Eur J Plant Pathol (2008) 122:413–424
DOI 10.1007/s10658-008-9308-z

Enhanced detection and isolation of the walnut pathogen *Brenneria rubrifaciens*, causal agent of deep bark canker

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Received: 11 September 2007 / Accepted: 20 March 2008 / Published online: 6 May 2008

Abstract Deep bark canker (DBC) of walnut is caused by the bacterium *Brenneria rubrifaciens* which produces the red pigment rubrifacine. This disease of English walnut trees, is characterized by deep vertical cankers which exude sap laden with *B. rubrifaciens*. Although DBC is not observed on young trees, it is hypothesized that *B. rubrifaciens* is present in host tissue years before symptom development. Therefore, a sensitive technique would be useful in detecting *B. rubrifaciens* in asymptomatic trees. Tn*5* mutants deficient in rubrifacine production (*pig* $^{-}$) were generated and DNA sequences from *pig* $^{-}$ mutants were used to design two primer sets; GSP1F–GSP1R and GSP2F–GSP2R. A third primer pair, BR1–BR3 was designed from the 16S rRNA gene. The three primer pairs did not amplify the diagnostic bands from members of the following bacterial genera: *Agrobacterium*, *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Rhizobium*. In addition, no amplification was observed using DNA from the following *Brenneria* species, *alni*, *nigrifluens, quercina*, or *salicis*. All three DNA primer sets detected *B. rubrifaciens* in spiked greenhouse soil and infiltrated walnut leaf tissue. PCR detection limits for BR, GSP1, and GSP2 primer pairs were 254, 254, and 2.54×10^4 colony forming units (CFU) respectively. Real-time PCR detection limit for BR primers was 8 CFU. The differential medium, yeast extract dextrose calcium carbonate agar (YDCA) was amended with novobiocin, and bacitracin, to enhance isolation from environmental samples. The improved detection and isolation methods described here will facilitate examination of *B. rubrifaciens* ecology under both nursery and orchard conditions.

Keywords PCR · Rubrifacine · Transposon · Walnut

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Introduction

Deep bark canker (DBC) of walnut, caused by the bacterium *Brenneria rubrifaciens* is a late onset disease that leads to a slow decline in productivity

of walnut trees (*Juglans regia*). DBC symptoms typically appear in mature trees and are characterized by development of deep longitudinal cracks on the, trunk, scaffolds, or larger branches which exude a dark sap containing *B. rubrifaciens* (Kado and Gardener 1977; Schaad and Wilson 1971; Wilson et al. 1967). Trunk cankers on walnut are also caused by *B. nigrifluens* (Hauben et al. 1998; Wilson et al. 1957). The dark canker on the trunk bark is limited to the outer bark for *B. nigrifluens* and to the inner bark and cambium for *B. rubrifaciens* (Wilson 1967). In addition to North America, both *B. nigrifluens* and *B. rubrifaciens* have been shown to cause disease in walnut trees across Europe (Gonzalez et al. 2002; Loreti et al. 2005; Mazzaglia et al. 2005; Menard et al. 2004). Forty-five of 54 English cultivars including Howe, Payne, Franquette, and Hartley are known to be susceptible to *B. rubrifaciens* with Hartley being the most susceptible cultivar (Schaad and Wilson 1971; Wilson et al. 1967) resulting in a decrease in the California acreage planted to this once popular cultivar. Currently, only 40% of the walnut acreage is planted to Hartley down from 90% in 1990. In California, Chandler is currently the dominant cultivar planted in new orchards.

Limited data suggest *B. rubrifaciens* may infect the vascular system of trees where it lies dormant until environmental conditions conducive to disease development occur years later (Teviotdale personal communication). This hypothesis is plausible since the bacterium has been shown to persist for extended periods of time in walnut trees that do not display DBC symptoms, such as the black walnut species *J. hindsii* and *J. nigra* (Kado et al. 1977). However, details of the infection process of *B. rubrifaciens* in walnut are poorly understood. Cracks created by mechanical shakers during harvesting and wounds created by birds and insects have been implicated as sites of bacterial entry and routes of transmission (Kado and Gardener 1977; Schaad and Wilson 1971). Graft transmission via infected budwood also has been determined to be another mode of pathogen spread (Teviotdale 1979; Teviotdale et al. 1991). Once trees are colonized with a quiescent *B. rubrifaciens* population, water stress has been identified as a key factor in eliciting the appearance of disease symptoms (Teviotdale and Sibbett 1982).

At present, there are no effective control measures for DBC in mature trees and no rapid means to detect latent infections in symptomless trees. Many of the Chandler orchards, which replaced Hartley orchards, are approaching the age (~15 years) when DBC symptoms are first observed on Hartley trees (Schaad et al. 1973). As a consequence, the increased occurrence of DBC-like symptoms in Chandler trees is a concern to the walnut industry (B. Beede personal communication).

Rapid PCR based detection of *B. rubrifaciens* in orchard/nursery soil and young walnut trees prior to symptom development will improve discrimination of DBC from other bark cankers, facilitate the development of DBC control strategies, and allow for the culling of seedlings with latent infections. Similarly, effective PCR-based detection methods for *Erwinia amylovora*, the causal agent of fireblight on apple (De Bellis et al. 2007; Llop et al. 2000), the fireblight biocontrol strain, *Pseudomonas fluorescens* (Pujot et al. 2006), the pear pathogen, *Erwinia pyrifoliae* (Kim et al. 2001), and the apple pathogen, *Pseudomonas syringae* pv. *papulans* (Vanneste and Yu 2006) have been developed and used to characterize the bacterial interaction with the plant host.

In vitro, *B. rubrifaciens* produces rubrifacine, a unique water soluble red pigment of unknown function (Feistner et al. 1984; Feistner and Budzikiewicz 1985; Wilson et al. 1967). Rubrifacine, an arylpyrrolindione, is distinct from ferrorosamine A, the red pigment produced by *Erwinia rhapontici* (Feistner et al. 1983). We hypothesized that the genes involved in rubrifacine biosynthesis, or export, would make suitable targets for PCR-based detection of *B. rubrifaciens*. Random Tn*5* mutagenesis was employed to generate *B. rubrifaciens* mutants deficient in pigment production (*pig*$^{-}$). Sequence analysis of host DNA adjacent to the transposon insertion site in selected *pig*$^{-}$ mutants was used to develop oligonucleotide primers for PCR-based detection of *B. rubrifaciens* in environmental samples.

To facilitate the isolation of *B. rubrifaciens* strains from environmental samples and complement the development of PCR-based detection tools reported here, a semi-selective medium was developed. This medium consists of YDCA amended with novobiocin and bacitracin, which dramatically reduces background microbial growth, facilitating *B. rubrifaciens* isolation from complex environments. By combining the PCR-based culture-independent approach with our improved semi-selective medium, careful examination

of *B. rubrifaciens* ecology and DBC epidemiology in walnut nurseries and orchards is now possible.

Materials and methods

Bacterial strains and cultivation

All bacterial strains, including those used for specificity testing of the oligonucleotide primers reported here are listed in Table 1. For DNA extraction, bacteria were grown in Luria–Bertani (LB) broth and LB agar (LBA) or BBL trypticase™ soy broth (TSB) and BBL trypticase™ soy agar (TSA) (Becton, Dickinson and Company, Sparks MD). Wildtype and *B. rubrifaciens* mutants were grown in yeast extract, dextrose, $CaCO_3$, (YDC) medium or on yeast extract, dextrose, $CaCO_3$ agar (YDCA) to facilitate production of rubrifacine. Colony-forming units (CFU) per unit mass or volume was determined by dilution plating on TSA plates. Antibiotic concentrations were as follows: 50 μg ml^{-1} kanamycin (Km) for *E. coli* containing rescue plasmids with the EZ-Tn5™ <R6Kγori/KAN-2> transposon flanked by *B. rubrifaciens* DNA, and 25 μg ml^{-1} kanamycin (Km) for *B. rubrifaciens* transposon mutants.

Antibiotic resistance screen

B. rubrifaciens 6D370, from an −80°C freezer stock, was plated on TSA and YDCA plates. Filter paper discs impregnated with a given antibiotic (Becton Dickinson, Sparks MD) were placed on TSA and YDCA plates containing a lawn of *B. rubrifaciens*. The plates were incubated at 28°C for 3 days after which the presence of a zone of inhibition was noted. Two discs per antibiotic were used in the screen. Nineteen antibiotics were examined in this fashion.

B. rubrifaciens recovery from spiked samples on modified YDCA (YDCA+)

YDCA amended with 30 mg l^{-1} novobiocin and 10 mg l^{-1} bacitracin was designated as YDCA+. *B. rubrifaciens* from an overnight culture grown at 28°C was serially diluted tenfold in walnut tree sap (cv. Hartley), non-sterile field soil suspension (0.1 g soil ml^{-1} H_2O), and sterile H_2O (control). Each dilution was spot-plated in 10 μl volumes in triplicate on YDCA+ and incubated at 28°C. The experiment was repeated once. Colonies were tallied after 2–3°days and detection limits determined.

DNA preparation and manipulation

Genomic DNA from *B. rubrifaciens* and other bacteria were extracted using the Masterpure Total DNA extraction kit (Epicentre, Madison, WI). Plasmid DNA was isolated using the Qiagen Miniprep kit (Valencia, CA). All oligonucleotide primers were obtained from Operon (Valencia, CA). Following heat inactivation of enzymes, DNA digestions and ligations were desalted using Microcon 30 spin columns (Microcon, Bedford, MA). The final sample volumes were adjusted to 12 μl with sterile milli-Q H_2O.

Transposon mutagenesis and pigment screen

Electrocompetent cells of *B. rubrifaciens* 6D370 were prepared as described previously using the method of Shen and Forde (1989). A transposon-based approach using the EZ-Tn5™ <R6Kγori/KAN-2> system (Epicentre, Madison, WI) was used to generate Tn5 mutants. One microliter of EZ::Tn5 <R6Kγori/KAN-2> transposome was used to transform *B. rubrifaciens* by electroporation at 9−10 kV cm^{-1} in an Eppendorf 2510 electroporator. Transformants were selected on LBA amended with 25 μg ml^{-1} Km. Each colony was used to inoculate 150 μl of YDC amended with 25 μg ml^{-1} Km in 96-well microtitre plates and incubated with shaking (200 rpm) at 28°C for 3 days. Mutants which exhibited no pigment production after three independent screenings were considered pig^- and retained for molecular characterization. DNA from *B. rubrifaciens* transposon mutants (3 μl/50 μl sample) was digested in 20 μl reactions overnight at 37°C with 5 units (U) EcoRV (Promega, Madison, WI). The digests were processed as described above (DNA manipulations and primer design) and self-ligated in 10 μl reactions with 5 U T4 DNA polymerase (Promega, Madison, WI) overnight at 15°C. Ligation mixtures were desalted and 3 μl of each genomic DNA ligation was used to electroporate 40 μl of *E. coli* pir 116^- cells (Epicentre, Madison, WI) in 0.2 cm cuvettes pulsed at 1.5 kV. Transformants were selected on LBA amended with 50 μg ml^{-1} Km. Plasmid DNA was extracted from the transformants for sequence analysis.

Table 1 Bacterial strains used and primer specificity

Primers				
Strain	BR	GSP1	GSP2	Strain source
Agrobacterium tumefaciens				
A356	−	−	−	Garfinkel et al. (1981)
ID135	−	−	−	Lin and Kado (1977)
68-3A	−	−	−	L. Epstein, U. C. Davis
B6	−	−	−	Stonier (1960)
Bacillus species	−	−	−	G. Browne USDA/ARS
Brenneria alni				
700182	−	−	−	ATCC
Brenneria nigrafluens				
13028	−	−	−	ATCC
Brenneria quercina				
29281	−	−	−	ATCC
Brenneria rubrifaciens				
2921	+	+	+	ATCC
6D381	+	+	+	Azad and Kado (1980)
6D380	+	+	+	Azad and Kado (1984)
6D379	+	+	+	Azad and Kado (1980)
6D378	+	+	+	Azad and Kado (1980)
6D377	+	+	+	Azad and Kado (1980)
6D376	+	+	+	Azad and Kado (1980)
6D375	+	+	+	Azad and Kado (1980)
6D372	+	+	+	Azad and Kado (1980)
6D371	+	+	+	Azad and Kado (1980)
6D370	+	+	+	Azad and Kado (1980)
6D368	+	+	+	Azad and Kado (1980)
6D366	+	+	+	Azad and Kado (1980)
6D360	+	+	+	Azad and Kado (1984)
6D356	+	+	+	Azad and Kado (1984)
6D348	+	+	+	Azad and Kado (1984)
6D344	+	+	+	Azad and Kado (1984)
6D34	+	+	+	Azad and Kado (1984)
6D339	+	+	+	Azad and Kado (1984)
6D331	+	+	+	Azad and Kado (1984)
6D3	+	+	+	Azad and Kado (1984)
6D327	+	+	+	Azad and Kado (1984)
1915	+	+	nt	ICPM (J. Vanneste)
1917	+	+	nt	ICPM (J. Vanneste)
TSP3	+	+	+	D. Kluepfel USDA/ARS
TSP4	+	+	+	D. Kluepfel USDA/ARS
TSP5	+	+	+	D. Kluepfel USDA/ARS
TSP6	+	+	+	D. Kluepfel USDA/ARS
TSP7	+	+	+	D. Kluepfel USDA/ARS
TSP8	+	+	+	D. Kluepfel USDA/ARS
WSBR1	+	+	+	D. Kluepfel USDA/ARS
WSBR2	+	+	+	D. Kluepfel USDA/ARS
Brenneria salicis				
15712	−	−	−	ATCC
Erwinia carotovora	−	−	−	M. Davis, U.C. Davis

Table 1 (continued)

Primers Strain	BR	GSP1	GSP2	Strain source
Escherichia coli				
JM109	–	–	–	Yanisch-Perron et al. (1985)
XL1-blue	–	–	–	Yanisch-Perron et al. (1985)
Pseudomonas synxantha				
BG33R	–	–	–	Kluepfel et al. (1993)
BG33R-216	–	–	–	Wechter et al. (2001)
BG33R-1233	–	–	–	Wechter et al. (2001)
Pseudomonas putida				
KT2440	–	–	–	Franklin et al. (1981)
Ralstonia solanacearum				
SC101	–	–	–	D. Kluepfel USDA/ARS
SC08	–	–	–	D. Kluepfel USDA/ARS
Rhizobium leguminosum				
bv. *vicae*	–	–	–	M. Eztler, U. C. Davis
bv. *phaseolica*	–	–	–	M. Eztler, U. C. Davis
Sinorhizobium meli loti	–	–	–	M. Eztler, U. C. Davis
Xanthomonas campestris	–	–	–	R. Gilbertson, U.C. Davis

DNA extracted from the bacteria listed were PCR amplified using the three primer pairs BR, GSP1, and GSP2. DNA samples which produced a PCR fragment of the predicted size are designated by a (+). A (−) designates no detectable PCR products.
nt Not tested, *ICPM* International Collection of Microorganisms from Plants (New Zealand), *ATCC* American Type Culture Collection

DNA sequence analysis and *B. rubrifaciens-specific* primer design

Host DNA adjacent to the *Tn*5 insertion in all *pig*$^-$ mutants was sequenced using primers KAN-2 FP-1 and R6 KAN-2RP-1 supplied by Epicentre (Madison, WI). The sequences were analyzed using Vector NTI software (Invitrogen, Carlsbad, CA) and contiguous sequences (contigs) were generated from the sequence data of linked mutants. A database search for homology was performed using nucleotide query BLASTn and translated query BLASTx searches of the NCBI sequence database (Altschul et al. 1990, 1994). Based on these sequence data, two *g*ene *s*pecific *p*rimer pairs (GSP) were designed; GSP1F (5′-TAGTGTTGCATTA GCCGATTTAG-3′) and GSP1R (5′-GCATTTAAAG ACTATGTTTCCTG-3′), and GSP2F (5′-CATTA CTGTTTCTCCTCGCTAATC-3′) and GSP2R (5′-GATGTAAATTAGCCATACACGGAATG-3′). Primers BR-1 (5′-CAGCGGGAAGTAGCTTGCTAC TTTGCCG G-3′) and BR-3 (5′-TGAAAAAGTC TCTCTTAAACCTTTCC-3′) were designed by evaluating the 16S rDNA sequences from 40 members of the *Enterobacteriaceae* including, *Enterobacter*, *Brenneria*, *Erwinia*, *Shigella*, and *Salmonella* species. The sequences were aligned and examined for unique stretches of sequences specific for *B. rubrifaciens*. The programme Primer3 was used to evaluate the sequence data and design the BR primer set (Rozen and Skaletsky 2000). As a PCR-positive control, universal eubacterial primers fD1 and rP1 were used to amplify a target from a highly conserved region of the 16S rRNA gene (Weisburg et al. 1991).

Species specificity of BR, GSP1 and GSP2 primer pairs

Genomic DNA was isolated as described above, from 15 plant-associated bacterial species and 32 strains of *B. rubrifaciens* collected from four counties in California over a period of 30 years (Table 1) and used as PCR template DNA (1 µl from a 30 µl DNA sample). All PCR mixtures had a final volume of 25 µl and contained 1× PCR buffer (Invitrogen, Carlsbad, CA), 0.2 mM of each deoxynucleotide triphosphate, 0.32 µM of each primer, and 1.0 U (0.2 µl) Taq polymerase (Invitrogen, Carlsbad, CA). PCR cycling conditions consisted of an initial denaturation step (94°C, 5 min), followed by 39 cycles at 94°C (15 s), 58°C (30 s), 72°C (30 s) and a final extension step of 2 min at 72°C. PCR products were

resolved on 2% (*w*/*v*) tris–borate–EDTA agarose gels and visualized with EtBr staining. The same reactions and cycling conditions were used for all primer pairs.

PCR sensitivity tests

After bacterial DNA extraction as described above, DNA from *B. rubrifaciens* was quantified using a Beckman DU800 spectrophotometer (Beckman Coulter, Fullerton, CA). A dilution series of the DNA from 200 ng to 2 fg was prepared and used to determine the limits of detection for each primer pair. This procedure was repeated once with an independent DNA dilution series. To estimate the detection limit of primer sets in CFU ml^{-1}, a ten-fold dilution series of *B. rubrifaciens* cells was generated. The CFU ml^{-1} of each dilution was determined by dilution plating in duplicate on TSA. DNA was extracted from the remaining bacterial suspension and suspended in 10 μl of water. One microliter was used as PCR template.

Detection of *B. rubrifaciens* from inoculated walnut tissue, tree sap, and soil

Juglans hindsii (black walnut) leaf discs (7 mm diameter, 3 mg) were vacuum-infiltrated with *B. rubrifaciens* suspensions ranging from 1×10^4 to 10^8 CFU ml^{-1}. A final infiltration suspension of 1 ml of 1×10^6 CFU ml^{-1} was used to infiltrate the leaf discs. Three leaf discs were infiltrated in four pulses for 5, 10, 15, and 15 min (45 min total) at 25°C with a pressure setting of 7 mm Hg in 1.5 ml microcentrifuge tubes. Control leaf discs were infiltrated with sterile distilled water. After infiltration, leaf discs were rinsed twice with 500 μl sterile water. Each disc was homogenized in 200 μl of sterile water with a sterile plastic pestle. Half of the homogenate was serially diluted on TSA to determine *B. rubrifaciens* populations. The experiment was repeated twice.

DNA was extracted from the remaining homogenate using the Masterpure total DNA extraction kit (Epicentre, Madison, WI) with two modifications. First, the slurry was ground in liquid nitrogen using a mortar and pestle prior to solubilization in lysis buffer. Second, the cleared lysate, after protein precipitation, was mixed with 30 μl of 5% (*w*/*v*) polyvinylpolypyrrolidone (PVPP) in 0.2 M potassium phosphate buffer pH 7.2 prior to isopropanol addition. DNA was extracted from walnut tree sap using the Masterpure DNA extraction kit amended with addition of PVPP as described above for leaf disc extraction. Sap was collected from walnut trees (cv. Hartley) in a Dixon, California walnut orchard exhibiting DBC symptoms. *Brenneria rubrifaciens* isolates were recovered from two of five samples after culturing dilutions of sap on YDCA.

One gram of sterilized greenhouse soil was spiked with 50–100 μl *B. rubrifaciens* from an overnight culture grown in TSB at 28°C. *B. rubrifaciens* CFU g^{-1} soil was determined by dilution plating. The tubes were spiked in duplicate. DNA was extracted from spiked soil samples (Wechter et al. 2003) and used as a template for conventional and real-time PCR. *Brenneria rubrifaciens* isolates were selected based on rubrifacine production and growth on semi-selective medium YDCA+ described above. *Brenneria rubrifaciens* was confirmed by PCR using the three primer pairs.

Real-time PCR conditions

Real-time PCR mixtures had a final volume of 20 μl and contained 1× Brilliant SYBR-Green QPCR mastermix (Stratagene, La Jolla, CA), 1 μM of each primer, and 1.0 μl DNA template. Reactions were run in optical clear tubes and caps (Stratagene, La Jolla, CA) on a Stratagene Mx 3000P real-time thermocycler. PCR cycling conditions consisted of an initial denaturation step (95°C, 10 min), followed by 40 cycles at 95°C (30 s), 55°C (30 s), 72°C (30 s) and a final dissociation segment of 95°C (60 s), 55°C (30 s), 95°C (30 s) for discriminating PCR product species. A standard curve was generated by running the ten-fold dilution series of purified DNA 200 ng−20 fg template and plotting the threshold cycle values over DNA samples (picogram) using two different DNA dilution series. DNA extracted from sap that was collected from a DBC symptomatic tree was analyzed using real-time PCR as described above. All standards and samples were run in triplicate in two separate experiments.

Results

Transposon mutagenesis and primer design

Six hundred and fifty Tn*5* mutants were generated. Twenty-two of these mutants were found to be pigment minus (pig^-). Pig^- mutants with overlapping sequences were assembled into contiguous sequences (contigs).

Contig 2 assembled from mutants Br413, Br348, and Br322 was chosen to provide the sequence for primer set GSP1. Primer set GSP2 was designed from the sequence data of mutant Br367. GSP1F and GSP1R primers showed no nucleotide homology to any published bacterial genes. Both the predicted GSP1 PCR fragment and the entire contig 2 sequence exhibited limited homology to known bacterial nucleotide sequences using nucleotide BLAST (BLASTn). Translated query BLAST (BLASTx) of the PCR fragment and the flanking host DNA sequence revealed significant matches to bacterial peptide synthetases, FenC (*Bacillus amyloliqufaciens e* value or $e=2\times10^{10}$) and plipastitn synthetase (*Bacillus subtilis* $e=2\times10^{28}$). GSP2F showed limited homology to a sequence from a *Serratia* species ATCC39006 ($e=0.32$). GSP2R was not similar to any known bacterial sequences. The predicted GSP2 PCR product had significant nucleotide (BLASTn) and amino acid (BLASTx) matches to a 'global regulator' ($e=2\times10^{-13}$ and $e=3\times10^{-16}$ respectively) from *Erwinia carotovora* subsp. *atroseptica* but only with 110/288 bp at the 3′ end of the DNA fragment. Similarly, no DNA homology was observed with the 1,250 bp of DNA adjacent to the transposon insertion site in Br367, but amino acid homology to Hor (Homologue of Rap) from *E. carotovora* subsp. *carotovora* was identified using BLASTx at the amino terminal end of the translated sequence corresponding to the 750 bp at the 5′ end.

Species specificity of *B. rubrifaciens* primers

Of the 15 bacterial species examined, only the 32 *B. rubrifaciens* strains produced DNA fragments of the predicted size with all three primer sets BR-1/BR-3 (409 bp), GSP1F/GSP1R (233 bp), and GSP2F/GSP2R (280 bp) (Table 1). The primers did not generate any PCR products from the other bacterial species tested. Universal eubacterial PCR primers fD1 and rP1 (Weisburg et al. 1991) amplified DNA from all the bacterial samples examined confirming the DNA targets were indeed bacterial in origin in addition to serving as positive controls for all PCR assays (data not shown).

In vitro primer sensitivity

Conventional PCR

DNA extracted from ten-fold serial dilutions of *B. rubrifaciens* cells was tested using all three primer pairs to determine detection limits. Limits of detection for BR, GSP1, and GSP2 primer pairs were 254, 254, and 2.54×10^4 CFU respectively.

Real-time PCR

The detection limit for BR, GSP1 and GSP2 primer pairs, using genomic DNA isolated from pure culture *B. rubrifaciens*, was 20 fg (Figs. 1 and 2), 2 pg, and

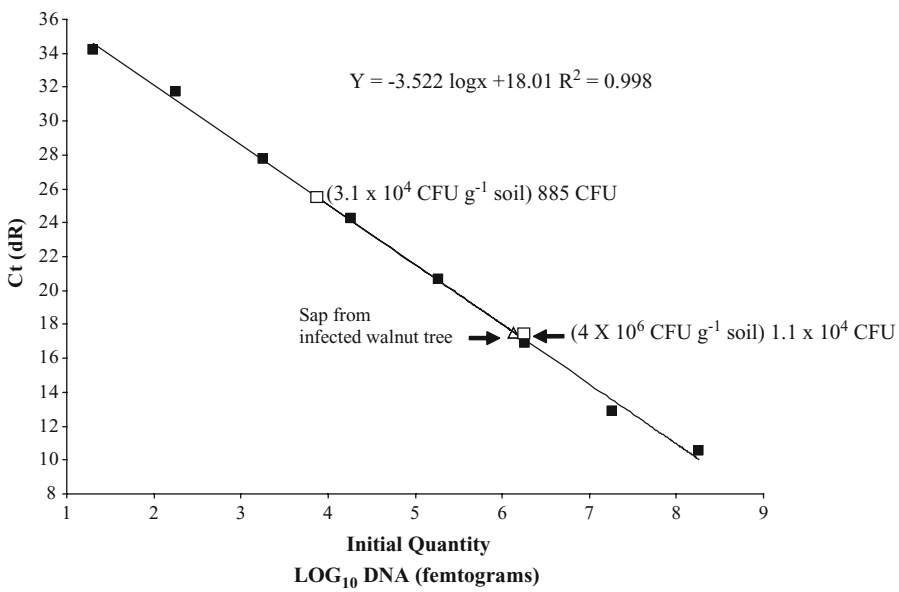

Fig. 1 Real-time PCR detection of *Brenneria rubrifaciens* DNA extraction from soil and tree sap. PCR target was detected from DNA extracted from walnut sap from a tree exhibiting DBC symptoms (*empty triangle*) an orchard soil spiked with *B. rubrifaciens* (*empty square*). Colony-forming units (*CFU*) values were determined by dilution plating on TSA. The calculated concentration of *B. rubrifaciens* in the sample is given in CFU per unit mass in parentheses adjacent to the calculated CFU in the reaction. DNA standards (*filled square*)

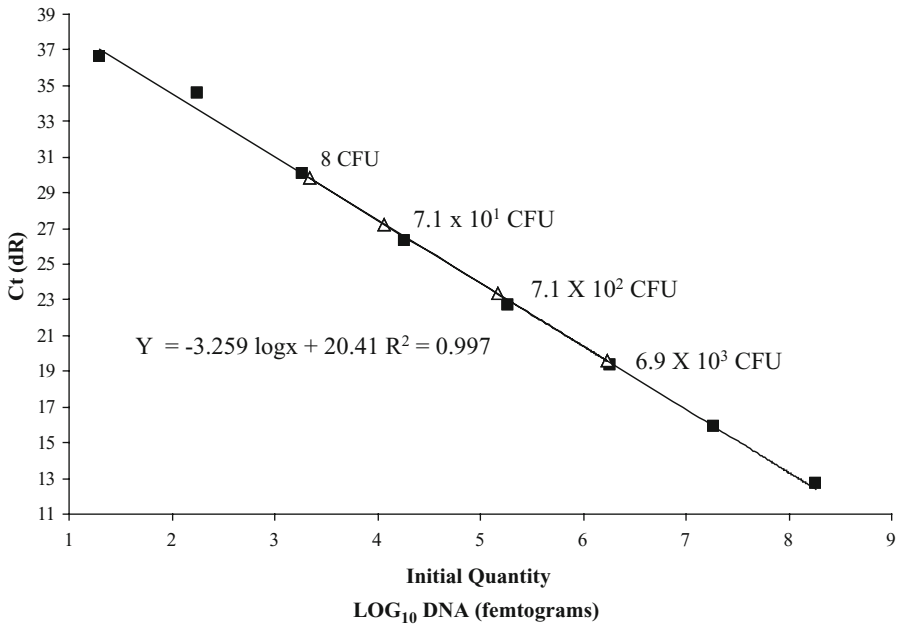

Fig. 2 Real-time PCR detection of *Brenneria rubrifaciens* with the BR primer set. PCR target DNA was extracted from a ten-fold dilution series of pure culture *B. rubrifaciens*. Samples extracted from the dilution series are designated with a (*empty triangle*). Colony-forming units (*CFU*) values were determined by dilution plating on TSA. The calculated concentration of *B. rubrifaciens* in the reaction is given in CFU. DNA standards (*filled square*)

2 pg respectively. Using DNA extracted from a *B. rubrifaciens* dilution series, BR primer pairs exhibited a detection limit of 8 CFU (Fig. 2). The other primer sets were not tested using DNA isolated from the dilution series.

PCR detection of B. rubrifaciens in walnut leaves

Primer sets, BR, GSP1, and GSP2 amplified their respective targets from DNA extracted from three infiltrated walnut leaf discs in two conventional PCR experiments. The mean CFU of *B. rubrifaciens* per milligram leaf disc (wet weight) was 182.

PCR detection in soil and sap

Three of five DNA samples extracted from five walnut sap samples from trees with DBC symptoms tested positive for *B. rubrifaciens* by conventional PCR using the BR primers. One of the three PCR-positive sap DNA extracts was analyzed in real-time PCR using the BR primer set. A *B. rubrifaciens* DNA concentration of 1 ng was detected. *B. rubrifaciens* isolates were recovered from the same BR-positive tree sap using YDCA+ and YDCA. Primer sets, BR, GSP1, and GSP2 amplified their respective targets from DNA extracted from a soil sample spiked with *B. rubrifaciens* at 5.2×10^4 CFU g^{-1} for BR and GSP1 primer sets and 5.4×10^6 CFU g^{-1} for the GSP2 primer set in two separate experiments. Using real-time PCR, BR primers detected as few as 885 CFU (Fig. 1) from a DNA extract from field soil spiked with *B. rubrifaciens* 6D370. GSP1 and GSP2 primers were not tested using real-time PCR analysis of DNA extracted from spiked soil.

Antibiotic resistance

B. rubrifaciens was susceptible to the following antibiotics: rifampin, kanamycin, vancomyin, gentamycin, chloramphenicol, neomycin, tetracycline, ampicillin, streptomycin, trimethoprim, tobramcyin, minocycline, nalidixic acid, carbencillin, cefoxitin, and cefoperazone, and penicillin. *Brenneria rubrifaciens* was found to be resistant to only two of 19 antibiotics tested. When tested individually, *B. rubrifaciens* grew in the presence of novobiocin, and bacitracin at 30, and 10 mg l^{-1} respectively. The limits of detection, determined by spot dilution plating, on YDCA+ was 1.6×10^3 CFU ml^{-1} for *B. rubrifaciens* suspended in water, 1.7×10^3 CFU ml^{-1} spiked in walnut tree sap, and 2.1×10^3 CFU ml^{-1} spiked in a suspension of field soil. Unspiked sap and field soil samples did not yield any *B. rubrifaciens*-like colonies on either YDCA or YDCA+. There was no difference in recovery of pure culture *B. rubri-*

faciens strains 6D344, 6D370, 6D371, 6D374, TSP3, TSP4, TSP5, TSP6, TSP7, WSBR1, and WSBR2 on YDCA versus YDCA+. However, YDCA+ reduced background microbial growth by tenfold as compared to YDCA (data not shown).

Discussion

Early observations suggest *B. rubrifaciens* infects young trees and becomes endophytic where it remains quiescent until bacterial populations or environmental conditions become conducive for disease development (Teviotdale 1979; Teviotdale et al. 1991). Alternatively, it has been hypothesized the bacteria may occur epiphytically on the leaf surface. However, researchers have been unable to isolate *B. rubrifaciens* from symptomless leaves and stems collected from orchard sites. When *B. rubrifaciens* was artificially inoculated into walnut leaves, they exhibited an atypical blackening and necrosis never observed under natural conditions (Schaad and Wilson 1971). Preliminary analysis of walnut leaves taken from symptomless orchard trees was negative for epiphytic *B. rubrifaciens* on the semi-selective medium YDCA +(unpublished data). These results indicate that if *B. rubrifaciens* survives as a leaf epiphyte or an endophyte in stems, it occurs below detection limits and causes no apparent changes in leaf and stem phenotype. Interestingly, *B. rubrifaciens* could be found persisting in the soil near diseased trees and from dried sap exudate residue (Schaad and Wilson 1971). In order to determine whether *B. rubrifaciens* is a resident of the endophytic or epiphytic community in symptomless walnut trees a more sensitive and specific detection method than culture based plating is required.

To facilitate our investigation into the ecology of *B. rubrifaciens* and its interaction with its plant host, we developed species-specific PCR primers for real-time PCR detection from environmental samples. The PCR primer pairs reported here, combined with direct DNA extraction, provide a robust and sensitive culture-independent method to detect *B. rubrifaciens* in environmental samples.

A diagnostic feature of *B. rubrifaciens* is the in vitro production of copious amounts of rubrifacine which distinguishes *B. rubrifaciens* from other *Brenneria* species grown on YDCA. We hypothesized that genes encoding proteins involved in the synthesis or export of rubrifacine would be unique to *B. rubrifaciens* and make suitable targets for PCR-based detection. Screening of *pig*$^-$ Tn5 mutants identified two key genetic regions involved in the production of rubrifacine. Analysis, at the amino acid level, revealed a contiguous DNA sequence adjacent to the Tn5 insertion in mutants Br322, Br348, and Br413, that was homologous to bacterial nonribosomal peptide synthetases (NRPS). These enzymes are involved in the synthesis of a variety of peptide-derived compounds including toxins, siderophores, and antibiotics (Finking and Marahiel 2004; Minowa et al. 2007; Walsh 2007).

The Tn5 insertion in mutant Br367 was in a region homologous, at the protein level, to the Hor (SlyA-like) protein, an *E. carotovora* homologue of the *r*egulation of *a*ntibiotic and *p*igment (Rap) protein from *Serratia marcescens*. Rap belongs to a conserved group of regulatory proteins controlling a variety of processes in bacterial pathogens (Thomson et al. 1997). At the nucleotide level, both loci used to design GSP1 and GSP2 primers had no significant matches in the NCBI database with any known peptide synthetase genes or other genes from plant pathogenic bacteria.

The GSP2 primers were less sensitive than BR and GSP1 primers in the detection experiments using DNA extracted from pure culture and in soil spiked with *B. rubrifaciens*. The GSP1 primers were equally as sensitive as the BR primers within the range of DNA template concentration examined using conventional PCR suggesting that the NRPS target sequence has a similar abundance or is as accessible as the BR primers are for the 16S rRNA sequence. When a DNA dilution series was used instead of the culture dilution series, the detection limit in conventional PCR was 0.27, 0.27, and 27 pg for the, BR, GSP1, and GSP2 primers respectively. Using the *E. coli* genome to estimate the *B. rubrifaciens* genome size, these mass values correspond to a detection limit of 60 CFU for BR and GSP1 primers. Real-time PCR led to an increase in the sensitivity of the BR and GSP2 primer pairs to 20 fg and 2 pg respectively, corresponding to <10 CFU.

Using a serial dilution of *B. rubrifaciens* cells combined with BR primers in real-time PCR, the limit of detection was approximately 8 CFU which is less than the minimum number of *B. rubrifaciens* cells

required for infection and DBC symptom development in walnut trees (Kado and Gardener 1977; Schaad et al. 1973). This level of sensitivity will facilitate detection of *B. rubrifaciens* from environmental samples such as sap samples from walnut trees lacking symptoms.

Walnut sap could not be used for PCR-based detection of *B. rubrifaciens* directly. Similar difficulties have been described for direct amplification of bacterial DNA from unpurified sap (Hauben et al. 1998; Katterman and Shattuck 1983). However, use of the Epicentre Masterpure DNA combined with PVPP treatment to adsorb polyphenolic contaminants facilitated PCR amplification of diagnostic DNA from diseased tree sap and infiltrated walnut leaves.

The limits of detection achieved using GSP1 and BR primer pairs are similar to those reported for other bacterial detection systems. Species-specific PCR detection of *Pseudomonas avellanae*, *Brenneria salicis*, and *Ralstonia solanacearum* was 2×10^3 CFU ml^{-1}, 20 CFU ml^{-1}, and 10^3 cells ml^{-1} respectively (Hauben et al. 1998; Loreti and Gallelli 2002; Poussier et al. 2002).

Though not selective, YDCA is commonly used for culturing *B. rubrifaciens* since it facilitates production of rubrifacine after 2–3 days of incubation (Kado and Heskett 1970; Wilson et al. 1967). Unfortunately, YDCA also supports the growth of related species such as *B. nigrifluens*, *B. alni*, and *B. salicis* (unpublished results). However, the three phenotypes: novobiocin resistance, bacitracin resistance combined with the production of rubrifacine, facilitate the use of YDCA+ as an effective semi-selective medium by reducing general background microbial growth and resolving *B. rubrifaciens* from all closely related *Brenneria* spp.

The BR, GSP1, and GSP2 primers described here can identify, detect, and quantify the deep bark canker pathogen in natural walnut tree sap, spiked walnut leaves, and spiked soil using either conventional or real-time PCR. The use of primers pairs targeting three different DNA targets will be useful in reducing the probability of false positives in complex environmental samples. Under the conditions described in this study, these primers generate unique diagnostic DNA fragments from *B. rubrifaciens* but not from other *Brenneria* species tested. This is important since *B. nigrifluens*, a related walnut pathogen, causes a similar disease known as shallow bark canker which is often observed on trees also displaying DBC symptoms (Menard et al. 2004). Armed with a robust culture-independent, real-time PCR detection system in combination with an enhanced semi-selective medium, we are prepared to examine the ecology of *B. rubrifaciens* in walnut nurseries and orchards and its interaction with *B. nigrifluens*.

Acknowledgements The authors wish to thank Dr. Patrick Wechter and Dr. Joel Vanneste for critical reading of this manuscript. This work was supported in part by the California Walnut Marketing Board and the USDA/ARS. We also wish to thank Joel Vanneste at the HortResearch Ruakura Research Centre in New Zealand for confirming the specificity of BR and GSP1 primer pairs on *B. rubrifaciens* strains in his collection.

References

Altschul, S. F., Boguski, M. S., Gish, W., & Wootton, J. C. (1994). Issues in searching molecular sequence databases. *Nature Genetics*, *6*, 119–129.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410.

Azad, H. R., & Kado, C. I. (1980). Numerical and DNA–DNA reassociation analyses of *Erwinia rubrifaciens* and other members of the Enterobacteriaceae. *Journal of General Microbiology*, *120*, 117–129.

Azad, H. R., & Kado, C. I. (1984). Relation of tobacco hypersensitivity to pathogenicity of *Erwinia rubrifaciens*. *Phytopathology*, *74*, 61–64.

De Bellis, P., Schena, L., & Cariddi, C. (2007). Real-time Scorpion-PCR detection and quantification of *Erwinia amylovora* on pear leaves and flowers. *European Journal of Plant Pathology*, *118*, 11–22.

Feistner, G., & Budzikiewicz, H. (1985). The structure of rubrifacine. *Canadian Journal of Chemistry*, *63*, 495–499.

Feistner, G., Korth, H., Budzikiewicz, H., & Pulverer, G. (1984). Rubrifacine from *Erwinia rubrifaciens*. *Current Microbiology*, *10*, 169–172.

Feistner, G., Korth, H., Ko, H., Pulverer, G., & Budzikiewicz, H. (1983). Ferrorosamine A from *Erwinia rhapontici*. *Current Microbiology*, *8*, 239–243.

Finking, R., & Marahiel, M. A. (2004). Biosynthesis of nonribosomal peptides. *Annual Review of Microbiology*, *58*, 453–488.

Franklin, F. C. H., Bagdasarian, M., Bagdasarian, M. M., & Timmis, K. N. (1981). Molecular and functional analysis of the Tol plasmid Pwwo from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. *Proceedings of the National*

Academy of Sciences of the United States of America, *78*, 7458–7462.

Garfinkel, D. J., Simpson, R. B., Ream, L. W., White, F. F., Gordon, M. P., & Nester, E. W. (1981). Genetic analysis of crown gall fine structure map of the T DNA by site directed mutagenesis. *Cell*, *27*, 143–154.

Gonzalez, R., Lopez-Lopez, M. J., Biosca, E. G., Lopez, F., Santiago, R., & Lopez, M. M. (2002). First report of bacterial deep bark canker of walnut caused by *Brenneria* (*Erwinia*) *rubrifaciens* in Europe. *Plant Disease*, *86*, 696.

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

Kado, C. I., & Gardener, J. M. (1977). Transmission of deep bark canker of walnuts by the mechanical harvester. *Plant Disease Reporter*, *61*, 321–325.

Kado, C. I., & Heskett, M. G. (1970). Selective media for isolation of *Agrobacterium*, *Cornyebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. *Phytopathology*, *60*, 969–975.

Kado, C. I., Dutra, J. C., Moller, W. J., & Ramos, D. E. (1977). An assessment of the susceptibility of various walnut cultivars to deep bark canker. *Journal of the American Society for Horticultural Science*, *102*, 698–702.

Katterman, F. R. H., & Shattuck, V. I. (1983). 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*, 347–359.

Kim, W. S., Jock, S., Paulin, J.-P., Rhim, S.-L., & Geider, K. (2001). Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. *Plant Disease*, *85*, 1183–1188.

Kluepfel, D. A., McInnis, T. M., & Zehr, E. I. (1993). Involvement of root-colonizing bacteria in peach orchard soils suppressive of the nematode *Criconemella xenoplax*. *Phytopathology*, *83*, 1240–1245.

Lin, B. C. & Kado, C. I. (1977). Evidence for the utilization of galactose by normal and transformed plant cells in culture. *Botanical Bulletin of Academia Sinica (Tapei)*, *18*, 71–77.

Llop, P., Bonaterra, A., Penalver, J., & Lopez, M. M. (2000). Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. *Applied Environmental Microbiology*, *66*, 2071–2078.

Loreti, S., & Gallelli, A. (2002). Rapid and specific detection of virulent *Pseudomonas avellanae* strains by PCR amplification. *European Journal of Plant Pathology*, *108*, 237–244.

Loreti, S., Galleli, A., Piccirillo, P., & Belisario, A. (2005). Bacterial bark canker on English walnut. *Acta Horticulturae (ISHS)*, *705*, 433–435.

Mazzaglia, A., Fabi, A., Belisario, A., Libriandi, I., Cefalo, G., Varvaro, L., & Anselmi, N. (2005). Bark cankers on English walnut: An emerging disease. *Acta Horticulturae (ISHS)*, *705*, 437–442.

Menard, M., Delort, F., Baudry, A., & Le Saux, M. (2004). First report of bacterial canker of walnut caused by *Brenneria nigrifluens* in France. *Plant Disease*, *88*, 220.

Minowa, Y., Araki, M., & Kanehisa, M. (2007). Comprehensive analysis of distinctive polyketide and nonribosomal peptide structural motifs encoded in microbial genomes. *Journal of Molecular Biology*, *368*, 1500–1517.

Poussier, S., Cheron, J. J., Couteau, A., & Luisetti, J. (2002). Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates. *Journal of Microbiological Methods*, *51*, 349–359.

Pujot, M., Badosa, E., Manceau, C., & Montesinos, E. (2006). Assessment of the environmental fate of the biological control agent of fire blight, *Pseudomonas fluorescens* EPS62e, on apple by culture and real-time PCR methods. *Applied and Environmental Microbiology*, *72*, 2421–2427.

Rozen, S., & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology*, *132*, 365–86.

Schaad, N. W., & Wilson, E. E. (1971). The ecology of *Erwinia rubrifaciens* and the development of phloem canker of Persian walnut. *Annals of Applied Biology*, *69*, 125–136.

Schaad, N. W., Heskett, M. G., Gardner, J. M., & Kado, C. I. (1973). Influence of inoculum dosage, time after wounding and season on infection of Persian walnut trees. *Phytopathology*, *63*, 327–329.

Shen, W.-J., & Forde, B. G. (1989). Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Research*, *17*, 8385.

Stonier, T. O. M. (1960). *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. *Journal of Bacteriology*, *79*, 889–898.

Teviotdale, B. L. (1979). Graft transmission of *Erwinia rubrifaciens* in English walnuts. *Phytopathology*, *69*, 921–921.

Teviotdale, B. L. & Sibbett, G. S. (1982). Midwinter irrigation can reduce deep bark canker of walnuts. *California Agriculture*, *36*, 6–7.

Teviotdale, B. L., Sibbett, G. S., Fitch, L., & Harper, D. H. (1991). Budwood transmission of *Erwinia rubrifaciens*, causal agent of deep bark canker disease of English walnut. *Plant Disease*, *75*, 360–363.

Thomson, N. R., Cox, A., Bycroft, B. W., Stewart, G. S. A. B., Williams, P., & Salmond, G. P. C. (1997). The Rap and Hor proteins of *Erwinia*, *Serratia*, and *Yersinia*: A novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. *Molecular Microbiology*, *26*, 531–544.

Vanneste, J. L., & Yu, J. (2006). Detection of *Pseudomonas syringae* pv. *papulans* in apple budwood. *New Zealand Plant Protection*, *59*, 146–149.

Walsh, C. T. (2007). Natural product enzymatic assembly lines: Novel features. *FASEB Journal*, *21*, A147–A148.

Wechter, W. P., Glandorf, D. C. M., Derrick, W. C., Leverentz, B., & Kluepfel, D. A. (2001). Identification of genetic loci in a rhizosphere-inhabiting species of *Pseudomonas* involved in expression of a phytoparasitic nematode ovicidal factor. *Soil Biology and Biochemistry*, *33*, 1749–1758.

Wechter, P., Williamson, J., Robertson, A., & Kluepfel, D. (2003). A rapid, cost-effective procedure for the extraction of microbial DNA from soil. *World Journal of Microbiology and Biotechnology*, *19*, 85–91.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S Ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*, 697–703.

Wilson, E. E., Starr, M. P., & Berger, J. A. (1957). Bark canker, a bacterial disease of Persian walnut tree. *Phytopathology*, *47*, 669–673.

Wilson, E. E., Zeiton, F. M., & Fredrickson, D. L. (1967). Bacterial phloem canker, a new disease of Persian walnut trees. *Phytopathology*, *57*, 618–621.

Yanisch-Perron, C., Vieira, J., & Messing, J. (1985). Improved M-13 phage cloning vectors and host strains nucleotide sequence of the M-13mp-18 and Puc-19 vectors. *Gene (Amsterdam)*, *33*, 103–119.