DEVELOPMENT OF A PCR-BASED METHOD FOR THE DETECTION OF *BRENNERIA RUBRIFACIENS*; THE CAUSAL AGENT OF DEEP BARK CANKER OF WALNUT

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ABSTRACT

Deep Bark Canker (DBC), caused by the bacterium *Brenneria rubrifaciens* (previously known as *Erwinia 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 throughout the tree that exude a bacterial-laden reddish brown sap. When cultured in artificial media, *B. rubrifaciens* produces a unique water-soluble red pigment of unknown function called rubrifacine. Given the unique nature of this pigment we choose to develop PCR primers specific to one of the genes involved in rubrifacine biosynthesis. Twenty-two transposon mutants deficient in rubrifacine production were generated from a pool of 603 mutants. Gene specific primers (GSP1) were designed to amplify a 233 bp region around the transposon insertion site in pigment-minus mutant 61. GSP1 primers failed to generate an amplification product from the genomic DNA isolated from closely related *Erwinia* and *Brenneria* species or from the 15 species in 6 different plant-associated bacterial genera that were examined. The limits of PCR-based detection, with GSP1 primers, was determined using both pure culture isolates of *B. rubrifaciens* and walnut leaves infiltrated with *B. rubrifaciens*. Genomic DNA from pure culture *B. rubrifaciens* was serially diluted and used as target DNA with GSP1 primers. The limit of PCR detection was 0.27 μg of *B. rubrifaciens* DNA which represents approximately 61 *B. rubrifaciens* cells. GSP1 primers also were able to detect as few as 14 *B. rubrifaciens* cells in 1 mg of bacteria-infiltrated walnut leaf tissue. These primers provide a new specific and sensitive tool for the detection of *B. rubrifaciens* strains in both sap and walnut plant tissue.

OBJECTIVES

Goal: Develop a culture independent based method for the detection of *B. rubrifaciens* from soil, walnut sap and walnut plant tissue.

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

2. Combine the use of a direct DNA extraction with a PCR-based method to detect the presence of *Brenneria rubrifaciens* in non-symptomatic plant tissue and orchard soil.

PROCEDURES

Generation and characterization of *B. rubrifaciens* pigment-deficient transposon mutants: Transposon mutants of *B. rubrifaciens* were generated using EZ::TN <R6Kγori/KAN-2> (Epicentre Madison, WI) as described by the manufacturer. Plasmid DNA also was generated from the *B. rubrifaciens* pigment deficient transposon mutants using the transposon, EZ::TN <R6Kγori/KAN-2> (Epicentre Madison, WI) as described by the manufacturer.

Transposon-specific primers were used to obtain the sequence of the flanking DNA (forward primer KAN-2 FP-1 and reverse primer R6 KAN-2RP-1 (Epicentre Madison, WI). The sequences were analyzed using Vector NTI software. Gene homology was assigned based on translated query BLAST results of sequence data in the NCBI database.

DNA sequence analysis and primer design

GSP1 primers, GSP1F and GSP1R were designed using DNA sequence data from pigment minus *B. rubrifaciens* mutant 61. Using the sequence for each primer, a nucleotide BLAST search of the NCBI data base was conducted. Primers were synthesized by Operon (Huntsville, AL).

Bacterial culture and DNA extraction conditions

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

Detection of *B. rubrifaciens* from inoculated walnut tissue or tree sap

Artificially inoculated leaf tissue was used for direct detection of *B. rubrifaciens* in walnut tissue. Walnut leaf discs (7mm dia, *Juglans hindsii*) were vacuum-infiltrated for 30 minutes at room temperature at 30 mmHg with *B. rubrifaciens* (GD370) suspensions ranging from 10^3 to 10^8 CFU/mL. Control leaf discs were infiltrated with sterile distilled water. After infiltration, leaf discs were rinsed twice and DNA was extracted from the discs using the masterpure total DNA extraction kit (Epicentre, Madison, WI) with two modifications; the leaf tissue was ground in liquid nitrogen prior to solubilization in cell lysis buffer and the cleared lysate, after protein precipitation, was extracted 1:1 (v/v) with 5% (w/v) polyvinylpolypyrrolidone (PVPP) prior to isopropanol precipitation. Serial dilution plating of the plant tissue grindate on TSBA was used to estimate the number of bacterial cells in both aqueous suspensions and infiltrated leaf discs. DNA from walnut tree sap was extracted using the masterpure total DNA extraction kit (Epicentre, Madison, WI) with the modifications described above.

GSP1 PCR conditions

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^{o}C, 5 minutes), followed by 35 cycles of 15 seconds at 94^{o}C, 30 seconds at 58^{o}C, 30 seconds at 72^{o}C and a final elongation step of 2 minutes at 72^{o}C.

16S rDNA PCR conditions

Purified DNA (1 µL or 2% 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^{o}C followed by 29 cycles of 30 seconds at 94^{o}C, 60 seconds at 50^{o}C, 90 seconds at 72^{o}C and a final elongation step of 3 minutes at 72^{o}C. Three 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.

RESULTS AND DISCUSSION

Currently, the cultivar Chandler dominates the California walnut industry. However, many of the Chandler orchards are approaching the age (≥15 years) when DBC symptoms are first observed on the most susceptible cultivar, Hartley. Consequently the increasingly common occurrence of Chandler trees exhibiting deep bark canker symptoms is a major concern (B. Beede personal communication). In order to examine the epidemiology of this disease and the ecology of its causative agent, *B. rubrifaciens*, we need to develop a robust method for *B. rubrifaciens* detection and quantification.

B. rubrifaciens currently, can be detected and identified, with some difficulty, using traditional culture based microbiological methods which require approximately 3-4 days (Kado and M.G. Heskett 1970, Schaad and Wilson 1970). A detection system based on PCR amplification of *B. rubrifaciens* could reduce the time required for detection to 5 hours.

Specific DNA primers for use in PCR-based detection are a necessary prerequisite to identify individual species with in a diverse community of microorganisms. This also is important for the detection and quantification of *B. rubrifaciens* in orchard samples since plant sap, leaves, stems and soil support heterogeneous bacterial populations. (Hauben et al. 1998, Loreti and Galleli 2002, Maes et al. 2002).

When cultured in artificial media, *B. rubrifaciens* produces a unique water-soluble red pigment of unknown function called rubrifacine (Feistner and Budzikiewicz, 1985). We choose to exploit the unique nature of this pigment by developing PCR primers specific to one of the genes involved in rubrifacine biosynthesis. The first step in this process was the generation of rubrifacine minus transposon mutants. Due to poor transformation efficiency, multiple independent transformations were required to assemble a collection of *B. rubrifaciens* transposon mutants. Twenty-two pigment-minus mutants were identified from a total of 603 mutants examined. The host DNA sequence flanking the transposon insertion site in the 22 pigment minus mutants were translated and submitted to a protein query pBLAST search of the NCBI data base. Eight of the 22 mutants were found to have a high homology to non-ribosomal peptide synthetases. This was the biosynthetic gene we choose to exploit in the development of species specific DNA primers GSP1F and GSP1R which amplify a 233 bp fragment of the putative peptide synthetase gene from *B. rubrifaciens*. (The BLAST results revealed this gene to be 53% similar and 36% identical to the non-ribosomal peptide synthetase gene from *Proprionobacterium acnes*). Both of these primers exhibited a low degree of homology to all entries in the NCBI data base indicating that the nucleotide sequence of this particular region is distinct from the synthetase encoding sequences found in other plant associated bacteria.

DNA extracted from three strains of *B. rubrifaciens* produced the expected size fragment of 233 bp after PCR amplification with GSP1F and GSP1R primers (Table 1). The primers did not amplify a PCR product from any of the other 14 bacterial species tested including two closely related *Brenneria* species (Table1).
PCR 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).

GSP1 primers amplified a PCR product of the expected size from 0.27 pg of purified genomic DNA template (represents approx. 61 *B. rubrifaciens* cells). Finally, the GSP1 primers also amplified the DNA target from DNA extracted from a walnut leaf disc (3.0 mg) infiltrated with as few as fourteen *B. rubrifaciens* cells/mg leaf tissue. These detection limits are significant because they are in the range of the 10 cells/mL inoculum which was shown to be sufficient to cause disease symptoms in wound inoculated walnut trees (Schaad et al. 1973).

DNA isolation and purification using the masterpure total DNA kit (Epicentre Madison WI), along with the addition of 5-10% (w/v) PVPP, provided suitable DNA for PCR analysis from both tree sap and walnut leaves. The requirement for DNA isolation and purification from these sources is probably due to the presence of such compounds as, tannins, phenolics, and polysaccharides which are known to inhibit the polymerase chain reaction (Katterman, and Shattuck, 1983).

We are continuing our efforts to increase the detection sensitivity and speed of our PCR-based system by developing real-time PCR protocols. In addition, we are also developing additional species-specific PCR primer sets and a real-time PCR system that will be useful in the detection and confirmation of *B. rubrifaciens* presence in asymptomatic plant tissue.

REFERENCES

Feistner, Gottfried and Herbert Budzikiewicz. On the structure of rubrifacine. Canadian Journal of Chemistry. 63: 1985. 495-499.

Loreti, Stefania and Gallelli, Angela. Rapid and specific detection of virulent *Pseudomonas avellanae* strains by PCR amplification. European Journal of Plant Pathology. 108 (3): 2002. 237-244.

Maes, M.; Baeyen, S.; De Croo, H.; De Smet, K.; Steenackers, M. Monitoring of endophytic *Brenneria salicis* in willow and its relation to watermark disease. Plant Protection Science. 38: 2002. 528-530.

Hauben, L., Steenackers, M., and Swings, J. PCR-based detection of the causal agent of watermark disease in willows (S*alix* spp.) Applied and Environmental Microbiology. 64 (10): 1998. 3966-3971.

Katterman, F.R.H. and V.I. Shattuck. An effective method for DNA isolation. From the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. Preparative Biochemistry. 13: 1983. 347-359.

Schaad N.W. and Wilson E.E. Survival of *Erwinia rubrifaciens* in soil. Phytopathological Notes 60: 1970. 557-558.

Schaad, Norman W., Heskett, M.G., Gardner J.M., and C.I. Kado. Influence of inoculum dosage, time after wounding and season on infection of Persian walnut trees. Phytopathology. 63: 1973. 327-329.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. 16S ribosomal DNA Amplification for Phylogenetic Study. 173: 1991. 697-703.

Table 1. Bacterial strains used to test GSP1 primer specificity.
DNA extracted from the bacteria listed was amplified with GSP1 and 16S rDNA primer sets by PCR. DNA samples which produced a PCR fragment are designated with a (+). Absence of the predicted fragment is indicated with a - .

Strains	Primer sets		Source
	GSP1	16S rDNA	
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 Tissue Culture Collectiion (ATCC)
Brenneria nigrafluens ATCC 13028	-	+	American Tissue Culture Collectiion (ATCC)
Erwinia carotovora	-	+	This study
Bacillus species	-	+	This study
Pseudomonas species	-	+	This study
			This study