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ORIGINAL ARTICLE

Quorum-sensing signals in the microbial community of the cabbage white butterfly larval midgut

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The overall goal of this study was to examine the role of quorum-sensing (QS) signals in a multispecies microbial community. Toward this aim, we studied QS signals produced by an indigenous member and an invading pathogen of the microbial community of the cabbage white butterfly (CWB) larval midgut (*Pieris rapae*). As an initial step, we characterized the QS system in *Pantoea* CWB304, which was isolated from the larval midgut. A *luxI* homolog, designated *panI*, is necessary for the production of *N*-acyl-L-homoserine lactones (AHLs) by *Pantoea* CWB304. To determine whether AHL signals are exchanged in the alkaline environment of the midgut, we constructed AHL-sensing bioluminescent reporter strains in *Pantoea* CWB304 and a *panI* mutant of this strain. In the gut of the CWB larvae, the reporter in an AHL-deficient *Pantoea* CWB304 detected AHLs when coinoculated with the wild type. To study the role of AHL signals produced by a community invader, we examined pathogenesis of *Pseudomonas aeruginosa* PAO1 in CWB larvae. Mortality induced by *P. aeruginosa* PAO1 was significantly reduced when signaling was interrupted by either a potent chemical inhibitor of QS or mutations in the *lasI* and *rhlI* AHL synthases of *P. aeruginosa* PAO1. These results show that AHLs are exchanged among bacteria in the alkaline gut of CWB larvae and contribute to disease caused by *P. aeruginosa* PAO1.
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Introduction

Quorum sensing (QS) is the cell density-dependent regulation of gene expression that enables members of a bacterial population to work together to accomplish tasks that would be impossible or useless for a single cell. Genes regulated by QS dictate a diversity of physiological traits in various bacteria that are often involved in the establishment of mutualistic symbioses (Lithgow *et al.*, 2000; Visick *et al.*, 2000) and pathogenic relationships (Cui *et al.*, 1995; Pearson *et al.*, 2000). In Proteobacteria, communication is mediated by *N*-acyl-L-homoserine lactone (AHL) signal molecules (Manefield and Turner, 2002) and homologs of LuxI and LuxR proteins (Fuqua *et al.*, 1994). AHLs are synthesized by LuxI-type synthases and interact with LuxR-type regulator proteins to regulate the expression of specific genes positively or negatively (Engebrecht and Silverman, 1984; von Bodman *et al.*, 1998). Although bacterial QS systems differ in the chemical structures of the signals, signal relay mechanisms and target genes they regulate, many bacteria share the ability to communicate with each other to coordinate gene expression and ultimately their behavior in the community (Miller and Bassler, 2001).

Despite advances in understanding the role of signaling in bacteria–host interactions, the role of signaling in multispecies bacterial communities remains largely unexplored. Some studies suggest that interspecies communication, signal mimicry and signal degradation play a role in interspecies

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interactions. Many bacteria produce AHLs that other species can recognize. In the rhizosphere, *Pseudomonas putida* responds to AHLs produced by indigenous bacteria (Steidle *et al*., 2001), in mixed biofilms, *Burkholderia cepacia* responds to AHLs produced by *Pseudomonas aeruginosa* (Riedel *et al*., 2001) and *Escherichia coli* and *Salmonella enterica* serovar Typhimurium both detect AHLs produced by other species with the SdiA receptor, a predicted LuxR homolog (Michael *et al*., 2001). Molecules that functionally resemble natural ligands also modulate signaling networks by signal mimicry. Natural and synthetic furanones that mimic AHLs regulate AHL-mediated QS systems in a positive and negative manner (Givskov *et al*., 1996; Martinelli *et al*., 2004). Signal mimics from uncultured bacteria associated with the midguts of gypsy moth larvae that induce QS have also been identified by a metagenomic analysis (Guan *et al*., 2007). Signal degradation may also influence signaling in multispecies communities (Leadbetter and Greenberg, 2000; Dong *et al*., 2002; Carlier *et al*., 2003; Lin *et al*., 2003; Park *et al*., 2003, 2005). These examples illustrate some of the mechanisms by which microorganisms respond to signal mimics and possibly modulate QS networks in multispecies communities. Given the importance of QS in coordination and regulation of gene expression in single species, it seems likely that it plays a prominent role in the context of the entire community.

To study signaling in a multispecies community, we chose the midgut of the cabbage white butterfly (CWB) larva because it harbors a community of only a few bacterial species. This environment is intriguing because AHLs are typically unstable in alkaline pH environments, and the average pH of the midgut of larvae fed sterile artificial diet has been measured to be 8.68 ± 0.53 (unpublished); other studies have reported the pH to be 7.3–7.6 (Berenbaum, 1980; Wittstock *et al*., 2004). The gut community can be manipulated by amending the insect's sterile artificial diet with compounds or bacterial strains. When the larvae are reared on sterile artificial diet, the community is estimated to contain an average of 14 species of bacteria, of which the majority are uncultivated Proteobacteria. A more detailed report of the CWB midgut community composition will be published elsewhere (CJ Robinson, unpublished data). We reasoned that AHL-mediated intraspecies and interspecies communication between these bacteria could be pervasive.

We also sought to investigate the role of AHL signaling by invasive pathogens in this community. CWB larvae are susceptible to the opportunistic pathogen *P. aeruginosa,* which uses AHL-mediated QS to control virulence gene expression during infection of diverse eukaryotic hosts including plants, nematodes, insects and mammals under the control of AHL-mediated QS (Pearson *et al*., 2000; Lesprit *et al*., 2003). *P. aeruginosa* produces distinct AHLs, *N*-butyryl homoserine lactone (Pearson *et al*., 1995) and *N*-(3-oxododecanoyl)-L-homoserine lactone (Pearson *et al*., 1994), which interact with the LasR, RhlR and QscR transcriptional regulators to direct the transcription of distinct but overlapping regulons that include virulence genes (Latifi *et al*., 1995; Pearson *et al*., 1997; Lequette *et al*., 2006).

In this study, we report that AHL-mediated QS is active in the microbial community of the CWB larval gut. Furthermore, we demonstrate that QS signals play a role in pathogenesis by *P. aeruginosa*, which can be attenuated by administering a non-native AHL antagonist. The study establishes the CWB larval midgut as a compelling model system for study of microbial community structure and function.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in Luria-Bertani (LB) medium at 28 °C. Antibiotics were added as required at final concentrations of 20 mg tetracycline per l, 50 mg trimethoprim per l and 25 mg nalidixic acid per l. Bacteria that were fed to CWB larvae were prepared from stationary phase cultures by centrifugation, washed twice and resuspended in 1/10 of the original volume in LB medium. Ten microliters of the washed and concentrated bacterial suspension was applied to each disk of insect diet.

Isolation and identification of AHL-producing bacteria

Bacterial strains were isolated from the midgut of CWB larvae and identified by 16S rRNA gene sequencing as described previously (Broderick *et al*., 2004). Isolates were screened for AHL production on LB agar plates in a cross-streaking assay against the AHL reporter strains *Chromobacterium violaceum* CV026 (McClean *et al*., 1997), *E. coli* pJBA132 (Andersen *et al*., 2001) and *P. putida* F117 pKRC12 (Steidle *et al*., 2001) as described previously (Williamson *et al*., 2005).

Random insertion mutagenesis of Pantoea *CWB304*

Random transposon mutations were generated in *Pantoea* CWB304 with the EZ-Tn5 ⟨DHFR-1⟩Tnp Transposome kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Transposase complexes were introduced by electroporation into *Pantoea* CWB304 electrocompetent cells as directed in the operating manual for the Gene Pulser transfection apparatus for *E. coli* (Bio-Rad Laboratories, Hercules, CA, USA), except that cultures were incubated at 28 °C for preparation of electrocompetent cells. Electroporated cells were allowed to recover for 2 h at 28 °C with shaking, plated on LB agar containing trimethoprim and incubated at 28 °C to select for transposon insertion.

Table 1 Bacterial strains and plasmids

Strain or plasmid	*Relevant characteristics*	*Source or reference*
Pantoea CWB301	Bacterial isolate from cabbage white butterfly larvae, AHL producer	This study
Pantoea CWB304	Bacterial isolate from cabbage white butterfly larvae, AHL producer, Nal^R CWB301 derivative	This study
Pantoea CWB304 *panI*::Tn*5*	Tn*5* mutant, Trm^R, Nal^R, AHL-deficient CWB304 derivative	This study
Pseudomonas aeruginosa PAO1	Wild-type strain, AHL producer	Pearson *et al.* (1997)
Pseudomonas aeruginosa PAO1-JP2	Δ*rhlI*::Tn*501-2*, Δ*lasI*::Tet, derivative of PAO1, Hg^R and Tet^R, AHL-deficient	Pearson *et al.* (1997)
Chromobacterium violaceum CV026	AHL-deficient, AHL reporter strain	McClean *et al.* (1997)
Escherichia coli DH5α	*recA1* and *endA1* cloning strain	Invitrogen, Carlsbad, CA, USA
Agrobacterium tumefaciens NTL4 pZLR4	AHL-deficient, AHL reporter strain, Gm^R	Piper *et al.* (1993)
Escherichia coli MT102 pJBA132	AHL-deficient, AHL reporter strain, Tet^R	Andersen *et al.* (2001)
Pseudomonas putida F117 pKRC12	AHL-deficient derivative of *P. putida* IsoF, Δ*ppuI*, AHL reporter strain	
	pKR-C12: pBBR1MCS-5 carrying P_{lasB}-*gfp*(ASV)-P_{lac}*lasR*; based on components of the *P. aeruginosa las* QS system; Gm^R	Steidle *et al.* (2001)
pSB401	P_{luxI}-*luxCDABE* transcriptional fusion, *luxR*$^+$, Tet^R	Winson *et al.* (1998)
pPanlux	P_{panI}-*luxCDABE* transcriptional fusion, Tet^R	This study
pGEM-5Zf(+)	Cloning vector, Amp^R	Promega
pGEM-T Easy	TA cloning vector, Amp^R	Promega

Abbreviations: AHL, *N*-acyl-L-homoserine lactone; Gm^R, gentamicin resistance; Hg^R, mercuric chloride resistance; Nal^R, nalidixic acid resistance; Tet^R, tetracycline resistance; Trm^R, trimethoprim resistance.

Identification and characterization of AHL-deficient Pantoea *mutants*

Trimethoprim-resistant *Pantoea* mutants were patched pairwise with the AHL reporter strain *C. violaceum* CV026. Presence of AHL is indicated by the production of violacein. *Pantoea* mutants that did not induce violacein production in *C. violaceum* CV026 were further analyzed by sequence analysis of the genomic DNA flanking the transposon. Genomic DNA from the *Pantoea* mutants was isolated with the Easy-DNA genomic DNA extraction kit (Invitrogen, Carlsbad, CA, USA). Genomic DNA was digested with *Sac*II (Promega, Madison, WI, USA) and ligated into pGEM-5Zf(+) (Promega), which was linearized with *Sac*II. The ligation products were electroporated into *E. coli* DH5α that was plated on LB agar containing trimethoprim and incubated at 37 °C to select clones containing the transposon with flanking DNA. Plasmid DNA was isolated from trimethoprim-resistant transformants and sequenced with primers complementary to the transposon. Primer walking with custom-designed primers obtained additional nucleotide sequences. BigDye Terminator (v. 3.1; Applied Biosystems, Foster City, CA, USA) sequencing was conducted at the University of Wisconsin–Madison DNA-sequencing facility. Sequence reads were assembled with Seqman (DNASTAR, Madison, WI, USA).

Characterization of AHLs produced by Pantoea *strain CWB304*

Culture extracts for thin layer chromatography (TLC) analysis were prepared from 50 ml cultures grown to stationary phase at 28 °C. Bacteria were removed by centrifugation, and the supernatants were extracted twice with 50 ml of acidified ethyl acetate (0.1 ml glacial acetic acid per l). Residues were dried, then dissolved in a final volume of 50 μl acidified ethyl acetate and stored at −20 °C. One-microliter samples of extracted culture supernatants and synthetic AHL standards were applied to C_{18} reversed-phase TLC plates (200-m layer; JT Baker, San Francisco, CA, USA) and developed with methanol/water (60:40, vol/vol). Solvents were evaporated, and the plates were analyzed for the presence of AHLs with an *Agrobacterium tumefaciens* AHL indicator strain NTL4 pZLR4 (Piper *et al.*, 1993). The AHL indicator was grown to stationary phase and 5 ml of culture was added to 95 ml of LB medium maintained at 50 °C amended with 7.5 g agar per l and 50 μg ml^{-1} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). The agar suspension containing the bacteria was mixed briefly with a vortex mixer and 50 ml was applied to the TLC plate by spraying (250-ml sprayer; Kontes, Vineland, NJ, USA). After the agar solidified, TLC plates were incubated at 28 °C for 18 h in Big Blue Trays (Stratagene, La Jolla, CA, USA). R_f values for samples were compared with

AHL standards, which were the gifts from Dr Ronald Binder (USDA ARS Pacific West Area).

Construction and characterization of Pantoea *QS reporters*
The *luxCDABE*-based AHL sensor plasmid, pSB401, was introduced by electroporation into the wild-type *Pantoea* strain and the *Pantoea* AHL-negative mutant to monitor QS activity. To monitor the regulation of *panI*, we constructed a *panI* transcriptional fusion with the bacterial luciferase reporter *luxCDABE* and introduced this construct into *Pantoea* CWB304. The promoter region and partial coding region of *panI* was PCR-amplified using the primers pantprofor (5′-AACAGCCCAGTCAAGTC GATCCTT-3′) and pantprorev (5′-TTGACGGGAC TTGTAGTTGCCCTT-3′). The 397-bp amplicon was TA-cloned into pGEM-T Easy (Promega) as suggested by the manufacturer to create plasmid pGTEpantpro. The reporter plasmid pSB401 was linearized by *Eco*RI digestion and purified by gel electrophoresis and gel extraction to remove the 1-kb region that encodes *luxR* and the *luxI* promoter, resulting in a linearized vector with a promoterless *luxCDBAE* operon. Plasmid pGTEpantpro was linearized by *Eco*RI digestion, and the *panI* promoter and partial coding region were purified by gel electrophoresis and gel extraction and ligated into the modified pSB401 promoterless vector to produce plasmid pPanlux, which was introduced by electroporation into *Pantoea* CWB304 *panI*::Tn5.

All of the reporter strains were monitored for bioluminescence activity in the presence and absence of synthetic 50 nM *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6 AHL). Bacterial cells from overnight cultures were concentrated, washed twice and inoculated into LB medium at an OD_{600} of 0.04. Cells were dispensed into 96-well Costar 3603 plates (Corning Inc., Corning, NY, USA) to a final volume of 100 μl. The cultures were incubated at 28 °C with constant agitation.

Bioluminescence and absorbance (OD_{600}) were measured with a Wallac Victor2 plate reader (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA), and specific luminescence was defined as relative luminescence per unit of absorbance (OD_{600}). Bioluminescence of *Pantoea* CWB304 pPanlux was also monitored during growth in LB medium, and the bioluminescence of *Pantoea* CWB304 *panI*::Tn5 pPanlux was measured in response to exogenous provision of *N*-hexanoyl-L-homoserine lactone (C6 AHL), *N*-octanoyl-L-homoserine lactone (C8 AHL), 3-oxo-C6 AHL and *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8 AHL) at final concentrations of 5, 10, 50, 250 and 500 nM.

CWB larvae rearing
CWB eggs (Carolina Biological Supply Company, Burlington, NC, USA) were surface-sterilized by soaking in a solution of 1% Tween 80 and 2% bleach for 3 min, rinsed twice with sterile distilled water and dried under a sterile air stream in a laminar flow hood. Sterile artificial diet (USDA, Hamden formula) was added to the petri plates containing the eggs, and fresh sterile artificial diet was supplied every 72 h or as it was consumed. Plates were partially sealed with parafilm and incubated in an environmentally controlled chamber maintained at 25 °C with a photoperiod of 16–8 h (light–dark).

Visualization of reporter gene activity in insecta
CWB larvae were reared to middle of the fourth instar growth stage and starved for 4 h. Thereafter, individual larvae were transferred to wells of 12-well Costar cell culture plates (Corning Inc.), which contained artificial diet disks to which various bacterial strains were added. Larvae were fed on the diet disks for 18 h. After feeding, larvae that had consumed the diet were starved for 6 h before imaging. Bioluminescence emitted from live larvae and intact guts dissected from larvae were measured with an IVIS BLI 100 CCD camera (Xenogen, Alameda, CA, USA). Dissection of intact guts was performed as previously described (Broderick *et al*., 2004).

Plasmid transfer in insecta
To determine whether strains of *Pantoea* transferred plasmids in the guts of CWB larvae, *Pantoea* CWB304 *panI*::Tn5 was mixed in a 1:1 ratio with *Pantoea* CWB304 pSB401 and fed to CWB larvae as described. After feeding, bacteria isolated from dissected guts were plated on selective media containing trimethoprim and tetracycline to determine whether plasmid transfer had occurred.

Growth of P. aeruginosa *PAO1 in CWB larvae*
To characterize the growth of *P. aeruginosa* PAO1 in CWB larvae, we monitored the population of *P. aeruginosa* PAO1 as a function of time. The guts from CWB larvae fed on unamended diet and diet amended with *P. aeruginosa* PAO1 were harvested at time intervals of 1, 3 and 5 days after feeding. Dissection of guts and quantification of bacteria in the guts were performed as previously described (Broderick *et al*., 2004). To determine whether *P. aeruginosa* PAO1 survived on sterile artificial diet, bacterial suspensions were applied to diet disks as described above.

P. aeruginosa *pathogenicity assays*
Diet disks were amended with various compounds and bacterial strains. The indole QS inhibitor compound, *N*-(indole-3-butanoyl)-L-homoserine lactone was dissolved in ethyl acetate at a concentration of 15 mg ml^{-1}. *N*-(indole-3-butanoyl)-L-homoserine lactone (referred to as ‘indole analog’

throughout) was synthesized and purified according to methods described previously (purity⩾95%) (Geske *et al*., 2005). A 10-μl portion of the indole compound solution or ethyl acetate (control) was added to each diet disk in two aliquots of 5 μl. Ethyl acetate was evaporated from the samples in a sterile laminar flow hood. A 10-μl aliquot of washed and concentrated bacterial suspension or LB medium (control) was applied to each disk. Larvae reared to the fourth instar were transferred to petri dishes that contained artificial diet disks with treatments after 4 h of withholding food. Each feeding treatment was replicated three times. Mortality of the larvae was assessed every 24 h until the larvae initiated pupation.

Nucleotide sequence accession numbers
The nucleotide sequence of *panI* and *panR* have been submitted to GenBank databases under accession numbers EU780668 and EU780669, respectively. The 16S rRNA gene sequence of *Pantoea* CWB304 has been submitted to GenBank databases under accession number EU780667.

Results

A larval gut inhabitant produces AHLs
As a first step in determining whether QS signals are important in the alkaline environment of the lepidopteran midgut, we surveyed the cultivated members of the CWB larval gut community for the ability to stimulate AHL biosensor strains by cross-streaking. We identified a bacterial isolate, CWB304, that induces violacein production in the *C. violaceum* CV026 AHL sensor and green fluorescent protein expression in the *E. coli* JB525 AHL sensor, both of which are responsive to short-chain AHLs, but did not stimulate the AHL sensor strain, *P. putida* F117 pKRC12, which responds to longer chain AHLs. The 16S rRNA gene sequence from CWB304 is 97% identical to *Pantoea* sp. PPE7. Thus, we have designated this strain *Pantoea* CWB304. Further phylogenetic analyses of the isolate will be presented elsewhere.

Ethyl acetate extracts of cell-free supernatant from *Pantoea* CWB304 cultures retained the ability to induce AHL reporter strains. Organic extracts applied to TLC and visualized with the application of the *A. tumefaciens* NTL4 pZLR4 AHL sensor revealed the presence of four different AHLs in multiple extractions and chromatographs (Figure 1). On the basis of the mobility of the AHLs detected and comparison to synthetic standards (Figure 1), we tentatively identified these molecules as C7 AHL, C8 AHL, 3-oxo-C6 AHL and 3-oxo-C8 AHL.

Characterization of a gene responsible for AHL production in Pantoea *CWB304*
Random transposon mutants of *Pantoea* CWB304 were screened for loss of production of AHL by testing them for the ability to induce violacein production in *C. violaceum* strain CV026 (Figure 2). Out of the 11 228 mutants screened, three failed to activate the CV026 AHL sensor strain. All three of the mutants contained transposon insertions in a homolog of the *luxI* synthase, which is divergently transcribed from a flanking *luxR* homolog. We designated the genes *panI* and *panR* based on the phenotype of the *panI* mutant and sequence similarity to *luxI* and *luxR* homologs, respectively. BlastX (Altschul *et al*., 1997) results indicate that the deduced amino-acid sequence of PanI is 59% identical and 74% similar to HslI from *Pectobacterium carotovorum*, and PanR is 45% identical and 69% similar to SmaR from *Serratia* sp. ATCC 39006. We failed to detect AHLs in cultures of the *panI* mutant after extraction with acidified ethyl acetate and TLC analysis (Figure 1).

Bioluminescent AHL biosensors in Pantoea *CWB304*
We constructed several reporter strains in which bioluminescence is a measure of AHL-mediated gene regulation to detect signal exchange in guts of CWB larvae. We introduced pSB401 into the wild-type *Pantoea* and the AHL-deficient mutant. The AHL reporter plasmid pSB401 is a transcriptional fusion of *luxI* promoter region and the bacterial luciferase reporter *luxCDABE* under transcriptional control of LuxR (Winson *et al*., 1998), which is activated by short-chain AHLs. Bioluminescence is

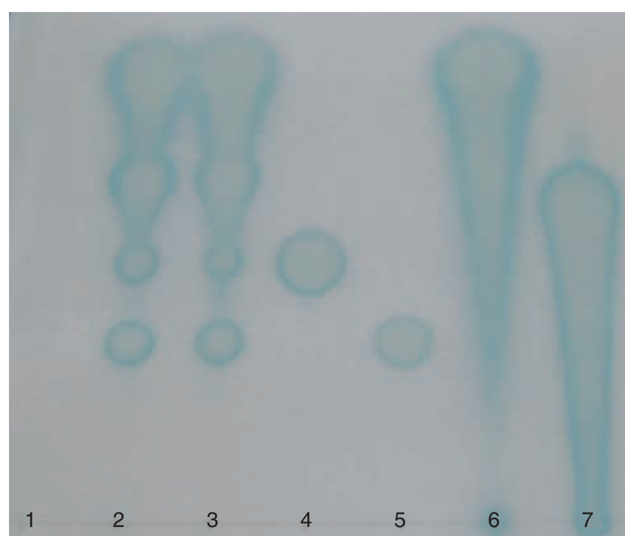

Figure 1 Chromatograph of extracts from *Pantoea* CWB304 and *Pantoea* CWB304 *panI*::Tn5. Samples extracted from culture supernatants of the wild-type, *panI* mutant and AHL standards were chromatographed on C_{18} reversed-phase TLC plates and visualized with the *A. tumefaciens* reporter strain NTL4 pZLR5. Samples are for the following extracts from bacterial strains and AHL standards: 1, *Pantoea* CWB304 *panI*::Tn5 (AHL-deficient); 2, *Pantoea* CWB304 extract A; 3, *Pantoea* CWB304 extract B; 4, C7 AHL; 5, C8 AHL; 6, 3-oxo-C6 AHL and 7, 3-oxo-C8 AHL. AHL, *N*-acyl-L-homoserine lactone; CWB, cabbage white butterfly; TLC, thin layer chromatography.

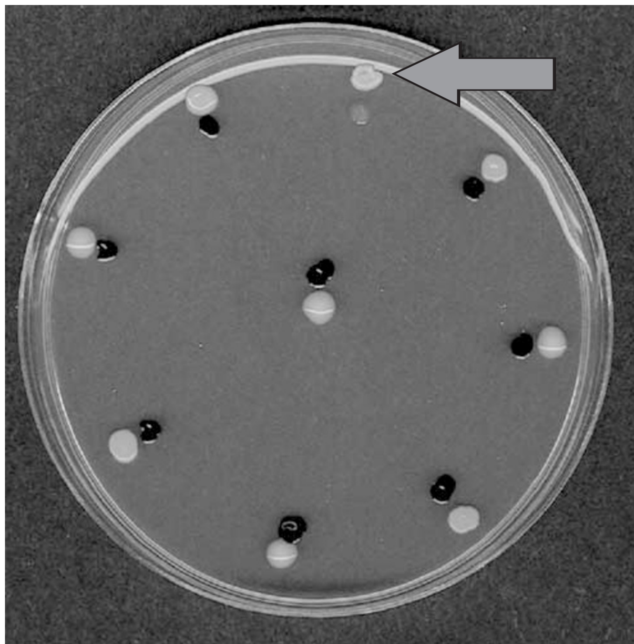

Figure 2 Identification of *Pantoea* mutants that do not produce AHLs. Each transposon mutant was patched near the *C. violaceum* CV026 AHL reporter to identify mutants that do not produce AHLs, which do not induce production of violacein (purple pigment) by *C. violaceum* CV026. An AHL-deficient mutant is indicated by the arrow. AHL, *N*-acyl-L-homoserine lactone.

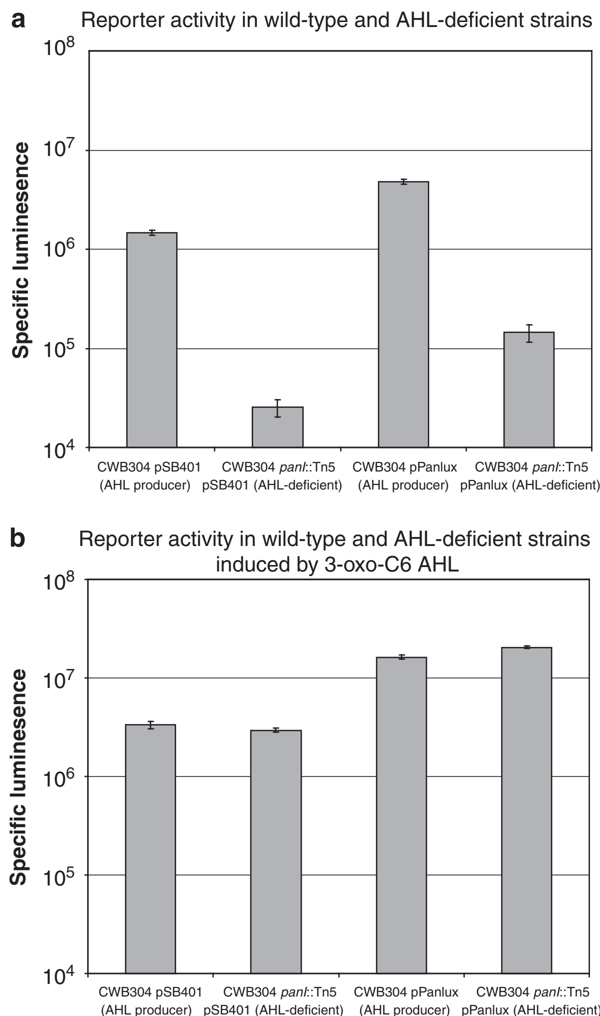

Figure 3 QS activity in *Pantoea* reporter strains. *Pantoea* strains were grown in LB media and specific luminescence induced by endogenous AHL production and background reporter activity (**a**) and the addition of 50 nM synthetic 3-oxo-C6 AHL (**b**) were measured. Values represent means of specific luminescence, which is relative luminescence per unit of absorbance (OD_{600}), of four replicates after 8 h of growth. Error bars indicate the s.d. AHL, *N*-acyl-L-homoserine lactone; QS, quorum sensing.

induced in *Pantoea* CWB304 pSB401 by its own AHLs (Figure 3a), whereas bioluminescence is induced more than 100-fold in *Pantoea* CWB304 *panI*::Tn5 pSB401 when exogenous AHLs are provided (Figures 3a and b).

We characterized the regulation and specificity of QS in *Pantoea* CWB304 by introducing pPanlux into the wild-type *Pantoea* and the *panI* mutant and tested the strains in the absence and presence of exogenous AHLs (Figures 3a and b). The reporter plasmid, pPanlux, carries a fusion of the *panI* promoter region and the promoterless *luxCDABE* reporter genes. The expression of *panI* in *Pantoea* CWB304 *panI*::Tn5 pPanlux was induced nearly 80-fold in late logarithmic growth phase (Figure 4), which suggests that *panI* is regulated in a density-dependent manner indicative of positive feedback regulation of the AHL synthase.

To determine the nature and specificity of QS regulation in *Pantoea* CWB304, we introduced the pPanlux construct into the AHL-deficient mutant generating *Pantoea* CWB304 *panI*::Tn5 pPanlux. Bioluminescence was induced by the exogenous addition of AHLs, further supporting the conclusion that *panI* is regulated in a cell density-dependent manner by AHLs (Figure 3b).

To assess the activities of AHLs that induce QS in *Pantoea* CWB304, we induced *Pantoea* CWB304 *panI*::Tn5 pPanlux with various concentrations of C6 AHL, C8 AHL, 3-oxo-C6 AHL and 3-oxo-C8 AHL (Figure 5). Synthetic 3-oxo-C6 AHL and 3-oxo-C8 AHL induced luminescence greater than 20-fold at concentrations as low as 5 and 250 nM, respectively after 4 h of growth. C6 and C8 AHLs failed to induce strong luminescence at concentrations up to 500 nM. These findings provide evidence that *panI* is positively regulated in a concentration-dependent manner and that 3-oxo-C6 AHL is likely the preferred ligand for the regulation of the PanIR QS system.

Cell–cell communication in the CWB larval midgut

To determine whether bacteria produce and respond to AHL signal molecules in insecta, bioluminescent reporter strains in various combinations were administered orally to CWB larvae reared under

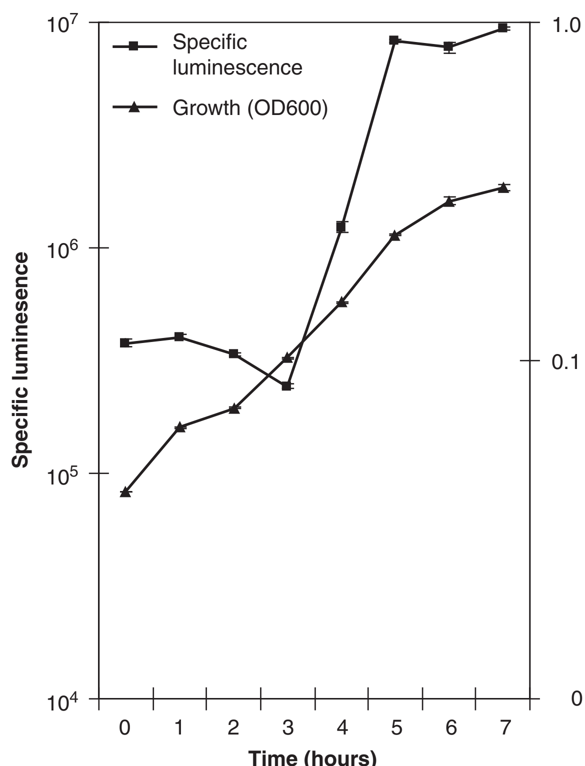

Figure 4 Cell density-dependent regulation of *panI*. CWB304 pPanlux, which contains a *panI-luxCDABE* transcriptional fusion, was grown in LB medium, and specific luminescence (■) and optical density (▲) were measured hourly. The specific luminescence of the bacterial cultures was quantified as relative light units (RLU) per unit of absorbance (OD_{600}). Mean values and s.d. are shown for three replicates. CWB, cabbage white butterfly; LB medium, Luria-Bertani medium.

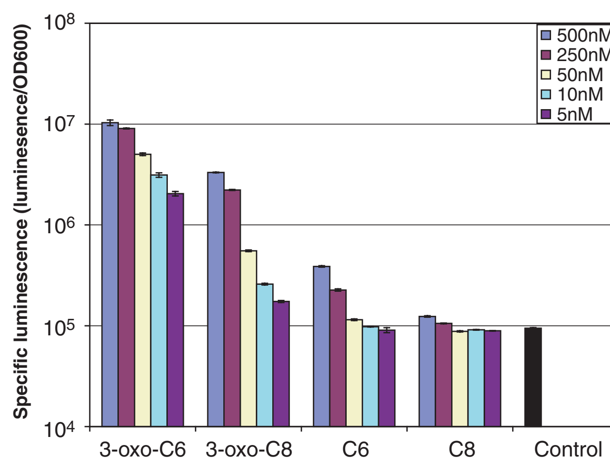

Figure 5 Response of *Pantoea* CWB304 *panI*::Tn5 pPanlux to synthetic AHLs. Specific luminescence of CWB304 *panI*::Tn5 pPanlux was measured in the presence of various synthetic AHLs. Specific luminescence is the luminescence divided by the optical density (OD_{600}) of the cultures measured at 4 h. Mean values and s.e. are shown for three replicates. AHL, *N*-acyl-L-homoserine lactone; CWB, cabbage white butterfly.

sterile conditions and luminescence was detected by an IVIS BLI 100 CCD camera *in vivo*. The wild type, which contained the AHL biosynthetic gene *panI*, and the biosensor, pSB401, were luminescent in the gut of the CWB larvae. This result indicates that *Pantoea* CWB304 pSB401 produces and responds to AHLs in the insect gut (Figure 6).

To determine whether strains exchange and respond to AHLs in the insect gut, the wild-type and the *panI* mutant harboring pSB401 were established in the gut and bioluminescence detected with a CCD camera (Figure 6). The AHL-negative *Pantoea* strain responded to AHLs produced by the wild-type strain, suggesting that AHL signals produced by one strain were perceived by the other strain in the alkaline environment of the CWB larval midgut. Bioluminescence was barely detectable in one of the four caterpillars in which the *panI* mutant harboring pSB401 was established (Figure 6), indicating a low level of background reporter activity or the limited presence of AHL-producing bacteria. Antibiotic resistance profiles of bacteria recovered from the gut indicate that plasmid transfer was not responsible for activation of the reporter. We detected no transconjugants within the limit of detection of this assay, which is 10 bacteria per gut. These results indicate that AHL signal exchange between strains is responsible for activation of the bioluminescent reporter.

Establishment of P. aeruginosa *PAO1 as a model pathogen of CWB larvae*

To establish *P. aeruginosa* PAO1 as a pathogen of CWB larvae, the colonization and pathogenesis of CWB larvae by *P. aeruginosa* PAO1 were evaluated. PAO1 colonized CWB larvae, establishing a population size of 9 log colony-forming units after 5 days. The population of *P. aeruginosa* PAO1 increased by more than 4 log colony-forming units between days 1 and 3 of the colonization experiment. To establish a causal relationship between *P. aeruginosa* PAO1 and the disease resulting in septicemia and larval death, Koch's postulates were fulfilled. Three independent *P. aeruginosa* isolates cultured from infected CWB larvae were fed to healthy CWB larvae. All of the larvae that were infected with the *P. aeruginosa* isolates developed septicemic symptoms associated with *P. aeruginosa* infection. Isolates of bacteria whose colony morphology resembled that of *P. aeruginosa* were cultured from infected larvae and their identity was verified as *P. aeruginosa* by 16S rRNA gene sequence. Control larvae that were not fed *P. aeruginosa* isolates remained healthy, and *P. aeruginosa* was not detected by culturing these larvae.

Role of AHLs in P. aeruginosa *virulence in CWB larvae*

After establishing that *P. aeruginosa* PAO1 is pathogenic to CWB larvae and AHL signals are exchanged in the CWB larvae gut, we explored the question whether AHLs govern bacterial behaviors,

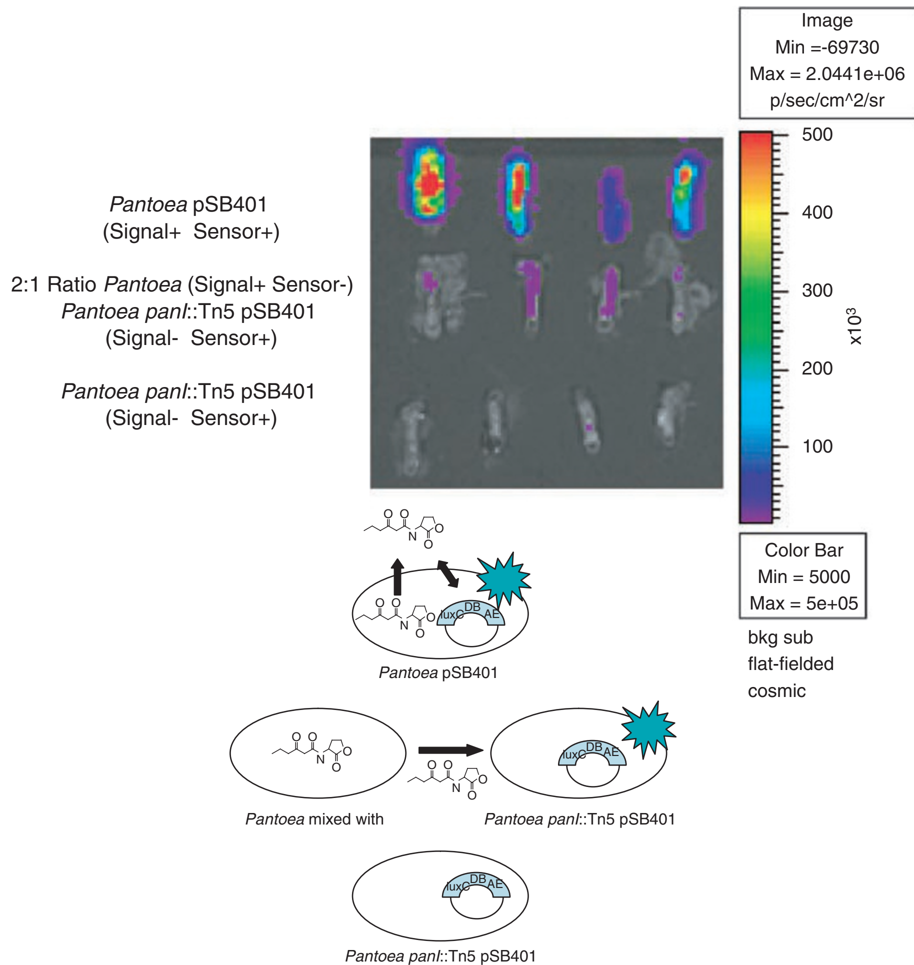

Figure 6 Detection of QS activity and signal exchange in the guts of CWB larvae. Bioluminescence detected in the individual guts of larvae fed *Pantoea* pSB401 (top row), *Pantoea* mixed with *Pantoea panI*::Tn5 pSB401 (middle row) and *Pantoea panI*::Tn5 pSB401 (bottom row). CWB, cabbage white butterfly; QS, quorum sensing.

specifically pathogenesis. *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1-JP2, a derivative of PAO1 lacking the genes for AHL synthesis (*lasI* and *rhlI*), were fed individually to larvae and mortality was evaluated. The AHL-deficient mutant, PAO1-JP2, induced significantly less mortality (47%) than the parent strain (88%), indicating that AHL signaling contributes to virulence (Figure 7). Moreover, a synthetic antagonist of LasR, the AHL indole analog *N*-(indole-3-butanoyl)-L-homoserine lactone (Geske *et al*., 2005), reduced virulence of the wild type to a level comparable to that of the AHL-deficient mutant (Figure 7).

Discussion

In this study, we show that QS signals are active in the guts of CWB larvae. Two lines of evidence support this conclusion. First, we demonstrate the exchange of AHL signals in insects in real time with luminescent reporter strains that detect AHLs. Second, mutations in *P. aeruginosa* that abolish the production of AHLs or treatment of the wild type with a QS inhibitor reduced mortality of larvae compared with larvae infected with wild-type *P. aeruginosa* with no inhibitor. These results are significant because it was previously unknown whether signal exchange occurs in the alkaline environment of the CWB larval midgut. *In vitro*, AHLs are degraded above pH 7 by lactonolysis, and the open-ring molecule is inactive (Yates *et al*., 2002). Bacteria residing in the larval guts may alter the pH of their local microenvironments or exist in biofilms with other organisms that reduce the pH.

Pantoea CWB304 isolated from the gut of CWB larvae produces AHLs that regulate expression of the *panI*-encoded AHL synthase, which is regulated by cell density. *Pantoea* CWB304 is most sensitive to 3-oxo-C6 AHL. It is unknown whether *panI* from *Pantoea* CWB304 produces all four of the AHLs detected in broth culture in the insect gut or whether there are additional AHL synthases in the genome of *Pantoea* CWB304 that are regulated by the products

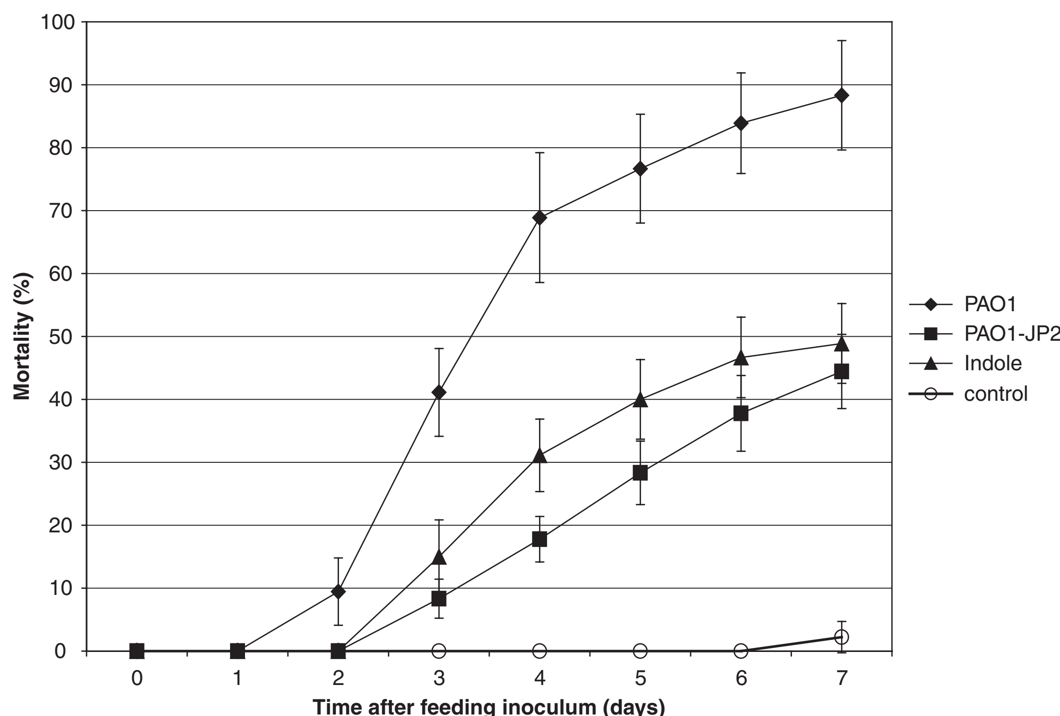

Figure 7 Mortality of cabbage white butterfly larvae fed *P. aeruginosa* strains and the QS analog indole inhibitor. Treatments include *P. aeruginosa* PAO1 (◆), *P. aeruginosa* PAO1 and indole inhibitor (▲), *P. aeruginosa* PAO1-JP2 (*lasIrhlI* AHL-deficient mutant) (■) and no *P. aeruginosa* PAO1 control (○). Values represent the mean mortality as a percentage of 18 larvae per treatment replicated in five independent experiments. Error bars are the s.e. AHL, *N*-acyl-L-homoserine lactone; QS, quorum sensing.

of *panI.* The production of other AHLs may be important for crosstalk or regulation of unknown LuxR-type receptors in *Pantoea*.

Our data indicate that QS signals contribute to pathogenesis of *P. aeruginosa* PAO1 in CWB larvae as in other host–pathogen systems (Tan *et al*., 1999; Pearson *et al*., 2000). However, the QS inhibitor and a *P. aeruginosa* mutant deficient in AHL signal production did not completely abolish pathogenicity, indicating that pathogenicity in *P. aeruginosa* is not controlled solely by the production and sensing of AHLs. It is possible that additional signaling systems are involved. The structurally dissimilar *P. aeruginosa* signal, 2-heptyl-3-hydroxy-4-quinolone, plays a significant role in the transcription of *P. aeruginosa* virulence genes (McGrath *et al*., 2004) and may play a partial role in *P. aeruginosa* pathogenicity of CWB larvae.

The majority of microorganisms exist in multispecies communities in which complex signaling networks are believed to regulate the behavior of the community. Studies of model systems have provided the foundation and principles underlying many microbial interactions, but deciphering the networks that govern the entire community is challenging. To address this challenge, we have developed the multispecies community of the CWB larval gut to study the largely unexplored role of signaling in communities.

Future work will focus on characterizing the *Pantoea* CWB304 genes that are regulated by QS in the guts of CWB larvae. We will investigate the use of other AHL analog inhibitors on QS inhibition *in vivo* by monitoring the activity of QS reporter strains in real time. The work reported here demonstrates the use of the CWB larval gut and its associated microbial community as a model system to study signaling interactions in communities. This model invertebrate system may also be used to study additional mechanisms of virulence regulation and the efficacy and non-target effects of possible therapeutics that modulate AHL-dependent QS systems.

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