

## Detection of *Ralstonia solanacearum* Strains with a Quantitative, Multiplex, Real-Time, Fluorogenic PCR (TaqMan) Assay

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**A fluorogenic (TaqMan) PCR assay was developed to detect *Ralstonia solanacearum* strains. Two fluorogenic probes were utilized in a multiplex reaction; one broad-range probe (RS) detected all biovars of *R. solanacearum*, and a second more specific probe (B2) detected only biovar 2A. Amplification of the target was measured by the 5' nuclease activity of *Taq* DNA polymerase on each probe, resulting in emission of fluorescence. TaqMan PCR was performed with DNA extracted from 42 *R. solanacearum* and genetically or serologically related strains to demonstrate the specificity of the assay. In pure cultures, detection of *R. solanacearum* to  $\geq 10^2$  cells ml<sup>-1</sup> was achieved. Sensitivity decreased when TaqMan PCR was performed with inoculated potato tissue extracts, prepared by currently recommended extraction procedures. A third fluorogenic probe (COX), designed with the potato cytochrome oxidase gene sequence, was also developed for use as an internal PCR control and was shown to detect potato DNA in an RS-COX multiplex TaqMan PCR with infected potato tissue. The specificity and sensitivity of the assay, combined with high speed, robustness, reliability, and the possibility of automating the technique, offer potential advantages in routine indexing of potato tubers and other plant material for the presence of *R. solanacearum*.**

*Ralstonia solanacearum* (Smith) (30) is the agent of bacterial wilt, infecting over 450 plant species, including many economically important crops (12). This species has been subclassified into biovars based on biochemical tests and host-dependent races. Biovar 2A (equivalent to race 3) is adapted to temperate climates, has a narrow host range, and is responsible for recent outbreaks of potato brown rot disease in several countries of Western Europe and elsewhere worldwide (13, 27). Although other biovars can also infect potatoes, biovar 2A is the most destructive phenotype in temperate areas.

*R. solanacearum* is listed as a quarantine organism in the European Union (EU) (2), where new legislation has been introduced to control and eradicate the organism (3). Latent infections in seed potato tubers (6) have led to the spread of the organism, both locally and internationally, and effective control of brown rot is dependent on the reliability of detection of the pathogen at this latent stage. For practical purposes, a detection assay is required which is rapid, specific, and sensitive to levels lower than those occurring in naturally infected potatoes and should be applicable to a crude sample of the specimen of interest (25). Serological techniques such as immunofluorescence (IF) microscopy, the enzyme-linked immunosorbent assay (ELISA) (10, 15, 21), and molecular techniques involving the PCR (9, 24) have been described for detection of *R. solanacearum*. An EU control directive (3) allows for a variety of detection methods to be employed. Briefly, a primary screening test (i.e., IF and/or selective isolation) is conducted with extracts from vascular tissue sampled from 200 tubers per 25-tonne lot. To confirm the presence of the pathogen, presumptive cultures isolated on a semiselective medium (8) are identified (e.g., by fatty acid profiling) (16, 26), and pathogenicity is confirmed by a host test on tomato seedlings (15). This procedure is labor intensive and time-consuming. In addition, the primary screening techniques are not

completely reliable, despite recent attempts to improve the specificity and sensitivity of *R. solanacearum* detection (8, 10, 29).

Fluorogenic PCR-based (TaqMan) assays have recently shown promise in the detection of a variety of organisms, including clinical bacteria (4, 5, 19) and plant pathogenic potato leaf roll virus (23). In addition, a TaqMan assay for detection of the potato ring rot bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*, has also been reported (22). TaqMan PCR exploits the 5' nuclease activity of *Taq* DNA polymerase (14) in conjunction with fluorogenic DNA probes (18). Each probe, designed to hybridize specifically to the target PCR product, is labelled with a fluorescent reporter dye and a quencher dye. During PCR amplification, the probe is digested by *Taq* DNA polymerase, separating the dyes, resulting in an increase in reporter fluorescence. Repeated PCR cycles result in exponential amplification of the PCR product and a corresponding increase in fluorescence intensity.

Because the TaqMan amplicon is generally between 60 and 70 bp, the reaction is also more efficient than a standard PCR in which target PCR products are required to be at least 200 bp in length to permit detection by electrophoretic separation techniques. This increase in efficiency and incorporation of a sequence-specific probe enhance sensitivity and specificity. Fluorescence can be measured throughout the PCR, providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets. The measurement of fluorescence throughout the reaction by a fluorometer (ABI Prism 7700 Sequence Detection system; PE Biosystems, Foster City, Calif.) eliminates the need for post-PCR processing steps, such as gel electrophoresis and ethidium bromide staining of target DNA, easing automation of the technique and large-scale sample processing. Thus, there is reduced potential for contamination of the PCR mixture with target DNA because the reaction tubes remain closed throughout the assay. Interpretation of the fluorometric data can be presented as a simple qualitative conclusion as to the presence or absence of amplified DNA within minutes of the end of the PCR. Alternatively, real-time analysis can facilitate quantification of the amount of

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sample DNA present in the reaction by ascertaining when (i.e., during which PCR cycle) fluorescence in a given reaction tube exceeds that of a threshold (threshold cycle [ $C_T$ ]). Comparison between reaction tubes and/or known standards can quantify the amount of DNA template present in a given tube.

This paper reports the development of a fluorogenic PCR-based assay which uses a probe-primer set (RS) to detect all known strains of *R. solanacearum* and another set (B2) specific for the biovar 2A genotype. A multiplex (RS-B2) reaction is described in which each probe is labelled with a different reporter dye, thus providing a single tube assay for both tests. The sensitivity of the assay and the effect of potential TaqMan PCR inhibitors present in potato tissue are also quantified. Finally, we report the detection of *R. solanacearum* in infected potato tissue by using a multiplex reaction which incorporates a third probe-primer set (COX) designed to detect the potato cytochrome oxidase gene sequence as an internal control for the TaqMan reaction.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *R. solanacearum* strains evaluated are listed in Table 1. Bacterial strains related to *R. solanacearum* or having previously cross-reacted with polyclonal antisera to *R. solanacearum* in IF or ELISA were also evaluated and are also listed in Table 1. Strains were grown (24 h at 27°C) on Casamino Acids-Peptone-glucose (CPG) agar (17). A single colony was then transferred to 100  $\mu$ l of sterile nucleic acid-free water, vortexed, heated to >96°C for 4 min, and placed on ice. Samples were finally diluted in 900  $\mu$ l of the sterile water and stored at -80°C until required. Previous work has indicated that this is an effective method for extracting DNA from *Ralstonia* spp. (8, 24).

**TaqMan probe design.** The sequences of the primers and TaqMan probes used are given in Table 2. The probes and primers were designed with Primer Express version 1.0 software (PE Biosystems, Foster City, Calif.) by adapting previous *R. solanacearum* PCR protocols. The broad-host-range *R. solanacearum* probe (RS-P) is partially homologous to 16S rRNA gene primer OLI1 (24), with primers (RS-I and RS-II) flanking this region. The primers and TaqMan probe for the biovar 2A-specific assay were elucidated from the biovar 2A-specific DNA sequence described by Fegan et al. (9), retaining the original forward primer 631 (B2-II) and selecting the probe (B2-P) and reverse primer (B2-I) from the upstream region. The internal positive control primer-probe combination was designed according to the published sequence (20) of the abundant constitutive cytochrome oxidase (COX) gene. RS-P was covalently labelled at the 5'-terminal nucleotide with the FAM (6-carboxyfluorescein) reporter dye and at the 3'-terminal nucleotide with the TAMRA (tetra-methylcarboxyrhodamine) quencher dye. B2-P and COX-P were labelled with the VIC (PE Biosystems) reporter dye at the 5'-terminal nucleotide and again with the TAMRA quencher dye at the 3'-terminal nucleotide. TaqMan probes were synthesized by PE Biosystems.

**5' Nuclease PCR assay.** The 5' nuclease PCR with a fluorogenic probe is run under generic cycling conditions, and so requires the optimization of primer concentration to take into account real differences in primer melting temperature. Different forward and reverse primer concentrations for each probe were evaluated, to ascertain the effect on  $C_T$  and endpoint fluorescence values. For each probe, a primer concentration of 300 nM was found to be most efficient, giving a high endpoint fluorescence and low  $C_T$  (data not shown). This primer concentration was used, in an RS-B2 multiplex reaction, to test *R. solanacearum* strains and related species.

PCR was performed in 25- $\mu$ l volumes using MicroAmp Optical 96-well reaction plates and MicroAmp Optical Caps (PE Biosystems) for each well. All reagents were obtained from the TaqMan Core PCR Reagent kit (PE Biosystems). The PCR mixture for the RS and B2 multiplex was as follows: 2.0  $\mu$ l of cell lysate; 2.5  $\mu$ l of 10 $\times$  TaqMan buffer A; 200 nM (each) dATP, dCTP, and dGTP; 400 nM dUTP; 0.025 U of AmpliTaq Gold DNA polymerase per ml; 0.01 U of uracil-N-glycosylase per ml (AmpErase UNG); 300 nM (each) primers RS-I, RS-II, B2-I, and B2-II; 25 nM TaqMan probe RS-P; and 50 nM TaqMan probe B2-P. The RS-COX multiplex PCR mixture was as follows: 2.0  $\mu$ l of infected tissue lysate; 2.5  $\mu$ l of 10 $\times$  TaqMan buffer A; 200 nM (each) dATP, dCTP, and dGTP; 400 nM dUTP; 0.025 U of AmpliTaq Gold DNA polymerase per ml; 0.01 U of uracil-N-glycosylase (AmpErase UNG) per ml; 150 nM (each) primers RS-I and RS-II; 100 nM (each) primers COX-F and COX-R; 25 nM TaqMan probe RS-P; and 50 nM TaqMan probe COX-P. An ABI Prism 7700 Sequence Detection system (PE Biosystems) was used for amplification and fluorescence measurement. All cycles began with 2 min at 50°C and then went to 10 min at 95°C, followed by 40 two-step cycles of 10 s at 95°C and then 1 min at 60°C.

**Post-PCR analysis.** The fluorescent intensities of each dye were measured by the ABI Prism 7700 Sequence detection system (PE Biosystems) at every temperature step and cycle during the reaction. Data acquisition and analysis were handled by Sequence Detector version 1.6 software (PE Biosystems). Briefly a

normalized reporter ( $R_n$ ) value is defined for each reaction tube and  $\Delta R_n$ , an indication of the magnitude of signal generated by the PCR, is calculated. The  $C_T$  value is the first cycle at which a statistically significant increase in  $\Delta R_n$  is detected and is based on average standard deviation of  $R_n$  during the early cycles, where no fluorescence is observed. Samples with a  $\Delta R_n$  value exceeding this threshold (and greater than 0.3) were considered positive.

**Sensitivity of *R. solanacearum* TaqMan assays.** *R. solanacearum* biovar 2A isolate (CSL3468) was grown on CPG medium (24 h at 27°C) and suspended in 10 ml of sterile water ( $\sim 10^9$  cells ml $^{-1}$ ). A 10-fold dilution series was made from the suspension ( $10^9$  to  $10^0$ ), and 100  $\mu$ l of each dilution was heated above 96°C for 4 min. Total cell counts in each diluted suspension were estimated by indirect immunofluorescence microscopy with anti-*R. solanacearum* polyclonal antiserum IACR-278 and fluorescein isothiocyanate-conjugated antirabbit immunoglobulin G (Sigma F6005; Sigma-Aldrich Co., Ltd., Poole, Dorset, United Kingdom) according to the method of Janse (15). TaqMan PCR (RS and B2) was performed on the  $10^7$ -to- $10^0$  dilutions. TaqMan PCR assays (RS and B2) were performed individually on the same dilution series. Three separate dilution series were tested.

**Potato tissue extracts.** For the RS-COX multiplex reaction, a core of vascular tissue ( $\sim 0.1$  g) was aseptically removed from the stolon end of a naturally infected potato tuber (cv. Cara) and homogenized in 1 ml of 50 mM phosphate buffer (pH 7.0). One hundred microliters of the resulting suspension was heated above 96°C for 4 min and cooled rapidly on ice. To this sample, 900  $\mu$ l of sterile water was added to complete sample preparation. Ten-fold serial dilutions were made from this suspension.

The effect of inhibitors present in crude potato tissue extract was assessed by spiking a dilution series of *R. solanacearum* into extracts prepared in accordance with official testing methods (3). Briefly, a sample of 200 tuber stolon-end vascular tissue cores (cv. Cara) was macerated with 30 ml of 50 mM phosphate buffer (pH 7.0) in a sterile stomach bag, allowed to stand for 30 min, and centrifuged at 180  $\times$  g for 10 min. The resultant supernatant was decanted and transferred to a fresh centrifuge tube before a second centrifugation step at 10,000  $\times$  g for 10 min. The pellet from this second centrifugation step was resuspended in 1 ml of 10 mM phosphate buffer (pH 7.2). The resuspended pellet was then used to prepare a decimal dilution series of *R. solanacearum* from  $10^7$  to 1 cell ml $^{-1}$  in undiluted pellet or pellet diluted 1:10 and 1:100. A similar dilution series was also prepared in sterile nucleic acid-free water. Each of the samples was thoroughly mixed before being heated above 96°C for 4 min to lyse the cells.

#### RESULTS

**TaqMan probe design and specificity.** For all *R. solanacearum* strains,  $\Delta R_n$  values between 0.68 and 1.75 were obtained by RS-P, indicating successful amplification (Table 1). The B2-P 5' nuclease assay resulted in  $\Delta R_n$  values between 1.26 and 1.45 being obtained for all biovar 2A strains tested. With biovar 2T and biovar 1, 3, 4, and 5 strains, no fluorescence was detected during the B2-P assay, indicating that amplification had not occurred (Table 1). A range of closely related bacterial strains, including strains that had cross-reacted with *R. solanacearum* polyclonal antisera in IF or ELISA (J. Elphinstone, unpublished data), were also evaluated. Several of these strains produced fluorescence during the RS-P assay, namely the banana blood disease bacterium, *Ralstonia syzygii* strains, and one (from three tested) *Pseudomonas* sp. strain (Taxon B). No fluorescence was detected from the B2-P assay on any of these strains. No elevated  $\Delta R_n$  values were detected for any of the other reference bacteria from either assay.

**Sensitivity of TaqMan PCR.** The sensitivities of the individual TaqMan PCR assays were measured with a 10-fold dilution series of *R. solanacearum* biovar 2A (isolate CSL3468). Target DNA was detectable in suspensions containing as few as  $10^2$  cells ml $^{-1}$  (0.2 cells per reaction) when 300 nM F-300 nM R primer concentrations were used for both TaqMan probes.  $C_T$  values increased at each dilution, demonstrating the validity of the assay as the target DNA concentration decreased and showing that quantification of target DNA is possible. For the RS-P fluorogenic probe, quantification was shown to be linear from  $10^7$  to  $10^4$  cells ml $^{-1}$  for suspensions diluted in both water and 1:10-diluted potato extract (Fig. 1 and 2). Similar results were observed with the B2-P probe (data not shown).

**TaqMan PCR from potato extracts.** Inhibition of TaqMan by high concentrations of potato extracts in the reaction mix

TABLE 1. *R. solanacearum* strains and strains related to *R. solanacearum* or having previously cross-reacted with polyclonal antisera to *R. solanacearum* in IF or ELISA<sup>a</sup>

Species	Collection <sup>b</sup>	Biovar	Origin	Yr isolated	Host or source	RS		B2	
						Mean $\Delta R_n^c$	Result	Mean $\Delta R_n^d$	Result
<i>Ralstonia solanacearum</i>	NCPPB1824	2A	Egypt	1966	Potato	1.12	+	1.43	+
<i>Ralstonia solanacearum</i>	CSL465	2A	Egypt	1990	Potato	0.68	+	1.36	+
<i>Ralstonia solanacearum</i>	CSL1328	2A	Egypt	1994	Potato	1.05	+	1.45	+
<i>Ralstonia solanacearum</i>	CSL3467	2A	England	1997	River water	0.96	+	1.51	+
<i>Ralstonia solanacearum</i>	CSL3468	2A	England	1997	Tomato	0.92	+	1.43	+
<i>Ralstonia solanacearum</i>	NCPPB1584	2A	Cyprus	1963	Potato	1.05	+	1.26	+
<i>Ralstonia solanacearum</i>	NCPPB2505	2A	Sweden	1972	Potato	1.08	+	1.48	+
<i>Ralstonia solanacearum</i>	RVP502	2A	Belgium	1989	Potato	1.07	+	1.39	+
<i>Ralstonia solanacearum</i>	NCPPB 4069	2A	Slovenia	1997	Potato	0.91	+	1.44	+
<i>Ralstonia solanacearum</i>	CIP310	2A	Colombia	Unknown	Potato	0.99	+	1.44	+
<i>Ralstonia solanacearum</i>	NCPPB325	1	United States	1953	Tomato	0.97	+	0.01	-
<i>Ralstonia solanacearum</i>	CIP226	2T	Brazil	Unknown	Potato	0.85	+	0.01	-
<i>Ralstonia solanacearum</i>	CIP49	3	Australia	Unknown	Potato	0.99	+	0.01	-
<i>Ralstonia solanacearum</i>	R470	4	Phillipines	Unknown	Ginger	0.98	+	0.01	-
<i>Ralstonia solanacearum</i>	R288	5	China	Unknown	Mulberry	0.94	+	0.19	-
<i>Ralstonia pickettii</i>	NCPPB3839		Unknown	Unknown	Unknown	0.03	-	0.00	-
<i>Ralstonia pickettii</i>	NCPPB 4076		Unknown	Unknown	Unknown	0.05	-	0.00	-
<i>Ralstonia pickettii</i>	CSL3466		Unknown	Unknown	Unknown	0.05	-	0.00	-
<i>Ralstonia pickettii</i>	CFBP2459		Unknown	Unknown	Unknown	0.00	-	0.00	-
<i>Pseudomonas</i> sp.	NCPPB928		Jamaica	1959	Sugarcane	1.75	+	0.01	-
<i>Pseudomonas</i> sp.	NCPPB927		United States	1956	Sugarcane	0.00	-	0.00	-
<i>Pseudomonas</i> sp.	NCPPB1695		Tanzania	1965	Sugarcane	0.00	-	0.00	-
<i>Ralstonia</i> sp.	PD2778		Unknown	Unknown	Unknown	0.01	-	0.00	-
Banana blood disease bacterium	NCPPB3726		South Sulawesi	1988	Banana	1.96	+	0.00	-
<i>Ralstonia syzygii</i>	NCPPB3790		North Sumatra	Unknown	Clove	1.48	+	0.00	-
<i>Ralstonia syzygii</i>	NCPPB3791		West Java	Unknown	Clove	1.53	+	0.01	-
<i>Ralstonia syzygii</i>	NCPPB3792		Central Java	Unknown	Clove	1.56	+	0.01	-
<i>Burkholderia andropogonis</i>	NCPPB1127		Zimbabwe	1961	<i>Bougainvillea</i> sp.	0.01	-	0.00	-
<i>Burkholderia caryophylli</i>	NCPPB353		United States	1954	Unknown	0.02	-	0.00	-
<i>Burkholderia cepacia</i>	NCPPB945		Unknown	1961	Unknown	0.02	-	0.01	-
<i>Burkholderia cepacia</i>	CFBP2227		Unknown	Unknown	Unknown	0.00	-	0.00	-
<i>Burkholderia glumae</i>	NCPPB3708		Japan	1967	Rice	0.05	-	0.00	-
<i>Burkholderia plantarii</i>	NCPPB3590		Japan	1982	Rice	0.06	-	0.00	-
<i>Bacillus polymyxa</i>	CFBP1954		Unknown	Unknown	Unknown	0.13	-	0.00	-
<i>Pseudomonas marginalis</i> subsp. <i>marginalis</i>	CFBP1538		Unknown	Unknown	Unknown	0.06	-	0.00	-
<i>Pseudomonas chloraphis</i>	NCPPB 4082		Unknown	Unknown	Unknown	0.03	-	0.00	-
<i>Enterobacteriaceae</i>	NCPPB 4078		Unknown	Unknown	Unknown	0.03	-	0.00	-
<i>Ratinella aquatilis</i>	NCPPB 4079		Unknown	Unknown	Unknown	0.14	-	0.21	-
<i>Ochrobactrum anthropi</i>	NCPPB 4080		Unknown	Unknown	Unknown	0.07	-	0.00	-
Unknown	CSL3461		Unknown	Unknown	Unknown	0.05	-	0.24	-
Unknown	CSL3462		Unknown	Unknown	Unknown	0.02	-	0.00	-
Unknown	CSL3463		Unknown	Unknown	Unknown	0.07	-	0.00	-
Unknown	CSL3464		Unknown	Unknown	Unknown	0.03	-	0.00	-
Unknown	PD2775		Netherlands	Unknown	Unknown	0.06	-	0.00	-

<sup>a</sup> Strains were evaluated and mean  $\Delta R_n$  values were generated during PCR with RS-I and RS-II primers plus the RS-P fluorogenic probe and B2-I and B2-II primers plus the B2-P fluorogenic probe.

<sup>b</sup> NCPPB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, Sand Hutton, York, United Kingdom; CSL, plant pathogenic bacteria culture collection, Central Science Laboratory; RVP, Rijkstasjon voor Plantenziekten, Merelbeke, Belgium; CFBP, Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; R, culture collection IACR-Rothamsted, Harpenden, Hertfordshire, United Kingdom; CIP, culture collection, International Potato Center, Lima, Peru; PD, Plantenziektenkundige Dienst, Wageningen, The Netherlands.

<sup>c</sup> Concentrations: 300 nM F-300 nM R primer, 25 mM RS probe.

<sup>d</sup> Concentrations: 300 nM F-300 nM R primer, 50 mM B2 probe.

was recorded (Table 3). Detection of *R. solanacearum* by using the generic TaqMan (RS) probe occurred with a lower limit of detection of  $10^4$  cells  $ml^{-1}$  in water and in potato pellet diluted 1:100. Detection with the biovar 2A-specific probe was approximately 10 times less sensitive, with a lower limit of detection of  $10^5$  cells  $ml^{-1}$ . In those samples prepared with potato extract diluted 1:10, the minimum limits of detection were between  $10^4$  and  $10^5$  cells  $ml^{-1}$  with the *R. solanacearum* species-specific probe and  $10^6$  cells  $ml^{-1}$  with the biovar 2A-specific probe.

Both probes were strongly inhibited by undiluted potato extract, with no positive reactions even at a cell concentration of  $10^7$   $ml^{-1}$ . TaqMan PCR was also completely inhibited in a set of samples prepared by lysing cells in 50 mM NaOH solution.

Conventional PCR was performed on the same spiked samples with the primers OLI-1 and Y-2 and the protocols of Seal et al. (24). With the samples prepared in water, a lower limit of detection of  $10^3$  cells  $ml^{-1}$  was recorded. Samples prepared with undiluted potato extract and potato extract diluted 1:10

TABLE 2. Characteristics of primers and TaqMan probes used to detect *R. solanacearum* and potato cytochrome oxidase DNA

Primer or probe <sup>a</sup>	Sequence (5'→3')	Length (nt)	Dye
RS-I-F	GCA TGC CTT ACA CAT GCA AGT C	22	
RS-II-R	GGC ACG TTC CGA TGT ATT ACT CA	23	
RS-P <sup>b</sup>	AGC TTG CTA CCT GCC GGC GAG TG	23	FAM
B2-I-F	TGG CGC ACT GCA CTC AAC	18	
B2-II <sup>c</sup> -R	AAT CAC ATG CAA TTC GCC TAC G	22	
B2-P	TTC AAG CCG AAC ACC TGC TGC AAG	24	VIC
COX-F	CGT CGC ATT CCA GAT TAT CCA	21	
COX-R	CAA CTA CGG ATA TAT AAG AGC CAA AAC TG	29	
COX-P	TGC TTA CGC TGG ATG GAA TGC CCT	24	VIC

<sup>a</sup> F, forward; R, reverse; P, probe.

<sup>b</sup> Partially homologous with OLI1 primer from Seal et al. (24).

<sup>c</sup> Primer 631 from Fegan et al. (9).

and 1:100 all showed amplification when the concentration of cells was  $10^4$  cells ml<sup>-1</sup> and greater.

Multiplex TaqMan PCR of infected potato extracts with the COX fluorogenic probe as an internal control simultaneously detected both *R. solanacearum* and potato cytochrome oxidase target DNA sequences (Table 4).

## DISCUSSION

The results presented above demonstrate the development of a fluorogenic 5' nuclease PCR-based (TaqMan) assay capable of detection and identification of *R. solanacearum* directly in potato tuber tissue with sensitivity and specificity equal to or greater than those achievable with existing PCR protocols (8, 9, 24). In routine laboratory studies, *R. solanacearum* can be detected in conventional PCR assays in aqueous suspensions ranging from  $10^3$  cells per ml (2 cells per PCR) by the method of Seal et al. (24) to  $10^5$  cells per ml (200 cells per PCR) by the method of Fegan et al. (9). However in potato extracts, detection limits for the former assay (24) have been shown to be increased to  $10^6$  cells per ml or higher (8).

The procedure is robust, rapid, automated, and quantitative, with high sample throughput potential, permitting analysis of up to 96 samples in 3 h. Avoidance of laborious post-PCR gel electrophoresis and greatly reduced opportunity for contamination of reaction mixtures with target DNA further increase the suitability of this assay for routine diagnostic testing.

With cells suspended in water, TaqMan PCR was able to detect *R. solanacearum* to  $10^2$  cells per ml<sup>-1</sup> using both the RS-P and B2-P probes, comparable to the results of Oberst et al. (19) in detecting *Escherichia coli* by using a fluorogenic 5' nuclease reaction. The lower C<sub>T</sub> values obtained from the RS-P assay, rather than those obtained from B2-P with the same extracts, indicate a higher concentration of RS target DNA than of B2 target DNA. This may be explained by RS target DNA being part of the multicopy 16S rRNA gene compared with the B2 target DNA, whose genomic location and function are not known (9).

For indexing potato tubers for freedom from *R. solanacearum*, two useful multiplex assays have been validated. The first

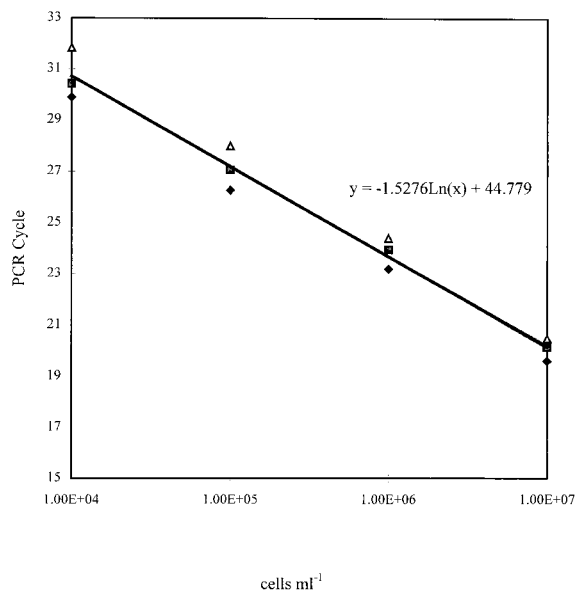


FIG. 1. Quantification of *R. solanacearum* biovar 2A cells (CSL3468) diluted in water from one experiment replicated three times with the RS-P probe. The trendline equation was calculated from the mean C<sub>T</sub> value by using Microsoft Excel. Primer concentration, 300 nM F–300 nM R; probe concentration, 50 nM.

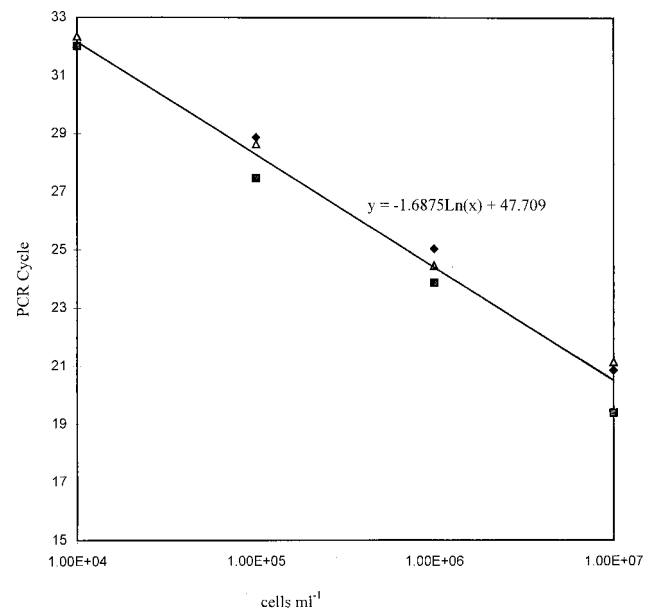


FIG. 2. Quantification of *R. solanacearum* biovar 2A cells (CSL3468) in diluted potato tissue, from one experiment replicated three times with RS-P probe. The trendline equation was calculated from the mean C<sub>T</sub> value by using Microsoft Excel. Primer concentration, 300 nM F–300 nM R; probe concentration, 50 nM.

TABLE 3. *R. solanacearum* biovar 2 dilution series, in water and potato extracts, and C<sub>T</sub> values generated during multiplex TaqMan PCR assay with the RS-I and RS-II primers plus RS-P fluorescent probe and B2-I and B2-II primers plus B2-P fluorescent probe

No. of <i>R. solanacearum</i> cells ml <sup>-1</sup>	C <sub>T</sub> value							
	Water		Pellet		Pellet (diluted 1:10)		Pellet (diluted 1:100)	
	RS (FAM)	B2 (VIC)	RS (FAM)	B2 (VIC)	RS (FAM)	B2 (VIC)	RS (FAM)	B2 (VIC)
10 <sup>7</sup>	19.31	20.76	—	—	22.59	25.74	19.46	21.64
10 <sup>6</sup>	22.08	24.08	—	—	26.89	31.18	24.36	26.13
10 <sup>5</sup>	26.17	28.36	—	—	28.67	—	27.56	29.45
10 <sup>4</sup>	29.37	— <sup>a</sup>	—	—	—	—	31.10	—

<sup>a</sup> —, no fluorescence above threshold recorded.

of these, using RS and B2 probe-primer sets, permits identification of the biovar 2A (race 3) phenotype without the need for isolation, purification, or biochemical phenotyping (requiring up to 3 weeks for completion). The second assay, using RS or B2 with COX probe-primer sets, provides simultaneous internal PCR control, permitting elimination of false-negative results due to inhibition of the reaction, an essential requirement for routine diagnostic applications.

As expected, the RS TaqMan primer-probe set detected all bacteria tested with known homology to the specific region of the 16S rRNA gene used for probe and primer design (24, 28). These included isolates representing all biovars and other infrasubspecific strains of *R. solanacearum* as well as the closely related banana blood disease bacterium and *R. syzygii* (the distribution of which has not been recorded outside of Indonesia). These results are consistent with those of the PCR protocol described by Seal et al. (24) using the 16S rRNA sequence upon which the RS assay is based. In addition, one isolate (from three tested) of *Pseudomonas* sp. strain Taxon B, commonly associated with sugar cane (11), also produced a positive result during the RS assay. This bacterium is known to have cellular fatty acid profiles closely resembling those of *R. solanacearum* (26). Other known *Ralstonia* spp. (*R. pickettii* and *R. eutropha*) not sharing homology with this specific region of the 16S rRNA gene were not detected.

In contrast, the B2 TaqMan primer-probe set detected only isolates of the biovar 2A phenotype, including strains of both restriction fragment length polymorphism groups 26 and 27 as described by Cook and Sequeira (7). As predicted, the DNA sequence used for probe and primer design in this assay is apparently unique to the biovar 2A genotype (9). However, the sensitivity of detection of *R. solanacearum* biovar 2A was sig-

nificantly higher when the TaqMan rather than the original PCR protocol was used (N. C. Smith, unpublished data). The B2 assay therefore ensures sensitivity and specificity of detection of the race 3 phenotype unattainable with other detection protocols (8–10, 15, 24, 29). Biovar 2T (which is believed to have originated in South America) has a significantly different genetic composition from that of other biovar 2A strains (7), accounting for the failure of the TaqMan probe B2-P to detect this strain.

TaqMan PCR using the COX-P probe as an internal PCR control and RS-P to detect the pathogen directly in infected potato extracts was demonstrated. Detection of *R. solanacearum* and potato cytochrome target DNA in 10-fold dilutions was observed to 10<sup>-2</sup> of the original extract. During the experimental work, evidence was accumulated to suggest that high concentrations of potato extract inhibit the TaqMan PCR. As a result, it is unlikely that this assay could be used for indexing potato tubers in combination with currently recommended sampling and extraction procedures (1, 3). However, it should be possible to simplify extraction procedures and to exploit the high assay sensitivity by eliminating the need for concentration of extracts prior to testing. Should the problem of assay inhibition continue, further steps for purification of target DNA or the use of pathogen enrichment procedures (8) may be required.

For indexing of potato tubers for freedom from *R. solanacearum*, a rapid response is required in order to prevent costly disruption to trade and to limit loss of this perishable commodity during testing. TaqMan PCR offers reliable detection within 1 day of receipt of samples, whereas current EU testing protocols that demand isolation, purification, identification, and demonstration of pathogenicity can take several days or weeks. Our assay questions the need for such time-consuming protocols. Because post-PCR processing steps are not required, the assay can be easily automated, unlike other PCR protocols. Automation will lead to high sample throughput, which, together with the high specificity and sensitivity of the assay, offers significant advantages over other current *R. solanacearum* detection techniques. As more TaqMan assays are developed to test for other potato pathogens and further fluorescent reporter dyes are developed, the potential of testing for several potato diseases in a single tube reaction is created. The probes described in this report, used to detect pathogens and to act as an internal PCR control, could be included in such a reaction, improving seed indexing procedures—the primary method of control for many potato pathogens.

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TABLE 4. Brown rot-infected potato extract dilution series and C<sub>T</sub> values generated during multiplex TaqMan PCR assay with the RS-I and RS-II primers plus RS-P fluorescent probe and COX-F and COX-R primers plus COX-P fluorescent probe

Potato tissue extract dilution	Mean C <sub>T</sub> value	
	RS (FAM) <sup>a</sup>	COX (VIC) <sup>b</sup>
Undiluted	14.84	24.46
10 <sup>-1</sup>	25.02	27.80
10 <sup>-2</sup>	29.83	32.36
10 <sup>-3</sup>	— <sup>c</sup>	—
10 <sup>-4</sup>	—	—

<sup>a</sup> Concentrations: 150 nM RS-F–150 nM RS-R primer, 25 mM RS-P probe. FAM, reporter dye.

<sup>b</sup> Concentrations: 100 nM COX-F–100 nM COX-R primer, 50 mM COX-P probe. VIC, reporter dye.

<sup>c</sup> —, no fluorescence above threshold recorded.

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## REFERENCES

1. Anonymous. 1990. Quarantine procedure: *Pseudomonas solanacearum*, inspection and test methods. Bull. OEPP/EPPO Bull. **20**:255–262.
2. Anonymous. 1995. Commission directive 95/4/EC. Off. J. Eur. Communities **L-182**:17–19.
3. Anonymous. 1998. Council directive 98/57/EC. Off. J. Eur. Communities **L-235**:1–39.
4. Bassler, H. A., S. J. A. Flood, K. J. Livak, J. Marmaro, R. Knorr, and C. A. Batt. 1995. Use of a fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. Appl. Environ. Microbiol. **61**:3724–3728.
5. Chen, S., A. Yee, M. Griffiths, C. Larkin, C. T. Yamashiro, R. Behari, C. Paszko-Kolva, K. Rahn, and S. A. De Gradis. 1997. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. Int. J. Food Microbiol. **35**:239–250.
6. Ciampi, L., L. Sequeira, and E. R. French. 1980. Latent infection of potato tubers by *Pseudomonas solanacearum*. Am. Potato J. **57**:377–386.
7. Cook, D., and L. Sequeira. 1991. The use of subtractive hybridisation to obtain a DNA probe specific to *Pseudomonas solanacearum*. Mol. Gen. Genet. **227**:401–410.
8. Elphinstone, J. G., J. Hennessy, J. K. Wilson, and D. E. Stead. 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum*. Bull. OEPP/EPPO Bull. **26**:674–678.
9. Fegan, M., G. Holoway, A. C. Hayward, and J. Timmis. 1998. Development of a diagnostic test based on the polymerase chain reaction (PCR) to identify strains of *R. solanacearum* exhibiting the biovar 2 genotype, p. 34–43. In P. H. Prior, C. Allen, and J. G. Elphinstone (ed.), Bacterial wilt disease: molecular and ecological aspects. Springer-Verlag, Berlin, Germany.
10. Griep, R. A., C. van Twisk, J. R. C. M. van Beckhoven, J. M. van der Wolf, and A. Schots. 1998. Development of specific recombinant monoclonal antibodies against the lipopolysaccharide of *Ralstonia solanacearum* race 3. Phytopathology **88**:795–803.
11. Hayward, A. C. 1962. Differentiation, taxonomy and nomenclature of the bacteria causing red stripe and mottled stripe diseases. Maurit. Sugar Ind. Res. Inst. Occas. Paper **13**:13–27.
12. Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. **29**:65–87.
13. Hayward, A. C., J. G. Elphinstone, D. Caffier, J. D. Janse, E. Stefani, E. R. French, and A. J. Wright. 1998. Round table on bacterial wilt (Brown Rot) of potato, p. 420–430. In P. H. Prior, C. Allen, and J. G. Elphinstone (ed.), Bacterial wilt disease: molecular and ecological aspects. Springer-Verlag, Berlin, Germany.
14. Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilising the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA **88**:7276–7280.
15. Janse, J. D. 1988. A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. Bull. OEPP/EPPO Bull. **18**:343–351.
16. Janse, J. D. 1991. Infra- and intraspecific classification of *Pseudomonas solanacearum* strains, using whole cell fatty acid analysis. Syst. Appl. Microbiol. **12**:335–345.
17. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on the tetrazolium medium. Phytopathology **39**:936–946.
18. Lee, L. G., C. R. Connell, and W. Bloch. 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. **21**:3761–3766.
19. Oberst, R. D., M. P. Hays, L. K. Bohra, R. K. Phebus, C. T. Yamashiro, C. Paszko-Kolva, S. J. A. Flood, J. M. Sargeant, and J. R. Gillespie. 1998. PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5' nuclease (Taq-Man) assay. Appl. Environ. Microbiol. **64**:3389–3396.
20. Quinones, V., S. Zanolungo, L. Holuigue, S. Litvak, and X. Jordana. 1995. The *cox1* initiation codon is created by RNA editing in potato mitochondria. Plant Physiol. **108**:1327–1328.
21. Robinson-Smith, A., P. Jones, J. G. Elphinstone, and S. M. D. Forde. 1995. Production of antibodies to *Ralstonia solanacearum*, the causative agent of bacterial wilt. Food Agric. Immunol. **7**:67–79.
22. Schaad, N. W., Y. Berthier-Schaad, A. Sechler, and D. Knorr. 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real time fluorescence detection system. Plant Dis. **83**:1095–1100.
23. Schoen, C. D., D. Knorr, and G. Leone. 1996. Detection of potato leafroll virus in dormant potato tubers by immunocapture and a 5' nuclease RT-PCR assay. Phytopathology **86**:993–999.
24. Seal, S. E., L. A. Jackson, J. P. W. Young, and M. J. Daniels. 1993. Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and the blood disease bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J. Gen. Microbiol. **139**:1587–1594.
25. Seal, S. E., and J. G. Elphinstone. 1994. Advances in identification and detection of *Pseudomonas solanacearum*, p. 35–58. In A. C. Hayward and G. L. Hartman (ed.), Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. CAB International, Wallingford, United Kingdom.
26. Stead, D. E. 1992. Grouping of plant pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. Int. J. Syst. Bacteriol. **42**:281–295.
27. Stead, D. E., J. G. Elphinstone, and A. W. Pemberton. 1996. Potato brown rot in Western Europe, p. 1145–1152. In Conference Proceedings—Brighton Crop Protection Conference—Pests and Diseases 1996. British Crop Protection Council, Farnham, Surrey, United Kingdom.
28. Taghavi, M., C. Hayward, L. I. Sly, and M. Fegan. 1996. Analysis of the phylogenetic relationships of strains of *Burkholderia solanacearum*, *Pseudomonas syzygii*, and the blood disease bacterium of banana based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. **46**:10–15.
29. Wullings, B. A., A. R. Van Beuningen, J. D. Janse, and A. D. L. Akkermans. 1998. Detection of *Ralstonia solanacearum*, which causes brown rot of potato, by fluorescent in situ hybridization with 23S rRNA-targeted probes. Appl. Environ. Microbiol. **64**:4546–4554.
30. Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi. 1995. Transfer of two *Burkholderia* and *Alcaligenes* species to *Ralstonia* gen. nov.—proposal of *Ralstonia pickettii* (Ralston, Palleroni & Doudoroff, 1973) comb. nov., *Ralstonia solanacearum* (Smith, 1896) comb. nov. and *Ralstonia eutropha* (Davis, 1969) comb. nov. Microbiol. Immunol. **39**:897–904.