REAL-TIME PCR DETECTION AND DEVELOPMENT OF A BIOASSAY FOR THE DEEP BARK CANKER PATHOGEN, *BRENNERIA RUBRIFACIENS*

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ABSTRACT

Deep Bark Canker (DBC), caused by the bacterium *Brenneria rubrifaciens* afflicts English walnut cultivars and is characterized by late onset of symptoms in trees greater than 15 years old. These symptoms include deep bleeding vertical cankers along the trunk and larger branches that exude a bacterial-laden reddish brown sap. *B. rubrifaciens* produces a unique water-soluble red pigment called rubrifacine when cultured in the laboratory. Here we describe the new primer pair, BR-1 and BR-3 that amplify a unique 409bp region of the 16S rDNA sequence that facilitates the sensitive and specific detection of *B. rubrifaciens.* Using these primers in a realtime-PCR system we were able to detect as few as 8 *B. rubrifaciens* colony forming units (CFU). A survey of 11 antibiotics revealed that *B. rubrifaciens* is resistant to erythromycin and novobiocin at 10 mg/L and 30 mg/L respectively. Amending the cultivation medium with these antibiotics has improved the semi-selective cultivation of *B. rubrifaciens* on solid media. Both walnut cultivars, Hartley and Chandler, grown in tissue culture are susceptible to infection by *B. rubrifaciens*. With in 21 days after inoculation Hartley shoots turned necrotic and died. Chandler shoots exhibited a similar phenotytpe 10wk after inoculation. This latter finding will be useful in our search for *Brennaria* genes involved in pathogenesis and the identification of walnut genotypes resistant to deep bark canker.

OBJECTIVES

1. Develop sensitive and species-specific DNA primers for use in a PCR based-detection system for *Brenneria rubrifaciens.*

2. Develop a semi-selective culture medium for *B. rubrifaciens*

3. Develop a rapid in-vitro pathogenesis bioassay for *B. rubrifaciens* on walnuts.

PROCEDURES

DNA sequence analysis and primer design

BR primers, BR-1 and BR-3 were designed using a DNA sequence alignment of ribosomal DNA sequences of various *Brenneria* and *Erwinia* species. Unique sequences were selected and used to design the primers. A nucleotide BLAST search of the NCBI data base with the primer sequence returned the highest identity matches to *B. rubrifaciens*. Primers were synthesized by Operon (Huntsville, AL).

Bacterial culture and DNA extraction conditions

DNA was extracted from *E. coli* strains cultured at 37°C in Luria-Bertani (LB) medium and from *Brenneria rubrifaciens* and other bacterial species grown at 28°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates. DNA was extracted using the masterpure total DNA extraction kit (Epicentre, Madison, WI).

Specific PCR using BR primers

All PCR mixtures had a final volume of 25 μL and contained 2 mM $MgCl_2$ 1x PCR promega B buffer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.4 mM GSP1F, and GSP1R, and 1.0 U (0.2 μL) Taq polymerase (Promega, Madison WI). PCR cycling conditions consisted of an initial denaturation step (94°C, 5 minutes), followed by 35 cycles of 15 seconds at 94°C, 30 seconds at 58°C, 30 seconds at 72°C and a final elongation step of 2 minutes at 72°C.

Realtime-PCR using BR primers

All PCR mixtures had a final volume of 20 μL and contained 1x Brilliant green QPCR mix (Stratagene) and 1μM BR-1 primer, 1 μM BR-3 primer. PCR cycling conditions consisted of an initial denaturation step (95°C, 10 minutes), followed by 40 cycles of 30 seconds at 95°C, 60 seconds at 55°C, 30 seconds at 72°C and a final denaturation cycle of 60 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 95°C.

General PCR using universal primers

Purified DNA (1.0 μL or 2% total DNA sample) from each bacterial isolate was used as template in 25 μL reactions containing 529 μM forward primer fD1, 591 μM reverse primer rD1, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1x PCR buffer (Invitrogen, Carlsbad CA) 1.25 U Taq polymerase (Invitrogen, Carlsbad CA). PCR cycling conditions for the 16S rDNA PCR target were as follows; denaturation for 2 minutes at 94°C followed by 29 cycles of 30 seconds at 94°C, 60 seconds at 50°C, 90 seconds at 72°C and a final elongation step of 3 minutes at 72°C.

3.0 μL from all PCR products were analyzed in 1.5% or 2% (w/v) agarose gels in 90 mM Tris-borate, 2 mM disodium EDTA(1x TBE), + 0.5 μg/mL ethidium bromide and photographed under shortwave UV illumination.

Template DNA for real-time PCR:

A dilution series of *B. rubrifaciens* DNA was prepared and used as standards for real-time PCR. An overnight culture was serially diluted in10% TSB. Each dilution was plated on TSA to determine CFU/mL. DNA was extracted from the remaining portion of the dilution and used as sample templates for real-time PCR.

Antibiotic resistance profiling:

B. rubrifaciens from -80°C freezer stocks was plated on TSA and yeast extract dextrose calcium carbonate (YDCA) plates. Ten different antibiotics were examined. Two filter discs impregnated with the same antibiotic were placed on both a streaked TSA and YDCA plate. The plates were incubated at 28°C for 3 days. Zones of inhibition on both media types were noted.

Tissue culture inoculation

Axenic Chandler walnut shoots (~5 cm tall) were inoculated with a sterile scalpel or a scalpel laden with *B. rubrifaciens* strain 6D 370 scrapped off a YDCA plate (3 days after being streaked). The walnut plants were left in their magenta boxes without transferal to fresh media for 10 -11 weeks at 25^{o}C, 40% relative humidity and a light level of 500 lux. Plant health was assessed visually.

RESULTS AND DISCUSSION

PCR based detection can greatly improve the speed, sensitivity and specificity of detecting microorganisms in complex environments. The GSP1 primers we designed previously target the genetic loci involved in production of the unique red pigment rubrifacine in *B. rubrifaciens*. However in PCR detection strategies it is useful to have multiple targets which improve/confirm specific identification of the desired organism, a critical feature when analyzing environmental samples containing complex mixtures of closely related microorganisms. The 16S ribosomal DNA (rDNA) gene, a relatively abundant gene was chosen as the new target. A well represented DNA region such as the 16S rDNA greatly increases the probability for detection from a small number of cells. The BR primers were designed to amplify a 409bp fragment from this region. They were specific for *B. rubrifaciens* and did not amplify a product from any other bacterial DNA tested (Table 1).

A BLAST search for sequence similarity with the BR-1 and BR-3 primers in the NCBI database returned with *B. rubrifaciens* as the highest match. DNA extracted from three *B. rubrifaciens* strains produced the expected size fragment of 409 bp after PCR amplification (Table 1). The real time PCR detection limit was between 8 CFU and 0.45 CFU (Table 2). This level of sensitivity is in the range found for other diagnostic DNA primers. The control primers, fD1 and rP1, are universal primers that amplify a highly conserved region of 16S ribosomal DNA from a wide range of eubacteria (Weisburg, W.G and et al. 1991). PCR amplification using these primers confirmed that the DNA targets were indeed bacterial in addition to serving as positive controls for all PCR assays (Table 1).

B. rubrifaciens was found to be resistance to only 2 of the 11 antibiotics tested; i.e., erythromycin and novobiocin at 15 and 30 mg/L respectively (Table 2). Both compounds affect different areas of bacterial physiology, erythromycin inhibits protein synthesis and novobiocin inhibits the DNA supercoiling enzyme DNA gyrase. Addition of both antibiotics to culture media such as YDCA, 10% TSA, LBA, or M9 minimal medium created an effective semi-selective medium for isolating *B. rubrifaciens* strains from tree sap or soil. Initial experiments examining the ability of the two antibiotics to improve the selectivity of the differential medium YDC was conducted. 0.5 mg/L orchard soil spiked with *B. rubrifaciens* was suspended in water and serially diluted in water. Dilutions were plated on YDCA+ novobiocin 30 mg/L and YDCA + erythromycin 15 mg/L + novobiocin 30 mg/L. 50% of the calculated CFUs was recovered on both media (data not shown). No *B. rubrifaciens* like colonies were isolated from non-spiked soil samples.

With two sensitive primer pairs for real-time PCR detection and a new semi-selective medium, we now have the tools to begin examining the prevalence of *B. rubrifaciens* in tree sap, soil, and asymptomatic plant tissue.

Both young Chandler and Hartley shoots growing in tissue culture were suscepetible to infection by wildtype *B. rubrifaciens*. In contrast, shoots of both cultivars that were mock inoculated with water appeared similar to the non inoculated controls. Chandler shoots inoculated with the non plant pathogenic bacterium, *E.coli* DH5α, also appeared similar to the non inoculated controls. Interestingly, Hartley shoots exhibited disease symptoms within 21 days after inoculation (data not shown) while Chandler shoots needed a 10 week incubation period before symptoms were visible (Table 4).

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Strains	Primer sets		Source
	BR1+BR3	fD1+rP1	
Brenneria rubrifaciens 6D 380	+	+	This study
Brenneria rubrifaciens 6D 371	+	+	This study
Brenneria rubrifaciens 6D 370	+	+	This study
Escherichia coli			
XL1-blue	-	+	This study
Pseudomonas synxantha			
BG33R	-	+	This study
Pseudomonas putida	-	+	This study
Ralstonia solanacearum			
SC08	-	+	This study
Rhizobium leguminosarum			
biovar viciae	-	+	This study
bivar phaseoli	-	+	This study
Xanthomonas campestris	-	+	This study
Rhizobium meliloti	-	+	This study
Agrobacterium tumefaciens			
C1 chico isolate	-	+	This study
B6	-	+	This study
Brenneria alni ATCC 700182	-	+	American Type Culture Collection
Brenneria nigrafluens ATCC 13028	-	+	American Type Culture Collection
Erwinia carotovora	-	+	This study
Bacillus species	-	+	This study
Pseudomonas species	-	+	This study

Table 1. Bacterial strains used to test BR primer set specificity.
DNA extracted from the bacteria listed was amplified with both the specific BR primer set for *B. rubrifaceins* target DNA and the general fD1-rP1 primers for all eubacteria16S rDNA target. DNA samples which produced a PCR fragment are designated with a (+). Absence of the predicted fragment is indicated with a - .

Antibiotic	**concentration (**mg/L)	**Zone of Inhibiton**
Rifampin	5	+
Kanamycin	30	+
Vancomycin	30	+
Gentamycin	10	+
Novobiocin	30	-
Chloramphenicol	30	+
Neomycin	30	+
Erythromycin	15	-
Tetracycline	30	+
Ampicillin	10	+
Streptomycin	10	+

Table 2. List of antibiotics tested for activity against *B. rubrifaciens.*
Discs containing the antibiotics were placed on TSA and YDCA plates containing fresh streaks of *B. rubrifaciens* strain 6D 370. The plates were incubated at 28^{o}C and checked for zones of inhibition after 2-3 days.

	DETECTION LIMIT (DNA template)	
BR primers	0.2 picograms	8 CFU

TABLE 3. Realtime PCR detection limit of *B. rubrifaciens* using primers BR-1 and BR-3.

The sensitivity of the BR primer set was evaluated using DNA extracted from *B. rubrifaciens*. These DNA samples were used to prepare a standard curve in the real-time PCR experiments using the BR primers. DNA was extracted from serial dilutions of known CFU/mL. These samples were also used as templates in real-time PCR with the BR primers and fitted to the standard curve. The detection limit in CFU/ reaction and mass (picograms) is given above.

Inoculation	**Living plants/total**
Mock	0/5 plants
B. rubrifaceins	5/5 plants

TABLE 4. Inoculation of Chandler walnut shoots with *Brenneria rubrifaciens*. 5 tissue culture shoots were cut along the simple stem with either a sterile scalpel or a scalpel laden with *B. rubrifaciens* 6D 370. The plants were incubated for 3 months in a growth chamber at 25^{o}C, 40% humidity, and a light level of 500 lux. Plant health was visually scored.. The values above are the average of three experiments.