Loop-Mediated Isothermal Amplification and Polymerase Chain Reaction Methods for Specific and Rapid Detection of *Rhodococcus fascians*

M. Serdani, M. Curtis, M. L. Miller, J. Kraus*, and **M. L. Putnam,** Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331

Abstract

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Rhodococcus fascians is a phytopathogenic actinobacterium which causes leafy galls and other plant distortions that result in economically significant losses to nurseries producing ornamental plants. Traditional assays for detection and identification are time-consuming and laborious. We developed a rapid polymerase chain reaction (PCR) diagnostic assay based on two primer pairs, p450 and fas, which target the *fasA* and *fasD* genes, respectively, that are essential for pathogenicity. We also developed a faster, more convenient, loop-mediated isothermal amplification (LAMP) assay targeting the *fasR* gene, which regulates expression of virulence genes. Both assays were evaluated for sensitivity and specificity in vitro and in planta. The p450 and fas primers amplified DNA only from pure cultures of pathogenic reference isolates of *R. fascians.* Nonpathogenic isolates and 51 other plant-associated bacteria were not amplified. The PCR primers correctly detected pathogenic *R. fascians* from 73 of 75 (97%) bacterial strains isolated from naturally infected plants. The PCR assay correctly discriminated between pathogenic *R. fascians* and other bacteria in 132 of 139 (95%) naturally infected plants, and in 34 of 34 (100%) artificially inoculated plants. The fas primers were slightly more accurate than the p450 primers. The LAMP assay accurately detected pathogenic *R. fascians* in 26 of 28 (93%) naturally infected plants and did not react with 23 asymptomatic plants. The LAMP primers also amplified product for DNA extracts of 40 of 41 bacterial strains isolated from plants with leafy galls. The detection limit of both the PCR and LAMP assays was approximately 10^3 CFU/30-µl reaction. These new tools allow fast, reliable, and accurate detection of *R. fascians* in vitro and in planta. The LAMP assay in particular is a significant advancement in rapid *R. fascians* diagnostics, and enables those with limited laboratory facilities to confirm the presence of this pathogen in infected plants.

The actinobacterium *Rhodococcus fascians* (Tilford) Goodfellow causes plant malformations such as leafy galls, witches'-brooms, shoot proliferation, adventitious growth, and distortion of underground storage tissues such as bulbs (24). *R. fascians* is a member of the family Nocardiaceae (suborder Corynebacterineae), and as such is Gram positive, does not form spores, and is variable in cellular morphology (nocardioform). This species is a pathogen of over 100 genera of primarily herbaceous hosts (19,24), most of which are grown as ornamentals. Cytokinins produced by the bacteria stimulate growth of dormant plant meristems, and cortical cells are reprogrammed to grow as new meristematic tissues (16). The combined effects of these hormonal perturbations are the formation of multiple shoot primordia, growth of adventitious tissues, leafy galls, and shoot proliferation, all of which disfigure plants grown specifically for their aesthetic appeal. Wholesale production nurseries have experienced significant financial losses due to either the cost of destroying diseased material and lost production time (24) or to implementing extraordinary disease-prevention tactics (e.g., installing filtration systems to purify irrigation water and disinfecting structures). *R. fascians* losses at one nursery were estimated at $150,000 over a 5-year period, and this is from a nursery with the most stringent phytosanitary standards. The pathogen lives as an epiphyte prior to tissue ingress and initiation of symptoms (3,11). Symptom expression may therefore be delayed for up to 6 months (12), which creates a difficult situation for growers who may be unknowingly maintaining infected plants.

Diagnosis of *R. fascians* is complicated by the similarity of its symptoms to other infectious agents, such as phytoplasmas, eriophyid mites, and other bacteria (11,24,26). In very young plants propagated from cuttings or tissue culture, the crown gall bacterium *Agrobacterium tumefaciens* can, in some hosts, cause stunted shoots, which emerge from enlarged masses of tissues, somewhat resembling a leafy gall. Additionally, symptoms produced by *R. fascians* can look very much like those produced by plant-growth regulators, which are used routinely in the industry to produce bushy, compact plants with good color that will not outgrow their pots prior to sale.

Our focus on *R. fascians* detection started in 2002, after the Oregon State University Plant Clinic received a request to test large numbers of plants for *A. tumefaciens*; however, the symptoms were clearly those of *R. fascians*. Traditionally, detection of *R. fascians* has been a difficult and time-consuming process. The pathogen grows slowly on nutrient media and may be quickly overgrown by other microflora. Isolation, purification, and confirmation by biochemical tests can take up to 4 weeks. Nonpathogenic forms of *R. fascians* are often present (13,24,35), and positive identification of such forms necessitates methods that can distinguish them from pathogenic strains. Inoculation to indicator hosts is the standard method to confirm pathogenicity, but this can take 2 to 4 weeks, the time required for unequivocal symptom development. To remedy this situation, we developed a rapid, specific, and sensitive polymerase chain reaction (PCR) assay for detection of the pathogen in planta.

Pathogenicity genes have been exploited in studies examining virulence (3–5,15,16,28,29), and may have utility for diagnostics. Pathogenicity genes are present at three different loci (*fas*, *att*, and *hyp*) on a linear conjugative plasmid, pFiD188 (4,5). The *fasciation* (*fas*) operon contains genes necessary for cytokinin biosynthesis, the products of which are essential for pathogenicity and symptom expression; inactivation of the *attenuation* (*att*) locus results in

Corresponding author: M. L. Putnam,
E-mail: putnamm@science.oregonstate.edu

*Deceased.

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reduced or attenuated leafy galls; whereas inactivation of one gene in the *hypervirulent* (*hyp*) locus results in increased virulence (4,15). The *fasD* region (a.k.a. *fas-1*) encodes an isopentenyltransferase and was the target for PCR primers developed to determine whether there was a consistent association of the presence of the gene with pathogenicity (27). While our work was in progress, PCR primers for eight gene regions, including *fasD*, were developed to characterize *R. fascians* virulence (21). However, none of these primers were tested in planta, nor were they evaluated as diagnostic tools.

In 2000, a novel means of highly specific DNA amplification, loop-mediated isothermal amplification (LAMP), was described (22). LAMP has been successfully applied to the rapid detection (within an hour) of numerous microbial pathogens, including phytopathogens (9,10,25,30,31,33,34). The reaction uses four to six primers, which simultaneously target six to eight nucleotide sequences, thereby enhancing specificity by minimizing nonspecific binding. The *Bst* polymerase used in the reaction has strand-displacement activity, thereby permitting target amplification at a constant temperature (e.g., 60 to 64°C). This eliminates the need for high-precision thermal cyclers and gel electrophoresis. DNA may be detected by addition of a double-stranded DNA-binding fluorochrome (e.g., PicoGreen) to the product or simply by visual evaluation (10) of the white precipitate by-product of amplification, magnesium pyrophosphate, which can also be quantified using a turbidimeter.

After years of diagnosing *R. fascians*–infected plants, it seemed that a LAMP assay would accommodate growers at wholesale nurseries who wanted their own means to assay suspect plants without the costs of purchasing specialized laboratory equipment. This paper describes the development and verification of PCR and LAMP assays for the detection of pathogenic *R. fascians*, and presents comparisons of their relative efficacy for diagnosing infection in planta.

Materials and Methods

Bacterial cultures and culture conditions. Plant-associated bacterial strains were either retrieved from freezer storage or collected as described below. *R. fascians* strains were maintained on Miller medium (MrM) consisting, in g/liter, of mannitol (10), casein (4), peptone (4), NH_4Cl (1), yeast extract (3), $MgSO_4 \cdot 7H_2O$ (0.3), NaCl (0.2), and KH_2PO_4 (0.5). The pH of the medium was adjusted to 6.5, and 15 g of agar was added prior to autoclaving for 20 min at 103.4 kPa. The sterile medium was cooled to 50°C, then 10 ml of sterile 2% cycloheximide (wt/vol) was added and mixed prior to pouring into petri dishes. Isolates were maintained in long-term storage at –80°C in Luria broth and 20% glycerol.

Isolation and identification of new bacterial strains. Plants with leafy galls, shoot proliferation, distortion, or other symptoms suggestive of infection by *R. fascians*, which were previously submitted to the Oregon State University Plant Clinic for diagnosis, were used for isolations. The isolates reported here were obtained from plant material submitted between 2002 and 2011. A sample of affected tissues was rinsed briefly with tap water to remove visible soil particles. Small pieces (3 to 5 mm in length) of symptomatic tissue were placed into each of two tubes containing 3 ml sterile 0.85% saline and 2% (wt/vol) cycloheximide, and incubated for 30 to 120 min at room temperature (21 to 23°C). The saline suspension was then streaked to plates of the semi-selective medium D2 (7) and incubated at 21 to 23°C. Plates were examined after 5 to 10 days. Domed, moist-looking, small, chrome-yellow colonies were selected, individually suspended in 3 ml of sterile deionized water (SDW), and streaked to MrM. Individual colonies were selected and re-streaked a minimum of three times or until apparently pure cultures were obtained, as determined by colony appearance and examination of stained bacterial smears at ×1,000.

All purified strains recovered from symptomatic plants were initially identified by colony morphology on D2, a positive Gram reaction, production of urease (14), and morphology at ×1,000 of 24-h cells grown at 27°C on tryptic soy agar. Individual cells of *R. fascians* are small rods, 0.5-0.9 × 1.5-4 µm, and are generally straight, but may be slightly clubbed at one end. Further identification was made by one or more of the following methods: substrate utilization using a commercially available system according to the manufacturer's directions (Biolog, version ML2, Biolog Inc., Hayward, CA); sequencing of a portion of the 16S rRNA gene; or assaying for virulence by inoculation to indicator plants.

Pathogenicity testing. Sweet pea (*Lathyrus odoratus*) or garden pea (*Pisum sativum*) have traditionally been used to determine virulence of *R. fascians* (11,12,20,32). To determine which species and cultivars were most suitable for assays, we used four pathogenic isolates of *R. fascians* to separately inoculate three cultivars of sweet pea (Elegance, Mrs. Collier, and Multiflora) and three of garden pea (Laxton Progress #9, Oregon Sugar Pod II, and Tall Telephone). Ten seeds of each cultivar were surface sterilized by first washing in running tap water for 1 h, then immersing in a 0.6% NaOCl solution for 15 min. The seeds were rinsed with SDW and placed onto moist, sterilized filter papers in sterile petri dishes to germinate. After germination, the seedlings were soaked in approximately 40 ml of a 10^8 CFU/ml suspension for 2 h. All seeds of one cultivar were then planted into a 1-gal pot containing a commercial peat-based potting mix (Sunshine SB 40, Sun Gro Horticulture Canada Ltd.) and incubated in the greenhouse at ambient temperature and light, supplemented with 16-h photoperiod (400 W sodium vapor lamps suspended 4 ft above the bench). Controls included peas inoculated with the nonpathogenic *R. fascians* isolate 02-416 and noninoculated seeds, which were immersed in water. Garden pea cv. Tall Telephone was less consistent in symptom expression at 4 weeks (*data not shown*) and so was not used further. Sweet pea seeds have a much thicker seed coat, take longer to germinate, and are more expensive than garden pea, so garden pea cvs. Laxton Progress #9 and Oregon Sugar Pod II were used for pathogenicity tests from 2002 through 2004.

Evening primrose, *Oenothera speciosa* 'Siskiyou', was also used as an indicator host, as it was more representative of plant samples from nurseries (24). For inoculations, *R. fascians* strains were grown for 2 to 3 days on MrM, and an aqueous suspension was adjusted to 10^8 CFU/ml (Klett colorimeter [clinical model, Scienceware, Pequannock, NJ] reading of 230 at 590 nm [blue filter]). For virulence testing, four plants in 4-in. pots were drenched with 25 ml of the suspension. Plants treated with the pathogenic strain A44a or water were used as positive and negative controls, respectively. Inoculated plants were covered with clear plastic bags to provide a moist environment for 2 weeks, after which the plants were evaluated for symptoms.

PCR detection of *R. fascians*. DNA template for PCR was prepared from either single bacterial colonies or symptomatic plant tissue. Bacterial strains were grown on MrM for 2 days, and an inoculating loopful of bacteria was suspended in 750 µl of molecular grade water (MGW) in a microfuge tube. Bacterial genomic DNA was then extracted using InstaGene matrix (Bio-Rad, Hercules, CA). Alternatively, template DNA was prepared by simple heat lysis of the cells. A sterile 10-µl pipette tip was touched to a single colony of bacteria, the cells were dislodged into 1.5 ml MGW, and heated at 100°C for 8 min. Both methods provided amplifiable DNA.

Two approaches were evaluated for detecting *R. fascians* on inoculated, symptomatic plant tissues: plant washes and tissue extracts. Plants were inoculated with known strains of *R. fascians* to confirm each method. The plant wash method involved incubating diseased tissue in a solution prior to assaying the liquid. Leafy galls initiated by *R. fascians* were aseptically removed from infected plants, lightly rinsed with tap water, and cut into pieces from 3 to 5 mm in length. Two pieces were placed into each of two tubes of 3 ml sterile 0.85% saline containing 2% (wt/vol) cycloheximide. After 1 to 3 days incubation at room temperature (21 to 23°C), the plant tissue was discarded from each tube and 1.4 ml of the incubation liquid was transferred to a 2-ml microfuge tube, to which 200 µl of InstaGene matrix was added for DNA extraction.

For the tissue extract method, the incubation step was omitted and DNA was extracted directly from symptomatic plant tissue.

Affected tissues were rinsed free of surface debris, and two subsamples of 100 to 200 mg each were homogenized using a FastPrep-24 (MP Biomedicals, Inc.) instrument for 40 s at a speed setting of 6.0. Total genomic DNA was isolated using a Fast DNA Spin Kit (MP Biomedicals, Inc.). To perform quality control checks on template preparations, "universal" primers (23) (Table 1) were used to amplify a 1,500-bp fragment of the bacterial 16S rRNA.

Primers for detecting *R. fascians* DNA were designed to target conserved regions of either the *fasA* (p450 primer set) or *fasD* (fas primer set) genes (GenBank Z29635; Table 1). A Basic Local Alignment Search Tool (BLAST) (1) analysis with each primer pair confirmed that only the *R. fascians* P450 monooxygenase or the isopentenyltransferase genes share sequence identity with both the forward and reverse primers of the p450 and fas pair, respectively.

PCR conditions were optimized by varying the annealing temperature, extension time, and amount of template used. One microliter of undiluted template DNA from plant washes, tissue extracts, or single bacterial colonies was used in 30 µl (total volume) of a PCR mix consisting of 22.8 µl of molecular grade water, 3.0 µl of 10× *Taq* buffer, 0.9 µl of 50 mM $MgCl_2$, 0.6 µl of a 10 mM mix of each dNTP, 0.6 µl of each primer (50 µM), 0.9 µl of a 1.2% bovine serum albumin solution, and 0.2 µl of 5 U/µl *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA). We used an MJ Research PTC 200 thermal cycler and a cycling profile of 94°C for 2 min followed by 33 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Ten microliters of product was then electrophoresed in 1.5% agarose gel at 100 V for 60 to 90 min and photographed after ethidium bromide staining. All sample assays included a nontemplate (MGW) control and DNA extracts of the known pathogenic strain A44a.

Primer specificity was tested using 51 strains of plant-associated bacteria and seven pathogenic and nonpathogenic *R. fascians* strains (Table 2). Template DNA was prepared by heat lysis as described above. From all 58 bacterial extracts, we observed a 1,500-bp amplicon with nonspecific 16S rRNA primers, indicating sufficient template DNA in the preparations. Sensitivity of the PCR primers was determined using a serial dilution of 2-day cultures of strain A44a added to plant tissues. Petunia (*Petunia* × *hybrida*) and evening primrose, two ornamentals commonly infected by *R. fascians* and useful as indicator hosts (17), were used to simulate naturally infected material and to assess whether PCR (or LAMP, below) is inhibited by plant extracts. One milliliter of a 10-fold serial dilution (10^{-1} to 10^{-8}) of each bacterial suspension was centrifuged at 6,000 rpm (Microfuge18 Centrifuge, Beckman Coulter) for 10 min, and 900 µl of supernatant was removed and discarded. The remaining 100 µl of resuspended cells for each dilution were added to 100 mg of leaf tissue, and total DNA was isolated using a Fast DNA Spin Kit. One microliter of the extract for each dilution was used as template in each of three assays. The concentrations of the dilutions were determined by triplicate plating 100 µl of the three most dilute cell suspensions onto MrM, and counting colonies after 2 days at 27°C.

A preliminary assay of the ability of our PCR primers to detect *R. fascians* in infected tissue was performed. Eight different pathogenic strains of *R. fascians* were inoculated to one or more cultivars of susceptible plants in duplicate. Plants used were *O. speciosa* 'Siskiyou'; *Campanula* 'Sarastro'; *Erysimum* 'Apricot Twist', 'Bowles Mauve', and 'Variagata'; *Veronica* 'Blue Fox'; *Tiarella* sp.; and *Iberis gilbraltarica*. A total of 34 plants were inoculated. Samples of symptomatic tissue were collected and suspended in sterile saline as described above (plant wash method), prior to DNA extraction and amplification. Samples were assayed using the *R. fascians*–specific p450 and fas primers, and the "universal" 16S primer set in separate PCR reactions.

The ability of the p450 and fas primers to detect *R. fascians* in naturally infected plants was determined by use on both symptomatic and asymptomatic Plant Clinic samples with either the plant wash method (samples received 2002 to 2007) or the tissue extract method (samples received 2008 to 2011; Table 3). Pathogenicity of a subset of the bacterial strains recovered from plants was determined by inoculation to garden pea (samples received prior to 2005) or evening primrose (samples received after 2005). For samples with no amplification, an aliquot of tissue extract was diluted 1:10 with MGW and re-assayed. Extracts of *Coreopsis*, *Echinacea*, *Erodium*, *Gaillardia*, *Heuchera, Lamium*, *Lavatera*, *Leucanthemum*, *Physocarpus,* and *Rosa* often required dilution.

LAMP detection of *R. fascians*. Inner (FIP, BIP) and outer (F3, B3) primers specific to regions of the *fasR* gene, an AraC-type regulator, which regulates transcription of the *fas* operon (29), were designed using PrimerExplorer V3 software (Eiken Co., Ltd., Japan). Out of the 20 primer sets evaluated, one set, number p16, was selected for further analysis; two optional loop-primers (F-Loop and B-Loop; 18) were designed in addition to the inner and outer primers (Table 1). DNA template for LAMP was prepared either from single bacterial colonies using heat lysis or from symptomatic plant tissue by homogenization (FastPrep-24, followed by Fast DNA Spin Kit, as described above). The standard LAMP reaction was performed in a 30 µl (total volume) reaction mixture containing 14.9 µl MGW, 3 µl 10× *Bst* buffer, 1.2 µl (100 mM) $MgSO_4$, 2.4 µl (5M) betaine (Sigma-Aldrich Corp, St. Louis, MO), 3 µl (10 mM) mix of each dNTPs, 0.35 µl (100 µM) each primers FIP and BIP, 0.3 µl (10 µM) each primers F3 and B3, 1.2 µl (8 U/µl) *Bst* polymerase (New England Biolabs), and 3 µl template DNA. The reactions, in clear, 0.2-ml PCR tubes, were incubated for 50 min in a water bath at 64°C. Immediately after, reactions were centrifuged 10 s at 6,000 rpm, visually inspected for a white pellet, and 10 µl of the reaction mixture was removed and added to 2 µl PicoGreen in a microfuge tube. A yellow color and

Table 1. Primers used in the course of this study

Primer name	Primer sequence (5′ to 3′)	Primer target	Product size (bp)	Reference
16S-F 16S-R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	16S ribosomal DNA	1,500	(23)
p450-F p450-R	TATCCTTGCTGCGGAGTTCT CAACCACCGCAATAATTCCT	*fasA*, P450 monooxygenase	538	This paper, (5)
fas-F fas-R	CAACACTACTTTGCCCAGCA GGCCAACTCCTCTGGTGTTA	*fasD*, isopentenyltransferase	195	This paper, (5)
16F3	ACTCGTCGACAGGTTGATCT	*fasR*, AraC-type regulator	191	This paper
16B3	GTGACTACCGCTAAACAGCT			
16FIP	TGCGACAGAATTACTTCGCCC GCGAAGCCGATCCACGATTC			
16BIP	TGAAGAGTGGAGGCATTGGC CGCACGGGTTACAGTCATTGT			
16 F-Loop 16 B-Loop	GACGTATTGAATCCTCACG CTATAGCGATTACAGTTAT			

Table 2. Bacterial strains of known identity assayed with *Rhodococcus fascians*–specific primers p450 and fas and the "universal" 16S rRNA polymerase chain reaction (PCR) primers[a]

Species	Strain ID	p450[b]	fas	16S
Acidovorax avenae	C-138	–	–	+
Agrobacterium radiobacter	A29/93	–	–	+
A. radiobacter	AS1/95	–	–	+
A. radiobacter	C1/81	–	–	+
A. radiobacter	K84	–	–	+
A. radiobacter	PM1	–	–	+
A. rhizogenes	15834	–	–	+
A. rhizogenes	A1/83	–	–	+
A. rhizogenes	A4	–	–	+
A. tumefaciens	BI2/95	–	–	+
A. tumefaciens	B68/95	–	–	+
A. tumefaciens	C58	–	–	+
A. tumefaciens	D106/87	–	–	+
A. tumefaciens	M53/79	–	–	+
A. tumefaciens	RR5	–	–	+
A. tumefaciens	T219/94	–	–	+
A. vitis	05-2	–	–	+
A. vitis	BR26/95	–	–	+
A. vitis	CG42	–	–	+
Burkholderia cepacia	PCA4	–	–	+
Clavibacter michiganensis subsp. *sepidonicus*	378	–	–	+
Curtobacterium flaccumfaciens	NE 21	–	–	+
Erwinia rhapontici	05-72	–	–	+
E. rhapontici	26420	–	–	+
Microbacterium testaceum	06-872	–	–	+
Pantoea agglomerans	26-SR-6-2	–	–	+
P. vagans	C9-15	–	–	+
Pectobacterium carotovorum	73-22	–	–	+
P. carotovorum subsp. *carotovorum*	CC301	–	–	+
P. chrysanthemi	B27	–	–	+
Pseudomonas agrici	ATCC25941	–	–	+
P. cichorii	4-211	–	–	+
P. fluorescens	A506	–	–	+
P. maltophilia	RM145	–	–	+
P. marginalis	PM7	–	–	+
P. putida	A12	–	–	+
P. savastanoi	10-1076	–	–	+
P. syringae pv. *phaseolicola*	HB-3	–	–	+
P. syringae pv. *syringae*	11E	–	–	+
Rhizobium japonicum	3164	–	–	+
R. japonicum	ATCC 11927	–	–	+
R. leguminosarum	175G11	–	–	+
R. meliloti	ATCC 10312	–	–	+
R. trifolii	ATCC10328	–	–	+
R. trifolii	T1	–	–	+
Rhodococcus coprophilus	06-620-1R	–	–	+
R. fascians	D-188	+	+	+
R. fascians	NCPPB 1488	+	+	+
R. fascians	NCPPB 1488c	–[c]	–	+
R. fascians	NCPPB 2551t2	+	+	+
R. fascians	NCPPB 469	+	+	+
R. fascians	PD302	–	–	+
R. fascians	VT141t1	–	–	+
Xanthomonas campestris	B498	–	–	+
X. campestris	426	–	–	+
X. campestris	ATCC 33913	–	–	+
X. hortorum pv. *carotae*	M801	–	–	+
X. translucens	148	–	–	+

[a] Bacterial isolates used here were part of the Oregon State University Botany and Plant Pathology bacterial culture collection. Primers for the 16S rRNA are from Pei et al. (23).

[b] A minus sign indicates assay results were negative; a plus sign signifies assay results were positive.

[c] Isolate NCPPB 1488c has been cured of the plasmid carrying the genes for pathogenicity. The remaining isolates of *R. fascians* which are listed as negative with the p450 and fas primers did not produce symptoms when inoculated to *Pisum sativum* in the course of this study.

bright fluorescence when exposed to UV-illumination indicated a positive LAMP reaction. LAMP amplification products were also electrophoresed in a 1.5% agarose gel and visualized by ethidium-bromide staining.

To confirm that primer set p16 amplified the target DNA, products were separated in a 0.8% agarose gel, and the smallest band (~200 bp) was excised, frozen, thawed, and the DNA extracted by centrifugation through glass wool. This LAMP product was then cloned into pCR 4-TOPO Vector (TOPO TA Cloning Kit for sequencing, Invitrogen), isolated from the transformed *Escherichia coli* DH5α (QIAprep Spin Miniprep Kit, Qiagen), and sequenced (Center for Genome Research and Biocomputing, Oregon State University).

To confirm the specificity of the p16 primers in the absence of additional loop primers, the LAMP assay was performed using DNA extracts from 13 reference strains of plant-associated bacteria and seven reference strains of pathogenic or nonpathogenic *R. fascians*. Template DNA was prepared by heat lysis of a single colony. Sensitivity of the LAMP primers was determined using 10-fold serial dilutions of strain A44a. Cell concentrations were confirmed by plating aliquots of the suspensions as described previously. Bacterial DNA was extracted by heat lysis using 100 µl of each dilution added to 200 µl MGW. Each dilution, in 100-µl aliquots, was also spiked into tissue samples of either petunia or evening primrose, and total DNA extracted (FastDNA SPIN kit). The LAMP assay used 3 µl of the resulting extracts as template in the reaction.

The optimal $MgSO_4$ concentration and time for precipitate formation were determined using a turbidimeter (Loopamp realtime turbidimeter, LA-320c, SA Scientific) for 70 min, set at 64°C. Sensitivity of primer set p16 was also tested using the optional primers 16 F-loop and 16 B-loop (Table 1) at a final concentration of 0.2 µM. To determine if excess nontarget DNA can interfere with detection of *R. fascians*, increasing numbers of *A. tumefaciens* cells (1.5-, 15-, and 150-fold more than *R. fascians*) were mixed with cells of strain A44a (final CFU/reaction: 1×10^4) prior to heat lysis and assaying with LAMP. *A. tumefaciens* is a ubiquitous soilborne bacterium that is often associated with plants affected by *R. fascians* (24). The comparative sensitivity of the PCR and LAMP primers was determined in parallel assays. Serial 10-fold dilutions of strain A44a were prepared and DNA of each dilution extracted using heat lysis; cell concentration was confirmed as described above. Additionally, bacterial DNA was spiked into tissue samples of petunia or evening primrose and total DNA extracted as previously described. Three microliters of the resulting extracts were used in LAMP and PCR reactions. The ability of the p16 LAMP primers, without the optional B-loop and F-loop primers, to detect *R. fascians* in naturally infected plant material was tested using 88 samples submitted to the Plant Clinic for diagnosis during the years 2002 to 2011 (Table 4). These same samples were also assayed using the p450 and fas PCR primers. Pathogenicity was determined for 44 of the 67 bacteria recovered from the samples by inoculation to indicator hosts.

Results

Identification of new bacterial strains. Of 33 bacterial strains which produced leafy galls when inoculated to indicator plants, the Biolog system failed to provide identification for 23. Of the 10 remaining, one was identified as *Corynebacterium nitrilophilus*, two as *R. coprophilus*, and one as *Rhodococcus* sp. Only six were correctly identified as *R. fascians* (Table 3).

Pathogenicity testing. All sweet pea seedlings inoculated with pathogenic *R. fascians* showed distorted growth and multiple shoots 4 weeks postinoculation. Garden pea cultivars inoculated with pathogenic strains were symptomatic, and only one-third the size of the water controls. Plants of both sweet and garden peas inoculated with nonpathogenic *R. fascians* looked similar to the noninoculated controls, which were normal. Pathogenic *R. fascians* strains inoculated to evening primrose 'Siskiyou' produced shoot proliferation and leafy galls beginning 2 weeks after inoculation

Table 3. Results of plant tissue extracts and bacterial strains assayed for *Rhodococcus fascians* using the p450 and fas polymerase chain reaction (PCR) primers[a]

Plant	Cultivar	Sample	State of origin	Plant tissue assayed		Bacteria assayed		Patho-genicity	Biolog identification
				p450	fas	p450	fas		
Acanthus mollis		05-2223-1	MI			+	+	+	*Rhodococcus fascians*
A. spinosus		07-1748	MI	–	–				No bacteria recovered
A. spinosus		07-1936	MI	–	–				No ID[b]
Acer japonicum		06-1083	OR	–	–				No bacteria recovered
Achillea millefolium	Paprika	08-584	MN	–	–	–	–		No ID
Agastache cana	Sonoran Sunset	06-1459	IA	–	–				No bacteria recovered
Agave geminiflora	Spaghetti Strap	08-1804	MN	–	–				No bacteria recovered
Ajuga reptans	Chocolate Chip	07-124	IA	–	–	–	–		No ID
Anthemis tinctoria	Sauce Hollandaise	06-1460	IA	–	–	–	–		No ID
Argyranthemum sp.		06-1789	CA	–	–				*R. coprophilus*
Aruncus aethusifolius		08-580	MN	+	+	+	+		No ID
Aruncus ×	Misty Lace	08-1862	MI	+	+				No ID
Aster amellus	Violet Queen	A73a	PA			+	+	+	*R. fascians*
A. chemosis	Woods Pink	04-516	FL			–	–	–	No ID
A. dumosus	Red Alert	06-1472	IA	–	–				No bacteria recovered
A. lateriflorus	Lady in Black	06-1132	IA	–	–				No bacteria recovered
Boltonia asteroides var. *latisquama*	Nana	06-1058	IA	–	–	–	–		No ID
Brassica oleracea	Botrytis group	08-1732	OR	–	–	–	–		No ID
Buddleia davadii	Nanho Blue	06-1466	IA	–	–				No bacteria recovered
Campanula persicifolia	Blue	07-488	IA	–	–	–	–		No ID
C. punctata	Plum Wine	08-601	OR	+	+	+	+	+	No ID
Campanula sp.		06-156-3	MI	+	+	+	+	+	No ID
Campanula sp.		06-156-3b	MI			+	+	+	No ID
Campanula sp.		06-1885	NY	–	–				No bacteria recovered
Campanula ×	Sarastro	02-815	MI			+	+	+	*Corynebacterium nitrilophilus*
Chionanthus virginiana		08-1481	OR	–	–				
Cleome hassleriana		07-2059	CA	–	–				No bacteria recovered
C. hassleriana		07-2060	CA	–	–				No bacteria recovered
Coreopsis sp.		08-443	MN	–	–				
Coreopsis verticillata	Zagreb	08-579	MN	–	–	+	+	+	No ID
C. verticillata	Moonbeam	08-583	MN	–	–				No bacteria recovered
C. verticillata	Crème Brulée	08-857	OR	–	–				No bacteria recovered
C. verticillata	Rosea	06-872	OH	–	–	–	–		*Microbacterium testaceum*
Cuphea sp.		08-543	CA	–	–				No bacteria recovered
Delosperma cooperi		07-2085	WA	–	–				No bacteria recovered
Dianthus caryophyllus		07-1243	CA	+	+	+	+		No ID
D. gratianopolitanus	Firewitch Raspberry	08-581	MN	–	–				No bacteria recovered
Dianthus sp.		07-214	CA	–	–	–	–		No ID
Dianthus ×	Firestar	06-1462	IA	–	–				No bacteria recovered
Dianthus ×	Strawberry Sorbet	06-1906	WA	–	–				No bacteria recovered
Echinacea purpurea		07-1839-1a	OR			–	–		No ID
E. purpurea	Little Magnus	08-1756	WA	–	–	+	+		No ID
Erodium reichardii		08-259	CA	–	–	+	+	+	*R. coprophilus*
E. reichardii		08-788	CA	–	–	–	–	–	*Rhodococcus* sp.
Euonymus japonica		08-237	CA	–	–				No bacteria recovered
Euonymus sp.		06-1475	IA	–	–				No bacteria recovered
Fuchsia ×	Indian Maid	05-2256-b-3	OR			+	+	+	No ID
Gaillardia grandiflora	Fanfare	07-178	IA	–	–	–	–		*Pseudomonas fluorescens*
Gaura lindheimeri	Siskiyou Pink	06-1463	IA	–	–				No bacteria recovered
Geranium sanguineum var. *striatum*		06-2169	WA	–	–				*Agrobacterium tumefaciens*
Heliopsis helianthoides	Loraine Sunshine	07-655	WA	+	+	+	+		*R. fascians*
H. helianthoides	Loraine Sunshine	A3b	MI			+	+	+	*R. fascians*
Herniaria glabra	Seafoam	07-1747	MI	+	+				*R. fascians*
Heuchera sanguinea	Snow Angel	07-339	WA	–	–				No bacteria recovered
Heuchera sp.		07-1895	IL	–	–				No bacteria recovered
Heuchera ×	Princess Anastasia	06-234-2a	MI	–	–	–	–	–	No ID
Hosta albomarginata	Gold Standard	07-1734	MI	–	–				No bacteria recovered
Hosta sp.		08-811-1-1a	MI	+	+	+	+	+	No ID
Hosta ×	Blue Umbrellas	05-339-1	MI			+	+	+	No ID

(*continued on next page*)

[a] Plant samples were submitted to the OSU Plant Clinic for diagnosis. The bacteria tested were isolated from the plants submitted. Selected isolates were also assayed for pathogenicity on either *Pisum sativum* or *Oenothera speciosa* as described in the text. Where bacteria were isolated, identity was attempted using the substrate utilization assay marketed by Biolog (Hayward, CA). A blank in a column indicates the assay was not performed; a plus sign signifies a positive assay; and a minus sign signifies the assay results were negative.

[b] No identity was obtained with the Biolog system according to the criteria set by the manufacturer.

Table 3. (*continued from previous page*)

Plant	Cultivar	Sample	State of origin	Plant tissue assayed		Bacteria assayed		Patho-genicity	Biolog identification
				p450	fas	p450	fas		
Hosta ×	Blue Umbrellas	08-1801	MN	–	–				No bacteria recovered
Hosta ×	Carnival	05-340-1	MI			+	+	+	No ID
Hosta ×	Carnival	05-340-2	MI			+	+	+	No ID
Hosta ×	Dress Blues	06-411-3	MI			–	–		*A. tumefaciens*
Hosta ×	Elegans	08-105	SC	–	–				No bacteria recovered
Hosta ×	Fragrant Blue	08-1860	MI	–	–				No bacteria recovered
Hosta ×	Fragrant Blue	08-1861	MI	+	+	+	+		No ID
Hosta ×	Hadspen Blue	08-235	MI	–	–				No bacteria recovered
Hosta ×	Praying Hands	07-1606	MI	+	+				*Rhodococcus* sp.
Hosta ×	Praying Hands	08-1802	MN	–	–				No bacteria recovered
Hosta ×	Sugar and Spice	08-1803	MN	–	–				No bacteria recovered
Isolepis cernua		06-1134	IA	–	–				No bacteria recovered
Lamium maculatum	Beacon Silver	08-578	MN	+	+	–	+	+	No ID
L. maculatum	White Nancy	06-1470	IA	–	–				No bacteria recovered
L. maculatum	White Nancy	08-577	MN	–	–	+	+	–	No ID
Lavandula angustifolia	Melissa Lilac	06-2193	WA	–	–				No bacteria recovered
L. angustifolia	Violet Intrigue	05-561	WA			–	–	–	
Lavatera × *clementii*	Barnsley	06-1059	IA	–	–	–	–		*Sphingobacterium thalpophilum*
Leucanthemum × *superbum*	Becky	06-468-2-2	MI			+	+	+	No ID
Leucanthemum × *superbum*	Becky	06-1968	MI	–	+				No bacteria recovered
Leucanthemum × *superbum*	Becky	06-1969	MI	–	+				No bacteria recovered
Leucanthemum × *superbum*	Becky	07-260	MN	+	+	+	+		*R. fascians*
Leucanthemum × *superbum*	Becky	08-368	OR	–	–	–	–		No ID
Leucanthemum × *superbum*	Becky	A22b	WA			–		–	No ID
Leucanthemum × *superbum*	Becky	A78	PA			+	+	+	No ID
Leucanthemum × *superbum*	Esther Read	06-325-1-1	WA			+	+	+	No ID
Leucanthemum × *superbum*	Gold Rush	08-87	WA	+	+			+	*R. fascians*
Leucanthemum × *superbum*	Gold Rush	08-1863	MI	–	–	+	+		*R. fascians*
Leucanthemum × *superbum*	Snow Cap	06-467-1	MI			+	+	+	No ID
Lilium sp.		07-68	WA	+	+	+	+		
Lilium sp.		07-69	WA	+	+	–	+		
Lilium sp.		07-168	WA	+	+				No bacteria recovered
Lilium sp.		07-169	WA	+	+				No bacteria recovered
Lilium sp.		07-170	WA	–	+				No bacteria recovered
Lilium sp.		07-171	WA	–	+				No bacteria recovered
Lilium sp.		07-1390	WA	+	+				
Lilium sp.		07-1596	WA	+	+				No ID
Lilium sp.		07-1597	WA	+	+				No ID
Lilium sp.		07-1598	WA	+	+				No ID
Lilium sp.		07-2032	WA	–	–				No bacteria recovered
Lilium sp.		07-2033	WA	–	–				No bacteria recovered
Lilium sp.		07-2034	WA	–	+				No bacteria recovered
Lilium sp.		07-2035	WA	–	–				No bacteria recovered
Lilium sp.		07-2036	WA	+	+				No bacteria recovered
Lobelia sp.		08-96	CA	–	–				No bacteria recovered
Lysimachia clethroides		06-1465	IA	–	–				No bacteria recovered
Malus domestica		07-340	OR			–	–	–	*Agrobacterium* sp.
Monarda didyma	Petite Delight	08-424	IN	–	–	–	–		
Nemesia sp.		06-621-1	CA	+		+	+	+	No ID
Oenothera speciosa	Siskiyou	08-89	WA	+	+			+	
Osteospermum sp.		08-77	NH	–	–				
Pelargonium sp.		07-273	KS	–	–				No bacteria recovered
Pelargonium sp.		07-1662	FL	–	–				No bacteria recovered
Pelargonium sp.		07-1788	IL	–	–				No bacteria recovered
Pentas lanceolata	Cranberry Punch	06-761	MD	–	–	–	–	–	*A. tumefaciens*
P. lanceolata		08-1920	CA	–	–				No bacteria recovered
Petunia × *hybrida*		07-1027	CA			–	–	–	

(*continued on next page*)

Table 3. (*continued from previous page*)

Plant	Cultivar	Sample	State of origin	Plant tissue assayed		Bacteria assayed		Pathogenicity	Biolog identification
				p450	fas	p450	fas		
Petunia × *hybrida*		08-437	CA	+	+	+	+	+	No ID
Phlox paniculata	Bright Eyes	07-208	MI	–	–				No bacteria recovered
P. paniculata	Pink Flame	06-871	WA	–	–	+	+		No ID
Phlox ×	Miss Candy	06-1471	IA	–	–				No bacteria recovered
Physocarpus opulifolius		08-858	OR	–	–	+	+	+	*Rhodococcus* sp.
Rhododendron sp.		07-177	WA	–	–				No bacteria recovered
Rhododendron ×	Aglo	08-1930	OR	–	–				No bacteria recovered
Rhododendron ×	Golden Lights	08-1946	OR	–	–	–	–		*R. fascians*
Rosa sp.	Yellow Brick Road	07-76	OR	–	–	–	–		*A. rhizogenes*
Rosa sp.	Morden Centennial	07-258	MN	–	–				*Agrobacterium* sp.
Rosa sp.	Prairie Sunrise	07-259	MN	–	–				*Agrobacterium* sp.
Rosa sp.	Yellow Brick Road	07-2062	OR	–	–				No bacteria recovered
Rosa sp.	Sweet Fragrance	07-2112	OR	–	–				No bacteria recovered
Rosa sp.	Paint the Town	07-2112	OR	–	–				No bacteria recovered
Rudbeckia sp.		07-213	CA	–	–				No bacteria recovered
Rudbeckia sp.		07-1242	NY	–	–				No bacteria recovered
Salix purpurea	Nana	07-257a	MT	–	–	–	–		*A. tumefaciens*
Salvia × *sylvestris*	Blauhügel	06-1473	IA	–	–				No bacteria recovered
Salvia × *sylvestris*	Mainacht	06-1474	IA	–	–				*R. fascians*
Scabiosa comumbaria	Butterfly	08-582	MN	–	–	–	–	–	*R. coprophilus*
Scabiosa sp.		08-444	MN	–	–	–	–	–	No ID
Sedum ewersii	Ewersii	06-1478	IA	–	–				No bacteria recovered
S. kamtschaticum var. *variegatum*		06-1467	IA	–	–				No bacteria recovered
S. spurium	Dragon's Blood	06-1468	IA	–	–				No bacteria recovered
Sequoia sempervirens		08-236	CA	–	–				No bacteria recovered
Sorbaria sorbifolia	Sem	08-445	MN	+	+	+	+	+	No ID
S. sorbifolia	Sem	08-446-3a	MN	+	+	+	+	+	No ID
S. sorbifolia	Sem	08-585	MN	+	+	+	+	+	*R. coprophilus*
Verbascum ×	Jackie	06-1891	MI	–	–				No bacteria recovered
Verbascum ×	Jackie in Pink	06-1893	MI	–	–				No bacteria recovered
Verbascum ×	Plum Smokey	07-1607	MI	–	–				No bacteria recovered
Verbena ×	Shauna Ann	05-2224-3a	MI			+	+	+	No ID
Veronica ×	Goodness Grows	06-469-2-1	MI			+	+	+	
Veronica spicata	Blue Charm	06-1476	IA	–	–				No bacteria recovered
V. spicata	Icicle White	06-1477	IA	+	+	+	+		*R. fascians*
V. spicata	Minuet	A44a	PA			+	+	+	No ID
V. spicata	Red Fox	06-873	OH	–	–	+	+		*R. fascians*
V. spicata	Royal Candles	05-2081	MI			+	+	+	
V. spicata	Royal Candles	06-418-1b	MI	+		+	+	+	No ID
V. spicata	Royal Candles	06-1100	MI	+	+				No bacteria recovered
V. spicata	Royal Candles	06-1970	MI	–	+				No bacteria recovered
V. spicata	Royal Candles	06-1971	MI	–	–				No bacteria recovered
V. spicata	Royal Candles	07-577	WA	–	+	+	+	+	*R. fascians*
V. spicata	Sunny Border Blue	05-2254-5	WA			+	+	+	*R. fascians*
V. spicata	Sunny Border Blue	06-470-1-2	MI			–	–	–	No ID
V. spicata	Sunny Border Blue	06-1133	IA	+	+				No bacteria recovered
V. spicata	Tickled Pink	06-516-1	FL			–	–	–	*Curtobacterium citreum*
V. spicata	Waterperry Blue	06-1461	IA	–	–				No bacteria recovered
Vinca minor		06-1479	IA	–	–				No bacteria recovered
Vinca sp.		07-1787	IL	–	–				No bacteria recovered
Viola ×	Purple Showers	02-816c	MI			+	+	+	No ID
Viola ×	Purple Showers	02-816d	MI			–	–	–	*Microbacterium terregens*
Vitis vinifera	Pinot Noir	07-1340	OR	–	–				*Pseudomonas* sp.

(Fig. 1). As evening primrose was easier to inoculate than pea, and because symptoms were more consistent with those of naturally infected nursery plants, this plant species replaced garden pea in pathogenicity assays after 2004.

PCR detection of *R. fascians*. PCR products of the correct size were obtained only for the four pathogenic reference strains of *R. fascians* (Table 2). No amplicon was obtained for one nonpathogenic *R. fascians* strain, which had been cured of the plasmid containing the virulence genes (NCPPB1488c), nor for isolates PD302 and VT141t1 (Table 2), which did not incite disease in garden pea. When petunia leaf tissue extracts were spiked with quantified cell suspensions of *R. fascians* strain A44a, the lower limit of detection was approximately 1,000 CFU/PCR reaction for both the p450 and fas primer sets (data not shown). The p450 primers were used successfully to detect *R. fascians* from all 34 inoculated plants (plant wash assay; data not shown). When frozen DNA extracts from most inoculated plants were later tested with the fas primers, all plants that had been inoculated with *R. fascians* ($n = 22$) produced a product of the expected size.

All 169 samples sent to our lab for diagnosis of either *A. tumefaciens* or *R. fascians* from 2002 to 2008 were assayed for *R. fascians* using PCR with the p450 and fas primer pairs (with DNA from plant tissue extracts or plant washes, the latter of which was for samples preceding number 08-89), culture-based detection, or both (Table 3). Considering the plant tissue extracts only, there was agreement between the p450 and fas primer sets for 132 of 139 (95%) plants (Table 3). Seven samples produced a PCR product with the fas primers only (i.e., were fas-positive). Six of the seven

Table 4. Results of plant tissues and bacterial cultures assayed for pathogenic *Rhodococcus fascians* with loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR)[a]

Plant name	Cultivar	Sample number	State or country of origin	Patho-genicity	LAMP results[b]	PCR results		Material assayed
						fas	p450	
Acanthus mollis		05-2223-1	MI	+	+	+	+	Bacteria
Achillea millefolium	Paprika	08-584	MN	NT	–	–	–	Bacteria
Angelonia ×	Actors White	09-2347-1	MI	NT	+	+	+	Bacteria
Angelonia ×	Actors White	09-2347-2	MI	NT	+	+	+	Bacteria
Aster amellus	Violet Queen	A73a	PA	+	+	+	+	Bacteria
Aster dumosus	Woods Pink	04-516	FL	–	–	–	–	Bacteria
Sutera cordata	Scopia Great Pink Ring	10-1523	UT	NT	+	+	+	Tissue
Brassica oleracea var. *botrytis*		08-1732	OR	NT	–	–	–	Bacteria
Campanula punctata	Plum Wine	08-601	OR	+	+	+	+	Bacteria
Campanula sp.		06-156-3b	MI	+	+	+	+	Bacteria
Campanula ×	Sarastro	02-815	MI	+	+	+	+	Bacteria
Cleome sp.		10-2022	CA	NT	–	–	–	Tissue
Coreopsis verticillata	Zagreb	08-579	MN	+	+	+	+	Bacteria
Coreopsis ×	Jive	10-212-2	MI	NT	–	–	–	Tissue
Dianthus ×	Cinnamon Red Hot	10-1976	CA	NT	–	–	–	Tissue
Dianthus ×	Cinnamon Red Hot	10-1976-1-1-ai	CA	NT	–	–	–	Bacteria
Dianthus ×	Cinnamon Red Hot	10-1976-1-1-aii	CA	NT	–	–	–	Bacteria
Dianthus ×	Cinnamon Red Hot	10-1976-1-1b	CA	NT	–	–	–	Bacteria
Dianthus ×	Cinnamon Red Hot	10-1976-2-2a	CA	NT	–	–	–	Bacteria
Echinacea purpurea		07-1839-1a	OR	NT	–	–	–	Bacteria
Echinacea ×	After Midnight	09-1953-1	WA	NT	–	–	–	Bacteria
Echinacea ×	After Midnight	09-1953-2	WA	NT	–	–	–	Bacteria
Echinacea ×	Gum Drop	10-999-1-1	OR	NT	–	+	–	Tissue
Erodium reichardii		08-259	CA	+	+	+	+	Bacteria
E. reichardii		08-788	CA	–	–	–	–	Bacteria
Fuchsia sp.		10-121a-1	OR	NT	+	+	+	Tissue
Fuchsia sp.		10-121a-2	OR	NT	+	+	+	Tissue
Fuchsia sp.		10-121a-1-3	OR	NT	–	+	+	Bacteria
Fuchsia sp.		10-121a-1-4	OR	NT	+	+	+	Bacteria
Fuchsia ×	Indian Maid	05-2256b-3	OR	+	+	+	+	Bacteria
Heliopsis helianthoides	Loraine Sunshine	A3b	MI	+	+	+	+	Bacteria
Heuchera sp.		11-42	MA	NT	–	–	–	Tissue
Heuchera sp.		11-43	MA	NT	–	–	–	Tissue
Heuchera sp.		11-44	MA	NT	–	–	–	Tissue
Heuchera sp.		11-45	MA	NT	–	–	–	Tissue
Heuchera sp.		11-46	MA	NT	–	–	–	Tissue
Heuchera ×	Caramel	11-205	OH	NT	–	–	–	Tissue
Heuchera ×	Dark Secret	11-204	OH	NT	–	–	–	Tissue
Heuchera ×	Princes Anastasia	06-234-2a	MI	–	–	–	–	Bacteria
Hibiscus rosa-sinensis	Carolina Breeze	10-718-2-1	FL	NT	–	–	–	Tissue
H. rosa-sinensis	Carolina Breeze	10-718-2-2	FL	NT	–	–	–	Tissue
Hosta sp.		08-811	MI	+	+	+	+	Bacteria
Hosta ×	Blue Umbrellas	05-339-1	MI	+	+	+	+	Bacteria
Hosta ×	Carnival	05-340-2	MI	+	+	+	+	Bacteria
Hosta ×	Dress Blues	06-411-3	MI	+[c]	–	–	–	Bacteria
Iberis semipervins	Purity	A24c	WA	–	–	–	–	Bacteria
Iberis ×	Absolutely Amethyst	10-1629	CA	NT	+	+	+	Tissue
Ipomoea ×	Green Yellow	10-710-1	CA	NT	+	+	+	Tissue
Ipomoea ×	Green Yellow	10-710-2	CA	NT	+	+	+	Tissue
Ipomoea ×	Green Yellow	10-931-1-1	CA	NT	–	–	–	Bacteria
Ipomoea ×	Green Yellow	10-931-2-1-2b	CA	NT	+	+	+	Bacteria
Lavandula angustifolia	Violet Intrigue	05-561	WA	–	–	–	–	Bacteria
L. angustifolia	Violet Intrigue	06-561-1	WA	–	–	NT	–	Bacteria
Leucanthemum sp.		11-176	CA	NT	–	–	–	Tissue
Leucanthemum × *superbum*	Becky	06-468-2-2	MI	+	+	+	+	Bacteria
Leucanthemum × *superbum*	Becky	09-2323-1	NY	NT	–	–	–	Bacteria
Leucanthemum × *superbum*	Becky	09-2323-2	NY	NT	–	–	–	Bacteria

(*continued on next page*)

[a] Plant samples were submitted to the Oregon State University Plant Clinic for diagnosis. Bacterial cultures (except the reference isolates) were obtained from infected plants submitted. Plus signs indicate the sample was positive and minus signs indicate the sample was negative in the assay. NT means the sample was not assayed for the test indicated.

[b] Results are the summary of duplicate assays.

[c] This sample was infected with a pathogenic strain of *Agrobacterium tumefaciens*.

[d] Isolates NCPPB 1488c and D-188c have been cured of the plasmid carrying the genes for pathogenicity. The remaining isolates which are listed as negative for pathogenicity did not produce symptoms when inoculated to *Pisum sativum* in the course of this study.

Table 4. (*continued from previous page*)

Plant name	Cultivar	Sample number	State or country of origin	Patho-genicity	LAMP results[b]	PCR results		Material assayed
						fas	p450	
Leucanthemum × *superbum*	Becky	A22b	WA	–	–	–	–	Bacteria
Leucanthemum × *superbum*	Becky	A78	PA	+	+	+	+	Bacteria
Leucanthemum × *superbum*	Broadway Lights	10-1795-1-3-2a	CA	NT	+	+	+	Tissue
Leucanthemum × *superbum*	Broadway Lights	10-1795-1-3-2b	CA	NT	+	+	+	Tissue
Leucanthemum × *superbum*	Esther Read	06-325-1-1	WA	+	+	+	+	Bacteria
Leucanthemum × *superbum*	Snow Cap	06-467-1	MI	+	+	+	+	Bacteria
Leucanthemum × *superbum*	Snow Cap	10-135-1	VA	NT	+	+	+	Tissue
Leucanthemum × *superbum*	Sunny Side Up	10-138-1	VA	NT	+	+	+	Tissue
Nemesia sp.		06-621-1	CA	+	+	+	+	Bacteria
Nemesia ×	Natalie	A25f	WA	+	+	+	+	Bacteria
Oenothera speciosa	Siskiyou	05-22552b-1	WA	+	+	+	+	Bacteria
O. speciosa	Siskiyou	08-89-1	OR	+	+	+	+	Bacteria
Osteospermum ×	Lemon Symphony	10-242	CA	NT	–	–	–	Tissue
Osteospermum ×	Orange Spark	10-243	CA	NT	–	–	–	Tissue
Pelargonium peltatum		10-121b	OR	NT	+	+	+	Tissue
P. peltatum		10-121b-1	OR	NT	+	+	+	Bacteria
Petunia × *hybrida*		08-437	CA	+	+	+	+	Bacteria
Petunia × *hybrida*	Surfina Baby Red	10-1077-1	UT	NT	–	–	–	Tissue
Petunia × *hybrida*	Surfina Baby Red	10-1077-2	UT	NT	–	–	–	Tissue
Petunia × *hybrida*	Vista Fuchsia	10-142-1	CA	NT	+	+	+	Tissue
Petunia × *hybrida*	Vista Fuchsia	10-142-2	CA	NT	+	+	+	Tissue
Phlox paniculata	Flame Lilac	10-743-1-1	PA	NT	+/–	–	–	Bacteria
P. paniculata	Flame Lilac	10-743-2	PA	NT	+	+	+	Tissue
P. paniculata	Flame Lilac	10-743-2-1	PA	NT	+	+	+	Tissue
Phlox sp	.	09-1941-1	OH	NT	–	–	–	Bacteria
Phlox sp.		09-1941-2	OH	NT	–	–	–	Bacteria
Physocarpus opulifolius		08-858	MN	+	+	+	+	Bacteria
Salix purpurea	Nana	07-257a	MT	–	–	–	–	Bacteria
Sorbaria sorbifolia	Sem	08-446-3a	MN	+	+	+	+	Bacteria
Torenia ×	Summer Wave Blue	11-207	CA	NT	–	–	–	Tissue
Verbena ×	Goodness Grows	06-469-2-1	MI	+	+	+	+	Bacteria
Verbena ×	Shauna Ann	05-2224-3a	MI	+	+	+	+	Bacteria
Verbena ×	Temari Cherry Red	10-1079-1	UT	NT	–	–	–	Tissue
Verbena ×	Temari Cherry Red	10-1082-1	UT	NT	–	–	–	Tissue
Veronica sp.		10-815-1-1	UT	NT	+	+	+	Bacteria
Veronica sp.		10-815-1-2	UT	NT	+	+	+	Tissue
Veronica sp.		10-815-2-1	UT	NT	+	+	+	Tissue
Veronica sp.		10-815-2-2	UT	NT	+	+	+	Tissue
Veronica spicata	Royal Candles	A76	MI	+	+	+	+	Bacteria
V. spicata	Royal Candles	05-2081	MI	+	+	+	+	Bacteria
V. spicata	Royal Candles	06-418-1a	MI	+	+	+	+	Bacteria
V. spicata	Royal Candles	06-418-1b	MI	+	+	+	+	Bacteria
V. spicata	Royal Candles	10-134-1	VA	NT	–	+	–	Tissue
V. spicata	Royal Candles	10-420-1a	WA	NT	+	+	+	Tissue
V. spicata	Royal Candles	10-420-1b	WA	NT	+	+	+	Tissue
V. spicata	Royal Candles	10-420-2a	WA	NT	+	+	+	Tissue
V. spicata	Royal Candles	10-420-2b	WA	NT	+	+	+	Tissue
V. spicata	Royal Candles	10-1468-1	MN	NT	–	–	–	Tissue
V. spicata	Royal Candles	10-1468-2	MN	NT	+	+	+	Tissue
V. spicata	Sunny Border Blue	06-470-1-2	MI	–	–	–	–	Bacteria
V. spicata	Sunny Border Blue	06-470-3	MI	+	+/–	+	+	Bacteria
V. spicata	Sunny Border Blue	05-2254-5	WA	+	+	+	+	Bacteria
V. spicata	Sunny Border Blue	10-139-1	VA	NT	+	+	+	Tissue
V. spicata	Tickled Pink	10-1509	PA	NT	+	+	+	Tissue
Veronica ×	Eveline	10-137-1	VA	NT	+	+	+	Tissue
Veronica ×	Eveline	10-137-1-3	VA	NT	+	+	+	Bacteria
Veronica ×	Minuet	A44a	PA	+	+	+	+	Bacteria
Viola ×	Purple Showers	02-816c	MI	+	+	+	+	Bacteria
Viola ×	Purple Showers	02-816d	MI	–	–	–	–	Bacteria

(*continued on next page*)

Table 4. (*continued from previous page*)

Plant name	Cultivar	Sample number	State or country of origin	Patho-genicity	LAMP results[b]	PCR results		Material assayed
						fas	p450	
Reference isolates of *R. fascians*								
Fragaria chiloensis var. *ananassa*		NCPPB 469	UK	+	+	+	+	Bacteria
Lathyrus odoratus		NCPPB 1488c	UK	–[d]	–	–	–	Bacteria
L. odoratus		NCPPB 2551	UK	+	+	+	+	Bacteria
Lilium speciosum	Rubrum	VT141t1	Belgium	–	–	–	–	Bacteria
Euphorbia pulcherrima		PD302	Netherlands	–	–	–	–	Bacteria
Chrysanthemum morifolium		D-188	UK	+	+	+	+	Bacteria
C. morifolium		D-188c	UK	–	–	–	–	Bacteria

Fig. 1. Symptoms of *Rhodococcus fascians* on an inoculated *Oenothera speciosa* plant. Shown is a massive leafy gall in the center of the pot, with normal leaves on an unaffected stem at the lower right.

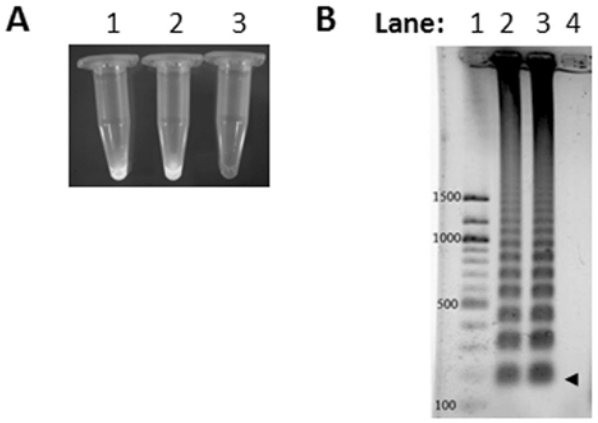

Fig. 2. Loop-mediated isothermal amplification (LAMP) of *Rhodococcus fascians* DNA. **A,** PicoGreen detection of positive LAMP reactions. LAMP was performed with primer set p16 without the optional loop primers using the standard condition (Materials and Methods). A DNA (~100 ng) extract of strain A44a was added to reactions 1 and 2. No DNA was added to reaction 3. An aliquot (5 µl) of each completed reaction was added to 1 µl PicoGreen, and fluorescence was excited using an ultraviolet-illuminator. **B,** Gel-electrophoresis of LAMP products amplified using primer set p16. LAMP was performed as described for A. Aliquots (10 µl) of completed reactions were run on a 1.5% agarose gel. Lane 1 is a 100-bp DNA ladder; lanes 2 and 3, A44a DNA as template; and lane 4, no DNA (water control). Arrowhead: example of LAMP-product purified for sequencing.

fas-positive samples were received in poor condition or had been treated with bactericides, and thus were not suitable for culture-based detection. One of the seven fas-positive samples (from *Veronica spicata*) was truly infected with pathogenic *R. fascians*, as determined by host inoculation, and hence was a false negative according to the p450 primers. However, there were no instances, when assaying plant tissue extracts, in which the p450 primers gave positive results and the fas primers gave negative results.

For identification of 75 bacterial strains isolated from 169 samples, results using the two primer pairs were consistent for 73 (97%) bacterial strains (Table 3). Two bacterial strains were positively identified as *R. fascians*, based on fas primers, but were p450-negative. Pathogenicity of the bacteria isolated from one of the fas-positive, p450-negative samples was not tested because the bacteria did not survive storage, but bacteria isolated from the other sample produced symptoms when inoculated to evening primrose, suggesting a false-negative with the p450 primers. Based on pathogenicity tests of 46 of the 75 bacterial isolates recovered, the p450 and fas primers correctly discriminated between 31 pathogenic and 12 nonpathogenic (43 total) strains. For the remaining three strains, there were the following inconsistencies between the primers and the pathogenicity tests: one fas-positive, p450-positive strain was nonpathogenic; one fas-negative, p450-negative strain was pathogenic; and one fas-positive, p450-negative strain was pathogenic. Finally, of the 169 plant samples assayed for *R. fascians*, there were eight from which extracts of bacteria recovered from the plants reacted with both the p450 and fas primers, but plant tissue extracts did not.

LAMP detection of *R. fascians.* From 20 sets of FIP/BIP and F3/B3 primer pairs, we selected one (p16) for further evaluation. The smallest product band (arrowhead, Fig. 2B) amplified using p16 was gel-purified, cloned, and sequenced, and found to be the expected DNA sequence. No amplicon was formed when set p16 was tested against DNA from 13 plant-associated bacteria, including other hyperplasia-inducing species. Tested were *Acidovorax avenae*, *Agrobacterium radiobacter* (two strains), *A. rhizogenes*, *A. tumefaciens*, *A. vitis*, *Bacillus uniflagellatus*, *Curtobacterium flaccumfaciens*, *Erwinia amylovora*, *Pectobacterium carotovorum*, *Pseudomonas maltophila*, *P. savastanoi*, and *P. syringae*. The p16 primers correctly discriminated between three pathogenic strains (NCPPB 469, NCPPB 2551, and D-188) and four nonpathogenic strains (NCPPB 1488c, VT141t1, PD302, and D-188c) of *R. fascians.* A precipitate formed in positive *R. fascians* LAMP (LAMP-positive) reactions, but only as a pellet after brief centrifugation. To enhance detection, we added PicoGreen to the reaction product, which produces a yellow color under daylight and fluoresces under UV light in a LAMP-positive reaction, and is orange and nonfluorescent in a LAMP-negative reaction (Fig. 2A). There was correlation between precipitate formation and fluorescence with PicoGreen in 148 of 158 reactions of known *R. fascians* cell concentrations (data not shown). In 10 reactions, there had been fluorescence but no precipitate, suggesting the dye was more sensitive at detecting amplification. Optimal time for precipitate formation was evaluated using a real-time turbidity meter for 70 min. Turbidity was dependent on $MgSO_4$ concentration and reached a maximum at 6 mM $MgSO_4$ (data not shown), the concentration used in our standard conditions. No product or precipitate formed when $MgSO_4$ was at or below 3 mM, and by 50 min the LAMP reaction was at an exponential phase of amplification, saturating at 70 min.

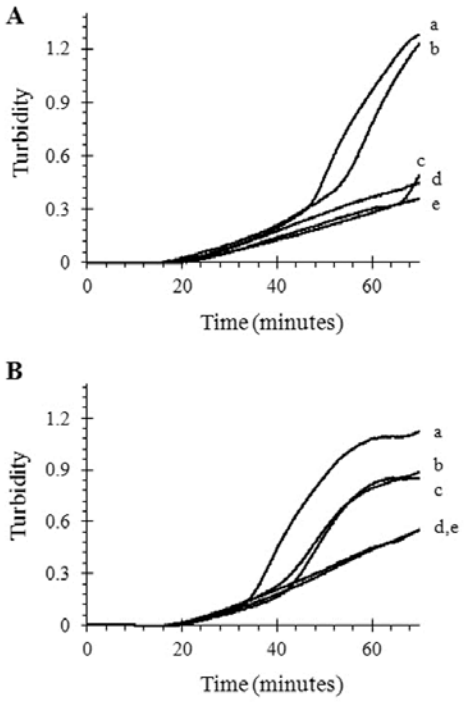

Fig. 3. Loop-mediated isothermal amplification (LAMP) sensitivity with and without loop-primers. LAMP reactions using primer set p16 were measured in real-time using a turbidity meter. DNA extracted from varying amounts of *Rhodococcus fascians* (strain A44a) colony-forming units was added to each reaction as follows: a, 5 × 10^4; b, 5 × 10^3; c, 5 × 10^2; d, 5 × 10^1; e, no DNA. **A,** LAMP in absence of loop primers. **B,** LAMP in presence of loop primers. LAMP reactions included 16 F-loop and 16 B-loop primers. Data is representative of four independent experiments.

Real-time turbidity was monitored using set p16 and extracts of known *R. fascians* cell concentrations. The detection limit for the p16 primers was 5 × 10^3 CFU per 30 µl LAMP reaction (Fig. 3A). Addition of F-loop and B-loop primers accelerated the LAMP reaction and shifted the exponential phase back to between 35 and 60 min. The loop primers increased sensitivity to 5 × 10^2 CFU/reaction (Fig. 3B). There was a gradual increase in turbidity in the absence of DNA template (Fig. 3A and B), and no LAMP product was detectable with PicoGreen or gel electrophoresis. Table 5 shows the detection limits of set p16 in the absence and presence of additional loop primers, which increased sensitivity of the assay in five of six cell concentrations to levels below that required for disease development (21). However, the frequency of false-positive reactions was unacceptably high (33%, Table 5). No false positives were generated in the absence of the loop primers, hence they were not used in our LAMP assays.

We found neither plant extracts nor extraneous bacterial DNA inhibited LAMP. *R. fascians* extracts with 1.5-, 15-, or 150-fold excess *A. tumefaciens* DNA still produced a LAMP-positive reaction, and we were consistently able to detect varying amounts of *R. fascians* DNA (≥1 × 10^3 CFU/30 µl reaction) added to petunia or evening primrose leaf tissue. When using *R. fascians* DNA combined with petunia tissue, there was no inhibition of the PCR reaction. In contrast, PCR of *R. fascians* DNA and evening primrose tissue was inhibited, and dilution was necessary to visualize product formation. The detection limit of both assays was comparable. LAMP detected *R. fascians* at ≥1 × 10^3 CFU/30 µl reaction in 80% of the reactions (Table 5), whereas p450 and fas detected *R. fascians* at ≥1 × 10^3 CFU/30 µl reaction for two of three experiments and ≥1 × 10^4 CFU/30 µl for the third.

LAMP and PCR were used to assay 49 symptomatic and asymptomatic plant tissue extracts and 67 bacterial strains isolated from 84 plant samples gathered over 10 years from 13 states (Table 4). The results of both assays agreed for 113 of 116 (97%) plant samples and bacterial strains, with the exceptions noted below. Of

Table 5. Sensitivity of the *Rhodococcus fascians*–specific loop-mediated isothermal amplification (LAMP) assay with and without additional loop primers

Loop primers[a]		No loop primers	
CFU[b]	**Frequency[c] (%)**	**CFU**	**Frequency (%)**
1-5 × 10^5	8/8 (100)	1-5 × 10^5	2/2 (100)
1-5 × 10^4	15/15 (100)	1-5 × 10^4	8/9 (89)
1-5 × 10^3	15/16 (94)	1-5 × 10^3	8/10 (80)
1-5 × 10^2	6/16 (38)	1-5 × 10^2	1/10 (10)
1-5 × 10^1	4/16 (25)	1-5 × 10^1	0/10 (0)
1–5	1/8 (12)	1–5	0/2 (0)
0.1–0.5	0/4 (0)	0.1–0.5	Not tested
False-positives[d]	4/12 (33)	False-positives	0/12 (0)
False-negatives[e]	1/39 (2)	False-negatives	3/21 (14)

[a] LAMP reactions included 16FIP/16BIP and 16F3/16B3 primers, and either no additional loop primers or the 16 F-loop/16 B-loop primers. Reactions were incubated for 70 min at 65°C.
[b] CFU = colony-forming units.
[c] Frequency = ratio of positive LAMP reactions (numerator) to the total number of reactions (denominator).
[d] False positives occurred when fluorescence was observed in the water controls (no DNA template) when PicoGreen was added to the reaction vessel at the end of the incubation period.
[e] Negative reactions occurred when there was no fluorescence with the addition of PicoGreen to the reaction vessels at the end of the incubation period when the template was ≥ 1 × 10^3 CFU reference strain of *R. fascians*.

28 tissue extracts from plants with leafy galls, 26 were PCR- and LAMP-positive. Two samples were LAMP- and p450-negative, but fas-positive (Table 4). Since the samples were of symptomatic plants, these results suggest the fas-positive was correct. Of 21 plant samples with either crown gall or no symptoms, all were PCR- and LAMP-negative. The p16 primers were used on DNA extracts of 67 bacterial strains recovered from the plant samples. Twenty-five of 26 strains isolated from asymptomatic plants were LAMP-negative. One strain produced mixed results between the two LAMP replicates and were p450- and fas-negative, suggesting it was not pathogenic *R. fascians*. Of 41 bacterial strains isolated from plants with leafy galls, 40 were LAMP-positive. One strain was LAMP-negative and p450- and fas-positive, suggesting a false negative with LAMP. Finally, LAMP results differed between the replicates for one strain which was pathogenic and p450- and fas-positive (Table 4). Usually when there was a discrepancy between replicate LAMP sample results, a repeat of the assay produced agreement between the replicates. Thirty-three of the 67 bacterial strains were pathogenic upon inoculation, and all were LAMP-positive, whereas all of the 11 nonpathogenic strains were LAMP-negative.

Discussion

Both PCR and LAMP assays were effective in amplifying DNA from pathogenic strains of *R. fascians* in vitro and in planta. The PCR primers were successful in identifying *R. fascians* from 169 plant samples, representing more than 70 plants species, originating from 17 states, and gathered over a period of 7 years. Both PCR primers accurately and specifically amplified only pathogenic reference cultures of *R. fascians*, and not 51 other plant-associated bacteria in vitro. PCR primers specific to virulence genes of *R. fascians* were previously published (21,27), but our work is unique in developing a rapid, reliable diagnostic tool for pathogenic *R. fascians* based on DNA-based detection of the virulence genes, and validated on plant samples from the field. The LAMP primers were successful in identifying *R. fascians* from 116 bacterial or plant extracts from naturally infected plant samples, representing 34 plant species, originating from 13 states, and gathered over a period of 10 years. The LAMP assay amplified only pathogenic reference cultures of *R. fascians*, and did not amplify 13 other plant-associated bacteria in vitro. There was excellent agreement between the LAMP p16 primers and the p450 and fas PCR pri-

mers, and between these DNA-based methods and the pathogenicity tests. Culture-based detection is the "gold standard" for detection of *R. fascians*. Given the importance of knowing the true status of propagation material (infected or not), isolation is easily done, albeit time-consuming. However, the PCR or LAMP assays described here not only detect *R. fascians*, but are specific to pathogenic strains, which is an improvement on culture-based detection. The DNA-based detection assays described here were developed with the typical equipment of either a molecular diagnostic laboratory (PCR assay) or a well-equipped large commercial nursery (LAMP assay). The simplicity and sensitivity of LAMP assay, in particular, opens up new means of ensuring biosecurity.

The ability to reliably detect pathogenic *R. fascians* in planta using PCR has streamlined our ability to confirm a diagnosis in 1 day compared to the four or more weeks for culture-based detection or inoculation to indicator hosts. The LAMP assay has shortened diagnosis to only 2 h. By adding loop primers (18) to our assay, we increased sensitivity, although with an unacceptably high level of false positives. The LAMP p16 primers (without the loop primers), as well as the PCR primers, had a lower detection limit of 1,000 CFU/30-µl reaction. We assume that quantification of DNA amplified from the virulence genes is approximate, however, because the proportion of pathogenic *R. fascians* cells varies within a population (21). Nonetheless, as 2.5×10^4 CFU/ml is the minimal concentration of *R. fascians* required to stimulate symptoms in pea seedlings (21), it is possible that our PCR and LAMP methods have sufficient sensitivity to detect *R. fascians* in the very early stages of infection.

A turbidimeter was useful in optimizing our LAMP reaction, but is not essential for accurate *R. fascians* detection. The addition of PicoGreen eased visualization of the reaction result, as the magnesium pyrophosphate precipitation was at times difficult to evaluate. However, PicoGreen has some drawbacks. LAMP reaction products are extremely stable, and opening tubes to add product to PicoGreen may increase the risk of contamination of laboratory equipment with LAMP amplicons, thereby increasing the risk of false positives (33). As such, tubes of LAMP amplicons should be opened away from the sample extraction and reaction preparation areas.

The LAMP and PCR primers target different gene regions, which increases their utility as diagnostic tools. The PCR primers we developed are specific to plasmid-borne genes intimately involved with cytokinin production (4,5,27,28), the primary means by which *R. fascians* causes plant disfigurement. We developed two primer sets rather than only one to increase diagnostic confidence in the case of nucleotide mutations that alter the primer binding site. Indeed, the p450 primers occasionally gave different results than the fas primers, the latter of which appear to be more accurate. The gene region targeted by the p450 primers may be more variable than the highly conserved *fasD* gene.

The eight samples from which bacterial extracts were positive by PCR, but from which tissue extracts were negative, was likely due to the presence of PCR inhibitors in the plant cells. Our plant wash method was meant to minimize cell disruption, but it prolonged assay time and did not remove PCR inhibitors, so this method was replaced by direct tissue extracts. Supplementing PCR reactions with BSA, and/or diluting template DNA depending on the plant species, reduced inhibition sufficiently to allow product amplification. LAMP is less susceptible to PCR inhibitors (8,22). Indeed, in our comparisons of PCR and LAMP using petunia and evening primrose tissues spiked with *R. fascians* DNA, we found that PCR, but not LAMP, was inhibited by compounds in evening primrose tissues. As this manuscript was in review, a real-time PCR protocol for detection of *R. fascians* was published (19), which may have potential, but requires validation with plant material, as direct tissue extracts do not always result in detection.

Although substrate-utilization assays for coryneform bacteria have been available and found accurate for medically important strains (2), this has not been the case for plant-pathogenic coryneforms. A commercial substrate utilization system (Biolog) gives inconsistent identification of *R. fascians* (6), and our findings of only six out of 31 accurate Biolog identifications support this observation. Harris-Baldwin and Gudmestad (6) used a supplemented database and found correct *R. fascians* identification for only 33% of samples. Individual *R. fascians* isolates may thus have greater flexibility in substrate utilization than is currently recognized, rendering nutritional requirements as identification criteria problematic.

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