Detection of *Ralstonia solanacearum* by Loop-Mediated Isothermal Amplification

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ABSTRACT

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Ralstonia solanacearum is a pathogenic bacterium that causes wilt in over 200 plant species. Here we report a rapid and sensitive detection of *R. solanacearum* using an isothermal method for copying DNA known as loop-mediated amplification (LAMP). A set of four primers was designed to replicate the gene coding for the flagellar subunit, *fliC*, and conditions for detection were optimized to complete in 60 min at 65°C. Magnesium pyrophosphate resulting from the amplification reaction could be detected optically as an increase in the solution turbidity, and the DNA products spread in a reproducible ladder-like banding pattern after electrophoresis in an agarose gel. Replication of the *fliC* gene was detected only from *R. solanacearum*. The detection limit of this LAMP assay was between 10^4 to 10^6 colony forming units/ml, and the technique may be useful for developing rapid and sensitive detection methods for the *R. solanacearum* pathogen in soil and water.

Additional keywords: gene-based diagnostics.

Ralstonia solanacearum Yabuuchi (Smith) is a pathogenic bacterium that causes wilt in over 200 plant species. Host plants include many important crops such as potato, tomato, banana, peanut, and ginger. Bacterial wilt from *R. solanacearum* results in great economic losses worldwide. Disease incidences of 15 to 55% have been reported in fresh market tomato in Taiwan, causing losses exceeding 12 million U.S. dollars annually (6). In Hawaii, ginger production was reported to have suffered losses of over 50% during 1998 and 1999 (34). The bacterium is well adapted to life in soil, where it waits for a host plant to inhabit, and there is no effective chemical control for *R. solanacearum*. In recent years, the increasing number of sites in Europe infested with potentially cold-adapted strains of *R. solanacearum* dramatically enhanced the threat posed to European potato crops (9,20, 30,32). Therefore, rapid early detection of this pathogen not only in tubers but also in soil or soil-related habitats is essential for disease management in the field to prevent losses and further pathogen spread.

R. solanacearum has been described as a species-complex, which was defined as a cluster of closely related strains with phenotypic and genotypic variation within the species (5). Based on 16S rDNA sequence analysis, the *R. solanacearum* species-complex was described as including two closely related organisms, the blood disease bacterium (BDB) and *Pseudomonas syzygii* (26). Bacterial identification has often been predicated on analysis of rDNA sequence, so that a large amount of sequence data is available which has been used to reassess phylogenetic relationships. Several PCR-based methods for the detection of *R. solanacearum* have been described in the literature based on ribosomal gene sequences (2,4,22,25,29,31). However, rDNA sequences from closely related bacteria are highly conserved, limiting the value of rDNA analysis for studies of population genetics, epidemiological analysis, or the development of strain-specific detection methods (11). For detection of *R. solanacearum*, more discriminating primers have been used targeting functional genes such as endoglucanase (4), *hrpB* (23), or a random fragment thought to be species specific (12).

The *fliC* gene coding for the flagellar subunit protein flagellin was used to develop a highly specific and sensitive polymerase chain reaction (PCR)-based detection system for *R. solanacearum* (24). Flagellin has a distinctive domainal structure, comprising conserved N- and C-terminal regions. A central domain may vary considerably in both amino acid sequence and size, and is widely understood to be responsible for flagellar antigenic variability. By comparing the flagellin gene sequences from a number of related strains it is frequently possible to design oligonucleotide primers specific for N-terminal and C-terminal conserved regions. The predicted *fliC* protein of *R. solanacearum* also had well-conserved N- and C-terminal regions, separated by a divergent central domain that may allow design of *R. solanacearum* subspecies-specific primer sets (27).

PCR methods are available and widely used for detection of *R. solanacearum* because of their sensitivity and specificity. In this study we sought alternative amplification methods that could be used in the field to facilitate detection without thermal cycling equipment. Loop-mediated amplification (LAMP), first described by Notomi et al. (19), was identified as a prospective method to achieve gene replication isothermally and without requiring denaturation of the template DNA. The LAMP method requires a set of four specially designed primers that recognize six distinct sequences and relies on autocycling strand displacing DNA synthesis by the *Bst* DNA polymerase large fragment. When combined with reverse transcription, this method can also amplify RNA sequences with high efficiency (7,21). Furthermore, single-stranded DNA can be isolated from LAMP products (18).

The primers essential for the LAMP reaction are the inner primers (FIP and BIP) and the outer primers (F3 and B3), and the reaction is initiated by inner primer (either FIP or BIP) hybridi-

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zation to its respective priming site (F2c or B2c) on the target DNA. The outer primer (F3 or B3) secondarily hybridizes to its priming site (F3c or B3c) on the target DNA and initiates synthesis of new complementary sequence that displaces DNA sequences already extended from the inner primer. The result is a DNA sequence which can form stem-loop structures at both ends. This autoprimed "dumb-bell" structure is the starting material for LAMP auto-cycling amplification. Recently, it has been reported that the LAMP reaction can be accelerated using additional primers, termed loop primers (17), which hybridize to sections of the loop which were transcribed from the target DNA template. This additional priming accelerates the reaction and improves the selectivity because it requires transcription of the correct starting material. The LAMP reaction can be conducted under isothermal conditions ranging from 60 to 65°C. The amplification products are stem-loop DNAs with several inverted repeats of the target, exhibiting cauliflower-like structure with multiple loops.

One of the characteristics of LAMP is its ability to rapidly synthesize a large amount of DNA. Accordingly, a large amount of by-product, pyrophosphate ion, is produced yielding a white precipitate of magnesium pyrophosphate and allowing rapid visual confirmation of the reaction (16). As in traditional PCR, the amplification products generated by LAMP can be verified with gel electrophoresis. The LAMP reaction produces many bands of different sizes, forming a ladder of DNA fragments of 100 bp and larger. Production of the bands depends on the presence of the inner primers, the template, and the DNA polymerase. Specific amplification can be distinguished from nonspecific amplification by differences in the ladder pattern. Restriction digests and sequencing are also useful in confirming the structure of the amplified products. In this study, we evaluated the performance of the LAMP method targeting the *fliC* gene for detection of *R. solanacearum*.

MATERIALS AND METHODS

DNA preparation. Genomic DNA samples used for evaluation of primer specificity were prepared from 19 different strains of *R. solanacearum*, five strains of BDB, and three nontarget bacteria, including one from the genus *Ralstonia* (*Ralstonia eutropha* strain H16), one common soilborne bacterium (*Enterobacter* strain A5150), and one plant pathogen (*Erwinia carotovora* subsp. *carotovora* strain CC26). One or two colonies from each strain were suspended in 100 µl of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 1.2% Triton X-100) and boiled for 10 min. The crude lysates were used as LAMP templates. Purified genomic DNAs were isolated with a commercial kit (Wizard Genomic DNA Purification Kit, Promega Corp., Madison, WI) according to the manufacturer's instructions. DNA concentrations were quantified photometrically (absorbance measurements at 260 and 280 nm with Nanodrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., Rockland, DE).

Primer design for LAMP. Four primers used for LAMP reactions were designed to target the *fliC* sequence of *R. solanacearum* strain GMI1000 (GenBank No. NC 003295, 003296). The structure of the LAMP primers and their complementarity to target DNA used in this study is shown in Figure 1. A forward inner primer (FIP) consisted of F1c (the complementary sequence

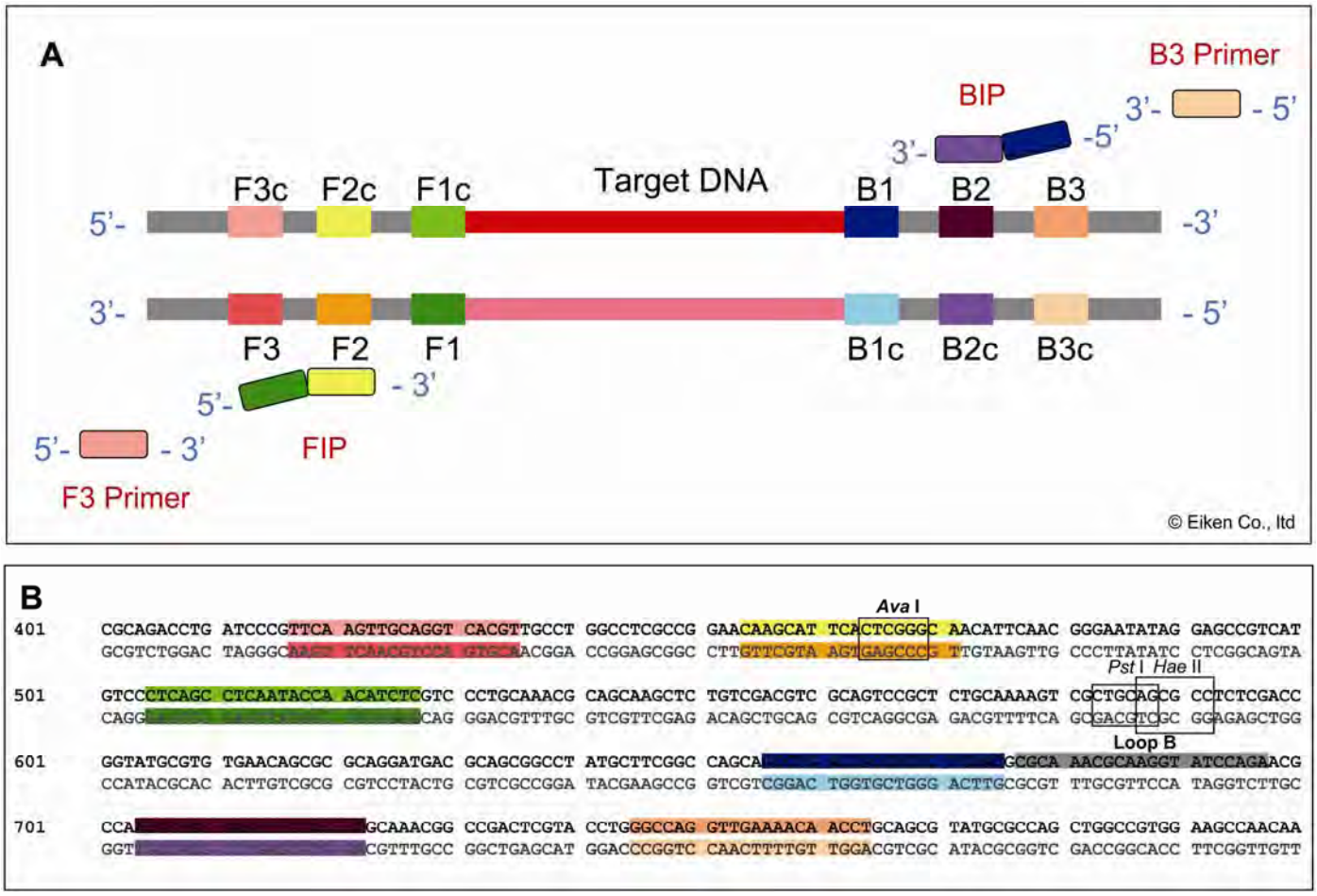

Fig. 1. Design of **A,** loop-mediated amplification (LAMP) primers, and **B,** *Ralstonia solanacearum* target DNA fragment. The target gene codes for the flagella subunit *fliC* of *R. solanacearum* strain GMI1000. The different colors correspond to the different primer sequences of the LAMP primer set shown in **A**. Restriction sites of enzymes *Ava*I, *Hae*II, and *Pst*I, which were used to confirm the LAMP products, are shown in rectangular boxes.

of F1) and F2, and a backward inner primer (BIP) consisted of B1c (the complementary sequence of B1) and B2. The outer primers F3 and B3 are required for initiation of LAMP reaction, and an additional loop primer was used in order to accelerate the process. To preclude the formation of hairpins or primer dimers that might result in false-positives, primer design software (PrimerExplorer V3, Eiken Co., Ltd., Japan) was used to identify a set of working primers and to ensure that specific primer combinations would not self hybridize. The software MFOLD (35), was used to verify the primer folding predictions. The resulting LAMP rsfliC primer set binds to an unambiguous region of the *fliC* gene that is unique from the binding sites of reported PCR primers such as Ral_*fliC*, Rsol_*fliC* primers (24). The sequences of each primer are shown in Table 1.

LAMP reaction. LAMP reactions were performed in 25 μl (total volume) reaction mixtures containing 1.6 μM FIP and BIP, 0.2 μM concentrations of the F3 and B3 primer, 0.4 μM concentrations of the loop B primer, 400 μM deoxynucleoside triphosphates (dNTPs), 1.0 M betaine (Sigma-Aldrich Corp, St. Louis, MO), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Triton X-100, and template DNA. The reactions were carried out in 0.2-ml microtubes, using a thermal cycler for temperature control. The mixtures were heated to 95°C for 5 min, and then chilled on ice prior to addition of 8 U of *Bst* DNA polymerase large fragment (New England Biolabs, Inc., Beverly, MA). Immediately after addition of the polymerase the mixture was incubated at 65°C for 60 min. Termination of the reaction was achieved by heating to 80°C to denature the polymerase.

Analysis of LAMP products. The amplified products were electrophoresed at 85V for 90 min through 2% agarose gel (1× Tris-acetate-EDTA), followed by staining with ethidium bromide, using appropriate size markers (Hyper ladder II; Bioline USA, Inc., Randolph, MA). In addition, to confirm the structure, the amplified products were digested with several restriction endonucleases and their sizes were analyzed by electrophoresis. Based on analysis of the predicted amplified gene sequence (Fig. 1B), cleavage at *Ava*I restriction site located on the FIP primer site would cause the LAMP product to be cut into 473-bp fragments by *Ava*I digestion. Likewise, digestion by *Hae*II would result in 225-bp predicted fragment size and digestion by *Pst*I would result in 215-bp predicted fragment size. To confirm these predictions, 2.0 μl of LAMP products digested with restriction enzyme were also subjected to electrophoresis on a 2% agarose gel. For comparison of the LAMP amplification to traditional PCR methods, the *fliC* gene was amplified using the primers and protocols as described in Schonfeld et al. (24).

Direct analysis of LAMP products. For visual assessment, white turbidity of the reaction mixture by magnesium pyrophosphate (by-product of LAMP) was detected by simple visual assessment.

Sensitivity of LAMP. A pure culture of *R. solanacearum* strain GMI1000 was plated on modified triphenyltetrazolium chloride (TZC) medium (0.8% peptone, 0.1% yeast extract, 1.8% agar, and 0.001% TZC) and incubated for 48 h at 28°C. One colony was transferred and grown in PY medium (0.5% dextrose, 1.0% peptone, 1.7% agar, and 0.001% TZC) at 28°C for 24 h. The culture was diluted (approximately 10^{-1} dilution) with distilled water in a test tube to give a predetermined optical absorbance at 600 nm (A_{600} of 0.1; approximately 10^8 CFU/ml) as a stock sample. A 10-fold dilution series (10^{-1} to 10^{-7}) in distilled water was made from the stock and plated onto TZC medium to estimate the colony number. Ten microliters of each dilution were further diluted by the addition of 80 μl of distilled water and 10 μl of 10× concentrated TE buffer (100 mM Tris-HCl pH 8.0, and 10 mM EDTA pH 8.0), incubated at 95°C for 10 min, and centrifuged at 18,120 × *g* and 4°C for 5 min. Five microliters of supernatant from each sample in TE buffer was then used for the LAMP process. Three replicates of each dilution were tested to assess the sensitivity of LAMP reaction.

To test the efficacy of the LAMP reaction in mixed cultures, supernatants from cultures of *R. solanacearum* strain GMI1000 were mixed with those from cultures of *Enterobacter* strain A5150 prior to assay with the LAMP process. Ten-microliter aliquots of the *R. solanacearum* cultures (from undiluted stock or from a 10^{-1} dilution in TE buffer) were mixed with different volumes (10 to 80 μl) of a culture of *Enterobacter* strain A5150, and subsequently diluted in TE buffer to the desired concentration of *R. solanacearum*. The mixed cultures were incubated at 95°C for 10 min, and centrifuged at 18,120 × *g* and 4°C for 5 min. Five microliters of the resulting supernatants was then assayed using the LAMP process, taking three replicates of each mixed culture.

Application of LAMP for effluent water from wilted edible ginger plant. Edible ginger plants (*Zingiber officinale*) infected by *R. solanacearum* strain A4515 were used to demonstrate the application of LAMP technology in a natural media.

Three-month-old ginger plants grown in soil collected from a ginger farm on the island of Hawaii were inoculated by pouring 25 ml of a culture of *R. solanacearum* A4515 strain (approximately 10^8 CFU/ml) over a wound cut into the rhizome with a scalpel. The plants were kept in pots in the greenhouse under regular sunlight and irrigated daily with 250 ml of water. After plants exhibited symptoms of bacterial wilt, drainage water (approximately 100 ml) was collected after each daily irrigation for laboratory analysis.

A filtration technique was used with the drainage water to concentrate the bacteria and remove inhibitors of LAMP reaction. A total of 1.0 ml of the sample was filtered on a commercial column with a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Ultra-free MC GV column, Millipore Corp., Bedford, MA) centrifuged at 1,000 × *g* for 2 min. To remove contaminants, the filter was then washed by applying 1.5 ml of TE buffer to the column and centrifuging at 1,000 × *g* for 2 min. The column was then crushed with a pair of pliers and the PVDF membrane was cut out with a set of sterilized forceps. The extracted membrane was then vortexed with 50.0 μl of TE buffer, incubated at 95°C for 5 min, and centrifuged at 20,400 × *g* for 5 min. Five microliters of the resulting supernatant was then assayed using the LAMP reaction as described previously.

Viable colony count of *R. solanacearum* from drainage water samples. Drainage water samples collected from daily irrigation were applied for viable colony count. A 10-fold dilution series (10^{-1} to 10^{-4}) in phosphate buffer (0.01 M phosphate, pH 6.9) was made from each sample and 100 μl of each dilution was plated on a modified semiselective agar medium (SMSA), South Africa, (1.0% peptone, 0.5% glycerol, 0.1% casein hydrolysate, 1.7% agar, 0.01% polymyxin B sulfate, 0.005% TZC, 0.0025% bacitracin, 0.0025% cycloheximide, 0.0005% chloromycetin, 0.0005% crystal violet, and 0.00005% penicillin) selective for *R. solanacearum* (3).

Measurement of turbidity. Optical measurements were taken on 2.0-μl samples using a commercial spectrophotometer (Nano-

TABLE 1. The rsfliC primer sequences for loop-mediated amplification replication of the flagellar gene *fliC*[a]

Primer	Sequence
rsfliC F3	5′-TTCAAGTTGCAGGTCACGT-3′
rsfliC B3	5′-AGGTTGTTTTCAACCTGGCC-3′
rsfliC FIP	5′-GAGATGTTGGTATTGAGGCTGAGCAAGCATTCA-CTCGGGCA-3′
rsfliC BIP	5′-GCCTGACCACGACCCTGAACAGGTACGAGTTCG-CACCGT-3′
rsfliC loop	5′-CGCAAACGCAAGGTATCCAGA-3′

[a] FIP consists of F2 and F1c sequences as shown on Figure 1. BIP consists of B2 and B1c sequences.

drop ND-1000 Spectrophotometer). Absorbance spectra of positive LAMP reactions diluted in the unreacted mixture showed that the LAMP products could be detected at any wavelength in the visible spectra, and showed especially strong absorbance towards the lower wavelength end of the spectrum (data not shown). For further analysis, a wavelength of 400 nm was used in order to avoid interference from the strong UV absorbance of the single nucleosides in the reaction mix. Absorbance readings were recorded for LAMP reaction mixtures from samples of the drainage water in the infected ginger experiments, and corrected for the background absorbance of a reaction mixture without template DNA.

Statistical data analysis. To determine an absorbance threshold for classification of positive LAMP reactions, 15 samples of sterile water were assayed using the LAMP reaction. The classification threshold was taken as three standard errors of the observed absorbance values above the mean absorbance values for all of the negative controls. This threshold corresponds to the value at which at least 99% of all negative samples will correctly be classified as negative.

To determine the detection limit for LAMP using absorbance measurements, a commercial graphing software (SigmaPlot 8.0, Systat Software Inc., San Jose, CA) was used to fit the observed absorbance data to the corresponding colony counts from the original samples according to a simple sigmoidal equation:

$$A_{400} = \frac{a}{1 + e^{\left(\frac{x_0 - x}{b}\right)}}$$

where A_{400} is the absorbance at 400 nm; a, b, and x_0 are fitted coefficients; and x equals the logarithm of the colony count y in CFU/ml: $x = \log_{10}(y)$.

The fitted colony count in CFU/ml corresponding to the absorbance value equal to three standard errors of the regression was taken as the detection limit, which by definition is the minimum amount of bacteria required to ensure that a positive reaction will be observed at least 99% of the time.

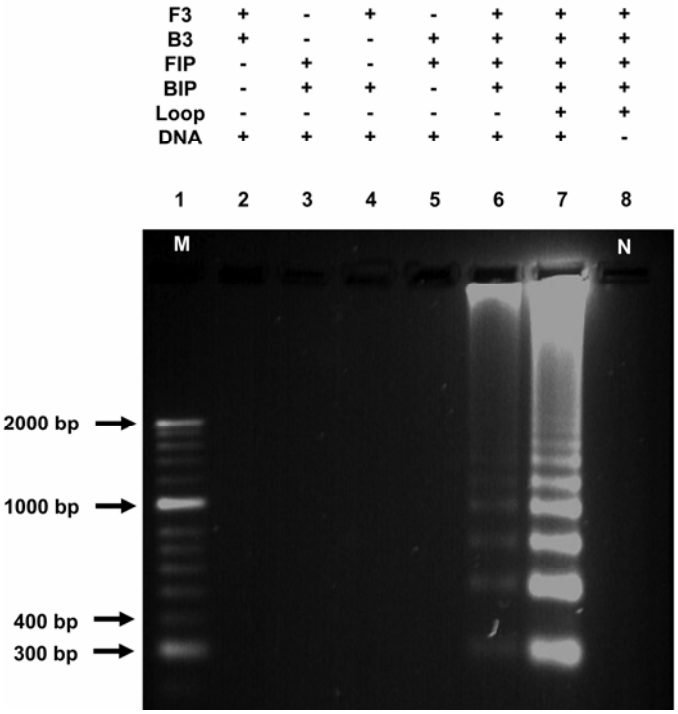

Fig. 2. Primer (F3, B3, FIP, BIP, and Loop) combinations applied to *Ralstonia solanacearum* genomic DNA (DNA) to determine the primers required for loop-mediated amplification (LAMP) and to observe LAMP acceleration by the loop primer. One microliter aliquots (out of 25 µl total in the reaction solutions) were applied to 2% agarose gel electrophoresis and stained with ethidium bromide. All four primers F3, B3, FIP, and BIP are required for successful LAMP reaction (lane 6). The LAMP reaction was accelerated by adding a loop primer (lane 7). Lane 1 (M) has size markers (BIOLINE), and lane 8 (N) is a control with no template DNA.

RESULTS

Primers required for LAMP reaction. A successful LAMP reaction with species-specific primers produced many bands of different sizes (Fig. 2) in a reproducible ladder-like pattern. When the sample tube did not contain selected target DNA or any of the primers, F3, B3, FIP, and BIP, no amplification was observed. To confirm that the amplification products had the predicted DNA structures, they were digested with restriction enzymes and the sizes of the fragments were analyzed by electrophoresis. *Ava*I cuts on the F2 fragment of *fliC* amplicons, and *Hae*II and *Pst*I both cut between the F1 (F1c) and the B1c (B1) fragments. The sizes of the fragments generated after digestion corresponded to the sizes predicted theoretically from the expected DNA structures: 473 bp for *Ava*I; 225 bp for *Hae*II, and; 215 bp for *Pst*I (Fig. 3).

Visual detection of LAMP reaction. Iwamoto et al. (8) reported that a LAMP reaction mixture containing amplified fragments turned green after the addition of SYBR Green I, whereas a solution with no amplicons retained the original orange color of SYBR Green I. Thus, the results of the LAMP reactions could be confirmed visually. We were unable to detect any differences between successful and unsuccessful amplifications based on visual inspection of SYBR Green I. We were, however, able to visually confirm successful LAMP reactions by observation of a magnesium pyrophosphate precipitate (Fig. 4). One of the characteristics of LAMP is its ability to amplify a large amount of target DNA rapidly, making it easy to identify through by-products of DNA strand synthesis such as pyrophosphate. The increase of white turbidity can be measured quantitatively in real-time (1,13–16,28,33).

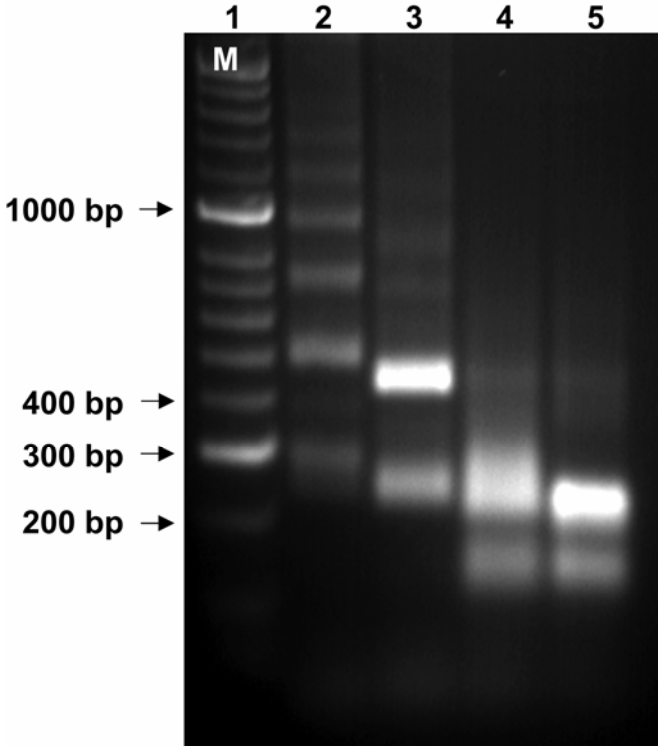

Fig. 3. Restriction enzyme digestion of loop-mediated amplification (LAMP) products. The LAMP products (lane 2) were digested by *Ava*I (lane 3), *Hae*II (lane 4), and *Pst*I (lane 5), applied to 2% agarose gel electrophoresis, and stained with ethidium bromide. Expected sizes of digested LAMP products were as follows: *Ava*I, 473 bp; *Hae*II, 25 bp; and *Pst*I, 215 bp. Lane 1 (M) has size markers (BIOLINE).

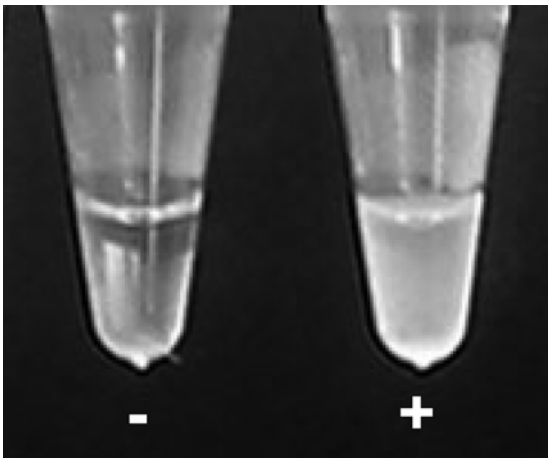

Fig. 4. Visual assessment of DNA proliferation by loop-mediated amplification. The turbidity indicating a positive reaction (+) is caused by insoluble pyrophosphate ions produced during DNA synthesis, whereas the negative control (–) remains clear.

Analysis of primer selectivity. The LAMP studies reported previously were conducted with purified genomic DNA from different strains of *R. solanacearum* (races 1, 2, 3, and 4, and *R. solanacearum* BDB) and other soilborne bacteria strains (*E. carotovora* subsp. *carotovora* strain CC26, *Enterobacter* strain A5150, and *R. eutropha* strain H16). A summary of all bacterial strains tested with the LAMP reaction is given in Table 2. With the exception of all five blood-disease strains, all strains tested that belong to the species complex of *R. solanacearum*, including the Race 3 strains, gave an amplification product with a ladder-like pattern typical of LAMP, whereas LAMP amplification products were not observed following LAMP reaction with other soilborne bacteria strains (Fig. 5).

Sensitivity of LAMP. The detection limit, determined as the minimum amount of culturable bacteria required to induce an observable LAMP reaction, was determined to be between 1.1×10^4 and 1.3×10^6 CFU/ml for *R. solanacearum* strain GMI1000 based on visual detection of amplicons run through an agarose gel. The detection limit for other positive *R. solanacearum* strains varied from 6.0×10^4 to 1.5×10^6 CFU/ml (data not shown). In

TABLE 2. A summary of strains used in this study

Species	Strain	No. of strains	Race	Host plants (no. of strains)	LAMP[a]
Ralstoia solanacearum		4	1	Tomato (2), squash (1), peanut (1)	+
		6	2	Banana (5), heliconia (1)	+
		5	3	Potato (2), geranium (2), *S. phurjea* (1)	+
		4	4	Ginger (4)	+
		5	BDB	Banana (5)	–
Ralstonia eutropha	H16	1	...	N/A	–
Erwinia carotovora subsp. *carotovora*	CC26	1	...	Potato	–
Enterobacter	A5150	1	...	N/A	–

[a] Loop-mediated amplification.

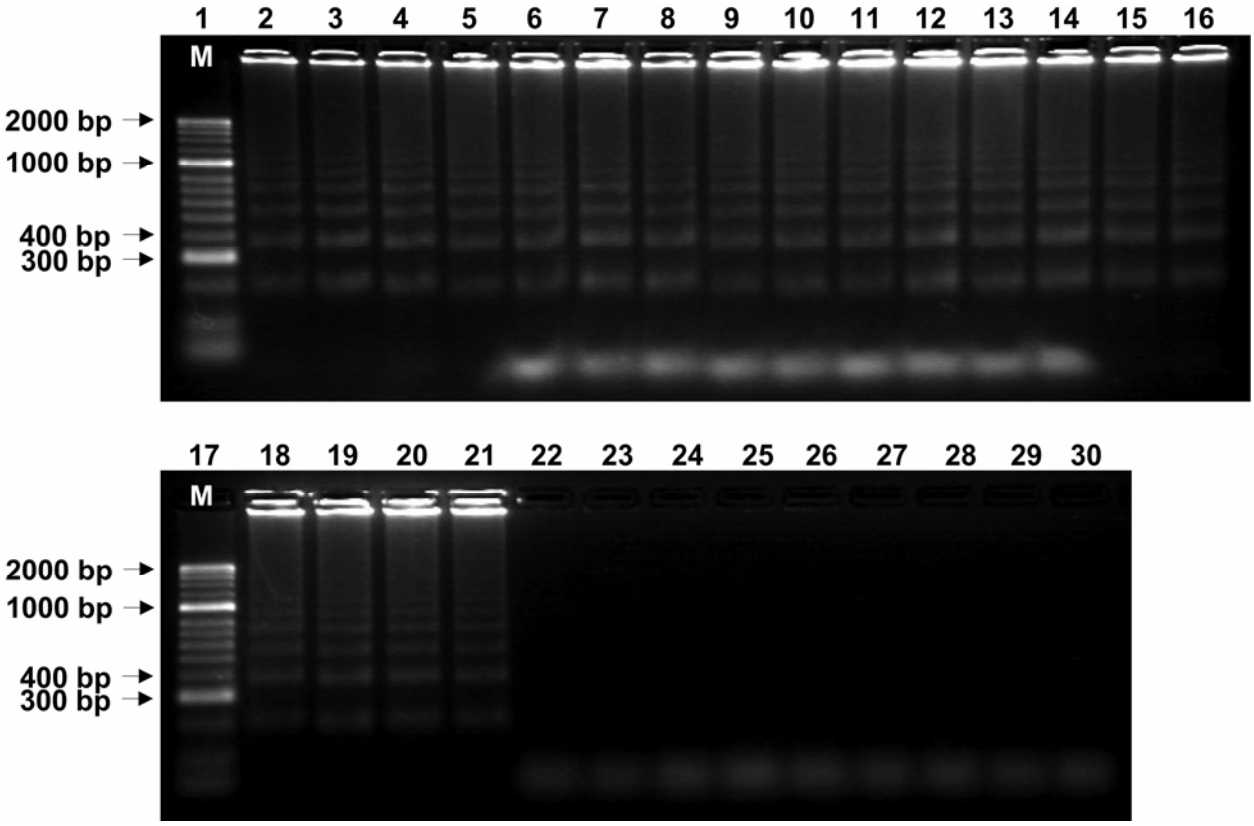

Fig. 5. Gel electrophoresis applied to loop-mediated amplification products from different strains of *Ralstonia solanacearum* (lanes 2 to 5: race 1; GMI1000, A3450, A3777, and A3780. Lanes 7 to 12: race 2; A3381, A3908, A4125, A4126, A4127, and A4128. Lanes 6 to 16: race3; A5286, A5289, A5290, and A5291. Lanes 18 to 21: race 4; A3786, A4515, A5190, and A5192), and nonspecific bacteria (lanes 22 to 26: BDB strains; A4606, A4608, A4609, A4611, and A4612. Lane 27: *Ralstonia eutropha*; H16. Lane 28: *Erwinia carotovora* subsp. *carotovora*; CC26. Lane 29: *Enterobacter*; A5150. Lane 30: negative control. Lanes 1 and 17 have size markers (BIOLINE).

mixed cultures the detection limit of the LAMP reaction was not affected by the presence of *Enterobacter* strain A5150, even at concentrations of up to 1000× that of *R. solanacearum*.

Application of LAMP for environmental samples. We observed that LAMP could reliably be used to amplify the target gene from effluent water samples with various concentrations of *R. solanacearum*. The filtration step was used to preconcentrate the pathogen and to remove inhibitors of the LAMP reaction. Statistical analysis of the turbidity data showed that the detection limit based on quantitative absorbance measurements was 10^5 CFU/ml, although positive test results were observed for samples down to near 10^2 CFU/ml (Fig. 6).

DISCUSSION

We used a novel nucleic acid amplification method called LAMP for detection of *R. solanacearum*. This technology has advantages over PCR and immunological methods. The most significant advantage of LAMP is its ability to amplify specific sequences of DNA at 65°C without thermal cycling. The reaction itself occurs within 60 min and only 3 h are required for a complete detection from cultured cells. These characteristics confer some advantages for incorporation of LAMP into a simple disposable detection system (10) for field detection of *R. solanacearum*. We developed an *R. solanacearum* species-specific LAMP method by designing a primer set "rsfliC" consisting of five primers; F3, B3, FIP, BIP, and loop. The sequences of the rsfliC primer set were designed based on the published sequence of *R. solanacearum* strain GMI1000 (GenBank nos. NC 003295, and 003296), targeting the flagellar subunit, flagellin gene *fliC*.

LAMP amplification products using the rsfliC primer set had multiple fragment sizes that were typical for the LAMP process. Amplification of the target sequence was confirmed by restriction enzyme digestion and DNA sequencing (data not shown) of the amplicons. The rsfliC LAMP primer set was shown to be *R. solanacearum* species specific as it resulted in amplification from *R. solanacearum* strains, but not from the related BD bacterial strains or other soilborne bacteria species such as *E. carotovora* subsp. *carotovora* strain CC26 and *Enterobacter* sp. strain A5150. Notably, the primer set was positive against all tested race 3 strains of *R. solanacearum* which are considered of high international impact. This result is consistent with results of PCR amplification of the same gene using Ral_*fliC* or Rsol_*fliC* primers, indicating that the flagellin gene *fliC* is a good candidate to target for species identification.

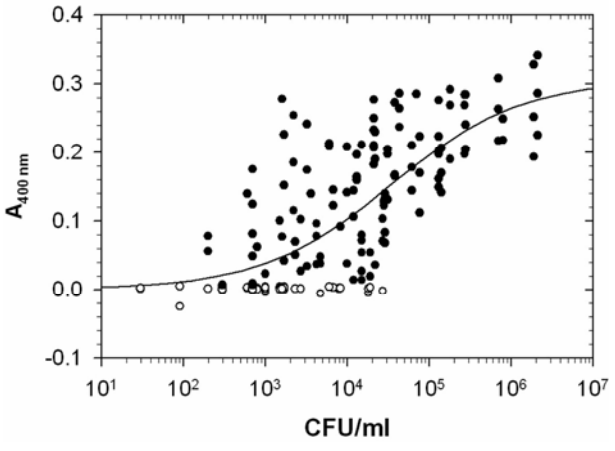

Fig. 6. Absorbance of loop-mediated amplification reaction mixture at 400 nm versus *Ralstonia solanacearum* population of collected drainage water samples. Solid circles indicate samples classified as "positive" for *R. solanacearum*, and open circles indicate samples classified as negative based on optical absorbance measurements. The line indicates the best fit sigmoidal curve of the form of equation 1 (R^2 = 0.54; standard error = 0.066 absorbance units; detection limit = 10^5 CFU/ml; a = 0.307; b = 0.791; x_0 = 4.541).

As in any DNA polymerization reaction, the LAMP process results in the insoluble by-product magnesium pyrophosphate. However, the high yield of the LAMP reaction results in a highly turbid solution with a large amount of precipitate that can be easily recognized by visual inspection. Our results confirm that a successful LAMP reaction could be identified visually or with the aid of a spectrophotometer, with only marginal improvement in classification accuracy using the analytical instrument (data not shown).

LAMP has high specificity and speed, attributed to autocycling amplification under isothermal conditions by using four primers. The rsfliC LAMP primer set used for *R. solanacearum* detection is highly sensitive, as it detects approximately 10^4 to 10^6 CFU/ml of *R. solanacearum* strain GMI1000 in less than 1 h under isothermal conditions. The visual assessment of LAMP products demonstrates the possibility of applying this method for field detection. However, more definitive confirmation methods such as restriction analysis, DNA hybridization, or DNA sequencing are still needed. Recently, protocols for isolation of single stranded DNA from LAMP products were reported (18). This may facilitate, for example, the adaptation of LAMP for use with an electrochemical DNA hybridization sensor (10) for more absolute confirmation in the field.

ACKNOWLEDGMENTS

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