Effects of AiiA-Mediated Quorum Quenching in *Sinorhizobium meliloti* on Quorum-Sensing Signals, Proteome Patterns, and Symbiotic Interactions

Mengsheng Gao,¹ Hancui Chen,² Anatol Eberhard,³ Matthew R. Gronquist,⁴ Jayne B. Robinson,⁵ Mary Connolly,⁵ Max Teplitski,¹ Barry G. Rolfe,² and Wolfgang D. Bauer⁶

¹Department of Soil and Water Sciences, 2159 McCarty Hall A, Institute of Food and Agricultural Sciences (IFAS), University of Florida, Gainesville 32611-0290, U.S.A.; ²Australian Research Council Centre of Excellence for Integrative Legume Research, Genomic Interactions Group, Research School of Biological Sciences, GPO Box 475, Australian National University, Canberra ACT 2601, Australia; ³Department of Microbiology, Wing Hall 359, Cornell University, Ithaca, NY 14853, U.S.A.; ⁴Chemistry Department, SUNY College at Fredonia, Fredonia, NY 14063, U.S.A.; ⁵Biology Department, University of Dayton, Dayton, OH 45460, U.S.A.; ⁶Department of Plant Sciences, Mail Stop #1, Plant and Environmental Sciences Bldg., University of California, Davis 95616, U.S.A.

Submitted 8 December 2006. Accepted 15 February 2007.

Many behaviors in bacteria, including behaviors important to pathogenic and symbiotic interactions with eukaryotic hosts, are regulated by a mechanism called quorum sensing (QS). A “quorum-quenching” approach was used here to identify QS-regulated behaviors in the N-fixing bacterial symbiont *Sinorhizobium meliloti*. The AiiA lactonase from *Bacillus* produced in *S. meliloti* was shown to enzymatically inactivate *S. meliloti*’s *N*-acyl homoserine lactone (AHL) QS signals, thereby disrupting normal QS regulation. Sixty proteins were differentially accumulated in the AiiA-producing strain versus the control in early log or early stationary phase cultures. Fifty-two of these QS-regulated proteins, with putative functions that include cell division, protein processing and translation, metabolite transport, oxidative stress, and amino acid metabolism, were identified by peptide mass fingerprinting. Transcription of representative genes was reduced significantly in the AiiA-producing strain, although the effects of AiiA on protein accumulation did not always correspond to effects on transcription. The QS signal-deficient strain was reduced significantly in nodule initiation during the first 12 h after inoculation onto *Medicago truncatula* host plants. The AiiA lactonase also was found to substantially inactivate two of the AHL mimic compounds secreted by *M. truncatula*. This suggests some structural similarity between bacterial AHLs and these mimic compounds. It also indicates that quorum quenching could be useful in identifying *Sinorhizobium* genes that are affected by such host QS mimics in planta.

Additional keywords: bacteroids, ESI/MS/MS, ExpR, SinI.

Many bacteria rely on a cell-to-cell communication and regulatory mechanism called “quorum sensing” (QS) to modulate gene expression and behavior within a microbial community (Bassler and Losick 2006; Keller and Surette 2006; Loh et al. 2002; von Bodman et al. 2003; Whitehead et al. 2001). Nearby cells are helped to act together in a coherent, cooperative manner by the exchange of QS signals. As the number of sibling

cells in a local population increases, so does the concentration of the signal, leading to changes in expression of some or many genes. In various gram-negative bacteria, the QS signals are commonly *N*-acyl homoserine lactones (AHLs) (Cha et al. 1998; Eberhard et al. 1981). Typically, AHLs are perceived by LuxR-type AHL receptors. The interaction of the cognate AHL signal with the AHL-binding domain of a LuxR homolog can stabilize the protein against proteolytic degradation (Zhu and Winans 1999, 2001) and facilitate its multimerization. The DNA-binding domains of the LuxR-AHL multimers bind to conserved sites within promoter regions of regulated genes to stimulate their expression (Winans 2002). QS regulation via production and exchange of AHLs has been shown to govern the in vitro expression of approximately 5 to 10% of the genes in bacteria such as *Pseudomonas aeruginosa* (Hentzer et al. 2003; Schuster et al. 2003; Wagner et al. 2003; Whiteley et al. 1999) and has been found crucial to behaviors such as motility, biofilms, antibiotic production, and the virulence of both plant and animal pathogens (Bassler and Losick 2006; Brenner and Winans 2005; Dudler and Eberl 2006; von Bodman et al. 2003; Whitehead et al. 2001). Symbiotic luminescence and host colonization phenotypes of *Vibrio* spp. also are dependent on QS regulation (Lupp and Ruby 2005; Lupp et al. 2003).

QS regulation also is involved in the establishment and maintenance of symbiotic relationships between leguminous plants and their N-fixing bacterial partners, the rhizobia. Among the symbiotically relevant functions known to be directly or indirectly QS regulated in various rhizobia are cell division, transfer of symbiotic plasmids, gene expression in the rhizosphere, symbiosome development, N fixation, and nodule number (Chen et al. 2003; Daniels et al. 2004; Gao et al. 2005; Hoang et al. 2004; Loh et al. 2002; Sanchez-Contreras et al. 2007; Teplitski et al. 2004; Wisniewski-Dye and Downie 2002). However, the mechanisms by which QS contributes to the regulation of these functions often are poorly understood and the role of QS in some crucial aspects of the symbiosis such as control of bacterial cell division are little explored. In general, rhizobial QS mutants still are able to infect the roots of host plants, form nodules, and fix nitrogen in exchange for fixed carbon (Gonzalez and Marketon 2003; Loh et al. 2002). However, AHL synthase and receptor mutants in various *Rhizobium* and *Sinorhizobium* strains are defective in the rate or efficiency of nodule initiation, the number

of nodules formed, or the efficiency of N fixation (Daniels et al. 2002; Gao et al. 2005; Sanchez-Contreras et al. 2007; Wisniewski-Dye and Downie 2002; Zheng et al. 2006). Thus, it appears that QS regulation may serve primarily as a mechanism for optimizing host interactions in a variety of small ways rather than any single stage or step.

As in other rhizobia, QS in *Sinorhizobium meliloti* is complex and only partially characterized (Sanchez-Contreras et al. 2007). One AHL synthase (SinI) and two AHL receptors (SinR and ExpR) have been identified clearly in *S. meliloti* (Marketon et al. 2002; Pellock et al. 2002). However, there are six other plausible AHL receptor homologs that have not been tested (Hoang et al. 2004) and there also may be a second AHL synthase (Gonzalez and Marketon 2003; Hoang et al. 2004; Marketon et al. 2002a). ExpR appears to be the most important AHL receptor of those tested in *S. meliloti*, at least in vitro. The genomically sequenced *S. meliloti* 1021 strain contains a native insertion sequence in the *expR* gene (Pellock et al. 2002).

Recently, global studies to identify QS-regulated functions by transcriptome and proteome analysis have been conducted with strain 1021, its isogenic *expR*⁺ derivative 8530, (Pellock et al. 2002), and with QS mutants of these strains. When activated by AHLs, ExpR regulates expression of genes required for synthesis of a symbiotically important exopolysaccharide (EPSII) and many other functions (Gao et al. 2005; Hoang et al. 2004; Pellock et al. 2002). Approximately 140 ExpR-dependent transcripts and over 50 ExpR-dependent proteins have been identified through studies in vitro (Gao et al. 2005; Hoang et al. 2004). The SinR AHL receptor has been shown to modulate expression of approximately 30 genes, including *sinI*, which encodes the AHL synthase (Hoang et al. 2004; Marketon et al. 2002). In addition, another 130 proteins were differentially accumulated in response to added SinI AHLs in strain 1021 (Chen et al. 2003; Teplitski et al. 2004). Because strain 1021 lacks a functional ExpR receptor, QS regulation of these proteins presumably is mediated by SinR or the other, as yet untested LuxR homologs.

In strains 1021 and 8530, the SinI AHL synthase catalyzes the synthesis of AHLs with long acyl side chains (C₁₂ to -18) including C₁₂-homoserine lactone (HSL), C₁₄-HSL, 3-oxo-C₁₄-HSL, C₁₆-HSL, C_{16:1}-HSL, 3-oxo-C_{16:1}-HSL, and C₁₈-HSL (Gao et al. 2005; Marketon and Gonzalez 2002; Teplitski et al. 2003). Most of these long-chain AHLs appear to be unique to *Sinorhizobium* spp. In most cases, it has not been established which of the SinI AHLs are perceived by which AHL receptors. However, there is evidence that different AHLs elicit changes in the accumulation of different sets of proteins (Chen et al. 2003; Gao et al. 2005), indicating some specificity of response.

At present, it is not clear whether strains 1021 or 8530 produce AHLs with short acyl side chains (C₆ to C₈), whether the SinI synthase can make short-chain AHLs, or whether there is a second, potentially cryptic AHL synthase capable of producing short-chain AHLs under certain conditions. Early studies with culture extracts from both the 1021 strain and a *sinI* mutant showed the presence of several substances capable of activating the *Agrobacterium tumefaciens* AHL reporter on thin-layer chromatography (TLC) plates with mobilities similar to those of C₆ to C₈ AHLs (Marketon et al. 2002). These *Agrobacterium*-active spots were assumed to be short-chain AHLs, leading to the conclusion that a second AHL synthase, not SinI, was responsible for production of short-chain AHLs (Marketon et al. 2002). Hoang and associates (2004) recently suggested that Smc0714 might encode such a synthase. However, heterologous expression of the SinI synthase in *Escherichia coli* gave TLC spots corresponding to both long- and short-chain AHLs (Marketon and Gonzalez 2002), suggesting that SinI might be capable of producing both long- and short-chain AHLs. More recent studies

using gas chromatography/mass spectrometry (GC/MS) and electrospray ionization tandem mass spectrometry (ESI/MS/MS) detected only long-chain AHLs in culture extracts of the parental 1021 strain (Gao et al. 2005). In contrast to the earlier study by Marketon and associates (2002), Gao and associates (2005) found that *Agrobacterium*-active spots moving with mobilities similar to those of short-chain AHLs were lost or diminished in the *sinI* mutant. These results are consistent with the possibility that such spots may correspond to QS-active compounds that are not short-chain AHLs, but are synthesized in a manner that depends on QS regulation via SinI AHLs.

In view of these uncertainties regarding the production of short-chain AHLs by *S. meliloti* 1021 and the possibility of a second AHL synthase, we have used a "quorum-quenching" approach (Dong and Zhang 2005) to specifically inactivate all AHLs regardless of chain length or synthase. The specific inactivation of AHLs was accomplished by expression of the *aiiA* gene from *Bacillus* spp. *aiiA* encodes a well-characterized AHL lactonase that hydrolyzes the homoserine lactone ring of AHLs (Dong et al. 2000). Synthesis of the AiiA AHL lactonase in heterologous hosts, including plants, has served as a valuable quorum-quenching tool for studying QS regulation in plant-microbe interactions (Cantero et al. 2006; Dong et al. 2001, 2004; Molina et al. 2005; Smadja et al. 2004), and is especially useful for studies of QS in bacteria where genomic sequences are not available or where AHL QS signals may be produced by novel or cryptic synthases (Wopperer et al. 2006). By comparing the proteomes of AiiA-producing and control strains, we detected over 60 QS-regulated proteins in *S. meliloti* that are regulated by AHL receptors other than ExpR. A number of functions were identified as differentially regulated by QS in early log versus stationary growth phases. The AiiA-producing strain was significantly reduced in its rate or efficiency of nodule initiation during the first 12 h of contact with the host.

RESULTS

Effects of AiiA production on QS signal levels in *S. meliloti*.

AHL reporter biosassays of high-performance liquid chromatography (HPLC) fractions from culture filtrate extracts revealed substantial differences in QS-active compounds in extracts from the AiiA-producing strain (1021 pE7-R3 = 1021 pLAFR3*aiiA*) (Table 1) and the vector control strain (1021 pLAFR3), indicating that essentially all AHLs were inactivated by AiiA (data not shown). GC/MS and ESI MS/MS analysis identified 3-oxo-C_{16:1}-HSL as the most abundant QS signal in culture filtrate extracts from the vector control strain *S. meliloti* 1021 pLAFR3. In addition, C_{16:1}-HSL and C₁₆-HSL were present at levels approximately 1/10 that of 3-oxo-C_{16:1}-HSL. A small amount of 3-oxo-C₁₄-HSL also was detected. C_{16:1}-HSL was the only AHL detected in extracts from the AiiA-producing strain, 1021 pLAFR3*aiiA*, and its concentration was approximately 10- to 12-fold lower than in culture filtrate extracts of *S. meliloti* 1021 pLAFR3. No AHLs with short acyl side chains were detected by GC/MS or ESI MS/MS in extracts from either strain. The greatly reduced accumulation of AHLs in the AiiA-producing strain is consistent with studies carried out in other organisms (Dong et al. 2000, 2002; Molina et al. 2005).

The detection of short-chain AHLs by mass spectrometry might have been masked by co-eluting components of the rich culture medium; therefore, culture extracts also were examined by TLC. Culture filtrate extracts from the vector-only control strain (Fig. 1, lane 4) gave a large spot (f) at the origin, whereas culture filtrates from the AiiA-producing strain gave only a faint spot at the origin (Fig. 1, lane 2). AHLs with long acyl side

chains remain at the origin under these TLC conditions (Cha et al. 1998; Gao et al. 2005; Marketon et al. 2002). Thus, it appears that almost all of the long-chain AHLs secreted by *S. meliloti* are inactivated by AiiA in the 1021 pLAFR3*aiiA* strain. Likewise, extracts of whole cultures (i.e., cells plus culture filtrate) of the vector-only control had a large spot (f) at the origin (Fig. 1, lane 5), whereas there was little or no spot detectable at the origin for whole culture extracts of the AiiA-producing strain (Fig. 1, lane 3). These TLC results are consistent with the ESI/MS/MS analysis above, which indicated that culture filtrate extracts of the AiiA-producing strain contain no long-chain AHLs except for C_{16:1}-HSL at approximately 10% of normal levels.

TLC analysis also revealed a number of QS-active spots (a to e) that migrated with mobilities similar to those of short-chain AHLs (Fig. 1). One spot that was detected by the *Agrobacterium* QS reporter, spot d, migrated with an mobilities similar to that of C₆-HSL and was seen in extracts of whole cultures and culture filtrates from both the AiiA-producing and vector control strains (Fig. 1, lanes 2 through 5). There was no appreciable difference in the size of spot d in culture filtrate from the AiiA-producing strain (Fig. 1, lane 2) and in culture filtrate from the vector-only control strain (Fig. 1, lane 4). Because AiiA inactivates short-chain AHLs even more efficiently than long-chain AHLs, the persistence of spot d in culture filtrate extracts of the AiiA-producing strain indicates that the active compound probably is not C₆-HSL or any other short-chain AHL. It is noteworthy that spot d is very faint in whole-culture extracts of the AiiA-producing strain (Fig. 1, lane 3), but appears to be quite large in whole-culture extracts of the vector control strain (Fig. 1, lane 5). This suggests that the active compound is highly concentrated inside the vector control bac-

teria, with little escaping into the external medium. Short-chain AHLs equilibrate rapidly across the cell membrane (Pearson et al. 1999); therefore, the accumulation of this *Agrobacterium*-active compound inside the cells provides further evidence that the compound is not likely to be a short-chain AHL. Two other *Agrobacterium*-active compounds, corresponding to spots c and e, were detected in extracts from the vector-only control strain (Fig. 1, lanes 4 and 5), but were absent in extracts from the AiiA-producing strain (Fig. 1, lanes 2 and 3). The loss of these spots in the AiiA-producing strain is consistent with the spots being short-chain AHLs. However, such loss also is consistent with the active compounds being synthesized in a manner that is dependent on QS mediated by long-chain AHLs. The presence of much larger spots for these two compounds in whole-culture extracts of the vector-only control strain (Fig. 1, lane 5 versus 4) suggests that the active compounds are highly concentrated in the bacterial cells and, therefore, probably are not short-chain AHLs. There also appear to be other, more rapidly migrating *Agrobacterium*-active compounds (spots a and b) present inside the vector-only control cells (Fig. 1, lane 5). These compounds do not appear to diffuse from or be secreted by the cells. They are not detectable inside the AiiA-producing cells (Fig. 1, lane 3). This suggests that their synthesis is likely to be QS-regulated in a manner dependent on SinI AHLs.

Quorum quenching affects the rate and efficiency of nodule initiation.

To test the in vivo effects of quorum quenching on the timing and efficiency of symbiotic infection, strains 1021, 1021 *sinI*, 1021 pLAFR3, and 1021 pLAFR3*aiiA* were inoculated onto 2-day-old seedlings of *Medicago truncatula* in growth pouches.

Table 1. Bacterial strains and plasmids

Strain, plasmid	Relevant characteristics ^y	Reference
<i>Sinorhizobium meliloti</i>		
1021	<i>expR102::ISRM2011-1</i> , Sm ^r	Galibert et al. 2001
8530	1021 <i>expR</i> ⁺ (formerly <i>expR101</i>), Sm ^r	Pellock et al. 2002
MG32	8530 with in-frame <i>sinI</i> deletion (<i>sinI-expR</i> ⁺), Sm ^r	Gao et al. 2005
MG75	1021 with in-frame <i>sinI</i> deletion (<i>sinI-expR</i> ⁻), Sm ^r	Gao et al. 2005
1021 (pE7-R3)	1021(<i>sinI+expR</i> ⁻) pE7-R3 (<i>aiiA</i> on pLAFR3), Sm ^r , Tc ^r	This study
1021 (pLARF3)	1021 (<i>sinI+expR</i> ⁻) pLARF3 vector, Sm ^r , Tc ^r	This study
MG80 (pE7-R3)	1021 <i>ftsZ1::gus aiiA</i> ⁺ , Sm ^r , Tc ^r , Nm ^r	This study
MG81 (pE7-R3)	1021 <i>gor::gus aiiA</i> ⁺ , Sm ^r , Tc ^r , Nm ^r	This study
MG83 (pLAFR3)	1021 <i>gor::gus</i> , Sm ^r , Tc ^r , Nm ^r	This study
MG84 (pLAFR3)	1021 <i>ibpA::gus</i> pLAFR3, Sm ^r , Tc ^r , Nm ^r	This study
MG85 (pE7-R3)	1021 <i>ibpA::gus aiiA</i> ⁺ Sm ^r , Tc ^r , Nm ^r	This study
MG89 (pE7-R3)	1021 <i>soxG::gus aiiA</i> ⁺ , Sm ^r , Tc ^r , Nm ^r	This study
MG90 (pLAFR3)	1021 <i>soxG::gus</i> pLARF3, Sm ^r , Tc ^r , Nm ^r	This study
MG94 (pLAFR3)	1021 <i>ftsZ1::gus</i> , Sm ^r , Tc ^r , Neo ^r	This study
<i>Escherichia coli</i>		
DH5α	<i>hisdR17 endA1 thi-1 gyrA96 relA1 supE44 ΔlacU169(φ80dlacZAM15)</i>	Invitrogen
AHL reporters ^z		
<i>Agrobacterium tumefaciens</i> NTI (pZLR4)	TraR receptor, cognate AHL = 3-oxo-C8-HSL, Gm ^r	Shaw et al. 1997
<i>Pseudomonas putida</i> 117 (pAS-C8)	CepR receptor, cognate AHL = C8-HSL, Gm ^r	Steidle et al. 2001
<i>E. coli</i> JM109 (pSB1075)	LasR receptor, cognate AHL = 3-oxo-C12-HSL, Ap ^r	Winson et al. 1998
<i>E. coli</i> JM109 (pSB401)	LuxR receptor, cognate AHL = 3-oxo-C6-HSL, Tc ^r	Winson et al. 1998
Plasmids		
pLAFR3	IncP broad host range cosmid vector, Tc ^r ,	Staskawicz et al. 1987
pE7-R3	pLAFR3 carrying <i>aiiA</i> , Tc ^r	Dong et al. 2000
pRK600	pRK2013 Nm ^r :: Tn9 Cm ^r	Long et al. 1988
pVMG	pVO155 with STOP codons in three possible reading frames of the promoterless <i>gus</i> reporter	Gao et al. 2005
pMG3	Internal 413-bp fragment of Smc00154 (<i>gor</i>) cloned into pVMG	Gao et al. 2005
pMG10	Internal 295-bp fragment of Smc03930 (<i>soxG2</i>) cloned into pVMG	Gao et al. 2005
pMG12	Internal 383-bp fragment of Smc04040 (<i>ibpA</i>) cloned into pVMG	This study
pMG13	Internal 489-bp fragment of Smc1874 (<i>ftsZ1</i>) cloned into pVMG	This study

^y Sm^r, Tc^r, Nm^r, Gm^r, Ap^r, and Cm^r = resistance to streptomycin, tetracycline, neomycin, gentamycin, ampicillin, and chloramphenicol, respectively.

^z AHL = *N*-acyl homoserine lactone.

Root hairs develop acropetally and remain susceptible to infection by rhizobia in various legumes primarily while they are still elongating (Bhuvaneswari et al. 1981). By marking the position of the root tip at the time of inoculation (as described below), one defines a set of *M. truncatula* root hairs that are initially susceptible, but then lose susceptibility to *Sinorhizobium* infection within approximately 8 to 12 h (Gao et al. 2005). Nodules that develop on the primary root below this mark (RT1) result from infections in root hairs that emerged and elongated at times progressively later than 12 h after inoculation. After 3 weeks, the wild-type *S. meliloti* 1021 strain and the vector control (*S. meliloti* 1021 pLAFR3) strain elicited, on average, 1.32 and 1.05 nodules/plant, respectively, above the RT1 mark (Table 2). The 1021 *sinI* mutant and the 1021 pLAFR3*aiiA* strain elicited, on average, 0.76 and 0.58 nodules/plant, respectively, above the RT1 mark. These values for the *sinI* mutant and *aiiA*-expressing strain are significantly lower than those for the 1021 wild-type and vector control strains (Table 2). The reduced number of nodules above RT1 formed by the AiiA-producing strain and the *sinI* mutant strain corresponds to an appreciably lower rate or efficiency of nodule initiation than the corresponding control strains during the first 12 h. The position of the root tip 24 h after inoculation also was marked (RT2). No significant differences were observed in the number of nodules initiated later on the primary root, between RT1 and RT2, or below the RT2 mark, between any of the inoculum strains, indicating that the effects of AiiA production on nodule initiation by strain 1021 were transient. Almost all the bacteria recovered from nodules

elicited by the AiiA-producing and vector control strains were resistant to tetracycline, indicating that the pLAFR3 plasmid was retained.

Bacteroids inside the uppermost (oldest) nodule located above RT1 taken from seven representative plants inoculated with *S. meliloti* 1021 pLAFR3*aiiA* and from four plants inoculated with *S. meliloti* 1021 pLAFR3 were examined by low-pressure scanning electron microscopy (Fig. 2). Among the 10 to 20 infected host cells observed for each nodule at relatively high magnification, host cells filled with smaller (1.1- to 1.3-micron), randomly oriented bacteria (Fig. 2A) were seen in sections of all four of the nodules generated by the 1021 pLAFR3 strain and in sections of five of seven nodules generated by the 1021 pLAFR3*aiiA* strain. However, bacteroids that were significantly elongated (2.6 to 7.5 microns) and oriented in a side-by-side array around the periphery of the infected host cells, leaving a "hole" of host vacuole or cytoplasm in the middle (Fig. 2B), were seen in sections of four of the seven nodules generated by the 1021 pLAFR3*aiiA* strain, but not in any of the four nodules generated by the vector control strain. Such peripherally located *S. meliloti* bacteroids normally are found in the zone of active N fixation (Haynes et al. 2004; D. Gage, *personal communication*).

Proteome analysis of quorum quenching in *S. meliloti*.

In order to globally identify QS-regulated functions affected by quorum quenching in *S. meliloti*, proteins were extracted from early log phase and early stationary phase cultures of the AiiA-producing and vector-only control strains. To facilitate comparison of results with earlier proteome studies (Chen et al. 2003; Teplitski et al. 2004) and to complement recent proteome characterization of ExpR-dependent QS regulation (Gao et al. 2005), the *expR*- strain 1021 was used as a base for our proteome analysis rather than the *expR*+ strain 8530. After electrophoretic separation on sets of two-dimensional gels with overlapping pH ranges, the proteins were stained and analyzed for differential accumulation (as described in below). Over 60 proteins were differentially accumulated in the control strain compared with the AiiA lactonase-expressing strain (Table 3). Fifty-two of these proteins were identified by peptide mass fingerprinting. All of these proteins were identified at the highest level of confidence (Weiller et al. 2001), except for those corresponding to Smc00972, 03121, 03930, 04262, and 04342, which all had the next highest confidence rating.

Fig. 1. Thin-layer chromatography (TLC) comparison of quorum sensing (QS)-active compounds from AiiA-expressing and vector control *Sinorhizobium meliloti* strains. Solvent extracts from culture filtrates (CF) and whole cultures (WC = cells + CF) of the AiiA-expressing strain 1021 pLAFR3*aiiA* (*aiiA*) and the vector control strain 1021 pLAFR3 (WT) were tested for the presence of compounds capable of activating the *Agrobacterium* NTL4 pZLR4 QS reporter after TLC separation. Lane 1 = *N*-acyl homoserine lactone standards (C₈-HSL, C₆-HSL, and 3-oxo-C₆-HSL), lane 2 = *aiiA* CF, lane 3 = *aiiA* WC, lane 4 = WT CF, and lane 5 = WT WC. Spots corresponding roughly to QS-active compounds in the WC extract from WT cells are designated a to f.

Table 2. Nodule initiation on *Medicago truncatula* primary roots by *Sinorhizobium meliloti* 1021 and mutant strains^a

Inoculum strain	Average no. of nodules/plant in given primary root zone			No. of plants
	Above RT1	RT1-RT2	Below RT2	
1021	1.3	1.2	1.5	97
1021 <i>sinI</i>	0.76**	1.4	1.3	92
1021 pLAFR3	1.1	1.4	1.3	116
1021 pLAFR3 <i>aiiA</i>	0.58*	1.1	1.4	119

^a Values are averages of combined data from two independent experiments with two independent cultures of each strain, two independently sterilized sets of seed, and 25 to 50 seedlings per strain in each experiment. Nodules on the primary roots above the position of the root tip at the time of inoculation (RT1), between RT1 and the position of the root tip 24 h after inoculation (RT2), and below RT2 were counted. Nodule initiation above RT1 was significantly lower at the *P* = 0.05 level (*) for *S. meliloti* 1021 pLAFR3*aiiA* compared with *S. meliloti* 1021 pLAFR3, and at the *P* = 0.008 level (**) for *S. meliloti* 1021 *sinI* compared with *S. meliloti* 1021. There was no significant difference between *S. meliloti* 1021 pLAFR3 and *S. meliloti* 1021 under the same conditions according to square-root-transformed data with Tukey's studentized range (highly significant difference) test.

A spot corresponding to the AiiA protein (MW 28.5 kDa, isoelectric point (pI) 4.77) was detected in cells of *S. meliloti* 1021 pLAFR3*aiaA* but not in the cells of the 1021 pLAFR3 vector control strain (Table 3). AiiA did not accumulate to high levels in the cells, having a spot volume corresponding to only 0.05% of the total resolved proteins (Table 3), but was clearly visible and separated from other proteins.

More proteins were differentially accumulated in early stationary phase cultures ($n = 51$) than in early log phase cultures ($n = 17$). Slightly over half of the proteins were accumulated to higher levels as a result of quorum quenching (Table 3). This indicates that QS regulation in *S. meliloti* 1021 normally serves to diminish the accumulation of specific proteins just as often as it enhances accumulation. An earlier proteome study of the *expR* and *sinI* mutants of *S. meliloti* also found that QS served to diminish or suppress the accumulation of approximately half of the QS-regulated proteins (Gao et al. 2005), and a quorum quenching study in *Rhizobium leguminosarum* likewise found reduced accumulation of approximately half the QS-regulated proteins (Cantero et al. 2006).

Only five proteins were differentially accumulated in both early log and early stationary phase cultures of the AiiA versus vector-only strains. Interestingly, these five proteins include all three isoforms of the GroESL5 chaperonin (Smb21566) and one isoform of the hypothetical conserved signal peptide (Smc01418). Quorum quenching affected the accumulation of the isoforms of both the GroESL5 chaperonin and the Smc01418 signal peptide to different extents (Table 3). The same is true for the two isoforms of 6-phosphogluconate dehydrogenase (Smc04262). These differences suggest that QS normally is involved in differentially regulating the formation or persistence of the different isoforms of these three proteins.

Transcriptional analysis of genes corresponding to QS-regulated proteins.

Single crossover chromosomal *gus* fusions in four genes encoding QS-regulated proteins were constructed to examine the effects of quorum quenching on expression of the genes (Table 4). The expression of all four genes was significantly higher in the vector control strain, which has QS signals, than in the AiiA-producing strain, where the QS signals have been destroyed. This indicates that AHLs in the bacterium normally serve to activate QS-regulated expression of these four genes. There was good correspondence between gene expression and protein accumulation for *ftsZ1*. However, the accumulation of

proteins encoded by *soxG*, *ipbA*, and *gor* was increased in the quorum-quenched strain, whereas *gus* fusion expression was reduced (Table 4). In a previous study, only 5 of 10 representative *gus* fusions showed good correspondence between protein accumulation and transcriptional activity in *sinI* and *expR* mutants or in response to added AHLs (Gao et al. 2005). The differences between protein accumulation and transcription observed by Gao and associates (2005) as well as those seen here could be due to the differences in growth phase or population density of cells taken for proteome versus *gus* fusion assays to post-transcriptional regulation (Arevalo-Ferro et al. 2003) or to other indirect effects of QS regulation on the accumulation of specific proteins.

The AiiA-lactonase inactivates AHL mimic compounds secreted by the host plant, *M. truncatula*.

Previous studies have shown that *M. truncatula* secretes a variety of compounds that effectively mimic AHLs and can either stimulate or inhibit AHL-mediated QS regulation (Gao et al. 2003; Teplitski et al. 2000). The preferential solubility of these "AHL mimic" compounds in methanol or methanol/water instead of ethyl acetate indicates that the *M. truncatula* compounds are chemically different from known bacterial AHLs (Gao et al. 2003). Nonetheless, if these *M. truncatula* AHL mimic compounds had a furanone or lactone ring structure similar to bacterial AHLs, it is possible that the AiiA lactonase might hydrolyze the active plant compounds. If so, the presence of AiiA inside *Sinorhizobium* cells might inactivate both endogenous AHL signals and host plant mimic compounds during symbiotic interactions. In order to explore this possibility, HPLC fractions containing two separate LuxR-stimulatory mimic compounds were treated with either crude soluble proteins from *E. coli* cells expressing AiiA or the proteins from vector control cells. AiiA in crude protein preparations substantially inactivated both of the host plant-derived LuxR-stimulatory activities tested (Table 5).

DISCUSSION

Effects of AiiA-mediated quorum quenching on QS signal levels in *S. meliloti*.

Although the amount of AiiA protein accumulated in *S. meliloti* cells was not large at either early log or stationary phase (Table 3), enough of the enzyme was present to reduce the levels of long-chain AHLs to levels that were nondetect-

Fig. 2. Bacteroid phenotype of *Sinorhizobium meliloti* cells expressing the AiiA *N*-acyl homoserine lactone lactonase. Representative 3-week-old nodules generated by *S. meliloti* 1021 pLAFR3*aiaA* and *S. meliloti* 1021 pLAFR3 in two separate experiments were excised from *Medicago truncatula* roots in the region above RT1, fixed in fresh glutaraldehyde, sectioned free hand with a razor, and examined with a Hitachi 3500 low-pressure scanning electron microscopy instrument. **A**, Bacteroids in an infected cell of a nodule formed by strain 1021 pLAFR3. **B**, Bacteroids in an infected cell of a nodule formed by strain 1021 pLAFR3*aiaA*. Dot scale at bottom edge = 1 μ m.

able or near detection limits using either mass spectrometry or the *Agrobacterium* reporter. AiiA inactivation of these AHLs was somewhat specific. In extracts of the vector control strain, the levels of 3-oxo-C_{16:1}-HSL were 10-fold greater than for C_{16:1}-HSL; however, AiiA inactivated 3-oxo-C_{16:1}-HSL completely, whereas it reduced the levels of C_{16:1}-HSL by only 90%.

It is not clear whether the residual levels of C_{16:1}-HSL secreted by the AiiA-producing strain are capable of eliciting significant QS responses in the bacterium. In previous studies,

a form of surface swarming was induced in an ExpR-dependent manner by 5-mM levels of C_{16:1}-HSL but not by comparable concentrations of other SinI AHLs (Gao et al. 2005). The QS-regulated synthesis of EPSII, triggered by C_{16:1}-HSL, was induced at surprisingly low population densities (approximately 10⁷ cells/ml) (Pellock et al. 2002). These results indicate that even very low levels of C_{16:1}-HSL might be sufficient to activate QS-regulated gene expression, at least in cells with a functional ExpR receptor. To test this possibility, the

Table 3. Protein identification of AiiA-regulated proteins in *Sinorhizobium meliloti*

Spot #	Acc. # ^x	Predicted Protein	Accumulation in AiiA cells ^u				Volume of spot as % total spot volume				Mascot ^y			MW/pI ^w	
			Early log		Stationary		Early log phase		Stationary phase						
			%	P	%	P	AiiA	WT	AiiA	WT	M ^z	Pep	Seq	Exp.	Theor.
146	Q9L8R8	aiaA, Putative metallohydrolase [Bacillus sp. strain 240B1]	New		New		0.064± 0.037	Missing	0.051 ± 0.012	Missing	65	4	15	28500/4.77	28019/4.74
36	SMa1450	Probable thiolase	50	0.003	50	<0.001	Missing	Missing	0.553 ± 0.085	1.173 ± 0.161	126	7	30	42250/5.90	40823/5.87
30	SMa1452	Probable dehydrogenase			60	<0.001	0.088 ± 0.004	0.067 ± 0.043	0.670 ± 0.071	1.171 ± 0.253	145	8	55	27455/5.65	26313/5.62
69	SMa1507	Conserved hypothetical protein					0.047 ± 0.006	0.087 ± 0.026	0.049 ± 0.013	0.081 ± 0.067	124	14	38	36262/5.65	38516/6.33
94	SMB20019	sucB, Dihydrolipoamide succinyltransferase			20	<0.001	Missing	Missing	0.026 ± 0.007	0.149 ± 0.018	98	6	24	46635/6.20	40122/6.16
41	SMB20020	pdh, Pyruvate dehydrogenase e1 component, alpha and beta subunits			40	<0.001	0.060 ± 0.044	0.051 ± 0.018	0.212 ± 0.094	0.505 ± 0.077	189	12	23	71834/5.66	75719/5.63
126	SMB20442	Periplasmic solute-binding protein			50	0.002	0.046 ± 0.023	0.038 ± 0.004	0.052 ± 0.015	0.110 ± 0.033	66	4	19	34596/5.69	36058/5.97
25	SMB20484	ABC transporter periplasmic sugar-binding protein			40	<0.001	0.015 ± 0.002	0.019 ± 0.006	0.050 ± 0.011	0.145 ± 0.030	106	6	27	32770/5.06	34865/5.40
112	SMB20660	Putative sugar uptake ABC transporter periplasmic solute-binding protein pre			30	0.002	Missing	Missing	0.053 ± 0.010	0.178 ± 0.082	93	6	25	42636/5.26	47150/5.71
86	SMB20753	(EC 1.3.99.-) Putative acyl-CoA dehydrogenase			60	<0.001	0.124 ± 0.062	0.130 ± 0.102	0.202 ± 0.062	0.346 ± 0.055	101	6	16	41031/5.68	41078/5.66
35	SMB20964	Putative anti-oxidant protein, AHPCTSA family			60	0.031	Missing	Missing	0.265 ± 0.124	0.453 ± 0.122	107	6	41	25800/5.85	24121/5.83
37	SMB21192	cbhA2 (EC 4.1.2.13) Putative fructose-bisphosphate aldolase			200	<0.001	0.386 ± 0.084	0.315 ± 0.065	0.257 ± 0.027	0.128 ± 0.014	82	6	30	39098/5.82	37608/5.73
145	SMB21304	(EC 3.5.3.18) Putative NG,NG-dimethylarginine dimethylaminohydrolase	220	0.003			0.174 ± 0.059	0.079 ± 0.025	0.152 ± 0.033	0.157 ± 0.016	51	3	11	29000/4.77	28619/4.79
16	SMB21566	groEL5, Putative heat shock protein groEL	390	<0.001	320	<0.001	0.106 ± 0.029	0.027 ± 0.003	0.320 ± 0.058	0.100 ± 0.021	76	4	45	12468/5.16	57847/5.23
21	SMB21566	groEL5, Putative heat shock protein groEL	190	0.027	350	<0.001	0.109 ± 0.043	0.058 ± 0.014	0.393 ± 0.054	0.112 ± 0.014	129	9	31	56236/5.16	57847/5.23
23	SMB21566	groEL5, Putative heat shock protein groEL	160	<0.001	230	<0.001	0.640 ± 0.059	0.394 ± 0.038	1.608 ± 0.342	0.701 ± 0.146	123	8	21	55464/5.21	57847/5.23
5	SMc00140	Amino-acid binding periplasmic protein			50	0.016	0.207 ± 0.027	0.167 ± 0.091	0.303 ± 0.135	0.665 ± 0.266	88	5	35	26381/4.62	28001/4.85
101	SMc00154	gor (EC 1.6.4.2) Glutathione reductase	160	0.012			0.337 ± 0.092	0.211 ± 0.039	Missing	Missing	114	6	16	53134/5.92	49891/5.81
26	SMc00324	pnp (EC 2.7.7.8) Polyribonucleotide nucleotidyltransferase			270	<0.001	0.233 ± 0.088	0.242 ± 0.115	0.095 ± 0.016	0.035 ± 0.014	127	9	18	77029/5.18	77593/5.16
40	SMc00641	serA (EC 1.1.1.95) D-3-phosphoglycerate dehydrogenase			150	0.008	1.168 ± 0.758	1.595 ± 0.293	0.614 ± 0.140	0.408 ± 0.068	68	4	10	56908/5.67	56136/5.60
14	SMc00770	potF, Putrescine-binding periplasmic protein			50	0.005	0.147 ± 0.048	0.085 ± 0.044	0.210 ± 0.095	0.408 ± 0.155	70	6	34	38000/4.52	40588/4.63
66	SMc00851	glyS (EC 6.1.1.14) Glycyl-trna synthetase beta chain	210	<0.001			0.127 ± 0.014	0.061 ± 0.013	0.058 ± 0.019	0.056 ± 0.006	129	7	15	78338/5.71	79016/5.67
132	SMc00860	ispB (EC 2.5.1.-) Octaprenyl-diphosphate synthase			180	<0.001	0.135 ± 0.007	0.128 ± 0.027	0.132 ± 0.015	0.072 ± 0.024	149	9	40	40364/5.15	36156/5.16
87	SMc00945	gpt (EC 2.4.2.22) Xanthine-guanine phosphoribosyltransferase			190	<0.001	0.486 ± 0.164	0.440 ± 0.129	0.238 ± 0.020	0.125 ± 0.025	54	3	18	23305/5.95	18418/5.91
93	SMc00972	dxs, 1-Deoxyxylulose-5-phosphate synthase			310	<0.001	0.069 ± 0.024	0.062 ± 0.024	0.058 ± 0.006	0.019 ± 0.003	48	3	6	74195/6.06	68601/6.00
137	SMc00993	purD (EC 6.3.4.13) Phosphoribosylamine--glycine ligase	200	<0.001			0.067 ± 0.004	0.034 ± 0.006	Missing	Missing	95	5	14	50500/4.83	44296/4.82

Continued on next page

^u % = protein accumulation in AiiA-producing cells as a percentage of accumulation in vector control cells. *P* = *P* value for statistical significance of differences in protein accumulation as determined by linear restricted maximum likelihood analysis.

^v Mascot search results. Pep = number of peptides matched. Seq = sequence coverage.

^w MW/pI = molecular weight and isoelectric point; Exp. = experimental; Theor. = theoretical.

^x Best matching gene product based on Mascot Blastx search.

^y M = Mowse score. Proteins with Mowse scores over 41 are considered significant matches.

^z No value is listed for a protein if its accumulation in AiiA-producing cells was not significantly different from accumulation in the vector control cells.

pLAFR3*aiiA* plasmid was transferred to the 8530 (*expR*+) strain and tested for ability to swarm. The 8530 pLAFR3*aiiA* strain swarmed in essentially the same manner as the 8530 wild-type strain, indicating that residual levels of C_{16:1}-HSL in the AiiA-producing cells seem to be sufficient to trigger at least this specific behavior (data not shown). Whether other receptors in the 1021 (*expR*-) pLAFR3*aiiA* strain respond significantly to the residual C_{16:1}-HSL is unknown.

The mass spectrometric and TLC data presented here are consistent with the conclusion that *S. meliloti* 1021 pLAFR3

synthesizes no AHLs with short acyl side chains, at least under the in vitro culture conditions employed. In previous studies with the *S. meliloti* *expR*+(8530) strain, culture filtrates were found to contain a number of compounds that were capable of activating AHL reporter strains and that migrated on TLC plates like short-chain AHLs, even though no short-chain AHLs were detected by mass spectrometry (Gao et al. 2005). Several of the *Agrobacterium*-active spots migrating in the region of short-chain AHLs in that study were diminished or absent in the *sinI* mutant, suggesting that they were either

Table 3. Continued from preceding page

Spot #	Acc. # ^x	Predicted Protein	Accumulation in AiiA cells ^u				Volume of spot as % total spot volume				Mascot ^v			MW/pI ^w	
			Early log		Stationary		Early log phase		Stationary phase		M ^z	Pep	Seq	Exp.	Theor.
			%	P	%	P	AiiA	WT	AiiA	WT					
96	SMc01005	folE (EC 3.5.4.16) GTP cyclohydrolase I			280	0.005	0.049 ± 0.012	0.054 ± 0.014	0.048 ± 0.022	0.017 ± 0.002	91	4	23	26872/6.26	22772/6.23
31	SMc01140	Sigma 54 modulation protein			50	<0.001	0.225 ± 0.210	0.195 ± 0.190	0.069 ± 0.023	0.150 ± 0.039	65	5	29	28827/5.61	21218/5.62
125	SMc01227	nerA (EC 1.-.-.-) Glycerol trinitrate (GTN) reductase			280	<0.001	0.505 ± 0.085	0.507 ± 0.076	0.096 ± 0.016	0.034 ± 0.003	211	10	49	40874/5.71	39900/5.71
50	SMc01322	nusG, Transcription antitermination protein			160	<0.001	0.544 ± 0.221	0.562 ± 0.208	0.411 ± 0.064	0.265 ± 0.024	108	6	47	23340/6.18	19871/6.13
1	SMc01418	Signal peptide hypothetical/global homology	50	0.031	40	<0.001	0.632 ± 0.434	1.187 ± 0.279	4.221 ± 1.577	10.86 ± 3.513	79	4	45	11656/4.65	13303/4.80
9	SMc01418	Signal peptide hypothetical/global homology			20	0.005	0.006 ± 0.001	0.009 ± 0.005	0.034 ± 0.009	0.228 ± 0.132	58	3	35	12019/4.73	13303/4.80
10	SMc01418	Signal peptide hypothetical/global homology			20	0.003	0.009 ± 0.006	0.012 ± 0.011	0.031 ± 0.012	0.209 ± 0.127	59	3	32	11865/4.71	13303/4.80
119	SMc01642	Periplasmic binding protein			40	<0.001	Missing	Missing	0.196 ± 0.101	0.511 ± 0.148	87	5	13	52051/5.44	59268/5.76
58	SMc01727	Conserved hypothetical protein	60	<0.001			0.028 ± 0.007	0.050 ± 0.010	0.048 ± 0.012	0.044 ± 0.011	130	7	35	27546/5.04	26916/5.08
24	SMc01874	ftsZ1, Cell division protein			60	0.022	0.171 ± 0.037	0.260 ± 0.143	0.128 ± 0.051	0.212 ± 0.053	181	11	30	65470/5.08	62935/5.06
78	SMc01934	proS (EC 6.1.1.15) Polyl-tRNA synthetase			210	<0.001	0.140 ± 0.069	0.225 ± 0.153	0.158 ± 0.022	0.076 ± 0.013	56	4	10	50129/5.99	49397/5.89
123	SMc02433	clpB, ATP-dependent protease (heat shock protein)			160	0.034	0.063 ± 0.010	0.057 ± 0.004	0.157 ± 0.049	0.097 ± 0.029	152	9	15	87681/5.52	95612/5.49
122	SMc02725	trpE (EC 4.1.3.27) Anthranilate synthase includes: glutamine amidotransferase			150	0.003	0.089 ± 0.037	0.096 ± 0.048	0.067 ± 0.018	0.045 ± 0.008	155	8	13	75669/5.50	80455/5.44
64	SMc02755	ahcY (EC 3.3.1.1) Adenosylhomocysteinase	30	<0.001			0.082 ± 0.062	0.268 ± 0.090	0.197 ± 0.102	0.101 ± 0.036	173	9	29	50284/5.45	50594/5.40
46	SMc02877	Conserved hypothetical protein			230	0.018	0.075 ± 0.017	0.087 ± 0.015	0.190 ± 0.087	0.082 ± 0.030	75	4	15	38518/6.18	35171/6.09
84	SMC03121	ABC transporter periplasmic binding protein			170	<0.001	0.305 ± 0.150	0.254 ± 0.159	0.207 ± 0.025	0.119 ± 0.019	41	3	7	48309/5.89	42751/5.21
6	SMc03131	ABC transporter amino acid-binding periplasmic protein	70	<0.001			0.221 ± 0.034	0.317 ± 0.017	2.336 ± 0.362	2.692 ± 0.110	79	5	25	27377/4.69	30073/4.87
4	SMc03157	Transmembrane outer membrane lipoprotein			210	0.007	0.314 ± 0.124	0.362 ± 0.229	0.348 ± 0.088	0.166 ± 0.105	84	5	29	29457/4.50	27536/4.65
63	SMc03846	acnA (EC 4.2.1.3) Aconitate hydratase	160	<0.001			0.063 ± 0.008	0.041 ± 0.007	0.040 ± 0.014	0.032 ± 0.032	158	9	13	91807/5.08	96852/5.14
109	SMc03878	phbB (EC 1.1.1.36) Acetoacetyl-CoA reductase	150	0.036			0.302 ± 0.080	0.196 ± 0.062	Missing	Missing	70	4	19	23523/6.05	25354/6.15
92	SMc03930	soxG2 (1.5.3.1) Sarcosine oxidase gamma subunit			220	<0.001	0.147 ± 0.043	0.136 ± 0.010	0.089 ± 0.004	0.040 ± 0.003	44	3	30	18235/6.02	18917/5.78
51	SMc04040	ibpA, Heat shock protein			620	<0.001	Missing	Missing	0.157 ± 0.058	0.025 ± 0.005	87	4	39	19351/6.10	17402/6.11
70	SMc04094	Conserved hypothetical protein	60	0.03			0.118 ± 0.044	0.186 ± 0.045	0.150 ± 0.079	0.165 ± 0.027	73	4	23	31352/5.50	25845/5.40
43	SMc04262	gnd (EC 1.1.1.44) 6-Phosphogluconate dehydrogenase (decarboxylating)			170	0.005	1.581 ± 0.285	1.444 ± 0.240	0.996 ± 0.209	0.599 ± 0.188	127	7	24	46965/6.02	50772/5.92
85	SMc04262	gnd (EC 1.1.1.44) 6-Phosphogluconate dehydrogenase (decarboxylating)			310	0.002	0.141 ± 0.089	0.159 ± 0.116	0.140 ± 0.060	0.046 ± 0.012	52	3	9	48995/5.86	50772/5.92
91	SMc04342	Methyltransferase			220	<0.001	0.261 ± 0.083	0.195 ± 0.039	0.157 ± 0.019	0.072 ± 0.008	47	3	19	35701/5.81	33692/5.84
80	SMc04386	aatB (EC 2.6.1.1) Aspartate aminotransferase B			130	0.007	0.308 ± 0.034	0.434 ± 0.129	1.027 ± 0.161	0.783 ± 0.080	104	7	28	44861/6.13	44347/6.05
18	No Good Match				50	0.02	0.977 ± 0.376	0.793 ± 0.254	0.302 ± 0.247	0.628 ± 0.133				21500/5.32	
20	No Good Match				60	0.011	0.065 ± 0.031	0.093 ± 0.071	0.238 ± 0.035	0.398 ± 0.121				31374/5.20	
27	No Good Match		140	<0.001	380	<0.001	0.063 ± 0.005	0.046 ± 0.002	0.166 ± 0.021	0.044 ± 0.006				57789/5.30	
33	No Good Match				30	<0.001	Missing	Missing	0.107 ± 0.012	0.329 ± 0.073				13282/5.80	
61	No Good Match		230	<0.001			0.127 ± 0.032	0.056 ± 0.019	0.074 ± 0.013	0.077 ± 0.014				17219/5.17	
88	No Good Match				160	0.002	3.111 ± 1.578	3.416 ± 0.509	1.383 ± 0.227	0.844 ± 0.254				7533/5.70	
97	No Good Match				40	<0.001	0.112 ± 0.051	0.087 ± 0.032	0.026 ± 0.006	0.064 ± 0.009				61182/5.88	
98	No Good Match				60	<0.001	0.068 ± 0.109	0.060 ± 0.019	0.041 ± 0.004	0.075 ± 0.005				63377/5.88	
115	No Good Match				30	<0.001	0.166 ± 0.052	0.127 ± 0.035	0.053 ± 0.023	0.178 ± 0.003				35792/5.33	
118	No Good Match				190	0.005	0.081 ± 0.054	0.057 ± 0.026	0.093 ± 0.030	0.050 ± 0.004				62017/5.51	
143	No Good Match				50	<0.001	Missing	Missing	0.058 ± 0.008	0.127 ± 0.042				42854/4.70	

AHLs synthesized by SinI or they were compounds whose synthesis depends on QS mediated by SinI AHLs. The results of Gao and associates (2005) are consistent with the reduced levels of spots c and e seen here in culture filtrates from the AiiA-expressing strain compared with the vector-only control (Fig. 1). The accumulation of these and other *Agrobacterium*-active substances inside the cells of the vector-only control, but not in the AiiA-expressing strain, argues that these compounds are synthesized in a QS-dependent manner but are not short-chain AHLs. Although these compounds apparently are able to affect QS in *Agrobacterium* spp., it is not yet clear whether any of them are able to affect QS in *S. meliloti*. Nor is it clear whether these compounds are produced or secreted in planta or in the rhizosphere. However, it does seem that AiiA-mediated quorum quenching eliminates or reduces most of these unknown compounds to low levels. If any short-chain AHLs were produced by *S. meliloti* 1021 under in vivo conditions via a cryptic AHL synthase, it is likely that they would be completely inactivated in the AiiA-producing strain.

Proteome analysis of quorum quenching mediated by the AiiA lactonase.

Surprisingly few (7 of 52) of the proteins identified as QS-regulated in this study (Table 3, bold) were identified previously in the other global studies of *S. meliloti* QS (Chen et al. 2003; Gao et al. 2005; Hoang et al. 2004; Teplitski et al. 2004). The reasons for this lack of overlap are not clear, although considerable differences between QS-regulated functions identified by different laboratories have been observed in *P. aeruginosa* (Arevalo-Ferro et al. 2003; Vasil 2003). The other *S. meliloti* proteome studies (Chen et al. 2003; Gao et al. 2005; Teplitski et al. 2004) used the same culture medium employed here and each involved or included studies of QS regulation in strain 1021, which lacks the ExpR AHL receptor. The transcriptome study of Hoang and associates (2004) grew cells in a different medium but did include a comparison of strain 1021 and the 1021 *sinI* mutant. Of the approximately 170 QS-regulated genes identified in the transcriptome study, expression of only 11 genes was found to be dependent on SinI in the absence of ExpR (Hoang et al. 2004), indicating that SinR and the other untested LuxR homologs in *S. meliloti* play only minor roles relative to the ExpR receptor under those conditions. However, the four proteome studies found roughly 150 proteins that are QS regulated in the absence of a functional ExpR receptor

(Chen et al. 2003; Gao et al. 2005; Teplitski et al. 2004) (Table 3), suggesting that the other AHL receptors are of comparable overall importance to ExpR and that the lack of overlap between studies of QS-regulated functions is not specific to ExpR. At this point, we believe that several factors contribute importantly to the overall lack of correspondence between QS-regulated functions identified in the several proteome studies as well as the large divergence between the transcriptome and proteome studies: i) differences in specific growth phase and treatment interval, ii) differences in culture medium or history, and iii) differences in the nature of the differential used to identify QS-regulated functions (e.g., responses to exogenous AHLs versus signal or receptor mutants versus quorum quenching). Taken together, the studies to date indicate that a large (and still expanding) number of functions is subject to QS regulation in *S. meliloti*. The most important goal in future studies will be to determine how such regulation contributes to the bacterium in specific natural environments.

Proteins with functions of potential symbiotic importance.

One of the symbiotically relevant proteins found to be QS regulated in the present study is FtsZ1, a cell-division protein with a potential role in septum initiation, probably interacting with FtsA (Margolin 2003; Margolin and Long 1994; Margolin et al. 1991). FtsZ1, encoded by Smc01874, was not differentially accumulated in early log cultures, but accumulated to reduced levels in the AiiA-producing strain at early stationary phase (Table 3). At the transcriptional level, expression of the *ftsZ1::gus* fusion was reduced approximately twofold in the AiiA-expressing strain in early to mid log (Table 4), consistent with the reduced accumulation of the FtsZ1 protein in the AiiA-producing strain (Table 3). These results suggest that QS regulation of FtsZ1 normally involves SinI AHLs acting through some AHL receptor other than ExpR to enhance accumulation of the cell division protein in early stationary phase. It is worth noting that the mechanism by which rhizobial cell division is regulated inside the host is one of the central, unresolved issues of the legume–*Rhizobium* spp. symbiosis. It is not yet clear how the growth of rhizobia inside infected host cells is controlled so that the host cells are filled with bacteria (Fig. 2A) that subsequently stop dividing despite access to plentiful nutrients. Nor is it clear how (or whether) cell division of *Sinorhizobium* bacteroids or undifferentiated siblings resumes inside senescing nodules so that the bacterial partner

Table 4. Effect of AiiA expression on activity of single-crossover *gus* gene fusions

Strain	GUS activity (Miller units) at differing culture optical densities (OD) ^y					
	<i>soxG2</i> (Smc03930)		<i>ibpA</i> (Smc04040)		<i>ftsZ1</i> (Smc01874)	<i>gor</i> (Smc00154)
	0.10	1.5	0.27	1.6	0.30	0.36 1.6
1021 pLAFR3	5 a	3,000 a	4,000 a	4,400 a	220 a	350 a 100
1021pLAFR3 <i>aiaA</i>	2 b	100 b	560 b	40 b	120 b	260 b 90
Protein accumulation (%) ^z	NSD	220	NSD	620 (at 1.0 OD)	50 (at 1.0 OD)	160 (at 1.0 OD) ...

^y Values are averages from three replicate cultures for each strain in a representative experiment; Averages for a given strain that are significantly different from each other at the $P = 0.05$ level by Tukey's studentized range test (HSD) are indicated by different letters. NSD = no significant difference in protein accumulation between AiiA-expressing strain and vector control.

^z Protein accumulation in AiiA+ (as percentage of wild type); values for the 1021 pLAFR3*aiaA* strain are from Table 3.

Table 5. AiiA lactonase inactivation of LuxR stimulatory *N*-acyl homoserine lactone mimic compounds from *Medicago truncatula*^z

Treatment	LuxR reporter response (net luminescence units)	
	HPLC fraction 46	HPLC fraction 51
Treated with crude cell preparation from vector control <i>Escherichia coli</i>	1,470	4,180
Treated with crude cell preparation from AiiA-producing <i>E. coli</i>	170	140

^z LuxR-active compounds were isolated from *M. truncatula* root exudate, and fractionated and treated with cell lysates as described in Materials and Methods. HPLC = high-performance liquid chromatography.

benefits from the association. QS regulation of FtsZ1 in *S. meliloti* may be related to the higher frequency of elongated bacteroids seen in nodules generated by the AiiA-producing strain (Fig. 2) and is consistent with other links between QS and the regulation of cell division in rhizobia. Hoang and associates (2004) determined that two genes involved in the inhibition of *Sinorhizobium* cell division, *minD* and *minE*, were subject to QS regulation. Expression of *minD* and *minE* was dependent on the ExpR receptor, but was independent of the *sinI* mutation. This could mean that some signal other than SinI AHLs interacts with the ExpR receptor to release the cells from inhibition by *minD* and *minE*. The QS signal of *R. leguminosarum*, 3-OH-C_{14:1}-HSL, acts to inhibit cell division in both *R. leguminosarum* and *R. etli* (Daniels et al. 2002; Gray et al. 1996; Thorne and Williams 1999; Wilkinson et al. 2002), also suggesting that QS can play an important role in regulating cell division in *Rhizobium* spp.

In an earlier study, interruption of the *Sinorhizobium soxG* gene (Smc03930) resulted in a substantially diminished rate or efficiency of nodule initiation on *M. truncatula* (Gao et al. 2005), indicating that it contributes a symbiotically important function. *soxG* encodes sarcosine oxidase, which catalyzes oxidation of sarcosine to glycine. Accumulation of SoxG in the present study was enhanced in early stationary cultures of the AiiA-producing strain (Table 3), whereas expression of the *soxG::gus* fusion was reduced in both early log and early stationary cultures of the AiiA-producing strain (Table 4). This indicates that SinI AHLs, acting through some AHL receptor other than ExpR, normally suppress SoxG accumulation in early log, but stimulate transcription of the *soxG* gene in both early log and stationary phase. SinI AHLs (specifically 3-oxo-C_{16:1}-HSL) also stimulated *soxG* expression in mid log and stationary phase in an ExpR-dependent manner (Gao et al. 2005). Thus, QS regulation of this symbiotically relevant function is complex and not well understood.

Oxidative stress.

Several proteins with roles in oxidative stress responses were affected by quorum quenching. Smc00154 encodes a predicted glutathione reductase, Gor. In other gram-negative bacteria, glutathione reductase is involved in responses to oxidative stress (Li et al. 2004). In *S. meliloti*, Gor contributes importantly to optimizing the rate or efficiency of nodule initiation (Gao et al. 2005). The results in Table 3 indicate that QS in the vector control strain acts to reduce Gor accumulation in early log phase and has no effect on Gor accumulation in stationary phase cultures. Quorum quenching had no significant effect on expression of the *gor* fusion in early stationary phase, but modestly reduced expression in early-mid log phase (Table 4). These quorum-quenching effects on *gor* transcription are contrary to the effects of AiiA on changes in Gor protein accumulation, as also seen in an earlier study (Gao et al. 2005). Thus, it appears that QS regulation of Gor is likely to involve more than one AHL receptor and transcriptional as well as post-transcriptional regulation. Accumulation of the antioxidant protein encoded by Smb20964 was inhibited by quorum quenching, whereas accumulation of IspB, a predicted octaprenyl-diphosphate synthase encoded by Smc00860, was promoted by quorum quenching (Table 3). In *E. coli*, IspB catalyzes production of ubiquinone, an antioxidant (Kainou et al. 2001). AHL-enhanced accumulation of IspB also was seen in an earlier study (Teplitski et al. 2004).

Transport.

QS appears to play a role in the accumulation of a number of proteins with putative functions associated with metabolite transport, including a predicted transporter of polar amino acids

encoded by Smc00140, a spermidine/putrescine transporter encoded by Smc00770, a predicted peptide/nickel transporter encoded by Smc01642, a putative branched-chain amino acid transporter encoded by Smc03121, and a putative methionine transporter encoded by Smc03157. The latter two proteins were accumulated to higher levels in the AiiA-expressing strain during entry into stationary phase. The others were reduced, indicating that QS normally serves to enhance the accumulation of these transporters during entry to stationary phase. Similarly, several proteins with predicted functions in the transport of sugars or carboxylic acids were reduced in stationary phase by quorum quenching, indicating that QS serves to enhance their accumulation in wild-type *Sinorhizobium* during entry into stationary phase. Specifically, a putative ABC sugar transporter component encoded by Smb20484, a predicted sugar-binding transporter most closely related to a family of maltose-binding proteins encoded by Smb20660, and a predicted C4-dicarboxylic-acid-binding protein or transporter encoded by Smb20442, were reduced in the AiiA-expressing strain. Quorum quenching also reduced accumulation of the protein encoded by Smc00324, a polyribonucleotide nucleotidyltransferase (RNase) (Table 3). In *E. coli*, the homolog of Smc00324 is associated with RNase E and is involved in degradation of the *ptsG* message (Morita et al. 2005). PtsG is a major glucose transporter. Other sugar-related transporters with greater accumulation in early stationary phase were identified by Chen and associates (2003), including those encoded by Smc03061 (α -glucoside), Smc04251 (mannitol), Smb20712 (inositol rhizopine), Sm20895 (sugar), and Smb21652 (lactose).

Protein synthesis and processing.

Accumulation of the GroEL5 chaperonin encoded by Smb21566, the ClpB protease (Smc02433), and the IbpA heat-shock protein (Smc04040) all were affected by quorum quenching (Table 3). The *groESL5* gene is regulated by RpoH1 in *S. meliloti* (Bittner and Oke 2006; Mitsui et al. 2004), but mutants have no obvious symbiotic defect. In previous studies, accumulation of the GroEL5 chaperonin was upregulated by addition of 3-oxo-C₁₆ for 2 h (Chen et al. 2003) and by treatment with a crude *S. meliloti* 1021 AHL extract and treatment with a purified AHL-mimic compound from *Chlamydomonas* spp. (Teplitski et al. 2004). In the present study, quorum quenching stimulated accumulation of the IbpA heat-shock protein (Table 3) even though it inhibited expression of the *ibpA::gus* transcriptional reporter in both early log and stationary phase cultures (Table 4). In *E. coli*, IbpA is a stationary-phase, *rpoS*-controlled protein (Kabir et al. 2004) that accumulates in response to stress. However, *S. meliloti* does not encode an *rpoS*; therefore, the role of IbpA in *Sinorhizobium* is unclear.

Regulation of translation.

AiiA quorum quenching affected the accumulation of two proteins with potentially opposite roles in translation. The protein encoded by Smc01140 is a putative sigma 54-modulation protein, which contains a ribosome-associated inhibitor A (RAIA) motif. In stationary-phase *E. coli* cells, RAIA-containing proteins bind to ribosomes and arrest translation (Agafonov et al. 1999; Maki et al. 2000). Accumulation of the RAIA-like protein encoded by Smc01140 was inhibited in early stationary phase by quorum quenching (Table 3) (i.e., its accumulation is stimulated by QS in the wild type). In contrast, the protein encoded by Smc01322 (NusG) is a transcriptional antitermination protein. NusG is a component of a complex that causes RNA polymerase to bypass transcript terminators in *rrn* operons (Grundy and Henkin 2004). NusG also is known to bind to a single site on Rho factors and to interact with RNA polymerase (Richardson and Richardson 2005), stimulating escape

of RNA polymerase from class II pauses. Therefore, RAI A and NusG seem to function in opposite directions (RAI A proteins promote stalling and NusG helps RNA polymerase overcome stalling). In this study, QS regulation of the RAI A-type protein Smc01140 and NusG was opposite: in stationary-phase cultures of the wild type, levels of the RAI A-like protein were up and levels of NusG were down, suggesting that AHL-mediated QS favors stalling of RNA polymerase in early stationary phase, consistent with the reduced accumulation of elongation factors in stationary phase seen by Chen and associates (2003).

Nutrient scavenging and amino acid metabolism.

Normal QS regulation in the vector control strain led to the accumulation of proteins corresponding to Sma1450 and Sma1452 (Table 3). Sma1452 is predicted to encode a 3-hydroxyacyl-CoA dehydrogenase. The reaction product of Sma1452-catalyzed dehydrogenation, 3-oxo-acyl-CoA, is a substrate for the acyl-CoA acyl transferase (thiolase) encoded by Sma1450. These proteins are predicted to function in the terminal reactions of amino acid, fatty acid, pyruvate, benzoate, propanoate, and butanoate degradation so that the products can be used in the citrate cycle. Accumulation of the thiolase previously was identified by Chen and associates (2003) as strongly upregulated in stationary phase, and enhanced accumulation of the thiolase was found to be *sinI*-dependent in late log cultures (Gao et al. 2005).

Accumulation of four other proteins (Smb20019, Smb20020, Smb21304, and Smb20753) with functions in amino acid degradation were upregulated by QS in stationary phase cultures. Smb20019 is predicted to encode dihydrolipoamide succinyltransferase, an enzyme of the TCA cycle that also is involved in lysine degradation. Smb20020 encodes a predicted 2-oxo-isovalerate dehydrogenase. It is a key enzyme in valine, leucine, and isoleucine metabolism, where it catalyzes their degradation to (S)-2-methyl-alkanoyl-dihydrolipoamides. Smb21304 encodes a putative N(G),N(G)-dimethylarginine dimethylaminohydrolase, which catalyzes the conversion of NG,NG-dimethyl-L-arginine to dimethylamine and L-citrulline and, therefore, is important for arginine degradation. Smb20753 encodes an acyl-CoA dehydrogenase with CaiA and IBD motifs. It is predicted to catalyze α , β -dehydrogenation of short, branched chains of acyl-CoA intermediates in valine metabolism.

In addition to increased accumulation of several proteins involved in amino acid degradation during stationary phase, QS regulation also led to reduced accumulation of various proteins involved in amino acid synthesis. Proteins encoded by Smc00641, Smc00851, and Smc03930 are predicted to function in glycine and serine synthesis whereas AatB, encoded by Smc04386, catalyzes conversion of L-aspartate to L-glutamate (Alfano and Kahn 1993). TrpE (Smc02725) catalyzes chorismate + L-glutamine = anthranilate + pyruvate + L-glutamate, a step in synthesis of tryptophan and histidine. The QS-regulated decrease in accumulation of TrpE in this study (Table 3) is consistent with results from *trpE-lacZ* transcriptional assays (Bae and Crawford 1990) and microarray analysis (Hoang et al. 2004) as *S. meliloti* enters stationary phase. The function of TrpE may not be limited to tryptophan or histidine synthesis. A *trpE* *Sinorhizobium* mutant was defective in nodulation even though mutants defective in other aspects of tryptophan synthesis or utilization showed normal nodulation (Barsomian et al. 1992).

Effects of AiiA-mediated quorum quenching on symbiotic interactions.

Based on the nodule initiation studies shown in Table 2, quorum quenching by AiiA significantly reduced the rate or

efficiency of nodule initiation on primary roots of the *M. truncatula* host plant during the first 12 h after inoculation, but did not measurably affect nodule initiation at later times. Interestingly, in similar experiments, Gao and associates (2005) found that the *sinI* mutation reduced nodule initiation for a 36-h period and more substantially in the 8530 (*expR*+) strain than seen here for the *sinI* mutation in the 1021 (*expR*-) strain. This suggests that a functional ExpR receptor is a more important contributor to QS regulation of the rate or efficiency of symbiotic nodule initiation than the other AHL receptors. Because the *sinI* mutation affected nodule initiation in strain 1021 at least as much as quorum quenching by AiiA (Table 2), it appears that either there is no second, cryptic AHL synthase, or that AHLs produced by such a synthase do not contribute appreciably to nodule initiation.

AiiA-expressing *S. meliloti* cells appear capable of forming normal numbers of normal bacteroids in infected host cells (Fig. 2). Preliminary examinations indicate that nodules generated by the 1021 pLAFR3*aiaA* strain seemed normal in size, pink color, and acetylene reduction rates (data not shown). The failure to detect elongated, peripherally located bacteroids in sections of nodules generated by the vector control strain and their presence in most of the nodules generated by the AiiA-producing strain (Fig. 2B) may be simply coincidental. Alternatively, it could reflect a greater abundance of such bacteroids in the QS-defective nodules, perhaps in compensation for reduced N-fixation efficiency. Further studies are required to clarify this uncertainty and to determine whether QS contributes importantly to other aspects of bacteroid function such as the rate or efficiency of bacteroid formation, the control of bacteroid cell division inside infected host cells, or to the control of metabolic changes related to nitrogen fixation or senescence.

Inactivation of host plant QS mimic compounds by AiiA.

The ability of AiiA to inactivate certain host plant AHL mimic compounds (Table 5) suggests that some of these compounds share a furanone ring structure in common with bacterial AHLs, consistent with the presence of a furanone ring in the QS-active compounds produced by the marine red alga *Delisea pulchra* (Givskov et al. 1996) and consistent with the recent identification of AiiA-sensitive QS effectors in rice (Degrassi et al. 2007). We note that the inactivation of some *M. truncatula* AHL mimic compounds by AiiA could prove to be a useful tool to determine whether such AiiA-sensitive AHL mimic compounds from the host play a role in manipulating QS-regulated genes or symbiotic behaviors in the bacterium. By comparing the in planta expression of specific QS-regulated genes in the *sinI* mutant, which produces no AHLs, with in planta expression of the same genes in the AiiA+ strain, which inactivates AHLs and some host mimic compounds, one potentially could identify symbiotic interactions that are subject to manipulation of QS regulation by AiiA-sensitive host mimic compounds. Such studies could be important to gaining an understanding of how each symbiotic partner uses QS regulation to integrate and optimize their interactions with the other partner.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *S. meliloti* strains were grown in a TA (Chen et al. 2000) or M9 minimal medium (Meade and Signer 1977) supplemented with sucrose (0.2% wt/wt) at 30°C, unless noted otherwise. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. For proteomic assays, TA medium was buff-

ered to pH 6.5 with 10 mM morpholinepropanesulfonic acid (MES) to minimize alkaline hydrolysis of AHLs. When needed, antibiotics were added to the following final concentrations: streptomycin at 500 µg/ml, tetracycline at 5 to 10 µg/ml, and neomycin at 50 to 200 µg/ml. The *aiiA* gene encoding the AHL lactonase from *Bacillus* sp.240B1 strain was present as an *Eco*RI fragment in the cosmid vector pLARF3 (Dong et al. 2000).

For AHL extraction and proteomic experiments, two independent cultures of *S. meliloti* strain 1021 pLARF3*aiiA* and 1021 pLARF3 were started directly from glycerol stocks and grown in 5 ml of MES-buffered TA with tetracycline overnight at 30°C with shaking (225 rpm) to an optical density at 600 nm (OD₆₀₀) of 2.0. Cells then were washed twice with sterile water and inoculated into 400 ml of MES-buffered TA in 2.8-liter wide-bottom flasks. Bacterial cultures were grown at 30°C with shaking and harvested for proteome studies when cultures entered early log (OD₆₀₀ = 0.1) and early stationary (OD₆₀₀ = 1.5) growth phases. The pH of the stationary phase cultures was between pH 6.5 and 6.8.

Strain construction.

The plasmids pE7-R3 (= pLARF3 *aiiA*) and pLARF3 were mobilized into *S. meliloti* 1021 by triparental mating with pRK2013 as a helper plasmid (Glazebrook and Walker 1991). Transconjugants were selected on M9 sucrose agar plates containing tetracycline and streptomycin. Isolates from the pE7-R3 mating were screened by polymerase chain reaction (PCR) for the presence of *aiiA* (*aiiA* 2L2: TGTATGTTGGATCATT CGTCTGT; *aiiA* 148R1: CAG GACCGGATTTTCTGTC). Fifteen isolates from each mating were further screened for AHL degradation activity *in vivo*. Cultures of 1021 transconjugants carrying pE7-R3 or pLARF3 were incubated overnight in a TA medium with 15 µM C₄-HSL at 30°C, and the degradation of C₄-HSL by AiiA then was estimated using a modified *C. violaceum* CV026 bioassay (McClellan et al. 1997; Teplitski et al. 2000).

The effect of *aiiA* on the expression of four genes identified by proteomic analysis was tested by construction of the corresponding chromosomal promoter-*uidA* (β-glucuronidase) reporter fusions. These reporters were constructed using the pVMG vector, which cannot replicate in *Sinorhizobium* spp. (Gao et al. 2005). All constructs were designed in such a way that the integration of the plasmid disrupted the chromosomally encoded gene. To construct pMG3, a 413-bp fragment in the middle of *Smc00154* (*gor*) was amplified by Pfu polymerase-catalyzed PCR using primers TTCGCGGCTGTGTAG CGAAGAA and ATCGCGGATCCCTCTATGTTTATGCC. Likewise, to construct pMG11, a 489-bp fragment of *Smc1874* (*ftsZ1*) was amplified with ATCGCGGATCCTATTTCCGATC TTCAC and TACGCGGATCCGACGACGGAAACCCC. This construct was designed so that integration of the reporter into the *ftsZ1* gene was downstream of the conserved *ftsZ* sequence as defined by Margolin and Long (1994). Because pVMG contains three STOP codons upstream of *uidA*, we expect that a truncated, but partially functional FtsZ protein can be made by the resulting reporter. To construct pMG10, a 295-bp fragment of *Smc03930* (*soxG2*) was amplified with ATCGCGGATCCG TCATGTTCTCTGGC and TACGCGGATCCAGACGCCCA ACACC. To construct pMG12, a 383-bp fragment of *Smc04040* was amplified using ATCGCGGATCCGTCATTTGATTTTTC and ATCGCGGATCCGGATATTGCGCAGA. The PCR-amplified fragments were digested with *Bam*HI and then cloned into the pVMG vector treated with *Bam*HI and calf intestinal phosphatase. The resulting plasmids were mobilized into *S. meliloti* strains (carrying pE7-R3 or pLARF3) by triparental mating using pRK600 as a helper, and selected on M9 with

sucrose, streptomycin, neomycin, and tetracycline. MG94 pLARF3 was constructed by mating pLARF3 into the MG94 strain by triparental mating using pRK600. The location and the orientation of the insertions were verified by PCR using a second pair of primers, one binding within the *uidA* gene and the other binding to an appropriate chromosomal sequence upstream of the insertion site. The presence of *aiiA* in the final constructs was confirmed by PCR as above.

Quantitative assay of β-glucuronidase reporter gene fusions.

β-Glucuronidase activity was measured using a fluorescence assay essentially as described (Swanson et al. 1993). Briefly, duplicate cultures of the reporter strains were grown overnight in TA at 30°C with shaking to OD₆₀₀ 1.5 to 2.0. Cells then were washed with sterile water and diluted 1:100 into fresh M9-sucrose medium and grown until the OD doubled (approximately 6 h). Each culture (100 µl) was washed twice with sterile water, transferred to microtiter plates, and mixed with an equal volume of 2× MUG buffer (50 mM sodium phosphate, pH7.0; 10 mM β-mercaptoethanol; 0.1% Triton x-100; 0.5% sodium dodecyl sulfate [SDS]; and 1 mM 4-methyl-umbelliferyl-glucuronide). The reaction mixes were incubated at 37°C and fluorescence was measured every 30 min by a Victor3 microtiter plate reader (Perkin-Elmer, Shelton, CT, U.S.A.) using an ex365 nm/em 455 nm filter set.

AHL purification and bioassay.

Two liters of culture filtrate (pH < 7.0) from early stationary phase (OD₆₀₀ = 1.5) cultures of 1021 pLADR3*aiiA* and 1021 pLARF3 were extracted twice with 500 ml of ethyl acetate acidified with glacial acetic acid at 2 ml/liter. Extracts were dried by rotary evaporation over a 40°C water bath, then dissolved in 1 ml of acetonitrile, with the residue further dissolved in 1 ml of 1:1 acetonitrile/water. These solutions were combined, centrifuged, and the supernatants injected onto a column (25-by-1 cm C18 Partisil 10 ODS-3; Alltech, Deerfield, IL, U.S.A.) equilibrated with 90:10 water/acetonitrile for separation by reverse-phase HPLC. Samples were eluted at 2 ml/min with a linear water-acetonitrile gradient to reach 90% acetonitrile after 55 min and 100% acetonitrile after 65 min, then 20 min with 100% acetonitrile. Aliquots (90 µl for 1021 pLADR3*aiiA* and 30 µl for 1021 pLARF3) of each HPLC fraction were tested for the presence of QS-active compounds using *E. coli*-based reporters (pSB1075, a LasR-based reporter and pSB401, a LuxR-based reporter). Bioassays with *E. coli* reporters were carried out as described earlier (Gao et al. 2005; Winson et al. 1998). Those fractions with biological activity were subjected to GC/MS and ESI/MS/MS analysis to identify the kinds and amounts of AHLs as described earlier (Marketon et al. 2002; Teplitski et al. 2003). For TLC analysis, 1021 pLADR3*aiiA* and 1021 pLARF3 were grown in 7 ml of TA to OD₆₀₀ = 2.0, then extracted twice with 3 ml of acidified ethyl acetate. The extracts then were evaporated to dryness, resuspended in 10 µl of methanol, separated by TLC, and the plates examined for compounds able to activate the *A. tumefaciens* NTL4 pZLR4 reporter, as described (Gao et al. 2005; Shaw et al. 1997).

Protein extraction, separation, and quantification.

Proteins were extracted from freeze-dried cells of replicate independent cultures of 1021 pLARF3*aiiA* and 1021 pLARF3. Protein concentrations then were normalized and the samples were subjected to two-dimensional (2-D) gel separation as previously described (Chen et al. 2000; Natera et al. 2000). Aliquots containing 1 mg of solubilized cellular protein were cup loaded onto the acidic end of each immobilized pH gradient strip with pH ranges of 4.0 to 5.0, 4.5 to 5.5, 5.0 to 6.0, and 5.5

to 6.7 and run for 295 kV · h. Chromatography in the second dimension was performed on precast 12 to 14% SDS-polyacrylamide gel electrophoresis gels (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Gels were stained with Coomassie brilliant blue (CBB) in a step-wise colloidal staining procedure (Neuhoff et al. 1988). The gels were scanned using a UMAX Astra 2400 scanner at a resolution of 600 dpi, black and white, and the image was stored as a TIF file. The scanned image of the gel was analyzed using ImageMaster software (GE Healthcare). The percent volume was calculated for each protein spot and used for protein spot quantification. Gels of four biological repeats were analyzed for each sample. A linear restricted maximum likelihood analysis using GenStat 7 (VSN International, Ltd., Hemel Hempstead, U.K.) was performed to test for statistical significance of differences in protein accumulation (using $P < 0.05$) between the different treatments and controls.

Protein identification.

Proteins were identified by tryptic digestion of the polypeptides isolated from the CBB-stained 2-D gels followed by peptide mass fingerprinting (PMF) with matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectrometry performed on a Micromass ToFSpec 2E time of flight mass spectrometer (Waters Corporation, Milford, MA, U.S.A.) at the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia), as described (Chen et al. 2003). Peptide mass fingerprints were identified by comparison against the *S. meliloti* 1021 proteomic database with Mascot software (Micromass; Waters Corporation) (Weiller et al. 2001). The search parameters for MALDI-TOF mass spectra included a maximum of 1 miss-cleavage, a peptide tolerance of 100 ppm, allowance of oxidation (M) modifications, and a peptide charge of 1+, 2+, and 3+. PMF matches were rated on the scale of Weiller and associates (2001) based on the following criteria: i) a minimum of four peptides matched within 100 ppm to the theoretical mass of the polypeptide without any protein modification, ii) matched peptides collectively compose >30% of the entire protein, iii) there is a good agreement between the actual and predicted molecular mass and pI, and iv) there were no other polypeptide matches. For smaller proteins (<15 kDa), three precisely matching nonmodified peptides were allowed, but a higher coverage (>40%) was considered. For proteins larger than 60 kDa, more than six peptides were required, but the coverage requirement was relaxed to 20%. A confidence rating of 3 was assigned if all criteria were met and a score of 2 was assigned if one criterion was not met. Predictions of protein functions were made based on the web-based Kyoto Encyclopedia of Genes and Genomes.

Rate or efficiency of nodule initiation.

Nodule initiation tests were conducted essentially as described (Gao et al. 2005). Surface-sterilized seed of *M. truncatula* A17 from U. Mathesius at the Australian National University were germinated on 2% agar containing one-quarter-strength N-free Hoagland's. Two-day-old seedlings were transferred to growth pouches (Mega International, Minneapolis, MN, U.S.A.) wetted with 8.5 ml of the sterile Hoagland's solution. After overnight growth, each seedling was inoculated with 200 µl of *S. meliloti* 1021, MG75 (1021 *sinI*), 1021 pLAFR3*aiiA*, or *S. meliloti* 1021 pLAFR3. The inocula were obtained by suspending cells growing for 1 to 2 days on TA agar, centrifuging and resuspending in fresh TA medium, shake culturing for 2 to 3 h, then washing and resuspending the cells in the Hoagland's solution at 0.1 OD₆₀₀. The position of the primary root tip at the time of inoculation (RT1) was marked on the pouch face for each seedling, as was the posi-

tion of the root tip 24 h later (RT2). After 3 weeks, the number of nodules that developed on primary roots above RT1, between RT1 and RT2, and below RT2 was counted for those seedlings with primary roots extending to the bottom of the pouch.

Effects of AiiA on the activity of AHL mimics from *M. truncatula*.

E. coli DH5α pDSK*aiiA* and a control strain, DH5α pDSK, were shake cultured in 1.5 ml of LB broth with kanamycin at 50 µg/ml and isopropyl-β-D-thiogalactoside at 20 µg/ml at 37°C to OD₆₀₀ = 1.7. Cells were harvested by centrifugation and resuspended in 3 ml of phosphate buffer (pH 7.0). Cells were sonically disrupted with 10 5-s pulses at 0°C, then centrifuged to remove cell debris. AiiA activity in the supernatants was verified by adding 3-oxo-C₆-HSL at various concentrations to wells containing 80 µl of the cell extract, incubating the mix at 30°C for 3 h, drying, and assaying with the *E. coli* pSB401 (LuxR) reporter (Winson et al. 1998). The same amount of crude AiiA extract was used to treat dried samples of plant compounds present in two separate HPLC fractions. To obtain the HPLC fractions, freeze-dried, aseptically produced root exudate from *M. truncatula* seedlings first was extracted with ethyl acetate, then the residue was extracted with methanol, and the remaining material was extracted with 1:1 methanol/water as described (Gao et al. 2003). The compounds solubilized by a second extraction with methanol/water were fractionated by reverse-phase HPLC as described (Gao et al. 2003). Samples equivalent to exudate from 50 seedlings were treated for 3 h with the crude AiiA or vector control cell preparations and then assayed for activity with the LuxR reporter.

ACKNOWLEDGMENTS

We thank D. Li for help with statistical analysis, J. Streeter for help with acetylene reduction assays, D. Fulton for help with electron microscopy, Ulrike Mathesius for *M. truncatula* seed, and D. Gage for advice and micrographs. The plasmids pE7-R3, pDSK*aiiA*, and pDSK519 were generous gifts from L.-H. Zhang. Support for these studies was provided by United States Department of Agriculture National Research Institute grants 2002-353991559 (to W. D. Bauer, J. B. Robinson, and B. G. Rolfe) and 2003-01177 (to W. D. Bauer, J. B. Robinson, and B. G. Rolfe), University of Florida School of Natural Resources and Environment grant (to M. Teplitski, M. Gao, and W. D. Bauer) and National Science Foundation Postdoctoral Fellowship DBI-0102085 (to M. Connolly). A. Eberhard thanks S. Winans for hospitality and partial support under grant GM042893-14. W. D. Bauer thanks D. A. Phillips for facilities, advice, encouragement, and partial support under NSF DEB-0120169. Partial support for salary and supplies was provided to W. D. Bauer by the Ohio Agricultural Research and Development Center. Horticulture and Crop Science manuscript HCS 07-02.

LITERATURE CITED

- Agafonov, D. E., Kolb, V. A., Nazimov, I. V., and Spirin, A. S. 1999. A protein residing at the subunit interface of the bacterial ribosome. *Proc. Natl. Acad. Sci. U.S.A.* 96:12345-12349.
- Alfano, J. R., and Kahn, M. L. 1993. Isolation and characterization of a gene coding for a novel aspartate aminotransferase from *Rhizobium meliloti*. *J. Bacteriol.* 175:4186-4196.
- Arevalo-Ferro, C., Hentzer, M., Reil, G., Gorg, A., Kjelleberg, S., Givskov, M., Riedel, K., and Eberl, L. 2003. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Environ. Microbiol.* 5:1350-1369.
- Bae, Y. M., and Crawford, I. P. 1990. The *Rhizobium meliloti* *trpE(G)* gene is regulated by attenuation, and its product, anthranilate synthase, is regulated by feedback inhibition. *J. Bacteriol.* 172:3318-3327.
- Barsomian, G. D., Urzainqui, A., Lohman, K., and Walker, G. C. 1992. *Rhizobium meliloti* mutants unable to synthesize anthranilate display a novel symbiotic phenotype. *J. Bacteriol.* 174:4416-4426.
- Bassler, B. L., and Losick, R. 2006. Bacterially speaking. *Cell* 125:237-46.
- Bhuvaneshwari, T. V., Bhagwat, A., and Bauer, W. D. 1981. Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. *Plant Physiol.* 68:1144-1149.

- Bitner, A. N., and Oke, V. 2006. Multiple *groESL* operons are not key targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*. J. Bacteriol. 188:3507-3515.
- Brencic, A., and Winans, S. C. 2005. Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. Microbiol. Mol. Biol. Rev. 69:155-194.
- Cantero, L., Palacios, J. M., Ruiz-Argueso, T., and Imperial, J. 2006. Proteomic analysis of quorum sensing in *Rhizobium leguminosarum* biovar *viciae* UPM791. Proteomics 6 (Suppl. 1):S97-S106.
- Cha, C., Gao, P., Chen, Y. C., Shaw, P. D., and Farrand, S. K. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. 11:1119-1129.
- Chen, H., Higgins, J., Kondorosi, E., Kondorosi, A., Djordjevic, M. A., Weinman, J. J., and Rolfe, B. G. 2000. Identification of *nolR*-regulated proteins in *Sinorhizobium meliloti* using proteome analysis. Electrophoresis 21:3823-3832.
- Chen, H., Teplitski, M., Gao, M., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. 2003. Proteomic analysis of wildtype *Sinorhizobium meliloti* responses to *N*-acyl homoserine lactone quorum sensing signals. J. Bacteriol. 185:5029-5036.
- Daniels, R., De Vos, D. E., Desair, J., Raedschelders, G., Luyten, E., Rosemeyer, V., Verreth, C., Schoeters, E., Vanderleyden, J., and Michiels, J. 2002. The *cin* quorum sensing locus of *Rhizobium elii* CNPAF512 affects growth and symbiotic nitrogen fixation. J. Biol. Chem. 277:462-468.
- Daniels, R., Vanderleyden, J., and Michiels, J. 2004. Quorum sensing and swarming migration in bacteria. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 28:261-289.
- Degrassi, G., Devescovi, G., Solis, R., Steindler, L., and Venturi, V. 2007. *Oryza sativa* rice plants contain molecules that activate different quorum-sensing *N*-acyl homoserine lactone biosensors and are sensitive to the specific AiiA lactonase. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 269:213-220.
- Dong, Y. H., and Zhang, L. H. 2005. Quorum sensing and quorum-quenching enzymes. J. Microbiol. (Spec. No.) 43:101-109.
- Dong, Y. H., Xu, J. L., Li, X. Z., and Zhang, L. H. 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acad. Sci. U.S.A. 97:3526-3531.
- Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., and Zhang, L. H. 2001. Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 411:813-817.
- Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L., and Zhang, L. H. 2002. Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. Appl. Environ. Microbiol. 68:1754-1759.
- Dong, Y. H., Zhang, X. F., Xu, J. L., and Zhang, L. H. 2004. Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. Appl. Environ. Microbiol. 70:954-960.
- Dudler, R., and Eberl, L. 2006. Interactions between bacteria and eukaryotes via small molecules. Curr. Opin. Biotechnol. 17:268-273.
- Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Neilson, K. H., and Oppenheimer, N. J. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry 20:2444-2449.
- Galibert, F., Finan, T. M., Long, S. R., Puhle, R. A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dreano, S., Federspiel, N. A., Fisher, R. F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kahn, M. L., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M. C., Pohl, T. M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F. J., Weidne, R. S., Wells, D. H., Wong, K., Yeh, K. C., and Batut, J. 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science 293:668-672.
- Gao, M., Teplitski, M., Robinson, J. B., and Bauer, W. D. 2003. Production of substances by *Mycobacterium tuberculosis* that affect bacterial quorum sensing. Mol. Plant-Microbe Interact. 16:827-834.
- Gao, M., Chen, H., Eberhard, A., Gronquist, M. R., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. 2005. *sinI*- and *expR*-dependent quorum sensing in *Sinorhizobium meliloti*. J. Bacteriol. 187:7931-7944.
- Givskov, M., Nys, R. D., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P. D., and Kjelleberg, S. 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. J. Bacteriol. 178:6618-6622.
- Glazebrook, J., and Walker, G. C. 1991. Genetic techniques in *Rhizobium meliloti*. Methods Enzymol. 204:398-418.
- Gonzalez, J. E., and Marketon, M. M. 2003. Quorum sensing in nitrogen-fixing rhizobia. Microbiol. Mol. Biol. Rev. 67:574-592.
- Gray, K. M., Pearson, J. P., Downie, J. A., Boboye, B. E., and Greenberg, E. P. 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: Autoinduction of a stationary phase and rhizosphere-expressed genes. J. Bacteriol. 178:372-376.
- Grundy, F. J., and Henkin, T. M. 2004. Kinetic analysis of tRNA-directed transcription antitermination of the *Bacillus subtilis* *glyQS* gene in vitro. J. Bacteriol. 186:5392-5399.
- Haynes, J. G., Czymmek, K. J., Carlson, C. A., Veereshlingam, H., Dickstein, R., and Sherrier, D. J. 2004. Rapid analysis of legume root nodule development using confocal microscopy. New Phytol. 163:661-668.
- Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J. W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoiby, N., and Givskov, M. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO (Eur. Mol. Biol. Organ.) J. 22:3803-3815.
- Hoang, H. H., Becker, A., and Gonzalez, J. E. 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. J. Bacteriol. 186:5460-5472.
- Kabir, M.S., Sagara, T., Oshima, T., Kawagoe, Y., Mori, H., Tsunedomi, R., and Yamada, M. 2004. Effects of mutations in the *rpoS* gene on cell viability and global gene expression under nitrogen starvation in *Escherichia coli*. Microbiology 150:2543-2553.
- Kainou, T., Okada, K., Suzuki, K., Nakagawa, T., Matsuda, H., and Kawamukai, M. 2001. Dimer formation of octaprenyl-diphosphate synthase (IspB) is essential for chain length determination of ubiquinone. J. Biol. Chem. 276:7876-7883.
- Keller, L., and Surette, M. G. 2006. Communication in bacteria: An ecological and evolutionary perspective. Nat. Rev. Microbiol. 4:249-258.
- Li, K., Hein, S., Zou, W., and Klug, G. 2004. The glutathione-glutaredoxin system in *Rhodobacter capsulatus*: Part of a complex regulatory network controlling defense against oxidative stress. J. Bacteriol. 186:6800-6808.
- Loh, J., Pierson, E. A., Pierson, L. S., 3rd, Stacey, G., and Chatterjee, A. 2002. Quorum sensing in plant-associated bacteria. Curr. Opin. Plant Biol. 5:285-290.
- Long, S., McCune, S., and Walker, G. C. 1988. Symbiotic loci of *Rhizobium meliloti* identified by random *TnphoA* mutagenesis. J. Bacteriol. 170:4257-4265.
- Lupp, C., and Ruby, E. G. 2005. *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. J. Bacteriol. 187:3620-3629.
- Lupp, C., Urbanowski, M., Greenberg, E. P., and Ruby, E. G. 2003. The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol. Microbiol. 50:319-331.
- Maki, Y., Yoshida, H., and Wada, A. 2000. Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase *Escherichia coli*. Genes Cells 5:965-974.
- Margolin, W. 2003. Bacterial division: The fellowship of the ring. Curr. Biol. 13:R16-18.
- Margolin, W., and Long, S. R. 1994. *Rhizobium meliloti* contains a novel second homolog of the cell division gene *ftsZ*. J. Bacteriol. 176:2033-2043.
- Margolin, W., Corbo, J. C., and Long, S. R. 1991. Cloning and characterization of a *Rhizobium meliloti* homolog of the *Escherichia coli* cell division gene *ftsZ*. J. Bacteriol. 173:5822-5830.
- Marketon, M. M., and Gonzalez, J. E. 2002. Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. J. Bacteriol. 184:3466-3475.
- Marketon, M. M., Gronquist, M. R., Eberhard, A., and Gonzalez, J. E. 2002. Characterization of the *Sinorhizobium meliloti* *sinR/sinI* locus and the production of novel *N*-acyl homoserine lactones. J. Bacteriol. 184:5686-5695.
- McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S., and Williams, P. 1997. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology 143:3703-3711.
- Meade, H. M., and Signer, E. R. 1977. Genetic mapping of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. 74:2076-2078.
- Mitsui, H., Sato, T., Sato, Y., Ito, N., and Minamisawa, K. 2004. *Sinorhizobium meliloti* RpoH1 is required for effective nitrogen-fixing symbiosis with alfalfa. Mol. Genet. Genomics. 271:416-425.
- Molina, L., Rezzonico, F., Defago, G., and Duffy, B. 2005. Autoinduction in *Erwinia amylovora*: Evidence of an acyl-homoserine lactone signal in the fire blight pathogen. J. Bacteriol. 187:3206-3213.

- Morita, T., Maki, K., and Aiba, H. 2005. RNase E-based ribonucleoprotein complexes: Mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev.* 19:2176-2186.
- Natera, S. H. A., Guerreiro, N., and Djordjevic, M. A. 2000. Proteome analysis of differentially displayed proteins as a tool for the investigation of symbiosis. *Mol. Plant-Microbe Interact.* 13:995-1009.
- Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9:255-262.
- Pearson, J. P., Van Delden, C., and Iglewski, B. H. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* 181:1203-1210.
- Pellock, B. J., Teplitski, M., Boinay, R. P., Bauer, W. D., and Walker, G. C. 2002. A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J. Bacteriol.* 184:5067-5076.
- Richardson, L. V., and Richardson, J. P. 2005. Identification of a structural element that is essential for two functions of transcription factor NusG. *Biochim. Biophys. Acta* 1729:135-140.
- Sanchez-Contreras, M., Bauer, W. D., Gao, M., Robinson, J. B., and Downie, A. J. 2007. Quorum-sensing regulation in rhizobia and its role in symbiotic interactions with legumes. *Phil. Trans. R. Soc.* doi:10.1098/rstb.2007.2041. Published online.
- Schuster, M., Lostroh, C. P., Ogi, T., and Greenberg, E. P. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: A transcriptome analysis. *J. Bacteriol.* 185:2066-2079.
- Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Jr., Rinehart, K. L., and Farrand, S. K. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. U.S.A.* 94:6036-6041.
- Smadja, B., Latour, X., Faure, D., Chevalier, S., Dessaux, Y., and Orange, N. 2004. Involvement of *N*-acylhomoserine lactones throughout plant infection by *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium atrosepticum*). *Mol. Plant-Microbe Interact.* 17:1269-1278.
- Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* 169:5789-5794.
- Steidle, A., Sigl, K., Schuhegger, R., Ihring, A., Schmid, M., Gantner, S., Stoffels, M., Riedel, K., Givskov, M., Hartmann, A., Langebartels, C., and Eberl, L. 2001. Visualization of *N*-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. *Appl. Environ. Microbiol.* 67:5761-5770.
- Swanson, J. A., Mulligan, J. T., and Long, S. R. 1993. Regulation of *syrM* and *nodD3* in *Rhizobium meliloti*. *Genetics* 134:435-444.
- Teplitski, M., Robinson, J. B., and Bauer, W. D. 2000. Plants secrete substances that mimic bacterial *N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol. Plant-Microbe Interact.* 13:637-648.
- Teplitski, M., Eberhard, A., Gronquist, M., Gao, M., Robinson, J. B., and Bauer, W. D. 2003. Chemical identification of *N*-acyl homoserine lactone quorum sensing signals produced by *Sinorhizobium meliloti* strains in defined medium. *Arch. Microbiol.* 180:494-497.
- Teplitski, M., Chen, H., Rajamani, S., Gao, M., Merighi, M., Sayre, R. T., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. 2004. *Chlamydomonas reinhardtii* secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. *Plant Physiol.* 134:137-146.
- Thorne, S. H., and Williams, H. D. 1999. Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. *phaseoli*: Identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary-phase survival. *J. Bacteriol.* 181:981-990.
- Vasil, M. L. 2003. DNA microarrays in analysis of quorum sensing: Strengths and limitations. *J. Bacteriol.* 185:2061-2065.
- von Bodman, S. B., Bauer, W. D., and Coplin, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. *Annu. Rev. Plant Pathol.* 41:455-482.
- Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., and Iglewski, B. H. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.* 185:2080-2095.
- Weiller, G. F., Djordjevic, M. J., Caraux, G., Chen, H., and Weinman, J. J. 2001. A specialised proteomic database for comparing matrix-assisted laser desorption/ionization-time of flight mass spectrometry data of tryptic peptides with corresponding sequence database segments. *Proteomics* 1:1489-1494.
- Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J., and Salmond, G. P. 2001. Quorum-sensing in gram-negative bacteria. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 25:365-404.
- Whiteley, M., Lee, K. M., and Greenberg, E. P. 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 96:13904-13909.
- Wilkinson, A., Danino, V., Wisniewski-Dye, F., Lithgow, J. K., and Downie, J. A. 2002. *N*-acyl-homoserine lactone inhibition of rhizobial growth is mediated by two quorum-sensing genes that regulate plasmid transfer. *J. Bacteriol.* 184:4510-4519.
- Winans, S. C. 2002. Bacterial esperanto. *Nat. Struct. Biol.* 9:83-84.
- Winston, M. K., Swift, S., Fish, L., Throup, J. P., Jorgensen, F., Chhabra, S. R., Bycroft, B. W., Williams, P., and Stewart, G. S. 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 163:185-192.
- Wisniewski-Dye, F., and Downie, J. A. 2002. Quorum-sensing in *Rhizobium*. *Antonie Leeuwenhoek* 81:397-407.
- Wopperer, J., Cardona, S. T., Huber, B., Jacobi, C. A., Valvano, M. A., and Eberl, L. 2006. A quorum-quenching approach to investigate the conservation of quorum-sensing-regulated functions within the *Burkholderia cepacia* complex. *Appl. Environ. Microbiol.* 72:1579-1587.
- Zheng, H., Zhong, Z., Lai, X., Chen, W. X., Li, S., and Zhu, J. 2006. A LuxR/LuxI-type quorum-sensing system in a plant bacterium, *Mesorhizobium tianshanense*, controls symbiotic nodulation. *J. Bacteriol.* 188:1943-1949.
- Zhu, J., and Winans, S. C. 1999. Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters in vitro and decreases TraR turnover rates in whole cells. *Proc. Natl. Acad. Sci. U.S.A.* 96:4832-4837.
- Zhu, J., and Winans, S. C. 2001. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc. Natl. Acad. Sci. U.S.A.* 98:1507-1512.