Advanced Journal of Microbiology Research Vol. 2017
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full length Research Paper

Direct detection of *Brenneria rubrifaciens* by (polymerase chain reaction) PCR-based assay using rubrifacine synthetic gene

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Accepted 2 August, 2017

A rapid high-throughput, specific and sensitive (polymerase chain reaction) PCR-based assay coupled with DNA hybridization technique for the detection of deep bark canker (DBC) pathogen *Brenneria rubrifaciens* directly from mixed bacterial cells and *in planta* was developed. These result shows that the specific genetic markers have a powerful potential to detect *B. rubrifaciens* directly from crude samples at field conditions.

Key words: *Brenneria rubrifaciens*, deep bark canker, PCR (polymerase chain reaction), rubrifacine.

INTRODUCTION

Brenneria rubrifaciens (Wilson et al., 1967) is the causal bacterium of deep bark canker (DBC) disease of walnut tree (*Juglans regia*) as well as many other cultivars. Importantly, it has been registered as a quarantine bacterium in Korea. Detection of *B. rubrifaciens* is difficult due to lack of active external symptoms of DBC on trees and also due to its endophytic life cycle. Therefore, rapid, cheap and highly reliable methods are required to detect possible infection from symptomless trees, to develop effective management strategies of this quarantine pathogen. *B. rubrifaciens* produces a water soluble red pigment “rubrifacine”, hypothesized to be related to its virulence (McClean and Kluepfel, 2009). Additionally, the rubrifacine synthetic gene is unique to *B. rubrifaciens*, and we confirmed its conservation in five of our tested strains. Thus, we hypothesized that this gene would be useful as an appropriate target as a genetic marker for the specific detection of *B. rubrifaciens* directly from infected or diseased tissues.

Previously, McClean et al. (2008) developed both conventional and real time PCR techniques to identify the ecological behavior of *B. rubrifaciens.* However, these protocols were developed only with purified DNAs from DBC saps and artificially inoculated leaf and soil. On the Other hand, the methods did not detect *B. rubrifaciens* directly, either from pure bacterial cells or from infected samples. Although PCR is a very rapid and tempting procedure especially in the diagnostic field with purified DNAs, it may gives poor results when performed directly on mixed or crude plant homogenates. Here, we report the development of genetic markers, the adaptation of a previous method which allows direct detection of *B. rubrifaciens* from bacterial cells, mixed bacterial cultures and infected branch tissues without DNA purification.

MATERIALS AND METHODS

A total of 30 bacterial strains were used for the evaluation of our PCR-based assay which included 5 *B. rubrifaciens* and 25 other phytopathogenic bacteria (Table 1). The strains were obtained from Korean Agricultural Culture Collection (KACC, Korea), American type culture collection (ATCC™, USA) and Belgian coordinated collections of microorganisms/LMG bacteria collection (BCCM™, Belgium). Two primer pairs (i) BrAF; 5’-ATGTACGCAGTCTCTATTTGG corresponding to position 33 to 54 and BrAR; 5’- CCATCAGCCTGAAATAACTCA corresponding to position 548 to 569 of *B. rubrifaciens* asparagine synthetase gene (Genbank accession no. FJ205695) and ii) 2BrIF; 5’-

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Table 1. List of bacteria used in this study and primer and probe specificity.

Bacterial species	Strain	Primer (BrAF/BrAR)	Primer (2BrIF/2BrIR)	Probe (BrA)	Probe (BrI)	Depositor
Brenneria rubrifaciens	ATCC29291[a]	+	+	+	+	M. P. Starr
B. rubrifaciens	ATCC29292	+	+	+	+	M. P. Starr
B. rubrifaciens	LMG5110	+	+	+	+	C. Kado
B. rubrifaciens	LMG5116	+	+	+	+	C. Kado
B. rubrifaciens	LMG5117	+	+	+	+	C. Kado
Pantoea stewartii subsp. *stewartii*	ATCC8199[a]	-	-	-	-	W. H. Burkholder
P. stewartii subsp. *stewartii*	LMG2712	-	-	-	-	L. Williams
P. stewartii subsp. *indologenes*	ATCC35396	-	-	-	-	J. M. Wells
P. stewartii subsp. *indologenes*	ATCC51785[a]	-	-	-	-	J. Margaert
P. agglomerans pv. *herbicola*	LMG2565[a]	-	-	-	-	E. Rosylcky
P. annanatis	LMG2665[a]	-	-	-	-	C. Robbs
P. dispersa	LMG2603[a]	-	-	-	-	F. Gavini
P. agglomerans pv. *gypsophilae*	KNUCPB301	-	-	-	-	C. K. Lim
Pectobacterium carotovorum subsp. *atrosepticum*	ATCC33260[a]	-	-	-	-	D. Graham
P. carotovorum subsp. *carotovorum*	ATCC15713[a]	-	-	-	-	E. Hellmers
P. carotovorum subsp. *wasabiae*	ATCC43316[a]	-	-	-	-	M. Goto
P. chrysanthemi	ATCC11663[a]	-	-	-	-	W. H. Burkholder
Erwinia rhapontici	ATCC29283[a]	-	-	-	-	M.P. Starr
E. rhapontici	2OP2	-	-	-	-	C. K. Lim
Ralstonia solanacearum	KNUCPB09	-	-	-	-	C. K. Lim
Xanthomonas axonopodis pv. *glycines*	8ra	-	-	-	-	E. J. Braun
X. campestris pv. *vesicatoria*	KNUCPB07	-	-	-	-	C. K. Lim
X. codiaei	LMG8678[a]	-	-	-	-	Kersters and Swings
E. amylovora	ATCC15580[a]	-	-	-	-	D. W. Dye
E. amylovora	LMG1877	-	-	-	-	J. Hockenhull
E. amylovora	LMG1946	-	-	-	-	R. Vantomme
E. pyrifoliae	WT3	-	-	-	-	C. K. Lim
E. pyrifoliae	Ep1	-	-	-	-	S. L. Rhim
Pseudomonas fluorescens	Gpf01	-	-	-	-	C. K. Lim
P. syringae	ATCC53543	-	-	-	-	Eastman Kodak Co.

[a]Type strain, ATCC, American Type Culture Collection ; KNUCPB, Kangwon National University Collection of Phytopaghogenic Bacteria ; LMG, Laboratorium voor Microbiologie.

CGGGATCCATGTTAGAAATATTCGATGTC and 2BrIR; 5' ATCAGCTGTCAAGCCTCTTCCTTTTTG (McClean and Kluepfel, 2009) designed from the autoinducer synthase gene involved in rubrifacine production were tested. Thus, we may expect the size of amplicons as 536 and 671bp to those of BrAF-R and 2BrIF-R, respectively. The PCR

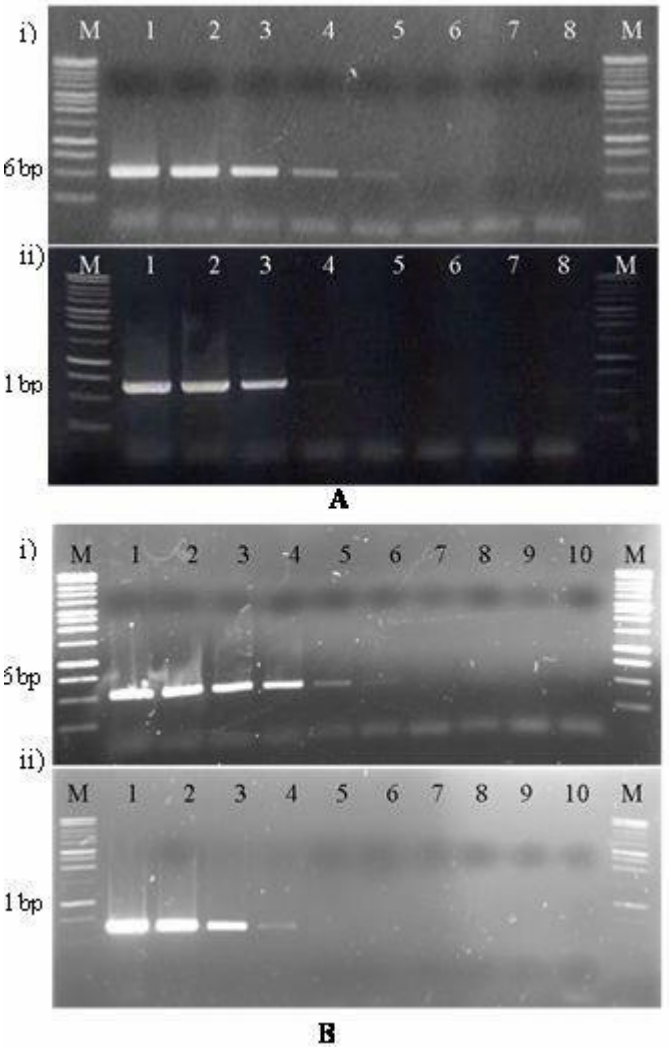

Figure 1. (A). PCR sensitivity assay from known concentrations of *B. rubrifaciens* DNA template with primers; i) BrAF/BrAR and ii) 2BrIF/2BrIR: Lane 1, 50 ng; lane 2, 5 ng; lane 3, 0.5 ng; lane 4, 50 fg; lane 5, 5 pg; lane 6, 0.5 pg; lane 7, 50 fg; lane 8, 5 fg; lane 9, 0.5 fg and lane 10, water control. (B) . Limit of detection from pure cell suspension; i) BrAF/BrAR and ii) 2BrIF/2BrIR: lane 1, 1×10^6 CFU ml $^{-1}$; lane 2, 1×10^5 CFU ml^{-1} ; lane 3, 1×10^4 CFU ml^{-1}; lane 4, 1×10^3 CFU ml^{-1} ; lane 5, 1×10^2 CFU ml $^{-1}$; lane 6, 1×10^1 CFU ml^{-1}; lane 7, 1×10^0 CFU ml^{-1}; lane 8, water control. Lane M, size marker (1kb DNA ladder, Promega™).

amplification was carried out in 25 µl reaction volumes, containing 20 pmol of each primer, 20 µM concentration of each dNTP (Promega, USA), 1 U of *Taq* polymerase (Biotools, Madrid, Spain) with 10 ng of DNA.

PCR analysis was performed with DNA thermal cycler (Bio-Rad, CA, USA). Amplification was carried out with initial denaturation at 94°C for 2 min, followed by 30 cycles consisting of denaturation of 94°C for 15 s, annealing at 64°C for 15 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. A 5 µl aliquot of each amplified PCR product was electrophoresed on a 0.7% agarose gel (Qbiogene, CA, USA) stained with ethidium bromide and visualized on a UV transilluminator.

To determine the sensitivity and limit of detection of primer pairs, pure bacterial cell suspensions (OD_{600} = 1.0) of *B. rubrifaciens* were serially diluted, 10 fold and chromosomal DNA serially diluted from 50 to 0.5 ng. 5 µl of bacterial cell suspension and 1 µl of DNA were used as PCR template.

To access the validity and robustness of our assay when analyzing mixed and plant samples, cell suspension of *B. rubrifaciens* LMG 5110 (1×10^4 CFU/ml) was mixed with cell suspension (1×10^7 CFU/ml) of *E. amylovora* ATCC 15580, *P. stewartii* LMG 2712, *E. pyrifoliae* WT3 and *P. carotovorum* ATCC 15713 and 5 µl of boiled mixed bacterial cells was used for PCR templates. Furthermore, walnut branches were wounded with scalpel approximately into epidemic cells to infect bacterium like infection naturally and then inoculated with *B. rubrifaciens* suspension (1×10^6 CFU/ml)**.** One week after infiltration, 1 g of artificially infected walnut branch tissue was cut and homogenized with mortar pestle in 9 ml sterile water and 5 µl of crude extracts were used for PCR templates. The PCR condition was used as described above.

For dot blot hybridization, DNA probes were random-primed labeled with Digoxigenin (DIG) -11-dUTP by using the DIG DNA Labeling Kit (Roche Diagnostics GmbH, Mannheim, Germany). The labeling procedures were carried out according to the manufacturer's specifications. Dot blots of the appropriate dilutions of genomic DNA were prepared by spotting 2.0 µl of denatured DNA (heated at 100°C for 10 min in equal volume of 0.2 N NaOH and immediately chilled on ice) on a Hybond-N membrane (Amersham, NJ, USA) and fixed to the membranes baking at 80°C in an oven for 2 h. Hybridization reaction was conducted overnight at 65°C with constant agitation. After hybridization, the membrane was washed 2 times for 5 min with 2 × SSC, 0.1% SDS at room temperature and twice with 0.2 × SSC, 0.1% SDS at 65°C for 15 min. Chemiluminescence detection was performed according to the manufacturer's protocol (Roche), and membranes were exposed to Kodak X-ray film (Eastman Kodak, Rochester, USA) for 1 to 30 min to detect the chemiluminescent reaction.

RESULTS AND DISCUSSION

All the *B. rubrifaciens* strains showed specific bands of size 536 bp (BrAF/BrAR) and 671 bp (2BrIF/2BrIR), but the other bacterial pathogens did not show any such bands (Table 1). The limits of detection were ~5×10^2 and 5×10^4 CFU/ml (~5 bacterial cells per reaction) of bacterial suspension and ~5 and ~50 pg of total genomic DNA, respectively (Figure 1). These detection limits are comparable with those described for other bacterial pathogens (Loreti and Gallelli, 2002; Loreti et al., 2008; McClean et al., 2008).

As expected, specific amplicons of size 537 and 671 bp were obtained from the mixed bacterial cells, and from artificially infected plant materials 7 days post inoculation (Figure 2). These results demonstrate that these primers could yield both high degree of sensitivity and specificity in detecting *B. rubrifaciens* from complex cultures containing four different species, which all are in former *Erwinia* genus. Moreover, these primers could also detect *B. rubrifaciens* from symptom less tissue indicating the feasibility of the primers for diagnosis of *B. rubrifaciens* when it present as form of ephiphytic and/or endophytic life.

Furthermore, we carried out dot blot hybridization for the sensitivity and validation of our assay. Two PCR products deduced from BrAF-R and 2BrIF-R primers

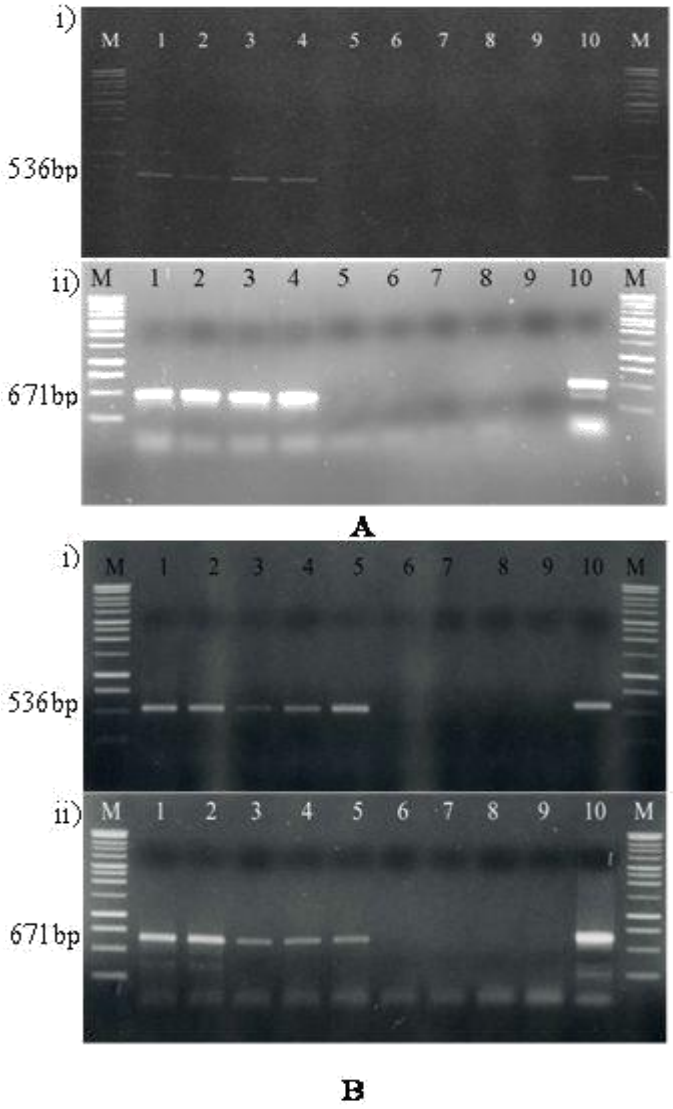

Figure 2. Specific detection of *B. rubrifaciens* with primers i) BrAF/BrAR and ii) 2BrIF/2BrIR: A) Mixed bacterial cell: Lane 1 to 4, *B. rubrifaciens* LMG 5110 and (WT3, ATCC 15580, LMG 2712 and ATCC 15713, respectively); Lane 5, ATCC 15580; Lane 6, LMG 2712; Lane 7, WT3; Lane 8, ATCC 15713; Lane 9, water; Lane 10, chromosomal DNA LMG 5110. B). Artificially infected branch: Lane 1 to 2, isolated DNA of *B. rubrifaciens* strains (LMG 5110 and LMG 5117); Lane 3 and 5, infected branch tissue suspension (LMG 5110, LMG 5116, and LMG 5117); Lane 6, LMG 2712; Lane 7, ATCC 15713, Lane 8, symptomless non inoculated branch; Lane 9, water; Lane 10, chromosomal DNA LMG 5110. Lane M, size marker (1kb DNA ladder, Promega™).

were used as probes. The hybridization results were accordance to those of PCR results (Table 1). The specificity of the probe sequence was confirmed with BLASTn searches, which failed to reveal similarities with any sequences beside *B. rubrifaciens* in the GenBank database. The probes more strongly hybridized to genomic DNA of all the *B. rubrifaciens* strains than other phytopathogenic bacteria. This finding again attests to the specificity of the assay and reaffirms the suitability for use as a diagnostic tool by non-radioactive reagents.

Taken together, BrAF-R is more sensitive than 2BrIF-R primer set however, both primers are useful for diagnosis of *B. rubrifaciens* in mixed bacteria and infected tissues.

ACKNOWLEDGEMENT

This work was supported by a grant provided by Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fishery grant (309015-04-1-HD130), Republic of Korea.

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