

Quorum Sensing in Nitrogen-Fixing Rhizobia

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INTRODUCTION

Bacterial populations coordinately regulate gene expression by producing diffusible signal molecules. These signals, known as autoinducers, accumulate extracellularly and interact specifically with a receptor protein to affect changes not related to their own metabolism. Production of autoinducers typically occurs at specific stages of growth or in response to changes in the environment and induces a concerted response once a critical concentration has been

reached. These diffusible signals frequently act to induce gene expression in response to bacterial cell density in a process often referred to as quorum sensing (8, 59, 60, 69, 120, 166, 178, 182, 185). Alternatively, autoinducer secretion and response may confer on the bacterium the ability to determine whether secreted molecules move away from the cell. This process, termed diffusion sensing by Rosemary Redfield, could allow the cells to regulate the secretion of effectors, such as degradative enzymes, antibiotics, surfactants, and siderophores, to minimize losses to extracellular diffusion (137). The best characterized quorum-sensing mechanism is found in gram-negative organisms and involves the use of acylated homoserine lactones (AHLs) as signal molecules (8, 59, 60, 63, 69, 120, 145, 166, 178, 182, 185).

Recent publications have shown that quorum sensing plays a

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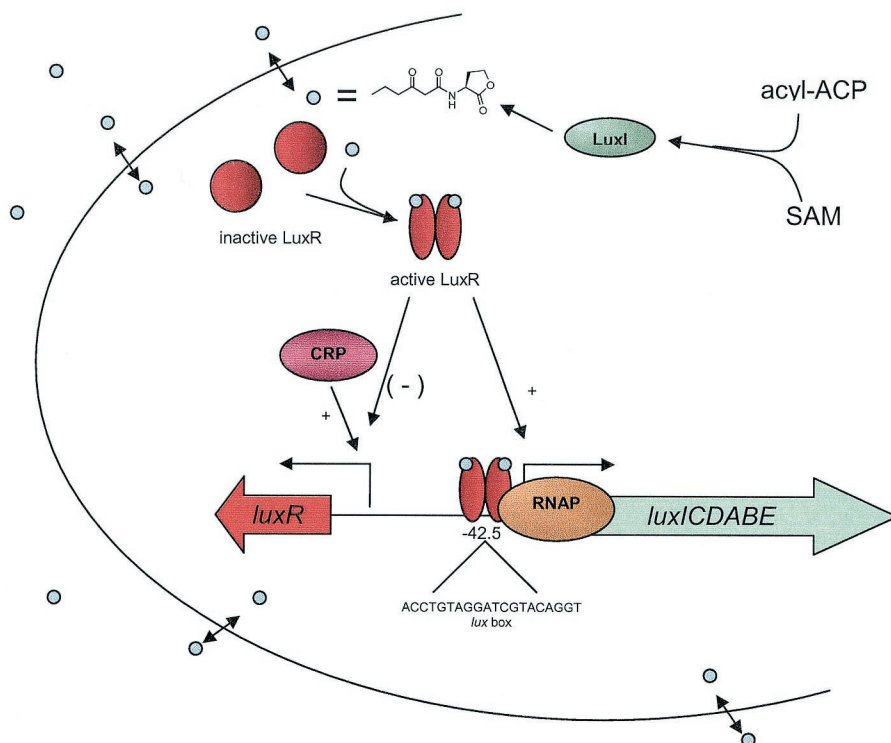


FIG. 1. Quorum-sensing model in *P. fischeri*. At low cell densities, transcription of the *luxICDABE* operon occurs at basal levels. LuxI encodes the AHL synthase, which synthesizes 3-oxo-C₆-HSL from acyl-ACP and SAM substrates. High cell densities lead to the accumulation of AHLs, which bind and activate the LuxR transcriptional activator. LuxR binds to an inverted repeat referred to as the *lux* box, which is centered at -42.5 from the transcriptional start site, and makes contact with the RNA polymerase to stimulate the expression of the *luxICDABE* genes. Expression of the *luxR* gene is regulated by several factors such as heat shock, catabolite repression, and even LuxR itself (only at very high cell densities).

major role in preparing and perhaps coordinating the symbiotic nitrogen-fixing rhizobia during the establishment of their interactions with the host plant.

HOW BACTERIA TALK TO EACH OTHER

AHL-Mediated Cell-Cell Communication

Historically, it was thought that bacteria were solitary individuals, each growing independently of the population. However, in 1970 Neelson et al. (114) discovered that bacteria can sense and respond to the rest of the population. This phenomenon is called quorum sensing and is defined as the cell density-dependent regulation of gene expression (for reviews, see reference 51, 59, 60, 120, and 181). One of the best-studied examples of quorum sensing is in *Photobacterium fischeri* (formerly *Vibrio fischeri*), a marine bacterium that is a symbiont of several marine fish and squids (Fig. 1) (142, 173). In this model organism, the basal-level synthesis of autoinducers occurs at low cell densities, like those found in seawater. The autoinducers, which belong to the AHL family of signal molecules, are thought to pass through the cell membrane by diffusion (86). As the cell density increases during the symbiotic association with the animal host, autoinducers accumulate in and around the cells (86).

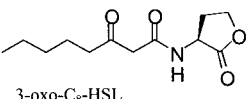
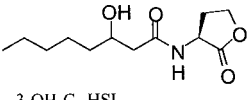
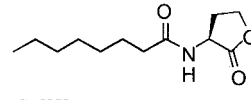
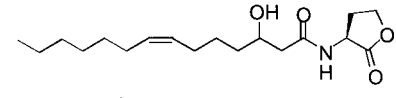
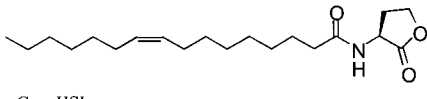
When a threshold level of AHLs (about 10 nM) is reached, the LuxR regulator is activated by binding the AHL (73, 86). LuxR, a transcriptional activator, then induces expression of

the *lux* operon (Fig. 1). The *lux* operon contains *luxI* (the AHL synthase) along with the genes necessary for luminescence (48, 49, 164). Activation of the *lux* operon leads to a rapid rise in the levels of autoinducer and creates a positive-feedback loop, which is followed by the onset of luminescence. LuxR is regulated at the transcriptional level by cyclic AMP receptor protein (41) and presumably at the posttranscriptional level by GroEL (37). At low AHL levels, LuxR activates its expression, while at high AHL levels, the active LuxR represses itself (152, 153).

Conjugal Transfer of the *A. tumefaciens* Ti Plasmid

Agrobacterium tumefaciens has become one of the paradigms for quorum sensing, especially as it relates to soil organisms. This plant pathogen induces crown gall tumors on susceptible plant hosts (181, 190). This is mediated by the transfer of oncogenic DNA fragments from its Ti plasmid directly into the nuclei of the host plant cell (63, 181). These DNA fragments encode the production and secretion of opines by the plant, which are then utilized by *A. tumefaciens* as nutrients. In addition, the Ti plasmids mediate their own conjugal transfer between associated agrobacteria (51, 128). This process is regulated in part by quorum sensing. The Ti plasmid carries the key quorum-sensing regulators: *traI* (a *luxI* homolog), *traR* (a *luxR* homolog), and *traM* (an antiactivator of TraR) (58, 62, 83, 84). TraI synthesizes an AHL, 3-oxo-C₈-homoserine lactone

TABLE 1. Characteristics of AHLs

Structure	Representative organisms	Reference(s)
 3-oxo-C ₈ -HSL	<i>A. tumefaciens</i> , <i>Rhizobium</i> sp. strain NGR234, <i>R. leguminosarum</i> , <i>S. meliloti</i> , <i>R. etli</i> CFN42	62, 78, 84, 107, 130, 170, 180; Marketon et al., submitted
 3-OH-C ₈ -HSL	<i>R. leguminosarum</i> , <i>S. meliloti</i> , <i>R. etli</i> CFN42	107, 170, 184; Marketon et al., submitted
 C ₈ -HSL	<i>R. leguminosarum</i> , <i>S. meliloti</i>	29, 107, 108, 139, 180, 184; Marketon et al., submitted
 3-OH-C _{14,1} -HSL	<i>R. leguminosarum</i>	95
 C _{16,1} -HSL	<i>S. meliloti</i>	107; Marketon et al., submitted

(3-oxo-C₈-HSL), which binds and activates TraR (135). TraR then goes on to activate its targets: *traAFB*, *traCDG*, and *traI-trb* (58, 62, 83, 130, 188). When the level of TraR is low, TraM binds to it and forms an inactive complex, thereby preventing plasmid transfer until the cell density is high (85, 104, 132, 165). The Ti plasmid copy number is also influenced by the quorum-sensing system. Transcription of the Ti *repABC* operon is regulated by TraR in combination with 3-oxo-C₈-HSL. This activation leads to an elevated Ti plasmid copy number and enhanced tumorigenesis (94, 118, 119). In addition to TraM, the *tra* genes are under another level of regulation. Opines secreted by crown gall tumors bind a receptor protein, AccR or OccR, depending on the strain (14, 72). The opine-receptor protein combination initiates the transcription of *traR* by either induction (OccR) or derepression (AccR) (131). Therefore, conjugal transfer of the Ti plasmid does not occur in the absence of opines. Thus, while the quorum-sensing network is similar to that of the *P. fischeri* model, an additional regulatory network, required for proper induction of conjugal plasmid transfer, is superimposed on the *tra* system.

AHL-Mediated Cell-Cell Communication Plays a Role in Symbiosis and Pathogenesis in Some Organisms

In the organisms characterized so far, the quorum-sensing mechanisms are similar to those in the *P. fischeri* paradigm but the activated target genes are diverse. Examples of genes regulated by quorum sensing include the *lux* (luminescence) genes in *P. fischeri*, the *tra* (Ti plasmid transfer) genes in *A. tumefaciens*, exoenzymes and virulence factors in *Pseudomonas aeruginosa* and *Erwinia carotovora*, swarming motility in *Serratia liquefaciens*, antibiotics and violacein pigment in *Chro-*

mobacterium violaceum, and exopolysaccharide production in *Pantoea stewartii* (43, 63, 145, 166). All of these organisms have one or more LuxR and LuxI homologues. In addition to sharing similar quorum-sensing mechanisms, most of the organisms establish symbiotic or pathogenic relationships with eukaryotic hosts. Therefore, it is not surprising that symbiosis, pathogenesis, and quorum sensing are intertwined in a complex story of gene regulation. Furthermore, the possibility has been raised that in natural habitats, different bacterial species communicate with one another to coordinate their behavior (7, 8). An example of this is the bacterial community that naturally colonizes the roots of tomato plants (156). It has been suggested that the AHLs act as signals for coordination of the functions of the different populations within this rhizosphere community. Further evidence for interspecies signaling through AHLs was shown by McKenney et al. (110), when they demonstrated that AHLs in spent culture supernatants from *P. aeruginosa* enhanced virulence factor production in *Burkholderia cepacia*.

MOLECULAR MECHANISMS

AHL Family of Autoinducers

AHL characteristics. AHLs consist of an HSL head group attached to a variable acyl side chain (Table 1). The amphiphathy of the AHL molecule seems to be a balance between the hydrophobic side chain and the hydrophilic HSL ring. These characteristics presumably allow the AHLs to traverse the phospholipid bilayer of the cell membrane and to navigate the aqueous intracellular and extracellular environments (60). The acyl chain varies in length, from 4 to 18 carbons in those AHLs identified so far (71, 77, 108, 134, 150). Variability also exists in

the third carbon position of the acyl chain, where there can be a hydrogen, hydroxyl, or oxo substitution (see Table 1). A few AHLs that have unsaturated acyl chains have also been identified (71, 134, 150). The overall length of the side chain and the chemical modification at the third carbon position provide the specificity to quorum-sensing signals. To add complexity, most organisms produce more than one type of AHL and different organisms can produce the same AHL (77). Therefore, there is some overlap in the production and recognition of AHLs by different organisms.

AHL Synthases

LuxI-type synthases. All LuxI-type proteins identified to date resemble the LuxI of *P. fischeri* and catalyze the ligation of *S*-adenosylmethionine (SAM) with an acylated acyl carrier protein (acyl-ACP), which form the HSL and acyl chain components, respectively, of the resulting AHL (60, 111, 147). The catalytic model proposed by Parsek, Greenberg, and coworkers suggests a nucleophilic attack on the C-1 position of the acyl-ACP molecule by the amino nitrogen of SAM, resulting in an amide linkage. This is followed by the lactonization of SAM together with amide bond formation, which results in ring formation and release of the AHL (42, 74, 122). LuxI homologues are about 200 amino acids in length, and disruption of certain conserved residues in the amino-terminal half of the protein leads to a reduction or loss of synthase activity. It has been suggested that conserved amino acids in the carboxy terminus may be necessary for acyl-ACP selection (76, 121). The recently determined crystal structure of EsaI, the LuxI homologue from *P. stewartii*, shows remarkable similarity to *N*-acetyltransferases, and its core catalytic fold has features essential for phosphopantetheine binding (175, 176). The structural analysis suggests that the *N* acylation of SAM is likely to include abstraction of an amine proton by a catalytic base. In addition, variable residues in the C-terminal half of the protein and the nature of the amino acid at position 140 constitute the basis for the acyl chain specificity (175, 176).

LuxM/AinS-type synthases. The products of the *luxM* gene from *V. harveyi* and the *ainS* gene from *P. fischeri* synthesize AHLs despite their lack of similarity to LuxI (10, 64). These AHL synthases direct the synthesis of 3-OH-C₄-HSL and C₈-HSL, respectively. These two proteins share a strong conserved region, and null mutations in either of these genes abolish synthesis of their respective AHLs. In vitro studies revealed that AinS catalyzes C₈-HSL synthesis from SAM and either octanoyl-ACP or octanoyl coenzyme A (octanoyl-CoA) conjugates (75). This is in contrast to LuxI-type proteins, which use acyl-ACP as their primary source of fatty acyl chains.

HdtS-type synthases. More recently, a third AHL synthase, HdtS, has been detected in *Pseudomonas fluorescens* F113 (87). When expressed in *Escherichia coli*, *hdtS* directs the production of three AHLs (3-OH-C_{14:1}-HSL, C₁₀-HSL, and C₆-HSL). The HdtS protein sequence does not show homology to either the LuxI or the LuxM families of AHL synthases (87). Instead, it seems to be related to the lysophosphatidic acid acyl transferase family, which is responsible for the transfer of an acyl chain from either acyl-ACP or acyl-CoA to lysophosphatidic acid, resulting in the production of phosphatidic acid. It has

been suggested that HdtS could transfer acyl chains from acyl-ACP or acyl-CoA to SAM to generate AHLs (87).

AI-2 Family of Autoinducers

LuxS-type synthases. A second autoinducer response system, independent of the AHL/LuxR-type system, controls the density-dependent expression of the luciferase (*lux*) operon in *V. harveyi* (7–9, 11). This system synthesizes and responds to a signal termed autoinducer-2 (AI-2), as opposed to the classical AHL (AI-1) based system. This second autoinducer is a furanosyl borate diester (25). AI-2 is also produced from SAM and requires three enzymatic steps (148). The use of SAM as a methyl donor produces *S*-adenosylhomocysteine (SAH). Hydrolysis of SAH results in adenine and *S*-ribosylhomocysteine (SRH), and LuxS catalyzes the conversion of SRH to 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine. DPD undergoes additional rearrangements, resulting in two fused five-member rings with a boron atom bridging the diester (25). Synthesis of AI-2 is dependent on the *luxS* gene (162), which has no homology to the *luxI*, *luxM/ainS*, or *hdtS* families. Highly conserved *luxS* homologues have been identified in both gram-negative and gram-positive bacterial species. Despite this, it is not clear that all bacteria that produce AI-2 use it as a signaling molecule. Some evidence suggests that AI-2 may be a metabolic waste product that could under some circumstances play a role in signaling (162, 163). So far, no *luxS* homologues have been found in the α -proteobacteria.

Quorum-Sensing Regulators

LuxR-type regulators. LuxR-type proteins share two regions of sequence conservation, an AHL binding domain and a DNA binding motif (154, 155). The current model suggests that LuxR acts as a dimeric protein (135, 192). LuxR regulators have an amino-terminal domain that binds AHLs and mediates protein oligomerization (28). The cytoplasmic carboxy-terminal domain includes a helix-turn-helix DNA binding region that is thought to be involved in transcriptional regulation (27, 28). Specific interactions between the cognate AHLs and purified LuxR homologues have been demonstrated by various laboratories (135, 177, 191). Studies of *A. tumefaciens* have suggested that TraR, on binding the AHL signal, undergoes a conformational change, dimerizes, and activates transcription (192). Transcriptional activation by LuxR-type proteins requires *cis*-acting DNA elements, normally referred to as *lux*-type boxes (34, 70). The typical *lux*-type box is an 18- to 22-bp inverted-repeat sequence centered at about –40 from the transcriptional start site (44). Although many target genes of the LuxR-type regulators contain *lux*-type boxes within their promoters, there are reports of targets lacking a discernible *lux* box (58, 61). Once bound, LuxR facilitates the binding of RNA polymerase to the target promoter (157–159), leading to activation of transcription. Although most instances show AHL-bound LuxR-type proteins to function as transcriptional activators, a few examples of these proteins have been reported to function as repressors (2, 13, 15).

A new and exciting development in the study of quorum sensing is in the area of structural biology. Recently the TraR protein was crystallized in the presence of 3-oxo-C₈-HSL and a

self-complementary oligonucleotide containing the canonical *tra* box sequence (171, 189). The crystal unit contains two TraR dimers, each binding two molecules of 3-oxo-C₈-HSL and one duplex DNA fragment. The N-terminal domain of each TraR monomer binds the AHL molecule, while the C-terminal domain contains a four-helix bundle that binds to half a *tra* box (171, 189). These two domains are joined by a 12-amino acid linker. The AHL binding domains seem to provide a significant dimerization interface, while the DNA binding domains add additional dimer interactions (189). Interestingly, the AHL molecule is fully embedded within the protein and shows no significant contact with solvent (171, 189). Earlier studies from the laboratory of Steve Winans suggested that TraR may require the AHL molecule as a scaffold that helps the protein to acquire a protease-resistant tertiary structure (191, 192). The recent structural studies seem to confirm the key role of the AHL in the correct folding of the nascent TraR protein (171, 189). Another interesting finding of the structural work is the overall asymmetry of the dimer complex. The TraR dimer displays a twofold symmetry axis in each domain, but the two axes of symmetry are at a 90° angle (171, 189).

The authors of one of these studies (189) propose that the autoinducer indirectly affects gene activation by increasing the stability of TraR and the formation of active dimers. The dimers are then predisposed to recognize specific TraR binding sites and activate transcription (189).

AHL Accumulation and Transport

AHL concentrations are thought to rise mainly as a result of the increase in population density (143). Although AHLs can clearly accumulate as a result of a simple increase in bacterial numbers, other factors could also influence the environmental concentration of these signal molecules. Bacterial aggregation, biofilm formation, and physical confinement could play roles in increasing the local concentration of AHLs (137). Other conditions such as high pH or enzymatic degradation could effectively decrease the concentration of signal molecules available for LuxR-type protein activation.

In the mid-1980s it was shown by Kaplan and Greenberg that radiolabeled 3-oxo-C₆-HSL from *P. fischeri* freely diffuses in and out of both *P. fischeri* and *E. coli* cells (86). Their studies showed that the distribution of 3-oxo-C₆-HSL was dictated largely by its concentration gradient across the bacterial membrane. AHLs from other bacteria were also thought to cross the bacterial envelope by diffusion, since exogenous AHLs activated LuxR-type proteins. However, with the identification of AHLs with long acyl chains, the idea that all AHLs are freely diffusible has come into question. Of particular significance is the observation by Iglewski and colleagues that the 3-oxo-C₁₂-HSL produced by *P. aeruginosa* does not rely solely on diffusion to exit the cells. In a series of elegant experiments, they showed that 3-oxo-C₁₂-HSL was subject to active export by a specific efflux pump encoded by the *mexAB-oprM* operon (124), a member of a large family of antibiotic transporters. In the absence of this pump, cellular levels of 3-oxo-C₁₂-HSL were much higher than external levels. Therefore, while the long-chain AHL was able to diffuse across the cell membranes, it was not very efficient in doing so and was likely to partition to the lipid bilayer (124). This is in contrast to the C₄-HSL also

produced by *P. aeruginosa*, which freely diffuses into and out of the cells (124).

Although active transport of an AHL has been demonstrated for 3-oxo-C₁₂-HSL only in *P. aeruginosa*, increasing numbers of organisms have been shown to produce long-chain AHLs (108, 146). It is not yet known if pumps similar to the MexAB-type pump are involved in AHL transport in those organisms. However, the efflux pump encoded by the *mexAB-oprM* operon belongs to the resistance/nodulation/cell division family, which includes a number of other multidrug resistance proteins (AcrB and AcrF from *E. coli* and MtrD from *Neisseria gonorrhoeae*) (116, 123). Interestingly, this family also includes the NolGHI system from *S. meliloti*, which may be involved in the export of nodulation signals (144).

ENVIRONMENTAL CONSIDERATIONS AND THE ECOLOGY OF QUORUM SENSING

Soil Relationships

A wide variety of soil- and plant-associated bacteria produce AHLs (24). Recent work has suggested that AHL production is more common in plant-associated than in soil-borne pseudomonads (46). In a survey study conducted in Stephen Farrand's laboratory, the pattern of AHLs produced by numerous plant-associated bacteria was analyzed by reverse-phase C₁₈ thin-layer chromatography (24). Although this technique cannot identify the exact AHL structures, it is interesting that several different species appeared to have one or more AHLs in common. For instance, *Erwinia carotovora* pv. atroseptica, several xanthomonads, *A. tumefaciens*, and several rhizobia all have an AHL with a mobility identical to 3-oxo-C₈-HSL (24). In addition, most rhizobia had an AHL that was strongly nonpolar and stayed at the thin-layer chromatography plate origin. It has been suggested that in the soil, various chemical signals serve as a bacterial Esperanto, helping microorganisms interact with or avoid each other in their quest to interrelate with their plant hosts. Elegant studies by Bassler and coworkers (7) suggested that quorum sensing modulates both intra- and interspecies cell-cell communication. It is interesting to speculate that evolution might have allowed the development of this type of chemical communication to ultimately increase cell survivability by coordinating interactions among potential bacterial competitors and their plant hosts.

AHL Mimics

A large number of bacteria associated with eukaryotes are known to regulate important phenotypes like motility, virulence, exoenzyme production, exopolysaccharide production, and antibiotic production by quorum sensing (63, 77). As stated above, a variety of plant-associated bacteria produce AHL quorum-sensing signals (24). Therefore, the potential exists for the eukaryotic hosts to disrupt this regulatory system by producing homologs (AHL mimics) to the quorum-sensing signals and thus protect themselves from pathogens by modifying bacterial behavior.

Pisum sativum (pea) and other higher plants produce AHL-mimicking compounds that interfere with the quorum-sensing-regulated behavior of several reporter strains (167). Another

example of a plant signal affecting quorum sensing in an associated bacterium is that of the Australian red alga *Delisea pulchra*, which interferes with the swarming motility of *Serratia liquefaciens*. *D. pulchra* produces halogenated furanones that are structurally similar to the AHL signals produced by *S. liquefaciens*. The furanones successfully inhibit swarming motility in *S. liquefaciens* (65). It was recently demonstrated that the halogenated furanones modulate LuxR activity through accelerated degradation of the transcriptional activator rather than by blocking or displacing the binding of the AHL signal (105).

There could be more such mechanisms that are utilized by eukaryotic hosts to interfere with the quorum-sensing behavior of associated bacteria. It would be very interesting and rewarding to investigate such systems, since they could prove to be a potential solution for the manipulation of pathogenic and symbiotic bacteria.

AHL Degradation

Another potential way to interfere with quorum sensing is through the degradation or inactivation of the AHL signal molecules. A strain of *Variovorax paradoxus* was isolated from soil based on its ability to utilize AHLs as the sole source of energy and nitrogen, an activity that could disrupt the signaling process of other bacteria sharing the same environment (88). More recently, Zhang and colleagues isolated a *Bacillus* sp. capable of inactivating AHLs. They cloned from this species a gene encoding an AHL-lactonase capable of inactivating AHLs by hydrolyzing the lactone bond (39). Furthermore, transgenic plants expressing the AHL-lactonase activity were found to be more resistant to *E. carotovora*, a plant pathogen that requires AHLs for expression of the genes necessary for pathogenicity (38).

BACTERIUM-PLANT SYMBIOTIC INTERACTIONS

The symbiotic relationships formed between the nitrogen-fixing rhizobia and their legume hosts are the result of an intricate signaling network between the host and symbiont. As yet, many aspects of the signal exchange are still a mystery; however, quorum sensing has been implicated as a key player in the symbiotic process (29, 30, 98, 106). The fact that quorum sensing regulates aspects of symbiosis is not surprising. The process of symbiosis (discussed below) leads to the concentration of bacteria in and around the plant's roots and nodules. This rise in rhizobial cell density, as determined by quorum sensing, is therefore an important component of the signaling process.

Nodulation by Rhizobia

The nodulation of leguminous plants by rhizobia is a complex and fascinating developmental phenomenon that requires a series of biochemical interactions between the bacterium and its host (18, 55, 101, 103, 140, 172) (Fig. 2). In the course of this association, the bacteria undergo chemotaxis toward the plant roots and alter the growth of the epidermal hairs on the surface of the roots such that they curl. Subsequently, the bacteria induce cell division in the normally quiescent cells of the inner

cortex of the root, which leads to the establishment of a nodule meristem. The bacteria trapped in the curled root hair induce the formation of an infection thread, a tube of plant origin, which penetrates the outer plant cells while the bacteria proliferate inside. As the nodule develops, infection threads ramify and penetrate individual target cells (79). The bacteria are surrounded by a membrane of plant origin and then released into the cytoplasm of these cells. Once released, the bacteria differentiate into morphologically altered forms termed bacteroids and begin to synthesize nitrogenase and the other proteins required for nitrogen fixation. The plant cells also differentiate and express a number of nodule-specific proteins termed nodulins, such as leghemoglobin. The symbiotic interaction results in the reduction of atmospheric dinitrogen to ammonia by the bacteroids, which is then utilized by the host plant.

Nodulation (Nod) Factors

Most of the research on rhizobia has focused on the *nod* genes (23, 100, 102), mutants of which do not induce root hair curling or nodule formation (101). Control of *nod* gene expression in rhizobia varies from strain to strain but is usually mediated by NodD, a transcriptional regulator that belongs to the LysR family. NodD binds to a conserved 47-bp region (the *nod* box) found upstream of the nodulation genes (*nod*, *nol*, and *noe*) (126). The presence of plant-produced flavonoids is generally necessary, but not essential, for the expression of the *nod* genes. Direct binding of flavonoids to NodD remains to be shown, but they clearly activate *nod* gene expression and subsequent Nod factor production (54, 112, 126, 127). The Nod factors produced by all rhizobia have the same generic lipochitooligosaccharide structure: generally, three, four, or five β -1,4-linked *N*-acetyl glucosamines, with the terminal nonreducing sugar *N* acylated by a fatty acid usually of 16 or 18 carbon atoms (35).

Biosynthesis of the Nod factor requires the *nodABC* genes. The NodC protein is a β -glucosaminyl transferase that links the UDP-*N*-acetyl glucosamine monomers. NodB removes an acetyl group from the terminal residue of the chitin-like backbone, while NodA catalyzes the transfer of a fatty acyl chain onto the resulting free amino group by using acyl-ACP from fatty acid biosynthesis (80). Many of the other nodulation genes determine the nature of the substitutions at the terminal residues and the structure of the acyl chain, both important features that play a role in determining host specificity (3, 32, 33, 45, 55, 151). The structure of the acyl moiety attached to lipochitooligosaccharide can contain one of a broad variety of acyl groups that also occur commonly as moieties of the phospholipids (for summaries, see references 21, 32, 33, 55, 92, and 138). Evidence suggests that the ratios of the common types of Nod factor acyl substituents reflect the composition of the fatty acyl pool that is present as components of the phospholipids.

Treatment of alfalfa seedlings with concentrations of purified Nod factor as low as 10^{-12} M produces an ion flow across the plant plasma membrane. This results in a depolarization of the membrane, causing periodic oscillations in intracellular calcium concentrations (calcium spiking) (for a review, see reference 40). This is followed by root hair deformation and the development of empty nodules (169) reminiscent of those

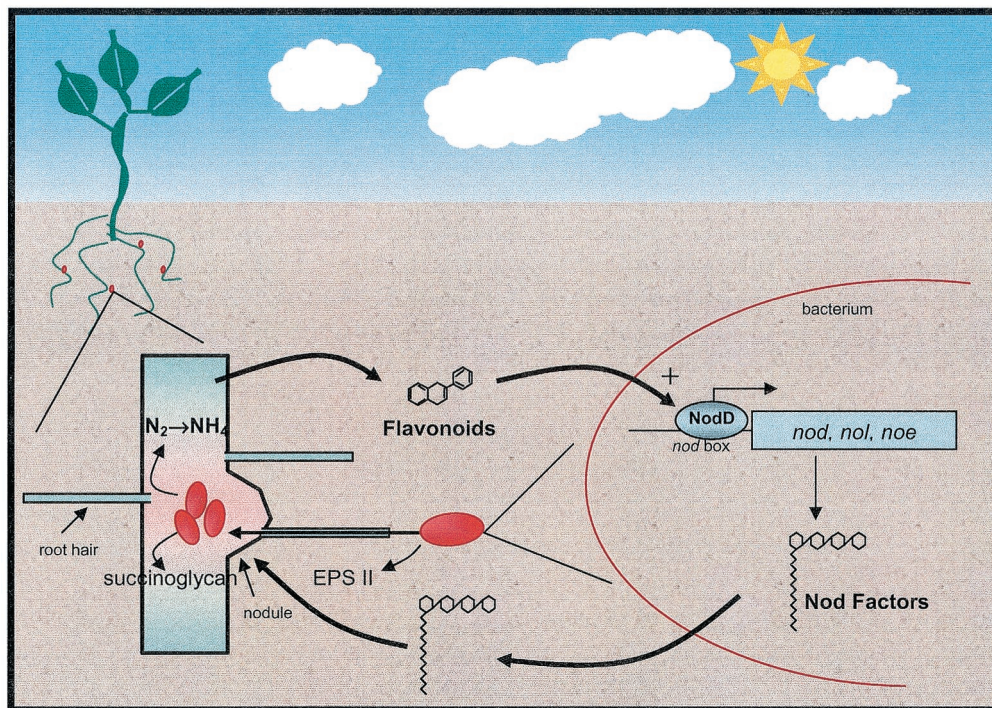


FIG. 2. *Rhizobium*-legume symbiosis model. The process of nodule invasion begins with the production, by the plant roots, of phenolic signals called flavonoids. Flavonoids serve as inducers for the NodD transcriptional activator, which then binds to conserved promoter elements, referred to as *nod* boxes, upstream of the nodulation genes (*nod*, *noe*, and *nol*). Expression of the nodulation genes results in the production of Nod factor molecules composed of a backbone of *N*-acetylglucosamine residues (two to five), a fatty acyl moiety with variable length and degrees of saturation, and various decorations on the backbone, all of which are species specific. Bacterial Nod factors then act on the plant roots to induce nodule formation and root hair curling. The bacteria are then able to invade the root hairs; this process requires the production of certain symbiotically active exopolysaccharides, which also vary depending on the species. Once inside, the bacteria undergo a differentiation process and begin expressing nitrogenase and other genes necessary for nitrogen fixation.

elicited by exopolysaccharide-deficient mutants of *Sinorhizobium meliloti* (see below). The identity of the Nod factor receptor(s) in legumes is unknown, but biochemical and genetic approaches have led to the characterization of various putative high-affinity binding sites. One interesting candidate is NFBS2, located in the plasma membrane of alfalfa cells. NFBS2 binds *S. meliloti* Nod factors with high affinity but does not select for the presence of sulfate on the reducing sugar. Sulfated *S. meliloti* Nod factors are required for optimal nodulation of alfalfa (17, 31, 115). A second candidate, Db-LNP, is an unusual Nod factor binding lectin with apyrase activity. It was shown that ATPase activity of Db-LNP increases on Nod factor binding to the lectin domain of the protein (50).

Another interesting approach to the study of Nod factor signaling is the analysis of nodulation-deficient plant mutants (160). Various plant hosts blocked in the early steps of symbiosis have been identified, and positional cloning of the plant symbiotic genes has been initiated (47, 149, 174). Analysis of these plant mutants promises to be a very active area of study in the near future.

Requirement for Exopolysaccharides in Nodule Invasion

Work from Graham Walker's laboratory and others has helped to focus attention on the importance of rhizobial exopolysaccharides in nodulation. Most rhizobia produce a variety of polysaccharides (12, 22, 89, 129), and it was hypothesized

that they might play roles in bacterium-plant interactions, such as being at least partially responsible for the host specificity of various *Rhizobium* species. Arguably, the best-characterized symbiotically important exopolysaccharides can be found in *S. meliloti*. *S. meliloti* is capable of synthesizing two different exopolysaccharides, succinoglycan and EPS II. The synthesis of at least one of these exopolysaccharides by *S. meliloti* Rm1021 is absolutely required for the development of normal nitrogen-fixing nodules (32, 33, 67, 68, 138). Mutants that are unable to synthesize either exopolysaccharide form empty nodules that lack bacteria and bacteroids (53, 90, 91, 186) and are similar to the empty nodules elicited by treatment of alfalfa roots with purified Nod factor (169). Root hair curling is delayed, and normal infection threads are not seen in the curled root hairs; infection threads are detected on sectioning, but these abort within the peripheral cells of the developing nodule (26, 91, 186). The empty nodules elicited by mutants unable to make either exopolysaccharide appear to be arrested at an intermediate state of nodule development and express only 2 of the 17 nodule-specific plant proteins (nodulins) that are synthesized in nodules containing wild-type bacteria (186).

Importance of Rhizobial Plasmids in Symbiosis

In different *Rhizobium* species, most nodulation, nitrogen fixation, and exopolysaccharide biosynthesis genes are present on one or more megaplasmids known as symbiotic (Sym) plas-

mids (4, 16, 82, 179). In contrast, *Bradyrhizobium*, *Azorhizobium*, and *Mesorhizobium* species carry most of the symbiotic information in clusters or islands found on the chromosome. Recent evidence suggests that the symbiosis island from *Mesorhizobium loti* can be transferred horizontally by conjugation from a nitrogen-fixing-proficient strain to a nonsymbiotic mesorhizobium (161). Several laboratories have demonstrated that these Sym plasmids can be cured under the proper permissive conditions without affecting growth and reproduction but that they are essential for effective symbiosis (117). In addition to the Sym plasmids, rhizobia can harbor various cryptic plasmids, most of them of unknown function.

In *S. meliloti*, for example, there are two classes of plasmids, megaplasmids and pRme plasmids (4, 5). *S. meliloti* typically contains two megaplasmids of approximately 1.4 and 1.7 Mb, termed pSymA and pSymB, respectively (6). pSymA carries genes for nitrogen fixation (*nif*) and for nodulation (*nod*), while the genes involved in the production of the symbiotically essential exopolysaccharides (*exo* and *exp*), thiamine (*thi*) biosynthesis, and dicarboxylic acid transport (*dct*) are found on pSymB (6). pSymA and pSymB are transmissible and stably maintained in new hosts, such as *A. tumefaciens* (4, 82). On the other hand, the pRme plasmids, whose number varies widely among strains, do not seem to be essential for effective nodulation and nitrogen fixation (4, 82). It is unclear what functions are carried on these plasmids, but many of them contain regions with extensive homology to the conjugal plasmid transfer genes from the Ti plasmids of *A. tumefaciens* (82). Most of these plasmids appear to be self-transmissible and could be involved not only in their own transfer but also in mediation of the low-frequency transfer of the symbiotic megaplasmids (4, 82).

A similar story can be found in *Rhizobium* sp. strain NGR234, *R. etli*, and *R. leguminosarum*. The broad-host-range strain NGR234 carries a symbiotic plasmid (pNGR234 *a*) with genes homologous to the plasmid replication (*rep*) and conjugal transfer (*tra*) genes of *Agrobacterium* Ti plasmids (56, 57). Recent work by Fuqua and colleagues showed that these genes are indeed involved in low-frequency plasmid transfer and that at least some of these *tra* genes are regulated by quorum sensing (78; also see below). *R. etli* also contains a large symbiotic plasmid (pSym) in addition to one or more "cryptic" plasmids, most of which remain uncharacterized (19, 136). Some of these plasmids are self-transmissible at a high frequency. pSym of *R. etli* CFN42 is also transmissible, but this event is dependent on the presence of the self-transmissible plasmids (20). In *R. leguminosarum*, most of the symbiotic genes are also located in a symbiotic plasmid designated pRL1JI. Recent work by Downie and colleagues has identified a cluster of genes on pRL1JI with homology to the *trb* operon and the *tra* regulators of *A. tumefaciens*, and they appear to be involved in transfer of this symbiotic plasmid (180; also see below).

QUORUM SENSING IN SELECTED RHIZOBIA

In addition to the well-characterized signal molecules (flavonoids, Nod factors, and exopolysaccharides) that are involved in the nodulation process, AHLs produced by bacterial quorum sensing can now be included in the list of symbiotic

signals. As discussed below, quorum sensing has recently been linked to various phenomena including nodulation efficiency, symbiosome development, exopolysaccharide production, and nitrogen fixation, all of which are important for the establishment of a successful symbiosis.

R. leguminosarum bv. *viciae*

Of the nitrogen-fixing rhizobia, quorum sensing is best characterized in *R. leguminosarum* bv. *viciae* (for a review, see reference 183). Several quorum-sensing systems (*rai*, *rhi*, *cin*, and *tra*) have been identified and are intertwined in a complex regulatory network (Fig. 3) (29, 95, 139, 180, 184). Early work focused on the *rhi* system, composed of *rhiR* (a *luxR* homolog), *rhiI* (a *luxI* homolog), and the *rhiABC* operon, all of which are located on the symbiotic plasmid pRL1JI (29, 139). It was demonstrated that *rhiABC* was controlled by RhiR and that flavonoids repressed the expression of both *rhiR* and *rhiABC* (29). Although the function of *rhiABC* is unknown, *rhiA* was shown to be highly expressed in the rhizosphere but not in bacteroids (36). Moreover, mutations in either *rhiA* or *rhiR* led to a decrease in the number of nodules, but only in combination with a *nodFE* mutant, leading to the hypothesis that the *rhi* operon may play a role in the early stages of the symbiotic process, as do the *nod* genes (29). Further investigation identified *rhiI* (139) and showed that it was responsible for the synthesis of several short-chain AHLs, including C₆-HSL, C₈-HSL, and another compound comigrating with C₇-HSL (139) (Table 2).

In addition to short-chain AHLs, a long-chain AHL, originally termed *small* for its bacteriocin-like activity (81, 179), was identified as 3-OH-C_{14:1}-HSL (71, 150) (Table 2). Initial experiments showed 3-OH-C_{14:1}-HSL to be an inducer of the *rhi* genes (71), but later data showing that *rhiI* and *rhiA* could be induced by short-chain AHLs (C₆-HSL, C₈-HSL, and 3-oxo-C₈-HSL) but not 3-OH-C_{14:1}-HSL suggested that the induction was indirect (139). The production of 3-OH-C_{14:1}-HSL is a result of the *cinRI* locus, located on the chromosome (95). *cinI*, the AHL synthase gene, is positively autoregulated by CinR and 3-OH-C_{14:1}-HSL. The mechanism of the regulation of *cinR* is unclear, since mutations in *cinR* or *cinI* or even the lack of the *rhi* and *tra* systems do not seem to affect *cinR* expression (95). However, *cinR* expression is cell density dependent (95). More important, though, is the observation that mutations in *cinR* and *cinI* led to decreased levels of all of the short-chain AHLs, suggesting that the *cin* system is situated at the top of the quorum-sensing network (95).

An additional plasmid (pIJ9001)-located locus, *raiRI*, has recently been identified (184); this gene synthesizes 3-OH-C₈-HSL as its major product, with C₆-HSL, C₇-HSL, and C₈-HSL as minor products (Table 2) (95). *R. leguminosarum* RaiI and RaiR have 93 and 88% identity to *R. etli* CNPAF512 RaiI and RaiR, respectively (184). As expected, *raiI* is positively autoregulated by RaiR and 3-OH-C₈-HSL but is also induced to a lesser extent by 3-OH-C_{14:1}-HSL and 3-oxo-C₈-HSL (184). These observations help explain the effect of *cinRI* mutations on the production of some of the short-chain AHLs and also suggest that an additional 3-oxo-C₈-HSL-producing locus may contribute to regulation of *raiRI* (184).

Recent work has also identified a cluster of genes on pRL1JI

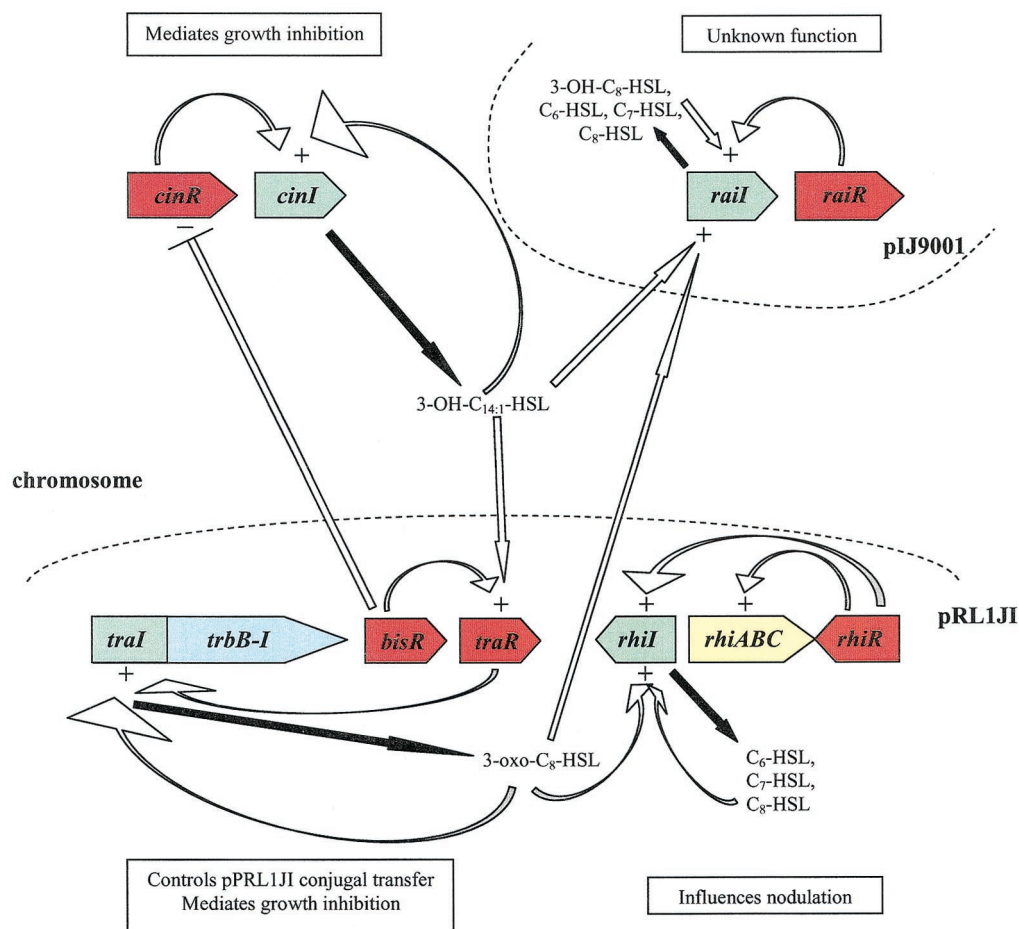


FIG. 3. *R. leguminosarum* by viciae quorum-sensing network. *R. leguminosarum* harbors four known quorum-sensing systems. The *cinRI* system resides on the chromosome and produces 3-OH-C_{14:1}-HSL, which positively influences the *tra* and *rai* systems. BisR plays a dual role in activating *traR* and repressing *cinR* in response to 3-OH-C_{14:1}-HSL, thereby linking the *cin* and *tra* systems. pRL1JI harbors both the *tra* and *rhi* systems, as well as the genes that confer growth sensitivity in response to 3-OH-C_{14:1}-HSL. The *tra* system is responsible for the production of 3-oxo-C₈-HSL and controls conjugal plasmid transfer, while the *rhi* system produces several short-chain AHLs and influences nodulation efficiency by an unknown mechanism. The *raiRI* locus resides on pIJ9001 and also produces several short-chain AHLs; however, little is known about the role of this quorum-sensing system.

with homology to the *trb* operon of *A. tumefaciens* (180). Wilkinson et al. identified *traI* (an AHL synthase), followed by the *trb* genes (which function in mating pore formation), as in *A. tumefaciens* and *Rhizobium* NGR234 (see below) (51, 57, 62, 130, 180). Interestingly, in addition to a *traR* regulator, *R. leguminosarum* carries a second regulatory gene, *bisR*, downstream of the *trb* operon. BisR has 59% identity to CinR, while TraR has 64% identity to NGR234 TraR. Furthermore, *traR* expression seems to be controlled by *cinRI* through the BisR regulator, presumably through the binding of BisR to 3-OH-C_{14:1}-HSL. TraR then goes on to control the conjugal transfer of pRL1JI by inducing the *trb* operon. Unpublished data from the laboratory of Alan Downie also suggests that *traI* is responsible for the synthesis of 3-oxo-C₈-HSL, as in *A. tumefaciens* and NGR234, which could link activation of the *tra* system to a small induction of the *rai* system. Another link in the regulatory network is the observation that BisR can repress *cinI*, suggesting a negative-feedback loop between the *cin* and *tra* systems.

Although much work has gone into characterizing the quorum-sensing network of *R. leguminosarum*, little is known about the role of these systems in the life cycle of the organism. Mutations in the *rai*, *cin*, and *tra* systems do not have any apparent defects in nodulation (95, 180, 184). The *rhi* system seems to play a role in nodulation efficiency, but no dramatic defect has been observed for *rhi* mutants that might suggest a possible mechanism (29, 139). The only system with a defined role is the *tra* system, since it was clearly shown to regulate the conjugal transfer of pRL1JI, a symbiotic plasmid (180). However, the advantage of having plasmid transfer under the control of the *cin* system is not apparent. Lastly, the growth-inhibitory role of OH-C_{14:1}-HSL and *cinRI* is still a mystery. This AHL-mediated growth inhibition was shown by Gray et al. (71) to result from an early induction of the stationary phase, but only strains carrying pRL1JI are sensitive to the growth inhibition. Furthermore, addition of OH-C_{14:1}-HSL has been shown to promote starvation survival of *R. leguminosarum* cultures that enter stationary phase at low cell density

TABLE 2. AHL production in rhizobia

Organism	AHL(s) present	System responsible for AHL production	Phenomenon regulated by quorum sensing	Reference(s)
<i>A. tumefaciens</i>	3-Oxo-C ₈ -HSL	<i>traR/traI</i>	Plasmid transfer	62, 84, 130
<i>R. leguminosarum</i> bv. <i>viciae</i>	C ₆ -HSL	<i>rhlR/rhlI</i>	Nodulation efficiency	29, 139
	C ₇ -HSL			
	C ₈ -HSL			
	3-OH-C ₈ -HSL	<i>raiR/raiI</i>	Unknown	184
	C ₆ -HSL			
	C ₇ -HSL			
	C ₈ -HSL			
	3-Oxo-C ₈ -HSL ^a	<i>traR/traI</i>	Plasmid transfer	180
	C ₈ -HSL ^a			
<i>R. etli</i> CNPAF512	3-OH-C _{14:1} -HSL	<i>cinR/cinI</i>	Growth inhibition	95
	Hydroxylated long chain AHL	<i>cinR/cinI</i>	Nitrogen fixation, symbiosome development, growth inhibition	141
	Short-chain AHLs	<i>raiR/raiI</i>	Nitrogen fixation, growth inhibition	30, 141
<i>R. etli</i> CFN42	3-Oxo-C ₈ -HSL ^a	<i>traR/traI</i>	Plasmid transfer	170
	3-OH-C ₈ -HSL	Unknown	Unknown	170
<i>Rhizobium</i> sp. strain NGR234	3-Oxo-C ₈ -HSL ^a	<i>traR/traI</i>	Plasmid transfer, growth inhibition	78
	Other short-chain and long-chain AHLs	Unknown	Unknown	78
<i>S. meliloti</i> Rm1021	C ₁₂ -HSL	<i>sinR/sinI</i>	Exopolysaccharide EPS II production	107, 108; Marketon et al., submitted
	3-Oxo-C ₁₄ -HSL			
	C _{16:1} -HSL			
	3-Oxo-C _{16:1} -HSL			
	C ₁₈ -HSL			
	C ₈ -HSL	<i>mel</i> (putative)	Unknown	108
	Other short-chain AHLs			
<i>S. meliloti</i> Rm41	3-Oxo-C ₁₄ -HSL	<i>sinR/sinI</i>	Exopolysaccharide EPS II production	107; Marketon et al., submitted
	C ₁₆ -HSL			
	C _{16:1} -HSL			
	3-Oxo-C _{16:1} -HSL			
	3-Oxo-C ₈ -HSL	<i>traR/traI</i>	Plasmid transfer	107; Marketon et al., submitted
	3-OH-C ₈ -HSL			
	C ₈ -HSL			
	Other short-chain AHLs	<i>mel</i> (putative)	Unknown	Marketon et al., submitted
<i>Bradyrhizobium</i> sp.	Bradyoxetin ^b	Unknown	Activation of <i>nod</i> genes	96, 98, 99

^a AHL designation is based on thin-layer chromatography characteristics but has not been confirmed by mass spectrometry or nuclear magnetic resonance spectroscopy analysis.

^b Not a AHL.

(168). Wilkinson et al. later showed that BisR, TraR, and the *tra* AHLs conferred sensitivity to OH-C_{14:1}-HSL, thus further complicating the reasoning for having the *cin* and *tra* systems intertwined (180).

R. etli CNPAF512

In contrast to *R. leguminosarum*, quorum sensing in *R. etli* is less well characterized but also seems less complex (Fig. 4). Although *R. etli* makes up to seven AHLs, only two quorum-sensing systems have been identified: *raiRI* and *cinRI* (30, 141). These two systems are responsible for the synthesis of all of the AHLs, since a *raiI cinI* double mutant produced no detectable AHLs (30). Rosemeyer et al. first identified *raiI* (a *luxI* ho-

molog) and *raiR* (a *luxR* homolog) and demonstrated the role of *raiI* in the synthesis of multiple, unidentified short-chain AHLs (141). In addition, this study provided some of the first evidence linking quorum sensing to symbiosis by showing that *raiI* mutants actually had a slight increase in the number of nodules per plant but that no other defects were apparent. Recent unpublished work suggests a more general role for the *rai* quorum-sensing system in nitrogen fixation (R. Daniels, personal communication).

In addition to the smaller AHLs, Rosemeyer et al. provided evidence that *R. etli* produced a long-chain AHL, related to the *R. leguminosarum* *small* (141). Although the structure of the *R. etli* AHL is still unknown, later work characterized it as a

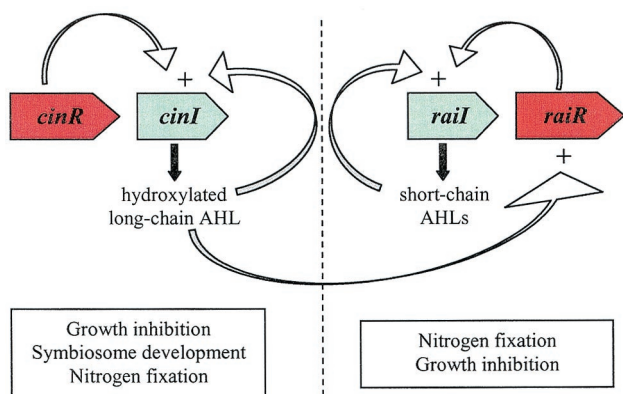


FIG. 4. Interaction of the *cinRI* and *raiRI* quorum-sensing systems in *R. etli* CNPAF512. *R. etli* CNPAF512 carries two quorum-sensing systems, *cinRI* and *raiRI*, both of which are located on the chromosome. The *cinRI* locus encodes a long-chain AHL and is involved in growth inhibition, as in *R. leguminosarum*. This locus is also required for efficient nitrogen fixation and proper symbiosome development. The *raiRI* system is positively regulated by *cinI*-produced AHL and is, in turn, responsible for the production of several short-chain AHLs. The *raiRI* locus also controls nitrogen fixation and mediates growth inhibition.

hydroxylated long-chain AHL, lacking the double bond seen in the 3-OH-C_{14:1}-HSL of *R. leguminosarum* (30) (Table 2). This long-chain AHL is synthesized by the *cinRI* locus, which has 96 and 95% identity to the *R. leguminosarum* CinR and CinI proteins, respectively (30). This 3-hydroxylated long-chain AHL has a growth-inhibitory effect on *R. leguminosarum* bv. *viciae* 248 (30).

Although the *rai* and *cin* systems affect the growth of *R. etli*, the most exciting data on these two systems came from in planta studies (30). Daniels et al. reported the first analysis of AHL production by bacteroids (30). These authors demon-

strated that compounds comigrating with the *cinI* AHL and some of the *raiI* AHLs could be extracted from *R. etli* bacteroids. Moreover, mutations in *cinI* and *cinR*, while causing no readily observable defect in nodulation, resulted in decreased nitrogen fixation as well as abnormal symbiosome development. Additionally, a *cinI raiI* double mutant had an exacerbation of the nitrogen-fixing defect. These observations provide some of the most compelling evidence supporting the idea that quorum sensing is involved in symbiosis, even though the mechanisms through which quorum sensing acts is still a mystery.

R. etli CFN42

R. etli strain CFN42 contains one chromosome and six plasmids (p42a to p42f). Plasmid p42a is self-transmissible at a high frequency (10^{-2}) and is required for mobilization of the symbiotic plasmid, p42d (19). Recently, a *traI-trb* operon, with high similarity to the transfer genes of pTi and pNGR234a, has been localized on p42a (170). Four regulatory genes, *traI*, *traR*, *cinR*, and *traM*, have also been found on this plasmid (Fig. 5). Only two AHLs have been described in *R. etli* CFN42, 3-oxo-C₈-HSL, synthesized by TraI, and a putative 3-OH-C₈-HSL, whose function and synthase remain to be identified (Table 2).

Transfer of p42a is regulated by the products of *traI*, *traR*, and *cinR*. TraI expression is dependent on itself and on the presence of TraR and CinR. The *traR* gene seems to be expressed constitutively, but expression of *cinR* requires an active TraI. The p42a plasmid also encodes a putative TraM-like antiactivator, but no expression of this gene was detected under the experimental conditions tested. Therefore, it seems that conjugal transfer of p42a is derepressed, which could account for its high transfer frequency (170). The *R. etli* CFN42 quorum-sensing system does not seem to be directly involved in the symbiotic process (derivatives of CFN42 lacking p42a

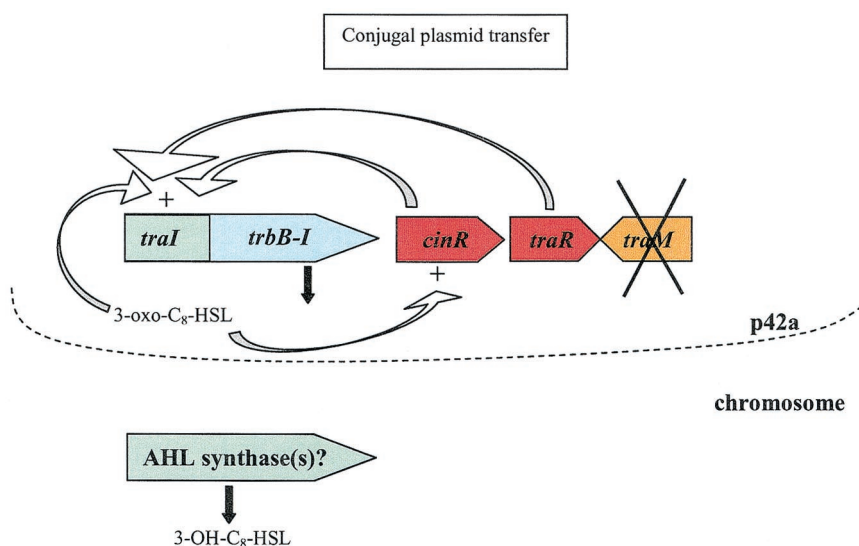


FIG. 5. Quorum sensing in *R. etli* CFN42. Quorum sensing in *R. etli* CFN42 controls conjugal plasmid transfer through the action of the *tra* genes, which are located on p42a. In this system, *traI* produces 3-oxo-C₈-HSL and is regulated by both CinR and TraR. The *traM* gene is present but does not seem to be expressed and therefore does not play a regulatory role in strain CFN42. A second AHL has also been described, but the locus responsible for its production has not been identified.

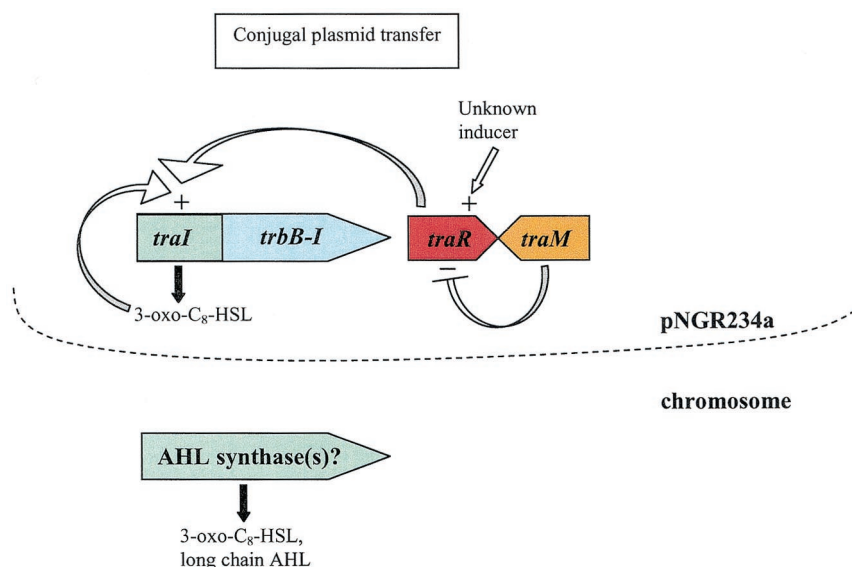


FIG. 6. Quorum sensing in *Rhizobium* sp. strain NGR234. NGR234 carries a large symbiotic plasmid, pNGR234a, which harbors the *tra* quorum-sensing system. The *tra* genes control the production of 3-oxo-C₈-HSL as well as the conjugal transfer of pNGR234a. At least one other quorum-sensing system may be present elsewhere in the genome and controls the production of 3-oxo-C₈-HSL and another long-chain AHL.

are able to effectively nodulate bean plants) (18), but it probably plays an indirect role by regulating the conjugal transfer of the p42d symbiotic plasmid.

Rhizobium sp. Strain NGR234

Rhizobium sp. strain NGR234 is unique among the rhizobia for its unusual ability to nodulate over 100 different legume species and at least one nonlegume (133). Sequencing of the symbiotic plasmid pNGR234a (57) showed a cluster of genes (*traI-trb* operon) with significant homology to the conjugal plasmid transfer (*tra*) system of *A. tumefaciens* (1, 52, 57, 93). In addition to these genes, which are required for the mating pore and DNA transfer, there were the quorum-sensing regulators, *traI*, *traR*, and *traM*, all of which are orthologues of the *A. tumefaciens* quorum-sensing regulators discussed above. Although there were no previous reports on quorum sensing in NGR234, the identification of these genes suggested that a quorum-sensing system might exist to regulate the transfer of pNGR234a, similar to the regulation of Ti plasmid transfer in *A. tumefaciens* (Fig. 6).

Recent work by He et al. showed that indeed the NGR234 *tra* system has striking similarities to that of *A. tumefaciens* (78). These authors showed that *traI* is responsible for the synthesis of an AHL that is likely to be 3-oxo-C₈-HSL. However, a *traI* mutant still produces a compound comigrating with 3-oxo-C₈-HSL and another that seems to be a long-chain AHL, suggesting that one or more additional AHL synthases may be present (Table 2). Since these remaining putative AHLs are also seen in a strain lacking pNGR234a, the synthase gene(s) must reside elsewhere in the genome. As expected, *traI* seems to be autoregulated by TraR and 3-oxo-C₈-HSL and TraR activity is inhibited by TraM, which corresponds to properties of the *A. tumefaciens tra* system. However, there are some interesting differences between the two systems. Whereas

transfer of the Ti plasmid normally occurs at a frequency of 10⁻², transfer of pNGR234a occurs at a frequency of 10⁻⁹ (78). While the cause of this extremely low transfer frequency is unknown, the authors speculated that an environmental signal, perhaps analogous to *A. tumefaciens* opines, may be required to induce higher levels of plasmid transfer. This finding is also in contrast to the transfer frequency (10⁻²) seen for *R. leguminosarum* and *R. etli*, which apparently does not require an extra signal to induce plasmid transfer. Therefore, although these three organisms carry a conserved cluster of genes involved in plasmid transfer, distinct forms of regulation have been imposed on the quorum-sensing regulators through evolution in order to customize the systems to the organisms' particular niches.

In addition to an extremely low plasmid transfer frequency, other interesting differences between the *A. tumefaciens* and NGR234 *tra* systems were observed (78). Through expression analysis of the expected TraR targets (*traI-trb*), He et al. found that the *traAFB* operon was not significantly expressed. This observation thus provides a possible explanation for the low transfer frequency of pNGR234a (78). Another possible explanation lies in the fact that the *trbE* coding sequence on pNGR234 is split into two separate reading frames, *trbE1* and *trbE2*. Although each has a reasonable start codon and Shine-Dalgarno sequence, it is possible that these do not function in conjugation, leading to the overall conjugal deficiency (C. Fuqua, personal communication). Furthermore, it appears that there may be differences in the role of TraR. Expression of TraR increased the production of the other AHLs produced by NGR234 and, in the presence of 3-oxo-C₈-HSL, resulted in growth inhibition, an observation reminiscent of the bacteriocin-like activity in *R. leguminosarum* bv. *viciae* (71, 180). Although the mechanism of the growth inhibition is unknown, data indicated that it requires one or more genes, which are not located on pNGR234a, in addition to *traI*, *traR*, and *traM* (78).

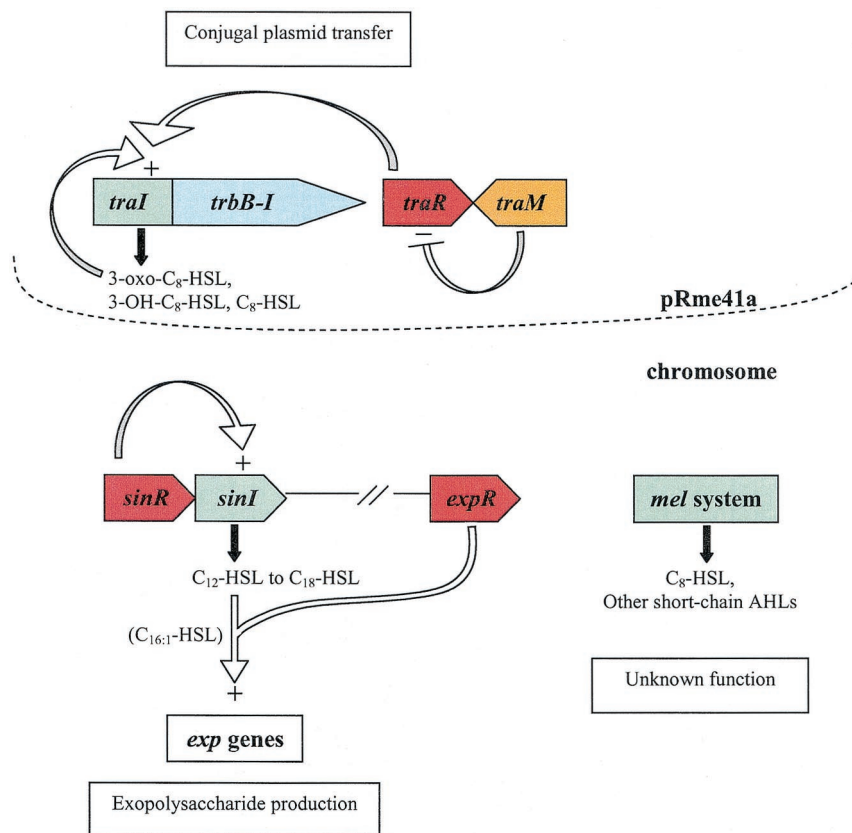


FIG. 7. *S. meliloti* quorum-sensing systems. *S. meliloti* strain Rm41 harbors three quorum-sensing systems, while strain Rm1021 carries only two systems. The *tra* system, which is carried on pRme41a and is present only in strain Rm41, produces several short-chain AHLs, including 3-oxo-C₈-HSL, and controls the conjugal transfer of pRme41a. The *sinRI* system resides on the chromosome and is present in both strains. *SinI* produces several long-chain AHLs, and at least one of them (C_{16:1}-HSL), along with *ExpR*, is required for the production of a symbiotically important exopolysaccharide, EPS II. The *mel* system, also present in both strains, produces several short-chain AHLs, but the corresponding genes remain to be identified.

S. meliloti

The well-characterized *S. meliloti* strain Rm1021 harbors at least two quorum-sensing systems (Fig. 7) (107, 108). The *sinR/sinI* locus is responsible for the production of several novel AHLs, ranging in size from C₁₂-HSL to C₁₈-HSL (108) (Table 2). Some of these are the longest AHLs identified so far. Disruption of the *sin* system correlates with a delay in the appearance of nitrogen-fixing nodules, as well as with an overall decrease in the number of pink nodules, suggesting a role for quorum sensing in establishing a successful symbiosis with *Medicago sativa* (108). More recently, it was shown that the *sinR* and *sinI* genes were required for synthesis of EPS II by a strain proficient in the production of this exopolysaccharide (106). The Rm1021 strain, which normally does not produce EPS II, has an insertion sequence that results in the disruption of *expR* (a *luxR* homologue) (125). Strains proficient in EPS II production, such as Rm41 and Rm8530 (Rm1021 *expR*⁺), possess an intact *expR* gene (106, 125). *ExpR* is a positive regulator of the *exp* genes, which are responsible for EPS II biosynthesis; however, it is unclear whether this regulatory effect is direct or indirect (66). In a *sinI* mutant, expression of several of the *exp* genes is abolished, and this deficiency can be fully complemented by the addition of either crude AHL extracts from

wild-type Rm1021 or synthetic C_{16:1}-HSL (106). Therefore, it seems that the *sinRI* locus controls EPS II production via *ExpR*. Regulation of EPS II production by *sinRI* was shown to be important for nodule invasion, since a strain that produces exclusively EPS II, combined with a *sinI* mutation, is no longer capable of forming nitrogen-fixing nodules. These results provide the first details of a mechanism for quorum sensing control in the development of symbiosis (106).

Disruption of the *sinI* gene abolishes the production of only the long-chain AHLs, while synthesis of short-chain AHLs, one of which was identified as C₈-HSL, remains unaffected (Table 2). It was proposed that these short-chain AHLs are part of a second quorum-sensing system in Rm1021, termed the *mel* system (108). While the components of the *mel* system have not yet been identified, a search of the Rm1021 genome database did not reveal any additional LuxI homologues. However, Rm1021 does carry a gene homologous to the *P. fluorescens* HdtS AHL synthase. Whether this HdtS homolog synthesizes AHLs in *S. meliloti* remains to be determined.

In addition to the *sin* and *mel* systems, a third quorum-sensing system has been identified in another commonly used *S. meliloti* strain, Rm41 (107). The *tra* system, named for its homology to the *tra* systems in *A. tumefaciens* and *Rhizobium*

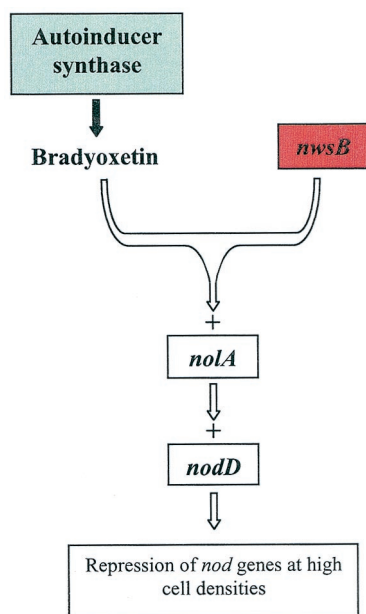


FIG. 8. Quorum sensing in *B. japonicum*. Quorum sensing in *B. japonicum* is involved in repressing the nodulation genes at high cell densities. The system, unlike other rhizobial quorum-sensing systems, is not controlled by AHLs. Instead, the autoinducer is a low-molecular-weight compound termed bradyoxetin. NwsB is part of a two-component system and is required for the detection of bradyoxetin. NwsB then induces *nola*, which in turn induces *nodD2*, leading to repression of the *nod* genes.

strain NGR234, resides on a plasmid called pRme41a. This plasmid is unique to Rm41 and therefore represents a quorum-sensing system that is present in Rm41 but not Rm1021. At least three regulatory genes (*traR*, *traI*, and *traM*), in addition to genes with homology to the *trb* operon, have been identified in pRme41a (M. M. Marketon, M. R. Gronquist, A. Eberhard, and J. E. González, submitted). TraI is an AHL synthase responsible for the production of at least three different AHLs, 3-oxo-C₈-HSL, 3-OH-C₈-HSL, and C₈-HSL (Marketon et al., submitted) (Table 2). This activity is regulated through the transcriptional activator TraR. TraM was shown to negatively regulate TraR activity in a manner analogous to the *tra* system in *A. tumefaciens*, to ensure that the *tra* system is active only at high cell densities. The *S. meliloti* Rm41 *tra* system also controls conjugal plasmid transfer, as in other organisms, by mediating the transfer of pRme41a (Marketon et al., submitted). Disruption of the *traR*, *traI*, or the *trb* genes abolishes plasmid transfer. Interestingly, transfer frequency, as in NGR234, also occurs at a fairly low frequency (about 10⁻⁷) (Marketon et al., submitted).

B. japonicum

While all the other nitrogen-fixing rhizobia characterized thus far utilize AHLs to mediate a quorum-sensing response, *Bradyrhizobium japonicum* appears to be unique (Fig. 8). Early work showed that *nod* genes seemed to be repressed at high cell densities, suggesting a quorum-sensing phenomenon, but to date no evidence of AHL production has been found (97,

99, 187). This population density-dependent control appeared to be mediated by an extracellular signal molecule termed CDF, for "cell density factor" (97, 98). The chemical structure of CDF was recently elucidated and shown to be 2-[4-[[4-(3-aminooxetan-2-yl)phenyl](imino)methyl]phenyl]oxetan-3-ylamine, also designated bradyoxetin (96–98) (Table 2). Bradyoxetin has structural similarity to certain antibiotics and to siderophores. Synthesis of bradyoxetin was found to be iron regulated, with maximal production under iron-depleted conditions (96, 98). Loh et al. identified a potential response regulator, *nwsB*, which is part of a two-component system and is required for detection of CDF (97). NwsB responds to a rise in cell density and autoinducer levels by inducing *nola* (97), which then activates the *nodD2* regulator (99). NodD2 is required for repression of the *nod* genes at high cell densities in the presence of flavonoids (97, 99). In planta studies showed that *nola* and *nwsB* were required for the repression of *nod* genes in plants (97), suggesting that quorum sensing in *B. japonicum* could mediate signaling events in the early to intermediate stages of the symbiotic process. Interestingly, bradyoxetin activity has been found in extracts of all the α -proteobacteria tested (96, 98). This suggests that compounds similar to bradyoxetin may play an important role not only in rhizobial symbiosis but also in other plant- and animal-bacterium interactions.

CONCLUSIONS AND PERSPECTIVES

Establishment of symbiosis between rhizobia and their legume hosts is a poorly understood and complex process. For example, bacterial exopolysaccharides are required for successful nodule invasion but their exact function is still unknown. Another area of uncertainty is the regulation of bacterial differentiation and host-bacterium signal exchange once the bacteria are inside the nodule. It is known, however, that the cell density of *Rhizobium* species must reach a threshold level around the plant roots before nodulation can occur (21). Therefore, it seemed likely that quorum sensing plays a role in regulating one or more stages of symbiosis. Nodule invasion requires the clustering of bacteria around the root hairs; it is therefore possible that the rise in cell density around the root hairs alters the expression of some genes. In addition, selected rhizobial strains are able to synthesize rhizopines, opine-like compounds reminiscent of those produced by *Agrobacterium* species. These compounds are synthesized by the bacteroids within the plant nodule and can be catabolized as a nutrient source by the free-living cognate rhizobia (113). These rhizopine-producing strains could potentially affect the dynamics of rhizobial soil populations.

In *R. leguminosarum* and *R. etli*, quorum sensing seems to be involved in restricting the number of nodules and in symbiosome development, although very little is known about the mechanism. In *S. meliloti*, quorum sensing controls the production of EPS II, an exopolysaccharide shown to be involved in the nodule invasion process. Most of the nitrogen-fixing rhizobia also seem to regulate conjugal plasmid transfer via quorum-sensing systems, as in *A. tumefaciens*. While these *tra* systems do not seem to be essential for nodulation, they may play a role in rhizosphere survival.

Recently, AHL-dependent gene regulation has received in-

creasing recognition as an important form of cell-cell communication in gram-negative bacteria. Often, the genes controlled by LuxR-LuxI-type quorum sensors are involved in important microbial processes, including microbe-host interactions such as pathogenesis and symbiosis. Quorum sensing also seems to regulate virulence in many human and plant pathogens. Presumably, in an attempt to avoid alerting the host immune system to their presence, quorum-sensing bacteria delay virulence factor production until their cell number is large enough that secretion of virulence factors will result in a productive infection. As our understanding of the biochemical mechanism by which bacteria synthesize and respond to AHLs increases, human intervention designed to manipulate this regulation and to harness or mollify quorum-dependent gene products will become possible. Furthermore, the observation that AHL-mediated regulation can act in a global fashion suggests that a wide range of functions may be targeted with a single strategy. It is therefore important to develop rigorous analyses of how bacteria communicate within and between species and how eukaryotic hosts talk back. The rhizobia serve as an excellent quorum-sensing model system for such studies. It seems that the seminal work of understanding the signal molecules and the regulatory systems involved in the production of those signals is now in place. The next few years should provide a wealth of information on how these quorum-sensing systems participate in the nodulation process. This information will help us elucidate bacterial cell-cell signaling in addition to the insufficiently understood prokaryotic-eukaryotic cell-signaling systems.

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