Genetic Loci Involved in Rubrifacine Production in the Walnut Pathogen *Brenneria rubrifaciens*

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ABSTRACT

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Brenneria rubrifaciens produces a unique red pigment known as rubrifacine that has been hypothesized to play a role in pathogenesis on walnut. Analysis of DNA flanking the Tn*5* insertion site in 20 rubrifacine minus (*pig*$^-$) mutants identified three regions required for rubrifacine production. The first region was homologous to nonribosomal peptide synthetases (NRPS), the second was homologous to autoinducer synthase genes (*expI* homologs), and the third region was homologous to the *slyA* gene of *Candidatus blochmania* and *Escherichia coli*. Pigment production was not necessary for elicitation of the hypersensitive response (HR) in tobacco and had little impact on virulence in tissue-cultured walnut plants. The *expI*-interrupted mutants exhibited reduced virulence on walnut and were HR negative on tobacco. Pigment production was restored in Br-212 when grown in the presence of wild-type *B. rubrifaciens*, *E. coli* carrying the cloned *expI*-like gene, or introduction of the cloned wild-type copy of the *expI*-like gene. Two *Brenneria* spp., *B. nigrifluens* and *B. salicis*, also restored pigment production in Br-212. These results demonstrate that rubrifacine production and virulence of *B. rubrifaciens* on walnut are under the control of a quorum-sensing system and are sensitive to signal molecules from other *Brenneria* spp.

Additional keywords: deep bark canker, transposon.

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 English (Persian) walnut trees. Typically, DBC symptoms are not observed on trees younger than 10 years old (47). More than 70% of the 54 English cultivars examined in pathogenicity assays were susceptible to the disease but, under field conditions, cv. Hartley was the most susceptible cultivar (22). This has contributed to cv. Chandler emerging as the dominant English cultivar in California walnut production, a position once held by Hartley. A significant concern to the walnut industry is the recent unpublished field observations indicating that Chandler, like many of the other cultivars examined, also is susceptible to DBC (R. Beede and J. Grant, *personal communication*).

It has been postulated that *B. rubrifaciens* enters an endophytic quiescent stage in susceptible walnut cultivars where it remains, perhaps for years, until environmental conditions facilitate a change to a pathogenic lifestyle. Water stress caused by extended drought conditions may be one environmental factor that triggers an increase in DBC symptoms. In fact, a limited control measure to reduce the appearance of symptoms in orchards is to increase irrigation in mid- to late winter (38). The mechanism involved in this interaction is unknown. Interestingly, HopAm1, a type III effector in the plant pathogen *Pseudomonas syringae*, has been shown to enhance virulence in water-stressed *Arabidopsis* plants (19). Similar type III effectors have been shown to occur in other plant-pathogenic members of the *Enterobacteriaceae* family (20, 39), which includes *Brenneria* spp.

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If *B. rubrifaciens* indeed persists in a quiescent state as postulated, a change in this state in response to an environmental change may be controlled by a global regulatory mechanism in the bacterial pathogen. Quorum sensing (QS) regulates many of the enzymes and other proteins involved in bacterial plant interactions in *Erwinia carotovora* subsp. *carotovora* (29). Given the late onset nature of DBC and its induction with water stress, we postulated that a similar regulatory process is involved in the appearance of disease symptoms in older walnut trees.

B. rubrifaciens is a gram-negative, rod-shaped bacterium with peritrichous flagellation and an optimal temperature growth range of 30 to 33°C (47). On yeast-dextrose calcium-carbonate agar (YDCA), *B. rubrifaciens* produces a red pigment called rubrifacine, which stains the medium but does not impart color to the bacterial colony. In vitro rubrifacine production is the key phenotypic feature that distinguishes *B. rubrifaciens* from the related walnut pathogen *B. nigrifluens*.

Plant-associated bacteria produce a diverse array of pigment molecules with a wide range of biological properties. Some of these functions include but are not limited to toxins, siderophores, signal molecules, oxidative stress reduction, and energy generation (5,11,30). It is also hypothesized that these pigments may facilitate competition in, and colonization of, susceptible host plant tissue (32,33). However, the biological role of rubrifacine, an arylpyrrolindione, remains unknown (16).

Here, we report on investigations into the characterization of genetic loci involved in rubrifacine production and regulation in addition to examining its possible role in *B. rubrifaciens* pathogenesis. Transposon mutants exhibiting a pigment-minus (*pig*$^-$) phenotype were generated and examined for their ability to elicit the hypersensitive reaction (HR) in tobacco (*Nicotiana benthamiana*) and necrosis in tissue-cultured walnut plants. Properties of the *pig*$^-$ mutants generated here provide insight into both the role of rubrifacine in walnut infection and the associated QS regu-

latory process that controls rubrifacine production and pathogenesis by *B. rubrifaciens*.

MATERIALS AND METHODS

Bacterial strains and cultivation. The bacterial strains used in this work are *B. nigrifluens* ATCC 13028, *B. salicis* ATCC 15712, *B. rubrifaciens* 6D370, *Escherichia coli* BL21 DE3 LysS, *E. coli* DH5α, and *E. coli* BL21 DE3 LysS: pRSETA-bruI. The *B. rubrifaciens* transposon mutants used in this work are Br-61, Br-62, Br-212, Br-258, Br-263, Br-322, Br-367, Br-379, Br-400, Br-401, Br-441, Br-470, Br-472, Br-501, Br-512, Br-555, Br-573, and Br-212C.

For DNA extraction, bacteria were grown in Luria-Bertani (LB) broth, LB agar (LBA), trypticase soy broth (TSB), or trypticase soy agar (TSA) (Becton, Dickinson, Sparks, MD). Wild-type and *B. rubrifaciens* mutants were grown in yeast-extract dextrose $CaCO_3$, (YDC) liquid medium or on YDCA to facilitate detection of rubrifacine production. Antibiotic concentrations were as follows: kanamycin (Km) at 50 μg/ml for *E. coli* containing rescue plasmids with EZ-Tn5 <R6Kγori/KAN-2> transposons flanked by *B. rubrifaciens* DNA, and Km at 25 μg/ml for *B. rubrifaciens* mutants with transposon EZ-Tn5 <R6Kγori/KAN-2> insertions.

Bacterial DNA preparation. Genomic DNA from all bacteria was extracted using the Masterpure Total DNA extraction kit (Epicentre, Madison, WI). Plasmid DNA was extracted using a Qiaprep spin miniprep kit (Valencia, CA) or a Wizard Plus SV miniprep kit (Promega Corp., Madison, WI).

DNA manipulations and primer design. 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) when necessary. Sample volumes were adjusted to 12 μl with sterile Milli-Q H_2O. All sequencing was performed at the University of California-Davis, College of Biological Sciences Automated DNA Sequencing Facility.

Transposon mutagenesis and pigment production screen. Electrocompetent cells of *B. rubrifaciens* 6D370 were prepared (44) and Tn*5* mutants were generated using the EZ-Tn5 <R6Kγori/KAN-2> system (Epicentre, Madison, WI). EZ::Tn5 <R6Kγori/KAN-2> transposome (1 μl) was used to transform *B. rubrifaciens* by electroporation at 9 to 10 kV/cm in an Eppendorf 2510 electroporator. Transformants were selected on LBA amended with Km at 25 μg/ml. Each colony was used to inoculate 150 μl of YDC amended with Km at 25 μg/ml in 96-well microtiter plates and incubated with shaking (200 rpm) at 28°C for 3 days. Mutants which exhibited no pigment production after three independent screenings were retained for molecular characterization (Fig. 1).

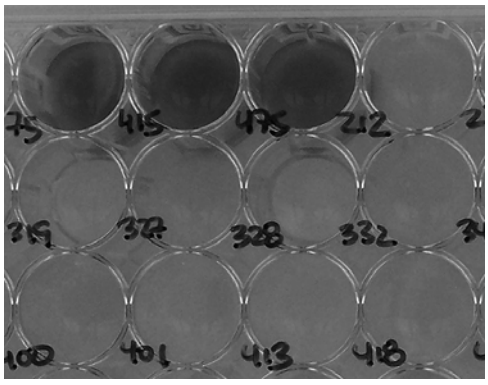

Fig. 1. Screen for mutants deficient in rubrifacine production. Transposon mutants were inoculated into 200 μl of yeast dextrose calcium carbonate (YDC) and screened for pigment production in 96-well Microtest tissue culture plate. Mutants were incubated at 28°C with shaking at 200 rpm. The first three wells in the first row show pigment positive mutants. The other wells contain pigment negative mutants.

Transformation of *B. rubrifaciens* DNA into *E. coli* pir-116. Genomic DNA from *B. rubrifaciens* transposon mutants (3 μl per 50-μl sample) was digested in 20-μl reactions overnight at 37°C with 5 units (U) of *Eco*RV (Promega Corp.). The digests were processed as described above (DNA manipulations and primer design) and self-ligated in 10-μl reactions with 5 U of T4 DNA polymerase (Promega Corp.) 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) in 0.2-cm cuvettes pulsed at 1.5 kV. Transformants were selected in LB amended with Km at 50 μg/ml. Plasmid DNA was extracted from *E. coli* transformants, digested with 5 U of *Eco*RV or 5 U of *Pst*I (Promega Corp.), and resolved in a 1% (wt/vol) Tris-borate-EDTA agarose gel followed by ethidium bromide (EtBr) staining. Larger rescue plasmids (>10 kb) were created by digesting genomic DNA from the Tn*5* mutants with *Not*I (Promega Corp.) instead of *Eco*RV and processed as described above.

DNA sequence analysis and identification of loci conferring pigment minus phenotype. In all, 20 *pig*$^-$ mutants from four independent transformations were identified from a pool of 650 transposon mutants. Host DNA adjacent to the Tn*5* insertion in *pig*$^-$ mutants was sequenced using primers KAN-2 FP-1 and R6 KAN-2RP-1 supplied by Epicentre. For some mutants, the flanking regions were also used to design primers to obtain additional DNA sequence data extending further into adjacent host DNA. Sequence data from the mutants were analyzed using Vector NTI software (Invitrogen, Carlsbad, CA) and BLASTx, or BLASTp programs (3,4) from the National Center Biotechnology Information (NCBI) website. Assemblies of contiguous sequences (contigs) were generated for the mutants.

HR bioassay. *N. benthamiana* tobacco plants were grown from seed in autoclaved potted soil under greenhouse conditions. Plants with expanded leaves were transferred to the lab for infiltration experiments. Frozen glycerol stocks (–80°C storage) of *B. rubrifaciens* were used to inoculate 10 ml of M9 minimal medium amended with 0.4% (wt/vol) glucose. The cultures were grown for 3 days at 28°C and 200 rpm in 50-ml corning tubes, subcultured 1:10 in 10 ml of fresh M9, and then incubated overnight under the same conditions. The cultures were centrifuged at 12,000 × *g* for 5 min at room temperature. The supernatant was discarded and the pellets were washed with 10 ml of sterile water and centrifuged again. The bacterial pellets were resuspended in 2 ml of sterile water and adjusted to an optical density at 600 nm = 1.0 in water using a Beckman Coulter spectrophotometer. The suspensions were pressure infiltrated into tobacco leaves using a sterile 1-ml syringe. The plants were kept in the lab under a light level of ≈380 lux, 40% relative humidity, and a temperature of 24°C. Results were scored visually at 24 and 36 h post-infiltration.

Virulence bioassay. Bacterial colonies used for walnut virulence assays were streaked from frozen stocks onto YDCA plates and grown for 4 days at 28°C. Walnut plants (cv. Hartley) were transferred into individual magenta boxes (one plant per magenta box) on DKW tissue culture medium (14). All plants were wounded using a sterile scalpel (size 22). Controls included non-wounded plants and plants wounded with the scalpel. Plants inoculated with wild-type *B. rubrifaciens* and mutants were wounded with a scalpel covered with bacteria scrapped from a bacterial culture grown on YDCA. All plants were incubated in a Conviron growth chamber for 30 to 36 days at 25°C, 40% humidity, and a regiment of 16 h of light and 8 h of darkness, with a light level of 1,525 lux.

In-situ complementation of pigment production. *Agrobacterium tumefaciens, B. salicis, B. alni, B. quercina, B. nigrifluens*, and *Pectobacterium carotovorum* were each streaked parallel (without touching) or cross-streaked (touching) to wild-type *B. rubrifaciens* or and *expI*-like-minus mutant, Br-212, grown on

YDCA. After 2 days at 28°C, the plates were visually examined for pigment production.

Both *E. coli* strain BL21 DE3 LysS and strain BL21 DE3 LysS with pRSETA-bruI containing the in-frame *expI*-like gene, designated here as *bruI*, were spotted in 5-µl drops on M9 minimal medium with 0.4% (wt/vol) glucose and 0.3% (wt/vol) agar and grown overnight at 37°C. The following day, a 5-µl drop of mutant Br-212 was pipetted adjacent to the *E. coli* culture drop but not in contact. Then, 5 µl of Br-212 and 5 µl of wild-type *B. rubrifaciens* were spotted separately onto M9 minimal media. All bacterial drops were from bacterial suspensions adjusted to an optical density of 600 nm = 0.5. The plates were incubated at 28°C and examined for pigment production after 3 to 4 days.

Oligonucleotide primers were designed to amplify the entire coding sequence of the open reading frame, *bruI*, disrupted by transposon insertion in mutant Br-212. The forward primer, 2BrI 5′, was designed from nucleotide sequence starting from the ATG start codon, basepair 1, continuing 3′ to basepair 21. The reverse primer, 2BrI 3′, was designed from the complement of the nucleotide sequence, starting 19 basepair 5′ of the stop codon and continuing to the TGA stop codon (basepair 19). Sequences for *Bam*HI and *Pvu*II restriction sites were included on the 5′ ends of primers 2BrI 5′ and 2BrI 3′, respectively.

The *bruI* gene was amplified by PCR from *B. rubrifaciens* 6D370 in the following cocktail: 0.4 µM 2BrI 5′-primer (5′-CGGGATCCATGTTAGAAATATTCGATGTC-3′), 0.4 µM 2BrI 3′-primer (5′-ATCAGCTGTCAAGCCTCTTCCTTTTTG-3′), 1× Flexi GoTaq PCR buffer, 2 mM $MgCl_2$, 200 µM each dNTP, 1 U of Flexi GoTaq, and ≈100 ng of *B. rubrifaciens* 6D370 DNA. The following cycle conditions were used: 94°C for 5 min; 2 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s; 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s; 1 cycle of 72°C for 2 min; and a hold at 4°C. The PCR product was digested as a *Bam*HI-*Pvu*II fragment and cloned in-frame into *Bam*HI-*Pvu*II-digested pRSETA to create pRSETA-*bruI*. DH5α and BL21 DE3 LysS competent *E. coli* cells (Invitrogen) were transformed with pRSETA-*bruI* according to the manufacturer's protocol. The cloned *bruI* open reading frame (ORF) was confirmed by sequence analysis and restriction digest (15-µl volumes) with 5 U of *Pvu*II and 5 U of *Bam*HI (Promega Corp.). *B. rubrifaciens* mutant Br-212C was generated by electroporating pRSETA-*bruI* into Br-212 using the method described for *B. rubrifaciens* 6D370. The intact *bruI* gene was verified by PCR as described above.

RESULTS

Nonribosomal peptide synthetase locus. Twenty *pig*$^-$ mutants from four independent transformations were identified from a pool of 650 transposon mutants. Eleven *pig*$^-$ mutants were found to be homologous to nonribosomal peptide synthetases (NRPS). Eight mutants with overlapping sequences formed a 4,907-bp contig. The BLASTp results from a 1,098-bp amino acid query yielded a match for *Bacillus cereus* ATCC 1459 peptide synthetase NP 832215.1 from the NCBI ref-seq prot database. BLASTp results from the Swiss-Prot and PDB databases resulted in strong matches to different domains, SP Q7OLM4 and pdb 1AMU A, respectively, of gramicidin synthetases (Table 1), which are NRPS proteins that synthesize broad-spectrum antibiotics. Protein domains were identified for the partial NRPS by searching the NCBI conserved domain database (CDD) (26). Three characteristic NRPS domains were found, including an adenylation (A) domain, a phosphopantetheine group (PP), also called a thioesterase (T) domain, and a condensation (C) domain. An enterobactin synthetase component F domain (EntF) was noted as well (Fig. 2A). Enterobactin synthetase is a nonribosomal peptide synthetase from *E. coli* that produces the strong iron-chelating siderophore, enterobactin. The remaining three mutants (Br-457, Br-465, and Br-573) had nonoverlapping sequences but were homologous to NRPS sequences from *Nostoc* sp. PCCv 7120 NP 486688.1 (Br-457 and Br-465 using BLASTx with a 767-bp query) and *Chromobacterium violaceum* ATCC 12472 NP 901383.1 for Br-573, also using BLASTx with a 712-bp query (Table 1). Mutants Br-457 and 465 were interrupted in the same genomic region.

Autoinducer synthase (*expI*-like locus). Two mutants, Br-212 and Br-512, are from independent transformation events and formed a two-member contig with insertions in an ORF homologous, in amino acid sequence, to autoinducer synthases (Fig. 2B). The most extensive matches from a protein blast using a 218-amino-acid (aa) query were to the AHL synthases YpeI (*Yersinia pestis* CO92) NP 405990, EchI (*Erwinia chrysanthemi*) SP Q46968, and EsaI (*Pantoea stewartii*) pdb 1K4J A from refseq-protein, Swiss-Prot, and PDB databases, respectively (Table 1). There was also significant amino acid sequence homology (62% identical) to ExpI from *E. carotovora* SP 33880 (data not shown) using BLAST 2 sequences (BLAST 2 S) (37).

TABLE 1. Sequence analysis of contiguous and noncontiguous sequences from pigment-minus (*pig*$^-$) transposon mutants

Locus, description	Database	*E* value	Mutants
Nonribosomal peptide synthetase (NRPS), accession no. FJ167368[a]			
Peptide synthetase *B. cereus* ATCC 14579	refseq-protein	8×10^{-131}	Br-61, Br-258, Br-263, Br-322, Br- 348,
LGRD BREPA linear gramicidin synthetase subunit D	Swiss-Prot	2×10^{-131}	Br-379, Br-413, and Br-555
Chain A phe activating domain of gramicidin A	PDB	4×10^{-56}	
Autoinducer synthase (BruI), accession no. FJ167366[a]			
N-AHL synthase YpeI (*Yersinia pestis* CO92)	refseq-protein	7×10^{-63}	Br-212 and Br-512
ECHI ERWCH AI synthase echI	Swiss-Prot	3×10^{-85}	
Chain A crystal structure AHL EsaI	PDB	2×10^{-54}	
SlyA, accession no. FJ167367[a]			
Transcriptional regulator SlyA *Wigglesworthia glossinidia*	refseq-protein	5×10^{-52}	Br-367, Br-400, Br-401, Br-418, Br-467,
Transcriptional regulator SlyA *Wigglesworthia glossinidia*	Swiss-Prot	3×10^{-72}	Br-470, and Br-472
NRPS, accession nos. FJ205693 and FJ205694[b]			
Peptide synthetase *Nostoc* sp PCC 7120	refseq-protein	4×10^{-15}	Br-457 and Br-465
NRPS, accession no. FJ205695[c]			
Asparagine synthetase, *Chromobacterium vioaleceum* ATCC 12472	refseq-protein	4×10^{-58}	Br-573

[a] Consensus amino acid sequences were analyzed using BLASTp and the reference proteins (refseq_protein) database from the National Center Biotechnology Information (NCBI), Swiss-Prot database, or the Brookhaven protein database (PDB). The nucleotide sequences used for the translations were determined using contigs formed from overlapping mutants.

[b] Consensus nucleotide sequences from both mutants were analyzed using BLASTx and the reference sequence (refseq_protein) database from NCBI.

[c] Nucleotide sequence from mutant Br-573 was analyzed using BLASTx and the (refseq_protein) database from NCBI. The nucleotide sequence analyzed did not share significant homology with sequences of the other NRPS-like sequences.

Transcriptional activator (*slyA*-like locus). The other common transposon insertion region contained seven mutants. A BLASTp search with the 145-aa query revealed homology to the transcriptional activator SlyA NP 871339.1 and SP Q9RBO9 from reseq-protein and Swiss-Prot databases, respectively (Table 1; Fig. 2C).

In planta assays. The *pig*$^-$ and pigment-positive (*pig*$^+$) transposon mutants were infiltrated into *N. benthamiana* tobacco leaves to assess their ability to elicit a HR (Table 2). Mutant Br-212 (*bruI*, *expI*-like interrupted ORF) was not able to induce an HR in tobacco. Both mutant Br-61, with an insertion in the NRPS-like locus, and mutant Br-470, with an insertion in the *slyA*-like ORF, were able to induce an HR. As controls, several random *pig*$^+$ Tn*5* mutants were examined (e.g., Br-62) and were found to induce an HR in tobacco. Walnut cv. Hartley grown in tissue culture was inoculated with *pig*$^-$ and several *pig*$^+$ transposon mutants (Fig. 3). Br-212 showed significantly reduced necrosis relative to wild-type *Brenneria rubrifaciens* while mutant Br-61 and Br-470 induced disease symptoms similar to those induced by wild-type *B. rubrifaciens*.

Complementation of pigment-producing phenotype. *B. salicis* and *B. nigrifluens* triggered pigment production in *bruI*-like disrupted *B. rubrifaciens* mutant Br-212 when grown in adjacent quadrants on YDCA plates. After 2 days, rubrifacine was visible

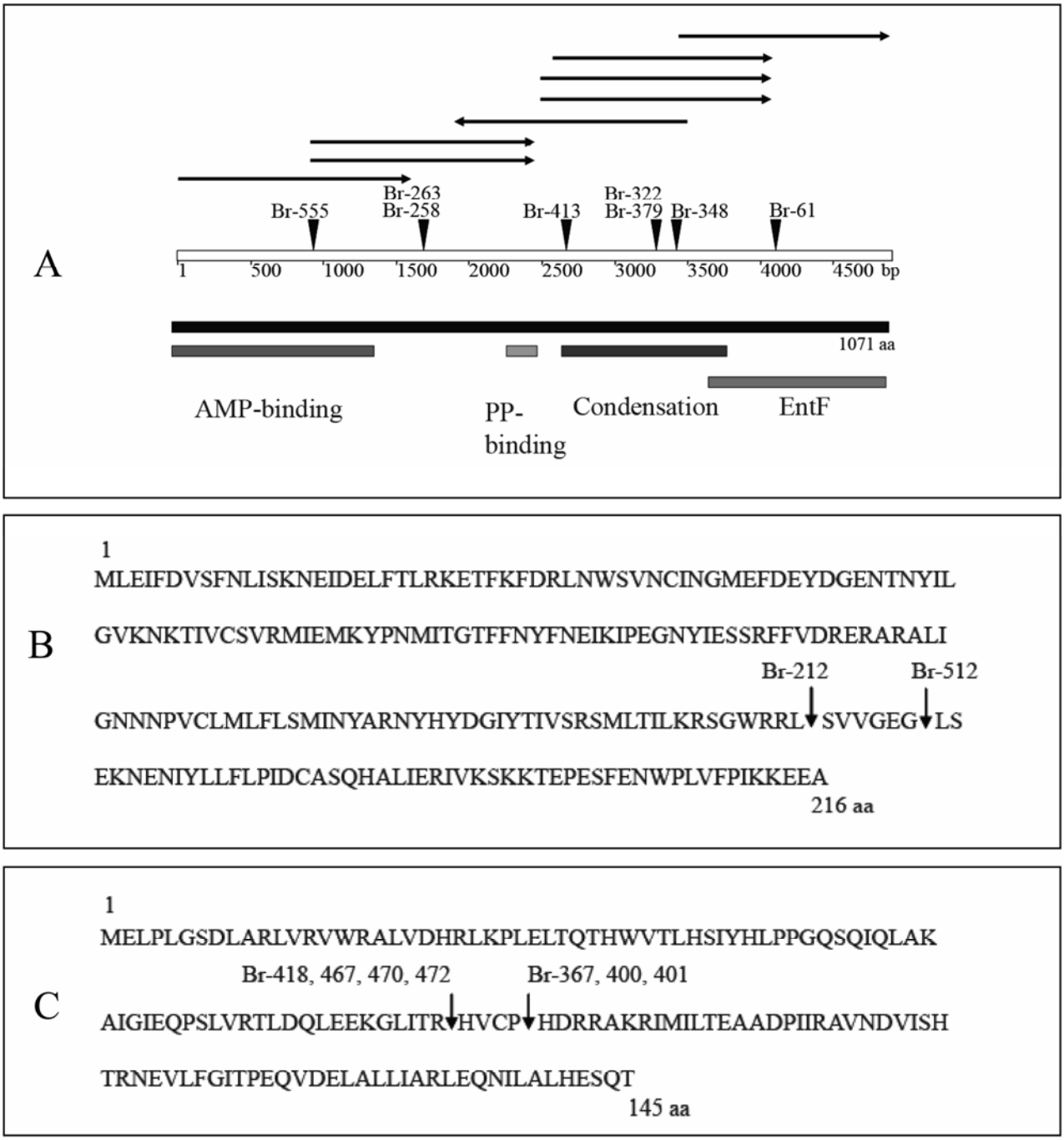

Fig. 2. Genetic characterization of transposon insertion sites in pigment-minus (*pig*$^-$) mutants. Transposon insertion sites of the *pig*$^-$ mutants organized into contiguous sequences. **A,** Nonribosomal peptide synthetases contig showing characteristic protein domains found in the conserved domain database (CDD). The vertical arrows indicate the transposon insertion sites for the specified mutants. The horizontal black arrows represent the host DNA sequence flanking the transposon insertion site. **B,** Acylhomoserine (AHL) synthetase gene amino acid sequence showing the Tn*5* insertion sites in mutants Br-212 and Br-512. **C,** SlyA open reading frame (ORF) from Br-470 showing the position of Tn*5* insertion sites of mutants Br-418, Br-467, Br-470, Br-472, Br-367, Br-400, and Br-401. The arrows pointing downward in B and C represent the transposon insertion sites for the specified mutants.

around the Br-212 colonies and the intensity of the pigment continued to increase over the next 3 days (Fig. 4). The *pig*$^-$ transposon mutants Br-61 (NRPS mutant) and Br-470 (*slyA*-like) also induced rubrifacine production in mutant Br-212 (data not shown).

Escherichia coli-bearing plasmid pRSETA-*bruI*, which includes the intact *B. rubrifaciens bruI* gene, complemented rubrifacine production by mutant Br-212 on M9 + 0.4% glucose + 0.3% (wt/vol) agar medium when cultivated adjacent to each other (Fig. 5). The pigment-production phenotype was not restored when the pRSETA-*bruI*-bearing *E. coli* was replaced with nonplasmid-bearing *E. coli* (data not shown). Plasmid pRSETA-*bruI*, when transformed into mutant Br-212 (*bruI*-interrupted ORF), created a recombinant strain Br-212C, in which pigment production was restored when grown in YDC (data not shown).

DISCUSSION

Structural and functional analyses revealed that rubrifacine production in *B. rubrifaciens* is dependent on multiple genetic loci. One of these regions is homologous at the amino acid level to nonribosomal peptide synthetases (NRPS) found in many bacteria and some fungi. NRPS are involved in the synthesis of a variety of peptide-derived compounds, including siderophores and toxins (7,17,23). Many of the homologous enzyme matches at the amino acid level were from gram-positive bacteria and cyanobacteria including, such genera as *Bacillus*, *Streptococcus*, *Streptomyces*, *Microcystis*, and *Nostoc*. Some of the distinctive domains of an NRPS module are evident in the translated sequence of the *B. rubrifaciens* NRPS contig, including an amino adenylation or activating domain (A), condensation domain (C), and thioesterase (T) or peptidyl carrier protein (PCP) domain (36).

NRPS proteins are large, multifunctional enzymes comprising modules that range in size from 100 to 1,600 aa (36). In *B. rubrifaciens*, the homologous peptide synthetase gene loci identified do not appear to be in the same ORF but may be in different ORFs or different modules located in the same gene cluster required for rubrifacine biosynthesis. NRPS-type enzymes have received extensive attention, primarily due to their potential application in generating novel antibiotics, antitumor compounds, and other bioactive compounds (1,8,13,21,40–42,49,50). The role of NRPS-encoding genes in host–pathogen interactions has recently begun to receive similar attention. Plant-pathogenic fungi such as *Cochliobolus heterostrophus* and *Magnaporthe grisea* contain NRPS genes with demonstrated roles in virulence and avirulence (10). In bacteria, some cyclic lipopeptides (CLPs) are produced using an NRPS system and function as virulence factors substantially increasing disease severity (31). Some classes of nonribosomally synthesized molecules also have multiple functions in that they are both phytotoxic and antimicrobial, such as the CLPs from some *Pseudomonas* spp. and the albicidins from *Xanthomonas albilineans*, a pathogen of sugarcane (9). However, our data suggest that rubrifacine production is not required for virulence on walnut in the bioassay used.

Other NRPS-generated molecules may offer protection against reactive oxygen species (ROS) created by host defense responses, as in the case of the blue pigment indigoidine synthesized by *Erwinia chrysanthemi* (33) and metabolites produced by the *pes1* gene of the human pathogen *Aspergillus fumigatus*. It will be interesting to explore the possibility of a similar role for rubrifacine in *B. rubrifaciens*.

Sequence analysis of the DNA flanking the *Tn* insertions in *pig*$^-$ mutants Br-367, Br-400, Br-401, BR-418, and Br-470 have a high degree of amino acid homology to the SlyA protein. This implies that the pigment biosynthetic genes lie in a regulon controlled by this regulator. SlyA is a member of MarR/SlyA family of transcriptional regulators (15). These proteins are found in over 45

TABLE 2. Hypersensitive response (HR) in tobacco bioassay[a]

Strain	Pigment production[b]	HR in tobacco[c]	Transposon insertion site (protein homology)
Brenneria rubrifaciens 6D370	+	+	Wild type
Tn*5*-mutants			
Br-61	–	+	*Bacillus cereus* (peptide synthetase)
Br-465	–	+	*B. cereus* (peptide synthetase)
Br-555	–	+	*B. cereus* (peptide synthetase)
Br-367	–	+	SlyA (transcriptional regulator)
Br-401	–	+	SlyA (transcriptional regulator)
Br-470	–	+	SlyA (transcriptional regulator)
Br-472	–	+	SlyA (transcriptional regulator)
Br-62	+	+	Unknown
Br-441	+	+	Unknown
Br-501	+	+	Unknown
Br-212	–	–	YpeI autoinducer synthetase
Br-512	–	–	YpeI autoinducer synthetase

[a] Pigment-minus *Brenneria rubrifaciens* transposon mutants were pressure infiltrated into *Nicotiana benthamiana* tobacco leaves at a concentration of 1.0 at an optical density at 600 nm. Some pigment-positive mutants were also tested and used as positive controls.
[b] Symbols: + and – denote rubrifacine production and no visible rubrifacine production, respectively.
[c] Symbols: + and – denote an HR and no HR, respectively, in tobacco leaves.

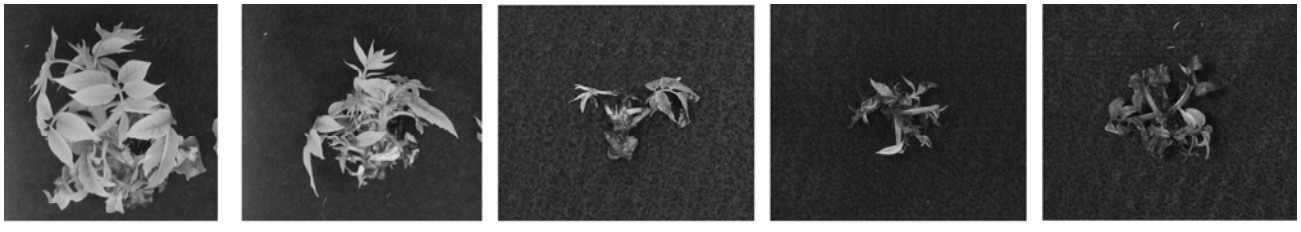

Fig. 3. Virulence bioassay on walnut plants. Tissue-cultured cv. Hartley plants were inoculated with pigment-minus mutants. Plants were wounded with a sterile scalpel (mock) or with a scalpel containing Br-212 (*bruI*$^-$), Br-470 (*slyA*$^-$), Br-61 (nonribosomal peptide synthetase$^-$), and wild-type *Brenneria rubrifaciens* 6D370. Photos were taken 1 month post inoculation.

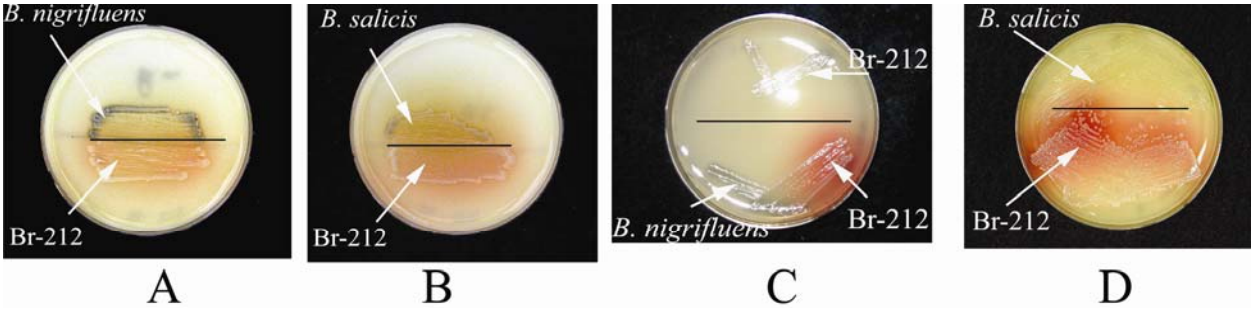

Fig. 4. Pigment phenotype complementation of autoinducer synthase mutant by other *Brenneria* spp. Br-212 was **A** and **B,** streaked parallel to and **C** and **D,** cross-streaked to *Brenneria nigrifluens* and *B. salicis*, respectively.

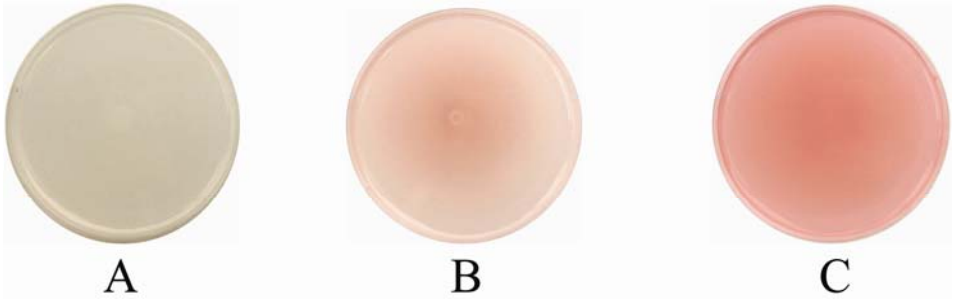

Fig. 5. Pigment phenotype in vitro complementation of autoinducer synthase mutant with *Escherichia coli*. Br-212 (5 µl) was spotted on **A,** M9 + 0.4% (wt/vol) glucose+0.3% (wt/vol) agar (M9M) and **B,** M9M + 5 µl of pRSET A +*bruI* in *E. coli* BL21DE3 LysS; and **C,** 5 µl of *Brenneria rubrifaciens* 6D370 wild-type was spotted on M9 +glucose+0.3% (wt/vol) agar as a positive control.

species of bacteria, where they act as transcription factors. Structurally, the proteins have a winged-helix DNA-binding motif and a protein-binding domain (2,46). They activate or repress the expression of genes in their respective regulon but some members also can activate or repress their own expression. Some MarR proteins act as regulators of virulence. In the animal pathogen *Y. enterocolitica*, the MarR-like protein RovA controls the expression of several genes, including *inv* which encodes invasion, the major invasion factor of enteropathogenic *Yersinia* spp. Similarly, SlyA in *Salmonella* spp. is a major regulator of genes required for virulence (28,35,48). However, not all deletions in MarR/SlyA regulators reduce virulence in bacterial pathogens. Mutations which disrupt in *E. chyrsanthemi*, *pecS* (a SlyA-like regulator), resulted in a hypervirulent phenotype (15). Other phenotypes controlled by MarR/SlyA-like genes include *E. carotovora* attachment to the gut of *Drosophila melanogaster* mediated by Hor (6), upregulated pigment production in *Serratia marcescens* by Rap, and repression of multiple drug resistance in *Escherichia coli* via MarR. Plants inoculated with the SlyA-like mutant Br-470 appeared to have more severe symptoms than wild-type inoculated plants but no significant differences in plant mass was observed in two experiments (data not shown).

The SlyA-like mutant Br-470 exhibited an in vitro growth phenotype similar to wild-type *B. rubrifaciens* 6D370 despite being defective in pigment production (Fig. 3). This suggests a regulatory role in pigment production similar to the *hor* homolog from *S. marcescens*. Additional experiments to evaluate the virulence of mutant Br-470 are ongoing.

The gene sequence flanking the inserted transposon in mutants Br-212 and Br-512 exhibited a high degree of homology, at the nucleotide level, to *expI* and *expR* from *Erwinia carotovora* (27). Both genes are homologs to the *lux* genes first described in *Vibrio* spp. which have been shown to encode proteins involved in cell-to-cell communication via QS (18,25,34,43). These phenomena regulate bacterial response to local cell density and environmental stimuli.

Genes regulating the induction of hydrolytic enzymes, a proteinaceous elicitor, virulence factors, and biofilm formation are controlled by QS in the phytopathogens *E. carotovora* subspp. *carotovora* and *atroseptica* and *P. stewartii* (12,24,29,45). The data presented here support the hypothesis that the QS locus we have identified in *B. rubrifaciens* is involved in regulating both virulence and rubrifacine production. Br-212 was unable to elicit the HR on tobacco and was attenuated in virulence on walnut, indicating that genes regulated by the *bruI* locus are important in these phenotypes. Expression of the *B. rubrifaciens expI* homolog, *bruI*, in *Escherichia coli* (in vivo), verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (data not shown), produced a diffusible signal that restored pigment production to Br-212 *in trans* on M9 agar. *E. coli* without *bruI* did not trigger pigment production in Br-212. Finally, transfer of the wild-type copy of *bruI* into *pig*$^-$ mutant Br-212 restored pigment production and HR elicitation activity, demonstrating that the *bruI* ORF is required for HR and pigment production phenotypes in *B. rubrifaciens*. Interestingly, *B. nigrifluens* and *B. salicis* also produced a diffusible signal that elicited pigment production in *B. rubrifaciens* mutant Br-212. *B. nigrifluens* and *B. rubrifaciens* cause similar diseases of walnut (shallow bark canker and deep bark canker of walnut, respectively) and often coexist in diseased walnut trees. This latter finding reveals that communication occurs between three *Brenneria* spp. which also has implications in the design of effective DBC control strategies.

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