Bacteria used in PCR amplification are listed in Table 1. Bacteria were grown on YGM plates (6) and preserved using cryopreservation beads (Technical Service Consultants, Ltd., Heywood Lanes, England) at –80°C.

DNA extraction. For pure cultures, a loopful of bacteria was suspended and washed once in sterile distilled water and pelleted by centrifugation in a 1.5-ml Eppendorf tube. The pellet was frozen at –20°C for 1 h and thawed at room temperature. After being treated with 100 µl of cold acetone (–20°C) for 10 min, the pellet was suspended in 500 µl of TE (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) buffer, followed by addition of 50 µl of 500 mM EDTA (pH 8.0), 50 µl of 14% sodium dodecyl sulfate, and 10 µl of 0.1% proteinase K (Sigma Chemical Co., St. Louis) and incubation for 1 h at 55°C or overnight at 37°C. An equal volume of 7.5 M ammonium acetate was then added to separate the DNA in solution from most cell debris, which precipitated, and was removed by centrifugation at 17,310 × *g* for 20 min. DNA in the supernatant was precipitated using isopropanol at –20°C for 30 min. The DNA was pelleted, washed with 70% ethanol, and vacuum-dried before dissolving in 100 µl of sterile distilled water.

TABLE 1. *Clavibacter michiganensis* subsp. *sepedonicus* (*C. m. sepedonicus*) strains and other bacteria tested in polymerase chain reaction (PCR) amplification using two pairs of PCR primers

Bacteria (no. of strains)	Culture code[a]	Geographic location	PCR amplification with[b] 1493f+23r	PCR amplification with[b] Sp1f+Sp5r
C. m. sepedonicus (24)	LMG 2889^T	Canada	+	+
	P45, 50/100[c]	Unknown	+	+
	CS3Rc, R8, 3M, 3NM, R14, RR1A	Alberta, Canada	+	+
	CS 2, 3-1, 3-2, 4, 7, 9, 12	Finland	+	+
	R5, R16, R17	BC, Canada	+	+
	Mutants 2, 3, 8, 9, 11	Finland	+	+
C. m. michiganensis (5)	LMG 7333^T	Hungary	+	–
	CM 3	Alberta, Canada	+	–
	CM 5, 6	ON, Canada	+	–
	CM 8	Kenya	+	–
C. m. insidiosus (7)	LMG 3663^T	USA	+	–
	CI 16	Kans.	+	–
	CI 102B	Calif.	+	–
	CI LETH, N, N53, 4	Unknown	+	–
C. m. nebraskensis (2)	LMG 5627^T, CN-USA	USA	+	–
C. m. tessellarius (2)	LMG 7295, ATCC 33566^T	USA	+	–
Rathayibacter rathayi (2)	CR 101^T	England	+	–
	CR 1	Unknown	+	–
R. iranicus (2)	CI 147, 148	Unknown	+	–
R. tritici (2)	CT 102	Egypt	+	–
	CT 104	Iran	+	–
Erwinia carotovora subsp. *astroseptica* (1)	ECA 439	Mexico	+	–
Bacteria isolated from potato (19)[d]	A, B1, B2, C1, C2, E, F, G, H, I1, I2, J, K, L, M1, M2, N, O1, O2	BC, Canada	+	–
Bacteria isolated from potato tubers (31)[e]	None	BC, Canada	+	–

[a] T = type strain.
[b] The two pairs of primers are specific for eubacteria and *C. michiganensis* subsp. *sepedonicus*, respectively.
[c] Antibiotic resistant strain.
[d] Unidentified isolates cross-reacted with polyclonal antisera or monoclonal antibodies produced against *C. michiganensis* subsp. *sepedonicus*.
[e] Isolates were not identified.

PCR amplification and DNA sequencing. *Taq* polymerase (Gibco-Bethesda Research Laboratories [Gibco-BRL], Gaithersburg, MD) and eubacterial universal primers were used to amplify the spacer region between 16S and 23S rRNA genes in PCR, as described previously (17). The universal primers were 1493 forward (1493f, 5′-AGTCGTAACAAGGTAGCCGT-3′, position 1493 to 1512: *Escherichia coli* numbering system for 16S rRNA) (24) and 23 reverse (23r, 5′-GTGCCAAGGCATCCACC-3′, position 23 to 39: *E. coli* numbering system for 23S rRNA) (3), derived from the conserved region of 16S and 23S rRNA genes, respectively (M. Fegan, *personal communication*). A reaction without template served as a negative control. PCR products were gel-purified using an Elu-Quick DNA purification kit (Schleicher & Schuell, Keene, NH) after electrophoresis in 1% agarose gel. Purified PCR products served as templates for sequencing reactions.

PCR-based sequencing reactions were carried out in a thermal cycler (Perkin-Elmer [Norwalk, CT] model 480) using a dye-deoxy chain terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions. The same pair of primers was used for all strains to sequence a fragment of approximately 500 bp. Nucleotide sequences were determined using an automated DNA sequencer (Applied Biosystems) by the Nucleic Acid-Protein Service Unit (NAPS, UBC, Vancouver, BC, Canada).

Selection of PCR primers for specific amplification. Sequence data of the spacer region between the 16S and 23S rRNA genes for the five subspecies of *C. michiganensis* were manually aligned. Comparisons also were made with published sequence data of the same region in other bacteria retrieved from GenBank (2). A primer analysis software, Oligo 4.0 (National Biosciences, Inc., Plymouth, MN), was used to select PCR primers targeting the spacer region of *Clavibacter michiganensis* subsp. *sepedonicus*, avoiding possible formation of primer dimers, self-priming, primer hairpin, and primer multiple binding sites. Pri were synthesized using an ABI 391 DNA synthesizer (Applied Biosystems) by NAPS and purified as described (22).

Optimal conditions for PCR were determined with a total of 20-µl volume containing 1× *Taq* buffer (supplied with *Taq* polymerase by Gibco-BRL), 0.1 mM dNTPs, 2.5 mM magnesium chloride, 0.5 units of *Taq* polymerase (Gibco-BRL), 0.2 µl of DNA extracts from pure culture, and 0.5 µM each primer. For each PCR mixture, 0.5 to 1 µl of blotto (10% skim milk) was used to minimize the possible effect of inhibitors in the DNA extracts from plants (X. Li and S. H. De Boer, *unpublished data*). The optimum amount of blotto varied with different batches of *Taq* enzyme. Two thermal cyclers (Perkin-Elmer model 480; and TwinBlock, Ericomp, Inc., San Diego, CA) were used and compared for PCR amplification with initial denaturation at 94°C for 3 min followed by 40 cycles at 62°C for 45 s, 72°C for 90 s, and 94°C for 60 s. A final elongation was performed at 72°C for 5 min. A positive control with DNA extract from strain P45 and a negative control without template were used in each PCR amplification experiment. PCR products were resolved in a 2% agarose gel stained with ethidium bromide.

The lower limit of sensitivity for PCR amplification was determined by testing dilution series of pure cultures. Loopfuls of bacteria, strains CS3Rc and R8, were suspended separately in 1 ml of

sterile distilled water and diluted 12 times in a 10-fold series. Concentration of bacteria was determined by a standard plate count procedure on YGM medium. Each dilution was tested by ELISA and immunofluorescence, and 100 µl of each dilution was used to extract DNA for testing by PCR.

PCR for detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers. Potato tuber samples were harvested from field plots planted with seed pieces, which had been inoculated prior to planting by cutting with a knife dipped in a suspension of about 10^7 cells per ml of inoculum. Harvested tubers were stored at 10°C for 4 to 6 months prior to sampling. Two batches of diseased potato tuber samples from commercial producers with symptoms of decay also were tested.

DNA was extracted from potato tuber tissue taken from the stolon end. Approximately 2 g of potato tissue was crushed and suspended in 1 ml of sterile distilled water, and 100 µl of the liquid phase was transferred to an Eppendorf tube. After centrifugation, DNA was extracted from the pellet by the procedure described above. The final pellet was resuspended in 20 µl of sterile distilled water.

For every set of DNA extractions from potato, at least one mock extraction without potato material and one potato tuber from the virus-free potato collection at the Pacific Agriculture Research Centre, Vancouver, BC, Canada, were used as two internal negative controls to detect whether contamination occurred during DNA extraction.

PCR amplification at optimal conditions, as described above, was carried out with 1 µl of each DNA extract from potato tubers. A positive control with DNA extract from strain P45, a negative control without template, and the two internal negative controls mentioned above were used in each PCR amplification experiment. For some DNA samples, PCR amplification experiments were repeated 2 to 3 times. PCR products were resolved in a 2% agarose gel as described above.

Serological tests. Potato tuber samples were visually inspected for internal symptoms before proceeding with ELISA, immunofluorescence, and DNA extraction. ELISA (9) and immunofluorescence (10) using MAbs 1H3 and 9A1, respectively, were applied to the same potato samples used to extract DNA for PCR amplification.

RESULTS

Sequence data and accession numbers. Almost the complete spacer region between 16S and 23S rRNA genes was sequenced for the five subspecies of *C. michiganensis*: *sepedonicus*, *michiganensis*, *insidiosus*, *nebraskensis*, and *tessellarius*. The sequence data were deposited in GenBank under accession numbers U09378 to U09382, except for that of subspecies *tessellarius* (L43095). Sequences for the five subspecies of *C. michiganensis* were similar to one another, but some base pair differences were present (Fig. 1).

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Subspecies                Sequences
-------------------------------------------------------------------------------
sepedonicus     NNNNNNNNNN NNNNNNACAG GAAGGTGTGG CTGGATCACC TCCTTTCTAA GGAGCATGTG
insidiosus      GTAACAAGGT AGCCGT..C. .......... .......... ......NN.. ..........
nebraskensis    GTAACAAGGT AGCCGT..C. .......N.. .......... .......... ..........
michiganensis   GTAACAAGGT AGCCGT..C. .....A.C.. N......... .......... ..........
tessellarius    .......... ......NNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN

sepedonicus     CACCTCTCCT CTGTATACAG GG-AGATCAC GGGTGCCAAG TCACGCGTCA GGCGTATGTT
insidiosus      .......... .......... ..T....... .......... .......... .....T...C
nebraskensis    .......... .......... .......... ..N....... .......... .....T...C
michiganensis   .......... .......... .........A .......... .......... .....C....
tessellarius    NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN

sepedonicus     CTGGCGGTGG CGCTCA-TGG GTGGAACATT GACATTGATG CCGGCTGATG TGCCGGGCTG
insidiosus      ....T..... .......... .......... ..T....... .......... ..T.......
nebraskensis    ....G..... A......... ......N... ..N....... .......... .NT.......
michiganensis   .......... .......... .......... .......... .......... ..T.......
tessellarius    NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNT..TN...

                          Sp1f
sepedonicus     CTAGTACGCC TCCTTGTGGG GTGGGAAAAT GGTCTGGTGT GTCGAGGGCA TGTTGCACGC
insidiosus      .......A.. .......... .......CG. .......... .......... ..........
nebraskensis    ....A..... ...A...... .......CGC ........N. .......... ........N.
michiganensis   .......... .......... .......CG. .......... .......... ..........
tessellarius    .......... .......... .......CG. ...GC..G.. .......... ..........

sepedonicus     TGTTGGGTCC TGAGGGACCG GGCCGCACCT TTTGG--TGT GTCTGGTTTC TTGTCGGACC
insidiosus      .......... .......... .A.....T.. ..C..GG... .......... .......G..
nebraskensis    .......... .......... .......... N.C..G.... .......... ..........
michiganensis   .......... .......... .......... .C...G.... .......... ..........
tessellarius    .......... .......... .......... .C-..G.... .......... ..........

sepedonicus     CTTTCCGTCG TCCCTTGAGT GGATGGTGGT GGGGTACCGC CCGTATATTG AGAACTACAC
insidiosus      .......... ....-G.... .......... .......... .......... ..........
nebraskensis    .......... ...T-.TC.. .......... .......... .......... ........N.
michiganensis   .......... ...TG.T-.. .......... .......... .......... ..........
tessellarius    .......... ....-..... .......... .......... .......... ..........

                                                          Sp5r
sepedonicus     AGTGGACGCG AGCATCTTAG ATTCACCGGT TTACCCGGTG GATCACAAAG ATCTATTTAT
insidiosus      .......... .......... .........G ..T..-.... .......... ..........
nebraskensis    .......... .......... .......... ..T..-.... .......... ..........
michiganensis   .......... .......... .......... ..T..-.... .......... ..........
tessellarius    .......... .......... .......... ..T..-.... .......... ..........

sepedonicus     AGATCATTGG TCAATTCTGT CTCCTCTAGG GGAGGCGAAA CGATTCAATC TCATGTGATT
insidiosus      .......... .......... .......... .......... ...A...... ..........
nebraskensis    .......... .......... .......... .......... .......... ..........
michiganensis   .......... .......... ....CT.C.. .......... .......... ..........
tessellarius    .......... .......... .......... .......... .......... ..........
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Fig. 1. Sequence alignment of the intergenic spacer region between the 16S and 23S rRNA genes of *Clavibacter michiganensis* subspecies. ACGU, standard nucleotide abbreviation; N, unsequenced region; dash (–), alignment gaps and base deletions; dot (·), identity with sequence of *C. michiganensis* subsp. *sepedonicus*. The shaded and underlined regions are specific primer sites.

Specific PCR amplification of *Clavibacter michiganensis* subsp. *sepedonicus*. On the basis of the sequence data, a pair of PCR primers, Sp1f (5′-CCTTGTGGGGTGGGAAAA-3′) and Sp5r (5′-TGTGATCCACCGGGTAAA-3′), were selected for specific amplification of *Clavibacter michiganensis* subsp. *sepedonicus* in a 40-cycle reaction. A 215-bp fragment was amplified for all strains of *Clavibacter michiganensis* subsp. *sepedonicus*, including strain P45, at an annealing temperature of 62°C (Fig. 2). Similar results were obtained consistently using both the Perkin-Elmer model 480 and Ericomp TwinBlock PCR thermocyclers (data not shown).

Primers Sp1f and Sp5r were subspecies-specific to *Clavibacter michiganensis* subsp. *sepedonicus*, and no PCR products of the same or a different size were obtained at 62°C using this pair of primers for bacteria, including four *C. michiganensis* subspecies, *Rathayibacter* spp., and *Erwinia carotovora* (Table 1). In addition, the 50 unidentified isolates from potato also were negative in PCR (Table 1). However, the entire spacer region of all these bacteria was amplified using eubacterial universal primers.

Results also were consistent when Sp1f and Sp5r were used with the 62°C annealing temperature for amplifying *Clavibacter michiganensis* subsp. *sepedonicus* strains from different geographic locations (Table 1). Mutant strains selected in Finland on the basis of reduced production of cellulase or amylase (K. Lehtila and M. Metzler, *personal communication*) also were amplified.

Detection sensitivity of PCR with our primers was 5 to 50 CFU per PCR reaction (approximately 500 to 5,000 CFU per ml) (Fig. 3), compared to 10^4 CFU per ml in immunofluorescence and 10^5 CFU per ml in ELISA in comparative tests.

PCR detection of *C. michiganensis* subsp. *sepedonicus* in potato tubers. A 215-bp amplification product was obtained using Sp1f and Sp5r in PCR on potato tuber samples infected with *Clavibacter michiganensis* subsp. *sepedonicus* (Figs. 4 and 5). PCR compared favorably with standard techniques, including visual inspection of potato tubers for symptoms, ELISA, and immunofluorescence (Table 2). Nine of eleven symptomless tubers from inoculated plants were negative in ELISA, but they all tested positive in PCR (Table 2). Five of these tubers also were positive in immunofluorescence on the basis of ≥5 cells per microscope field. However, even the immunofluorescence "negative" tubers had some fluorescing bacterial cells but at levels too low (average of 0.6 to 1.8 cells per field) to be considered a positive test result

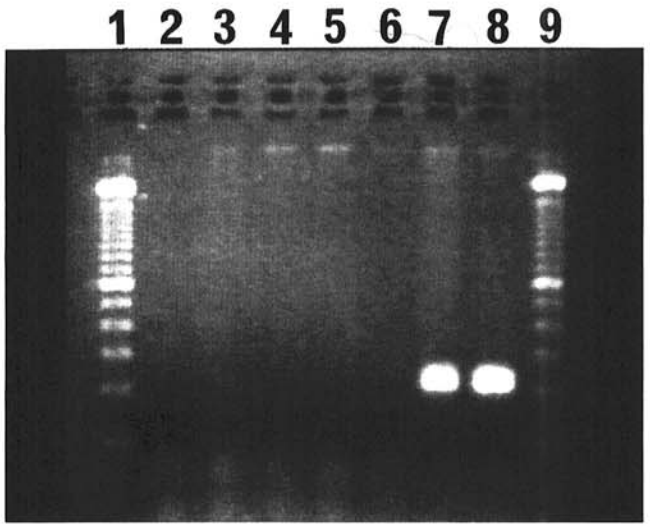

Fig. 2. Ethidium bromide-stained polymerase chain reaction products amplified using primers Sp1f and Sp5r from genomic DNA of lane 3, *Clavibacter michiganensis* subsp. *tessellarius*, ATCC 33566; lane 4, *C. michiganensis* subsp. *michiganensis*, LGM 7333; lane 5, *C. michiganensis* subsp. *nebraskensis*, LGM 5627; lane 6, *C. michiganensis* subsp. *insidiosus*, LGM 3663; lanes 7 and 8, *C. michiganensis* subsp. *sepedonicus*, strains P45 and LGM 2889, respectively. Lanes 1 and 9 are 100-bp ladders, and lane 2 is a negative control.

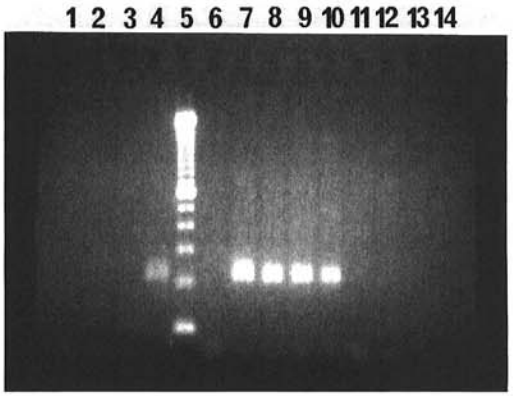

Fig. 4. Ethidium bromide-stained polymerase chain reaction products amplified using primers Sp1f and Sp5r from DNA extracts of potato tubers (lanes 7–14). Lanes 1–4, *Clavibacter michiganensis* subsp. *michiganensis* (LGM 7333), *nebraskensis* (LGM 5627), *insidiosus* (LGM 3663), and *sepedonicus* (P45, a positive control), respectively; lanes 5 and 6, 100-bp ladder and a negative control, respectively; lanes 7 and 8, artificially infected potato tubers using *C. michiganensis* subsp. *sepedonicus* strain R14; lanes 9 and 10, potato tubers 87-1 and 87-2; lanes 11 and 12, potato tubers 93-1 and 93-2; lanes 13 and 14, potato tubers from the virus-free potato collection at the Pacific Agriculture Research Centre, Vancouver, BC, Canada.

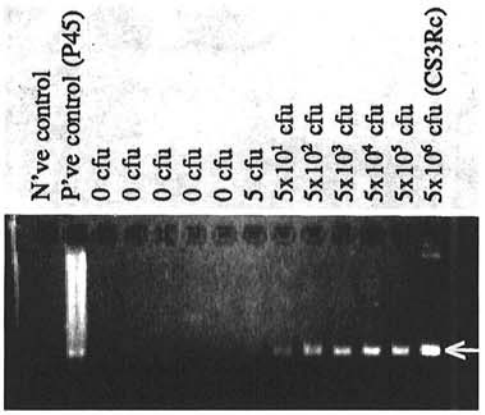

Fig. 3. Ethidium bromide-stained agarose gel electrophoresis of amplified polymerase chain reaction (PCR) products from DNA extracts of a dilution series of *Clavibacter michiganensis* subsp. *sepedonicus* strain CS3Rc. Numbers at the top indicate CFU per 20-µl PCR reaction. Strain P45 served as a positive control. Arrow indicates molecular weight of 215 bp.

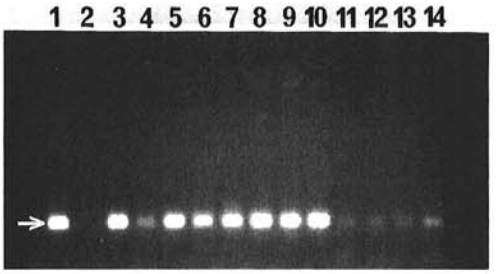

Fig. 5. Ethidium bromide-stained agarose gel electrophoresis of amplified polymerase chain reaction products from DNA extracts of potato tubers (lanes 4–14). Lanes 1 and 2, positive (P45) and negative controls, respectively; lane 3, DNA extract from an asymptomatic potato tuber (lane 4) mixed with *Clavibacter michiganensis* subsp. *sepedonicus* strain CS3Rc cells. Fluorescing cells were present in immunofluorescence for samples from which DNA was extracted for lanes 4 and 11–14, but the number of cells per microscope field was too low to be considered positive at currently set thresholds. Samples used for extracting DNA in lanes 5–10 were strongly positive in immunofluorescence. Arrow indicates molecular weight of 215 bp.

(9). Potato tubers 93-1 and 93-2 showed soft decay in tissue, which was atypical compared to the ring rot symptom caused by *Clavibacter michiganensis* subsp. *sepedonicus*. Consistent negative test results in ELISA, immunofluorescence, and PCR indicated that the symptoms were caused by organisms other than *Clavibacter michiganensis* subsp. *sepedonicus*. All 12 healthy tubers from the virus-free potato collection at the Pacific Agriculture Research Centre were negative in PCR, ELISA, and immunofluorescence.

DISCUSSION

The eubacterial intergenic spacer region between the 16S and 23S rRNA genes has been considered an ideal region for developing specific PCR primers that can differentiate bacteria, because it contains extensive sequence variation (1). Variation in this region among closely related bacteria is probably the result of minimal selective pressure to maintain sequences, in contrast to sequences within genes that have important functional roles such as the rRNA genes. The spacer region separating 16S and 23S rRNA genes has been utilized previously for developing PCR primers for other bacteria (1), but to our knowledge, our study is the first to target the spacer region for developing PCR primers specific for a plant-pathogenic bacterium.

The degree of similarity (about 95%) within the nucleotide base sequences of the spacer region among the *C. michiganensis* subspecies was not unexpected in light of previous studies (21). Although the five subspecies are highly host specific and have distinctive colony characteristics, they share characteristic properties such as high G+C% values, similar cell wall composition, and similar, but distinct, protein profiles (4,5). A close relationship also is suggested by serological cross-reactivity (10), DNA cross-hybridization (20), and similarity of the nucleotide sequence of the 16S rRNA genes (X. Li and S. H. De Boer, *unpublished data*). In contrast, fatty acid analysis supported species status for each of the *C. michiganensis* subspecies (14). Regardless of the appropriate taxonomic status of these plant pathogens, sufficient differences occurred in the spacer region to design specific PCR primers for *Clavibacter michiganensis* subsp. *sepedonicus*. The two base pair differences at the 3′ end of the Sp1f primer and the one base pair difference plus one insertion near the 3′ end of the Sp5r primer were sufficient to prevent amplification of the other *C. michiganensis* subspecies (Fig. 1).

The primers selected for PCR amplification of *Clavibacter michiganensis* subsp. *sepedonicus* also failed to amplify species of the closely related *Rathayibacter* genus (Table 1). *Rathayibacter* spp. were once considered members of the *Clavibacter* genus because they contain 2,4-diaminobutyric acid and lack arabinose in their cell wall peptidoglycans but were later differentiated into a separate genus on the basis of cell wall composition, physiological characteristics, DNA base composition and DNA/DNA hybridization (25), and 16S rRNA homology (21). Strains of the other *Clavibacter* species, *C. xyli*, were not available to us, but sequence differences in the corresponding spacer region, as determined by M. Fegan and A. C. Hayward (*personal communication*), were greater than among the *C. michiganensis* subspecies. Furthermore, an exhaustive search of corynebacterial DNA sequences in GenBank (2) indicated that primers Sp1f and Sp5r would not hybridize with other more distantly related coryneform bacteria. In addition, the 50 unidentified isolates from potato, including 19 cross-reacting with antibodies to *Clavibacter michiganensis* subsp. *sepedonicus* and 31 saprophytic bacteria, were all negative in PCR, which further confirmed the specificity of the primers we developed (Table 1).

The advantage of a PCR detection test based on bacterial genomic sequences is that detection does not depend on the presence of a plasmid. Other PCR tests for *Clavibacter michiganensis* subsp. *sepedonicus* all utilized plasmid sequences for primers (23,13) and, therefore, failed to amplify strain P45, the strain known to lack a plasmid (19). However, one pair of primers derived from a plasmid of *Clavibacter michiganensis* subsp. *sepedonicus* successfully amplified strain P45, raising a question about the absence of plasmid sequences in strain P45 (20). Moreover, these primers also amplified a similar size DNA fragment for all strains of *Clavibacter michiganensis* subsp. *insidiosus* (20). Some DNA probes based on genomic sequences were not specific to the ring rot pathogen (18) because they targeted functional genes that are similar among the *C. michiganensis* subspecies (X. Li and S. H. De Boer, *unpublished data*). The primers selected in this study amplified all strains of *Clavibacter michiganensis* subsp. *sepedonicus* tested from several geographic areas (Table 1) and, hence, would be useful tools for indexing potatoes for the disease. In addition, mutation or loss of plasmid by *Clavibacter michiganensis* subsp. *sepedonicus* strains would not be a problem when these primers are used.

Detection sensitivity for *Clavibacter michiganensis* subsp. *sepedonicus* by PCR was similar and, in some cases, greater than for immunofluorescence and ELISA and was borne out by tests on asymptomatic potato tubers selected from ring rot-infected plants (Table 2). Several tubers from inoculated plants with negative results in ELISA and immunofluorescence at currently set thresholds yielded the characteristic amplification product when subjected to PCR. Control tubers from known healthy plants did not yield any amplification product, and this lends credence to the validity of the PCR results in the absence of alternative methods

TABLE 2. Comparison of polymerase chain reaction (PCR) with visual inspection, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF) techniques for detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tuber samples

Sample	Symptom rating[a]	ELISA[b]	IF[c]	PCR[d]
1	1	0.059	0.7	++
2	2	0.054	1.2	+
3	3	0.040	~40	+
4	1	0.047	~40	+
5	1	0.244	>50	+++
6	3	0.287	>50	+++
7	2	0.253	>50	+++
8	2	0.098	>50	+++
9	1	0.082	8.0	+++
10	2	0.172	~40	+++
11	2	0.261	>50	+++
12	2	0.275	>50	+++
13	1	0.103	7.0	+
14	2	0.052	8.0	+
15	1	0.040	9.0	+
16	4	0.272	>50	+++
17	3	0.097	7.0	++
18	3	0.198	>50	+++
19	1	0.080	6.0	++
20	3	0.318	>50	+++
21	1	0.093	1.8	+
22	1	0.066	1.0	+
23	1	0.063	0.6	+
24	1	0.045	1.0	+
25-30[e]	1	0.067	0	–
93-1[f]	3	0.059	0	–
93-2[f]	3	0.062	0	–
87-1[f]	4	0.327	>50	+++
87-2[f]	4	0.324	>50	+++
VF-1-12[g]	1	0.051	0	–

[a] Rating scheme 1, no symptom; 2, slight symptom; 3, obvious symptom; 4, rot.
[b] Mean of duplicate readings at 405 nm. Threshold: ≥0.1, positive.
[c] Average number of fluorescing cells per microscope field, based on 30 fields. Threshold: ≥5, positive.
[d] The intensity of the 215-bp PCR product in ethidium bromide-stained 2% agarose gel: +, weak band; ++, moderate band; +++, strong band; –, no band.
[e] Six healthy potato tubers uninoculated from control plants.
[f] Samples from commercial producers. Sample 87 showed typical ring rot symptoms, whereas sample 93 showed atypical soft decay.
[g] Twelve samples from the virus-free potato collection at the Pacific Agriculture Research Centre, Vancouver, BC, Canada.

for confirming the presence of *Clavibacter michiganensis* subsp. *sepedonicus* in the serologically negative tubers.

The procedure we used to prepare DNA for PCR amplification, avoiding the use of organic solvents for separating DNA from cell debris, was relatively easy to use on a large number of samples. Successful amplification of DNA extracted from various tubers with and without extensive decay indicated wide applicability of the test. Similarity of results with different thermocyclers also attested to the robustness of the procedure. Nevertheless, it is not expected that PCR will replace serological tests in routine potato indexing because of the considerably greater amount of time and effort involved in preparing samples for PCR compared to serology. It is not clear at this time if the additional sensitivity of PCR is necessary for seed indexing in ring rot eradication programs. PCR is expected to be useful, however, for confirming the presence of *Clavibacter michiganensis* subsp. *sepedonicus* in seed lots with questionable serological results. Furthermore, PCR will be a useful tool in research to elucidate the potential of *Clavibacter michiganensis* subsp. *sepedonicus* to persist in agricultural soils outside potato plant tissue.

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