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. picketti* and four environmental *Ralstonia* isolates also yielded amplicons. The *Ral fliC* PCR products obtained with 12 strains (*R. solanacearum*, *R. picketti*, 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. picketti* 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 10^5 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. picketti*, 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 *C. pneumoniae* (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...
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. solanacearum* or *R. pickettii* 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 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. 10^5 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).
**RESULTS AND DISCUSSION**

*Primer development.* TheRal*flf*C primer gave a single product of 724 bp not only with DNA of *R. solanacearum* but also with *R. piettii* and with four environmental isolates identified by FAME as *R. piettii* 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] DSMZ29544), and group F (*R. piettii* 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*flf*C sequences with 36 homologous *flf* sequences obtained from the GenBank database, two of them being published *R. solanacearum* *flf* 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 DSM5944 and 1609. This primer system, denoted RsoL*flf*C, 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. piettii* 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*flf*C showed mismatches with the sequences of all nontarget bacteria for which *flf* 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*flf*C reverse primer, all *R. piettii* 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* *flf* sequences of strains GMI1000 (AL460078) and K60 (AF283285). Surprisingly, the nucleotide sequence similarities of *flf* sequences of all species of the related genus *Burkholderia* included in the alignments to the *R. solanacearum* *flf* sequences were very low, ranging from 30.2 to 34% sequence identity. In contrast, we assumed that the *flf* 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*flf*C. Furthermore, a 400-bp PCR product was obtained from the *Pseudomonas syzygi* strain JV1010 (one strain), whereas no PCR products were observed with the strains of *R. piettii* (five strains), *R. eutropha* (one strain), *R. basilisrix* (one strain), and *R. manitobolytica* (one strain). Hence, the PCR-based approach was most likely specific for *R. solanacearum*. False-positive reactions with bac-
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/H14061*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 of 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 Schönheld 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).

<table>
<thead>
<tr>
<th>Primers</th>
<th>5′ Rsol*flIC_for</th>
<th>3′ Rsol*flIC_rev</th>
<th>Position in <em>flIC</em> of GM11000</th>
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</thead>
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<tr>
<td><em>R. solanacearum</em> K60</td>
<td>CA............CG</td>
<td>GGGGCGCTTCAGGGAGGTC</td>
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<td>1095</td>
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<tr>
<td><em>R. solanacearum</em> 267</td>
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<td>GGGGCGCTTCAGGGAGGTC</td>
<td>1076</td>
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<tr>
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<td>GGGGCGCTTCAGGGAGGTC</td>
<td></td>
</tr>
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<tr>
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<td>GGGGCGCTTCAGGGAGGTC</td>
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<td></td>
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<td>Salmonella enteritidis</td>
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<td>GGGGCGCTTCAGGGAGGTC</td>
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<tr>
<td>Clostridium difficile</td>
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<td>CA............CG</td>
<td>GGGGCGCTTCAGGGAGGTC</td>
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Hybridization of Southern-blotted PCR products obtained with the Rsol\_flC primers by using a probe generated from a Rsol\_flC PCR of the introduced \textit{R. solanacearum} strain 1609 revealed that cell densities of \textit{R. solanacearum} down to ca. \(10^3\) cells g of soil\(^{-1}\) could be detected (Fig. 2B).

To demonstrate that Rsol\_flC PCR in combination with Southern hybridization is a useful tool for estimating unknown concentrations of \textit{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 \(10^6\) \textit{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 (\(10^6\) \textit{R. solanacearum} cells g of soil\(^{-1}\)) and 62 days after inoculation (unknown titer of \textit{R. solanacearum}) was used to amplify the 400-bp \textit{R. solanacearum}-specific fragment. A decline of \textit{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 \textit{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\_flC PCR (\(10^2\) \textit{R. solanacearum} cells g of soil\(^{-1}\)) (Fig. 3a). Strong hybridization signals were observed at \(10^3\) cells g of soil\(^{-1}\), but differences in relative intensities were still distinguishable (Fig. 3b). No hybridization signal was detected in uninoculated soil samples (data not shown).

The Rsol\_flC PCR combined with Southern hybridization was applied to detect \textit{R. solanacearum} of different biovars in a survey of soil samples from various geographic sites. Rsol\_flC 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 \textit{R. solanacearum} could not be detected, indicating that \textit{R. solanacearum} was either not present or was present at concentrations of <\(10^3\) cells g of soil\(^{-1}\).

Considering that the regulatory network \textit{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 flC fragment down to a level of \(10^3\) \textit{R. solanacearum} cells g of soil\(^{-1}\), it can be assumed that the pathogen is detectable by Rsol\_flC PCR even if no symptoms of brown rot occur. A promising application of the Rsol\_flC PCR in combination with Southern hybridization is in monitoring the fate of the pathogen over time. Shifts in the \textit{R. solanacearum} population size can be detected by quantifying the Rsol\_flC PCR product, for example, by real-time PCR. Thus, one of the main

![FIG. 3. (a) Agarose gel of Rsol\_flC PCR products amplified from soils from a field experiment in The Netherlands obtained 0 or 66 days after inoculation with \textit{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\_flC probe derived from flC PCR of strain 1609.](http://aem.asm.org/)
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-fltC fragment is detectable from strains which exhibit limited virulence in planta.

Variability within the 400-bp Rsol-fltC fragment. From sequence alignments, it appeared that the 400-bp fltC fragment of all R. solanacearum strains sequenced in the present study corresponds to the region between positions 696 and 1095 of the fltC sequence of R. solanacearum GM11000. As representatives of three different biovars, fltC sequences of the six R. solanacearum strains were analyzed: DSM9544 and K60 (biovar 1); 1609, 1737, and 267 (biovar 2); and GM11000 (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. GM11000 (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 GM11000 were more pronounced: strain 1609 differed from DSM9544 in 15 positions and from K60 in 7 positions. GM11000 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 fltC 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 fltC 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 fltC fragments in DGGE. A single strong and sharp band was observed in DGGE of Rsol-fltC 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-fltC 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 corresponding to 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-fltC PCR-DGGE offers an alternative method to
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 sufficient 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. picketti*. The PCR system can be used for cultivation-independent detection of the pathogen in DNA directly extracted from environmental samples. Since

**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. picketti* DSM6297 as an outgroup. (B) Sequence comparison of the 400-bp fliC fragments of strains belonging to different biovars.
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 *Rsol* flaIC 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 flaIC genes of *R. solanacearum* strains under different conditions in soil by *Rsol* flaIC PCR amplification of reverse-transcribed RNA that was directly extracted from soil.

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