AHL Signals Induce Rubrifacine Production in a *bruI* Mutant of *Brenneria rubrifaciens*

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ABSTRACT

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Several members of the bacterial genus *Brenneria* are pathogenic on different tree species. Cell-free extracts from the bacterial phytopathogens *Brenneria rubrifaciens*, *B. salicis*, and *B. nigrifluens* induced production of the red pigment rubrifacine in the *B. rubrifaciens* *bruI* insertional mutant Br-212. Analysis of the *bruI* locus identified an adjacent open reading frame, designated *bruR*, with homology to *luxR*. High-performance liquid chromatography and mass spectrometry analysis of ethyl acetate extracts from wild-type *B. rubrifaciens* and *Escherichia coli* expressing the *bruI* gene identified two acyl homoserine lactone (AHL)

peaks, *N*-(3-oxohexanoyl)-homoserine lactone (3OC6HSL) and *N*-hexanoyl-homoserine lactone (C6HSL). Addition of synthetic 3OC6HSL and C6HSL at 10 μ M to the *bruI* mutant, strain Br-212, induced rubrifacine production and the ability to elicit a hypersensitive reaction (HR) in tobacco leaves. Synthetic C6HSL was less effective at inducing pigment production than 3OC6HSL at 10 μ M. The *bruI* mutant Br-212 did not produce detectable AHLs, indicating that C6HSL and 3OC6HSL are the major AHLs produced by this species. The AHLs *N*-heptanoyl-DL-homoserine lactone (C7HSL), *N*-octanoyl-DL-homoserine lactone (C8HSL), and *N*-(3-oxooctanoyl)-DL-homoserine lactone (3OC8HSL) also induced pigment production in Br-212 and restored its ability to elicit an HR in tobacco, suggesting that cross-talk with other bacterial species may be possible.

Microorganisms sense other microorganisms in the environment using a variety of mechanisms. One of the best-studied chemical communication systems in bacteria is quorum sensing (QS), a type of intercellular communication which depends on the production of and response to low molecular weight inducers in a cell-density-dependent manner (10,29). These signaling molecules are called autoinducers (AIs). They are classified into two groups: AI-1 molecules, used primarily for intraspecies communication, and AI-2 compounds, thought to be important for interspecies communication (5,11,65).

The best-studied AI-1 molecules from many gram-negative bacteria are fatty acid derivatives (type I), whereas peptide derivatives (type II) are well defined for gram-positive bacteria. The type I system consists of three major parts, acyl homoserine lactone (AHL) signaling molecules, the synthases that produce the AHLs, and receptors that bind AHLs and alter gene expression (28,68). QS was first described in the marine symbiont *Vibrio fischeri* (22,24). LuxI, an acyl homoserine synthase, produces the AHLs *N*-(3-oxohexanoyl)-homoserine lactone (3OC6HSL) (major AHL) and *N*-hexanoyl-homoserine lactone (C6HSL) (35,54). These AHL signal molecules freely diffuse out of the cells and increase in proportion to the number of bacterial cells until sufficient AHLs accumulate to bind LuxR, an AHL receptor and

transcription factor. LuxR dimerizes to modulate the expression of select genes, leading to changes in the behavior of the bacterial symbiont and subsequent production of bioluminescence in the mollusk host (16,17,57). Several LuxI and LuxR homologs have been identified and characterized from a wide range of free-living, symbiotic, and pathogenic gram-negative bacterial species (66,69). These proteins regulate a diverse range of phenotypes, including antibiotic production, toxin generation, synthesis of enzymes involved in secondary metabolism, virulence, motility, and biofilm formation (8,21,46).

Brenneria rubrifaciens is the causal agent of deep bark canker (DBC) of walnut, first described in California (71). DBC typically afflicts mature (≥ 15 years old) English walnut trees and is one of three commercially important bacterial diseases of walnut along with crown gall (caused by *Agrobacterium tumefaciens*) and walnut blight (caused by *Xanthomonas arboricola* pv. *juglandis*). It is characterized by vertical cracks in the central trunk and large scaffold branches that exude a discolored sap and serve as an infection site for other microorganisms. Disease symptoms are not observed on young trees but the pathogen can be found in sap of symptomless trees of various ages, including cultivars and species used for budwood and rootstock production, suggesting a widespread distribution of natural populations of *B. rubrifaciens* (62,63). Intensity of symptoms on walnut is cultivar specific (32), with the once dominant ‘Hartley’ being replaced in orchards due to its susceptibility to DBC. Hartley once accounted for the majority of walnut trees planted in California and is now the second most planted cultivar, occupying 17% of the total acreage, while ‘Chandler’ occupies 33% of the total acreage (33). Seventeen other commercially important cultivars vary from moderately susceptible to highly susceptible to DBC (32). Adaptation of improved midwinter irrigation regimens and modified tree shaker methods by the walnut industry has helped reduce disease inci-

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dence in many orchards but recent reports of DBC have been described on Chandler, the dominant cultivar in newer orchards. The late-onset nature of DBC and presence of the pathogen in symptomless trees indicate the importance of some stimuli or possible QS regulation which mediates pathogenesis.

When cultured in vitro, *B. rubrifaciens* produces copious amounts of the water-soluble red pigment rubrifacine, which has an unknown role in pathogenesis (25). *B. rubrifaciens* transposon mutants defective in rubrifacine production were characterized previously (42). One of these pigment-deficient mutants, Br-212, was disrupted in the *luxI* homolog, *bruI*, and was unable to produce rubrifacine or elicit a hypersensitive reaction (HR) in tobacco. Br-212 was also attenuated in virulence on tissue-cultured walnut plants, implicating the involvement of QS in the regulation of these phenotypes. Preliminary work revealed two other *Brenneria* spp., *B. nigrifluens* and *B. salicis*, that also induce pigment production in Br-212 on solid media, suggesting that these species produced similar signaling compounds (42). *B. nigrifluens* causes the disease shallow bark canker in walnut (70). Symptoms are characterized by small cracks in the trunk of the tree that lightly ooze sap. *B. salicis* is the causal agent of water-mark disease in willow trees, characterized by wilted leaves and discolored bark (39). Recently, the major AHL of *B. salicis* was identified as 3OC6HSL (31). Here, we report the major AHLs of *B. rubrifaciens* as 3OC6HSL and C6HSL, which were identified by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis. In addition, a genetic locus with open reading frames (ORFs) designated *bruI* and *bruR* homologs to *luxI* and *luxR* are described. Addition of various synthetic AHLs and cell-free extracts from *B. salicis* and *B. nigrifluens* resulted in restored pigment production in the *B. rubrifaciens* mutant Br-212, and the ability to elicit an HR in tobacco are also described.

MATERIALS AND METHODS

Bacterial strains and media used. Bacterial strains used are listed in Table 1. The media types used were YDC (yeast extract at 10 g/liter, dextrose at 20 g/liter, and calcium carbonate at 20 g/liter), YDCA (YDC plus agar at 15 g/liter), and TSB (BBL trypticase soy broth at 30 g/liter [Becton Dickinson, MD]).

Rubrifacine induction by crude extracts. Ethyl acetate (EtOAc) extracts were prepared by growing *Brenneria* spp. and mutant Br-212 in 250 ml of TSB at 28°C for 2 days, after which the cells were pelleted by centrifugation. The culture fluid was filtered through a 0.2-μm filter and extracted twice with an equal volume of acidified ethyl acetate (0.1 ml HOAc/liter of EtOAc). The extract was reduced to a volume of 1 ml by rotary evaporation and stored at -20°C. Each extract (30 μl) was spread on YDCA plates (in duplicate) and subsequently plated with Br-212. Rubrifacine production was assessed visually after incubation at 28°C for 48 h. Alternatively, a 50-μl aliquot from each extract was added to 10 ml of YDC and inoculated with Br-212 (optical density at 600 nm [OD₆₀₀] = 0.05 or ≈10⁸ CFU/ml). All samples were examined for pigment production by visual examination. Selected samples were analyzed for absorbance by scanning at a

wavelength of 300 to 700 nm using a DU 800 spectrophotometer (Beckman, CA). Experiments were done in duplicate.

Rubrifacine induction with synthetic AHLs. N-butyl-L-homoserine lactone (C4HSL), C6HSL, 3OC6HSL, N-heptanoyl-DL-homoserine lactone (C7HSL), N-octanoyl-DL-homoserine lactone (C8HSL), N-(3-oxooctanoyl)-DL-homoserine lactone (3OC8HSL), N-decanoyl-DL-homoserine lactone (C10HSL), and N-dodecanoyl-DL-homoserine lactone (C12HSL) were purchased from Sigma-Aldrich (St. Louis) and dissolved in acidified EtOAc to a working concentration of 10 mM. Synthetic AHL preparation (5 μl) plus 10 μl of sterile water was spread on YDCA plates and allowed to air dry. Subsequently, Br-212 was streaked from frozen stocks directly on the plates. Samples were examined for rubrifacine production after incubation at 28°C for 48 h. Experiments were done in triplicate.

Each of the synthetic AHL preparations (10 μl) was added to YDC liquid medium at a concentration of 10 μM. The AHL-amended YDC was inoculated with Br-212 (OD₆₀₀ = 0.05). Nonamended YDC medium, YDC inoculated with Br-212 alone, and wild-type *B. rubrifaciens* 6D370 (WT) were used as controls. All cultures, in triplicate, were incubated at 28°C on a gyratory shaker (211 rpm) and examined after 72 h for rubrifacine production. All samples were examined visually for pigment production. For spectrophotometric analysis, 1 ml of each culture was transferred to a 1.5-ml microcentrifuge tube and centrifuged at maximum speed (20,000 × g) for 2 min. The samples were analyzed as described above but at a wavelength of 400 to 700 nm. Experiments were done in duplicate.

HPLC analysis. *B. rubrifaciens* 6D370 and mutant Br-212 were grown in 10-ml YDC cultures at 28°C to OD₆₀₀ = 2. The cultures were extracted twice in acidified EtOAc and the extracts dried to completion under nitrogen gas. Both samples were reconstituted in 200 μl of 50% methanol and separated using C18 reverse-phase HPLC (10 to 100% methanol gradient, flow rate 1 ml/min, duration 70 min). Fractions were assayed for acyl-HSL activity using the *Escherichia coli* reporter strain EC21 (4). *B. rubrifaciens* 6D370 extract was spiked with 3OC6HSL and C6HSL and separated using C18 reverse-phase HPLC as described above to quantify total endogenous AHLs. Standard curves for both AHLs were generated using synthetic 3OC6HSL (1 to 4 nM) and C6HSL (8 to 20 nM) and reporter strain *E. coli* EC21.

Thin-layer chromatography analysis. EtOAc extracts (0.4 to 1.0 μl) containing AHLs from *Brenneria* strains were spotted on 10-by-10-cm partisil KC18 reverse-phase thin-layer chromatography (TLC) glass plates (Whatman, NJ) and developed with 60:40 methanol/H₂O in a glass TLC chamber. AHL standards C6HSL and 3OC6HSL were prepared in acidified EtOAc at 0.5 and 1 mM concentrations. *B. salicis* crude extract was diluted (1:4) in acidified EtOAc before spotting. Extracts from *B. rubrifaciens* and *B. nigrifluens* were applied undiluted. Plates were overlaid with 52 ml of 0.8% Luria-Bertani agar and kanamycin at 25 mg/liter seeded with 2 ml of an overnight culture of the AHL indicator strain *Chromobacterium violaceum* CV026 and incubated at 28°C for 12 to 16 h (43).

Liquid chromatography-mass spectrometry analysis. *B. rubrifaciens* and *B. nigrifluens* EtOAc extracts were diluted 1:5 prior to liquid chromatography-mass spectrometry (LCMS) analysis. All spectra were acquired on an LTQ Orbitrap XL mass spectrometer equipped with a Surveyor HPLC system and an electrospray ionization source (ThermoFisher, San Jose, CA), operating in the positive ion mode. The mobile phase was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Analytical samples were separated on a Symmetry C18 column (3.5 μm, 2.1 by 150 mm) (Waters, Milford, MA) using a linear gradient of 5 to 90%, solvent B/solvent A, over 60 min at a flow rate of 200 ml/min. Mass spectra were acquired using Xcalibur, version 2.0.5 (ThermoFinnigan). The spectra were externally calibrated using malto-

TABLE 1. Bacterial strains used in this study^a

Strains	Source
<i>Chromobacterium violaceum</i> CV026	McClean et al. (43)
<i>Escherichia coli</i> EC1	Antunes et al. (4)
<i>Brenneria nigrifluens</i> ATCC 13028	ATCC
<i>B. salicis</i> 15712	ATCC
<i>B. rubrifaciens</i> 6D370	Azad and Kado (6)
Br-212 <i>bruI</i> mutant	McClean and Kluepfel (42)
Br-415 <i>rpoN</i> -like mutant	This study

^a ATCC = American Type Culture Collection.

oligosaccharides and then further calibrated internally to <2 ppm with the lock mass tool (19).

Tobacco leaf infiltrations. TSB cultures (10 ml each) of *B. rubrifaciens* 6D370 (wild type) and mutant strains Br-212 and Br-415 (a *B. rubrifaciens* 6D370 transposon mutant impaired in HR induction but not in rubrifacine production) were grown for 3 days at 28°C, subcultured 1:10 in fresh TSB, and incubated overnight at 28°C on a rotary shaker at 211 rpm. The cultures were centrifuged at 12,000 × *g* for 3 min at room temperature. The supernatants were decanted and the pellets suspended in 2 ml of sterile milli-Q water and adjusted to OD₆₀₀ = 1.0 (≈10⁹ CFU/ml). AHL extracts were added to 2 ml of bacterial suspension to generate AHL⁺ samples (10 μM AHL). The AHL⁻ controls were not amended with any AHLs. Each suspension was pressure infiltrated into at least two leaves on different *Nicotiana benthamiana* tobacco plants using 1 ml Kendall tuberculin syringes (Tyco, MA). The infiltrated areas were circled with a black marker and checked for HR development at 36 to 48 h postinoculation. The plants were kept under laboratory lighting and temperature for the duration of the experiment. Experiments were done in triplicate.

Cloning and genetic analysis. The ORF *bruR* was identified by sequencing 3' of the Tn5 (Epicentre) insertion in the *bruI* gene. The *bruR* gene was amplified by polymerase chain reaction (PCR) from *B. rubrifaciens* 6D370 in the following cocktail: 0.4 μM 5' primer BruR-F (5'-CGGGATCCATGCTTTTTT TGGTCT-3') and 0.4 μM 3' primer BruR-R (5'-ATCAGCTG TCAAAAAGGAAGAGGCTTG-3'), 1× GoTaq colorless mastermix, ≈100 ng of *B. rubrifaciens* 6D370 genomic DNA, and nuclease-free water up to 25 μl. The following cycle conditions were used: 94°C for 5 min; 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 45 s; 1 cycle of 72°C for 2 min; and a hold at 4°C. The PCR product was cloned into pGEM-T (Promega Corp., Madison, WI) according to the manufacturer's instructions and submitted for sequence analysis at the University of California DNA Sequencing Facility (University of California, Davis). The resulting nucleotide sequence was edited using Sequencher 4.7 (Gene Code Corp., MI). Blast analysis was performed using the BlastN and BlastX servers (3).

RESULTS

Brenneria extracts were analyzed for rubrifacine induction in *B. rubrifaciens* mutant Br-212. Extracts from *B. rubrifaciens*, *B. salicis*, and *B. nigrifluens* restored pigment production to the *bruI* mutant strain Br-212 in assays using both solid and liquid

Fig. 2. Thin-layer chromatography (TLC) analysis of *Brenneria* spp. culture filtrate extracts. Crude ethyl acetate extracts were resolved on C₁₈ reverse-phase TLC glass plates. Acyl homoserine lactones (AHLs) were visualized by gel overlay of the TLC plates with reporter strain *Chromobacterium violaceum* CV026. Spots indicate pigment production in the presence of AHLs by CV026. A, *N*-(3-oxohexanoyl)-homoserine lactone (3OC6HSL); B, *N*-hexanoyl-homoserine lactone (C6HSL); C, *Brenneria nigrifluens* ATCC 13028; D, *B. rubrifaciens* 6D370; E, *B. salicis* ATCC 15712; and F, Br-212.

Fig. 1. Methanol-gradient high-performance liquid chromatography (HPLC) separation of acyl homoserine lactones (AHLs) from *Brenneria rubrifaciens* 6D370 and co-elution with standards *N*-(3-oxohexanoyl)-homoserine lactone (3OC6HSL) and *N*-hexanoyl-homoserine lactone (C6HSL). HPLC analysis of ethyl acetate-extracted culture fluid from *B. rubrifaciens* 6D370. AHLs containing fractions were identified using an *Escherichia coli* reporter strain EC21. Peak sizes correlate with overall abundance of AHL product. AHL-induced green fluorescent protein fluorescence is reported in fluorescence intensity units. The solid line indicates the percent methanol. Closed squares (■) indicate the *B. rubrifaciens* 6D370 extract, open diamonds (◇) indicate the C6HSL standard, and open circles (○) represent the 3OC6HSL standard.

medium. Extracts from *bruI* mutant Br-212 did not induce pigment production on YDCA and in YDC liquid medium (data not shown).

HPLC analysis of wild-type *B. rubrifaciens* 6D370 culture supernatant revealed two abundant AHL species, 3OC6HSL and C6HSL (Fig. 1). Detection of the AHLs was confirmed using the AHL reporter strain *E. coli* EC21. HPLC analysis of mutant Br-212 extracts did not reveal any detectable peaks (data not shown). Using EC21 and synthetic AHLs, we generated a standard curve and determined that the concentrations of 3OC6HSL and C6HSL in a 10-ml culture volume were 612 and 14.8 nM, respectively. TLC analysis of crude EtOAc extracts from the culture fluid of *B. rubrifaciens*, *B. nigrifluens*, and *B. salicis*, using a *C. violaceum* CV026 reporter strain, showed the same AHL patterns corresponding to 3OC6HSL and C6HSL (Fig. 2). Extract prepared from *bruI* mutant Br-212 did not show any spots on the TLC plate (Fig. 2). The AHLs 3OC6HSL and C6HSL were identified in extracts of both *B. rubrifaciens* 6D370 and *B. nigrifluens* American Type Culture Collection (ATCC) 13028 by LCMS. Spectra from extracted ion chromatograms from both extracts revealed masses (*m/z* 214 and 200) identical to 3OC6HSL and C6HSL, respectively (Figs. 3 and 4).

We next explored whether the synthetic AHLs 3OC6HSL, C6HSL, C7HSL, C8HSL, and 3OC8HSL were sufficient to induce rubrifacine production in Br-212 using both plate-based

(YDCA) and liquid (YDC) growth assays (Figs. 5 and 6). 3OC6HSL strongly induced rubrifacine production and this response was stronger than C6HSL at the same concentration of 10 μ M in YDC (Fig. 6). C7HSL, C8HSL, and 3OC8HSL also restored pigment production in Br-212 (Table 2). Previously, we showed that *bruI* was required for the ability of *B. rubrifaciens* to induce HR formation in tobacco leaves (42). Br-212 amended with 3OC6HSL or C6HSL could now induce HR on tobacco. Intriguingly, C7HSL and C8HSL also induced an HR in tobacco colonized with Br-212 similar to the wild-type strain. Br-212 alone did not induce an HR (Table 2). Br-415, an HR⁻ mutant, with an insertion in a genetic region with homology to genes encoding RpoN type σ factors, remained HR⁻ even after addition of 3OC6HSL, C6HSL, or C8HSL, indicating that the restorative phenotype was restricted to *bruI* mutant Br-212. C4HSL, C10HSL, and C12HSL, when amended at 10 μ M, did not induce rubrifacine production in Br-212 cultured in YDC (Table 2).

Sequence analysis of the 651-bp *bruI* locus in mutant Br-212 identified another ORF in the reverse orientation (Fig. 7), revealing a *luxI-luxR* like genetic locus. This additional 735-bp ORF, labeled *bruR*, exhibited nucleotide homology (74% identical, *E* value = 5×10^{-55}) to *expR* (729 bp) from *Pectobacterium carotovorum* subsp. *carotovorum* strain BC1 using BlastN. The predicted BruR (244 amino acids) shared the highest amino acid identity (59%) with YpeR from *Yersinia pestis* (NP_669049.1)

Fig. 3. Liquid chromatography-mass spectrometry (LCMS) analysis of *Brenneria rubrifaciens* and *B. nigrifluens* culture filtrate extracts for the presence of N-hexanoyl-homoserine lactone (C6HSL). C6HSL was identified in extracted ion chromatograms from *Brenneria* sp. extracts and analyzed by high resolution MS. Peaks for C6HSL at *m/z* 200 (indicated by the arrows) and *m/z* 201 (13C mass) are observed in **A**, the synthetic standard; **B**, *B. nigrifluens*; and **C**, *B. rubrifaciens*, respectively.

using BlastX. Another LuxR-like protein, EchR (Q46967.1) from *Dickeya chrysanthemi*, exhibited the highest match to BruR in the Swissprot database (72% identity, E value = 5×10^{-98}).

DISCUSSION

Bacteria in soil or associated with plants occur in complex microbial communities (20,58). Chemical signaling between the different bacterial species in environments such as plant sap or the rhizosphere can influence the composition and behavior of the resident bacterial community (5,7,27). AHLs are one class of chemical signals utilized by bacteria for both intra- and interspecies communication (45,49). Production and detection of particular AHLs by bacterial species is highly variable, with some species producing multiple AHLs or responding to exogenous AHL signals produced by other bacteria (14,60). This may be particularly advantageous when a bacterial species resides in a competitive environment containing both related and unrelated species.

Different bacterial species from the same environment or host often produce similar or identical AHLs, providing the opportunity for cross species communication. Human pathogenic strains of *Pseudomonas aeruginosa* PAO1 and *Serratia liquefaciens* synthesize the same major AHL molecule, C4HSL (23,72), and the maize pathogens *D. zea* and *Pantoea stewartii* both produce 3OC6HSL (44). Soilborne species such as the legume-

nodulating *Rhizobium leguminosarum* bv. *viciae* 8401 and the animal pathogen *Burkholderia pseudomallei* produce a wide variety of AHLs and one, C8HSL, is produced by both (56,73). Other soil microbes producing C8HSL include *Sinorhizobium meliloti*, *Mesorhizobium meliloti*, and *Burkholderia glumae* (61), facilitating cross-talk across different bacterial genera. One clear example of AHL-mediated cross-talk among different bacterial

Fig. 5. Rubrifacine induction in Br-212 on yeast extract-dextrose-calcium carbonate agar (YDCA) medium. *Brenneria rubrifaciens* *bruI* mutant Br-212 was streaked from frozen stock on **A**, YDCA plates alone or **B**, YDCA plates prespread with 10 μ l of 10 mM *N*-(3-oxohexanoyl)-homoserine lactone (3OC6HSL) in acidified ethyl acetate across the surface. Samples were examined for pigment production after 3 days at 28°C.

Fig. 4. Liquid chromatography–mass spectrometry (LCMS) analysis of *Brenneria rubrifaciens* and *B. nigrifluens* culture filtrate extracts for the presence of *N*-(3-oxohexanoyl)-homoserine lactone (3OC6HSL). 3OC6HSL was identified in extracted ion chromatograms from *Brenneria* sp. extracts and analyzed by high resolution MS. Peaks for 3OC6HSL at m/z 214 (indicated by the arrows) and m/z 215 (13C mass) are observed in **A**, the synthetic standard; **B**, *B. nigrifluens*; and **C**, *B. rubrifaciens*, respectively.

species has been described between *Burkholderia cepacia* and *Pseudomonas aeruginosa*, phylogenetically distinct species that colonize the same environment (i.e., the airways of cystic fibrosis patients) (13,51). *P. aeruginosa* can activate the expression of virulent factors in *B. cepacia* in mixed biofilms in vivo and in vitro, indicating that cell-to-cell communication between different genera occurs in mixed communities.

Closely related bacteria sometimes use similar AHL signals even if they occupy different environments. Plant pathogens *Erwinia amylovora* and *Pectobacterium carotovorum* have different hosts but still synthesize 3OC6HSL and C6HSL as AHL products (8). Similarly, the two *Brenneria* spp., *Brenneria salicis* and *B. rubrifaciens*, where QS has been described, exhibit different host ranges yet produce structurally identical AHLs. *B. salicis*, the causal agent of watermark disease of willow (*Salix* spp.), also occurs as an endophyte in alder and poplar trees (38,39). Yet only willow trees support high *B. salicis* populations and develop disease symptoms. The mechanism underlying disease development is not understood but QS has been implicated in the induction of cellulase activity in *B. salicis* isolates (31). In a similar manner, *B. rubrifaciens* and *B. nigrifluens* are also found in symptomless walnut trees (32).

QS is one regulatory pathway that may play a role in disease occurrence in these species. *B. salicis* extract induces pigment production in *B. rubrifaciens* mutant Br-212 because it contains molecules with chemical structures (3OC6HSL and C6HSL) identical to those produced by *B. rubrifaciens* 6D370 (Fig. 2). However, these signals may modulate very different effects in each species. *Brenneria alni* and *Brenneria quercina* were not able to induce rubrifacine production in Br-212 on YDCA plates but, interestingly, crude extract from *B. nigrifluens* did. TLC analysis using reporter strain *C. violaceum* CV026 demonstrates that *B. nigrifluens* ATCC 13028 extract contains compounds with the same mobility as AHLs from *B. rubrifaciens* 6D370 and *B. salicis* ATCC 15712 (Fig. 4).

Three complimentary methods—HPLC, TLC, and LCMS—confirm the signal molecules produced by *B. rubrifaciens* to be 3OC6HSL and C6HSL (Figs. 1 to 4), with the former being the most abundant species produced in 40-fold excess of the latter. TLC and MS results confirmed that *B. nigrifluens* also produces similar AHLs (3OC6HSL, and C6HSL) (Figs. 3 and 4) and therefore, unsurprisingly, extracts from this species induce pigment production in mutant Br-212. Both *B. rubrifaciens* and *B. nigrifluens* share the same restricted host range (walnut) and both

cause distinctive bark canker diseases (6,70,71). However, severe cases of shallow bark canker have symptoms very similar to DBC (37,52) and both *B. nigrifluens* and *B. rubrifaciens* have been isolated from trees with DBC (71). Because both diseases sometimes occur in adjacent tissue, cross-talk may occur between *B. rubrifaciens* and *B. nigrifluens* during colonization of the walnut host.

Heterologous expression of *bruI* in *Escherichia coli* complements the pigment deficient phenotype of mutant Br-212 in plate bioassays (42), indicating that *bruI* expression mediates production of signaling molecules required for rubrifacine production. C7HSL, C8HSL, and 3OC8HSL were not detected in extracts from *B. rubrifaciens* 6D370 but still induced rubrifacine production in Br-212 (Table 2), demonstrating that the mutant *B. rubrifaciens* Br-212 responds to AHLs which it is unable to synthesize. The *bruR* ORF is the only *luxR* homolog identified from this

TABLE 2. Induction of wild-type phenotypes in Br-212 by various synthetic acyl homoserine lactones (AHLs)^a

AHL added ^b	Rubrifacine production ^c	HR in tobacco ^d
C4HSL	–	–
C6HSL	+	+
3OC6HSL	+	+
C7HSL	+	+
C8HSL	+	+
3OC8HSL	+	+
C10HSL	–	–
C12HSL	–	–
None	–	–

^a AHLs were purchased from Sigma-Aldrich, suspended in acidified ethyl acetate, and added to *Brenneria rubrifaciens* mutant Br-212 in 10 ml of yeast extract-dextrose-calcium carbonate at 10 μM. Samples were evaluated visually for pigment production after 3 days at 28°C and rotary shaking at 215 rpm.

^b C4HSL = N-butyl-DL-homoserine lactone, C6HSL = N-hexanoyl-homoserine lactone, 3OC6HSL = N-(3-oxohexanoyl)-homoserine lactone, C7HSL = N-heptanoyl-DL-homoserine lactone, C8HSL = N-octanoyl-DL-homoserine lactone, 3OC8HSL = N-(3-oxooctanoyl)-DL-homoserine lactone, C10HSL = N-decanoyl-DL-homoserine lactone, and C12HSL = N-dodecanoyl-DL-homoserine.

^c + and – denote presence or absence of rubrifacine, respectively. *Nicotiana benthamiana* (tobacco) leaves were pressure-infiltrated with optical density at 600 nm suspensions = 1.0 of *B. rubrifaciens* mutant Br-212 and evaluated 36 to 40 h postinoculation for the occurrence of a hypersensitive reaction (HR).

^d + and – denote HR or no reaction, respectively.

Fig. 6. Rubrifacine production in *Brenneria rubrifaciens* mutant Br-212 by synthetic N-(3-oxohexanoyl)-homoserine lactone (3OC6HSL) and N-hexanoyl-homoserine lactone (C6HSL). Absorbance scans from cell-free supernatants of wild-type *B. rubrifaciens* 6D370 (WT), mutant Br-212, and mutant Br-212 amended with 10 μM 3OC6HSL and 10 μM C6HSL. Samples were evaluated using spectrophotometric analysis after 3 days of incubation at 28°C and rotary shaking at 215 rpm. Each sample was prepared in triplicate.

species and is the best candidate for mediating the response to the small-chain AHLs. Several LuxR homologs have been shown to respond to multiple AHLs, including TraR and CviR from *A. tumefaciens* and *C. violaceum*, respectively (43,55). Alternatively, bacteria can also respond to foreign AHLs by employing orphan LuxR homologs similar to those found in *Pseudomonas aeruginosa* (18), *E. coli* (14), and *Burkholderia cenocepacia* (40).

Regulation of rubrifacine production by AHL-mediated QS suggests a role for the pigment at high population densities. *B. rubrifaciens* populations reach 10^{10} CFU/ml in diseased trees (53). No function in virulence has yet been attributed to rubrifacine (42). The indigoidine pigment of *D. chrysanthemi* and ferrosamine A of *Pectobacterium rhapontici* function as a scavenger of oxygen radicals and siderophore, respectively, which has been suggested to confer a selective advantage to these phytopathogens. (26,50). Therefore, rubrifacine may also confer a similar selective advantage for *Brenneria rubrifaciens* in walnut.

Many plant-pathogenic bacteria trigger an HR in non-host plants species (34). This HR phenomenon, a type of programmed plant cell death, is regulated in many species by the type III secretion system (T3SS) encoded by hypersensitive response and pathogenicity (*hrp*) genes (1). The T3SS translocates bacterial effector proteins directly into eukaryotic host cells, allowing for modulation of the host signaling and defense (15). QS-mediated control of TSS3-mediated HR has been described for other species, including *Erwinia amylovora*, *Dickeya dadantii*, *Pantoea stewartii* subsp. *stewartii*, and *Pectobacterium atrosepticum* (36, 47,67). Application of exogenous AHLs enabled *B. rubrifaciens* mutant Br-212 to elicit an HR in tobacco leaves similar to wild-type *B. rubrifaciens*. *E. amylovora* and *D. chrysanthemi hrp* mutants are defective in HR and attenuated in virulence, similar to Br-212 (9,12). The HR results indicate that *B. rubrifaciens* also has genes involved in virulence and HR elicitation regulated by AHL-mediated QS.

Host cultivar-specific chemical profiles may contribute to walnut disease susceptibility. Many eukaryotes can influence AHL-mediated QS regulation in bacteria by utilizing either small-molecule AHL mimics or degradative enzymes. QS inhibitory compounds have been described from *Penicillium* spp. and algae, most notably *Delsia pulchra*. (41,48,59). Characterization of the chemical nature of QS compounds that influence bacterial QS in many terrestrial plants is still in the preliminary stages but QS-modulating activity has been described from some plants, including *Acacia nilotica* L., garlic bulb, *Medicago truncatula*, *Quercus virginiana*, and *Combretum albiflorum* (2,30,64). Given these findings, it is possible that specific walnut cultivars (32) contain compounds that modulate bacterial QS, contributing to the striking differences in disease susceptibility observed between walnut cultivars. Elucidation of this interaction will facilitate future breeding of disease-resistant cultivars.

Fig. 7. Genetic locus containing *bruI* and *bruR* genes in *Brenneria rubrifaciens* 6D370. Orientation of *bruI* and *bruR* open reading frames in *B. rubrifaciens* 6D370 depicted to scale using vector NTI software. National Center for Biotechnology Information accession number *bruI-bruR* locus: FJ167366.

The *bruI-bruR* locus described here is the first description of a *luxI-luxR* pair described for any *Brenneria* spp. The production of the same structural AHLs, 3OC6HSL and C6HSL, by *B. nigri-fluens*, *B. salicis*, and *B. rubrifaciens* and the induction of rubrifacine in Br-212 with *B. salicis* and *B. nigri-fluens* extracts suggest that *luxI* homologs are present in both species. In addition, mutant Br-212 will be useful as a new pigment-based bioreporter strain for short-chain AHLs. Understanding the role of QS in *B. rubrifaciens* is a prerequisite for understanding DBC and may provide insights into the long latency period of this disease, which has been poorly understood for decades.

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