

Specific and Sensitive Detection of *Ralstonia solanacearum* in Soil on the Basis of PCR Amplification of *fliC* Fragments

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Ralstonia solanacearum is the causative agent of bacterial wilt in many important crops. A specific and sensitive PCR detection method that uses primers targeting the gene coding for the flagella subunit, *fliC*, was established. Based on the first *fliC* gene sequence of *R. solanacearum* strain K60 available at GenBank, the Ral-*fliC* PCR primer system was designed; this system yielded a single 724-bp product with the DNAs of all of the *R. solanacearum* strains tested. However, *R. pickettii* and four environmental *Ralstonia* isolates also yielded amplicons. The Ral-*fliC* PCR products obtained with 12 strains (*R. solanacearum*, *R. pickettii*, and environmental isolates) were sequenced. By sequence alignment, Rsol-*fliC* primers specific for *R. solanacearum* were designed. With this primer system, a specific 400-bp PCR product was obtained from all 82 strains of *R. solanacearum* tested. Six strains of *R. pickettii* and several closely related environmental isolates yielded no PCR product; however, a product was obtained with one *Pseudomonas syzygii* strain. A GC-clamped 400-bp *fliC* product could be separated in denaturing gradient gels and allowed us to distinguish *P. syzygii* from *R. solanacearum*. The Rsol-*fliC* PCR system was applied to detect *R. solanacearum* in soil. PCR amplification, followed by Southern blot hybridization, allowed us to detect about one target DNA molecule per PCR, which is equivalent to 10³ CFU g of bulk soil⁻¹. The system was applied to survey soils from different geographic origins for the presence of *R. solanacearum*.

Ralstonia solanacearum is the causal agent of bacterial wilt in solanaceous crops but has also been recorded to infect a large range of more than 200 species representing over 50 families of plants (17). Traditionally, the pathogen has been classified in five biovars according to carbon source utilization (16, 18) and in six races based on host range (8, 26). *R. solanacearum* is supposed to be a soil-borne bacterium originating from the tropics, subtropics, and warm temperate regions (15), but strains causing brown rot of potato in geographic regions with a temperate climate are possibly adapted to lower temperatures (20, 24). In recent years, the increasing number of sites infested with potentially cold-adapted strains of *R. solanacearum* in several places in Europe dramatically enhanced the threat posed to European potato crops (20, 24, 43). Thus, reliable methods to detect the pathogen not only in tubers but also in soil or soil-related habitats are required. Several PCR-based methods for the detection of *R. solanacearum* have been described in the literature. These approaches are usually based on the amplification of ribosomal gene sequences (i.e., 16S or 16S-23S intergenic spacer region of the ribosomal DNA [rDNA]) (5, 12, 25, 35, 42, 44). However, due to the high degree of conservation of the ribosomal genes within the genus *Ralstonia*, 16S rDNA sequence similarities between species can

be as high as 98% (30, 35, 38). This can lead to positive signals with related species, such as *R. pickettii*, and thus rRNA-based methods have drawbacks. In other studies, the low level of resolution of 16S rDNA-based sequence analysis has been circumvented by using primers targeting functional genes such as endoglucanase and *hrpB* (28) or a random fragment that was claimed to be *R. solanacearum* specific (21).

In the present study, the *fliC* gene coding for the flagellar subunit protein flagellin was used to develop a highly specific and sensitive PCR-based detection system for *R. solanacearum*. The suitability of flagellin *fliC* genes for taxonomic applications has been shown in a number of studies for a large variety of bacterial species of several major bacterial groups: for α -proteobacteria (36); for β -proteobacteria, to which *R. solanacearum* belongs (11, 14); for low-G+C gram-positive bacteria (40); for the genus *Pseudomonas* (4, 23); and most notably for most enterobacterial species (3, 22). Flagellin genes have been used for detection, studies of population genetics, and epidemiological analyses (46). Due to their structure, which is conserved in the terminal regions that flank a variable, central region, flagellin genes are regarded as good candidates for PCR-based detection (46).

The main goal of the present study was to develop specific primers for amplification of a flagellin gene fragment that target all subgroups of the *R. solanacearum* species complex and to investigate their application to the detection of *R. solanacearum* in soil. Therefore, special attention was paid to achieving a sensitive amplifiability of the *fliC* gene fragments from DNA directly extracted from soil. The sensitivity of the detection system was enhanced by Southern blot hybridization. The novel method allowed the specific and sensitive detection

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TABLE 1. Strains used in this study^a

Group	Species	Strain	No. of strains	Biovar(s)	Host plants (no. of strains)	Geographic origin (no. of strains)	Source or reference
A	<i>R. solanacearum</i>		39	2	<i>Solanaceae</i> : potato (28), tomato (4), egg plant (1), and bittersweet (1); soil (1); ND (4)	Europe (39)	41
B	ND ^c		7	ND	<i>Solanaceae</i> : potato (6) and nightshade (1)	Europe: Friesland (6) and Aquitaine (1)	41
C	<i>R. solanacearum</i>		27	2	<i>Solanaceae</i> : potato (21), tomato (2), pepper (2), egg plant (1), and soil (1)	Africa (12), South America (14), Indonesia (1)	41
D	<i>R. solanacearum</i>		9	1, 3, 4	Musaceae (3), <i>Solanaceae</i> (4), <i>Eupatorium odoratum</i> (1), and ginger (1)	America (7), Australia (1), Cyprus (1)	41
E	<i>R. solanacearum</i>	DSM1993	1	3	Common bean	Mauritius	DSMZ
	<i>R. solanacearum</i>	DSM9544	1	1	Tomato	United States	DSMZ
	<i>R. solanacearum</i>	DSM50905	1	?	Banana	Costa Rica	DSMZ
	<i>R. solanacearum</i>	JS740	1	1	Potato	Colombia	27
	<i>R. solanacearum</i>	JS778	1	3	Potato	Réunion Island	27
	<i>R. solanacearum</i>	JS783	1	1	Tomato	United States	27
	<i>R. solanacearum</i>	JS841	1	4	Tomato	Sri Lanka	27
F	<i>P. syzygii</i>	JV1010	1		Clove	Indonesia	27
	<i>R. pickettii</i>	JR660	1				27
	<i>R. pickettii</i>	DSM6297	1				DSMZ
	<i>R. pickettii</i>	LMG5942	1				LMG
	<i>R. pickettii</i>	LMG6871	1				LMG
	<i>R. pickettii</i>	LMG7001	1				LMG
	<i>R. mannitolytica</i>	LMG6866 ^T	1				LMG
	Environmental isolates	Q3-8/14, OV203, OV225, and L3L	4		Potato rhizosphere	Germany and Brazil (L3L)	This study
	<i>R. eutropha</i>	DSM531	1				DSMZ
	<i>R. basilensis</i>	DSM	1				DSMZ

^a Genomic DNA of *Ralstonia* isolates, which was also used in the study by Timms-Wilson et al. (41), was applied in order to test the specificity of the primer systems *R. sol fliC_for/rev*; group E contains additional *R. solanacearum* strains that were not tested by Timms-Wilson et al. (41), and group F contains strains belonging to the *Ralstonia* genus but not to the *R. solanacearum* species complex.

^b DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; LMG, Laboratorium voor Microbiologie Gent, Universiteit Gent.

^c ND, not determined.

of this major bacterial pathogen in soils and related habitats. Sequence heterogeneities of *fliC* DNA fragments amplified from different *R. solanacearum* strains were detected by using denaturing gradient gel electrophoresis (DGGE).

MATERIALS AND METHODS

Bacterial strains. A list of all bacterial strains used to amplify *fliC* sequences is given in Table 1. Four environmental isolates from agricultural field soils in Germany (OV203, OV225, and Q3-8/14) or Brazil (L3L) (group F, Table 1) were included in the present study. These environmental isolates were identified by fatty acid methyl ester analysis (FAME) either as *R. pickettii* (Q3-8/14) or as *R. solanacearum* (OV203 and OV225) or by 16S rDNA sequencing (*Escherichia coli* positions 968 to 1401) as *R. solanacearum* (L3L).

Soil used for testing the sensitivity of the detection system. Samples of bulk soil used to test the sensitivity of the method originated from either (i) an experimental field site in The Netherlands, where *R. solanacearum* was initially added to a final level of ca. 10⁶ cells/g of soil, or (ii) a microcosm experiment performed at Plant Research International, Wageningen, The Netherlands. From the latter experiment, immunofluorescence colony-staining counts (IFC) (42) of *R. solanacearum* are available for all sampling time points (34).

Survey of soil samples originating from different geographic areas. To assess the prevalence of *R. solanacearum* cells in soils, a set of soil samples was tested. The samples originated from Brazil (six composite samples from a maize field), Thailand (six composite samples from a tomato field with infected plants), Cuba (four composite samples each from a sugarcane and a tobacco field and from forest soil), The Netherlands (six composite samples from a potato field with *R. solanacearum* infections), Spain (six composite samples from a potentially infested potato field), and Germany (six composite samples from a noninfested potato field from which strains OV203 and OV225 were isolated).

DNA extraction. Total DNA of all soil samples was either extracted following the method of Smalla et al. (37) or by using the UltraClean soil DNA kit (MoBio Laboratories, Solana Beach, Calif.). Both procedures include a combined enzymatic and bead-beating step (cell homogenizer; Braun, Melsungen, Germany) for cell lysis.

Genomic DNA of strains was obtained by sodium dodecyl sulfate and proteinase K cell lysis, selective precipitation of cell debris and polysaccharides with CTAB (cetyltrimethylammonium bromide), and isopropanol precipitation of DNA according to the protocol of Wilson et al. (45).

The amplifiability of DNA was checked by PCR amplification of the eubacterial 16S rDNA fragment between positions 968 and 1401 published in Heuer et al. (19).

Primer development. Based on the first *fliC* gene sequence of *R. solanacearum* strain K60 (available at GenBank under accession number AF283285 [submitted in 2000 and published in 2001]) (39), the *Ral_fliC* primer system (forward [5'-CCTCAGCCTCAATASCAACATC-3'] and reverse [5'-CATG TTCAGCTTTCMGAWGC-3']), resulting in an amplicon size of 724 bp, was derived and optimized by using the Oligo program (version 4.0.). The sequences of amplicons obtained with *R. solanacearum*, *R. pickettii*, or the four environmental isolates (Table 1) were used together with all of the *fliC* sequences available in the database to design the *R. solanacearum*-specific primers *Rsol_fliC* (forward [5'-GAACGCCAACGGTGCCTGAACT-3'] and reverse [5'-GGCGGCCTTCAGGGAGGTC-3']) giving an amplicon size of 400 bp. For DGGE separation of the *Rsol_fliC* amplicons, the GC-rich sequence described by Heuer et al. (19) was attached to the 5' end of the reverse primer to prevent complete melting. Both primer sets were analyzed by basic local alignment search tool (BLAST) (2).

PCR amplifications. For PCR amplification of *R. solanacearum fliC* gene fragments, the reaction mixture contained Stoffel buffer (10 mM KCl, 10 mM Tris-HCl [pH 8.3]), 0.2 mM deoxynucleoside triphosphates, 3.75 mM MgCl₂, 4% [wt/vol] acetamide, 100 nM concentrations of each forward and reverse primer, and 2 U of AmpliTaq Stoffel fragment/25 μ l. For amplification of environmental DNA extracted from soils, bovine serum albumin (1.25 μ g/25 μ l) was used to prevent inhibition. The PCR was carried out as follows. After an initial denaturation step for 5 min at 94°C, amplification was performed by using 25 cycles for DNA from isolates and 35 cycles for environmental DNA. Cycles consisted of a 30-s denaturation at 94°C, 2 min of primer annealing at 60°C for the *Ral_fliC* system and at 63°C for *Rsol_fliC* system, and a 1-min primer extension at 72°C, followed by a final step at 72°C (10 min) and cooling to 4°C. Products were analyzed by electrophoresis in 1% (wt/vol) agarose gels and ethidium bromide staining (32).

Southern blot hybridization. Probes were generated from the *Rsol_fliC* PCR product obtained with either strain *R. solanacearum* 1609 (race 3/biovar 2), strain DSM9544 (race 1/biovar 1), or strain DSM1993 (race 1/biovar 3) by labeling the PCR product, which was excised from the agarose gel after electrophoresis with digoxigenin. The probes were used either separately or as a mix of the three probes. Southern blotting was done according to the method of Sambrook et al. (32). Hybridization was performed under conditions of medium stringency following the protocol given in Fulthorpe et al. (13). Hybridization of Southern blotted *fliC* PCR products obtained from soil DNA was performed with a mix of the three separately prepared probes.

DGGE. DGGE systems (D-Code System; Bio-Rad, Inc., Hercules, Calif.) were used according to the protocols previously published by Heuer et al. (19). Polyacrylamide gels were composed of 0.17% (vol/vol) TEMED (*N,N,N',N'*-tetramethylethylenediamine), 0.047% (wt/vol) ammonium persulfate, a 60:1 ratio of acrylamide/*N,N'*-methylene bisacrylamide, 1 \times buffer, and 2% glycerol. Denaturing gradients of 30 to 70% of denaturant substances (100% denaturant corresponds to 7 M urea plus 40% [vol/vol] deionized formamide) were used. Gradients were poured by using a gradient former and a peristaltic pump, and gels were allowed to polymerize for at least 2 h. Electrophoresis was performed in 0.5 \times Tris-acetate-EDTA buffer at 58°C at a constant voltage of 220 V for 5.5 h. Samples of the PCR mixture (varying between 2 and 8 μ l to adjust differences in DNA concentration to ~40 ng) were applied to the polyacrylamide gels.

Silver staining. An acid silver-staining protocol was used for detection of DNA in DGGE gels (29). Gels were dried and digitalized with a translucent scanning device (Epson Deutschland GmbH, Düsseldorf, Germany).

Cloning and sequencing of *fliC* fragments. For cloning of *Ral_fliC* fragments, 12 bacterial strains of groups A, D, E, and F (Table 1) were chosen. The 724-bp PCR product was ligated into the pGEM-T vector (Promega, Madison, Wis.) and introduced into competent *E. coli* JM109 cells (Promega) via transformation. Sequencing was done with the standard primers SP6 and T7 (IIT GmbH, Bielefeld, Germany).

Sequence analysis. Analysis of cloned sequences was performed by using the software package BioEdit available from the internet (www.mbio.ncsu.edu/BioEdit). Multiple alignments were done applying the CLUSTALW tool delivered by the package. Maximum-likelihood phylogenetic trees were calculated by using the fastDNAmI algorithm, an accessory application within BioEdit.

Sequence accession numbers. All *fliC* sequences generated in this study were deposited in the GenBank database under accession numbers AY192716 to AY192727. The 16S rDNA sequences generated in the present study were deposited in the GenBank database under accession numbers AY216797 (OV225), AY216796 (*R. solanacearum* 1609), and AY216798 (Q3-8/14).

RESULTS AND DISCUSSION

Primer development. The *Ral_fliC* primer gave a single product of 724 bp not only with DNA of *R. solanacearum* but also with *R. pickettii* and with four environmental isolates identified by FAME as *R. pickettii* or *R. solanacearum* (Table 1). The PCR products obtained from 12 strains belonging to group A (*R. solanacearum* strains [biovar 2] 1609, 737, and 267), group E (*R. solanacearum* strain [biovar 1] DSM9544), and group F (*R. pickettii* strains LMG5942, LMG6871, LMG7001, and DSM6297 and environmental isolates OV203, OV225, Q3-8/14, and L3L) were cloned and sequenced.

A comparison of the cloned *Ral_fliC* sequences with 36 homologous *fliC* sequences obtained from the GenBank database, two of them being published *R. solanacearum fliC* sequences (31, 39), revealed four regions that were potentially specific for all *R. solanacearum* sequences analyzed. Based on these regions, a primer system was designed that gave single product of defined length (400 bp) after PCR amplification with *R. solanacearum* strains DSM9544 and 1609. This primer system, denoted *Rsol_fliC*, was further used in the study. Comparison of aligned target sequences for primer annealing revealed that a product of 400 bp would theoretically be obtained with all *R. solanacearum* strains, since both primers matched the corresponding target sequences in the first 17 nucleotides counted from the 3' end. In contrast, none of the other sequences, most notably the *R. pickettii* strains and the four environmental isolates, were expected to give a signal by PCR with this primer system. At least three of five bases at the 3' end of the forward primer *Rsol_fliC* showed mismatches with the sequences of all nontarget bacteria for which *fliC* sequences are available in GenBank (*Aquifex*, low-G+C gram-positive bacteria, α -proteobacteria, β -proteobacteria, *Pseudomonas* spp., and enterobacteria). In respect to the *Rsol_fliC* reverse primer, all *R. pickettii* sequences share a mismatch at position 5 from the 3' end of the primer. Sequences of the other bacterial taxa analyzed (see above) had additional mismatches at the 3' end. Thus, the forward primer was predicted to be the most important for the specificity of the primer system (Fig. 1).

A BLAST search performed with both primers on 25 September 2002 revealed that the only significant matches (100% identity) produced were with the two available *R. solanacearum fliC* sequences of strains GMI1000 (AL646078) and K60 (AF283285). Surprisingly, the nucleotide sequence similarities of *fliC* sequences of all species of the related genus *Burkholderia* included in the alignments to the *R. solanacearum fliC* sequences were very low, ranging from 30.2 to 34% sequence identity. In consequence, we assumed that the *fliC* gene offered a good phylogenetic resolution at the species level.

Testing the primer system with isolates. All 82 strains tested that belong to the species complex of *R. solanacearum* (groups A, C, D, and E, Table 1) gave an amplification product of 400 bp in PCR with the primer system *Rsol_fliC*. Furthermore, a 400-bp PCR product was obtained from the *Pseudomonas syzygii* strain JV1010 (one strain), whereas no PCR products were observed with the strains of *R. pickettii* (five strains), *R. eutropha* (one strain), *R. basilensis* (one strain), and *R. mannitolytica* (one strain). Hence, the PCR-based approach was most likely specific for *R. solanacearum*. False-positive reactions with bac-

	5'	Rsol_fliC_for	3'	5'	Rsol_fliC_rev	3'
Primers		GAACGCCAACGGTGC	AACT	GGCGGCCCTTCAGGGAGGTC		
<i>R. solanacearum</i> K60	CA	CG	AG	
<i>R. solanacearum</i> GMI1000	CA	CG	AG	
<i>R. solanacearum</i> DSM9544	CA	CG	AG	
<i>R. solanacearum</i> 267	CA	CG	A.....	AG	
<i>R. solanacearum</i> 1609	CA	CG	A.....	AG	
<i>R. solanacearum</i> 1737	CA	CG	A.....	AG	
L3L, isolate Brazil	CAAAC..AC.G.	CG	T..T.....CT..C.	AG	
<i>R. pickettii</i> LMG6871	CAAAC..AC.G.	CG	A..T.....CT..C.	AG	
Q3-8/14, isolate Germany	CA	...T....CAG..ACTG.	CG	...C.A.....TT...	AG	
<i>R. pickettii</i> LMG7001	CA	...T....CAG...TG.	CG	A..C.....TT...	AG	
<i>R. pickettii</i> LMG5942	CA	...T....CAG...TG.	CG	A..C.....TT...	AG	
<i>R. pickettii</i> DSM6297	CA	...T....CAG...TG.	CG	A..C.....TT...	AG	
OV203, isolate Germany	CAAAC..CGCG.	CG	C..C.A.....TTACC.	AG	
OV225, isolate Germany	CAAAC..CGCG.	CG	C..C.A.....TTACC.	AG	
<i>Bordetella bronchiseptica</i>	CG	C.....AC.GC.T..	CG	CTGTT.GT...C..CCT..	AT	
<i>Burkholderia mallei</i>	TCG...AC.GCGTG.	CG	CAGATTG..G.CC.TC.CG	AG	
<i>B. pseudomallei</i>	TCG...AC.GCGTG.	CG	CAGATTG..G.CC.TC.CG	AG	
<i>B. thailandensis</i>	TCG...AC.GCGTG.	CG	CAG..TTG..G.CC.TC.CG	AG	
<i>B. cepacia</i>	TCAC.GCGTG.	CG	CA..TTG...CC.TCTG.	AG	
<i>Pseudomonas fragi</i>	GC	A...CT..T.A.AC.G.CGTA	AC	AT.CAG.AGATC.ACAC..	AG	
<i>Escherichia coli</i> (B38)	CG	T.....AC.GT.TT.	CT	.AATTTG.CG.T...T.CA	AT	
<i>Salmonella enteritidis</i>	CG	T....T...AC.GC.TT.	CT	AA.T..G.C..CTTTT.A.	AA	
<i>Aquifex aeolicus</i>	AG	...ATTG.GTTC.GT.TT.	CC	TAT..TG.CA.CTTGTC.T	AC	
<i>Clostridium difficile</i>	AG	A...TTC.A.A..GT.TA.	CT	T.AT.AG..T.A.CTCT.A	AG	
<i>Rhodobacter sphaeroides</i>	CGGC.G.AC.GC..GA	AC	C..ACTGGC.G.TCTTC.AG	AG	
		↑	↑	↑	↑	
Position in <i>fliC</i> of GMI1000		696	715	1095	1076	

FIG. 1. Sequence comparison of the primers Rsol_fliC_for (forward) and Rsol_fliC_rev (reverse) with the corresponding sequences of target and other species. The alignment was based on amino acid sequences. Strain names in boldface indicate that the sequence was generated in the present study. Bases identical to those in the primer sequences are shown as dots.

terial strains outside the genus *Ralstonia* are not likely to occur since, for instance, the sequence similarity within the *fliC* fragment to the proximate genus *Burkholderia* was surprisingly low. In particular, the corresponding priming sites did not match the primer sequence of Rsol_fliC.

The finding that a PCR product of the expected size was amplified from *P. syzygii* strain JV1010 genomic DNA confirms the assumption that *P. syzygii* is part of the *R. solanacearum* species complex (12). Until now, there have been no PCR-based detection systems available to differentiate between *P. syzygii* and *R. solanacearum* in a fast and simple way.

Use of the Rsol_fliC primer system to check the identification of presumptive *R. solanacearum* isolates. Colonies isolated on R2A medium from field soils have been from time to time identified by fatty acid methyl ester analysis as *R. solanacearum* or *R. pickettii*. Thus, strains highly related to *R. solanacearum* can be isolated from uninfested soils. Since *R. solanacearum* is a quarantine organism, its isolation from field soils would be alarming and of enormous economic consequences for farmers. Strains OV225 and Q3-8/14 both gave positive signals with the primer system described by Seal et al. (35). The complete 16S rDNA sequence (positions 8 to 1513 based on *E. coli* numbering [6]) was determined for strain OV225 (which has identical BOX fingerprints as strain OV203) and strain Q3-8/

14. The 16S rDNA sequence of strain OV225 showed the highest sequence similarity to the 16S rDNA sequence of *R. solanacearum* 1609 (97.34%), whereas the 16S rDNA sequence of strain Q3-8/14 had 99.45% similarity to *R. pickettii* MSP3. Although all four environmental *Ralstonia* isolates yielded PCR products with the Ral_fliC primer system, no PCR products were obtained with the Rsol_fliC primer system. Thus, the latter PCR system allows to differentiate these environmental isolates from *R. solanacearum*.

Sensitivity of Rsol_fliC PCR applied to DNA extracted from soil samples. Special emphasis was placed on the applicability of Rsol_fliC PCR on soil DNA. The PCR amplifiability of all DNA samples directly extracted from soil was confirmed by the amplification of 16S rDNA fragments (positions 968 to 1401 [*E. coli* numbering]). The sensitivity of Rsol_fliC PCR detection of *R. solanacearum* in soils was evaluated with DNA extracted from soils from a microcosm experiment performed in The Netherlands; this microcosm had been inoculated with *R. solanacearum* at an initial concentration of 10⁸ cells g of soil⁻¹. IFC were available for five sampling time points at days 0, 33, and 54 after inoculation (Fig. 2; selected IFC data from Schönfeld et al. [34]). The detection limit of the direct Rsol_fliC PCR in soil was ca. 10⁵ cells g of soil⁻¹ when the PCR products were detected in ethidium bromide-stained agarose gels (Fig. 2A).

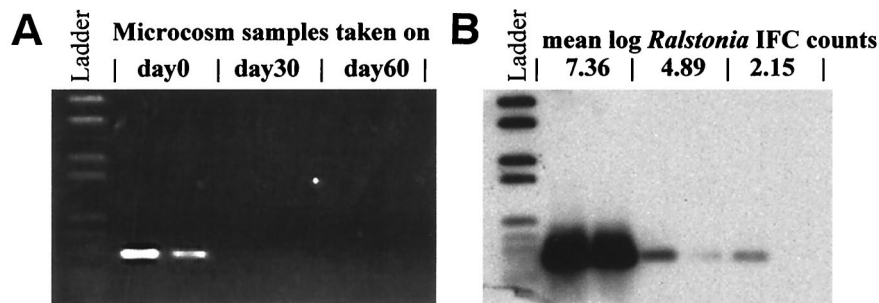


FIG. 2. Agarose gel (A) and corresponding Southern blot (B) of *Rsol_fliC* PCR products obtained from PCR amplification of microcosm soils with IFC available for each time point of sampling. Positive and negative controls are not shown.

Hybridization of Southern-blotted PCR products obtained with the *Rsol_fliC* primers by using a probe generated from a *Rsol_fliC* PCR of the introduced *R. solanacearum* strain 1609 revealed that cell densities of *R. solanacearum* down to ca. 10^3 cells g of soil⁻¹ could be detected (Fig. 2B).

To demonstrate that *Rsol_fliC* PCR in combination with Southern hybridization is a useful tool for estimating unknown concentrations of *R. solanacearum* cells, a set of samples from a field experiment performed in The Netherlands were analyzed (T. Gorissen et al., unpublished data). The soil had been inoculated with 10^6 *R. solanacearum* cells g of soil⁻¹ and was treated afterward by different measures (i.e., water-amended control and a combination of piggery manure amendment and solarization). DNA extracted from samples taken directly after inoculation (10^6 *R. solanacearum* cells g of soil⁻¹) and 62 days after inoculation (unknown titer of *R. solanacearum*) was used to amplify the 400-bp *R. solanacearum*-specific fragment. A decline of *R. solanacearum* cells over time was suggested by agarose gel and in the corresponding blot, as the band intensity declined (Fig. 3a). However, no differences in band intensities could be observed between control and treated samples (i.e., a combination of piggery manure amendment and solarization). However, comparison of relative band intensities of samples at days 0 and 62 allowed an estimation of the presumptive cell densities of *R. solanacearum* in soil. In most day 62 samples very faint bands were detected in the agarose gel, indicating that the cell densities were near the detection limit of direct *Rsol_fliC* PCR (10^5 *R. solanacearum* cells g of soil⁻¹) (Fig. 3a). Strong hybridization signals were observed at 10^5 cells g of soil⁻¹, but differences in relative intensities were still distin-

guishable (Fig. 3b). No hybridization signal was detected in uninoculated soil samples (data not shown).

The *Rsol_fliC* PCR combined with Southern hybridization was applied to detect *R. solanacearum* of different biovars in a survey of soil samples from various geographic sites. *Rsol_fliC* PCRs and subsequent Southern hybridization with a mixed probe composed of probes for biovars 1, 2, and 3 were applied to DNA extracted directly from soils originating from three continents. All four soil samples originating from an infested field site in The Netherlands showed an amplification of the specific 400-bp fragment, as evidenced by agarose gel electrophoresis (Fig. 4). Symptoms of bacterial wilt were reported for this field. Positive signals were also found in DNA from soil samples from Thailand. These soil samples originated from a tomato field in which wilted tomato plants were observed (K. Wydra, unpublished data). In soil samples from the other regions, vestiges of *R. solanacearum* could not be detected, indicating that *R. solanacearum* was either not present or was present at concentrations of $<10^3$ cells g of soil⁻¹.

Considering that the regulatory network *R. solanacearum* uses for virulence is activated only at cell densities of $>10^7$ ml⁻¹ (7) and since it was possible to detect the 400-bp *fliC* fragment down to a level of 10^3 *R. solanacearum* cells g of soil⁻¹, it can be assumed that the pathogen is detectable by *Rsol_fliC* PCR even if no symptoms of brown rot occur.

A promising application of the *Rsol_fliC* PCR in combination with Southern hybridization is in monitoring the fate of the pathogen over time. Shifts in the *R. solanacearum* population size can be detected by quantifying the *Rsol_fliC* PCR product, for example, by real-time PCR. Thus, one of the main

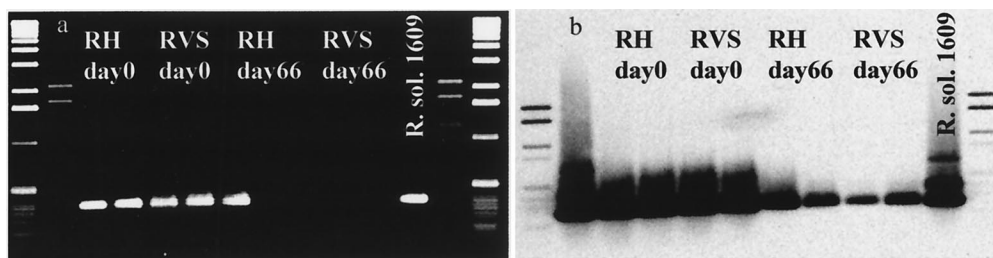


FIG. 3. (a) Agarose gel of *Rsol_fliC* PCR products amplified from soils from a field experiment in The Netherlands obtained 0 or 66 days after inoculation with *R. solanacearum* strain 1609 at an initial concentration of 10^6 cells g of soil⁻¹. Duplicates of samples are shown. Soil treatments are abbreviated as follows: RH, inoculated with strain 1609, untreated, water added; RVS, inoculated with strain 1609, manure amendment plus solarization. (b) Corresponding Southern blot of the agarose gel in panel a hybridized with an *Rsol_fliC* probe derived from *fliC* PCR of strain 1609.

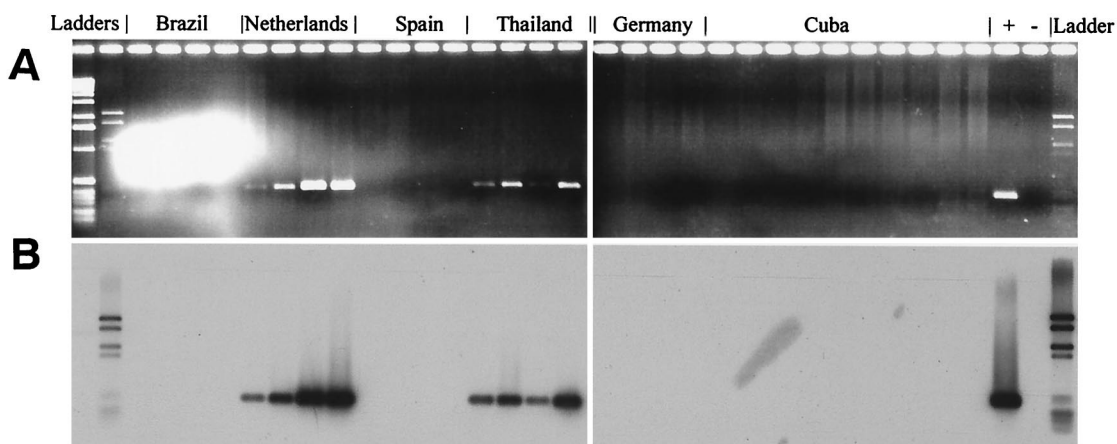


FIG. 4. Agarose gel (A) and corresponding Southern blot (B) of *Rsol_fliC* PCR products obtained from PCR amplification of soil samples originating from three continents. For hybridization, a mixed probe generated from three strains representing *R. solanacearum* biovars 1, 2, and 3 was used. The origins of the samples are indicated above the agarose gel image. A selection of the samples analyzed (four samples for each origin; 10 samples from Cuba) is presented.

advantages of the approach is the possibility to follow the fate of the pathogen without the necessity of introducing a special marker.

Another useful aspect of this approach is the possibility of detecting avirulent forms of the pathogen. Although flagellum-dependent motility was shown to play an important role in virulence during the early stages of disease manifestation, i.e., invasion and dissemination (39), the genes needed for flagellum constitution are not directly involved in virulence, i.e., they are not part of the virulence regulatory network (1, 9, 33). Therefore, the 400-bp *Rsol_fliC* fragment is detectable from strains which exhibit limited virulence in planta.

Variability within the 400-bp *Rsol_fliC* fragment. From sequence alignments, it appeared that the 400-bp *fliC* fragment of all *R. solanacearum* strains sequenced in the present study corresponds to the region between positions 696 and 1095 of the *fliC* sequence of *R. solanacearum* GMI1000. As representatives of three different biovars, *fliC* sequences of the six *R. solanacearum* strains were analyzed: DSM9544 and K60 (biovar 1); 1609, 1737, and 267 (biovar 2); and GMI1000 (biovar 3). Considerable sequence variability was found among these *R. solanacearum* strains (Fig. 5A). Although biovar 2 strains 1609, 1737, and 267 had identical sequences over the whole 400 bp, biovar 1 strains DSM9544 and K60 differed in two positions. GMI1000 (biovar 3) and 1609 (biovar 2) showed differences in eight positions. The differences of the two biovar 1 strains DSM9544 and K60 to biovar 2 strain 1609 and biovar 3 strain GMI1000 were more pronounced: strain 1609 differed from DSM9544 in 15 positions and from K60 in 7 positions. GMI1000 differed from DSM9544 in 19 positions and from K60 in 17 positions (see also Fig. 5A). All differentiating positions, occurring as single sites were found in the region from positions 73 to 400 of the PCR product (Fig. 5B).

The selected 400-bp fragment is located within the central region of the *fliC* gene, where the greatest sequence variability between strains can be expected because this region codes for a nonfunctional domain of the protein (flagellin) (46). Furthermore, the *fliC* gene is not a part of a pathogenicity island, nor was it found to be transmitted by horizontal gene transfer (46).

Therefore, it may represent a good phylogenetic marker, such as the 16S rDNA, but with a different scale of resolution and, presumably, a better discriminatory power, when closely related species or subgroups within a species are compared. Regarding the distinct positional variability within the 400-bp fragment, the application of DGGE as a sequence-based separation technique seemed to be promising.

Separation of *fliC* fragments in DGGE. A single strong and sharp band was observed in DGGE of *Rsol_fliC* fragments of each of the 82 *R. solanacearum* strains and one *P. syzygii* strain analyzed. According to their melting behavior in DGGE, at least seven different band positions could be identified (Fig. 6). Of the 66 biovar 2 strains analyzed by *Rsol_fliC* PCR-DGGE, 63 had an electrophoretic mobility identical to that of strain 1609 (Fig. 6, b and g). The biovar 2 strains originating from potato plants (52 strains), tomato plants (6 strains), soil (2 strains), egg plants (2 strains), and bittersweet (1 strain) were obtained from various geographic regions. Only two isolates from pepper, originating from Brazil and Indonesia (bands with the electrophoretic mobility of bands a and c [results not shown in Fig. 6]), and one potato isolate had a different electrophoretic mobility. Although only eight biovar 1 isolates were included in the present study, four different electrophoretic mobilities corresponding to band g (UW20 [banana, Venezuela], UW28 [origin not available], and DSM9544 [3]), band f (UW25 [tomato, United States] and JS783 [tomato, United States]), band e (UW70 [plantain, Colombia] and JS740 [potato, Colombia]), and band b (UW136 [heliconia, Costa Rica]) were observed (Fig. 6). Thus, in contrast to biovar 2 strains, a higher genetic diversity can be supposed for biovar 1 isolates. This observation confirms the finding reported by Poussier et al. (27). All four biovar 3 strains (UW8 [*Eupatorium odoratum*, Costa Rica], UW255 [pepper, Australia], JS778 [potato, Réunion Island], and DSM1993) had the same electrophoretic mobility (band b) as for two of the three biovar 4 isolates (JS841 [potato, Sri Lanka] and UW27 [tobacco, United States]). The *P. syzygii* product had an electrophoretic mobility different from that of all *R. solanacearum* strains. Thus, *Rsol_fliC* PCR-DGGE offers an alternative method to

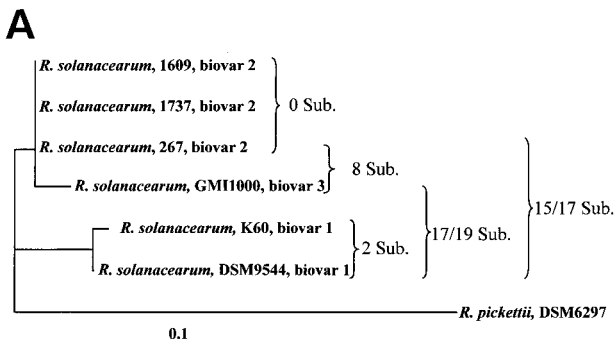


FIG. 5. (A) Neighbor-joining tree based on a comparison of 400-bp fragments of the *fliC* gene. The number of nucleotide substitutions between sequences is given at the branches. Sub., substitutions. The tree was rooted with *R. pickettii* DSM6297 as an outgroup. (B) Sequence comparison of the 400-bp *fliC* fragments of strains belonging to different biovars.

B

GMI1000, bv. 1	1	GAAGGCCAACGGTGCGA	ACTCGTACCTGCAAAACGGCCGACTCGTACCTGGGCCAGGTTGA	60
K60, bv. 1	1	GAAGGCCAACGGTGCGA	ACTCGTACCTGCAAAACGGCCGACTCGTACCTGGGCCAGGTTGA	60
DSM9544, bv. 3	1	GAAGGCCAACGGTGCGA	ACTCGTACCTGCAAAACGGCCGACTCGTACCTGGGCCAGGTTGA	60
1609, bv. 2	1	GAAGGCCAACGGTGCGA	ACTCGTACCTGCAAAACGGCCGACTCGTACCTGGGCCAGGTTGA	60
1737, bv. 2	1	GAAGGCCAACGGTGCGA	ACTCGTACCTGCAAAACGGCCGACTCGTACCTGGGCCAGGTTGA	60
267, bv. 2	1	GAAGGCCAACGGTGCGA	ACTCGTACCTGCAAAACGGCCGACTCGTACCTGGGCCAGGTTGA	60
GMI1000, bv. 1	61	AAACAACCTGCAACG	TATGCGCCACTGGCTGTGGAATCCAACAACGGCGGTCTGTGCGC	120
K60, bv. 1	61	AAACAACCTGCAACG	TATGCGCCACTGGCTGTGGAATCCAACAACGGCGGTCTGTGCGC	120
DSM9544, bv. 3	61	AAACAACCTGCAACG	TATGCGCCACTGGCTGTGGAATCCAACAACGGCGGTCTGTGCGC	120
1609, bv. 2	61	AAACAACCTGCAACG	TATGCGCCACTGGCTGTGGAATCCAACAACGGCGGTCTGTGCGC	120
1737, bv. 2	61	AAACAACCTGCAACG	TATGCGCCACTGGCTGTGGAATCCAACAACGGCGGTCTGTGCGC	120
267, bv. 2	61	AAACAACCTGCAACG	TATGCGCCACTGGCTGTGGAATCCAACAACGGCGGTCTGTGCGC	120
GMI1000, bv. 1	121	AGCCGACCAGACCAAC	CTGGACAAGGAATACCAACAGCTGGCAACGGCCAAACAAGAACAT	180
K60, bv. 1	121	AGCCGACCAGACCAAC	CTGGACAAGGAATACCAACAGCTGGCAACGGCCAAACAAGAACAT	180
DSM9544, bv. 3	121	AGCCGACCAGACCAAC	CTGGACAAGGAATACCAACAGCTGGCAACGGCCAAACAAGAACAT	180
1609, bv. 2	121	AGCCGACCAGACCAAC	CTGGACAAGGAATACCAACAGCTGGCAACGGCCAAACAAGAACAT	180
1737, bv. 2	121	AGCCGACCAGACCAAC	CTGGACAAGGAATACCAACAGCTGGCAACGGCCAAACAAGAACAT	180
267, bv. 2	121	AGCCGACCAGACCAAC	CTGGACAAGGAATACCAACAGCTGGCAACGGCCAAACAAGAACAT	180
GMI1000, bv. 1	181	CGAAACCAACGGCAAC	TACAACGGCAACAAGCTGTTGACGGCTCGGTGGCTTCGACGAC	240
K60, bv. 1	181	CGAAACCAACGGCAAC	TACAACGGCAACAAGCTGTTGACGGCTCGGTGGCTTCGACGAC	240
DSM9544, bv. 3	181	CGAAACCAACGGCAAC	TACAACGGCAACAAGCTGTTGACGGCTCGGTGGCTTCGACGAC	240
1609, bv. 2	181	CGAAACCAACGGCAAC	TACAACGGCAACAAGCTGTTGACGGCTCGGTGGCTTCGACGAC	240
1737, bv. 2	181	CGAAACCAACGGCAAC	TACAACGGCAACAAGCTGTTGACGGCTCGGTGGCTTCGACGAC	240
267, bv. 2	181	CGAAACCAACGGCAAC	TACAACGGCAACAAGCTGTTGACGGCTCGGTGGCTTCGACGAC	240
GMI1000, bv. 1	240	CTTCCAAATAGGGCC	GAGAACGCAAGCCACGGACCTGACCACGGTCACCAACGTCACATGTC	300
K60, bv. 1	240	CTTCCAAATAGGGCC	GAGAACGCAAGCCACGGACCTGACCACGGTCACCAACGTCACATGTC	300
DSM9544, bv. 3	240	CTTCCAAATAGGGCC	GAGAACGCAAGCCACGGACCTGACCACGGTCACCAACGTCACATGTC	300
1609, bv. 2	240	CTTCCAAATAGGGCC	GAGAACGCAAGCCACGGACCTGACCACGGTCACCAACGTCACATGTC	300
1737, bv. 2	240	CTTCCAAATAGGGCC	GAGAACGCAAGCCACGGACCTGACCACGGTCACCAACGTCACATGTC	300
267, bv. 2	240	CTTCCAAATAGGGCC	GAGAACGCAAGCCACGGACCTGACCACGGTCACCAACGTCACATGTC	300
GMI1000, bv. 1	298	GACCTTCGGCACGCT	CACCGGCACGAGCGTGACCAGCGCTGGCAACGGCAGCCGAGCCCA	360
K60, bv. 1	298	GACCTTCGGCACGCT	CACCGGCACGAGCGTGACCAGCGCTGGCAACGGCAGCCGAGCCCA	360
DSM9544, bv. 3	298	GACCTTCGGCACGCT	CACCGGCACGAGCGTGACCAGCGCTGGCAACGGCAGCCGAGCCCA	360
1609, bv. 2	298	GACCTTCGGCACGCT	CACCGGCACGAGCGTGACCAGCGCTGGCAACGGCAGCCGAGCCCA	360
1737, bv. 2	298	GACCTTCGGCACGCT	CACCGGCACGAGCGTGACCAGCGCTGGCAACGGCAGCCGAGCCCA	360
267, bv. 2	298	GACCTTCGGCACGCT	CACCGGCACGAGCGTGACCAGCGCTGGCAACGGCAGCCGAGCCCA	360
GMI1000, bv. 1	358	GGCCGCGATCGATAC	CGAATCTGACCTCCCTGAAGGCCGCT	400
K60, bv. 1	358	GGCCGCGATCGATAC	CGAATCTGACCTCCCTGAAGGCCGCT	400
DSM9544, bv. 3	358	GGCCGCGATCGATAC	CGAATCTGACCTCCCTGAAGGCCGCT	400
1609, bv. 2	358	GGCCGCGATCGATAC	CGAATCTGACCTCCCTGAAGGCCGCT	400
1737, bv. 2	358	GGCCGCGATCGATAC	CGAATCTGACCTCCCTGAAGGCCGCT	400
267, bv. 2	358	GGCCGCGATCGATAC	CGAATCTGACCTCCCTGAAGGCCGCT	400

the approach previously described by Poussier et al. (27) for differentiating this species from *R. solanacearum*. Although the limited number of isolates belonging to biovars 1, 3, and 4 that were included in the present study does not allow us to fully evaluate the discriminatory power of the *Rsol-fliC* PCR-DGGE approach, it might be particularly useful to analyze mixed infections with strains of different biovars of *R. solanacearum* in the plant or in the soil on the condition that sufficiently high numbers of *R. solanacearum* cells are present.

Conclusion. The *Rsol-fliC* primer system offers a specific and, in combination with Southern blot analysis, a very sensitive detection system for *R. solanacearum*. Although PCR products were obtained with *R. solanacearum* strains belonging to different biovars, no *Rsol-fliC* products were obtained with environmental soil isolates that seemed to be closely related to the *R. solanacearum* or *R. pickettii*. The PCR system can be used for cultivation-independent detection of the pathogen in DNA directly extracted from environmental samples. Since

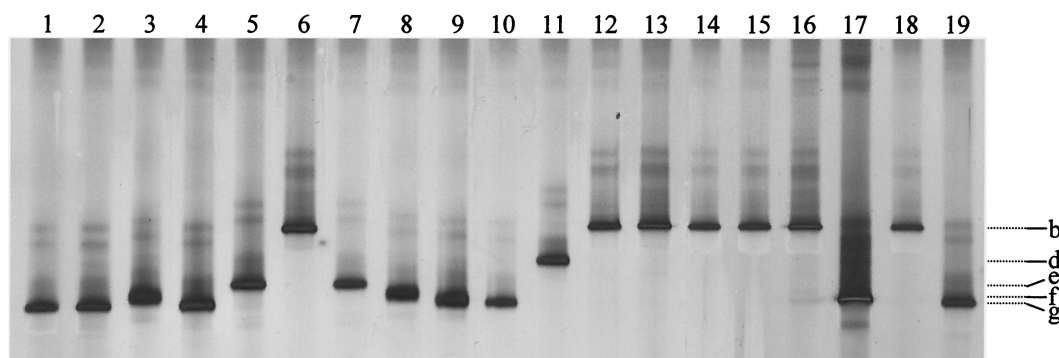


FIG. 6. Electrophoretic separation of GC-clamped *RsoI*-*fliC* PCR products of a selection of strains of *R. solanacearum* and *P. syzygii* strain JV1010 by 30 to 70% DGGE. Lanes (biovar/host plant/origin [NA = not available]): 1 and 19, strain 1609 (biovar 2/potato/The Netherlands); 2, strain UW20 (biovar 1/banana/Venezuela); 3, strain UW25 (biovar 1/tomato/United States); 4, strain UW28 (biovar 1/NA/NA); 5, UW70 (biovar 1/plantain/Colombia); 6, strain UW136 (biovar 1/heliconia/Costa Rica); 7, strain JS740 (biovar 1/potato/Colombia); 8, strain JS783 (biovar 1/tomato/United States); 9, strain DSM9544 (biovar 1/tomato/United States); 10, strain DSM50905 (biovar NA/banana/Costa Rica); 11, *P. syzygii* strain JV1010 (clove/Indonesia); 12, strain UW8 (biovar 3/*E. odoratum*/Costa Rica); 13, strain UW255 (biovar 3/pepper/Australia); 14, strain JS778 (biovar 3/potato/Réunion Island); 15, strain DSM1993 (biovar 3/*Phaseolus vulgaris*/Mauritius); 16, strain UW27 (biovar 4/tobacco/United States); 17, strain UW151 (biovar 4/ginger/Australia); 18, strain JS841 (biovar 4/potato/Sri Lanka). For further information on the above listed strains, see studies by Cook and Sequeira (10), Poussier et al. (27), and Timms-Wilson et al. (41).

environmental stresses such as low temperature are known to induce the viable-but-nonculturable state in *R. solanacearum* (43), the use of cultivation-independent detection techniques such as the *RsoI*-*fliC* detection system, which allows a sensitive and specific detection of the pathogen, are crucial. Furthermore, this system could be a valuable tool for monitoring the expression of the *fliC* genes of *R. solanacearum* strains under different conditions in soil by *RsoI*-*fliC* PCR amplification of reverse-transcribed RNA that was directly extracted from soil.

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