

Nested PCR for Ultrasensitive Detection of the Potato Ring Rot Bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*

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Oligonucleotide primers derived from sequences of the 16S rRNA gene (CMR16F1, CMR16R1, CMR16F2, and CMR16R2) and insertion element IS1121 of *Clavibacter michiganensis* subsp. *sepedonicus* (CMSIF1, CMSIR1, CMSIF2, and CMISR2) were used in nested PCR to detect the potato ring rot bacterium *C. michiganensis* subsp. *sepedonicus*. Nested PCR with primer pair CMSIF1-CMSIR1 followed by primer pair CMSIF2-CMSIR2 specifically detected *C. michiganensis* subsp. *sepedonicus*, while nested PCR with CMR16F1-CMR16R1 followed by CMR16F2-CMR16R2 detected *C. michiganensis* subsp. *sepedonicus* and the other *C. michiganensis* subspecies. In the latter case, *C. michiganensis* subsp. *sepedonicus* can be differentiated from the other subspecies by restriction fragment length polymorphism (RFLP) analyses of the nested PCR products (16S rDNA sequences). The nested PCR assays developed in this work allow ultrasensitive detection of very low titers of *C. michiganensis* subsp. *sepedonicus* which may be present in symptomless potato plants or tubers and which cannot be readily detected by direct PCR (single PCR amplification). RFLP analysis of PCR products provides for an unambiguous confirmation of the identity of *C. michiganensis* subsp. *sepedonicus*.

Phytopathogenic coryneform bacteria have been recently classified into two genera, *Clavibacter* and *Rathayibacter* (3, 5, 30). Some species and subspecies in these genera cause serious diseases in various plant species (5). Potato ring rot disease of potato, caused by *Clavibacter michiganensis* subsp. *sepedonicus*, is one such serious disease. Because potato ring rot has been assigned a zero tolerance level for both import and export of seed potatoes in Canada, the United States, and countries in the European Community (20–22, 27, 29), an ability to detect very low titers of *C. michiganensis* subsp. *sepedonicus* in seed potatoes is indispensable for disease control.

Enzyme-linked immunosorbent assay and immunofluorescence based on monoclonal antibodies have been used widely in potato indexing for detection of bacterial ring rot in commercial seed potatoes (6–8, 10, 12). Recently, DNA-based techniques involving DNA probes in DNA hybridization or involving primers based on cloned DNA sequences in PCR have been developed to specifically detect the ring rot bacterium (4, 10, 11, 13, 18, 20, 23, 25, 29). PCR with primers based on the sequences of randomly cloned DNA probes or of plasmid DNA probes or from the 16S-23S rRNA spacer region is more sensitive and more specific than serological procedures and DNA hybridization assays (10, 23, 29).

Due to a zero tolerance level of potato ring rot and the possible presence of reaction inhibitors in tissue extracts (24, 28), a more sensitive PCR assay is needed to increase the sensitivity of detection. The present study was undertaken to develop nested PCR assays with primers designed based on the highly repeated segment of DNA, the insertion element IS1121, present in the chromosome of *C. michiganensis* subsp. *sepedonicus* (4, 19, 20) and based on 16S rDNA sequences for an ultrasensitive detection of the potato ring rot bacterium.

(Preliminary reports on these studies were presented previously [14, 15].)

MATERIALS AND METHODS

Bacterial strains. The species and subspecies of *Clavibacter* and *Rathayibacter* and other prokaryotes used in this study and their sources of origin are shown in Table 1.

Primer pairs and PCR conditions. The primer pairs CMR16F1-CMR16R1 and CMR16F2-CMR16R2 were designed based on unique 16S rDNA sequences commonly present in all subspecies of *C. michiganensis*. The primer pairs CMSIF1-CMSIR1 and CMSIF2-CMSIR2 were designed based on the sequence of the 1.3-kb insertion element IS1121, a highly repeated segment of DNA that is present in plasmid pCS1 and in the chromosome of *C. michiganensis* subsp. *sepedonicus* (4, 19). For PCR amplification, 35 cycles were conducted in an automated thermocycler (Hybaid, Teddington, United Kingdom) with AmpliTaq or AmpliTaq Gold polymerase. PCR were performed as described previously (26) in mixtures containing each deoxynucleoside triphosphate at a concentration of 200 μ M and each primer at a concentration of 0.4 to 1.0 μ M (CMI series). The following parameters were used: denaturation at 94°C for a 1 min (2 min with AmpliTaq or 12 min with AmpliTaq Gold for the first cycle), annealing for 2 min at 62°C, and primer extension for 3 min (10 min in the final cycle) at 72°C. Formamide (final concentration, 3%) was added to the reaction mixture when the primer pairs CMSIF1-CMSIR1 and CMSIF2-CMSIR2 were used. The oligonucleotide sequences of the eight primers and base locations are as follows: CMSIF1, 5'-tgt act cgg cca tga cgt tgg-3' (bases 6 to 26 of RS sequence [4]); CMSIR1, 5'-tac tgg gtc atg acg ttg gt-3' (bases 1052 to 1071); CMSIF2, 5'-tcc cac ggt aat gct cgt ctg-3' (bases 105 to 125); CMSIR2, 5'-gat gaa ggg gtc aag ctg gtc-3' (bases 969 to 989); CMR16F1, 5'-gtg atg tca gag ctt gct ctg geg gat c-3' (bases 68 to 95, according to the numbering system in *Escherichia coli* [2]); CMR16R1, 5'-gta cgg cta cct ttg tac gac tta gt-3' (bases 1489 to 1514); CMR16F2, 5'-ccc cga ctc tgg gat aac tgc ta-3' (bases 136 to 158); and CMR16R2, 5'-cgg tta ggc cac tgg ctt cgg gtg tta ccg a-3' (bases 1420 to 1450).

The PCR products (5 to 10 μ l) were analyzed by electrophoresis on a 1% agarose gel followed by staining with ethidium bromide and visualization of the DNA bands with a UV transilluminator.

Restriction fragment length polymorphism (RFLP) analyses of PCR products. The CBR16F2-CBR16R2 PCR products, 16S rDNA sequences, amplified from five subspecies of *C. michiganensis* were analyzed by restriction endonuclease digestions. Between 3 and 5 μ l (100 to 200 ng of DNA) of each PCR product was digested separately with two restriction enzymes, *Hae*III and *Hha*I (GIBCO BRL, Gaithersburg, Md.). The restriction products were then separated by electrophoresis on a 5% polyacrylamide gel and stained with ethidium bromide (16).

DNA extraction and nested PCR assay. Strains of *Clavibacter* and *Rathayibacter* were grown in RSD broth (5). A 5-ml volume of each culture was centrifuged, and the pellet was resuspended for 1 h at room temperature in TEN buffer (10 mM Tris, 6 mM NaCl, 1 mM EDTA [pH 8.0]) containing 4 mg of lysozyme per ml. The extraction then proceeded according to the procedure for preparation of genomic DNA from bacteria as described by Ausubel et al. (1). The bacterial nucleic acid was resuspended in 50 μ l of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). A 1- μ l volume of diluted (1:30) nucleic acid preparations was used for

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TABLE 1. Species and subspecies of *Clavibacter* and *Rathayibacter* and other prokaryotes tested in PCR amplification with primer pairs designed based on sequences from 16S rRNA gene and repeated segment of chromosomal DNA of *C. michiganensis* subsp. *sepedonicus*

Bacterium ^b	Abbreviation ^b	Source	Geographic location	Detection by PCR amplification with:			
				CMR16F1-CMR16R1 (direct)	CMR16F2-CMR16R2 (nested ^d)	CMSIF1-CMSIR1 (direct)	CMSIF2-CMSIR2 (nested ^d)
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Cms						
AS1		B. Mogen	United States	+	+	+	+
P45		B. Mogen	Unknown	+	+	+	+
3R		B. Mogen	Canada	+	+	+	+
106		B. Mogen	United States	+	+	+	+
33113		ATCC ^c	Canada	+	+	+	+
<i>C. michiganensis</i> subsp. <i>insidiosus</i>	Cmi						
239		B. Mogen	United States	+	+	+	-
10253		ATCC	United States	+	+	ND ^d	ND
<i>C. michiganensis</i> subsp. <i>michiganensis</i>	Cmm						
492		ATCC	United States	+	+	-	-
7429		ATCC	United States	+	+	ND	ND
<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Cmm						
27822		ATCC	United States	+	+	-	-
27794		ATCC	United States	+	+	ND	ND
<i>C. michiganensis</i> subsp. <i>tessellarius</i>	Cmt						
33566		ATCC	United States	+	+	-	-
<i>C. xyli</i> subsp. <i>cynodontis</i>	Cxc						
FB1A		M. Davis	China	-	-	ND	ND
TB1A		M. Davis	China	-	-	ND	ND
33973		ATCC	China	-	-	-	-
<i>C. xyli</i> subsp. <i>xyli</i>	Cxx						
F1A		M. Davis	United States	-	-	ND	ND
L1A		M. Davis	United States	-	-	ND	ND
<i>Rathayibacter rathayi</i>	Rr						
13659		ATCC	United Kingdom	-	-	-	-
19379		ATCC	United Kingdom	-	-	ND	ND
<i>Rathayibacter tritici</i>	Rt						
11402		ATCC	India	-	-	-	-
11403		ATCC	Egypt	-	-	ND	ND
<i>Rathayibacter iranicus</i>	Ri	M. Davis	Iran	-	-	ND	ND
<i>Acholeplasma laidlawii</i>	Al	D. T. Kingsbury	United States	-	-	-	-
<i>Mycoplasma gallisepticum</i>	Mg	D. T. Kingsbury	United States	-	-	-	-
<i>Mycoplasma capricolum</i>	Mc	D. T. Kingsbury	United States	-	-	-	-
<i>Pseudomonas syringae</i>	Ps	C.-H. Liao	United States	-	-	-	-
<i>Erwinia carotovora</i>	Ec	C.-H. Liao	United States	-	-	-	-
<i>Xanthomonas campestris</i>	Xc	C.-H. Liao	United States	-	-	-	-
<i>Agrobacterium tumefaciens</i>	At	C.-H. Liao	United States	-	-	-	-
<i>Spiroplasma citri</i>	Sc	I.-M. Lee	United States	-	-	-	-
<i>Bacillus cereus</i>	Bc	D. T. Kingsbury	United States	-	-	-	-
<i>Streptococcus pneumoniae</i>	Sp	D. T. Kingsbury	United States	-	-	-	-
Phytoplasma AY1 ^e		R. E. Davis	United States	-	-	-	-
Phytoplasma CX ^e		L. N. Chiykowski	Canada	-	-	-	-

^a Nested PCR was performed with CMR16F1-CMR16R1 followed by CMR16F2-CMR16R2 and with CMSIF1-CMSIR1 followed by CMSIF2-CMSIR2-, respectively.

^b Abbreviations are those used in Fig. 1 to 3.

^c ATCC, American Type Culture Collection.

^d ND, not done.

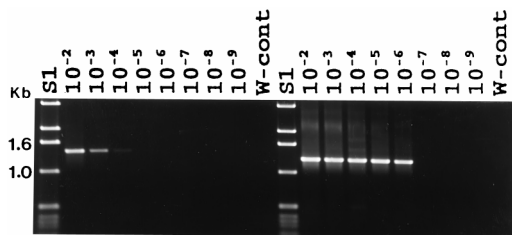


FIG. 4. Direct and nested PCR amplification of a 16S rDNA sequence from various titers of *C. michiganensis* subsp. *sepedonicus* in potato tissue. Direct PCR amplification was performed with CMR16F1-CMR16R1; nested PCR amplification was performed with CMR16F1-CMR16R1 followed by CMR16F2-CMR16R2. Lane S1 contains a 1-kb DNA ladder; lane W-cont. contains the water control.

these pathogens. The potato ring rot bacterium, *C. michiganensis* subsp. *sepedonicus*, could be differentiated from other subspecies of *C. michiganensis* by RFLP analysis (with restriction enzymes *Hae*III and *Hha*I) of the nested PCR products. For example, *C. michiganensis* subsp. *sepedonicus* has RFLP patterns distinct from those of the other four subspecies, *C. michiganensis* subsp. *michiganensis*, *nebraskensis*, *tessellarius*, and *insidiosus* (Fig. 3). Furthermore, since no subspecies other than *C. michiganensis* subsp. *sepedonicus* was reported to infect potato, this set of primer pairs is as good as the *C. michiganensis* subsp. *sepedonicus*-specific primers for detection of *C. michiganensis* subsp. *sepedonicus* in potato tissue.

The two primer pairs CMR16F1-CMR16R1 and CMR16F2-CMR16R2 were used in nested PCR to detect *C. michiganensis* subsp. *sepedonicus* in potato tissues. Under our experimental conditions, nested PCR detected (visible DNA bands on agarose gel) titers as low as 10^{-6} dilution (about 100 bacteria/reaction mixture) whereas direct PCR did not readily detect *C. michiganensis* subsp. *sepedonicus* when its titers in the potato tissue were below 10^{-4} dilution (Fig. 4). Taking into consideration the dilution factor (100-fold) of template DNA prepared

for nested PCR, we expect that nested PCR can detect fewer than 100 bacteria/reaction mixture. Compared to direct PCR assays, the sensitivity of detection increased 100- to 1,000-fold in nested PCR. The results underscore the necessity to apply more sensitive means such as nested PCR in screening for the potato ring rot pathogen in commercial seed potatoes. Direct (single-amplification) PCR is sensitive enough to detect as few as 5 to 50 CFU of *C. michiganensis* subsp. *sepedonicus* (pure culture) per 20- μ l PCR mixture (29), but the sensitivity of detecting *C. michiganensis* subsp. *sepedonicus* in potato tissues by direct PCR may decrease because of the presence of potential reaction inhibitors in potato tissue extracts. Due to a zero tolerance policy for potato ring rot pathogen in potato, a very sensitive means is needed to detect very low titers of *C. michiganensis* subsp. *sepedonicus* in commercial seed potatoes.

The results of PCR assays for the detection of *C. michiganensis* subsp. *sepedonicus* in commercial potato tubers or tubers obtained from experimental field plots are shown in Fig. 5 and summarized in Table 2. Nested PCR was needed to determine the presence of *C. michiganensis* subsp. *sepedonicus* in more than 40% of the symptomless tubers tested, consistent with a low titer of the bacterium in these tubers. In PCRs with either set of nested PCR primers, all duplicate or triplicate reaction mixtures yielded positive *C. michiganensis* subsp. *sepedonicus* detection in tests of symptomatic tubers. *C. michiganensis* subsp. *sepedonicus* DNA was detected in all duplicate or triplicate reaction mixtures for most symptomless tubers. RFLP analyses confirmed that only *C. michiganensis* subsp. *sepedonicus* was detected in all samples listed in Table 2 (data not shown).

The results also reflected a difference in the efficiency of *C. michiganensis* subsp. *sepedonicus* detection depending on the primers used in nested PCR. For example, *C. michiganensis* subsp. *sepedonicus* was detected in 14 of 17 symptomless tubers when nested PCRs were primed with CMSIF1-CMSIR1 followed by CMSIF2-CMSIR2 but in only 11 of the 17 symptomless tubers when nested PCRs were primed with CMR16F1-

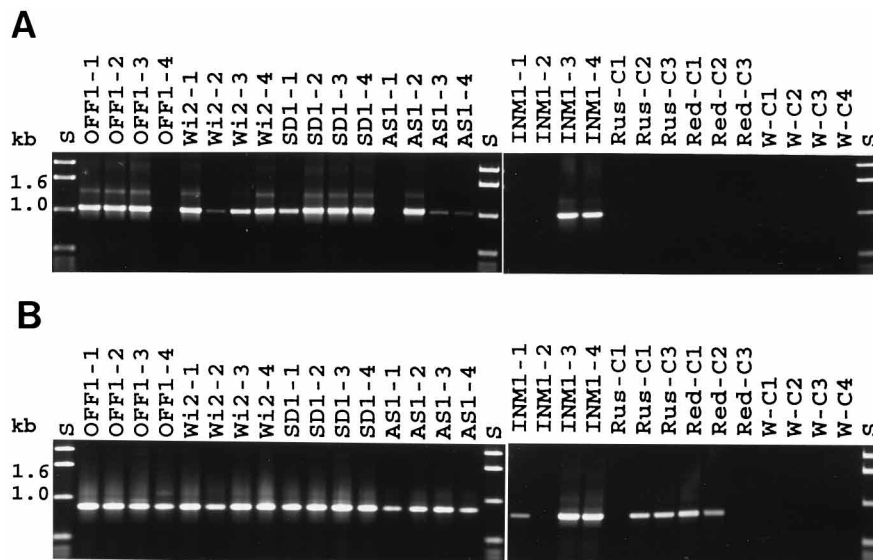


FIG. 5. Direct and nested PCR amplification of the 16S rDNA sequence of *C. michiganensis* subsp. *sepedonicus* in nucleic acid extracts prepared from potato tubers collected from experimental plots artificially inoculated with various strains of *C. michiganensis* subsp. *sepedonicus*. (A) Direct PCR with primer pair CMSIF1-CMSIR1; (B) nested PCR with CMSIF1-CMSIR1 followed by CMSIF2-CMSIR2. Lane S contains 1-kb DNA ladder; lanes Rus-C1 to Rus-C3 and Red-C1 to Red-C3 contain symptomless samples purchased from a local supermarket in Maryland; lanes W-C1 to W-C4 contain water controls. *C. michiganensis* subsp. *sepedonicus* strains: mucooid, OFF-1 and AS1; nonmucooid, SD1; intermediate, INM1 and Wi2.

TABLE 2. Results of direct and nested PCR assays for detection of *C. michiganensis* subsp. *sepedonicus* in field-collected potato tubers

Sample ^a	Cultivar	Symptom rating ^b	Bacterial detection by ^c :			
			Direct PCR		Nested PCR	
			I	II	I	II
1 (OFF1-1)	Russet Burbank	0	+	+	+	+
2 (OFF1-2)	Russet Burbank	3	+	+	+	+
3 (OFF1-3)	Russet Burbank	2	+	+	+	+
4 (OFF1-4)	Russet Burbank	0	-	-	+	+
5 (Wi2-1)	Russet Burbank	1	+	+	+	+
6 (Wi2-2)	Russet Burbank	0	+	-	+	+
7 (Wi2-3)	Russet Burbank	0	+	+	+	+
8 (Wi2-4)	Russet Burbank	0	+	+	+	+
9 (SD1-1)	Russet Burbank	0	+	+	+	+
10 (SD1-2)	Russet Burbank	2	+	+	+	+
11 (SD1-3)	Russet Burbank	1	+	+	+	+
12 (SD1-4)	Russet Burbank	1	+	+	+	+
13 (AS1-1)	Norchip	1	-	-	+	+ ^d
14 (AS1-2)	Norchip	0	+	+	+	+
15 (AS1-3)	Norchip	0	+	-	+	+
16 (AS1-4)	Norchip	0	+	+	+	+
17 (INM1-1)	Russet Burbank	0	-	-	+ ^d	-
18 (INM1-2)	Russet Burbank	0	-	-	-	+ ^d
19 (INM1-3)	Russet Burbank	2	+	+	+	+
20 (INM1-4)	Russet Burbank	1	+	+	+	+
21 (Rus-C1)	Russet Burbank	0	-	-	-	-
22 (Rus-C2)	Russet Burbank	0	-	-	+	-
23 (Rus-C3)	Russet Burbank	0	-	-	+ ^d	-
24 (Red-C1)	Red	0	-	-	+	+
25 (Red-C2)	Red	0	-	-	+ ^d	-
26 (Red-C3)	Red	0	-	-	-	-

^a Samples 1 to 20 were harvested from a potato field experimentally inoculated with various strains of *C. michiganensis* subsp. *sepedonicus*. OFF1 and AS1 are mucoid strains; INM1 and Wi2 are intermediate strains; and SD1 is a nonmucoid strain. Samples 21 to 26 were purchased from a local supermarket.

^b Symptom rating: 0, symptomless; 1, slightly brownish discoloration of vascular ring; 2, obvious discoloration; 3, severe symptom with canker rot.

^c I, primer pair CMSIF1-CMSIR1 for direct PCR; CMSIF1-CMSIR1 followed by CMSIF2-CMSIR2 for nested PCR. II, primer pair CMR16F1-CMR16R1 for direct PCR; CMR16F1-CMR16R1 followed by CMR16F2-CMR16R2 for nested-PCR.

^d Samples in which *C. michiganensis* subsp. *sepedonicus* could not be readily detected in every duplicate test. The frequency of detection ranged from 50 to 75% for all duplicate tests.

CMR16R1 followed by CMR16F2-CMR16R2. Presumably, the higher frequency of *C. michiganensis* subsp. *sepedonicus* detection in the PCRs with CMSI primers was due to the greater abundance of template sequences for these primers, since the CMSI primers were designed on the basis of the nucleotide sequence of insert element IS1121, a highly repeated (up to 50 repeats) sequence in the chromosome of *C. michiganensis* subsp. *sepedonicus* (4, 19).

It is also noteworthy that *C. michiganensis* subsp. *sepedonicus* was detected in only one of two duplicate reaction mixtures for three tubers (tubers 17, 23, and 25) when the CMSI primers were used and for two tubers (tubers 13 and 18) when the CMR16 primers were used (Table 2). Although the use of higher template DNA concentrations in PCRs for *C. michiganensis* subsp. *sepedonicus* detection would conceivably reduce the number of negative assays, such a step was not carried out because it would potentially increase the concentration of PCR inhibitors in the reaction mixture. Because of the inconsistency, we carried out an experiment in which four reaction mixtures were prepared for each of the five symptomless tubers. The frequency of positive assays ranged from 50 to 75%

for these five samples. The experiment included two other symptomless tubers that consistently tested negative in previous experiments and eight water blanks (PCR control mixtures devoid of added template DNA), none of which yielded the PCR product, indicating that positive assays were genuine and were not due to cross-contaminations with template. Based on dilution factors in the preparation of template DNA used in the test reactions, low levels (as low as 1.5×10^3 organisms per g of tuber tissue) could be detected when duplicate assays were carried out for a given sample. Since a single PCR mixture could fail to detect the presence of *C. michiganensis* subsp. *sepedonicus* at such low levels in tubers, the results underscore the need for duplicate or triplicate assays of samples suspected to contain low titers of the target template.

The nested PCR assays developed in this study provided a means for ultrasensitive detection of the potato ring rot bacterium, *C. michiganensis* subsp. *sepedonicus*, at very low titers in tubers. These assays should facilitate the screening of imported germplasm for the presence of the pathogen and aid the production of *C. michiganensis* subsp. *sepedonicus*-free commercial seed potato stock. The use of pathogen-free seed potato in commercial production could be the most efficient avenue to achieve the goal of zero tolerance.

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