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Quorum-sensing regulation in rhizobia and its role in symbiotic interactions with legumes

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Legume-nodulating bacteria (rhizobia) usually produce *N*-acyl homoserine lactones, which regulate the induction of gene expression in a quorum-sensing (or population-density)-dependent manner. There is significant diversity in the types of quorum-sensing regulatory systems that are present in different rhizobia and no two independent isolates worked on in detail have the same complement of quorum-sensing genes. The genes regulated by quorum sensing appear to be rather diverse and many are associated with adaptive aspects of physiology that are probably important in the rhizosphere. It is evident that some aspects of rhizobial physiology related to the interaction between rhizobia and legumes are influenced by quorum sensing. However, it also appears that the legumes play an active role, both in terms of interfering with the rhizobial quorum-sensing systems and responding to the signalling molecules made by the bacteria. In this article, we review the diversity of quorum-sensing regulation in rhizobia and the potential role of legumes in influencing and responding to this signalling system.

Keywords: *Rhizobium*; *Sinorhizobium*; *Bradyrhizobium*; homoserine lactone; nodulation; rhizosphere

1. INTRODUCTION

(a) *Quorum sensing in plant growth-promoting bacteria*

As described in the accompanying reviews, quorum sensing is classically a population-density-dependent signalling mechanism that allows bacteria to assess the size of their population and to behave coordinately (Dong *et al.* 2007; White & Winans 2007; Williams *et al.* 2007; Barnard *et al.* 2007; Bjarnsholt & Givskov 2007). Often the gene encoding the enzyme that synthesizes the signalling molecule is activated by quorum sensing, leading to the term 'autoinducer'. In this review, we will focus mainly on quorum sensing mediated by *N*-acyl homoserine lactones (AHLs). Quorum-regulated genes identified to date are usually involved in adaptive changes in physiology of the bacterial population enabling them to modify aspects of their behaviour that are best undertaken when there are several other related bacteria nearby. Some of the adaptations include light production, antibiotic production and conjugation, but quorum-sensing-mediated gene expression can allow complex interactions between bacteria of different species and also with some eukaryotic organisms with which they can closely interact.

Quorum sensing is common among Gram-negative plant-associated bacteria (Pierson *et al.* 1998*b*; Parsek & Greenberg 2000; Whitehead *et al.* 2001*b*; Loh *et al.* 2002*c*; Dong *et al.* 2007; White & Winans 2007; Barnard *et al.* 2007; Bjarnsholt & Givskov 2007) and regulates several physiological traits associated with plant–bacterial interactions. AHL production by plant-associated bacteria, belonging to the genera *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Pantoea*, *Erwinia*, *Pseudomonas* and *Xanthomonas*, was assessed and at least one isolate from each genera displayed AHL production (Cha *et al.* 1998). Most rhizobia tested were AHL producers, including isolates from *Rhizobium fredii*, *Rhizobium leguminosarum* bv. *viciae*, bv. *phaseoli* and bv. *trifolii* and *Sinorhizobium meliloti*. Different bacterial species can produce the same AHLs or AHLs with similar structures and properties suggesting that crosstalk between populations occurs and it is evident that quorum sensing via AHLs is more common among plant-associated bacteria than the general population of soil bacteria (Elasri *et al.* 2001; d'Angelo-Picard *et al.* 2005).

There are four broad classes of plant growth-promoting bacteria in soil: (i) those which enter into symbioses including the rhizobial–legume nitrogen-fixing symbioses (Wisniewski-Dye & Downie 2002; Gonzalez & Marketon 2003), (ii) the associative interactions in which bacteria such as pseudomonads suppress the growth of deleterious micro-organisms (Lugtenberg *et al.* 2001), (iii) the root-associated bacteria which can provide nutrients and/or growth-stimulating hormones as seen with species of *Azospirillum* and related bacteria (Dobbelaere *et al.* 2003), and (iv) bacteria which degrade environmental pollutants (Kuiper *et al.* 2004).

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One contribution of 12 to a Theme Issue 'Bacterial conversations: talking, listening and eavesdropping'.

Both the legume-nodulating rhizobia and the pseudomonads suppressing the growth of other microorganisms use quorum-sensing gene regulation in relation to their stimulation of plant growth (Whitehead *et al.* 2001*b*; Wisniewski-Dye & Downie 2002; Gonzalez & Marketon 2003). In pseudomonads, one of the key biocontrol traits is the production of phenazine antibiotics, the expression of which is under quorum-sensing control via the AHL synthase encoded by *phzI* and the regulator encoded by *phzR* (Pierson & Pierson 1996; Pierson *et al.* 1998*b*; Chancey *et al.* 1999; Chin-A-Woeng *et al.* 2003). The production of these antibiotics also involves other regulators such as GacA, GacS, PsrA and RpeA (Lugtenberg *et al.* 2001; Whistler & Pierson 2003; Girard *et al.* 2006). It can also be negatively affected by the production of the fungal metabolite fusaric acid (van Rij *et al.* 2005) illustrating the intimate interactions between these bacteria and the fungal pathogens they suppress. In addition to influencing the growth of other microorganisms, plant rhizosphere bacteria can activate the systemic plant defence system thereby conferring resistance to fungal pathogens. A strain of *Serratia liquefaciens* on the roots of tomato induced systemic resistance against the fungal leaf pathogen *Alternaria alternata* and this required the production of AHLs (Schuhegger *et al.* 2006). An important aspect of the ability of such bacteria to enhance plant growth is their ability to colonize roots and essentially form biofilms on the surface of roots, and this can be influenced by quorum-sensing regulation (Steidle *et al.* 2002; Ramey *et al.* 2004; Dubern *et al.* 2006).

Legume-nodulating rhizobia have several different quorum-sensing regulatory systems affecting plasmid transfer, symbiotic interactions, surface polysaccharide, growth inhibition and stationary-phase adaptation (Wisniewski-Dye & Downie 2002; Gonzalez & Marketon 2003). In this review, we will focus on the quorum-sensing regulatory systems that have been identified in a variety of different rhizobia. Most rhizobia tested were AHL producers, including isolates from *R. fredii*, *R. leguminosarum* bv. *viciae*, bv. *phaseoli* and bv. *trifolii* and *S. meliloti*. When compared with other genera, rhizobia produced the greatest diversity of AHLs, some of which were predicted to have very long acyl side chains (Cha *et al.* 1998; Brelles-Marino & Bedmar 2001). Where possible, we will relate the AHLs to genes and phenotypes that have been shown to be regulated by quorum sensing. We will also discuss how quorum-sensing regulation can directly or indirectly affect the interaction between rhizobia and the leguminous plants they nodulate.

(b) *Acyl homoserine lactone synthases and regulators*

There are two known protein families that have been shown to catalyse the synthesis of AHLs. The first group, and most widespread, is the LuxI family. These enzymes catalyse the ligation of homoserine from *S*-adenosylmethionine with an acylated acyl-carrier protein yielding acyl-homoserine lactone (More *et al.* 1996; Schaefer *et al.* 1996; Fuqua *et al.* 2001; Withers *et al.* 2001). A second set of AHL-biosynthetic proteins, the LuxM family, has been identified in *Vibrio harveyii* spp. (Bassler *et al.* 1994). AinS from *Photobacterium* (*Vibrio*) *fisheri* and VanM from *Vibrio anguillarum* are homologous to LuxM (Gilson *et al.* 1995; Milton *et al.* 2001), but no similar genes have been identified in rhizobia. All the AHL-response regulators identified to date in rhizobia belong to the LuxR protein family.

In many bacteria, quorum-sensing regulated genes identified to date are genes involved in bacterial interactions with their hosts (Fuqua *et al.* 1994, 2001; Salmond *et al.* 1995; Swift *et al.* 1996; Gray 1997; Fuqua & Greenberg 1998; Parsek & Greenberg 2000; Whitehead *et al.* 2001*a*; Withers *et al.* 2001; Bassler 2002; Lazdunski *et al.* 2004; Waters & Bassler 2005) and this is also true for rhizobia (Wisniewski-Dye & Downie 2002; Gonzalez & Marketon 2003).

(c) *Acyl homoserine lactone reporters*

The identification of AHL-based quorum-sensing systems in diverse bacteria and our understanding of how these systems work have been made possible through the use of AHL bioreporters. A variety of these reporter systems exist, the two most widely used being those in *Chromobacterium violaceum* and *Agrobacterium tumefaciens*, both often being used for visualization of AHLs separated by thin-layer chromatography.

In *C. violaceum*, the quorum-sensing genes *cviI*/*cviR* regulate a number of phenotypes including the production of the purple pigment violacein (McClean *et al.* 1997). A *C. violaceum* AHL-synthase (*cviI*) mutant cannot produce any AHL (and thus no violacein), but can produce only violacein in response to exogenous AHLs or to compounds that mimic AHLs, thus making a good bioreporter which is particularly sensitive to unsubstituted short-chain AHLs (C4 to C8; McClean *et al.* 1997).

The *A. tumefaciens* bioreporter is a non-AHL-producing strain carrying a *traG–lacZ* fusion and the LuxR homologue TraR, which induces *traG* in response to AHLs. This system detects 3-oxo, 3-hydroxy (3-OH) and unsubstituted AHLs with acyl side chains from C6 to C16 in length. This reporter is very sensitive, detecting AHLs at very low concentrations (Shaw *et al.* 1997). A reporter for detecting long-chain AHLs has been developed in *S. meliloti* (Llamas *et al.* 2004). A *sinI::lacZ* fusion integrated in the chromosome of *S. meliloti* lacking a functional *sinI* gene allows the detection of AHLs ranging from C12 to C18. The sensitivity can be increased, without loss of specificity, by overexpressing *sinR*.

Reporters based on luminescence have been constructed in *Escherichia coli* based on plasmids carrying the *lux* genes lacking the gene encoding the LuxI AHL synthase (Winson *et al.* 1998), but carrying genes (*lasR*, *luxR* or *ahyR*) encoding specific AHL receptors that activate the expression of the *luxCDAB genes*. An advantage is that they can be used for *in situ* analysis of gene expression in plants (Teplitski *et al.* 2000).

The use of green fluorescent protein reporter plasmids in non-AHL-producing strains of *Pseudomonas putida* and *S. liquefaciens* has made it possible to visualize AHL-mediated communication in the tomato rhizosphere (Steidle *et al.* 2001). Each of these strains was co-inoculated onto tomato roots with an

AHL-producing bacterial strain. Visualization of fluorescence demonstrated that the bioreporter could sense the AHLs produced by the AHL-producing strain, even on some occasions when the colonies were separated by some distance. These reporter strains also made it possible to monitor AHL production by indigenous bacteria from non-sterile soil in the tomato rhizosphere. Cross-species communication has also been demonstrated between *Pseudomonas* and *Burkholderia* in mixed species biofilms and cultures (McKenney *et al.* 1995; Riedel *et al.* 2001) and between bacteria in a native wheat rhizosphere (Pierson *et al.* 1998*a*).

(d) *Acyl homoserine lactone degradation*

In the soil environment, micro-organisms form communities in which AHL producers interact with bacteria capable of degrading AHLs, and thus interfere with quorum sensing. AHL-degrading enzymes, classified as lactonases or acylases depending on their mode of action, have been found in different species of soil bacteria (Zhang 2003) including bacteria from the *Rhizobiaceae*. In *A. tumefaciens*, *attM* encodes a lactonase that can degrade AHLs (Zhang *et al.* 2002*a*) thereby affecting signal turnover and thus quorum sensing. Gamma-butyrolactones, molecules that are natural substrates of AttM, can interfere with AHL accumulation by inducing the expression of *attM* (Carlier *et al.* 2004) and related genes are found in rhizobia. This implies that rhizobia can degrade AHLs as discussed for agrobacteria in the accompanying paper by Dong *et al.* (2007).

2. QUORUM-SENSING GENES IN RHIZOBIA

One of the central features of quorum sensing in rhizobia is that of diversity, because no two strains that have been analysed in detail have the same complement of quorum-sensing systems, even when such comparisons are limited to two different field isolates from within the same species. This diversity suggests that there may be no unifying paradigm of what is controlled by quorum sensing in the rhizobia. There are clearly quorum-sensing regulators and AHL synthases conserved among different species and genera, but it appears even then that different groups of genes may be controlled differently by orthologous synthases and regulators in different species. The range of genes regulated is just beginning to be identified in cultured rhizobia, but little is known about the range of rhizobial genes regulated by quorum sensing in the soil, rhizosphere or the plant environments. Most of the identified rhizobial quorum-sensing regulation systems seem to be based on AHL synthesis and perception. The LuxS-dependent biosynthetic pathway that leads to autoinducer-2 (AI-2) formation is absent in rhizobia (Winzer *et al.* 2002*a*,*b*). In this section, we will briefly summarize the AHL synthases, and quorum-sensing regulatory genes and the genes regulated in different rhizobia investigated so far. The various genes signalling AHLs and associated phenotypes in different rhizobia are summarized in table 1.

(a) Rhizobium leguminosarum

There are three different biovars of *R. leguminosarum*: one is bv. *viciae*, which nodulates peas, vetch and lentils, the others are bv. *trifolii*, which nodulates clover, and bv. *phaseoli*, which nodulates *Phaseolus* beans. Most research has been done on *R. leguminosarum* bv. *viciae* and thus far four different LuxI-type AHL synthase genes have been identified in different isolates of *R. leguminosarum* bv. *viciae* (Wisniewski-Dye & Downie 2002). Each of these AHL synthase genes has a dedicated regulator encoded by a gene (usually) closely linked to the AHL synthase gene.

(i) cinI *and* cinR

Common to all the analysed strains of *R. leguminosarum* are the *cinI* and *cinR* genes, which are located on the chromosome (http://www.sanger.ac.uk/Projects/R_leguminosarum; Lithgow *et al.* 2000). CinR regulates the expression of *cinI* in response to CinI-made 3-OH-C14:1-HSL and there appears to be co-regulation of adjacent genes (Lithgow *et al.* 2000). Mutations in *cinI* or *cinR* reduce the expression of all other AHL synthase genes (Rodelas *et al.* 1999; Lithgow *et al.* 2000; Wisniewski-Dye *et al.* 2002; Danino *et al.* 2003) and it appears that the *cinI*/*cinR* system acts as an overall switch potentially influencing many aspects of rhizobial physiology (Wisniewski-Dye & Downie 2002). Nevertheless, the growth rate of *cinI* and *cinR* mutants is normal in different laboratory growth media and nodulation is relatively normal (Lithgow *et al.* 2000); therefore, the phenotypic changes are probably subtle, perhaps relating to survival and growth in soil, rhizosphere or *in planta*.

It appears that CinI-made 3-OH-C14:1-HSL plays a role in adaptation to stationary phase. Following starvation by nutrient exhaustion of C or N source, cultures entering stationary phase at high cell densities show no loss of viability over long periods (20–60 days), while cultures entering stationary phase at low cell densities lost viability rapidly. Exogenous 3-OH-C14:1-HSL promoted starvation survival in cultures of *R. leguminosarum* bv. *phaseoli* entering stationary phase at low cell density (Thorne & Williams 1999).

In *R. leguminosarum*, growth inhibition due to a 'small bacteriocin' was found to be due to 3-OH-C14:1-HSL (Gray *et al.* 1996; Schripsema *et al.* 1996), which is produced by CinI and causes growth arrest in susceptible strains (Lithgow *et al.* 2000; Wilkinson *et al.* 2002). 3-OH-C14:1-HSL growth inhibition is maximal when C8-HSL or 3-oxo-C8-HSL is added together with 3-OH-C14:1-HSL (Wilkinson *et al.* 2002). Resistant strains of *R. leguminosarum* and *A. tumefaciens* become susceptible when carrying cloned *bisR* and *traR*. One protein induced by BisR/TraR has been identified, this being the protein translation factor Ef-Ts (Wilkinson *et al.* 2002). This suggests the possibility that the observed growth inhibition may be related to the adaptation to stationary phase.

(ii) raiI *and* raiR

The *raiI* and *raiR* genes are located on a large (non-symbiotic) plasmid in *R. leguminosarum* bv. *phaseoli* strain 8002, but are absent from the genome of

Table 1. Quorum-sensing systems in the *Rhizobiaceae*.

organism and strain	gene and location	signalling molecules	phenotype regulated	references
Agrobacterium tumefaciens	*traR*/*traI* (pTi)	3-oxo-C8-HSL	plasmid transfer	Piper *et al.* (1993); Fuqua *et al.* (1994) and Hwang *et al.* (1995)
Rhizobium leguminosarum				
bv. *viciae*	*cinR*/*cinI* (chromosome)	3-OH-C14:1-HSL	growth inhibition	Lithgow *et al.* (2000)
	rhiR/*rhiI* (pRL1JI)	C6-HSL, C7-HSL, C8-HSL	nodulation efficiency	Cubo *et al.* (1992) and Rodelas *et al.* (1999)
	traR/*traI* (pRL1JI)	3-oxo-C8-HSL, C8-HSL	plasmid transfer	Wilkinson *et al.* (2002); Danino *et al.* (2003)
	expR (chromosome)	unknown	unknown	Wisniewski-Dye & Downie (2002, unpublished)
bv. *phaseoli*	*raiR*/*raiI* (non-symbiotic plasmid)	3-OH-C8-HSL, C8-HSL	unknown	Wisniewski-Dye *et al.* (2002)
Rhizobium etli				
strain CNPAF512	*cinR*/*cinI* (chromosome)	3-OH-(slc)-HSL	nitrogen fixation, symbiosome development, growth inhibition	Daniels *et al.* (2002)
	raiR/*raiI* (chromosome)	short-chain AHLs	nitrogen fixation, growth inhibition	Rosemeyer *et al.* (1998) and Daniels *et al.* (2002)
strain CFN42	*traR*/*traI* (p42a) unknown	3-oxo-C8-HSL, 3-OH-C8-HSL	plasmid transfer, unknown	Tun-Garrido *et al.* (2003)
Sinorhizobium meliloti				
strain Rm1021	*sinR*/*sinI* (chromosome)	3-oxo-C14-HSL, C16:1-HSL, 3-oxo-C16:1-HSL, 3-oxo-C16-HSL, C18-HSL, C12-HSL	EPSII production, swarming	Marketon & Gonzalez (2002); Marketon *et al.* (2002, 2003); Teplitski *et al.* (2003) and Gao *et al.* (2005)
	expR (chromosome)	C16:1-HSL	EPSII production, swarming	Pellock *et al.* (2002); Gao *et al.* (2005)
	mel (putative)	C8-HSL, other short-chain AHLs	unknown	Marketon *et al.* (2002)
strain Rm41	*traR*/*traI* (pRm41a)	3-oxo-C8-HSL	plasmid transfer	Marketon & Gonzalez (2002) and Gonzalez & Marketon (2003)
RU10/406	*visN*/*visR* (chromosome)	unknown effector	motility (flagellar regulon: *fli*, *mot*, *fla* and *che* genes)	Sourjik *et al.* (2000)
Rhizobium sp.				
strain NGR234	*traR*/*traI* (pNGR234a)	3-oxo-C8-HSL	plasmid transfer	He *et al.* (2003)
	unknown genes (chromosome)	other AHLs	growth inhibition	
Bradyrhizobium				
japonicum USDA110	unknown	bradyoxetin	*nod* gene control	Brelles-Marino & Bedmar (2001); Loh *et al.* (2001, 2002*a*,*b*) and Pongsilp *et al.* (2005)
japonicum USDA 110/290 and *B. elkanii*	unknown	several AHLs detected (bioreporter)	unknown	

the sequenced strain of *R. leguminosarum* bv. *viciae* (http://www.sanger.ac.uk/Projects/R_leguminosarum) and appear to be absent from some other analysed strains of *R. leguminosarum* bv. *viciae* (Lithgow *et al.* 2000). As might be expected from their patchy distribution, mutations in these genes do not have much effect on the phenotype of *R. leguminosarum* either during free-living or symbiotic conditions. RaiR regulates the expression of *raiI* in response to the RaiI-made AHLs 3-OH-C8-HSL and C8-HSL (Wisniewski-Dye *et al.* 2002), but other genes regulated by RaiR are yet to be identified.

(iii) rhiI *and* rhiR

The *rhiR* gene was one of the earliest sequenced quorum-sensing regulators in the bacterial kingdom and was originally identified because it was very close to the genes (*nod*) required for legume nodulation and is required for the expression of the *rhiA* gene, which is highly expressed in the rhizosphere (Dibb *et al.* 1984; Economou *et al.* 1989; Cubo *et al.* 1992). RhiR regulates the expression of *rhiI* and *rhiABC* operon in response to RhiI-made C6-HSL, C7-HSL and C8-HSL (Rodelas *et al.* 1999). Mutations in *rhiA* or *rhiR* can cause a significant reduction in nodulation in strains already compromised for nodulation ability (Cubo *et al.* 1992), but although the sequence of the *rhiABC* genes has been known for several years, no function has been demonstrated for the gene products, which show no close similarities to proteins of known function. The observations that the *rhi* genes are closely linked to nodulation and nitrogen fixation genes and that the *rhi* genes are found only in bv. *viciae* but not in other biovars of *R. leguminosarum* suggest that they probably play a role in growth and/or survival in association with specific legume hosts.

(iv) traI *and* traR

TraR induces *traI* in response to TraI-made 3-oxo-C8-HSL. These genes are located on the symbiosis plasmid pRL1JI and together with *bisR* (encoding another LuxR-type regulator) are required to induce the plasmid transfer genes (see below). However, in the sequenced strain *R. leguminosarum* bv. *viciae* 3841, no equivalent genes were found on the symbiosis plasmid (which is called pRL10JI in that strain; http://www.sanger.ac.uk/Projects/R_leguminosarum). Homologues of *traI* and *traR* in strain 3841 are found on pRL7JI and pRL8JI, respectively, but their role in plasmid transfer has not been reported.

(v) *Other LuxR-type regulators*

In addition to the genes described above, there are three other LuxR-type regulators encoded in the genome of *R. leguminosarum* strain 3841. One is ExpR, which is located on the chromosome and is the orthologue of *expR* from *S. meliloti* (see below). Another gene of undefined function is located on the chromosome and yet another is on a plasmid. There are no LuxI-type (or any other candidate) AHL synthase genes associated with these regulators (http://www.sanger.ac.uk/Projects/R_leguminosarum) and their targets have not been identified.

(b) Rhizobium etli

Two different isolates of *Rhizobium etli* have been analysed, strains CNPAF512 and CFN42. It is clear that these are different from each other but share orthologous genes identified in different strains of *R. leguminosarum*.

(i) cinI *and* cinR

These genes in CNPAF512 seem to be very similar to those described above in *R. leguminosarum*, and CinI is responsible for the production of a long-chain AHL, similar to 3-OH-C14:1-HSL and referred to as 3-OH-(slc)- HSL. This AHL can inhibit the growth of a small bacteriocin-sensitive strain of *R. leguminosarum*, thus showing similar properties to 3-OH-C14:1-HSL from *R. leguminosarum* (Daniels *et al.* 2002). However, there is a distinct difference between *R. leguminosarum* bv. *viciae* and *R. etli* because mutations in *cinI* or *cinR* cause an increased lag phase and slower growth of *R. etli*, and this is correlated with abnormal development of nitrogen-fixing bacteria in nodules (Daniels *et al.* 2002). Mutations in the *R. leguminosarum cinRI* locus do not affect nodulation of pea or vetch (Lithgow *et al.* 2000), whereas mutations in the *R. etli cinR* or *cinI* genes (which are 95% identical to those of *R. leguminosarum*) dramatically decreased nitrogen fixation (Daniels *et al.* 2002). Nitrogen fixation decreased by more than 50% in both *cinR* and *cinI* mutants of *R. etli* and decreased even further in a *cinR*, *cinI* double mutant. The *cinI* mutant showed abnormal symbiosome development in nodules, decreased numbers of bacteroids packed within the symbiosome membrane and the *cinI* gene was shown to be expressed during the symbiosis, particularly in infection threads. (Daniels *et al.* 2002). The isolation of AHLs from bacteroids suggests that quorum sensing may play a role in the mature nodule (Daniels *et al.* 2002). At least three compounds, one of them likely to carry a long-chain AHL, were extracted from bacteroids present in nodules of bean inoculated with *R. etli*. Although the role, if any, played by quorum sensing has not been established, it is possible that quorum sensing may be involved in the regulation of processes that prepare bacteria for a return to a free-living state.

Mutation of *cinR* abolished swarming on agar plates, and this swarming could be reactivated by exogenous surfactant (Daniels *et al.* 2004) suggesting that genes regulated by CinR may regulate genes involved in surfactant biosynthesis as has been shown in *S. liquefaciens* (Lindum *et al.* 1998).

(ii) raiI *and* raiR

These genes in CNPAF512 appear to be orthologues of the *R. leguminosarum* bv. *viciae* genes described above (Rosemeyer *et al.* 1998). However, in *R. etli*, mutation of *raiI* caused an increase in the numbers of nodules and a parallel increase in nitrogenase activity. However, no significant net increase in symbiotic nitrogen fixation could be demonstrated based on the analysis of the growth of plants inoculated with the mutant (Rosemeyer *et al.* 1998). Surprisingly, while mutation of *raiI* increased nodulation, mutation of *raiR* had no effect, suggesting that RaiI-made AHLs may be involved in the suppression of nodulation (Daniels *et al.* 2002).

(iii) traI *and* traR

The arrangement of plasmid transfer genes on plasmid p42a of *R. etli* CFN42 (Tun-Garrido *et al.* 2003) and pRL1JI from *R. leguminosarum* bv. *viciae* (Danino *et al.* 2003) seem to be nearly identical and are presumably regulated in the same way (see below).

(c) Sinorhizobium meliloti

Quorum-sensing regulation has been examined in two different strains of *S. meliloti*, but has been much more fully analysed in 1021, the genomically sequenced strain, than in the other (Rm41/AK631).

(i) sinI *and* sinR

The *sinI* gene product produces diverse long-chain AHLs, including C12-HSL, C14-HSL, 3-oxo-C14-HSL, C16-HSL, C16:1-HSL, 3-oxo-C16-HSL, 3-oxo-C16:1-HSL and C18-HSL, with the mixture varying with the culture media used (Marketon *et al.* 2002; Teplitski *et al.* 2003). The specificity and concentration dependence of responses to different SinI-made AHLs have been little explored, but numerous specific responses to particular SinI-made AHLs have been observed (Chen *et al.* 2003; Gao *et al.* 2005). It appears that SinI may be the only AHL synthase in strain 1021. A second AHL synthase that produces short-chain AHLs, including C6-HSL, 3-oxo-C6-HSL and C8-HSL, had been suggested earlier (Marketon & Gonzalez 2002), but this was not confirmed in subsequent studies (Gao *et al.* 2005, in press). Mutation of *sinI* led to changes in the accumulation of over 35 different proteins (Gao *et al.* 2005) and the expression of over 100 genes (Hoang *et al.* 2004) in laboratory cultures. Phenotypically, *sinI* mutants were (i) defective in mucoidy (Hoang *et al.* 2004), (ii) significantly reduced in the rate or efficiency of nodule initiation, and (iii) defective for swarming on agar plates (Gao *et al.* 2005). The swarming could be restored by the addition of 5 nM C16:1-HSL (Gao *et al.* 2005).

s*inR* is adjacent to *sinI* on the chromosome, and SinR induces *sinI* expression in response to SinI-made AHLs, thereby enhancing AHL production 3–10-fold *in vitro* (Marketon *et al.* 2002). Slightly delayed nodulation has been reported for the *sinR* mutant. In microarray studies, three *sinI*-dependent genes had altered expression in the *sinR* mutant, consistent with SinI–AHL-dependent regulation via SinR (Hoang *et al.* 2004). Curiously, the expression of another 23 genes, including symbiotically relevant *fixS1*, was dependent on *sinR*, but independent of *sinI*, raising the question of what signal molecules besides SinI-made AHLs might be responsible for regulating the expression of these genes via SinR (Hoang *et al.* 2004).

(ii) expR

In strain 1021, the *expR* gene encoding a LuxR-type regulator is interrupted by a native insertion sequence (Pellock *et al.* 2002). Spontaneous excision of the insertion sequence generates a functional ExpR that regulates the expression of many genes, including genes in the *exp* operon required for the synthesis of EPSII (Pellock *et al.* 2002; Marketon *et al.* 2003; Hoang *et al.* 2004; Gao *et al.* 2005). EPSII is one of the three *S. meliloti* exopolysaccharides capable of eliciting responses in the host that permit infection (Gonzalez *et al.* 1996). Most wild-type strains of *S. meliloti* tested (Pellock *et al.* 2002) have an uninterrupted *expR* gene. Although the *expR* mutant has no obvious nodulation phenotype, the mutant is significantly altered in the levels of over 50 proteins and the expression of over 80 genes in the laboratory cultures (Gao *et al.* 2005; Hoang *et al.* 2004). These genes and proteins encompass a broad range of functions including central metabolism, regulation, transport, transposases, motility and symbiotically related behaviours. Remarkably, there was virtually no overlap of the 80 *expR*-dependent genes identified by microarray and the 50 *expR*-dependent proteins identified by proteome analysis (Gao *et al.* 2005). The observed lack of overlap may be due to specific culture conditions. However, if culture conditions so strongly influence which sets of genes are significantly affected by quorum sensing, then the really important focus in the future will be to identify the set of genes in rhizobia that are quorum-sensing regulated in natural environments—the soil, rhizosphere and host plant.

(iii) *Other LuxR-type regulators*

In addition to SinR and ExpR, the genome sequence for strain 1021 indicates the presence of six other proteins with good homology to LuxR-like regulators (Smc0658, 0877, 0878, 3015, 3016 and 4032), all chromosomally located (http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/). None of these six has been carefully tested, but if they prove to be functional quorum-sensing receptors, then quorum-sensing regulation in strain 1021 could be potentially much more complex than outlined above, especially in view of evidence for the formation of heterodimers as well as homodimers of the three AHL receptors in *P. aeruginosa* (Ledgham *et al.* 2003; Ventre *et al.* 2003). There is good evidence that many genes in strain 1021 may be regulated via LuxR-type regulators other than ExpR. Two earlier proteome studies (Chen *et al.* 2003; Teplitski *et al.* 2004) identified over 75 proteins in strain 1021 that were responsive to added SinI-made AHLs. In addition, another 60 proteins in strain 1021 that showed AHL-dependent accumulation have been identified (Gao *et al.* in press). Since strain 1021 lacks a functional ExpR, these results suggest that either SinR and/or one or more of the six putative AHL receptors may be either directly or indirectly responsible for the AHL responsiveness of this diverse and symbiotically relevant group of approximately 135 proteins. In this regard, it is worth noting that Smc3015 and 3016 in strain 1021 correspond to *visN* and *visR* in *S. meliloti* strain SU10/406. The *visNR* genes in SU10/406 encode LuxR-like regulators that are active as global regulators of flagellar motility and taxis, probably functioning as a heterodimer (Sourjik *et al.* 2000). The motility genes regulated by *visNR* in SU10/406 were not affected by added culture filtrate from stationary-phase cells, but the effects of added SinI AHLs were not tested. Thus, the role of *visNR* as AHL receptors remains uncertain.

(iv) traI, traR *and* melI

While strain 1021 lacks *traI* and *traR*, strain Rm41 (and its derivative AK631) has *traI* and *traR* next to plasmid transfer genes (Marketon & Gonzalez 2002). In addition, strain Rm41 has an AHL synthase encoded by *melI* (Marketon & Gonzalez 2002), which produces a range of AHLs with short acyl side chains in addition to the *sinI*-determined long-chain AHLs, including several in common with the SinI-made AHLs of strain 1021 (Teplitski *et al.* 2003).

(d) Rhizobium *sp. NGR234*

It appears that the transfer of the symbiotic plasmid of this strain has the potential to be under quorum-sensing regulation because *traI* and *traR* genes are on the plasmid pNGR234a. TraI synthesizes 3-oxo-C8-HSL, and two more AHLs have been detected in a *traI* mutant, indicating that the corresponding synthase(s) is encoded elsewhere in the genome (He *et al.* 2003). Quorum-sensing-regulated growth inhibition has also been reported in *Rhizobium* sp. NGR234. The inhibitory effect appears to be a result of TraR-dependent inhibition of growth in response to *traI*-made 3-oxo-C8-HSL. Addition of 3-oxo-C8-HSL to this strain leads to a significantly reduced growth rate. The inhibition requires *traR* and *traI* genes from pNGR234a and undefined genes elsewhere in the NGR234 genome (He *et al.* 2003).

(e) Mesorhizobium

Two LuxI-type AHL synthase genes are present in the genome of *Mesorhizobium loti*, one adjacent to a LuxR-type regulator gene and other genes that might be associated with conjugal transfer (http://www.kazusa.or.jp/rhizobase/); however, the effects of mutations have not yet been reported. A strain of *Mesorhizobium huakuii* has been shown to produce AHLs (Zhu *et al.* 2003) and reduced AHL production in this strain was correlated with the formation of a thinner biofilm (Wang *et al.* 2004).

(f) Bradyrhizobium

Approximately 20% of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains tested using an AHL-detection bioassay made detectable amounts of AHLs (Pongsilp *et al.* 2005); but only a little has been done with regard to the identification of the AHL synthesis genes or the genes regulated by AHLs (Brelles-Marino & Bedmar 2001). However, in *B. japonicum* strain USDA110, the nodulation genes are expressed in a population-density-dependent manner and this is regulated by a factor in the growth medium called bradyoxetin. This compound was shown to be an iron chelator and so is probably not among the classic types of quorum-sensing regulation signals (Loh & Stacey 2001; Loh *et al.* 2002*a*).

Bradyoxetin is similar in structure to the siderophore mugineic acid (Loh *et al.* 2002*a*). The production of bradyoxetin is iron regulated, being maximal in iron-depleted conditions (Loh *et al.* 2002*a*; Loh & Stacey 2003). At high cell densities, the *nod* genes are repressed by a cascade initiated by the regulator NswB in response to high concentrations of bradyoxetin. NswB functions by inducing NolA that activates NodD2, which then represses the *nod* genes (Loh *et al.* 2001, 2002*b*). This repression of *nod* genes has been demonstrated *in vitro* and *in planta* (Loh *et al.* 2002*b*). Bradyoxetin has also been found in other Alphaproteobacteria (Loh *et al.* 2002*a*; Loh & Stacey 2003), but its role in these species is yet to be established.

3. PLASMID TRANSFER

The paradigm for quorum-sensing control of plasmid transfer in the *Rhizobiaceae* was established in *A. tumefaciens*, which contain plasmids that carry genes for pathogenesis and transformation of plants (White & Winans 2007). *Agrobacterium tumefaciens* strains carrying the appropriate plasmids can induce crown gall disease on susceptible plant hosts. This disease is caused by the transfer of a part of the pathogenesis (Ti) plasmid into plant cells, where it recombines with the chromosome and initiates the production of a gall and metabolites (opines) that can be selectively metabolized by agrobacteria in the rhizosphere. The spread of this tumour-inducing (Ti) plasmid can allow horizontal transfer of the pathogenicity traits to other *Agrobacterium* strains lacking such characteristics (Zhu *et al.* 2000). The plasmid transfer genes on the Ti plasmid are strongly induced only in the presence of opines and only when the bacteria enter late exponential phase and are at an appropriate population density. This is achieved due to the action of the regulator TraR, which is transcriptionally induced by opines and activated by the accumulation of AHLs (principally 3-oxo-C8-HSL) produced by TraI (Piper *et al.* 1993; Zhang *et al.* 1993). TraR bound to the AHL has been crystallized and it is clear that the AHL is almost completely encased within the dimers of TraR (Zhang *et al.* 2002*b*). This TraR–AHL complex induces plasmid transfer operons and is much more stable to proteolytic degradation than TraR lacking the AHL (Zhu & Winans 2001). One of the induced operons contains *traI* as the first gene and so there is positive feedback that enhances the production of 3-oxo-C8-HSL and TraR-mediated gene induction (Zhu *et al.* 2000). Clearly, any such positive feedback system requires some kind of governor to prevent it from being induced prematurely. The *traM* gene product plays such a role: TraM forms a dimer (Chen *et al.* 2004; Qin *et al.* 2004) and binds to TraR–AHL preventing the induction of plasmid transfer operons. However, as the levels of AHL-activated TraR accumulate, these titrate out the available TraM and so can then initiate gene induction and the positive feedback effect on TraI-made AHLs.

It is clear that a similar quorum-sensing-based control of plasmid transfer is at the heart of the mechanisms of induction of transfer of several different plasmids in rhizobia. The systems best understood include various symbiotic plasmids such as pRL1JI from *R. leguminosarum* bv. *viciae* (Wilkinson *et al.* 2002; Danino *et al.* 2003), pNGR234 from *Rhizobium* sp. NGR234 (Freiberg *et al.* 1997; He *et al.* 2003) and pRme41a from *S. meliloti* (Marketon & Gonzalez 2002; Gonzalez & Marketon 2003), and non-symbiotic plasmids such as p42a from *R. etli* strain CFN42 can also use such a plasmid transfer induction system

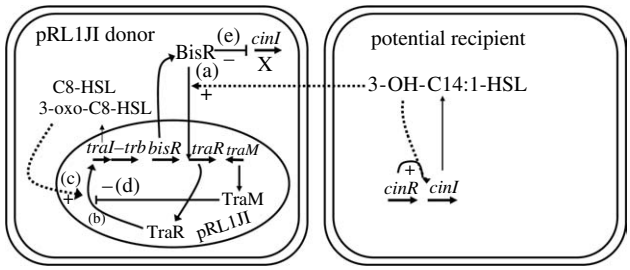

Figure 1. Model for the regulation of the plasmid transfer *traI–trb* operon on pRL1JI in *R. leguminosarum* bv. *viciae*. The effects of AHLs on activation or inhibition of regulators are shown as dashed lines, and the inducing or repressing effects of regulators are shown as solid lines. (a) In cells that act as donors of pRL1JI, BisR induces *traR* in response to 3-OH-C14:1-HSL. This occurs predominantly in response to 3-OH-C14:1-HSL from potential recipients of pRL1JI. (b) Induction of *traR* produces TraR, which induces the *traI–trb* operon in response to 3-oxo-C8-HSL and C8-HSL. (c) There is autoinduction of the *traI–trb* operon by the TraI-made AHLs, 3-oxo-C8-HSL and C8-HSL. (d) Induction of the *traI–trb* operon by low levels of TraR is inhibited by TraM. The TraM-mediated repression of *traI* expression is overcome following induction of *traR* by BisR (a), in response to 3-OH-C14:1-HSL from potential recipients. (e) Endogenous production of 3-OH-C14:1-HSL is greatly reduced because BisR represses the expression of the chromosomal gene *cinI*, whose product produces 3-OH-C14:1-HSL. This repression enables the pRL1JI donors to respond primarily to potential recipient strains lacking *bisR* (and hence pRL1JI) but not to strains carrying pRL1JI. This figure was modified and reproduced (with permission) from a previous publication (Danino *et al.* 2003).

(Tun-Garrido *et al.* 2003). All of these systems share *traI*, *traR* and *traM* genes and use TraI-made 3-oxo-C8-HSL to activate TraR, although other AHLs such as C8-HSL and 3-OH-C8-HSL can also activate TraR (Wilkinson *et al.* 2002; Danino *et al.* 2003; Gonzalez & Marketon 2003). It is important to note that this is not the only mechanism of regulation of plasmid transfer in rhizobia; for example, *R. etli* strain CFN42 contains another plasmid whose regulation is mediated via two genes, *rctA* and *rctB*, and whose control appears to be independent of quorum-sensing regulation (Perez-Mendoza *et al.* 2005).

A critical part of the induction of plasmid transfer is the control of *traR* expression, because unless *traR* is induced, the quorum-sensing induction of plasmid transfer does not occur. In *Agrobacterium* strains, the *traR* genes are co-induced with the opine catabolism genes induced in response to the opines secreted from transformed plant cells (Kim & Farrand 1998; Zhu *et al.* 2000). The mechanism of regulation of *traR* expression in the rhizobial strains analysed is different. As shown in figure 1, on the *R. leguminosarum* bv. *viciae* plasmid pRL1JI, the expression of *traR* is under the control of a second LuxR-type regulator called BisR (*bi*functional *s*ignalling *r*egulator). The *bisR* gene is on pRL1JI upstream of *traR* (Wilkinson *et al.* 2002), and BisR specifically induces *traR* expression in response to 3-OH-C14:1-HSL (there is no induction of *traR* by TraI-made 3-oxo-C8-HSL or C8-HSL; Danino *et al.* 2003). The 3-OH-C14:1-HSL is made by another AHL synthase encoded by the chromosomal gene *cinI*, which is strongly expressed in most strains of *R. leguminosarum* and is under the control of CinR, which induces *cinI* expression in response to CinI-made 3-OH-C14:1-HSL (Lithgow *et al.* 2000). However, in those strains carrying pRL1JI, little or no 3-OH-C14:1-HSL is made because BisR represses *cinI* expression. Consequently, there is little induction of *traR* by BisR (because the appropriate AHL is not present), even though BisR is present in the cells. Any low-level induction of *traR* is inhibited by the production of TraM, which by analogy with *A. tumefaciens* TraM (Chen *et al.* 2004; Qin *et al.* 2004) titres out TraR (Danino *et al.* 2003). When bacteria carrying pRL1JI come close to other strains of *R. leguminosarum* that produce 3-OH-C14:1-HSL, BisR detects this AHL and induces *traR* resulting in enough TraR to titre out TraM. The result is strong induction of plasmid transfer genes under TraI–TraR quorum-sensing control. This system can result in very high rates of plasmid transfer (up to 1 transconjugant per 100 recipients) under optimal conditions (Danino *et al.* 2003). This recipient-induced mode of plasmid transfer relies on the bifunctional (inducer and repressor) nature of BisR and can promote the spread of pea and vetch nodulation characteristics into *Rhizobium* strains that do not have a plasmid that carries *bisR* and so does not repress *cinI* expression. Based on the identified genes (Tun-Garrido *et al.* 2003), it is probable that the non-symbiotic plasmid p42a in *R. etli* CFN42 has a similar mechanism of plasmid transfer, but this is yet to be demonstrated.

In contrast to the high rates of plasmid transfer observed with pRL1JI and p42a, only low rates of plasmid transfer (around 1 transconjugant per 10^7–10^9 recipients) have been observed with pNGR234 from *Rhizobium* sp. NGR234 (He *et al.* 2003) or pRme41a from *S. meliloti* strain Rm1021 (Gonzalez & Marketon 2003). The mechanism of regulation of transfer of these plasmids is different from pRL1JI because these plasmids lack a *bisR* gene (Freiberg *et al.* 1997). Possibly these low frequencies occur because optimal induction of *traR* has not been achieved, or possibly because some other component of the plasmid transfer system is not fully functional (He *et al.* 2003).

4. RESPONSES OF EUKARYOTIC HOSTS TO BACTERIAL QUORUM SENSING

Quorum-sensing mutants of bacterial pathogens such as *P. aeruginosa* and *Erwinia carotovora* have much reduced virulence in their plant and animal hosts (Winzer & Williams 2001; Von Bodman *et al.* 2003), while quorum-sensing mutants of *Vibrio fisheri*, the bioluminescent symbiont of squid (Lupp & Ruby 2004; Nyholm & McFall-Ngai 2004) and some rhizobia (see above) are impaired in establishing normal symbiotic interactions with their hosts. Thus, both bacterial pathogens and symbionts have come to rely on quorum sensing during interactions with their hosts. It seems reasonable to expect that host organisms, in turn, have evolved mechanisms for neutralizing, disrupting or manipulating this regulation in the bacteria they encounter. Recent studies provide evidence that eukaryotic hosts can indeed suppress or

manipulate quorum sensing in a diversity of bacteria by synthesizing compounds that mimic the signals used by these bacteria. In addition, there is evidence that eukaryotes can detect bacterial quorum-sensing signals and make sophisticated responses to them, including altered regulatory, metabolic and defence responses. Both plant and animal hosts have also been shown to possess enzymes that rapidly and rather specifically inactivate bacterial AHL quorum-sensing signals. An examination of what has been learned about these capabilities of eukaryotic hosts may be valuable in appreciating the potential role of legume hosts in affecting AHL-mediated regulation in rhizobia.

(a) *Eukaryotic agonists and antagonists of bacterial quorum sensing*

Eukaryotic hosts that have no immune system often rely on the production of diverse secondary metabolites that help protect them from pests and pathogens. As described more fully in the accompanying review by Bjarnsholt & Givskov (2007), some very potent antifouling substances were recently found in a marine red alga, *Delisea pulchra*. This alga was remarkably free of the thick layer of bacteria and other organisms that colonize most biotic and abiotic surfaces in marine waters. The antifouling substances were identified as a set of approximately 30 different halogenated furanones. These furanones have structural similarity to AHLs and are potent and specific inhibitors of quorum sensing in many bacteria (Givskov *et al.* 1996; Manefield *et al.* 2000; Hentzer *et al.* 2002, 2003*a*,*b*; Hentzer & Givskov 2003; Martinelli *et al.* 2004). The furanones appear to interact directly with LuxR-type regulators and promote their proteolysis (Manefield *et al.* 1999, 2002; Koch *et al.* 2005), effectively preventing AHLs from activating gene expression. Mutations affecting AHL binding by LuxR had relatively little effect on inhibition by the furanones (Koch *et al.* 2005) suggesting that the furanones and AHLs may not compete for the same binding site.

Higher plants, including various legumes, rice, garlic and tomato, also secrete compounds that affect bacterial quorum sensing (Teplitski *et al.* 2000; Daniels *et al.* 2002; Gao *et al.* 2003; Keshavan *et al.* 2005; Rasmussen *et al.* 2005*a*; Bjarnsholt & Givskov 2007). Plant roots appear to secrete sufficient amounts of the active compounds to elicit changes in quorum-sensing-regulated gene expression in bacteria on the root surface (Teplitski *et al.* 2000). The set of plant-secreted effectors appears to change during seedling development, and bacteria may be exposed to a different set of AHL 'mimic' compounds once they get inside the root (Gao *et al.* 2003).

The active compounds from plants have not yet been chemically identified, so their mode of action and possible structural similarity to known quorum-sensing signals are unknown. However, the plant compounds were detected by their ability to activate or inhibit AHL-mediated gene induction in reporter bacteria that produce no AHLs. Thus, the active plant compounds appear to be substances that interact directly with AHL receptors in the reporters. Most of the active compounds secreted by pea and *Medicago truncatula* were soluble in methanol extracts of freeze-dried root exudates, but were less soluble or insoluble in ethyl acetate (Teplitski *et al.* 2000; Gao *et al.* 2003, in press) suggesting that these plant compounds are probably not AHLs. L-Canavanine, present in seed exudates of alfalfa, was shown to inhibit quorum-sensing-regulated gene expression in *Chromobacterium* and *Sinorhizobium* (Keshavan *et al.* 2005). However, canavanine is an analogue of arginine that can be incorporated into proteins, thereby causing polypeptide misfolding and inhibiting bacterial growth. The effects of canavanine on quorum sensing in bacteria may therefore be an indirect and general result of protein misfolding on various transcriptional regulators, including AHL regulators.

In conventional screens, quorum-sensing antagonists are difficult to distinguish from compounds that inhibit growth or are toxic to reporter bacteria. A novel screening method that distinguishes between quorum-sensing inhibitors and toxic substances has been developed (Persson *et al.* 2005). Several plant extracts and off-the-shelf chemicals were found to inhibit quorum sensing (Rasmussen *et al.* 2005*a*), with garlic extract and 4-nitro-pyridine-*N*-oxide being the most potent. Transcriptome analysis indicated that both of these rather specifically affected quorum-sensing-regulated gene expression in *P. aeruginosa* (Rasmussen *et al.* 2005*a*). The same kind of screen for antagonists revealed that most of the 50 *Penicillium* species surveyed produced compounds that inhibited AHL-mediated quorum sensing (Rasmussen *et al.* 2005*b*). Penicillic acid and patulin were among the active compounds and both of these have structural similarity to the *Delisea* furanones. Thus, it appears that fungi, as well as plants and algae, produce compounds that can alter quorum sensing in the bacteria they encounter.

Similar to higher plants, the green alga *Chlamydomonas* secretes agonists and antagonists of both AHL- and AI-2-mediated quorum-sensing gene expression in bacteria (Teplitski *et al.* 2004). A purified *Chlamydomonas* compound rather specifically targeted quorum sensing-regulated protein accumulation in *S. meliloti*. This agonist, like *S. meliioti's* own AHLs, stimulated the accumulation of some proteins; however, it also prevented AHL-induced accumulation of other proteins (Teplitski *et al.* 2004). This suggests that individual agonist or antagonist compounds may have markedly different effects on different AHL-regulated functions.

Another AHL-receptor agonist from *Chlamydomonas* has been chemically identified as lumichrome, which is a normal degradation product of the vitamin riboflavin (W. D. Bauer *et al.* 2007, unpublished data). Interestingly, lumichrome had previously been identified as a compound secreted by *S. meliloti* that was able to stimulate root respiration and shoot growth in alfalfa (Phillips *et al.* 1999). Thus, lumichrome may contribute to signalling and regulation in both *S. meliloti* and its legume hosts during symbiotic interactions.

Most of the active compounds detected after HPLC fractionation of pea, *M. truncatula* and *Chlamydomonas* exudates affected only one or two of the half-dozen reporter strains used. Both the plant and algal compounds therefore appear to interact rather specifically with certain AHL receptors and not others.

This is in contrast with the ability of the *Delisea* furanones to inhibit regulation mediated by most (but not all) of the AHL regulators tested. Many of the quorum-sensing active compounds produced by plants and algae may act by a different mechanism than the furanones.

Pea, *M. truncatula* and *Chlamydomonas* make several chromatographically separable compounds that act on specific AHL or AI-2 reporters. For example, *Chlamydomonas* produces about half a dozen substances that stimulate the LasR AHL receptor and produces a different half-dozen compounds that stimulate the CepR AHL receptor from *Burkholderia* (Teplitski *et al.* 2004). Why should eukaryotic hosts produce several compounds that affect the same receptor? The answer may lie in natural variation in binding specificity among the AHL receptors. Since different bacteria are likely to have receptors that differ somewhat in amino acid sequences and binding specificity, their hosts may need to produce several structural variants of their active compounds to ensure that at least one will act strongly in each of the different bacteria they encounter. If host organisms do employ a 'shotgun' approach like this to disrupt quorum sensing, how do highly co-evolved symbionts such as rhizobia avoid being hit? One possibility is that rhizobia may use signals such as the long-chain AHLs so that receptors for these signals are not much affected by 'signal-mimic' compounds that target other bacteria. Plant-made metabolites that influence quorum-sensing gene expression are described in the accompanying article by Bjarnsholt & Givskov (2007).

Most of the plant compounds so far detected in root exudates by reporter strains are agonists rather than antagonists of AHL signalling (Teplitski *et al.* 2000; Daniels *et al.* 2002; Gao *et al.* 2003, in press). Biologically, the ability of plant hosts to specifically stimulate gene expression in the bacteria they encounter greatly increases the potential for sophisticated host manipulation of bacterial behaviour. Instead of simply preventing the activation of gene expression by bacterial quorum-sensing signals, the stimulatory plant compounds could enable the host directly to induce bacterial genes, inducing changes that are beneficial to the host. The host may have a limited window of opportunity to influence gene expression when it makes first contact with low numbers of bacteria that are not yet quorate. One of the central questions regarding the role of quorum sensing in the *Rhizobium*–legume symbiosis is whether compounds produced by the plant host serve to stimulate events such as infection initiation and bacteroid formation and, perhaps, block others such as unrestrained bacterial multiplication within the host.

(b) *Responses of hosts to bacterial quorum-sensing signals*

Exposure of *M. truncatula* roots to physiological (nanomolar) concentrations of bacterial AHLs significantly altered the accumulation of over 7% of the root proteins resolved by two-dimensional gels (Mathesius *et al.* 2003). These results suggest that plants 'listen' very actively to bacterial conversations. The global responsiveness of the plant to bacterial AHL signals was unexpected and the biological consequences of the responses are not at all clear yet. Various proteins related to host defences, hormones, regulation, metabolism, protein processing and cytoskeleton were reduced in level, while others increased. Certain proteins changed levels in response to one AHL but not another, and many changes were time dependent (Mathesius *et al.* 2003). In addition, some of the responses to bacterial signals are tissue specific while others are systemic (Hartmann *et al.* 2003; Mathesius *et al.* 2003; U. Mathesius 2006, unpublished data).

If both the bacteria and the hosts can respond to the same signals, then the role of quorum sensing in modulating the outcome of interactions becomes much more complex. Animals respond in a variety of ways to AHL signals including modulation of immune responses, blood-vessel relaxation, selective apoptosis and chemotaxis (Lawrence *et al.* 1999; Joint *et al.* 2002; Smith *et al.* 2002; Williams 2002). Thus, eukaryotic responses to quorum-sensing signals are likely to have been established for a considerable time in host–bacterium interactions. As yet, no 'receptors' for the signalling molecules have been identified in any eukaryote and there have been no studies to establish the consequences of defective host perception of bacterial quorum-sensing signals on the outcome of host–bacterial interactions.

One can imagine that natural selection might favour the development of additional feedback layers of related interactions between specific partners. For example, perception of certain quorum-sensing signals might induce a host to produce a different set of agonistic or antagonistic signals (Mathesius *et al.* 2003). Alternatively, compounds from the host may stimulate or inhibit the synthesis of quorum-sensing signals by the bacterium. The antagonism between *Candida albicans* and *P. aeruginosa* provides an interesting example of such feedback-layered responses. Both of these microbes are opportunistic pathogens frequently co-cultured from cystic fibrosis patients. *P. aeruginosa* 3-oxo-C12-HSL inhibits the dimorphic shift of *C. albicans* from yeast-to-mycelial forms (Hogan *et al.* 2004), thus mimicking the action of farnesol, the yeast's own signal. In turn, farnesol is able to strongly suppress AHL synthesis in *P. aeruginosa* (J. Robinson 2006, unpublished data).

Another potentially important facet of host responses to quorum sensing is the ability of enzymes in both plants and animals to inactivate AHL signals (Chun *et al.* 2004; Delalande *et al.* 2005; Yang *et al.* 2005; Dong *et al.* 2007). If the primary function of these enzymes proves to be the disruption of quorum sensing in bacteria or modulation of host responses to related bacterial signals, then the production, location and specificity of the signal-degrading enzymes become rather important factors in the pattern of reciprocal action and reaction between hosts and bacteria.

5. PERSPECTIVES, QUESTIONS AND CONCLUSIONS

Various rhizobia establish fairly effective symbioses even when genes for AHL synthases and certain AHL receptors are mutated. Thus, rhizobia do not appear

to be as dependent as many bacterial pathogens on quorum sensing for successful interactions with their hosts. At present, it appears that quorum-sensing-regulated functions in the bacterium and host serve primarily to optimize various interactions between the partners; for example, the enhancement of infection initiation by *R. leguminosarum* (Cubo *et al*. 1992) and *S. meliloti* (Gao *et al*. 2005) and the enhancement of the number of *R. etli* bacteroids in symbiosomes (Daniels *et al*. 2002). There are many opportunities for both the host and the bacterium to modify the levels of many symbiotically relevant genes and proteins in both partners. This has been artificially done in relation to plant pathogenesis using transgenic plants expressing AHL-degrading enzymes; as reviewed in the accompanying article by Dong *et al*. (2007), such plants have increased resistance to some bacterial pathogens. The induction of systemic resistance dependent on quorum sensing in a bacterial pathogen illustrates the types of changes that can occur naturally in plant–bacterial interactions (Schuhegger *et al*. 2006). Furthermore, it has been shown that a bacterial AHL synthase targeted to potato and tobacco plastids can result in transgenic plants that produce AHLs, which can be released into the rhizosphere and can influence interactions with pathogens (Fray *et al*. 1999; Toth *et al*. 2004; Scott *et al*. 2006). In principle, similar experiments with transgenic legumes carrying genes to produce or degrade AHLs could be used to analyse the role of quorum sensing in their interactions with rhizobia.

In addition to a role in optimizing interactions with a host, quorum sensing in rhizobia may yet prove to be one of the required facets of successful symbiosis. All the rhizobia tested so far have additional LuxR-type regulators that have not been mutated and so the roles of quorum sensing in regulating some crucial aspects of the symbiosis are yet to be fully explored. For example, while AHLs inhibit cell division in *R. leguminosarum* and affect expression of cell division related genes in *S. meliloti*, the regulation of rhizobial cell division *in planta* is still unclear either when bacterial replication stops in mature nodules or reinitiates in senescing nodules. It will be interesting to learn from future studies whether all the diverse strategies for quorum-sensing regulation seen among different isolates and strains of rhizobia contain some elements in common that are required to establish and maintain mutually beneficial relations with their legume partners.

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