QUORUM SENSING IN \textit{SINORHIZOBIUM MELILOTI} AND EFFECT OF PLANT SIGNALS ON BACTERIAL QUORUM SENSING

DISSERTATION

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ABSTRACT

Many bacteria exchange $N$-acyl homoserine lactone (AHL) signal molecules to coordinate the behavior of individual cells. Such quorum sensing regulation controls many bacterial behaviors. The nitrogen-fixing symbiotic bacterium *Sinorhizobium meliloti* was found to produce AHLs with $C_{14}$-$C_{16}$ alkanoyl, alkenoyl and 3-oxo-alkenoyl side chains. In addition, some strains of *S. meliloti* also produced AHLs with $C_6$-$C_{10}$ alkanoyl and 3-oxo-alkanoyl side chains. Exposure of early log phase cells of wildtype *S. meliloti* 1021 to purified $C_{14}$-HL and 3-oxo-$C_{16:1}$-HL changed the accumulation of over a hundred polypeptides involved in stress responses, nitrogen and carbon cycling and utilization, nucleotide and secondary metabolite synthesis. These results provide the first global analysis of quorum sensing regulation in *S. meliloti*.

Seedlings of pea, *Medicago truncatula*, soybean, rice and tomato and cells of the unicellular alga *Chlamydomonas reinhardtii* were capable of producing substances that mimicked bacterial AHLs in well-characterized AHL reporter strains. This is the first demonstration of AHL signal-mimic compounds in eukaryotes after their discovery in a marine alga, and indicates that plants and algae may have a general ability to disrupt quorum sensing regulation in associated bacteria through secretion of quorum sensing signal mimics. Initial HPLC fractionation of AHL-mimics from plants separated about a half-dozen different activities, each affecting a different set of AHL receptors. AHL-mimics from plants may have important agricultural and industrial applications in controlling bacterial communities.
Exudates of pea seedlings were separated based on molecular weight. Low molecular weight components included the AHL-mimic compounds while the high molecular weight fraction contained root mucilage (complex polysaccharide-glycoprotein material). *Rhizobium leguminosarum*, a bacterial symbiont of pea, could use pea root mucilage and plant polysaccharides as sole carbon sources. Growth of *R. leguminosarum* on root mucilage, but not on the polysaccharides, was enhanced by the addition of micromolar concentrations of *nod*-gene inducing plant flavonoids. Several mutants of *R. leguminosarum* with altered glycosidase activity were isolated, none of which was affected in the ability to grow on root mucilage or other plant polysaccharides.
Dedicated to my grandparents
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CHAPTER 1

INTRODUCTION

The main goal of my doctoral research was to study signal exchange between rhizosphere organisms. Six chapters of this dissertation deal with various aspects of such communication. For most of the studies, acyl-homoserine lactone-mediated quorum sensing was chosen as a best studied model of bacterial communication. Initial characterization of quorum sensing in the soil bacterium *Sinorhizobium meliloti* is presented in Chapter 3. Effects of plant-secreted compounds on bacterial quorum sensing are presented in Chapters 4 and 5. Studies on metabolism of pea root mucilage by *Rhizobium leguminosarum* bv. *viciae* and roles of flavonoid signaling molecules in affecting polysaccharide degradation are presented in Chapter 6. Specific experimental goals of this doctorate research and relevant background information are presented in this chapter. Detailed descriptions of the materials and methods are presented in Chapter 2. Each chapter has a synopsis of relevant background information and an abbreviated description of materials and methods.

**Quorum sensing (QS) regulation of gene expression in bacteria**

The term "quorum sensing" describes various forms of population density-dependent communication leading to changes in gene expression (rev. Fuqua et al., 1996; Miller and Bassler, 2001). Since its discovery in a squid symbiotic bacterium *Vibrio fischeri*, QS mediated by *N*-acyl-homoserine lactone (AHL) signals has been described in many gram-
negative bacteria (Eberhard et al., 1981; Cha et al., 1998; rev. Whitehead et al., 2001; Fuqua et al., 2001). Population density-dependent gene expression is not limited to gram-negative bacteria. Gram-positive bacteria use a variety of QS signals (Khokhlov et al., 1967; Kondo et al., 1989; Loo et al., 2000; rev. Horinouchi and Beppu, 1992; Dunny and Leonard, 1997; Kleerebezem et al., 1997; Goldberg, 2002).

Both gram-positive and gram-negative bacteria use QS to coordinately express genes within a bacterial population. QS-dependent gene expression probably plays an important role in structuring of bacterial communities. For example, in both pseudomonads and streptococci, distinctly different forms of QS-dependent gene expression led to formation of biofilms, i.e. highly structured, stress-resistant multicellular aggregates (Fig. 1 and 2; Davies et al., 1998; Parsek and Greenberg, 1999; Loo et al., 2000; Lynch et al., 2002; Wen and Burne, 2002; Li et al., 2002). QS is not limited to bacterial biofilms, however. Fig. 1 and 2 illustrate the complex QS regulatory hierarchies, and give examples of well-characterized genes and behaviors known to be under QS control in the gram-negative species \textit{Pseudomonas aeruginosa} and the gram-positive species \textit{Streptococcus mutans}. In this dissertation, however, only QS-dependent gene regulation of gram-negative bacteria will be discussed in detail.

**Quorum Sensing System-1**

In gram-negative bacteria, there are at least two distinct quorum sensing systems. The first system is mediated by \textit{N}-acyl-L-homoserine lactones (AHLs), and requires perception of the AHLs by their cognate receptors, transcriptional regulators homologous to LuxR (Zhang et al., 2002a; rev. Fuqua et al., 1996). AHLs are synthesized by transfer of the acyl moiety from an acyl carrier protein onto S-adenosyl methionine (Fig. 1). Typically, this reaction is catalyzed by AHL synthases homologous to the prototypical AHL synthase LuxI of \textit{V. fischeri}. LuxM and AinS of \textit{V. harveyi} and \textit{V. fischeri}, and HdtS of \textit{P. fluorescens} represent two other classes of AHL synthases, distinct in their aminoacid sequence from each other and from all known LuxI homologs.
Synthesis of AHLs is autoregulated in many bacterial species. AHLs are produced constitutively at low population densities. When a population reaches its “quorum”, expression of the luxI-homologs is induced and more AHLs are synthesized (Nealson, 1977; rev. Fuqua and Eberhard, 1999). Such autoinduction allows amplification of gene expression in response to increasing population density.

In bacteria with multiple quorum sensing systems (e.g., *P. aeruginosa, Rhizobium leguminosarum*), there exist hierarchies where the dominant quorum sensing system controls production of other AHLs (Fig.1A.; Pearson et al., 1997; Pesci et al., 1997; Lithgow et al., 2000). Quorum sensing systems themselves can be under control of two-component environment sensing systems (Chancey et al., 1999), or stationary phase sigma factors (Fig. 1; Flavier et al., 1998; Whiteley et al., 2000; Diggle et al., 2002).

In *P. aeruginosa*, several higher level regulators further “time” the induction of AHL synthesis (Fig.1). A LysR-type regulator MvfR (Cao et al., 2001b) contributes to regulation of 3-oxo-C$_{12}$-HL and quinolone (PQS) synthesis without affecting synthesis of another major AHL (C$_{4}$-HL). MvfR is membrane-associated at low population densities. When a population reaches mid-late exponential phase, MvfR is cleaved and the inactivated protein is then delivered to the extracellular space. MvfR is itself negatively autoregulated through translocation and modification (Cao et al., 2001b). Two other novel regulators, RsmA and MvaT, affect AHL synthesis and the expression of other quorum sensing-regulated genes until the culture reaches high population density (Pessi et al., 2001; Diggle et al., 2002).

**Quorum Sensing System-2**

A second common quorum sensing system requires a signal (autoinducer-2, AI-2) which is perceived by a LuxP receptor. Perception of AI-2 then triggers a two-component system (Chen et al., 2002). Chemically, AI-2 was identified as a furanosyl borate diester (Chen et al., 2002). In *Vibrio harveyi*, a gram-negative model for studying QS system-2, AI-2 synthesis is directed by a LuxS protein (Lewis et al., 2001;
Hilgers and Ludwig, 2001; Ruzheinikov et al., 2001) which catalyzes cleavage of homocysteine from S-ribosylhomocysteine to yield 4,5-dihydroxy-2,3-pentanedione (DPD) (Schauder et al., 2001; Chen et al., 2002). It is further proposed that the cyclized DPD reacts with borate to yield an active AI-2 signal (Chen et al., 2002).

QS system-2 is widespread, and is present in both gram-negative and gram-positive bacteria: LuxS homologs and AI-2 signals were identified in vibrios, shigellas, salmonellae, escherischiae, porphyromonads, pasteurellaceae, clostridia, cocci, and bacilli (Day and Maurelli, 2001; Frias et al., 2001; Hilgers and Ludwig, 2001; Joyce et al., 2000; Malott and Lo, 2002; Ohtani et al., 2002; Schauder et al., 2001; Surette et al., 1999; Wen and Burne, 2002).

Other small molecules can also contribute to the population density-dependent communication in Gram negative bacteria. *Ralstonia solanecearum* produces volatile 3-hydroxypalmitic acid methyl ester (3-OH-PAME) (Clough et al., 1997). Mutants defective in production of 3-OH-PAME were also defective in production of extracellular polysaccharide I, endoglucanase, pectin methyl esterase, and AHL synthesis (Clough et al., 1997; Flavier et al., 1997a) suggesting a role of 3-OH-PAME in hierarchical regulation of quorum sensing (Flavier et al., 1997b). *Pseudomonas aeruginosa* PAO1 synthesizes 2-heptyl-3-hydroxy-4-quinolone (PQS) that binds the AHL receptors (Fig. 1; Pesci et al., 1999). Unlike AHLs, PQS is synthesized in stationary phase, and probably provides an additional layer of population density-dependent gene regulation (Pesci et al., 1999; Calfee et al., 2001). *P. aeruginosa* and numerous other bacterial species can produce cyclic dipeptides that can bind to the AHL receptors and affect quorum sensing-regulated behaviors (Holden et al., 1999). However, binding of cyclic dipeptides to the AHL receptors is 10-100 fold less efficient than that of the cognate AHLs. The role of cyclic dipeptides in quorum sensing is still unclear.
Objective 1: Purification and chemical identification of AHLs produced by *S. meliloti* strains.

Members of the *Rhizobiaceae* family, including *Rhizobium, Sinorhizobium, Bradyrhizobium, Mesorhizobium*, and *Agrobacterium* can establish intricate symbiotic or pathogenic relationships with plants. Symbiotic rhizobia infect cortical cells of legumes, where bacteria differentiate into bacteroids to form nitrogen-fixing nodules (rev. Oke and Long, 1999b; Long, 2001). Agrobacteria can infect parenchymatous plant cells, and integrate parts of their DNA into plant cells' genomes. The most common disease caused by pathogenic rhizobia is *Agrobacterium* crown gall. The formation of the gall is directed by the Ti plasmid-encoded bacterial auxin and cytokinin synthases injected by *A. tumefaciens* into the plant cells and integrated into the plant’s genome. Within the gall, the plant’s cellular metabolism is further redirected by the Ti plasmid-encoded genes to produce opines. Opines are sugar-aminoacid conjugates used as a C- and N- source for the infecting bacteria (rev. Zhu et al., 2000).

Establishment of the plant-rhizobium symbiosis or disease is a complex process involving multiple recognition signals from both partners (Fisher and Long, 1992; Peters et al., 1986; rev. Long, 2001; Zhu et al., 2000). Roles for AHL-mediated communication in symbiosis and crown gall tumorigenesis have been suggested (Hwang et al., 1995; Rosemeyer et al., 1998; rev. Zhu et al., 2000). Important similarities and differences exist in quorum sensing systems of *A. tumefaciens, R. leguminosarum* and other rhizobia.

In *A. tumefaciens*, the only characterized QS system is borne on the Ti plasmid and directs the plasmid’s conjugal transfer (rev. Zhu et al., 2000). The TraI AHL synthase catalyzes synthesis of 3-oxo-C$_8$-HL (Zhang et al., 2002a), which is perceived by the cognate receptor TraR. The AHL is bound by the $\alpha/\beta/\alpha$ sandwich at the N-terminal of TraR. Binding of 3-oxo-C$_8$-HL leads to the dimerization of the protein. The helix-turn-helix DNA binding motif at the C-terminus of the receptor can then bind to the palindromic DNA sequences (*tra* boxes) at the promoter regions of the regulated genes (Fuqua and Winans, 1996; Zhang et al., 2002a).
QS in *A. tumefaciens* is regulated at multiple levels. The TraR protein controls expression of *traI*. In turn, *traR* expression is regulated by opine catabolism regulator AccR and is autoregulated by TraR (Piper et al., 1999). TraR is also controlled at the functional level by an antiactivator TraM, which interacts with the DNA binding C-terminal domain of TraR. Interactions of TraM with TraR were shown to be independent of the AHL levels and seem to depend on the relative concentrations of the two proteins (Luo et al., 2000). An orthologous plasmid-borne TraR/TraI/TraM QS system was recently reported in a strain of *S. meliloti* (Marketon and Gonzalez, 2002). BLAST searches revealed homologous plasmid-borne *traR/traI/traM* sequences in *Rhizobium vitis, Rhizobium sp.NGR234, Rhizobium rhizogenes*, suggesting that the TraR/TraI/TraM QS system maybe wide spread in the *Rhizobiaceae*.

Additionally, an AHL-inactivating metalloenzyme, AiiB (AttM), was identified in *A. tumefaciens* (Zhang et al., 2002a). AiiB degrades AHL signals when cells reach stationary phase, thus controlling quorum sensing at the level of signal availability. Based on BLAST searches, an aiiB(attM)-like metallo-β-lactamase was also present in *R. etli*, but not in *S. meliloti* 1021.

In contrast with *A. tumefaciens* C58, *R. leguminosarum, R.etli* and *S. meliloti* have at least one chromosomally-encoded QS system. These three organisms share an orthologous *cinRI*(*sinRI* in *S. meliloti*) QS system. The CinR and CinI from *R.etli* and *R.leguminosarum* share 95% aminoacid identity and a common genetic organization of the cluster (Fig 3A, B). CinI proteins from both species may synthesize the same AHL (Daniels et al., 2002; Lithgow et al., 2000).

In *R. leguminosarum*, the AHL-synthase CinI directs synthesis of 3-OH-C_{14:1}-HL (Lithgow et al., 2000). 3-OH-C_{14:1}-HL can arrest cell division (Schripsema et al., 1996) and facilitate carbon-starvation survival and entry into stationary phase (Gray et al., 1996; Thorne and Williams, 1999) in cells carrying various symbiotic plasmids (pSym). Lithgow et al., (2000) reported that 3-OH-C_{14:1}-HL is the dominant AHL in the quorum
sensing hierarchy: it stimulates production of short chain AHLs by a putative RaiI AHL synthase and positively regulates the pSym-borne \textit{rhi} and \textit{tra} quorum sensing systems.

The Rai QS system is also common to \textit{R. leguminosarum} and \textit{R. etli}. In both species, the AHL synthase RaiI catalyzes synthesis of AHLs with medium-length acyl side chain (Rosemeyer et al., 1998; Lithgow et al., 2000). In \textit{R. etli}, both \textit{rai} and \textit{cin} mutants had altered (~2x) growth rates with extended lag and log phases (Daniels et al., 2002).

Strains of \textit{Rhizobium spp.} carry different symbiotic plasmids which determine their host-specificity. There is evidence that the different symbiotic plasmids can encode additional quorum sensing systems (Lithgow et al., 2001). For example, in \textit{R. leguminosarum} 8401 \textit{bv viciae} pRL1JI, the symbiotic plasmid pRL1JI carries at least two quorum sensing systems: TraRI and RhiRI. The Tra quorum sensing system of \textit{R. leguminosarum} is similar to the Tra quorum sensing system of \textit{A. tumefaciens} and was implicated in regulating plasmid transfer (Lithgow et al., 2000). \textit{rhi} QS system induces expression of the \textit{rhiABC} operon leading to production of an abundant rhizosphere protein with unknown functions (Rodelas et al., 1999) and has a moderate effect on nodulation.

QS is probably important in symbiosis. QS mutants of \textit{R. etli} had 30-40\% reduced rates of acetylene reduction (Daniels et al., 2002; Rosemeyer et al., 1998). Nodules formed by \textit{cinI} mutants of \textit{R. etli} were histologically different from those formed by the wild type. CinI mutant bacteroids were always individually packed within the symbiosome membrane, while wild type symbiosomes contained multiple bacteroids (Daniels et al., 2002). Mutation in \textit{raiI} moderately reduced nodulation and acetylene reduction (Rosemeyer et al., 1998).

The purification and identification of AHLs produced by \textit{S. meliloti} 1021 and \textit{S. meliloti} AK 631, and their effects on polypeptide accumulation in the wild type strain \textit{S. meliloti} 1021 are described in Chapter 3 of this dissertation.
**Objective 2: Role of quorum sensing in interactions between bacteria and eukaryotes. Isolation of AHL-mimics from plants.**

Functional quorum sensing systems are required for successful interactions of many gram-negative bacteria with their eukaryotic hosts. For example, various quorum sensing mutants of *P. aeruginosa*, a broad host-range opportunistic pathogen, were less infective than the wild type bacteria in plant, nematodes, amoeba, and mice models (Rahme et al., 2000; Rahme et al., 1995; Cao et al., 2001a). Based on a screen of transcriptional fusions constructed in an AHL synthase mutant, at least 200 genes were under quorum sensing control in this bacterium (Whiteley et al., 1999). Many quorum sensing-regulated genes of *P. aeruginosa* code for known virulence factors (Whiteley et al., 1999; Rahme et al., 1995; Rahme et al., 2000; rev. Cao et al., 2001a).

Plant pathogens (*Erwinia* spp. and *Pantoea* spp.) rely on quorum sensing to coordinately produce cell-wall degrading enzymes and exoproducts (Pirhonen et al., 1993; Beck von Bodman and Farrand, 1995; Bainton et al., 1992; McGowan et al., 1995). This strategy allows *Erwinia* spp. and *Pantoea* spp. to multiply within the plant tissues without triggering plant defense responses until the bacterial populations are high enough to swamp defenses of the plant hosts.

Because AHLs are used by many bacteria that attack or interact with a eukaryotic host, it is important to ask whether eukaryotes have evolved the means to detect AHLs and respond appropriately. AHLs from pathogenic bacteria were able to affect physiology and gene expression in several mammal systems. AHLs were active in picomolar concentrations, but the responses to AHLs differed between the systems (Telford et al., 1998; Saleh et al., 1999; Lawrence et al., 1999; Smith et al., 2002).

AHLs also have effects on plants. Our laboratory has shown that exposure to micromolar concentrations of synthetic AHLs (3-oxo-C$_{12}$-HL and C$_{6}$-HL) affected accumulation of over 100 polypeptides in a model legume, *Medicago truncatula* (Mathesius et al., 2002 submitted). Some of the polypeptides that were differentially accumulated in *M. truncatula* in response to the AHLs were also accumulated in response
to bacterial infections. These initial studies of eukaryotic perception of bacterial quorum sensing signals only begin to tackle the question of AHL recognition by higher organisms.

Since it appears that eukaryotes can detect and respond to bacterial AHLs, it is also important to ask whether eukaryotes can actively interfere with bacterial quorum sensing. In this regard, pioneering studies by the Kjelleberg lab have shown that a marine red alga, Delisia pulchra, produces a diversity of halogenated furanones that are structurally related to AHLs (Fig. 4; de Nys et al., 1993). These furanones specifically inhibit AHL-regulated QS in a variety of bacteria (Givskov et al., 1996; Kjelleberg et al., 1997). This suggests that eukaryotes not only "listen" to bacterial communications, but can also disrupt their signaling.

We discovered that various terrestrial plant and algal species can produce molecules capable of affecting or disrupting quorum sensing in various associated bacteria (Teplitski et al., 2000). An initial characterization of AHL-mimics produced by terrestrial plants and algae, and their effects on gene expression and behavior of different bacteria are presented in Chapters 4 and 5.

Objective 3: Utilization of plant polysaccharides as a sole carbon source by wildtype Rhizobium leguminosarum bv. viciae and mutants.

There has been considerable interest in the synthesis and secretion of polysaccharide degrading enzymes by rhizobia, with the thought that such enzymes from the bacteria might be essential for locally degrading the host root hair cell walls at the site of penetration and infection (Callaham and Torrey, 1981). R. leguminosarum bv. trifolii, for example, has been shown to synthesize an extracellular pectinase and a cellulase (Jimenez-Zurdo et al., 1996; Mateos et al., 1992), enzymes known to be crucial to the maceration of primary cell walls. Recent electron microscopy studies by Mateos et al. (2001) demonstrated that rhizobial cell-associated glycanases play an important role in degrading non-crystalline polymers of the cells walls. Degradation of the cell wall matrices lead to formation of a pit and facilitated infection of the cortical cells by R.
leguminosarum bv trifolii (Mateos et al., 2001). Rhizobial polysaccharide-degrading enzymes were thought to be important for local degradation of the cell walls to facilitate cell-cell transmission of the infection thread (van Spronsen et al., 1994). We sought to determine whether glycanases produced by R. leguminosarum bv. viciae could contribute to its saprophytic survival.

We focused on rhizobial degradation of pea root mucilage. It appears that high molecular weight mucilage secreted by the root cells is probably an important source of carbon in the rhizosphere. Chaboud et al. (1983) estimated that about 20%-25% of the total reduced carbon released by maize roots is in the form of high molecular weight root mucilage. Thus, mucilage may represent a significant carbon source for soil microbes. Structure of pea root mucilage has been recently characterized (Knee, 2001).

Knee et al (2001) reported that several soil bacteria, including strains of R. leguminosarum, could use plant polysaccharides and pea root mucilage as a sole carbon source. Interestingly, Knee et al (2001) reported higher cells yields of R. leguminosarum grown on pea root mucilage with the addition of a plant flavonoid naringenin. These findings suggested a role for plant signals in regulation of polysaccharide degradation by R. leguminosarum. I further wanted to test whether such plant-derived signals affected growth of R. leguminosarum on plant polysaccharides. The results of these studies are presented in Chapter 6. The roles of several bacterial glycanase mutants in pea root mucilage degradation are also discussed.
Figure 1: Regulatory QS hierarchy in *Pseudomonas aeruginosa*. In a bacterial cell, entering late log phase, the synthesis of the dominant AHL (3-oxo-C₁₂-HL, shown as a lavender-colored octagon) is catalyzed by the LasI AHL synthase from SAM (S-adenosyl-methionine) and 3-oxo-C₁₂-acyl carrier protein. The AHL receptor, LasR (pink oval) binds its cognate signal, 3-oxo-C₁₂-HL, and this complex regulates expression of the downstream genes, including *rhlI*. RhlI catalyzes synthesis of another AHL, C₄-HL (shown as an orange octagon). Regulators (Vfr, RsmA, RsaL, RpoS, DksA, MvfR) further regulate QS as described in text. Synthesis of PQS (2-heptyl-3-hydroxy-4-quinolone, shown as a purple octagon) from anthranilate and β-keto-decanoic acid is also shown, though the synthesis of PQS probably does not take place until entry into stationary phase. Thinner gray arrows (bars) indicate transcriptional activation (repression), orange arrows represent protein synthesis, lime-green arrows indicate activation of a receptor. Transport of the molecules across the membrane is indicated by multicolored thick arrows.
Figure 2. Hypothetical two-receptor model of *Streptomyces spp* quorum sensing (based on Li et al., 2002). The CSP (competence-stimulating peptide) is synthesized from the precursor peptide, encoded by *comCDE*. The CSP is perceived by a receptor-kinase ComD. A second hypothetical receptor is proposed to control bacterial cell separation.
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Figure 3A. ClustalW multiple alignment of CinI/SinI sequences of R. etli, R. leguminosarum and S. meliloti 1021. The sequence of ORF SMc 00168 (putative AHL synthase SinI) is from the genomic database (Galibert et al., 2001). Identical aminoacid residues are shaded in black, similar residues are shaded in gray. The sequences were aligned using ClustalW Multiple algorithm version 1.4 (Thompson et al., 1994).
Figure 3B. ClustalW multiple alignment of CinR/SinR sequences of *R. etli*, *R. leguminosarum* and *S. meliloti* 1021. The sequence of ORF SMc 00170 (putative AHL receptor SinR) is from the genomic database (Galibert et al., 2001). Identical aminoacid residues are shaded in black, similar residues are shaded in gray. The sequences were aligned using ClustalW Multiple algorithm version 1.4 (Thompson et al., 1994).
Figure 4. Structural comparison of an AHL (A = 3-oxo-C₆-HL) with other molecules that can bind to the AHL receptor proteins. B = halogenated furanone produced by *D. pulchra*, possible R-groups are indicated. C = 2-heptyl-3-hydroxy-4-quinolone (PQS) of *P. aeruginosa*. D = cyclo(L-Phe-L-Pro), a QS dipeptide isolated from *P. aeruginosa* cultures.
CHAPTER 2

ISOLATION, DETECTION, PURIFICATION AND SYNTHESIS OF BACTERIAL N-ACYL-HOMOSERINE LACTONES

ABSTRACT

N-acyl-homoserine lactones (AHLs) are quorum sensing signal molecules. AHLs were isolated by extraction from the cell-free bacterial culture filtrates with acidified ethyl acetate. AHLs and AHL-mimics were purified by reverse phase high performance liquid chromatography (HPLC) using water: acetonitrile gradients. The signalling molecules were detected by bioassay with a set of bacterial AHL reporters that utilize receptors for different AHLs. Collectively, these reporters can identify a wide range of AHLs and AHL-mimic compounds. Protocols for AHL and AHL-mimic purification and detection are described.

INTRODUCTION

As discussed in Chapter 1, N-acyl L-homoserine lactones (AHLs) are the best-studied of the quorum sensing signals. Both chemical and biological methods have been developed for the detection of AHLs and related molecules (Ravn et al., 2001; Schaefer et al., 2000; Charlton et al., 2000; Cha et al., 1998).
Chemical assays most frequently rely on identification of the AHL signature mass spectral peaks. Fragments \( m/z \) 102 \((\text{NH}_7\text{C}_4\text{O}_2)\), and 143 \((\text{NH}_8\text{C}_6\text{O}_3)\) are typical for all \(N\)-acyl homoserine lactones, and \( m/z \) 185 is generated only by 3-oxo-AHLs. Mass spectral analysis of the bacterial supernatants allows identification of small AHL amounts when they were reasonably pure.

Another sensitive method for identification of AHLs with a keto group at \(C_3\) was developed by Charlton et al. (2000). It is based on the chemical conversion of 3-oxo-HLs to their penfluorobezlyoxime derivatives, followed by electron capture-negative ion gas chromatography mass spectrometry (GC MS). The method has great advantages: derivatization can be carried out with very small, rather crude samples, and allows accurate identification of 100-200 fmoles of 3-oxo-HLs. Detected concentrations are generally 2 to 3 orders of magnitude lower then those identified with the bioassays described below. This assay, however, requires an electron capture detector which was not available. Alkanoyl-HLs and alkenoyl-HLs cannot be derivatized, and therefore cannot be identified by this method.

Biological methods to detect AHLs and related compounds were developed (McClean et al., 1997; Cha et al., 1998; Swift et al., 1997; Winson et al., 1998). The first AHL bioassays exploited changes in phenotypes (pigment production and motility) when the reporter bacteria were exposed to certain AHLs (McClean et al., 1997; Wood and Pierson, 1996; Givskov et al., 1996). Recently, a variety of chimeric reporter constructs was developed. These reporters consist of AHL receptors linked to luminescent, gfp or \(\beta\)-galactosidase cassettes (Winson et al., 1998; Steidle et al., 2001; Andersen et al., 2001; Cha et al., 1998) so that addition of an exogenous AHL to the reporter bacterium (which makes no AHLs of its own) increases luminescence, fluorescence or color development. Unlike chemical identification methods, bioassays are very cheap and require minimal instrumentation. Most importantly, bioreporters allow in situ (often real-time) detection of nM concentrations of AHLs or related molecules. Cyclic dipeptides, quinolones, AHL-mimics from \(D.\ pulchra\) (Fig. 4), -- biologically-
active molecules that bind to AHL receptors -- were detected using biological reporters (Holden et al., 1999; Givskov et al., 1996). Detection of AHLs using the reporters is usually coupled with thin layer (TLC) or HPLC. The TLC plates or individual HPLC fractions are then tested for biological activity.

REPORTERS AND BIOASSAYS FOR AHL DETECTION

**Chromobacterium violaceum reporter.** As evident from its name, *C. violaceum* produces dark-purple pigment, violacein. By subjecting the wild type strain to random mini-Tn5 mutagenesis, an AHL-deficient mutant of *C. violaceum* was isolated (McClean et al., 1997). This mutant, CV026 (Str<sup>+</sup> miniTn5 Hg<sup>+</sup> cviI::Tn5xylE Km<sup>+</sup>) does not produce its cognate AHL (C<sub>6</sub>-HL) and therefore does not produce violacein (Table 1). Addition of AHLs with a short alkanoyl (C<sub>4</sub>-C<sub>8</sub>) side chain restores violacein production. The AHL reporter strain *C. violaceum* CV026 responds to 0.03-0.05 nmoles of C<sub>6</sub>-HL and C<sub>6</sub>-homocysteine thiolactone, to nmoles of C<sub>4</sub>- and C<sub>8</sub>-HL, 3-oxo-C<sub>6</sub>- 3-oxo-C<sub>8</sub>-HL and their homocysteine thiolactones, to 10-20 n mole of 3-oxo-C<sub>4</sub>-HL, and 3-oxo-C<sub>6</sub>-D-HL (McClean et al., 1997). In addition to AHLs, 100 µM solutions of bacterial cyclo(L-Pro-L-Val), and nM levels of some synthetic acyl-homocysteine thiolactones can induce violacein production in this reporter (Holden et al., 1999; McClean et al., 1997). Induction of violacein synthesis by short-chain AHL<sub>s</sub> can be semi-reversibly inhibited by addition of 1-5 nM of AHL<sub>s</sub> with a long (C<sub>10</sub>-C<sub>12</sub>) alkanoyl or 3-oxo-alkanoyl side chain. Likely, this inhibition is achieved by (competitive) binding of the AHL<sub>s</sub> to the receptor, CviR. Synthetic D-isomers were at least 10-100x less active than *N*-acyl-L-homoserine lactones (McClean et al., 1997). Algal furanones also inhibited AHL-stimulated violacein production in this reporter (Kjelleberg et al., 1997). In our experiments, the reporter was insensitive to a range of concentrations of flavonoids (naringenin, luteolin, daidzein, 4’,7-dihydroxyflavone, and
3’,5’-methoxyflavone), homoserine, homoserine lactone or the dipeptide \( \gamma \)-glutamyl alanine. All these compounds were earlier identified in pea exudates (Van Egeraat, 1975a; Knee, 2001; Rolfe, 1988).

In our experiments, violacein production was inconsistent in Luria-Bertani (LB) shake cultures. In LB solidified with 0.3-0.5% agar, violacein was produced consistently in the top 1-2 mm of the agar. To estimate violacein production, several organic solvents (acetone, acetonitrile and 95% ethanol) were tested for their ability to extract violacein. All three solvents extracted the pigment in one step. There were some spectral differences in the violacein preparations, e.g. The spectrum of the acetone extract was shifted toward red. Ethanol (95%) was selected for further experiments because it extracted violacein completely (with the absorbance maximum at 575 nm, Fig. 5) and was generally easy to work with.

In addition to violacein synthesis, production of hydrogen cyanide, extracellular chitinases and exoprotease are all under quorum sensing control in \textit{C. violaceum} (Chernin et al., 1998; Winson et al., 1994). I tried to quantify HCN production in CV026 cultures using Microquant 14798 CN detection kit (E.Merck, Darmstadt, Germany). However various medium components significantly interfered with the assay. Chitinase and exoprotease activity were quantified using colorimetric substrates as in Chapter 3.

**Pseudomonas aureofaciens reporters.** Strain 30-84 of \textit{P. aureofaciens} is used as a biocontrol agent against the fungal pathogen \textit{Gaeumannomyces graminis}, causal organism of take-all in wheat. The antifungal biocontrol properties of \textit{P. aureofaciens} are due, in part, to the production of phenazine, an orange antibiotic (Pierson and Thomashow, 1992; Mazzola et al., 1992; rev. Pierson and Pierson, 1996). Phenazine production is controlled by \( C_6 \)-HL via PhzR in a population density-dependent manner (Wood and Pierson, 1996; Zhang and Pierson, 2001; Pierson et al., 1994). Wood et al. (1997) isolated an AHL-deficient mutant, \textit{P. aureofaciens} 30-84I (\textit{phzI::kan Kan}^r). The mutant (Table 1) does not produce phenazine, unless exposed to \( C_6 \)-HL or high concentrations of \( C_4 \)-HL.
In our experiments, *P. aureofaciens* 30-84I was generally insensitive to other AHLs. For quantification, phenazines could be extracted as described (Pierson and Thomashow, 1992; Mazzola et al., 1992).

*Agrobacteriun tumefaciens* reporter. When exposed to tumor metabolites (opines), *A. tumefaciens* C58 produces 3-oxo-C₈-HL, which is perceived by the TraR AHL receptor protein transcribed from the *traR* gene, located on the Ti virulence plasmid (Zhu et al., 2000; Fuqua and Winans, 1994). TraR regulates transcription of the downstream *tra* operon, involved in conjugal transfer of the virulence plasmid to the cells lacking the plasmid. Cha et al. (1998) constructed a TraR-based reporter by curing the wild type strain of its Ti plasmid. *traR* and *traG::lacZ* were independently cloned into the broad host range plasmid pBBR1MCS5 (Kovach et al., 1995), resulting in pZLR4. The reporter strain, *A. tumefaciens* NT1 (pZLR4) (Amp/Car, Gm), is sensitive to a broad range of alkanoyl (except C₄-HL), alkenoyl, 3-oxo-alkanoyl, and 3-hydroxy-alkanoyl-HLs (Table 1; Cha et al., 1998). In detection of 3-oxo-HLs, *A. tumefaciens* NT1 (pZLR4) was generally 10x more sensitive than *C. violaceum* CV026 (Shaw et al., 1997). This reporter also responds to 50-100 µM concentrations of cyclic dipeptides isolated from bacterial culture filtrates (Holden et al., 1999).

*A. tumefaciens* NT1 (pZLR4) was grown at 27°C in AB medium supplemented with 0.2% glucose (Chilton et al., 1974). For our bioassays, AB glucose was solidified with 0.3% agar. Five hundred microliters of an exponentially growing culture (OD₆₀₀=0.4-0.8) were suspended in 5ml of AB glucose agar with 40 µg X-gal (Cha et al., 1998; Shaw et al., 1997). Eighty microliters of this agar suspension was then added to the wells of a microtiter plate with the residue of HPLC fractions to be tested. X-gal hydrolysis - an indication of the reporter activity in response to AHLs - was evaluated visually. For more quantitative bioassays, I used the reporter in a liquid culture containing *p*-nitro-phenyl-β-D-galactopyranoside or the fluorescent substrate MU-GAL.
However, the reporter was significantly (10-100x) less sensitive in a liquid culture, a phenomenon we observed with other AHL reporters (S. liquefaciens PL10, C. violaceum CV026, P. aureofaciens 30-841).

**Serratia liquefaciens reporters.** *S. liquefaciens* is a common bacterium causing opportunistic infections in plants, animals and humans (Grimont and Grimont, 1978; Steidle et al., 2001). The wild type *S. liquefaciens* MG1 was isolated from a rotten cucumber by Givskov et al. (1997). The AHL synthase SwrI of *S. liquefaciens* MG1 produces C$_4$-HL and minor amounts of C$_6$-HL. Binding of these AHLs to SwrR induces synthesis of a lipopeptide surfactant, serrawettin, and facilitates surface swarming of the cells (Lindum et al., 1998). SwrR-C$_4$-HL quorum sensing also regulates accumulation of at least 26 polypeptides (Givskov et al., 1998). Mutation in SwrI abolishes synthesis of the AHLs (both C$_4$- and C$_6$-HL), and, consequently, the synthesis of serrawettin (Givskov et al., 1996; Lindum et al., 1998). The resulting mutant, MG44 (*luxI::kan*, Kan$^r$) forms swarm colonies, but with 2-3 days delay, compared to the wild type (Givskov et al., 1997). The delay could be overcome by addition of C$_4$- or C$_6$-HL. Swarming in this mutant could also be facilitated with exogenous surfactants.

MG44 was then randomly mutagenized with a miniTn$^5$:luxAB. The mutants luminescing in response to AHLs addition were isolated. One of these mutants, PL10 (*swrI*, *swrA::Tn5luxAB Kan$^r$ Tet$^r$) was characterized (Lindum et al., 1998; Table 1). A surfactant synthesis gene is disrupted in *S. liquefaciens* PL10. The mutant swarms in response to added surfactants (Lindum et al., 1998), and luminesces when exposed to C$_4$- or C$_6$-HL.

For our swarming assays, *S. liquefaciens* MG44 and PL10 were grown overnight in AB with Casamino acids and glucose (ABCAG) with ampicillin (50 µg/ml) or tetracycline (10 µg/ml) at 27°C to late log phase. The cells were then pelleted, and resuspended in 10 volumes of sterile water, and stab inoculated or 2-5µl were spotted
onto a glass fiber disk, and incubated on ABCAG solidified with 0.6% agar (as in Givskov et al., 1998). Swarm colonies were photographed with a Polaroid camera. Swarm assays were also conducted in 24 well microtiter plates followed by overnight incubation.

Our luminescence assays with PL10 were generally carried out and quantified as described below for *E. coli* lux reporters. Unlike *E. coli* lux reporters described below, *S. liquefaciens* PL10 required incubation in the *n*-decanal-saturated atmosphere inside a plastic container for 1-5 minutes to provide the substrate for the luciferase. Both *S. liquefaciens* reporters were sensitive to C₄- or C₆-HL. Other AHLs (3-oxo-C₆-HL and 3-oxo-C₁₂-HL) did not induce swarming or luminescence of the reporters, and did not inhibit these phenotypes in the cells already induced with same concentrations of C₄-HL. AHL-mimicking furanones from *D. pulchra* affect both of these reporters, presumably by binding to SwrR and inhibiting swarming initiation in the wild type, and interfering with AHL-induced phenotypes in the reporters (Givskov et al., 1996). Micromolar levels of some cyclic dipeptides produced by bacteria were likewise able to inhibit swarming initiation in MG44 in a medium with 150 nM of C₄-HL (Holden et al., 1999). By themselves, 15nM-15µM of the same cyclic dipeptide did not stimulate swarming of the *S. liquefaciens* mutants.

**E. coli lux reporters.** A set of highly sensitive chimeric luminescence reporters was constructed recently (Swift et al., 1997; Winson et al., 1998; Table 1). The plasmid constructs are introduced into *E. coli* JM109 (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac-proAB) mcrA, a bacterium that doesn’t synthesize AHLs. The reporters are constructed by cloning a gene coding for an AHL receptor from *Vibrio fischeri* (*luxR*), *P. aeruginosa* (*lasR*), or *Aeromonas hydrophila* (*ahyR*) next to a proper promoter sequence (from the corresponding AHL synthase gene) and a *luxCDABE* cassette, resulting in three multicopy reporter plasmids, pSB401, pSB1075 and pSB536 (respectively). Recognition
and binding of an AHL to its receptor leads to transcription of the lux operon, synthesis of luciferase and its substrate and the production of light. These reporters do not require exogenous luciferase substrate.

Responses to synthetic AHLs for reporters containing pSB401 and pSB1075 reporter plasmids were reported by Winson et al. (1998). *E. coli* JM109 (pSB401) (*luxRI'::luxCDABE Tc*) luminesces strongly in response to 10^{-7}-10^{-3} M solutions of *N*-alkanoyl (*C_6*-C_{12}*)-HLs and 10^{-9}-10^{-3} M solutions of 3-oxo-alkanoyl (*C_6*-C_{12}*)-HLs. This reporter was shown to detect non-AHL signalling molecules. For example, cyclic L-Met-L-Pro at mM concentrations also significantly activated *E. coli* JM109 (pSB401). The same levels of other cyclic dipeptides (cyclo (L-Pro-L-Val), cyclo (L-Leu-L-Pro), cyclo(L-Met-L-Pro) extracted from culture filtrates of *P. aeruginosa* antagonized stimulation of the reporter by a cognate 3-oxo-*C_6*-HL (Holden et al., 1999).

*E. coli* JM109 (pSB1075) (*lasRI'::luxCDABE Ap*) is activated by 10^{-9}-10^{-3} M solutions of *N*-alkanoyl (*C_{12}-C_8*)-HLs and 10^{-11}-10^{-3} M levels of 3-oxo-alkanoyl (*C_{12}-C_8*)-HLs. This reporter is significantly less sensitive to shorter chain (*C_6*-C_{4}) alkanoyl and 3-oxo-alkanoyl HLs. It has a dynamic response range in only 10^{-5}-10^{-3} M solutions of the short chain AHLs. In experiments with bacterial AHL-mimics, *E. coli* JM109 (pSB1075) was activated by µM concentrations of cyclo (L-Met-L-Pro) and cyclo(Δ-Ala-L-Val) (Holden et al., 1999).

*E. coli* JM109 (pSB536) (*ahyRI'::luxCDABE Ap*) responds to short chain alkanoyl AHLs, most strongly to its cognate C_{4}-HL. Swift et al. (1999) reported that alkanoyl and 3-oxo-alkanoyl *C_{10}-C_{14}*-HLs can antagonize binding of the cognate AHL to the AHL receptor AhyR.

For our bioassays to detect AHLs or plant AHL mimics, the luminescent reporters were grown at 37°C overnight in LB with appropriate antibiotics. The cultures were then diluted 200-fold, grown for 2 h, and diluted 100-fold again in fresh LB with antibiotics. The culture was grown to OD_{600}=0.2 then centrifuged and the pellet re-
suspended in 10 original volumes of LB. Eighty microliters of this reporter suspension were then added to the wells of black microtiter plates containing dried HPLC fractions, and incubated at 37°C for 3 to 4 hrs inside a Ziploc bag to minimize evaporation. The luminescence was measured with Wallac Victor-2 multimode microtiter plate reader (PerkinElmer). Relative luminescence was calculated as luminescence in a fraction (counts per second) minus an average of background luminescence of the “reporter only control”, divided by the background luminescence of the reporter. In general, while absolute luminescence counts were variable, calculated relative luminescence was very consistent.

**Vibrio harveyi reporters.** *V. harveyi* is a luminescent marine pathogen of fish, crustaceans and molluscs (Diggles et al., 2000; McDougald et al., 2000). Two different quorum sensing systems contribute to regulating luminescence in *V. harveyi* strains. The first quorum sensing system is based on production and perception of 3-hydroxybutanoyl homoserine lactone (3-OH-C₄-HL) (Cao and Meighen, 1989). This AHL is synthesized by AinS, an AHL-synthase apparently unique to *vibrios*. LuxR homologue perceived 3-OH-C₄-HL contributes to the regulation of the lux operon. *V. harveyi* also produces another quorum sensing signal. This signal, a product of a LuxS protein, is referred to as AI-2 (Schauder et al., 2001). Recently, the AI-2 signal was purified from a receptor-signal complex. Chemically, AI-2 is a furanosyl borate diester, distinct from other known quorum sensing molecules (Chen et al., 2002). In the same study, LuxP was identified as the AI-2 receptor in *V. harveyi*. QS system-2 acts through a phosphorelay two-component system LuxPQ, which contributes to regulation of luminescence (Bassler et al., 1994).

It appears that luminescence and virulence in *V. harveyi* are coordinately regulated (Manefield et al., 2000; Liu and Lee, 1999). Manefield et al., 2000 reported that furanones, produced by *D. pulchra*, interfere with luminescence of *V. harveyi* and attenuate virulence in the tiger prawn host model. Another putative AHL-mimic, [1-(2'-methylpropoxy)-2-hydroxy-2- methylpropoxy]butane from *V. angustum* can interfere with luminescence induction in *V. harveyi* (de Nys et al., 2001).
Because *V. harveyi* luminesces in response to quorum sensing molecules, we used wild type strains BB120, 403, 404, 697, and ATCC 14126 (Joyce et al., 2000; Pedersen et al., 1998; Table 1) for the bioassays. Two defined reporter strains BB886 and BB170, generous gifts from Dr. Bassler, were also used in our studies. BB886 is a *luxQ*:Tn5 mutant deficient in perception of AI-2, and therefore works as a luminescent AHL reporter. BB170 is a *luxN*:Tn5 mutant, that does not perceive its congnate AHL and therefore serves as a specific AI-2 reporter.

For our bioassays, *V. harveyi* strains were grown overnight from glycerol stocks in Autoinducer Bioassay (AB) medium (Joyce et al., 2000), then diluted 5,000 times. As a positive control for BB170 bioassays, 10-20 µl of AI-2-containing culture filtrate of wild type *S. enterica* Typhimurium 14028 were added to one of the wells. The culture filtrates were prepared as described elsewhere (Joyce et al., 2000; Surette et al., 1999). Eighty microliters of the diluted cultures were added to the wells of a microtiter plate, shaken in the microtiter plate reader and incubated at 27°C sealed inside a Ziploc® bag. After 2 h incubation, the plate was transferred into the chamber of the microtiter plate reader, and luminescence measurements were taken every 20 min until the relative luminescence reached a maximum. The data were recorded and the relative activity calculated when the difference between the negative and positive control was the greatest.

For plate overlays, the overnight culture was diluted into AB medium solidified with 0.3% agar. The overlays were incubated for 3-4 hrs, and the luminescence was measured with Hamatsu CCD camera. False color images were overlaid in Adobe Photoshop 5.0.
<table>
<thead>
<tr>
<th>Reporter strains</th>
<th>AHL-regulated phenotype</th>
<th>Receptor</th>
<th>Cognate signal</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. violaceum</em> CV026</td>
<td>Violacein, exoprotease, exochitinase, HCN</td>
<td>CviR</td>
<td>$C_6$-HL</td>
</tr>
<tr>
<td><em>P. aureofaciens</em> 30-84I</td>
<td>phenazine</td>
<td>PhzR</td>
<td>$C_6$-HL</td>
</tr>
<tr>
<td><em>S. liquefaciens</em> MG44</td>
<td>Surfactant production and surface spreading</td>
<td>SwrR</td>
<td>$C_4$-HL, $C_6$-HL</td>
</tr>
<tr>
<td><em>S. liquefaciens</em> PL10</td>
<td>Luminescence (chimeric construct)</td>
<td>SwrR</td>
<td>$C_4$-HL, $C_6$-HL</td>
</tr>
<tr>
<td><em>A. Tumefaciens</em> NT1 (pZLR4)</td>
<td>β-galactosidase (chimeric construct)</td>
<td>TraR</td>
<td>3-oxo-$C_8$-HL</td>
</tr>
<tr>
<td><em>E. coli</em> JM109 (pSB401)</td>
<td>Luminescence (chimeric construct)</td>
<td>LuxR</td>
<td>3-oxo-$C_6$-HL</td>
</tr>
<tr>
<td><em>E. coli</em> JM109 (pSB536)</td>
<td>Luminescence (chimeric construct)</td>
<td>AhyR</td>
<td>$C_4$-HL</td>
</tr>
<tr>
<td><em>E. coli</em> JM109 (pSB1075)</td>
<td>Luminescence (chimeric construct)</td>
<td>LasR</td>
<td>3-oxo-$C_{12}$-HL</td>
</tr>
<tr>
<td><em>V. harveyi</em> wild types (BB120, 403, 404, 697, and ATCC 14126)</td>
<td>luminescence</td>
<td>LuxR and a putative AI-2 receptor</td>
<td>AHL, AI-2</td>
</tr>
<tr>
<td><em>V. harveyi</em> BB 886</td>
<td>luminescence</td>
<td>LuxR</td>
<td>3-OH-$C_4$-HL</td>
</tr>
<tr>
<td><em>V. harveyi</em> BB 170</td>
<td>luminescence</td>
<td>LuxP</td>
<td>Furanosyl borate diester</td>
</tr>
</tbody>
</table>

Table 1. Relevant characteristics of the QS signal reporter strains used throughout these studies.
AHLs were extracted from late-exponential or early-stationary phase bacterial cultures. It is thought that the accumulation of AHLs is maximal at this point (Nealson, 1977; Schaefer et al., 2000), however there exist important temporal differences in the kinds and amounts of AHLs produced in some bacteria (Blosser-Middleton and Gray, 2001). We harvested early stationary phase cultures by centrifugation and extracted the cell-free supernatants twice with the same volume of acidified ethyl acetate (0.1ml of acetic acid per liter of ethyl acetate). Acidification may significantly contribute to the AHL recovery (Ravn et al., 2001), however the importance of acidification to prevent lactone hydrolysis of AHLs has been disputed (A.Eberhard, personal communication). Organic extracts were then dried over anhydrous sodium sulfate, filtered, and the solvents removed by rotary-evaporation over a 40°C water bath. The resulting yellow oily preparations were then stored at -20°C.

For semi-preparative reverse phase HPLC, the extracts were brought up in 1ml of 50% acetonitrile:water, spun down and injected onto a 10 x 250 mm C\textsubscript{18} column (ODS-3, Alltech). The samples were eluted with an acetonitrile: water gradient at 2 ml/min. Different gradients were used, depending on the purpose of the purification. Specific conditions for different gradients are described in the subsequent chapters. Absorbance was monitored at 210 nm since AHLs absorb weakly at that wavelength. Individual fractions were collected into glass tubes. Prior to HPLC, the glass tubes were washed in a 10% solution of KOH in 95% ethanol, washed extensively with tap water, and the residual KOH was neutralized by a 30-min soak in 5% HCl. The tubes were then rinsed extensively in tap water, and then rinsed with distilled water. This washing procedure is important in preventing AHLs from becoming bound to the impurities on glass surfaces.
SYNTHESIS OF $C_4$-HL

$C_4$-HL was synthesized essentially as described by Chhabra et al. (1993). One mmole of triethyl amine was stirred overnight with 1 mM aqueous solution of L-homoserine lactone hydrochloride, and 3 mmoles of butyric anhydride. The resulting solution was dried *en vacuo*, then partitioned against water:ethyl acetate mix (1:4). The organic phase was collected, washed first with 5% NaHCO$_3$, then with 1M KHSO$_4$, followed by a brine (saturated NaCl solution in water) wash. Triethyl amine, butyric anhydride, L-HL-hydrochloride were purchased from Sigma Chemical Co. (St Louis, MO). Purity of the synthetic $C_4$-HL was confirmed by HPLC and gas chromatography, and the biological activity tested with the appropriate AHL reporters. The structure was confirmed by EI-MS. Other synthetic AHLs were either purchased from Aurora Sciences (Coralville IN) or were generous gifts from Dr. Anatol Eberhard (Ithaca College, NY)
Figure 5. Ultraviolet light absorption spectrum by violacein pigment produced by *C. violaceum* CV026 treated with C₄-HL as described in this Chapter for the *Chromobacterium* reporter.
CHAPTER 3

PLANTS SECRETE SUBSTANCES THAT MIMIC BACTERIAL N-ACYL HOMOSERINE LACTONE SIGNAL ACTIVITIES AND AFFECT POPULATION-DENSITY DEPENDENT BEHAVIORS IN ASSOCIATED BACTERIA

ABSTRACT

In gram-negative bacteria, many important changes in gene expression and behavior are regulated in a population-density dependent fashion by N-acyl homoserine lactone (AHL) signal molecules. Exudates from pea (*Pisum sativum*) seedlings were found to contain several separable activities that mimicked AHL signals in well characterized bacterial reporter strains, stimulating AHL-regulated behaviors in some strains while inhibiting such behaviors in others. The chemical nature of the active mimic compounds is presently unknown, but all partitioned differently in organic solvents than did common bacterial AHLs. Various species of higher plants, in addition to pea, were found to secrete AHL mimic activities. The AHL signal-mimic compounds could prove to be important in determining the outcome of interactions between higher plants and a diversity of pathogenic, symbiotic and saprophytic bacteria.
INTRODUCTION

In recent years, it has become clear that many important behaviors in bacteria are regulated in a population-density dependent manner, including those that are crucial to attacking or colonizing plant, and animal hosts (Chapter 1; rev. Whitehead et al., 2001; Cao et al., 2001a). A recent study has revealed that transcription of over 250 genes is affected by the AHL signaling system in Pseudomonas aeruginosa (Whiteley et al., 1999). In P. aeruginosa, which can be a pathogen of both plants and animals, it has been shown that AHL receptor (LasR) mutants of the bacterium have reduced ability to induce disease symptoms in Arabidopsis (Rahme et al., 2000), similar to the reduced virulence of LasR mutants in animals (de Kievit and Iglewski, 1999; Cao et al., 2001a).

The synthesis of AHL signals is common among plant-associated bacteria (Chapter 1; Cha et al., 1998; von Bodman et al., 1998; Lithgow et al., 2000) and AHL signaling has been well studied in several of these bacteria (Pierson et al., 1999 for review). A given bacterial species can commonly synthesize several AHL signals that differ from one another in the length of the N-acyl side chain (4-16 carbons), the presence or absence of double bonds, or side chain substituents (keto, hydroxyl) (Swift et al., 1999a). There is evidence that AHLs secreted by cells of one species can induce population-density dependent responses in cells of other bacterial species in natural rhizosphere environments (Pierson et al., 1998).

Furthermore, bacteria from Salmonella, Escherichia and Klebsiella genera do not produce their own AHLs, but have functional AHL receptors. Michael et al. (2001) have shown that an AHL receptor from S. enterica detects and binds a variety of AHLs produced by other bacteria, and this detection leads to changes in its gene expression. Such interpopulation AHL signaling may be significant to structuring of the mixed bacterial communities.

An important recent study has demonstrated that halogenated furanones produced by the marine red algae Delisea pulchra are able to disrupt AHL-regulated behaviors in
*Serratia liquefaciens*, *Agrobacterium tumefaciens*, *Erwinia carotovora* and other bacterial species (Givskov et al., 1996; Kjelleberg et al., 1997; Manefield et al., 2001; Chapter 1). This ability could be a very valuable, co-evolved adaptation for any plant, enabling it to favor potential bacterial symbionts and deter potential pathogens. The discovery of AHL signal-mimic compounds in an alga raises the possibility that higher plants might also synthesize and secrete compounds that mimic the activity of bacterial AHL signal compounds. A recent study by Daniels et al. (2001) detected AHL-mimics produced in the nodules of a common bean, and in the roots of aseptically grown plants. The secretion of AHL mimic compounds could have important effects on bacterial colonization and infection of host plants, and might be of considerable medical and agricultural interest.

In an attempt to learn whether higher plants secrete substances that mimic AHL signal molecules in regulating population-density dependent behaviors, we have relied on several bacterial reporter strains carefully developed by others to detect exogenous AHLs, specifically *Chromobacterium violaceum* strain CV026 (McClean et al., 1997), *S. liquefaciens* strains MG44 and PL10 (Givskov et al., 1996; Lindum et al., 1998), *Pseudomonas aureofaciens* 30-84I (Wood and Pierson, 1996), *A. tumefaciens* (pZLR4) (Cha et al., 1998) and plasmid AHL reporters in *E. coli* (Winson et al., 1998; Swift et al., 1997) carrying a luxCDABE cassette activated by the LuxR receptor of *Vibrio fischeri*, the AhyR receptor of *Aeromonas hydrophila*, or the LasR receptor of *P. aeruginosa*. Strains of *P. aureofaciens*, *P. aeruginosa*, *A. tumefaciens*, *C. violaceum* and *S. liquefaciens* are often found in association with plants and the latter four species have also been recognized as opportunistic pathogens of humans (e.g., Rahme et al., 1997; Gupta et al., 1999; Hussain and Vancura, 1970; Grimont and Grimont, 1978; Miller et al., 1988; Hulse et al., 1993; Zhu et al., 2000). Our results indicate that the AHL signal-mimic compounds capable of affecting AHL-regulated behaviors in bacteria are common among higher plants.
**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Stock cultures of *S. liquefaciens* MG1 wild type and the reporter strains MG44 and PL10, generously provided by M. Givskov and S. Kjelleberg, were cultured on AB medium with glucose and Casamino acids as described by Givskov et al. (1997) with 50 µg/mL kanamycine (km50) or 10 µg/mL tetracycline (tc10) respectively. Cultures of *C. violaceum* wild type ATCC31532 and the reporter strain CV026 were generously provided by P. Williams, and cultured in LB broth. *A. tumefaciens* (pZLR4), a gift from Dr. S.Farrand, was grown at 27°C in AB medium supplemented with glucose as a C-source and gentamicin at 50 µg/mL (Cha et al., 1998). Lux plasmid AHL reporters *E. coli* JM109 (pSB401) (*luxRI'::luxCDABE Tc*), *E. coli* JM109 (pSB536)(*ahyRI'::luxCDABE Ap*), and *E. coli* JM109 (pSB1075)(*lasRI'::luxCDABE Ap*), a generous gift from S.Swift, were grown in LB broth with appropriate antibiotics as described by Winson et al. (1998). *P. aureofaciens* 30-84 and its *phzI* mutant, 30-84I (Wood and Pierson, 1996; Wood et al., 1997), a gift of F. Gong, were cultured on LB. See Table 1 for other relevant strain characteristics. All strains were maintained as glycerol stocks at -80°C and small samples were subcultured into fresh medium with antibiotics (when necessary) for the assays.

**Plant material.** Pea (*Pisum sativum*) ‘Extra Early Alaska’ (Livingston Seed Co., Columbus, OH) seeds were surface sterilized for 90 min in 95% ethanol, followed by 60 min in 70% commercial Chlorox bleach. Crown vetch (*Coronilla varia*) ‘Penngift’ seeds (Livingston Seed Co., Columbus, OH) were scarified for 10 min in concentrated sulfuric acid, and then surface sterilized in 95% ethanol for 30 min followed by 50% Chlorox for 30 min. Seeds of *Medicago truncatula* genotype A17 (a gift from Dr. D. Cook) were surface sterilized for 60 min in 95% ethanol, followed by 10 min treatment with full strength commercial Chlorox bleach. Soybean (*Glycine max*) ‘Flint’ (from Dr. S. St. Martin), *Arabidopsis thaliana* ColO (from Dr. J.C. Jang), tomato (*Lycopersicon*
esculentum) ‘Rutgers Select VFA’ (Geo. W. Park Seed Co., Inc, Greenwood SC), rice (Oryza sativa) ‘Nipponbare’ (from Dr. G.L. Wang) and romaine lettuce (Lactuca sativa) ‘Paris Island’ (Livingston Seed Co., Columbus, OH) were surface sterilized for 30 s in 95% ethanol and 15 min in 50% commercial Chlorox bleach.

The surface-sterilized seeds were extensively rinsed with sterile tap water, imbibed for 4-6 h in sterile water and germinated in the dark on moist Whatman #1 paper. After the seedlings were harvested, the germination paper was touched to the surface of an LB agar plate to check for microbial contamination. Only uncontaminated seedlings were used for the bioassays.

Preparation of pea exudates and extracts. Batches of ca. 100 aseptically grown, 7-d-old pea seedlings (and the washed, autoclaved Whatman filter paper used to maintain moisture for the seedlings) were flooded with 100 mL of sterile water and gently swirled for several min to collect the crude seedling rinsate. One hundred microliter samples of the rinsates were plated on LB agar to check for microbial contamination and contaminated batches were discarded.

Contaminant-free rinsates were spun down, filtered, frozen and lyophilized. On the average, 5-10 mg of dry crude exudate were collected per one hundred seedlings. Lyophilized exudate preparations were mixed with 50% methanol:water (1 mL per 10 mg of lyophilized material) and centrifuged after 15 min to remove insoluble materials. The 50% methanol extract was used for routine assays of violacein, swarming, and exoenzyme activity, as shown in Figures 7, 8 and 9. For further purification of activities, an additional volume of 100% methanol was added to the 50% methanol extract to bring the extracts to 95% methanol. Isopropanol (one volume of isopropanol to four volumes of 95% methanol:water) was then added to precipitate inactive contaminating materials. After 15-20 min incubation, the insoluble material (approximately 25% by mass) was removed by centrifugation. All extraction steps were carried out at room temperature.
The organic solvents were removed by rotary evaporation at 30°C. The resulting yellow, viscous exudate extract was dissolved in 1.0 mL water per 10 mg dried residue for the assays in Figures 7, 8 and 9.

**High performance liquid chromatography of pea extract.** For reverse phase HPLC chromatography, 1 mL of a 30% methanol:water solution of the isopropanol-precipitated extract prepared as described above, containing exudate from about 650 seedlings, was injected onto a water-equilibrated semi-preparative C\textsubscript{18} column (Whatman Partisil 10 ODS-3). The column was eluted for 15 min in water, followed for 45 min with a linear water:acetonitrile gradient at a flow rate of 3 mL/min. Absorbance was monitored at 254 nm. Fractions were collected every minute and bioassayed with the reporters as described below.

An amino analytical column (Alltech Associates Inc., Deerfield, IL) and an analytical guard-column packed with loose NH\textsubscript{2}-bonded silica (35-75 microns, Alltech Associates Inc., Deerfield, IL) were equilibrated in 15:75 (water:methanol). Biologically-active fractions eluting in the void volume of C\textsubscript{18} column were pooled, lyophilized and brought up in 90% methanol:10% water. The activity was eluted at 0.5mL/min isocratically for five min with 15%:75% (water:methanol), and then with linear gradient for 10 min to reach 100% methanol, and then isocratically for five minutes. Aliquots of the individual fractions were bioassayed as described above.

**Bioassays for AHL mimic compounds from plants.**

**Violacein and phenazine production.** *C. violaceum* CV026, *P. aureofaciens* 30-84 and its C\textsubscript{6}-HL\textsuperscript{-} mutant 30-84I were grown in LB broth overnight on a shaker at 27°C. Five hundred microliters of the cultures, containing ca 2-3 x10\textsuperscript{9} bacteria were spun down and resuspended in 5 mL of warm LB containing 0.5% agar. For indirect CV026 bioassays to measure inhibition of violacein synthesis by seedlings or exudate, 3.5 µg of C\textsubscript{4}-HL
dissolved in 7 µl of ethyl acetate were added to the soft agar suspension of bacteria. For assays with live seedlings (Fig. 6, Table 2), the bioassay agar suspensions were poured on a surface of regular LB agar, and aseptically grown seedlings were placed on the thin layer of soft agar containing the bacteria. As controls, glass fiber disks containing appropriate AHLs were placed on the surface of the bioassay agar. To semi-quantitatively estimate the ability of seedling exudates or exudate extract preparations to inhibit C₄-HL-induced pigment synthesis, 20-30 µl samples from a 2- or 3-fold serial dilution of the exudate or extract (with organic solvents removed by evaporation) were added to the wells of a 96-well Falcon microtiter plate and mixed with 50 mL of the warm bioassay agar containing CV026 and C₄-HL (or P. aureofaciens 30-84I with no AHL) as described above. The plates were incubated for 15-25 h at 27°C. 

Violacein was extracted from the wells with 700 mL of 95% ethanol, and absorbance measured at 575 nm as described in Chapter 2. The reversibility of pea exudate-induced inhibition of violacein synthesis was tested by adding an additional 0.5 µg/mL of C₄-HL either 0 or 4 h after mixing of CV026 bioassay agar with one seedling equivalent of the pea exudate extract and incubating for 20h.

To test whether compounds in pea root exudates can degrade or immobilize C₄-HL, HPLC fractions containing violacein synthesis inhibiting activity from 25 pea seedlings were re-dissolved in 250 mL of sterile distilled water and mixed with 50 mL of a solution containing 25 mg of C₄-HL in water and incubated overnight. As a control, 300 mL of sterile distilled water containing 25 mg of C₄-HL were incubated similarly. C₄-HL was extracted twice with 2 mL of ethyl acetate and the solvent removed by rotary evaporation. The C₄-HL residue was re-dissolved in sterile distilled water and samples from a 2-fold dilution series were assayed by the semi-quantitative microtiter plate method described above.
Luminescence bioassays using the CCD camera. *S. liquefaciens* PL10 was grown overnight at 27°C in AB with glucose and casamino acids (Eberl et al., 1996b) with tc10. *E.coli* JM109 (pSB401) (LuxRI’), *E.coli* JM109 (pSB536) (AhyRI’), and *E.coli* JM109 (pSB1075) (LasRI’) were grown at 37°C in LB broth with 10 µg/ml of tetracycline or 100 µg/ml of ampicillin. The bacterial suspensions were then centrifuged, and the pellets resuspended in 10 volumes of sterile water to an A$_{600}$ of ca 0.10. Aseptically grown, etiolated seedlings were completely submerged in the suspension of bacteria, then gently blotted on a piece of sterile absorbent tissue and incubated on the surface of AB agar at 27°C (for PL10) or LB agar at 37°C (for the plasmid reporters) for 5 to 9 h. As controls, suspensions of the reporter strains were inoculated onto small slabs (3x20x70mm) of nutrient agar, incubated and then observed at the same time as the seedlings. For the positive controls, the agar slabs contained a high, maximally activating concentration of C$_4$-HL for the LuxRI’ and AhyRI’ (2 µM) and PL10 (6 µM) reporters or 3-oxo-C$_{12}$-HL (5 µM) for the LasRI’ reporter. No AHL was added to the agar slabs of the negative controls. Images of the seedlings and luminescing bacteria were taken with a Hamamatsu C2400 intensified CCD camera and analyzed with an Argus-20 image processor. The false color images were superimposed on the digital images of the seedlings in Adobe Photoshop v.4.0.1. The total number of photons emitted and counted per second from the entire surface area of the seedling (or corresponding streak colonies for the controls) was determined. The relative luminescence of the reporter strain growing on an inoculated seedling was then calculated by subtracting the background luminescence of the uninduced reporter strain (growing on agar containing no AHL), and then expressing this net luminescence as a percentage of the luminescence of the positive controls. Relative luminescence values were normalized to equal numbers of reporter bacteria based on recovery of the reporter bacteria from seedling and agar surfaces and plating on agar to determine the number of colony forming units.
For quantitative bioluminescence bioassays, 20-30 µl of seedling exudate extract or HPLC fractions were placed onto 11 mm diameter Whatman GFC glass fiber discs. After evaporating the samples to dryness in a laminar flow hood, the discs were inoculated with 10 µl of 10-fold diluted cultures of the luminescence reporters and then incubated for 5-6 h in the shallow wells of a 96-well microtiter plate lid with 25 µl of AB glucose/Casamino acids medium (PL10) or LB (plasmid reporters) prior to CCD camera measurement of luminescence. Luminescence bioassays with PL10 required exposure to n-decanal vapor (30 µl in a large lidded box for 8-20 min) prior to luminescence measurements.

**Swarm assays.** *S. liquefaciens* MG44 and PL10 were grown overnight in AB with Casamino acids and glucose with amp50 or tc10 at 27°C to a late log phase. The cells were then pelleted, and resuspended in 10 volumes of sterile water. Aseptically grown seedlings or sterile toothpicks were dipped in the reporter suspensions, rinsed in sterile water, blotted on a sterile absorbent tissue and incubated for 10-15 h on a surface of AB agar with glucose and Casamino acids as in Givskov et al. (1997). Swarm colonies were photographed with a Polaroid camera. Swarm assays were also conducted in 24 well microtiter plates by inoculating the surface of agar containing seedling exudate or extract with the MG44 reporter strain followed by overnight incubation.

**Protease assays.** Late log phase suspensions of *C. violaceum* CV026 (10 µl) were added to 1 mL of fresh 1/10 strength LB medium containing 0.5 µg/mL C₄-HL and 0, 1, 5 or 15 seedling equivalents of pea exudate extract, with the organic solvents previously removed by evaporation. After 20 h of shake culture growth at 27°C, the bacteria were removed by centrifugation and the culture supernatants assayed for protease activity with azocasein (Sigma Chemical Co.) as described (Gerhardt et al. 1994). Similarly, *C. violaceum* wild type was inoculated into 1/10 LB medium with or without pea exudate extract and with or without 3-oxo-C₁₂-HL (2.3 µg/mL) and assayed after 20 h incubation for protease activity on azocasein.
Chitinase assays. For exochitinase (p-nitrophenyl-β-D-N-acetylglucosaminidase) activity, 10 µl of CV026 overnight LB shake culture were inoculated into 1/10 strength LB based SM medium with colloidal chitin prepared as described by Chernin et al. (1998) containing 0.5 µg/mL of C₄-HL and 0, 1, 5 or 15 seedling equivalents of a pea exudate extract. After 20 h incubation on a shaker at 27°C, exochitinase activity in culture supernatants was measured with p-nitrophenyl-β-D-N-acetylglucosaminide (Sigma Chemical Co., St.Louis, MO) as described in Chernin et al. (1998). Endochitinase activity was assayed in 1/10 LB based SM agar with colloidal chitin as in (Chernin et al., 1998) containing 0.5 µg/mL C₄-HL and 0, 1, 5 or 15 seedling equivalents of a pea exudate extract per 1 mL of the medium. The plates were stab inoculated with CV026 and chitin degradation was estimated by clearing of the medium surrounding the bacterial colonies after 3-4 d incubation.

Phospholipase assay. S. liquefaciens cultures (MG44 and PL10) incubated with pea seedling extracts, phospholipase activity was measured in egg yolk agarose as described by Givskov and Molin (1993).

In general, growth of bacteria was monitored in shake cultures by both turbidimetric measurements at A₆₀₀ and by dilution platings of gently sonicated cultures on LB agar. β-galactosidase production by A. tumefaciens (pZLR4) was measured as described by Cha et al. (1998). All assays were done in duplicates and all experiments were repeated at least twice with essentially the same results. The C₄-HL used in this study was synthesized as previously described (Chhabra et al., 1993) and verified by HPLC fractionation and MS analysis (Chapter 2), while the 3-oxo-C₁₂-HL was generously provided by B. Iglewski. Serrawettin was extracted from Serratia liquefaciens MG1 wild type cultures as described by Lindum et al (1998).
RESULTS

*C. violaceum* wild type and **CV026 reporter**. The CV026 reporter strain is a mutant of wild type *C. violaceum* ATCC 31532 which cannot synthesize *N*-hexanoyl-*L*-homoserine lactone (= C$_6$-HL). It is thus reliant on exogenous AHLs for activation of its AHL receptor, CviR, to induce synthesis of the purple antibiotic violacein and a number of exoenzymes (McClean et al., 1997; Chernin et al., 1998; Swift et al., 1999b). Violacein production in CV026 is induced by AHLs with a short (C$_4$-C$_8$) alkanoyl or 3-oxo-alkanoyl side chain or by a number of their chemical analogs (Fig. 6A and McClean et al., 1997; Cha et al., 1998). Addition of an AHL with a long alkanoyl or 3-oxo-alkanoyl side chains (e.g. 3-oxo-C$_{12}$-HL) competitively inhibits violacein synthesis induced by short side chain AHLs such as *N*-butanoyl-*L*-homoserine lactone (= C$_4$-HL), as shown in Fig. 6B and McClean et al. (1997). Thus, CV026 is an excellent reporter for a wide range of bacterial AHLs (or AHL signal-mimic compounds from plants).

AHL-regulated behaviors in *Chromobacterium* CV026 were strongly inhibited by substances secreted by pea seedlings. When 5-d-old, aseptically grown pea seedlings were placed on the surface of nutrient agar colonized by a lawn of the CV026 reporter strain, the seedlings induced no detectable violacein synthesis (Fig. 6C), in contrast to the positive C$_4$-HL control (Fig. 6A). However, when placed on agar containing 4 µM C$_4$-HL, the pea seedlings visibly inhibited C$_4$-HL-induced violacein synthesis in the lawn of CV026, leaving colorless areas around the seedlings (Fig. 6D). No inhibition of bacterial growth around the seedlings was observed, and none was detected in subsequent assays of CV026 growth in shake cultures containing pea exudates. The inhibition of violacein synthesis in areas adjacent to the seedlings was similar to that caused by the addition of a long chain AHL to a lawn of C$_4$-HL-induced CV026.

Substances that inhibited AHL-induced violacein synthesis in *Chromobacterium* CV026 were present in crude exudates collected by gentle rinsing of aseptically grown pea
seedlings and in methanol extracts of lyophilized exudate. As shown in Figure 7A, C₄-HL-inducible violacein synthesis was inhibited by a methanol extract of pea exudate in rough proportion to the amount of extract added. Inhibition of violacein synthesis by the extract could be partially prevented or reversed by an initial addition of twice as much C₄-HL to the assay wells or to the area adjacent to live seedlings (data not shown). Little restoration of violacein synthesis was seen if additional C₄-HL was added after 4 h or later. We have observed that CV026 cells remain responsive to C₄-HL induction of violacein synthesis for a limited time while they are actively growing (data not shown), perhaps accounting for the failure of delayed additions of C₄-HL to more fully restore exudate-inhibited violacein synthesis. The progressively greater inhibition of AHL-induced violacein synthesis by greater amounts of pea exudate and the partial reversal of such inhibition by additional C₄-HL both suggest, but do not prove, that pea seedlings secrete one or more substances capable of specifically interfering with the perception of C₄-HL by CV026.

To more rigorously test the possible disruption of AHL signaling in *C. violaceum* by substances in pea exudates, we examined the effects of methanol extract preparations on extracellular protease and chitinase activities, both of which are also regulated by the same receptor for AHL signals as violacein synthesis in *C. violaceum* (Chernin et al., 1998; Swift et al., 1999b). As shown in Figures 7C and 7D, the addition of seedling exudate extract substantially inhibited both C₄-HL-inducible protease and *N*-acetylglucosaminidase (exochitinase) activities in CV026. When assayed on colloidal chitin plates as described by Chernin et al. (1998), pea seedling exudates also substantially inhibited AHL-dependent endochitinase activity (data not shown). Growth of CV026 was not appreciably altered by pea exudates in these experiments, as determined by plate counts and absorbance measurements (data not shown). In other control experiments, CV026 cultures were first incubated with C₄-HL to induce extracellular protease and *N*-acetylglucosaminidase, then mixed with methanol-extracted
substances from pea exudate and immediately assayed. Addition of the pea exudate extract after induction by C₄-HL had no effect on these activities, indicating that the extracts did not contain substances that directly inhibited the enzymes. When C₄-HL was incubated overnight at room temperature with HPLC purified pea extracts containing the violacein inhibitory activity, it was found that the C₄-HL could be fully recovered from the mixture by ethyl acetate extraction (data not shown), suggesting that the active materials in seedling exudates did not degrade or immobilize the exogenous C₄-HL. Based on these results, it seems unlikely that the co-ordinate inhibition of AHL-induced violacein, protease and chitinases by pea exudates seen in Fig.7 is an artifact of some nonspecific interference with growth, violacein biosynthesis, exoenzyme synthesis or activity, or C₄-HL availability. Reasonable mechanisms of action to consider include direct interactions of the plant compounds with the receptor, CviR (as expected for true signal-mimic compound like the Delisea furanones) or indirect effects such as reduced bacterial uptake of the exogenous C₄-HL.

As shown in Figure 8, AHL-regulated extracellular protease activity was inhibited by pea exudate extracts in wild type C. violaceum to about the same extent as it was in the CV026 reporter strain supplied with exogenous C₄-HL. Pea exudate from five seedlings was about as effective as 4 µM exogenous 3-oxo-C₁₂-HL, a known competitive inhibitor of violacein and exoenzyme synthesis (McClean et al., 1997). The addition of pea exudate extract or 3-oxo-C₁₂-HL had no appreciable effect on growth of the bacteria (Fig. 8). These results are important because they suggest that the normal synthesis of cognate AHLs (C₆-HL in this case) by wild type bacteria may not be able to mask or overwhelm the effects of secreted plant signal-mimic compounds in natural encounters. Further studies are clearly needed to explore the question of just how effectively the plant compounds can compete with endogenously synthesized AHLs to affect behavior in wild type bacteria.
**Serratia reporters.** In wild type *S. liquefaciens* MG1, the initiation of swarming requires surface-stimulated induction of the *flhDC* operon, leading to formation of hyperflagellated cells (Givskov et al., 1997). Surface swarming also depends on C₄- and/or C₆-HL-induced synthesis of serrawettin, a lipopeptide surfactant (Eberl et al., 1999). *S. liquefaciens* strain MG44 is a *swrI*:Tn5 mutant of MG1 that cannot synthesize its own C₄-HL and C₆-HL and therefore requires exogenous AHLs for induction of serrawettin synthesis and swarming motility (Lindum et al., 1998).

Kjelleberg et al. (1997) used MG44 as a reporter to show that the halogenated furanones of *D. pulchra* inhibited AHL-induced surface swarming and other AHL-regulated behaviors in *S. liquefaciens* (as well as violacein synthesis in *C. violaceum*). They subsequently showed that serrawettin synthesis required expression of the *swrA* gene and then they isolated PL10, a *swrA*::Tn5*luxAB* mutant derivative of MG44. Thus, PL10 serves as a specific transcriptional bioluminescence reporter for the presence of exogenous AHLs capable of binding to the AHL receptor (SwrR) that activates the *swrA* gene and serrawettin synthesis (Lindum et al., 1998).

As shown in Figure 9, *S. liquefaciens* swarming was strongly affected by substances secreted by pea seedlings. The reporter strain MG44, unable to make its own AHLs, grew rapidly on the agar surface, but did not swarm (Fig. 9A). When aseptically grown pea seedlings were inoculated with MG44, placed on nutrient agar and incubated overnight, the seedlings induced the bacteria to swarm outward over the agar surface (Fig. 9B). As controls, toothpicks were inoculated with MG44 in the same manner. The inoculated toothpicks developed dense bacterial growth along their length, but did not induce surface swarming (Fig. 9C) unless previously dipped in a solution of C₄-HL (Fig. 9D). A methanol extract of pea seedling exudate, equivalent to the amount secreted by one 7-d-old pea seedling, strongly stimulated swarming of MG44 when present on a filter disc with the bacteria (Fig. 9E).
*S. liquefaciens* strain PL10 was used to test whether the plant-induced swarming of MG44 (Fig. 9B and 9E) was caused by secretion of a surfactant by the seedlings. Since PL10 is defective in both AHL and serrawettin synthesis, it does not swarm even in the presence of exogenous AHLs. However, PL10 can be stimulated to swarm by exogenous serrawettin or other surfactants (Lindum et al., 1998). PL10 thus provides a direct bioassay for exogenous surfactants. As shown in Figure 9F, pea seedlings did not induce surface swarming of PL10, indicating that pea does not secrete a surfactant capable of stimulating swarming.

While pea seedlings and methanol extracts of seedling exudate had no effect on swarming of PL10, they did stimulate AHL-dependent luminescence in this reporter, as described below.

**Other AHL reporters.** Another set of AHL reporters was recently developed for sensitive, quantitative, real time detection of exogenous AHLs (Swift et al., 1997; Winson et al., 1998; Cha et al., 1998). These AHL reporters normally function as multicopy plasmids in *E. coli* (a species which does not synthesize AHLs). The *E. coli*-based reporters carry plasmids with a gene for an AHL receptor protein (the LuxR receptor from *V. fisheri*, AhyR from *A. hydrophila*, or LasR from *P. aeruginosa*). Upon addition of an appropriate AHL, the receptor is activated so that it binds to its cognate *luxI* promoter and initiates transcription of a *luxCDABE* cassette, generating luminescence that is proportional to the concentration of added AHL (Winson et al., 1998). For example, the LuxRI’ reporter (pSB401) responds strongly to nanomolar concentrations of 3-oxo-C₆-HL and to micromolar concentrations of C₆-HL and 3-oxo-C₁₂-HL, whereas the LasRI’ reporter (pSB1075) responds strongly to nanomolar concentrations of 3-oxo-C₁₂-HL and C₁₂-HL (Winson et al., 1998). The AhyRI’ reporter (pSB536) responds most strongly to C₄-HL, its cognate AHL signal (Swift et al., 1997). Thus, collectively, these reporters can detect a fairly broad diversity of AHLs (or AHL signal-mimic compounds).
Figure 10 shows the luminescence of the three *E. coli* plasmid reporters and that of *S. liquefaciens* PL10 after inoculation onto live, 4-day-old pea seedlings. The digital images of the seedlings were overlayed with false color luminescence images taken with a very sensitive Hamamatsu charged couple device (CCD) camera 8-9 hours after inoculation. The luminescence induced in the reporter strains by the pea seedlings was generally 2- to 20-fold above levels of the no-AHL controls. The pea seedlings induced luminescence in each reporter that was an appreciable fraction (10% to 75%) of the maximal levels obtainable with high concentrations of an appropriate AHL. This suggests that the concentration of putative AHL mimic compounds at the plant surface may be high enough to affect AHL-regulated gene expression in wild type bacteria in natural encounters.

Plant-to-plant variation in luminescence was most frequently within about 2-fold for each of the reporters. The intensity of luminescence was normally greatest on the lower 2/3 of the pea root for each of the four AHL luminescence reporters. Luminescence was usually low on the upper portion of the root, the epicotyl and the cotyledons. It will be of interest to determine whether this pattern of luminescence reflects differences in the relative amounts of putative AHL mimic compounds secreted by the plant in these different parts of the seedlings or reflects differences in the number or metabolic vigor of associated bacterial reporter cells.

The ability of pea seedlings to stimulate the *E. coli* plasmid reporters provides prima facie evidence that at least some of the active plant compounds interact directly and specifically with the AHL receptor protein and affect its ability to initiate transcription of the *luxCDABE* cassette. The seedling-induced luminescence of PL10 is also consistent with direct interaction of the plant substances with the SwrR receptor, with resultant transcriptional activation of *swrA::Tn5luxAB*.

**AHL signal-mimic activity in other plant species.** Aseptically grown, uncontaminated seedlings of several other plant species were assayed for possible secretion of AHL mimic activity with the above reporter strains in the same manner as
shown in Figs. 6, 9 and 10 for pea. None of the plant species tested were able to 
stimulate detectable violacein synthesis in *Chromobacterium* CV026 or phenazine 
synthesis in the *P. aureofaciens* 30-84I reporter (data not shown). Only pea and crown 
vetch secreted compounds that inhibited AHL-induced synthesis of violacein in the 
*Chromobacterium* CV026 reporter strain, doing so without any apparent inhibition of 
growth of the reporter strain (Table 2). Rice, soybean, tomato, crown vetch and *Medicago 
truncatula* all activated AHL-dependent swarming in *Serratia* MG44 (Fig. 11A, *M. 
truncatula*), the colony morphology of *S. liquefaciens* MG44 was typical to that of a 
swarming colony (Fig. 11B). Seedlings of *Arabidopsis thaliana* ColO and lettuce did not 
stimulate swarming in this reporter (Fig. 11C, *Arabidopsis thaliana* ColO shown). As 
indicated in Table 2, these plant species also stimulated luminescence in *Serratia* PL10 
and in at least one of the three *E.coli* plasmid reporters. Neither lettuce nor *A. thaliana 
ColO* stimulated significant activity in any of the reporter strains under our assay 
conditions. The results from this small survey suggest that secretion of AHL signal-mimic 
compounds may be widespread among higher plants and at least quantitatively variable 
among species. This is what one might expect if AHL mimic synthesis by plants were an 
important and co-evolved mechanism for dealing with associated bacteria.
<table>
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<th>Plant</th>
<th>CV026&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PL10</th>
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<th>LasRI&lt;sup&gt;‘&lt;/sup&gt;</th>
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<sup>a</sup> Photon counts per second from two individual 4-d-old seedlings inoculated with a luminescence-based reporter strain were measured with the CCD camera as described in Materials and Methods. The relative luminescence of each seedling is reported individually and was calculated by subtracting background luminescence of the no-AHL controls from the luminescence of each seedling and expressing the remainder as a percentage of the luminescence of the +AHL controls, normalized to equal numbers of reporter bacteria. Similar results were obtained in repeated experiments.

<sup>b</sup> Relative inhibition of AHL-induced violacein synthesis.

**Table 2. Responses of AHL reporter strains to substances secreted by seedlings of various plant species.**
Initial purification and characterization of the AHL signal-mimic substances. The chemical nature of the substances from pea that affected AHL-inducible behaviors in the Chromobacterium, Serratia, Agrobacterium and E. coli plasmid reporters is presently unknown. The addition of homoserine at concentrations equivalent to, or 10-fold higher than, those found in pea root exudates (Van Egeraat, 1975a) had no effect in the Serratia MG44 swarming assay or Chromobacterium CV026 violacein assays. When 10 mg samples of lyophilized pea seedling exudate were successively extracted with 20 mL portions of chloroform, ethyl acetate, 100% methanol, and 1:1 methanol-water, no activity was detectable in the chloroform or ethyl acetate extracts by either the direct or indirect Chromobacterium assays or by the Serratia swarming or luminescence assays. Chloroform and ethyl acetate can extract the known AHLs made by bacteria, and did extract exogenously added C₄-HL from crude pea exudate preparations (data not shown). Thus, it appears that at least some of the plant substances with AHL signal activity are chemically different from bacterial AHLs.

Almost all of the activity in crude pea exudate preparations and detectable by the Serratia swarming and bioluminescence assays was present in the 100% methanol extracts. Microtiter plate dilution end point assays could detect about 1/4 of one seedling’s activity in the methanol extracts using the PL10 luminescence reporter as described in Materials and Methods (data not shown). Swarm plate assays with MG44 reached a dilution end point at extract concentrations roughly 5-fold higher than in the luminescence assays, and were considerably more variable. The activity detectable by the Chromobacterium CV026 indirect assay was usually present in the 50% methanol:water extract, although it could be fully, or almost fully, extracted into 100% methanol if freshly lyophilized material was used. The CV026 inhibitory activity, further purified by NH₂-bonded silica HPLC as described in Materials and Methods, was soluble in 100% acetonitrile. About 1/8 of one seedling’s activity could be detected by dilution endpoint assays of violacein production with the CV026 reporter, as described in Materials and Methods (data not shown). Thus, essentially all of the AHL mimic substances secreted
by pea and detectable with these reporters could be extracted with 100% methanol. From these semi-quantitative estimates of recovered activity, it seems that the mimic substances may be secreted in sufficient quantities to affect AHL-mediated behaviors of bacteria associated with the plant surface in a natural environment. This is consistent with the effects of intact, live seedlings on the behavior of the reporter strains (Figs. 6, 9 and 10).

As shown in Figure 12, some important separation of different substances with AHL mimic activity was obtained by chromatographic fractionation of pea exudate extract on a C$_{18}$ reverse phase column. The absorbance of fractions at 254 nm is shown as an (arbitrary) indication of where the bulk of the material in the exudate extract eluted. None of the fractions from the C$_{18}$ column contained substances that appreciably stimulated violacein synthesis in CV026 (data not shown). This is consistent with the failure of live pea seedlings to induce detectable violacein synthesis (Fig. 6C). When samples of each fraction equivalent to exudate from approximately 2 seedlings were assayed with CV026 and the luminescence reporters, all of the activity detectable by the CV026 indirect assay and some of the activity detected with the PL10, LasRI’ and AhyRI’ reporters and the Serratia MG44 reporter eluted in or near the void volume (fractions 8-15), suggesting that the compounds responsible for these activities could not bind to the C$_{18}$ column probably because they are hydrophilic or ionic. Several activities detectable with the PL10, MG44, LasRI’, AhyRI’ and LuxRI’ reporters eluted in fractions near the middle of the acetonitrile gradient (fractions 22-45). In addition, activities detectable with the AhyRI’ and LasRI’ reporters appeared in fractions near the end of the gradient (fr. 50-57).

When larger samples of the HPLC fractions were assayed (equivalent to exudate from six seedlings), one major new peak of PL10 activity was detectable in fr. 50-53 (data not shown). On a per seedling basis, the relative luminescence induced by material in this peak was about 70% of the +AHL control, likely accounting for most of the PL10
luminescence seen with inoculated seedlings (Fig. 10). It will be of interest to determine whether fractions that stimulated responses in two or three different reporter strains contain one compound acting on two or three reporters or several different compounds, each acting on a separate reporter.

To choose a type of the resin for further HPLC fraction of CV026- and PL10-activities eluting in the void volume of the C_{18} column, I passed the active fractions from the C_{18} column through commercially available C_{1}, C_{4}, C_{8}, NH_{2}, CN and ion-exchange sample prep cartridges (Alltech Associates Inc, Deerfield, IL) according to the manufacturer’s instructions. The activity was not retained on the reverse phase silica gels or anion exchange resin. It was either bound or destroyed by strong cation exchangers. The CV026 activity could be bound to and eluted from NH_{2} cartridges when it was equilibrated in acetonitrile and eluted with water. We exploited that property of the CV026 activity to purify it further using an analytical NH_{2} column (Alltech Associates Inc, Deerfield, IL). The CV026 activity was retained on the NH_{2} column when eluted with a water: methanol gradient (Fig. 13). The CV026 activity, when present in a crude pea exudate is (co)-precipitated or not fully soluble in organic solvents stronger than 100% MeOH. However, when it is purified through several HPLC runs, it is freely soluble in 100%AcN. Further purification and analysis to identify the active substances is in progress.

**DISCUSSION**

The halogenated furanones from Delisea pulchra provided the first clear example of AHL mimic compounds produced by a eukaryote (Givskov et al., 1996; Kjelleberg et al., 1997; Manefield et al., 2001). While quite limited, our initial examination of various plant species indicates that the synthesis of AHL signal-mimic substances may prove to be fairly general among higher plants. The apparent synthesis and secretion of AHL
mimic compounds by diverse plant species substantially broadens the range of
plant-microbe interactions where signal-mimic compounds might play important roles.
Future investigations to detect AHL mimic compounds in additional plants could be
valuable in exploring this possibility and in the search for new compounds to protect
plants as well as humans and other animals from pathogenic bacteria.

The AHL signal-mimic activities detected in pea exudate and with live seedlings do
not appear to be artifactual responses of the bacterial reporter strains. Pea seedlings and
exudates inhibited three different QS-controlled behaviors (violacein, protease and
exochitinase synthesis) in C. violaceum. We were initially puzzled by only partial
restoration of the disrupted violacein synthesis by additional C4-HL (Fig. 7A). Recent
biochemical studies with the AHL receptors from different bacteria suggest two plausible
explanations for this observed effect. Binding of the AHL-mimics from pea to CviR may
have led to accelerated turnover of this AHL receptor, similarly to the effect of algal
furanones on LuxR (Manefield et al., 2002). Or, like bacterial AHLs (Zhang et al.,
2002b), AHL-mimics could be firmly embedded within the α/β/α sandwich in the
N-terminus of the receptor, thus preventing displacement of the mimic by an AHL.

In S. liquefaciens MG44, the initiation of swarming depends on surface-stimulated
induction of the flhDC operon, leading to formation of hyperflagellated cells, as well as on
AHL-regulated serrawettin synthesis. Thus swarming is not as specifically dependent on
AHL binding as PL10 bioluminescence (Givskov et al., 1997; Lindum et al., 1998). In
control experiments, the addition of live pea seedlings or exudate extract preparations did
not appear to stimulate the flhDC operon, as measured by phospholipase assays (data
not shown). Phospholipase is under control of the flhDC operon (Eberl et al., 1996a). A
further indication that swarming of Serratia MG44 was specifically induced by plant
compounds acting as AHL mimics is the tightly parallel induction of MG44 swarming
and PL10 bioluminescence by live seedlings of pea (Fig. 9, 10), by other plants (Fig. 11)
tested in Table 2, and by at least three separate fractions of pea exudate (Fig. 12). Based
on such parallel induction of both swarming and swrA gene expression, and on evidence
for the absence of serrawettin-like surfactants in the exudate, or compounds that activated the *flhDC* operon, or compounds that affected growth of the reporter strains, it seems likely that pea seedlings secrete compounds that specifically activate the SwrR receptor governing *swrA* expression and serrawettin synthesis.

Similarly, the activation of the LuxRI’, AhyRI’ and LasRI’ plasmid reporters by seedlings of various plant species and by methanol extracts of pea seedling exudate suggests that these plants secrete compounds that specifically activate the AHL receptors, most likely by binding directly to the receptor protein. Our results with the PL10 and *E. coli* plasmid reporters appear to provide the first example of positively acting AHL signal-mimic compounds produced by eukaryotic organisms. It is worth noting, however, that others have shown that compounds which inhibit AHL-stimulated gene expression in cells with single copies of a LuxR-like receptor can show stimulatory activity when the receptor is present in multiple copies (Zhu et al., 1998). Thus, plant mimic compounds that stimulate the multicopy plasmid AHL reporters may act as competitive inhibitors of AHL-induced behaviors in wild type bacteria.

Further studies, preferably with pure mimic compounds, will be needed to rigorously establish the molecular mechanism(s) by which the AHL mimic compounds act. For example, plants might secrete “pseudo” mimic compounds that interfere with AHL synthesis or with AHL transport as well as “real” mimic compounds, like the halogenated furanones, that interact directly with the AHL receptor protein. It is of interest that *Pseudomonas aeruginosa* secretes cyclic dipeptides that act as AHL mimic compounds (Holden et al., 1999; Holden et al., 2000). The bacterial dipeptides appear to interact directly with AHL receptors and can either activate or inhibit receptor-mediated transcription, depending on the receptor. Another example of bacterial AHL-mimics is [1-[(2'-methylpropoxy)-2-hydroxy-2-methylpropoxy]butane produced *Vibrio angustum* S14 (de Nys et al., 2001). Unlike cyclic dipeptides, [1-[(2'-methylpropoxy)-2-hydroxy-2-methylpropoxy]butane does not share structural similarities with AHLs even though it affects some bacterial AHL reporters (de Nys et al., 2001).
Our initial attempts to purify the AHL mimic activity in pea exudates indicate that pea secretes at least one substance with inhibitory activity in the CV026 reporter and perhaps half a dozen or more different substances that stimulate one or more of the other AHL reporter strains. The sum of the relative activities observed in the HPLC fractions for each reporter agrees reasonably well on a per seedling basis with the total relative activity seen in Fig. 10 with live pea seedlings, indicating that most of the activities present on the seedling surfaces was probably recovered and detected after fractionation. However, given the limited range of concentrations tested and the unknown sensitivity of the reporters towards particular mimic compounds, we suspect that the relative luminescence elicited by substances in these HPLC fractions may not be a very reliable indicator of either the amount or potency of the AHL mimic substances present. We also note that the mimic activities detected in fractions from the C18 column probably represent just those compounds that can interact with the CviR, LuxR, AhyR, LasR and SwrR receptor proteins. The use of additional reporter strains that have AHL receptors with different binding specificities or affinities might well reveal additional AHL mimic activities. The ecological and evolutionary implications of AHL-mimics will be further considered in Chapter 7.

One of the central questions raised by our studies is whether the plant signal-mimic activities detectable with the AHL reporter strains are likely to affect wild type bacteria in natural encounters with the plant. There is no satisfactory general answer to this question yet. The AHL reporter strains which were used to detect the active plant compounds are unable to make AHLs of their own. Such reporters are useful for detection of substances that might affect AHL signaling, but they leave open the question of whether the mimic compounds detected by these reporters can compete with the endogenous AHLs made by wild type bacteria and effectively modify bacterial behaviors. In exploring this question, we found that pea exudate extracts were able to affect AHL-regulated protease production in wild type Chromobacterium cells, inhibiting the exoprotease activity to almost the same extent as in the AHL-deficient reporter strain,
This encourages one to think that the activities detected by the AHL reporter strains might actually affect the outcome of interactions of the plant with normal bacteria in natural environments. But it would be even more convincing and useful to show that many different AHL-regulated behaviors in wild type bacteria were affected by purified mimic substances at realistic concentrations. One approach to this goal would be the proteomic analysis of responses to purified mimics compounds mentioned above. A second approach would be to use promotorless Tn5 reporter mutants in AHL-regulated genes, like those of Whiteley et al. (1999), as reporters to detect AHL mimic compounds in a host plant. Such reporters would be useful both in vitro with purified mimic compounds and in planta to learn when and where a bacterium encounters sufficient concentrations of a mimic to affect the expression of an AHL-regulated gene.

Substances that can block quorum sensing in bacteria have been recently discussed as potentially valuable anti-infective agents for medicine (Finch et al., 1998). Their potential industrial and pharmaceutical applications will be discussed in Chapter 7.
Figure 6: Induction and inhibition of violacein synthesis in *Chromobacterium CV026* by AHLs and pea seedlings. **A. Direct assays.** Filter discs containing either 3.5 mg of C$_4$-HL (top) or no AHL (bottom) after 18 h incubation on a lawn of CV026 in a layer of LB soft agar on a base of LB agar. **C.** Aseptically grown 7-d-old pea seedlings 18 h after contact with a lawn of CV026 in a layer of LB soft agar on a base of LB agar. **Indirect assays. B.** Filter discs containing either 3 mg of 3-oxo-C$_{12}$-HL (top) or no AHL (bottom) after 18 h incubation on a lawn of CV026 in a 5 mL layer of LB soft agar containing 3 µM C$_4$-HL on a base of 15 mL LB agar. **D.** Pea seedlings 18 h after contact with a lawn of CV026 in a 5 mL layer of LB soft agar containing 3 µM C$_4$-HL on a base of 15 mL LB agar.
Figure 7: Effects of pea seedling exudates on violacein synthesis and exoenzyme activities in the CV026 reporter strain.  (A) Violacein production induced by 0.5 µg/mL (3 µM) C₄-HL in the presence or absence of pea exudate was assayed colorimetrically after 20 h incubation.  (B) An additional 0.5 µg/mL of C₄-HL was added either 0 or 4 h after suspension of CV026 cells in fresh medium, with or without one seedling equivalent of the pea exudate extract, and violacein production assayed colorimetrically after 20 h. (C) Extracellular protease activity induced after 20 h by 0.5 µg/mL C₄-HL in the presence or absence of pea exudate. (D) Exochitinase (p-nitropheny1-β-D-N-acetylglucosaminidase) activity induced after 20 h by 0.5 µg/mL C₄-HSL in the presence or absence of pea exudate.
Figure 7
Figure 8: Effects of pea seedling exudate on AHL-regulated extracellular protease production (A) and growth (B) in wild type C. violaceum. A. Solid bars show the average extracellular protease activity (+/-SE) in the supernatant of wild type C. violaceum cells after 20 h in the presence or absence of pea exudate extract or 4 µM 3-oxo-C\textsubscript{12}-HSL. B. Bars show growth of wild type C. violaceum (+/-SE) after 20 h in the presence or absence of pea exudate extract or 4 µM 3-oxo-C\textsubscript{12}-HSL. Growth and protease activity on azocasein were assayed in duplicate in repeated experiments as described in Materials & Methods.
Figure 9: Effects of pea seedlings and seedling exudates on AHL-dependent swarming of the Serratia MG44 reporter strain. Aseptically grown pea seedlings and toothpick controls were inoculated with a suspension of S. liquefaciens MG44 cultured as described in Materials & Methods. Inoculated seedlings or toothpicks were then placed on AB glucose-Casamino acid agar and incubated for 18-20 h. A. Filter disc inoculated with MG44. B. Pea seedling inoculated with MG44. C. Sterile toothpick inoculated with MG44. D. Sterile toothpick dipped in 0.5 µg/mL C₄-HL, then inoculated with MG44. E. Filter disk containing 1 seedling equivalent of an evaporated 50% methanol-water extract of pea seedling exudate and inoculated with MG44. F. Pea seedling inoculated with PL10.
**Figure 10: Pea seedling-induced luminescence of AHL reporter strains.**

Luminescence of the reporter strains on the seedling surfaces was measured and imaged as described in Materials and Methods. The intensity of luminescence is indicated by color (red>yellow>green>blue) and by numbers (photon counts/sec). Relative luminescence of the reporters on the seedlings is also given as a percentage of the maximum luminescence inducible by exogenous AHL, normalized to equal numbers of bacteria. The number of *E. coli* plasmid reporter cells recovered from the seedlings was generally about 1-2 x 10^6 per seedling, while the number of PL10 cells was typically about 5 x 10^7. Results are representative of several independent experiments. Control values (+/- AHL) were measured on toothpicks (with or without AHLs) inoculated with the reporter bacteria as described in Materials and Methods.
**Figure 11: Effects of seedlings on AHL-dependent swarming of the *Serratia* MG44 reporter strain.** Aseptically grown *M. truncatula* and *A. thaliana* seedlings were inoculated with a suspension of *S. liquefaciens* MG44 cultured as described in Materials and Methods. Inoculated seedlings were then placed on AB glucose-casamino acid agar and incubated for 18-20 h. **A.** *M. truncatula* seedling inoculated with MG44. **B.** A close-up view of the swarming colony formed by MG44 on *M. truncatula* seedling. **C.** *A. thaliana* seedling inoculated with MG44. Because the seedling did not induce swarming of the mutant bacteria, the bacteria formed a non-swarming colony over the seedling.
Figure 12. High performance liquid chromatographic fractionation of AHL mimic activities present in methanol extracts of pea seedling exudate. Fractions from a C$_{18}$ reverse phase HPLC column were collected and samples equivalent to exudate from about two seedlings were assayed for activity as described in Materials & Methods. Luminescence was measured with the CCD camera. Fractions that induced Serratia PL10 luminescence are indicated by yellow bars. These same fractions induced swarming of MG44 (not shown). Fractions that induced luminescence in the LuxRI’ reporter (pSB401) are indicated in black, the AhyRI’ reporter (pSB536) in red, and the LasRI’ reporter (pSB1075) in green. Relative inhibition of C$_{4}$-HL-induced violacein synthesis in Chromobacterium CV026 is shown in purple, and was equal to 100% in fractions 8 and 9. The thick grey line indicates the gradient of increasing acetonitrile concentration (0-100%) used for elution and the thin black line indicates A$_{254}$ (0-2). Similar results were obtained in repeated fractionations.
Figure 13: *C. violaceum* CV026 bioassay of NH$_2$-HPLC fractions of pea seedling exudate. CV026 fractions from the void volume of the initial C$_{18}$-HPLC run were re-chromatographed using NH$_2$ HPLC as described in Materials and Methods. Inhibition of C$_4$-HL-induced violacein synthesis in *Chromobacterium* CV026 bioassay is shown. Bottom left corner well contains an aliquot of the sample that was subjected to HPLC, the rest of the fractions in that row are bioassay controls of CV026+C$_4$-HL. Similar results were obtained in repeated fractionations.
CHAPTER 4

INITIAL CHARACTERIZATION OF QUORUM-SENSING MIMICS FROM CHLAMYDOMONAS REINHARDTII AND THEIR EFFECTS ON BACTERIAL BEHAVIOR

ABSTRACT

Culture filtrates and colonies of a unicellular alga *C. reinhardtii* were found to produce several separable activities that mimicked AHL signals in well-characterized bacterial reporter strains. The chemical nature of the active mimic compounds is presently unknown. Initial work on separation and characterization of the algal AHL-mimics is presented. The AHL signal-mimic compounds could prove to be important in determining the outcome of interactions between algae and bacteria, and may have important industrial applications.

INTRODUCTION

The marine alga *D. pulchra* and higher land plants were shown to produce signals that act to disrupt bacterial AHL-mediated quorum sensing (Givskov et al., 1996; Manefield et al., 1999; Chapter 3). Quorum sensing of Gram-negative bacteria regulates
expression of dozens of genes in a population density-dependent manner (Whiteley et al., 1999; Chapter 5; rev. Chapter 1; Whitehead et al., 2001). Many of these genes are important for bacterial invasion or colonization of their eukaryotic hosts (Tan et al., 1999; Eberl et al., 1996b; rev.de Kievit and Iglewski, 2000). It makes good evolutionary sense for the eukaryotes not only to recognize bacterial AHL signals (Telford et al., 1998; Smith et al., 2002; Mathesius et al., submitted), but to actively interfere with bacterial communication (rev.Kjelleberg et al., 1997; Bauer and Teplitski, 2001). The first eukaryotic molecules identified as mimics of bacterial AHLs were halogenated furanones of a red alga *D. pulchra* (cf. fimbriata) (de Nys et al., 1993; Givskov et al., 1996).

Halogenated furanones from *D. pulchra* are genuine AHL mimics. The furanones are structurally similar to bacterial AHLs (Fig. 4A, 4B, and de Nys et al., 1993; Givskov et al., 1996). Biochemical studies by Manefield et al., 1999 reported that algal furanones can bind to the bacterial AHL receptor (LuxR). Binding of the furanone to the LuxR protein alters stability of the protein-ligand complex and leads to a rapid turnover of the molecule (Manefield et al., 2002). Such interference with the bacterial signal perception by the furanones leads to specific disruption of bacterial quorum sensing gene regulation. As demonstrated by proteomic gels, algal furanones do not grossly disrupt bacterial metabolism, but rather specifically affect quorum sensing (Givskov et al., 1996; Manefield et al., 1999).

*D. pulchra* produces the AHL-mimicking furanones when grown in seawater, or in a medium supplemented with bromine. Vesicle-mediated release of the halogenated furanones to the thallus surface builds furanone levels up to ~100 ng cm$^{-2}$ near the plant's apical tips (Dworjanyn et al., 1999). These concentrations were found to be effective in disrupting colonization of the algal thalli by Gram-negative bacteria, and affecting the spectrum of bacterial colonizers (Dworjanyn et al., 1999; Steinberg et al., 1997). Furanone production by *D. pulchra* differs qualitatively and quantitatively among geographically distant populations. Statistical analyses of the occurrence and production
of the furanones by the algal populations suggest that the variation is a heritable evolutionary trait (Wright et al., 2000). *D. pulchra*, unfortunately, is not a suitable model organism for genetic studies.

After the discoveries that higher plants and marine algae produce AHL-mimics, we wanted to concentrate on finding tractable plant or algal models to study the ecological roles of AHL-mimics. *M. truncatula* is one such model allowing us to study potential effects of AHL-mimics on plant-bacterial interactions (Bauer and Teplitski, 2001). Significantly, the genome of this legume is being sequenced. In addition, studies on the proteomics and metabolomics of *M. truncatula* are underway (Cook, 1999; Mathesius et al., submitted). However, growing large amounts of seedlings for the identification of AHL-mimics may be challenging. We decided to investigate AHL-mimic production by a unicellular alga, *Chlamydomonas reinhardtii*. This well known alga can grow heterotrophically or photosynthetically with a generation time of ~12 h in shake cultures, allowing us to produce large volumes of culture. *C. reinhardtii* is haploid throughout much of its life cycle, which facilitates genetic studies (Harris, 2001) to identify mutants. We discovered that *C. reinhardtii* produces a variety of AHL mimics that affect AHL-regulated behaviors in bacteria. Studies on initial characterization of the AHL mimics from *C. reinhardtii* are presented in this chapter.

**MATERIALS AND METHODS**

**Organisms and growth conditions.** *Chlamydomonas reinhardtii* wild type 2137 (CC-1021) (Harris, 2001) was maintained on TAP agar (1.5%) under continuous illumination (40 µmol photons m\(^{-2}\) sec\(^{-1}\) of white light) at 25\(^{\circ}\)C. Individual colonies were restreaked on a fresh TAP agar plate every two months. Individual colonies were inoculated into TAP (pH 6.6-6.8) liquid shake cultures, grown axenically in light for 3-5
days to $OD_{750}=0.8$. The cells were then spun down, and re-suspended in three volumes of HS minimal medium without carbon source. The cultures were incubated for a week on a shaker (175 rpm) in continuous white light.

TAP and HS media was prepared as described in “The Chlamydomonas Sourcebook” (Harris, 1989). The pH of the media was adjusted to 6.6-6.8 prior to autoclaving. TAP liquid and solid media were buffered with TRIZMA®-Base (Sigma Chemical Co, St.Louis, MO). Bacto-agar (1.5%) was added to solidify TAP when needed. To avoid occasional formation of precipitate in TAP and HS, the stock solutions of Hunter’s Trace Metals was added as a filter-sterilized solution after the media components were combined and autoclaved.

Contamination of the algal cultures by bacteria was carefully avoided. To check for contamination, aliquots of each shake culture were plated on Luria-Bertani (LB), TAP, Soybean-Casein Digest Tryptic Soy Medium (TSA), Tryptone Agar (TA), high salt AB medium, and Corn Meal Agar (CMA). LB and TA were prepared as described in Chapter 2, high salts AB medium was prepared as in Joyce et al. (2000); CMA (Sigma Chemical Co, St.Louis, MO) and TSA (Difco Laboratories, Detroit, MI) were prepared according to the manufacturer’s instructions. Contamination checks on all these media allowed detection of most culturable bacteria that could conceivably cause contamination of the algal cultures. Any contaminated cultures were discarded.

AHL reporter bacteria were grown as described in Chapter 2. Bioassays to detect AHL mimics were done as described in Chapters 2, 3, and 4.

Protease assays. For use in the protease assays, HS cultures of $C. reinhardtii$ CC2137 were supplemented with 1 ml/L of glacial acetic acetate (initial pH 6.6-6.8) and were grown for a week. Cell-free culture filtrates were passed (20 ml at a time) through a water-equilibrated reverse phase silica ($C_1$) cartridge (Fisher Scientific, Pittsburgh, PA). The retained substances were eluted with five bed volumes of acetonitrile, and evaporated to dryness. Protease activity on azocasein was measured as in Chapter 3.
An effect of algal preparations on protease activity was measured in three strains of *C. violaceum*: CV026, CV017, and the wild type strain, ATCC 31532. CV026 is a mutant deficient in production of AHLs (McClean et al., 1997; Chernin et al., 1998). In CV026, purple pigment violaceum and exoenzymes are produced only when exogenous AHLs are supplied. CV017 is a mutant constitutively producing AHLs (Chernin et al., 1998), so that violaceum and exoenzymes are produced constitutively. In the ATCC 31532 wild type, AHL and exoenzyme synthesis are growth-phase regulated. The strains were cultured as described in Chapter 3.

**Extraction and purification of AHL-mimics from *C. reinhardtii***. TAP-grown cultures of *C. reinhardtii* CC-2137 (OD$_{750}$ = 0.8-1.0) were subcultured into three volumes of fresh HS medium without a carbon source and incubated in light at 25°C for a week to attain OD$_{750}$ = 0.3-0.4. Residue of spent TAP medium were removed by centrifugation prior to the subculture into the HS medium. The HS-grown cultures were then pelleted, and the culture filtrates (pH 6.4-6.8) collected and filtered through a 0.45 µm filter if necessary. The cell-free culture filtrates were then extracted twice with an equal volume of ethyl acetate, and the extract was rotary evaporated to dryness over a 40°C water bath. Care was taken to release the vacuum immediately after the solvent was evaporated; exposure to bright lights was avoided when possible. The dry residue was stored in glass vials at -20°C. For HPLC analysis, the residue from 5 to 7 L of culture was brought up in 1 ml of acetonitrile, spun down and the precipitate extracted with 1 ml of acetonitrile:water (1:1). The samples were injected onto a 10% acetonitrile:90% water-equilibrated semi-preparative C$_{18}$ column (Whatman Partisil 10, ODS-3), fitted with a guard column and equilibrated with 10:90 acetonitrile:water. The column was eluted at 2 ml/min with a linear water:acetonitrile gradient starting at 10% acetonitrile to 100% acetonitrile over 70 min, followed by additional 10 min with 100% acetonitrile. Fractions were collected at 1 min intervals. Individual HPLC fractions were assayed for AHL-mimic activity as described in Chapter 2.
For mass spectral analyses, bioactive fractions were pooled, organic solvents were rotary evaporated and the remaining water was sublimated by freeze-drying. The samples were sent to Cornell University for GC-MS and ESI-MS analysis. For GC-MS, the dried samples were dissolved in 100 µl of a 3:1 mix of acetonitrile and 1% formic acid. GC-MS was carried out as for *S. meliloti* AHLs described in Chapter 5.

**Initial work on establishing a mutant screen.** As outlined in the introduction to this chapter, *C. reinhardtii* is an attractive model eukaryote because it can be manipulated genetically. Large-scale mutant screens can be conducted relatively easily with the alga (Harris, 2001). One of the long term goals of the research in our lab is to identify plant or algal mutants unable to produce AHL-mimics. Therefore, a preliminary study to establish conditions for such a screen was conducted. To set up mock screens, TAP agar plates with colonies of *C. reinhardtii* or membranes (described below) were overlaid with a suspension of the *V. harveyi* BB170 AI-2 reporter strain, luminescent *E. coli* AHL reporter strains, *P. aureofaciens* strains, and *C. violaceum* CV026 (Chapter 2, Table 1).

For indirect assays to detect inhibitory substances, *E. coli* ((pSB1075)) cell suspension was supplemented with 0.5 x 10^-6 mg/ml of 3-oxo-C_{12}-HL, and incubated for 3-4 hrs. *V. harveyi* strains were grown overnight in high salt AB medium (Joyce et al., 2000), then diluted 5,000-fold in high salt AB medium solidified with 0.3% bacto agar. *E. coli* luminescent reporters were grown as described in Chapter 2, and then re-suspended in LB agar (0.3%). Images were analyzed with a CCD camera as described in Chapter 3. Pigment production by *P. aureofaciens* and *C. violaceum* was evaluated visually.

Alternatively, 5-10 µl of the algal cultures were applied to a sterile Whatman #1 9-cm filter and incubated on the surface of a TAP agar plate. For different mock screens, Westran PVDF membrane (Schleicher & Schuell, Keene, NH) or C₈-impregnated paper disk (3M Empore Filtration Products, St. Paul, MN) was placed between the filter and the surface of the TAP agar.
To test whether various materials can selectively retain different AHL-mimics, cell-free algal culture filtrates were passed under vacuum through Photogene Nylon membrane (Life Sciences Products, Inc. Denver, CO), 0.45 microne Nytran and Westran PVDF membranes (Schleicher & Schuell, Keene, NH), or C₈-impregnated Whatman paper. Westran PVDF membrane was first moistened with 95% ethanol, and then washed with distilled water. In some cases, membranes were rinsed with 5 ml of distilled water after the sample application and before it was subjected to bioassays. Colonies or cultures of wild type *E. carotovora* (a 3-oxo-C₆-HL producer) were grown similarly as a control for mock screens.

**RESULTS**

**Effect of algal preparations on protease synthesis in *C. violaceum***. The CV026 strain is a mutant of wild type *C. violaceum* ATCC 31532 which cannot synthesize *N*-hexanoyl-L-homoserine lactone (= C₆-HL). Exogenously added AHLs activate its AHL receptor, CviR, to induce synthesis of violacein and several exoenzymes (McClean et al., 1997; Chernin et al., 1998; Swift et al., 1999b). As discussed in Chapter 3, violacein production in CV026 is induced by AHLs with a short (C₄-C₈) alkanoyl or 3-oxo-alkanoyl side chain or by a number of their chemical analogs (Fig. 6A and McClean et al., 1997). Addition of an AHL with a long alkanoyl or 3-oxo-alkanoyl side chain (e.g. 3-oxo-C₁₂-HL, Fig. 6B), algal furanones (Kjelleberg et al., 1997) or some plant AHL-mimics inhibits violacein and exoenzyme synthesis induced by AHLs (Chapter 3).

Addition of CV026+C₄-HL to the plates with the algal colonies inhibited bacterial pigment synthesis. I further tested the effects of the algal preparations on other AHL-regulated phenotypes in the *Chromobacterium* strains. Unlike the inhibitory furanones of *D. pulchra* or pea AHL-mimics, preparations of *C. reinhardtii* culture filtrates had a stimulatory effect on protease synthesis in *C. violaceum* CV026 when the culture was
already treated with 3µM C₄-HL. As shown in Fig. 14, addition of C₄-HL to the mutant restored protease synthesis to the wild type levels, similar to the results observed in Chapter 3. Cultures of CV026 treated with 3µM C₄-HL and algal culture filtrate preparation synthesized twice as much protease. Addition of algal culture filtrates to the CV026 culture did not significantly affect protease synthesis in this mutant. CV017 is an un-characterized regulatory mutant of C. violaceum, constitutively producing C₆-HL, violacein pigment and exoproducts (Chernin et al., 1998). Levels of protease synthesis in CV017 were similar to those of wild type cells. Addition of algal culture filtrates or culture filtrates + C₄-HL to CV017 doubled protease synthesis. Treatment with 3 µM C₄-HL or algal culture filtrates in various combinations did not have any effect on protease synthesis or activity of wild type cells (data not shown). Protease induction experiments were carried out three times with different preparations from algal culture filtrates with essentially the same results. Representative results from one of the experiments are presented in Fig. 14.

To determine whether addition of algal preparations interfered with secretion of protease in Chromobacterium strains, protease activity was measured in both culture filtrates and in cell lysates. There was no significant effect of the algal preparations on the levels of cell-associated protease in any of Chromobacterium strains. The effect of the algal culture filtrate preparations on the pigment production in the strains of C.violaceum was also evaluated, however consistent patterns of interference were not observed. Addition of algal preparations did not appreciably affect growth of the bacterial strains.

**Initial purification and characterization of the AHL signal-mimic substances.** The chemical nature of the substances from C. reinhardtii that affected AHL-inducible behaviors in the AHL reporters is presently unknown. AHL-mimic activities from C. reinhardtii partitioned into the organic phase when the culture filtrate was extracted with
ethyl acetate. However, based on the initial mass spectral analyses, the preparations of algal culture filtrates did not contain known AHLs or AHL "signature" peaks (data not shown).

Based on HPLC separation, two peaks with LasR stimulatory activity and three peaks of LuxR stimulatory activity were detected in culture filtrates of HS-grown cultures (Fig. 15). Much lower levels of activity, eluting with similar retention times, were detected in the extracts from TAP-grown cultures (data not shown). As shown in Fig. 15, some important separation of different substances with AHL mimic activity was obtained by chromatographic fractionation of *C. reinhardtii* culture filtrate extracts on a C$_{18}$ reverse phase column. None of the fractions collected from the C$_{18}$ column contained substances that appreciably affected violacein synthesis in *C. violaceum* CV026, phenazine synthesis in *P. aureofaciens* strains, or affected luminescent AhyR-based reporter (data not shown). Likewise, no CvR-, PhzR- or AhyR-active substances were produced by the algal colonies.

Two peaks of activity (LuxR-active fractions 77-83 and LasR-active fractions 57-71 from the run in Fig. 15) were pooled and submitted for mass spectral analyses. No m/z peaks typical to AHL mass spectra (see Chapters 2 and 5) were detected. We also could not detect m/z corresponding to molecules with several halogen atoms, like halogenated furanones of *D. pulchra*.

**Preliminary work on mock mutant screen.** When reporter bacteria were overlaid on top of the algal colonies growing on TAP agar, the *V. harveyi* BB170 AI-2 reporter luminesced. But pigment production by *P. aureofaciens* and *C. violaceum* was not visibly affected (data not shown). Luminescence was actually inhibited in the *E. coli* (pSB401) (LuxR-based) and *E. coli* (pSB1075) (LasR-based) reporters directly over the *C. reinhardtii* colonies (Fig. 16). Because individual HPLC fractions of *C. reinhardtii* culture filtrates were able to stimulate the *E. coli* reporters, we sought ways to remove the inhibitory activity while retaining the stimulatory AHL-mimics. To do that, aqueous culture filtrates were passed through several types of retentive membranes.
After passing through the membranes, as described in Materials and Methods, LuxR-activating materials were retained on Westran PVDF membrane. The overlays of the LasR-based reporter were inhibited over the Westran PVDF membrane. Washing of the PVDF membrane with one ml of distilled water removed the inhibitory activity, and *E. coli* (pSB1075) (LasR-based) reporter was activated (Fig. 17). C₈-impregnated paper only retained LasR-inhibitory substances. In the control experiments, Westran PVDF and C18-impregnated paper retained enough AHLs from *E. carotovora* to fully activate the LuxR-based reporter (data not shown).

**DISCUSSION**

Within bacterial populations, AHL-regulated genes are important for regulation of various stress responses, and in structuring of bacterial biofilms and mixed bacterial communities (Davies et al., 1998; Michael et al., 2001; rev. Parsek and Greenberg, 1999; Whitehead et al., 2001). AHL-dependent quorum sensing is also important in coordinate regulation of the genes required for bacterial interactions with eukaryotic hosts (rev. Chapter 1). It is now becoming clear that eukaryotes are capable of perceiving and responding to bacterial AHLs (Telford et al., 1998; Smith et al., 2002; Lawrence et al., 1999; Saleh et al., 1999; Mathesius et al., submitted). Isolation of AHL-mimics from plant and algal species (Givskov et al., 1996; Manefield et al., 1999; Teplitski et al., 2000; Daniels et al., 2002) suggests that plants can manipulate bacterial signalling by producing and releasing signals that mimic bacterial signals.

Studies with furanones from *D. pulchra* have shown that AHL-mimics can affect populations of bacteria that colonize the thallus surface (Steinberg et al., 1997). Furanones of *D. pulchra* demonstrated that multicellular algae are capable of producing chemicals that interfere with bacterial communication by affecting the bacterial receptor stability (Manefield et al., 2000). Results presented in this chapter suggest that cultures of *C. reinhardtii* can also produce substances that disrupt bacterial AHL-dependent QS.
Based on the initial mass spectral analyses, these substances do not contain halogen atoms and are therefore different from *D. pulchra* furanones (data not shown).

Algal culture filtrates contained substances that affected protease synthesis in mutants of *C. violaceum*. Because protease synthesis in this strain is regulated by quorum sensing, it seemed possible that algal AHL-mimics are somehow involved in the observed stimulation of the protease synthesis. However, in the AHL-deficient mutant CV026, algal preparations by themselves did not affect protease synthesis and had additive effect when the cultures were treated with both AHLs and algal preparations. There was no significant effect of the algal preparations on the wild type protease. Because there was no observed effect on protease secretion in any of the *C. violaceum* strains, it is possible that the compound(s) in the algal preparations affects stability of the AHL receptor, alter transport or uptake of the AHLs, or interferes with the protease synthesis regulation at some other level.

Chemical identification of the AHL-mimics from *C. reinhardtii* is needed to elucidate their mode of action. AHL-mimics from *C. reinhardtii* culture filtrates could be chromatographed with C$_{18}$ reverse phase silica HPLC. The consistently detected LuxR- and LasR-active AHL-mimics were separated as shown in Fig. 15. In several batches of HS-grown cultures, an additional peak of LuxR activity was detected in the early fractions (eluting in 15-20% acetonitrile). Mass spectral analysis of that activity peak indicates that it contained several volatile molecules. No good explanation for the inconsistency of LuxR detection of the activity in fractions eluting at 15-20% acetonitrile can be provided at present. There may be real differences in the culture growth stage or metabolic state. Or it could be an artifact of the extraction procedure: extractability of the relatively hydrophilic activity with ethyl acetate may be relatively poor.

Initial experiments with the colony screens suggest that a relatively simple screen to identify algal mutants can be set up. It should be feasible to grow algal colonies on Whatman filters over the surface of a solid medium. The filter then could be lifted, placed over a Westran PVDF membrane on top of a solid medium for several days to allow
production and release of the AHL-mimics by the colonies. The PVDF membrane could then be bioassayed directly for LuxR-stimulatory activities. To assay for LasR-stimulatory activity, the filter will need to be rinsed with the distilled water, and then bioassayed. The diffusion of the AHL-mimics from individual colonies may present a problem in such a screen, however. Alternatively, individual mutant cultures can be grown in the 1 ml wells of microtiter plates, then centrifuged to sediment the cells, and the culture filtrates passed through the PVDF filters in 96-well extraction module (Whatman Co or 3M Empore Filtration Products, St.Paul, MN). The activities could be then eluted from the filter with an appropriate solvent into the wells of a black microtiter plate, for the bioassays with the appropriate reporters.

Once algal AHL-mimics are identified and their mode of action is established, AHL-mimics may have broad industrial and medical applications as discussed in (Bauer and Robinson, 2002) and Chapter 7. There is currently a great interest in non-toxic antifouling agents. For example, Hellio et al. (2001) reported that several antifouling agent-producing marine microalgae have been identified in a large-scale screen.
Figure 14: Effect of preparations from *C. reinhardtii* cultures on protease induction in *C. violaceum* wild type (ATCC 31532), and QS mutants CV017 and CV026.

When shown, 0.5 µg/mL of C\textsubscript{4}-HL was added to the cultures of the mutants to evaluate the ability of algal preparations to antagonize protease induction with C\textsubscript{4}-HL. Algal culture were grown in HS medium with acetate and then extracted as described in Materials and Methods.
Figure 15. High performance liquid chromatographic fractionation of AHL mimic activities present in the ethyl acetate extracts of *C. reinhardtii* culture filtrates.

Fractions from a C\textsubscript{18} reverse phase HPLC column were collected and aliquots were assayed for activity as described in Materials & Methods. Fractions that induced luminescence in the LuxRI’ reporter ((pSB401)) are indicated with hatched bars, the AhyRI’ reporter (pSB536) in red, and the LasRI’ reporter ((pSB1075)) solid black bars. The thick grey line indicates the gradient of increasing acetonitrile concentration (10-100%) used for elution and the thin black line indicates. These activities were consistently present in repeated fractionations. In some culture preparations, a strong LuxR-activity was detected in 15-20% acetonitrile.
Figure 16: Luminescence of *E. coli* (pSB1075), a LasRI'-based reporter (A) and *E. coli* (pSB401), a LuxRI'-based (B) reporter over the colonies of *C. reinhardtii* CC2135. Thick white arrows point to the *C. reinhardtii* colonies, all of which inhibit luminescence of the reporters. *C. reinhardtii* were cultured on TAP agar for a 3-5 days, and then overlaid with a suspension of the bacterial reporters as described in Materials and Methods.
Figure 17: Luminescence of the LasRI'-based reporter when inoculated on Westran PVDF membranes with algal compounds. A. Fifteen ml *C. reinhardtii* culture filtrates were passed through a PVDF membrane, and assayed with *E. coli* (pSB1075) reporter as described in Materials and Methods. B. An equivalent *C. reinhardtii* culture was passed through the Westran PVDF membrane, then rinsed with five ml of distilled de-ionized water. The rinsed membrane was then bioassayed with *E. coli* (pSB1075). Luminescence of the reporter on both membranes (A and B) was measured with a Hamamatsu CCD camera simultaneously.
CHAPTER 5

PROTEOMIC ANALYSIS OF WILDTYE SINORHIZOBIIUM MELILOTI RESPONSES TO AHL QUORUM SENSING SIGNALS

ABSTRACT

Sinorhizobium meliloti 1021 is a N-fixing bacterial symbiont of alfalfa and medics. It was found to synthesize N-(tetrahydro-2-oxo-3-furanyl)-Z-9-hexadecenamide (C_{16:1}-HL), 3-oxo-N-(tetrahydro-2-oxo-3-furanyl)hexadecenamide (3-oxo-C_{16:1}-HL), N-(tetrahydro-2-oxo-3-furanyl)tetradecanamide (C_{14}-HL), and 3-oxo-N-(tetrahydro-2-oxo-3-furanyl)tetradecanamide (3-oxo-C_{14}-HL), but did not make AHLs with shorter acyl side chains under the conditions tested. Proteomic analysis was used to characterize the responses of the early log phase wildtype S. meliloti 1021 cultures to purified C_{14}-HL and 3-oxo-C_{16:1}-HL. Significant differences were observed in the accumulation of over fifty polypeptides in response to these AHLs. Almost all polypeptides were accumulated in response to 3-oxo-C_{16:1}-HL. AHL-responsive polypeptides corresponded to ~10-15% of the polypeptides normally accumulated in stationary phase. AHL responsive polypeptides involved in metabolite transport, bacterial energy cycles, DNA synthesis, polypeptide turnover, and carbon and nitrogen metabolism were identified. It appears that proteomic analysis can be a powerful global approach to the identification of quorum sensing regulated functions and regulation in wildtype bacteria.
Introduction

We have been interested in contributing to the systematic characterization of quorum sensing regulation in the nitrogen-fixing bacterial symbiont, S. meliloti. The genomic sequences of the circular chromosome (SmC) and two megaplasmids (SmA and SmB) of the strain 1021 have been recently published (Galibert et al., 2001). Proteomic reference maps have been developed for both free-living S. meliloti 1021 (Guerreiro et al., 1999; Weiller et al., 2001) and differentiated nitrogen-fixing bacteroids (Natera et al., 2000). Its symbiotic partner, Medicago truncatula, is a model legume plant (Cook, 1999) that was recently shown to produce compounds that can mimic AHL signals and disrupt AHL-mediated quorum sensing in bacteria (Bauer and Teplitski, 2001; Mathesius et al., submitted). Examination of the S. meliloti 1021 genomic sequence indicates the presence of a LuxI AHL synthase homolog (Smc00168, Fig. 3A), a HdtS AHL synthase (Laue et al., 2000) homolog (Smc00714), and perhaps five potential LuxR AHL receptor homologs (Galibert et al., 2001; Pellock et al., unpublished), suggesting that AHL-mediated quorum sensing is both important in S. meliloti and likely to be complex. The gene for one of the putative AHL receptors in strain 1021, ExpR (ORF Smc 03896) was recently shown to be interrupted by a native insertion sequence (IS) (Pellock et al., in press). Spontaneous excision of this IS element resulted in enhanced production of EPS II in response to a specific AHL produced by S. meliloti 1021.

Strains of S. meliloti appear to produce a diversity of AHLs (Gray et al., 1996; Puskas et al., 1997; Cha et al., 1998; Marketon and Gonzalez, 2002). Studies by Marketon and Gonzalez (2002) suggest that SinI AHL synthase of S. meliloti 1021 produces at least six AHLs detectable by thin layer chromatography. Novel AHLs with long (C_{16}-C_{18})-acyl side chains, including predominantly C_{16:1}-HL and 3-oxo-C_{16:1}-HL, and several short-chain AHLs were subsequently identified by Marketon et al. (in press) using mass spectral analysis in studies conducted concurrently with ours.
Proteomic analysis was conducted in cooperation with the Genomics Interaction Group at the Australian National University to obtain a global overview of the number and nature of AHL-regulated functions in *S. meliloti* 1021, and to learn which functions might be affected by specific AHLs. As an initial study, prior to comparisons with potential AHL synthase and receptor deletion mutants or with plant-derived AHL signal-mimic compounds, we have exposed wildtype *S. meliloti* to various AHLs to identify the proteins that differentially accumulate. Studying quorum sensing responses in a wildtype background, rather than in an AHL synthase mutant background, requires that the level of endogenous AHLs be minimized so that the effects of the added AHLs can be reliably detected. Our approach was to repeatedly wash and regrow low density cultures of the bacterium to reduce the levels of endogenous AHLs, then expose the cultures briefly (ca. one generation) to levels of AHLs that they would encounter normally only at late log or early stationary phase. We identified over fifty proteins that accumulated to significantly different levels in response to added AHLs. Different AHLs induced unique as well as common changes in protein levels.

**Experimental Procedures**

**Bacterial strains, media and growth conditions.** For AHL extraction, *Sinorhizobium meliloti* strains 1021, *S. meliloti* AK631, *S. meliloti* 8501 were grown in a defined NM medium (Robinson et al., 1992) supplemented with 3g/L of glucose, 0.505 g/L of KNO$_3$ and Gotz vitamins. To prevent precipitation, MgSO$_4$$\cdot$7H$_2$O, CaCl$_2$, and FeSO$_4$-citric acid stocks were autoclaved separately. The pH of the medium was adjusted to 6.5-6.7 before autoclaving and was monitored throughout growth to ensure that bacteria grew normally and that AHLs in the cultures were not exposed to alkaline conditions. *S. meliloti* strains were grown in TA (YTB) medium (Chen et al., 2000b) for all other
purposes. The AHL reporter strains, their culture and use in bioassays are described in previous chapters. All bacteria were maintained as frozen glycerol stocks and subcultured into appropriate media as necessary.

**AHL extraction, purification, bioassay and identification.** Cultures (1.5 L) of *S. meliloti* were grown in 2.8 L wide-bottom flasks to early stationary phase (OD$_{600}$ = 1.4-1.8). Cell-free culture supernatants of *S. meliloti* (pH 6.4-7.1) were extracted twice with 1/2 volume of ethyl acetate containing 0.1 ml/L of glacial acetic acid, then dried over anhydrous sodium sulfate, filtered and rotary evaporated over a 40°C water bath. To estimate AHL concentrations within the cells, cell pellets were also extracted. The pellets were homogenized by vortexing with 10 ml of 95% ethanol, then extracted twice with 100 ml of acidified ethyl acetate, filtered and rotary evaporated.

For reverse phase HPLC, the extracts from 6 L of the cell-free culture supernatants or cells were dissolved in 1 ml of acetonitrile and injected onto semi-preparative C$_{18}$ column (Whatman Partisil 10, ODS-3), fitted with a guard column and equilibrated with 10:90 acetonitrile:water. The column was eluted at 2 ml/min with a linear water:acetonitrile gradient to reach 100% acetonitrile after 65 min, followed by additional 20 min in 100% acetonitrile. Synthetic AHLs were chromatographed on the same gradient to establish their retention times. Synthetic 3-oxo-C$_{12}$-L-HL, C$_{6}$-L-HL, 3-oxo-C$_{6}$-L-HL, and C$_{14}$-HL were purchased from Quorum Sciences (Corville, IA). C$_{4}$-L-HL was synthesized as described in Chapter 2. Synthetic C$_{16:1}$-HL (N-(tetrahydro-2-oxo-3-furanyl)-Z-9-hexacenamide) and C$_{14:1}$-HL (N-(tetrahydro-2-oxo-3-furanyl)-Z-9-tetradecenamide) were generous gifts from A. Eberhard.

As an alternative AHL extraction procedure, 9 L of cell free culture filtrates were passed through water-equilibrated Mega BondElut (Varian Inc., Palo Alto, CA) cartridge packed with C$_{18}$-bonded silica. The samples were passed through the cartridge at 1 L/hr, 1.5 L per run. After each run, the cartridge was eluted sequentially with 300 ml of
distilled water, then with 50ml of 10%:90% acetonitrile:water, 50%:50% acetonitrile:
water, acetonitrile and ethyl acetate. To estimate AHL recovery, synthetic C₄-HL was
mixed with NM medium, then either extracted with ethyl acetate or passed through the
Mega BondElut cartridge. The solvents were evaporated, and the samples were subjected
directly to mass spectral analyses.

To detect AHLs or AI2 in culture filtrates from S. meliloti, samples of the HPLC
fractions above were aliquoted into 96-well black microtiter plates (Life Sciences Inc,
Denver, CO), dried in a laminar flow hood, and then mixed with 80 µl of a reporter
suspension in LB. After approximately 3 h of incubation at 37°C, the luminescence of
the E. coli reporters was measured with a microtiter plate reader (Wallac Victor-2,
PerkinElmer Inc, Gaithesburg, MD). Pigment production by C. violaceum CV026 and
P. aureofaciens was assayed as described in Chapter 2. Vibrio harveyi BB170 assays
were performed as described by Joyce et al. (2000) with culture filtrates of Salmonella
enterica serovar Typhimurium 14028 as a positive control. The small bacteriocin assay
(Schripsema et al., 1996) was scaled down for microtiter plate analysis. HPLC fractions
were dried in clear-bottom 96-well microtiter plates, then overlaid with a suspension of
the bacteriocin sensitive R. leguminosarum ANU248 (OD₆₀₀=0.1) in semisolid TA
(0.3%) agar. Growth of the strain was quantitated using the Wallac Victor 2 reader every
12 h. Extracts of small bacteriocin-producing R.leguminosarum 8401 served as a control.

For identification of AHLs in S. meliloti cultures, the HPLC fractions above were
pooled in sets of 4-6 sequential fractions, dried and each set of pooled fractions subjected
to electrospray ionization mass spectrometry (ESI MS/MS). During the ESI MS/MS
runs, m/z 102 and 101 were monitored as described in Marketon et al. (2002, in press).
All fractions were also subjected to GC/MS. GC/MS was performed with a
Hewlett-Packard 5890 Gas Chromatographer at Cornell University and Ithaca College.
Samples were dissolved in ethyl acetate prior to injection. The resulting chromatograms
and mass spectra were compared to those of synthetic AHLs for identification. ESI/MS and GC-MS were carried out by Drs. M. Groenquist (Cornell University) and A. Eberhard (Ithaca College).

**Treatment of bacteria for proteomic analysis.** To determine the effects of exogenously added AHLs on wild type *S. meliloti* 1021 cells, it was important to reduce the concentration of endogenous AHLs as much as possible by growing the bacteria for several cycles at low population densities, washing and resuspending the cells at each cycle. A starter culture of *S. meliloti* 1021 was grown to $OD_{600} = 1.7$, then 1 ml was pelleted, resuspended and inoculated into 1 L of TA medium in a 2.8 L wide bottom glass flask and cultured overnight at 28°C to $OD_{600} = 0.2$ on a gyratory shaker at 200 rpm. A 100 ml portion was then centrifuged, and the pellet resuspended in 1 L of TA medium, and the suspension incubated on a shaker for 5 h to $OD_{600} = 0.05$. Three hundred mL portions of this low cell density culture were washed again and the pellets inoculated into flasks containing 1.5 L of TA supplemented with either $C_{14}$-HL or $C_{16:1}$-HL purified from early stationary phase *S. meliloti* 1021 cultures. The concentrations of the two purified *S. meliloti* AHLs were adjusted to be approximately equal to those present in an early stationary phase culture of the bacterium grown in NM supplemented with glucose and potassium nitrate (= 1 culture volume equivalent). The treated cultures were incubated for 2 h at 28°C and 200 rpm to a final $OD_{600} = 0.03-0.04$, yielding 0.1 g of dry cells per 1.5 L of culture. Duplicate control cultures with no added AHLs were grown under the same conditions. All centrifugations were conducted at room temperature to minimize stress. Viability and growth of the cells were monitored throughout the induction experiment by dilution plating. In addition, another low density culture of wild type 1021 was exposed to 3-oxo-C16:1-HL for 8 h. The cell density of this culture
reached a final OD$_{600} = 0.08$. Additional control cultures were grown to early stationary phase culture (24 h old, OD$_{600} = 1.8$) under the same conditions. All treatments and controls were duplicated.

**Protein extraction, and two-dimensional gel electrophoresis.** Proteins were extracted from freeze-dried cells as described earlier (Chen et al., 2000b; Chen et al., 2000a). Protein concentration in the cell lysates was quantitated based on a modified Bradford protein assay (Guerreiro et al., 1997). Protein concentrations were normalized, and the samples were subjected to the proteomic analysis as described by (Chen et al., 2000b). Isoelectric focusing was performed with linear immobilized pH 4-7 IPG strips (Amersham Pharmacia Biotechnology, Uppsala, Sweden). Aliquots containing 1 mg of solubilized cellular protein were cup-loaded onto the acidic end of each IPG strip and run for 200 kVh. Chromatography in the second dimension was performed on pre-cast polyacrylamide ExcelGel® XL SDS 12-14 gel from (Amersham Pharmacia Biotechnology, Uppsala, Sweden) according to the manufacturer’s instructions. Proteomic analyses were carried out by H.-C. Chen, M. Djordjevic and B. Rolfe in Genomic Interactions Group at the Australian National University.

**Image processing and protein identification.** Preparative gels were stained with Coomassie Brilliant Blue in a stepwise colloidal staining procedure described by (Neuhoff et al., 1988). Proteins on analytical gels were visualized by silver staining as described by (Chen et al., 2000b; Natera et al., 2000). Digitized images (600dpi) of the stained gels were quantified using MELANIE 3 image analysis software (Bio-Rad, Hercules, CA). Positions of the spots were compared to the ten landmark proteins, and matched against a specialized proteomic database for *S. meliloti* 1021 (Weiller, et al., 2001). Optical density (OD) of each spot over its area (volume) as a percentage of the relative OD of the gel image (% vol.) was used to quantify each spot as described by Natera et al. (2000). Digitized spot images were statistically analyzed using GenStat 4.2 software as described
in Mathesius et al. (submitted). A polypeptide was deemed "differentially accumulated" if Chi-square was less than 0.05. Confidence ratings were assigned as described in Weiler et al (2001).

Polypeptides from preparative gels were identified by comparison with the reference proteomics gels carefully developed by the Genomic Interactions Group at the Australian National University as described by Weiller et al. (2001).

RESULTS

\textit{S. meliloti strains produce multiple quorum sensing signals.} Fractionation of the AHLs present in the extracts from \textit{S. meliloti} 1021 by reverse-phase HPLC gave a broad peak of substances in the hydrophobic region of the elution profile (fractions 49-75) that stimulated responses in the LasR- and the LuxR-based AHL reporters (Fig. 18A). The putative AHLs detected by these reporters eluted after the retention time of 3-oxo-C\textsubscript{12}-HL (Fig. 18A). Refractionation of the substances in this broad activity peak resulted in the separation of two peaks of material capable of activating these reporters (Fig. 18B). One of these peaks (fractions 60-63) was further resolved by a third HPLC fractionation (Figs. 18D).

Pooled fractions from the peaks detected by the AHL reporters, and sets of fractions showing no activity with the AHL reporters, were examined by our collaborators Professor Anatol Eberhard and Dr. Matt Gronquist for the presence of AHLs by tandem electrospray ionization mass spectrometry (ESI-MS/MS). Professor Eberhard developed the (currently unpublished) ESI-MS/MS method for AHL identification.

Compounds with \(M + 1\) molecular ions corresponding to AHLs were detected only in those fractions in Figs. 18A-D which elicited responses in the AHL reporters. Compounds with mass spectral features characteristic of five hydrophobic AHLs were detected in those fractions that stimulated the LasR-, and LuxR-based reporters (Table 3). ESI-MS/MS and GC-MS indicated that the culture filtrates of strain 1021 grown on a
defined medium contained C\textsubscript{16:1}-HL, 3-oxo-C\textsubscript{16:1}-HL, C\textsubscript{14}-HL and 3-oxo-C\textsubscript{14}-HL in an approximate ratio of 10:2:1:1. Traces of \textit{N}-(tetrahydro-2-oxo-3-furanyl)-hexadecanamide (C\textsubscript{16}-HL) were also detected. The mass spectral comparison of C\textsubscript{14}-HL from 1021 and the standard C\textsubscript{14}-HL synthesized by Dr. Eberhard is shown in Fig. 19.

An AHL activity profile similar to that seen for the culture filtrates (Fig. 18A) was also obtained after extraction of the cell pellets. However, the activity from the cell pellets was about 10- to 100-fold less than in the corresponding HPLC fractions from culture filtrate extracts (data not shown). Only C\textsubscript{16:1}-HL and 3-oxo-C\textsubscript{16:1}-HL were present in amounts sufficient for chemical identification by mass spectrometry.

None of the HPLC fractions contained substances that detectably affected growth of the \textit{small} bacteriocin-sensitive \textit{R. leguminosarum} strain ANU248 when added at 2 to 10 culture volume equivalents. MS analysis of the HPLC fractions confirmed that \textit{S. meliloti} strains do not produce the 3-OH-C\textsubscript{14:1}-HL, the \textit{small} bacteriocin of \textit{R. leguminosarum} (Gray et al., 1996). None of the fractions consistently activated the AhyR-, PhzR- or CviR-based AHL reporters, although traces of C\textsubscript{4}-HL and C\textsubscript{8}-HL were detected in two batches out of the nine that were rigorously analyzed by ESI-MS/MS.

\textit{S. meliloti} strain AK631, derived from a Hungarian field isolate Rm41 (Kondorosi et al., 1991), had a very different AHL profile, consistent with the reports by Cha, et al 1998. In our studies, HPLC fractionation separated several activity peaks from \textit{S. meliloti} AK631 culture filtrate extracts were detected with either the LasR-, LuxR-, CviR-, PhzR- or AhyR-based reporters. Based on their retention times and mass spectra, the following AHLs were present in culture filtrates of AK631 in a ratio of roughly 10:5:5:1: \textit{N}-(tetrahydro-2-oxo-3-furanyl)-octanamide (C\textsubscript{8}-HL), 3-oxo-\textit{N}-(tetrahydro-2-oxo-3-furanyl)-octanamide (3-oxo-C\textsubscript{8}-HL), 3-hydroxy-\textit{N}-(tetrahydro-2-oxo-3-furanyl)-octanamide (3-OH-C\textsubscript{8}-HL), and \textit{N}-(tetrahydro-2-oxo-3-furanyl)-hexanamide (C\textsubscript{6}-HL) (Table 3). In addition, smaller amounts of \textit{N}-(tetrahydro-2-oxo-3-furanyl)-Z-2-octenamide (C\textsubscript{8:1}-HL), 3-oxo-\textit{N}-(tetrahydro-2-oxo-3-furanyl)-decanamide (3-oxo-C\textsubscript{10}-

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HL), 3-hydroxy-N-(tetrahydro-2-oxo-3-furanyl)-decanamide (3-OH-C\textsubscript{10}-HL), N-(tetrahydro-2-oxo-3-furanyl)-decanamide (C\textsubscript{10}-HL), 3-oxo-C\textsubscript{14}-HL, 3-oxo-C\textsubscript{16:1}-HL, and C\textsubscript{16:1}-HL were also detected. The C\textsubscript{8:1}-HL was most likely artifactually derived from 3-OH-C\textsubscript{8}-HL during extraction and chromatographic procedures. Based on the bioassay results, HPLC fractions from the cell extracts of \textit{S. meliloti} AK631 contained 10-100 fold less AHLs than the corresponding culture filtrates. Only C\textsubscript{8}-HL and 3-oxo-C\textsubscript{16:1}-HL were present in cell extracts in amounts sufficient for mass spectral identification.

Proteins affected by the addition of AHLs to early log phase culture of \textit{S. meliloti} 1021. The accumulation of 110 polypeptides was significantly affected by exposure to either 3-oxo-C\textsubscript{16:1}-HL or C\textsubscript{14}-HL. Of these 110 polypeptides, 57 proteins (Table 4) were identified by matching to the proteomic database of \textit{S. meliloti} 1021 (Weiller et al., 2001). As shown in Table 4, of these 57 polypeptides, 49 showed changes of 2- to 10-fold in accumulation after 2 h exposure to one or both AHLs. An additional eight proteins were affected by the exposure of the early log phase culture to 3-oxo-C\textsubscript{16:1}-HL for 8 h. Two hour exposure to 3-oxo-C\textsubscript{16:1}-HL affected the accumulation of only six of the same polypeptides affected by 8 h exposure to the same AHL. Exposure to C\textsubscript{14}-HL affected accumulation of fourteen polypeptides, five of which were also affected by a 2-hr exposure to 3-oxo-C\textsubscript{16:1}-HL, and additional four proteins were affected by the prolonged exposure to 3-oxo-C\textsubscript{16:1}-HL.

Since AHLs typically accumulate to high levels and activate gene expression in late log and stationary phase cultures (Schaefer et al., 2000), it is reasonable to expect that AHL-mediated QS might contribute importantly to the regulation of gene expression or protein accumulation in older cultures. To learn whether the addition of AHLs to early log phase cultures would induce the same changes in protein accumulation as maturation of the cultures from early log to early stationary phase, we determined which proteins
differentially accumulated during the transition from early log to early stationary phase. As shown in Table 5, over 80 polypeptides of diverse function were differentially accumulated in S. meliloti 1021 cells from early stationary phase cultures. Thirteen of these proteins were also differentially accumulated in response to either C_{14}-HL or 3-oxo-C_{16:1}-HL.

**Discussion**

**AHLs produced by S. meliloti.** S. meliloti strains appear capable of synthesizing an especially diverse array of AHLs. In their initial survey, Cha et al. (1998) used the A. tumefaciens and CV026 reporters coupled with thin layer chromatography to show that the two Sinorhizobium strains used in this study (1021 and AK631, a derivative of Rm41) produced remarkably different sets of AHLs. We found 3-oxo-C_{14}-HL, C_{16:1}-HL and 3-oxo-C_{16:1}-HL and C_{14}-HL were produced in appreciable quantities by late-log phase cultures of S. meliloti 1021 grown in a minimal medium. We have confirmed the presence of C_{14}-HL in 1021 culture filtrates by both retention time and ESI MS/MS comparison with the synthetic compound. None of these AHLs have been identified in other bacteria, except for 3-oxo-C_{14}-HL previously isolated from P. aeruginosa biofilms (Charlton et al., 2000). In studies conducted concurrently by the Gonzales laboratory at UT Dallas in collaboration with Professor Eberhard, strain 1021 grown on a rich medium was found to produce C_{8}-HL, as well as several long chain AHLs, including 3-oxo-C_{14}-HL, C_{16:1}-HL, 3-oxo-C_{16:1}- and C_{18}-HL (Marketon et al., 2002, in press). They did not see production of C_{14}-HL in their cultures and we did not detect C_{12}-HL, C_{18}-HL or C_{8}-HL or other short chain AHLs in our cultures. Since AHLs in both studies were identified by the same methods and instruments, it is likely that different culture conditions account for the different AHLs detected. This view is
consistent with the observation by Marketon et al. (in press) that strain 1021 made no detectable short chain AHLs when cultured, as we did, on a defined medium, but made several short chain AHLs when cultured on a rich medium. The culture medium/environment dependence of AHL synthesis evident in strain 1021 indicates the need for some caution in making assumptions about which AHL signals are really present and important during growth of the bacterium in natural environments. The recent discoveries that *P. aeruginosa* biofilms produce high concentrations of 3-oxo-C_{14}-HL in addition to the well-known 3-oxo-C_{12}-HL (Charlton et al., 2000), and different AHL profiles of *R. etli* shake cultures and bacteroids (Daniels et al., 2002) illustrate the kind of surprises that growth under more natural conditions may provide. The AHLs secreted and used for quorum sensing regulation by *S. meliloti* under natural conditions may be different from those produced in vitro, and may be significantly different in soil, rhizosphere and host tissue environments.

Marketon et al., 2002 (in press) speculated that long chain AHLs such as those detected in *S. meliloti* may not have been seen in other bacteria because they might have partitioned into the cell membrane and not been released substantially into the culture supernatant. We found, however, that extraction of the cell pellets of *S. meliloti* led to recovery of approximately 10% of the long chain AHL activity recovered by extraction of the culture supernatants. While AHLs with long side chains may indeed accumulate in the bacterial cells and membranes, it seems that the extracellular levels of these signals are quite sufficient for both chemical detection and biological function. Additionally, AHL extraction efficiency studies by A. Eberhard (unpublished) indicate that conventional ethyl acetate extraction procedure completely recovers C_{16}-HL, but only about 46% of C_{4}-HL and 3-OH-C_{6}-HL. More hydrophilic AHLs, e.g., 3-OH-C_{4}-HL and 3-oxo-C_{4}-HL did not partition into ethyl acetate at all. This may help to explain why the more hydrophilic short chain AHLs seen by Cha et al. (1998) in culture filtrates of *S. meliloti* Rm41 were not detected in our extracts from AK631. We explored the use of
an alternative extraction procedure to estimate the range of AHLs produced by *S. meliloti* 1021 (the Mega BondElut C\textsubscript{18}-packed cartridge as described in Materials and Methods). The 100% acetonitrile eluent contained C\textsubscript{14}-HL, C\textsubscript{16:1}-HL and C\textsubscript{16}-HL. However, this procedure was even less efficient than extraction with ethyl acetate. Only 10% of C\textsubscript{4}-HL extractable by ethyl acetate was retained by the cartridge.

Bioassay of HPLC fractions from culture filtrates of a *S. meliloti* Ω5301, a Tn5-233 (Driscoll and Finan, 1997) insertion mutant in ORF170, a putative AHL receptor (Galibert et al., 2001), indicated that the mutation caused a 10-fold reduction in the levels of AHLs (data not shown). The diminished secretion of long chain AHLs by this mutant is in good agreement with the reduced AHL synthesis phenotype of the *sinR* mutant described by Marketon et al 2002, although in both cases the reduced synthesis of AHLs may be due to downstream polar effects of the mutations.

The *V. harveyi* BB170 reporter did not detect any AI-2-like activity in cultures of *S. meliloti* 1021 or AK631 grown to mid-exponential or early-stationary phase in NM glucose or TA media (data not shown). The lack of AI-2 activity is consistent with the absence of a LuxS homolog in the genome of *S. meliloti* 1021 (Galibert et al., 2001).

Strain AK631 appears to make an impressive diversity of at least ten AHLs, predominantly the short chain C\textsubscript{8}-HL, 3-OH-C\textsubscript{8}-HL, and 3-oxo-C\textsubscript{8}-HL, but also lesser amounts of C\textsubscript{6}-HL, 3-OH-C\textsubscript{10}-HL and 3-oxo-C\textsubscript{10}-HL, plus the three long chain AHLs seen by both our lab and Marketon et al in strain 1021: 3-oxo-C\textsubscript{14}-HL, C\textsubscript{16:1}-HL and 3-oxo-C\textsubscript{16:1}-HL. Because 3-oxo-C\textsubscript{16:1}-HL is produced by both *Sinorhizobium meliloti* strains and not by any other tested species, we decided to further investigate their signaling role using proteomics. For comparison protein level responses to purified C\textsubscript{14}-HL were also identified.
Proteomic analysis of *S. meliloti* responses to AHLs. Proteomic analysis is a potentially important global approach to the analysis of quorum sensing regulation in bacteria, complementary to the random mutagenesis and genomic transcriptional approaches. Givskov et al. (1998) used pulse labeling with $^{35}$S- methionine to detect 28 proteins that were differentially accumulated in an AHL synthesis deficient mutant of *Serratia liquefaciens* in response to the addition of C$_4$-HL, the dominant AHL produced by that strain. These workers detected proteins that were differentially accumulated or modified within just a few minutes after addition of the AHL. Other researchers (Whiteley et al., 1999), used various mutant strains to estimate global physiological effects of QS. We wanted to learn about the effects of exogenously added AHLs on a wild type strain. To obtain biologically relevant information, it was important to maintain the wild type culture at a very low population density and remove AHLs by repeated washes.

Our initial proteomic study of quorum sensing in *S. meliloti* was conducted in a wild type background rather than in an AHL synthase mutant background in order to determine whether or not quorum sensing regulation could be analyzed effectively in wild type bacteria. In addition, we hoped to avoid the problems associated with the potentially invisible accumulation of second site mutations in AHL synthase mutants and the potential downstream polar effects of mutant constructs in the synthase genes (Beatson et al., 2002). We also wanted to start exploring the question of whether AHL synthase mutants go through the same progression of regulatory states as wild type cells during growth. The extensive and AHL-specific changes in protein level seen in this study indicate that treating washed, early log phase cultures of wild type bacteria with purified AHLs can indeed be an effective approach to the analysis of quorum sensing regulation. Additional proteomic studies in an AHL synthase mutant background are now needed to test the uncertainties regarding second site mutations, potential construct problems and regulatory states.
The addition of the two purified AHLs to an early log phase culture of *S. meliloti* 1021 was found to significantly affect the accumulation of over a hundred polypeptides, which represent roughly 5% of the total polypeptides resolved on the gels. This percentage is similar to the proportion of the AHL-responsive genes identified by Whiteley et al. (1999) in the AHL synthase minus mutant of *P. aeruginosa*.

Of the hundred AHL-responsive polypeptides, only 57 were matched to reference gels and presented in Table 4. The other 53 AHL-responsive polypeptides have not been identified in the cultures of *S. meliloti* 1021 grown under laboratory conditions. These 53 polypeptides may very well affect behaviors important to the strain under some natural condition, not normally encountered in the shake-cultures. The reliance upon protein profiles of bacteria in shake cultures is an important limitation of the currently available proteomic database. However, careful experiments are being conducted to catalog the physiological differences between the shake cultures and *S. meliloti* cells in various natural habitats (Natera et al., 2000; Oke and Long, 1999a). For this Discussion, only the identified proteins will be considered further.

The addition of purified 3-oxo-C\textsubscript{16:1}-HL and C\textsubscript{14}-HL to 1021 cells affected the accumulation of two quite distinct sets of proteins (Table 4). These sets of proteins help to define the functional quorum sensing regulons for each signal. Future studies with synthetic 3-oxo-C\textsubscript{16:1}-HL and C\textsubscript{14}-HL at defined concentrations will be needed to confirm and extend the definition of these regulons. The addition of 3-oxo-C\textsubscript{16:1}-HL affected the levels of a larger number of polypeptides after 2 h (42) than did C\textsubscript{14}-HL (14), and the changes in level of accumulation were generally larger after 3-oxo-C\textsubscript{16:1}-HL addition than after C\textsubscript{14}-HL addition. These differences in response to the two AHLs are consistent with the possibility that the two AHLs are primarily recognized by two different receptors or that the recognition of 3-oxo-C\textsubscript{16:1}-HL is dominant in a regulatory hierarchy over C\textsubscript{14}-HL, similar to the observation of Whiteley et al., 1999 (see also Fig. 1).
showing that the dominant AHL (3-oxo-C$_{12}$-HL) activated the majority of the
AHL-responsive fusions. In the future, it will be important to confirm these possibilities
through studies with knockout mutants of the AHL receptors and synthases.

It is noteworthy that 3-oxo-C$_{16:1}$-HL, which is synthesized by both $S$. meliloti strains
and no other known bacterium, also induces significant changes in the accumulation of
over 150 different root proteins in the host legume, $M$. truncatula (Mathesius et al.
unpublished).

Addition of AHLs to early log phase cultures resulted, either initially or after 8 h, in
the diminished accumulation of approximately 1/3 of the AH-responsive proteins (Table
4). It is not yet clear how this result should be interpreted. Added AHLs have been
reported to reduce the accumulation of polypeptides in other bacteria (Throup et al.,
1995, Givskov et al., 1998), so the phenomenon is not unique to $S$. meliloti. AHLs are
normally activators of gene expression through interactions with and stabilization of their
cognate receptors (Miller and Bossler, 2001; Zhang et al., 2002). The immediate
consequence of AHL addition should thus be enhanced transcription and increased
accumulation of the corresponding protein. However, the addition of AHLs was found to
directly repress transcription of genes in some bacteria (Koiv and Mae, 2001). In
addition, C$_8$-HL in $V$. fischeri was found to antagonize transcriptional activation via LuxR
by its other cognate AHL, 3-oxo-C$_6$-HL, thus reducing the accumulation of certain
polypeptides (Eberhard et al., 1986). Givskov et al (1998) observed that AHL addition
diminished the accumulation of certain proteins in $S$. liquefaciens within just 5 min, which
may also reflect reduced transcription. Thus, it seems possible that the addition of 3-oxo-
C$_{16:1}$-HL and C$_{14}$-HL may have reduced the transcription of the genes corresponding to
at least some proteins listed in Table 4 rather then reducing their level via enhanced
proteolysis or through other downstream effects. The repression of transcription and
protein levels by AHLs may prove to be a biologically important yet underappreciated
aspect of quorum sensing regulation. One can speculate that, in making the transition

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from low to high population density situation, it may be just as important for bacteria to reduce the levels of proteins useful at low densities as it is to increase the levels of proteins required for new functions at high densities.

Several functional classes of proteins were affected by addition of AHLs to wild type cells. One important class of AHL-responsive proteins is related to nitrogen assimilation and cycling (e.g., glutamine synthase I, glycine dehydrogenase, O-succinylhomoserine sulfhydrolase, D-threonine dehydrogenase, arginine/threonine antiporter, amidase, aspartate-semialdehyde dehydrogenase, nitrogen regulatory protein II, and an ABC aminoacid transporter). A second major class of AHL-responsive proteins is related to nucleotide synthesis and turnover (e.g., deoxyuridine 5'-phosphatase nucleotidohydrolase dUTPase, guanylate kinase, 2, 3, 4, 5, -tetrahydropyridine-2-carboxylate N-succinyl transferase, orotate phosphoribosyl-transferase, inosine-5'-monophosphate dehydrogenase, and a regulator of nucleoside diphosphate kinase). The two AHLs were also found to affect the levels of several proteins involved in stress-responses (e.g., peroxiredoxin, inosine-5'-monophosphate dehydrogenase) protein processing (e.g., Clp proteases, GroEL, chaperon protein Smc04456), carbon metabolism and assimilation (e.g., phosphoglycolate phosphatase, NADP-dependent malic enzyme, ribose-5-phosphate isomerase isoforms), phosphoenolpyruvate carboxykinase, phosphoglycerate mutase I, and aconitate hydratase), as well as proteins involved in secondary product synthesis and turnover (fatty-acid-CoA ligase, glutaryl-CoA dehydrogenase, amidase, precorrin-2 C20-methyltransferase, precorrin-8x methylmutase).

The 2D map of polypeptides found to differentially accumulate in late log phase cultures of strain 1021(Guerreiro et al., (1999) was quite similar to that obtained during our comparison of early log vs early stationary phase polypeptides (data not shown). The 80 “stationary phase” proteins identified in Table 4 were quite diverse in their functions, and included 14 transporters (all upregulated), five regulatory proteins (up- and down-regulated), and three elongation factors (all down-regulated). Differences between results from the two studies can be attributed to somewhat different culture
conditions and media. The gene corresponding to D-β-hydroxybutyrate dehydrogenase was reported to be upregulated in stationary phase cultures of *S. meliloti* (Aneja and Charles, 1999), consistent with the differences we found in polypeptide accumulation.

Only about 10-15% of the proteins that differentially accumulated during the transition to stationary phase were also affected by the addition of AHLs to early log phase cultures. There are several possible explanations for the observation that 3-oxo-C\textsubscript{16:1}-HL and C\textsubscript{14}-HL affected the accumulation of only 13 of the 80 stationary phase proteins listed in Table 4. Although one would expect quorum sensing regulation to be especially important in older cultures, this may not be true in *S. meliloti*. In this regard, Pellock *et al* (in press) found evidence of quorum sensing regulation of EPSII synthesis in *S. meliloti* at unusually low cell densities. It is also possible that more stationary phase proteins would be affected by AHL addition if different AHLs or higher AHL concentrations or longer exposure times were used. Alternatively, it may be that early log phase cultures of *S. meliloti* are not yet ready to respond fully to these AHLs.

Work elsewhere has shown that even though treatment with AHLs can stimulate various physiological changes characteristic of a stationary phase culture (eg. Nealson, 1977; Gray *et al*., 1996; Wilkinson *et al*., 2002), additional regulators and stationary phase sigma factors are required for full activation of certain AHL-regulated genes (Flavier *et al*., 1998; Whiteley *et al*., 2000; rev. Pearson, 2002). Further studies are required to determine whether AHL responsiveness in *S. meliloti* is similarly conditioned by such global, growth phase dependent regulators. The *S. meliloti* 1021 genomic database (Galibert *et al*., 2001) does not appear to contain any sequences similar to those of the regulators described in *P. aeruginosa* (MvaT, RsmA, RsaL, RpoS).

Our results suggest that bacteria in early log phase cultures can respond to the exogenously added AHLs, but these responses can differ both qualitatively and quantitatively from the responses of the older cultures. The responses of low density
cultures of the wild type bacteria to the AHLs have been studied relatively little (Nealson, 1977), but they may represent a type of ecologically important interaction between solitary bacteria and AHL-producing bacterial colonies or biofilms.

The two AHLs used for these proteomics experiments were chosen based on the observation that their structure may be unique to some strains of *S. meliloti*. We do not have any evidence to suggest that the same AHLs are produced by this bacterium in other environments. In fact, strains of *S. meliloti* are capable of producing an impressive diversity of AHLs (as described in (Cha et al., 1998; Marketon and Gonzalez, 2002, and above). The major finding of this study, though with its limitations as outlined in this discussion, is that wild type low population density cultures of soil bacteria can perceive AHLs and alter their physiological responses accordingly.
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Table 3. N-acyl homoserine lactones produced by strains of *S. meliloti* grown in minimal medium.
* fragments detected by ESI MS/MS
** fragments detected with GC MS
Figure 18: HPLC fractionation of *S. meliloti* 1021 AHLs. LuxR- and LasR-active fractions 49-63 (Fig. 18A) were pooled and rechromatographed (Fig. 18B) on the C\textsubscript{18} Partisil column with a water:acetonitrile step gradient (thick dark line) to separate major activities. The substances present in the two peaks indicated by arrows (fractions 47-52 and 60-63, Fig. 18B) were subsequently re-chromatographed on shallow water:acetonitrile gradients (Fig. 18C, Fig. 18D). Fractions representing peaks of biological activity (Fr. 48-49 Fig. 18C, and Fr. 32-34 and 43-45, Fig. 18D) were pooled and analyzed by ESI-MS. Substances in fractions indicated by dark bars (48-49 of Fig. 18C and fractions 32-34 of Fig. 18D) were identified by ms as 3-oxo-C\textsubscript{16:1}-HL and C\textsubscript{14}-HL, respectively and used to treat 1021 cells for the proteomic studies. Numbered arrows indicate elution times for synthetic AHL standards: 1 = C\textsubscript{4}-HL, 2 = 3-oxo-C\textsubscript{6}-HL, 3 = C\textsubscript{6}-HL, 4 = 3-oxo-C\textsubscript{12}-HL, 5 = C\textsubscript{12}-HL, 6 = C\textsubscript{14}-HL.
**Figure 19:** Comparison of the collisionally-induced dissociation (CID) spectrum for the precursor ion observed at m/z=312 in the natural sample to that of an authentic sample.  
(A) Daughter scan of synthetic \(N\)-(tetrahydro-2-oxo-3-furanyl)-tetradecanamide recorded for precursor ion m/z=312.  (B) Same scan carried out on the semi-purified natural material.
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Table 4. Polypeptides differentially accumulated in log phase cultures of *S. meliloti* treated with either 3-oxo-C<sub>16:1</sub>-HL or C<sub>14</sub>-HL for 2 or 8 hours.
Table 4 (continued)

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CHAPTER 6

UTILIZATION OF PLANT POLYSACCHARIDES AS A SOLE CARBON SOURCE BY WILDTYPE RHIZOBIUM LEGUMINOSARUM BV. VICIAE AND MUTANTS.

ABSTRACT

Plant roots secrete a complex polysaccharide mucilage which may serve as a significant source of carbon and/or facilitate colonization of roots by beneficial or pathogenic rhizosphere microbes. Root exudate from peas was fractionated to remove low molecular weight components easily metabolized by microbes. The high molecular weight mucilage polysaccharide and several commercial polysaccharides were used as sole carbon sources for growth of R. leguminosarum in minimal salts medium. When the plant flavonoid naringenin was added to the growth medium, the ability of the legume symbiont, R. leguminosarum bv. viciae 8401 (pRL1JI), to grow on root mucilage was enhanced to approximately 50% of the level of growth on glucose. Addition of flavonoids had no effect on the ability of R. leguminosarum to grow on commercial polysaccharides or glucose. Several mini-Tn5-induced mutants with altered ability to hydrolyze p-nitro-phenyl glycosides were isolated. None of these mutants was affected in ability to grow on plant polysaccharides or purified root mucilage from pea.
INTRODUCTION

Root mucilage is the high molecular weight material in plant root exudates. Mucilage is secreted primarily by root cap cells and by the border cells that detach and differentiate from the cap (Hawes and Brigham, 1992). The mucilage secreted by these cells can form a layer over the surface of younger parts of the root and may diffuse into the surrounding soil and help to form a “rhizosheath” of aggregated soil particles (McCully, 1995; Perkins et al., 1984). The function(s) of root mucilage remain to be firmly established, although mucilage is most commonly thought to act as a lubricant to protect the root tip as it pushes through the soil (Greenland, 1979). Studies have also indicated that root mucilage enhances the stability of soil aggregates (Morel et al., 1987; Morel et al., 1991). In addition, it has been suggested that mucilage may carry gravitropic signals from the root cap to the root tip (Moore et al., 1990) and that it may protect roots from the toxicity of aluminum and other ions (Horst et al., 1982; Mensch et al., 1987).

Previous studies have shown that root mucilage from several plant species is composed of approximately 95-97% sugars and 3-5% amino acids (Bacic et al., 1986; Chaboud, 1983; Chaboud and Rougier, 1984; Moody et al., 1988). Glycosidic linkages between sugar residues in maize mucilage proved to be surprisingly complex. The galactose, glucose, arabinose, fucose and xylose residues in maize mucilage were all present in 3 or 4 different patterns of glycosidic linkage to other sugars (Bacic et al., 1986). Overall, the diversity of linkages in the mucilage was comparable to the diversity of linkages between sugars in the various polysaccharides of plant cell walls (Zablackis et al., 1995). Based on the glycosidic linkage patterns in mucilage from maize, rice, wheat and cowpea, it seems likely that the mucilage from each plant species is comprised of different and possibly unique sets of hetropolysaccharides and glycoproteins.

Knee et al. (2001) reported that high molecular weight pea root mucilage was generally similar in its composition to the mucilage of cowpea, wheat and maize. The sugar composition of pea mucilage was most similar to that of cowpea, although cowpea
mucilage had higher amounts of fucose and glucose, and had no glucuronic acid. Pea and cowpea root mucilages had 3-5 times more uronic acids than the mucilages of wheat and maize. Unlike other mucilages, pea mucilage contained high amounts of galacturonic acid and hydroxyproline. Arabinose and galactose residues accounted for 60% of the sugars detected in pea mucilage. These findings prompted us to believe that pea root mucilage is composed primarily of arabinogalactan proteins (AGPs). AGPs have been identified by chemical and antigenic tests in the mucilage from maize (Bacic et al., 1986) and on roots of pea (Gollotte et al., 1995). They are recognized for their high water binding and gel formation properties (Fincher et al., 1983) that could serve in lubrication and retention of water around roots.

Chaboud et al. (1983) estimated that about 20%-25% of the total reduced carbon released by maize roots is in the form of high molecular weight root mucilage. Thus, it may represent a significant carbon source for soil microbes. Crude high MW maize mucilage was mineralized after addition to non-sterile soil (Mary et al., 1993), indicating that mucilage can be utilized at reasonable rates by microbes in the soil. Knee et al. (2001) recently reported that strains of *R. leguminosarum*, *P. fluorescens* and *Burkholderia cepacia* can utilize high molecular weight pea root mucilage as a sole carbon source when grown in mineral minimal medium. The ability to utilize root mucilage may be an important factor in the competitive colonization of roots by microbes. In addition, any microbial degradation of root mucilage could affect proposed mucilage functions such as lubrication, signal transmission and protection from toxic metal ions.

Synthesis and secretion of the polysaccharide-degrading enzymes in *R. leguminosarum* is likely to be complex. Knee et al. (2001) provided the first evidence that root mucilage degrading enzymes are normally repressed and require additional signals. Addition of 1 µM of the flavonoid naringenin increased mineralization of pea root mucilage by ~3 fold. The effect of naringenin was independent of NodD, a flavonoid receptor, indicating that there might exist another flavonoid receptor that in some way up-regulates enzymes involved in mineralization of pea root mucilage.
Rhizobium glycanases may be regulated at other levels. For example, Zorreguieta et al., (2000) suggested that an uncharacterized surface-associated component of \textit{R. leguminosarum} cells controls the release of two glycanases. This component was specific to \textit{R. leguminosarum} and was associated with the ability of the strain to produce extracellular polysaccharide.

\textit{R. leguminosarum} was selected for further studies for several reasons. First, strains of \textit{R. leguminosarum} were shown capable of producing an impressive array of polysaccharide degrading enzymes (Mateos et al., 1992; Knee, 2001). Second, strains of \textit{R. leguminosarum} that carry different symbiotic plasmids infect different legume hosts (Downie et al., 1983; Johnston et al., 1978) thus allowing us to study roles of mucilage degradation in specific plant-microbe interactions. Third, \textit{R. leguminosarum} can be subjected to miniTn5 mutagenesis and standard genetic manipulations.

Enumeration and analysis of all the carbohydrate-degrading enzymes secreted by \textit{R. leguminosarum} was not the objective of this study. I sought to identify only those enzymes that are involved in root mucilage digestion. In preliminary experiments, \(\alpha\)-mannosidase, \(\beta\)-galactosidase, \(\alpha\)-glucosidase, \(\alpha\)-rhamnosidase, \(\alpha\)-arabinosidase, and \(\beta\)-glucosidase were induced in \textit{R. leguminosarum} 8401 bv. \textit{viciae} (pRL1JI) cells grown in minimal medium supplemented with purified high molecular weight pea mucilage. The research described in this chapter focuses on the search for mutants with altered glycosidase activity. The ability of the wild type \textit{R. leguminosarum} bv. \textit{viciae} and various glycosidase mutants to grow on a variety of model plant polysaccharides is also described.

**MATERIALS AND METHODS**

**Preparation of purified root mucilage.** Root mucilage from pea (\textit{Pisum sativum}) cv. Little Marvel was collected as described by Knee et al. (2001). Briefly, surface-sterilized seeds were germinated in sterile sand without light. Roots of the week-old seedlings were
gently rinsed in sterile water. The rinsates were then filtered, and lyophilized. For purification of the root mucilage, the crude lyophilized root exudate was dissolved in 50 mM ammonium acetate buffer, pH 5.2. Any insoluble material was removed by centrifugation prior to HPLC gel filtration on a Toyopearl HW-50 column. Fractions were collected at a flow rate of 2 ml/min and elution of root exudate components monitored with a Shodex RI71 refractive index detector. The high molecular weight material, referred to as purified pea root mucilage, eluted from this column in the void volume fractions. Fractions containing purified mucilage were pooled and exhaustively lyophilized to remove ammonium acetate by sublimation. Chromatographic separation of root mucilage was carried out at Oklahoma State University, in Dr. Andrew Mort's lab.

**Bacterial strains and growth studies.** *R. leguminosarum* 8401 bv. *viciae* (pRL1JI) and other strains of *R. leguminosarum* were cultured in TY broth with the appropriate antibiotic for 24 h at 28°C, washed by centrifugation and then transferred to NM minimal salts medium (Robinson et al., 1992). The bacteria were then starved for 4 d in this medium to reduce internal nutrient reserves. Approximately 10⁶ starved cells were inoculated into NM minimal medium containing 1 mg/ml of a carbon source, either glucose, purified mucilage, or a commercial polysaccharide. Samples were taken from the cultures at each time point and the appropriate dilutions were prepared in water and plated on TY agar (1%) using a Spiral plater (Spiral Systems, Cincinnati, OH). Colony forming units were counted after incubating the plates at 28°C for 48 hours. For treatments with flavonoids, a 1mM stock solutions were added to the medium to give a final concentration of 1 µM and the appropriate concentration of ethanol was added to the mineral salts medium control. Flavonoids were purchased from Extrasynthese (Z. I. La Rechassiere, Genay) and dissolved in appropriate solvents as recommended by the manufacturer at a concentration of 10 mM.

Pectin and larch arabinogalactan were obtained from Sigma Chemical Co. (St. Louis, MO). Purified arabinan, de-branchcd arabinan, arabinoxylan, pectic galactan,
xyloglucan, rhamnogalacturonan were purchased from Megazyme (Sydney, Australia). Polysaccharide stock in sterile double-distilled water were prepared as described in Knee et al., 2001.

**mini-Tn5 mutagenesis.** *R. leguminosarum* and *E.coli* S17-1 λpir or *E.coli* Sm10 λpir carrying mini-Tn5: lacZ2 constructs (de Lorenzo et al., 1990) were grown in TY broth with streptomycin (200 µg/ml) or kanamycin (50 µg/ml) respectively. Matings were carried out as described elsewhere (de Lorenzo et al., 1990). Transconjugants were selected on TY agar supplemented with 200 µg/ml of streptomycin and 50 µg/ml of kanamycin, and were further purified on the same medium. To estimate the mutation efficiency, 500 mutants were evaluated for their ability to swim in TY supplemented with 0.3% agar. Transconjugants resistant to both streptomycin and kanamycin were aliquoted into the wells of 96-well microtiter plates (Falcon) with LB broth supplemented with 15% glycerol, sealed and frozen at -80°C.

To screen the library for mutants defective in their ability to cleave chromophoric \( p \)-nitrophenyl glycosides, transconjugants were replica-plated from their glycerol stocks into microtiter plates with TY broth, grown until uniform population density. Aliquots of the cultures were then diluted 15x into NM medium without a carbon source. The plates were then incubated for two days. \( p \)-nitro-phenyl glycosides were then added, and the assays carried out as described in Miller (1992). Production of yellow color was estimated visually, putative mutants were isolated, and the bioassays with \( p \)-nitro-phenyl substrates were repeated and quantified as in Miller (1992).

**RESULTS**

**Growth of R. leguminosarum on purified root mucilage and model polysaccharides.** *R. leguminosarum* 8401 grew on 0.1% purified root mucilage to about 16% of the cell numbers seen on 0.1% glucose. This growth was about 25 times higher than the controls with no added carbon. *R. leguminosarum* 8401 bv. *viciae* (pRL1JI) grew to 13% of the
cell numbers in glucose controls on purified pea mucilage, similar to the growth of 8401 cells carrying no symbiotic plasmid. These observations were consistent with the previous report by Knee et al. (2001).

R. leguminosarum 8401 (pRL1JI) was also able to utilize a number of commercially available plant polysaccharides for growth (Fig. 20). Maximal cell densities varied depending on the polysaccharide. Rhamnogalacturonan and debranched arabinan provided the highest final cell densities, while pectin and pectic galactan supported much lower levels of growth. Supplementing the culture medium with 10 nM to 1µM naringenin had no significant positive or negative effect on growth rates or growth yields of R. leguminosarum on any of the polysaccharides tested, nor on the growth rates or yields of R. leguminosarum 8401 on either glucose or NM salts containing no carbon source. A mix of seven flavonoids (daidzein, chrysin, genistein, naringenin, luteolin, 4’, 7-dihydroxyflavone, 3’,5’-dimethoxyflavone) did not affect growth of the strain on any commercial polysaccharides (Fig. 21, arabinogalactan only shown). Likewise, addition of the flavonoid cocktail did not significantly affect growth of the strain in minimal medium without carbon source. Addition of 0.05mg/ml "total" root exudate did not lead to higher final cell yield on commercial polysaccharides (Fig. 21, arabinogalactan shown).

Identification of glycosidase mutants of R. leguminosarum 8401 bv. viciae (pRL1JI). Using the p-nitro-α-D-phenyl-mannopyranoside substrate, five mutants were identified. Three mutants (R. leguminosarum 1G2, R. leguminosarum 17H4, and R. leguminosarum 1G12) had moderately decreased ability to cleave the substrate, and two (R. leguminosarum 3E4 and R. leguminosarum 1H4) had increased ability to cleave the substrate (Fig. 22). No mutants with abolished α-D-mannopyranosidase activity were identified.

A screen with the p-nitro-α-L-phenyl-arabinofuranoside substrate identified a mutant, R. leguminosarum 16D7, with an increased ability to cleave the substrate. The arabinofuranosidase activity in this mutant and the wild type R. leguminosarum was further characterized. It appears that the activity is cell-associated: no
arabinofuranosidase activity was found in the culture supernatant of the mutant or the wild type (Fig. 23). Arabinofuranosidase activity was low in young cultures of the wild type grown in complex media or with glucose as a C-source. Arabinofuranosidase activity was increased in starved wild type cells, in cells grown in the presence of pea root mucilage, arabinose, or arabitol (data not shown). In the mutant 16D7, arabinofuranosidase activity seemed to be constitutive (not shown). Growth rates and yields of the mutant on arabinan, de-branched arabinan, a 50:50 mix of arabinan and de-branched arabinan, root mucilage or glucose were identical to those of the wild type strain (data not shown).

**DISCUSSION**

The results presented above and in Knee et al (2001) show that the N-fixing symbiont of pea, *R. leguminosarum* 8401 (pRL1JI), is capable of using purified pea root mucilage and a variety of complex plant polysaccharides as sole carbon sources for growth. When starved *R. leguminosarum* 8401 cells were cultured on minimal salts medium containing purified mucilage, they were initially able to grow rapidly, at rates comparable to the glucose control for the first day, indicating that some amounts of the carbon and energy present in mucilage polymers was readily accessible to the bacteria. Nevertheless, the majority of the carbon and energy in pea mucilage polymers was inaccessible to *R. leguminosarum* 8401 under these conditions, since final cell numbers were about 6-fold lower than the glucose controls.

Knee et al. (2001) reported that growth of *R. leguminosarum* on mucilage was stimulated by the addition of micromolar concentrations of naringenin. This induced ability to grow on pea root mucilage was independent of the presence of NodD, a well-characterized flavonoid receptor (Mulligan and Long, 1989; Long, 1996). The flavonoid stimulation of mucilage utilization appears to be quite specific to this carbon source, since the flavonoids had no observable effect on utilization of glucose or the various plant polysaccharides tested (Fig. 21).
Since flavonoid-stimulated degradation of pea mucilage was extensive, R. leguminosarum 8401 must be able to produce quite a diversity of glycanases, presumably one (or two, α,β) for most of the major types of glycosidic linkage in pea mucilage. Production of a diversity of glycanases is consistent with the ability of the bacterium to grow on the array of plant polysaccharides tested in Fig. 20 and to hydrolyze a variety of p-nitrophenyl glycosides.

Using several p-nitrophenyl glycosides, several exoglycanase mutants were identified. Three mutants had reduced ability to cleave the substrates, three mutants were more efficient than the wild type in the cleavage of the substrates. Arabinofuranosidase activity in the mutant R. leguminosarum 16D7 was 5 to 10 times higher than in the wild type grown in glucose or TY broth. However, when grown on arabinan, arabitol or arabinose, both the mutant and the wild type produced the same levels of arabinofuranosidase activity.

It is, perhaps, not surprising that the glycanase mutants isolated in the mutant screen were not different from the wild type in their ability to grow on plant polysaccharides and root mucilage. First, R. leguminosarum probably has multiple glycanases with redundant functions, as evident for A. tumefaciens C58 with at least four putative α-L-arabinofuranosidase genes (Wood et al., 2001). Isolation of three mini-Tn5-induced mutants with reduced, but not abolished mannopyranosidase activity can probably be explained by the presence of mannopyranosidase isozymes. Second, Knee et al., 2001 reported that pea root mucilage is a highly complex polymer comprised of sugar and aminoacid residues, connected by a variety of chemical bonds. A mutation in a single glycanase will probably not significantly affect degradation of such complex polymers.
Figure 20: Growth of *R. leguminosarum* 8401 on plant polysaccharides. As a control, cells were cultured on glucose (Glc) and minimal NM salts (MS). Polysaccharides used as carbon sources are indicated as RG = rhamnogalacturonan, open triangle), dbA = debranched arabinan (open square), A = arabinan (inverted black triangle), PG = pectic galactan (black triangle), AX = arabinoxylan (open square), XG = xyloglucan (black square).
Figure 21: Growth of *R. leguminosarum* 8401(pRL1JI) on arabinogalactan (upside down open triangle), arabinogalactan supplemented with small amounts of crude pea root exudate (AG+RE, open square) an on arabinogalactan supplemented with 1μM flavonoid cocktail (AG+FC, upside down black triangle). As a control, cells were grown in glucose (Glc, black circle), minimal NM salts (NM, open circle), minimal salts supplemented with small amount of crude pea root exudate (NM+RE, open triangle), or minimal salts with 1μM flavonoid cocktail (NM+FC, black square).
Figure 23: Localization of \( \alpha \)-L-arabinofuranosidase activity in *R. leguminosarum* 8401 (pRL1JI) wild type and 16D7 mutant. From left to right: arabinofuranosidase activity in lysed cultures of the wild type, in culture filtrates of the wild type, in lysed cells of the wild type, in the lysed cultures of 16D7 mutant, in the culture filtrates of 16D7 mutant, in the lysed cells of 16D7 mutant. \( \alpha \)-L-arabinofuranosidase activity was measured with \( p \)-nitro-phenyl-\( \alpha \)-L-arabinofuranoside as described in Materials and Methods. The enzymic activity is expressed in Miller units (Y-axis).
CHAPTER 7

WHAT ROLES DO PLANT-PRODUCED SIGNALS PLAY IN THE RHIZOSPHERE?

The complexities of plant-bacteria interactions described in this dissertation are not at all unexpected from the evolutionary point of view for several reasons. Experimental evidence exists that plants have evolved means to recognize signals produced by both symbiotic and pathogenic bacteria and fungi (rev. Hulbert et al., 2001; Geurts and Bisseling, 2002; Long, 2001). For decades, plants were reported to secrete compounds that affect the composition of the associated bacterial communities (Van Egeraat, 1975b; Van Egeraat, 1975c; Faucher et al., 1988). Bacteria were shown capable of producing extracellular signals that help them in coordinately regulating physiological changes within a population (Khokhlov et al., 1967; Nealson, 1977; Eberhard et al., 1981).

Initial characterization of the plant signals that interfere with bacterial QS communication is the major finding of my research. Additionally, AHL signals produced by S.meliloti and their effect on polypeptide accumulation in low population density cultures of S.meliloti are complementary contributions. The discovery of AHL-mimics from higher plants presents several questions which will be addressed in this Chapter.
What is the role of plant AHL-mimics in bacterial quorum sensing? The first compounds to be recognized as mimics of bacterial AHL signals were the halogenated furanones of *D. pulchra*, a marine red alga (Givskov et al., 1996; rev. Chapter 1). All algal furanones identified to date inhibit bacterial quorum sensing, either by binding to the AHL receptor and exposing it to proteolysis (Manefield et al., 2002) or by affecting QS in some other way (Manefield et al., 2001). Plants, on the other hand, appear to produce both stimulatory and inhibitory AHL-mimics (Chapters 3, 4). A unicellular alga *C. reinhardtii* also produces a compound(s) that affected QS-regulated exoprotease induction in *C. violaceum* only when bacterial AHLs were also present.

These three types of effects on QS (stimulatory, inhibitory and additive) may represent different, but evolutionary and ecologically reasonable strategies for a given plant to deal with the bacterial populations. First, interactions of the inhibitory AHL-mimics with the AHL receptors could lead to destabilization and proteolysis of the receptor protein (not unlike furanone-LuxR interactions). Binding of both inhibitory and stimulatory AHL-mimics to the AHL-receptor can prevent binding of the native AHLs, and thus disrupt normal bacterial quorum sensing. Plant compounds could also affect transport of AHLs across bacterial membranes, or chemically modify bacterial AHLs.

Partial activation of bacterial QS by the stimulatory AHL-mimics (e.g. those produced by pea, *M. truncatula, C. reinhardtii*) may lead to the premature expression of the QS-regulated exoenzymes and thus induce plant defenses. Oligomers of plant cell-walls are known to trigger plant defense responses (rev. Mohnen and Hahn, 1993). Some bacterial phenotypes, normally repressed in low density cultures, may be beneficial to plants. For example, antifungal biocides are produced by pseudomonads only when they are exposed to AHLs (Pierson and Pierson, 1996). Plant stimulatory AHL-mimics may recruit even single bacterial cells to produce fungicides which may protect plants from infection. Other QS-regulated behaviors that might be of "interest" to plants include production of chelating agents (Lewenza and Sokol, 2001), various antibiotics (Thomson et al., 2000), fungicidal exoenzymes (Chernin et al., 1998).
The role for AHL-mimics and QS in legume-rhizobium interactions will not be firmly established until defined QS and AHL-mimic mutants are carefully constructed. There is evidence, however, that QS can play a role in the symbiosis. QS mutants of *R. etli* formed atypical symbiosomes occupied by single bacterioids (Daniels et al., 2002). Two proteins (GroEL5, and PckA, phosphoenolpyruvate carboxykinase) of the AHL-affected proteins in *S. meliloti* (Chapter 5) were also reported to be regulated within nodules formed by *S. meliloti* (Oke and Long, 1999a). Plant AHL-mimics from *M. truncatula* (Bauer and Teplitski, 2001) and *Phaseolis vulgaris* (Daniels et al., 2002) may play a role in restricting the nodule occupancy or stimulating symbiotically-important bacterial enzymes (like PckA).

**The nature and number of AHL mimic compounds in plants.** AHL mimic compounds in higher plants may prove to be of considerable biological and practical interest, but their study will not have a solid foundation until at least some active compounds have been identified chemically and the structures confirmed, as established for the furanone mimics from *D. pulchra*. Our HPLC purifications of the AHL mimic compounds present in plant seedling rinsates and *C. reinhardtii* culture filtrates have led to the separation of about half-dozen activities detectable with the AHL reporter strains used. Further purification of several of these activities is in progress. Based on these observations, individual species of higher plants may prove to synthesize at least as many different AHL signal-mimic compounds as *Delisea*, which secretes about 30 different halogenated furanone mimic compounds (Kjelleberg and Steinberg, 2002; Nys et al., 1993). This multiplicity of mimic compounds makes some sense when considering the dozen or so different AHLs identified in bacteria thus far and the very broad diversity of bacteria that a plant encounters.

None of the plant species tested elicited the same pattern of responses in the four lux-based AHL reporter strains. Lettuce and *Arabidopsis* did not appear to secrete any AHL mimic compounds capable of stimulating these reporters. It can be tentatively
concluded from this small survey that the production of AHL mimic compounds may be widespread among plants and variable among species. This is what one might expect if AHL mimic synthesis by plants were an important and co-evolved mechanism for dealing with associated bacteria.

**What factors determine the synthesis and secretion of AHL mimic compounds?** In the alga *D. pulchra*, the furanone AHL mimics are synthesized and released in special vesicles to the surface of the plant. The concentration of vesicles and furanone mimics on the surface is highest near the growing tips (Dworjanyn et al., 1999; Kjelleberg et al., 1997). In higher plants, the synthesis and secretion of AHL signal-mimics may not be constitutive. For example, we found no detectable mimic activity in the exudates of germinating pea seeds. The amount of stimulatory mimic activity secreted by young, germinated seedlings increased throughout the first 10 days, with little activity in seedlings less than four days old (data not shown). Four-days-old seedlings of *M. truncatula* induced luminesce of the lux AHL reporters within a few hours by mimic substances released at the plant surface. However, we could not recover detectable mimic activity in rinsates from uninoculated *M. truncatula* seedlings, as we had from pea. When aseptically grown *M. truncatula* seedlings were extracted with methanol to recover substances from inside the plants, a number of AHL mimic activities were detected, consistent with the activities seen with the reporters inoculated onto the surface. Some of these activities were released only after the seedlings were exposed to AHLs (Matthesius et al., submitted). From these results, it appears that *M. truncatula* seedlings (and possibly seedlings from other plants) synthesize several AHL mimic compounds, but do not secrete them until they are exposed to an environmental cue.

Of the variety of the plants tested, only pea (*Pisum sativum*) and crown vetch (*Coronilla varia cv Penngift*) could inhibit QS-regulated behaviors in *C. violaceum*. Both plants belong to the *Papilionoideaee* subfamily. Week-old seedlings of other *papilionoideaee* (soybean, *M. truncatula*, broad bean (*Vicia faba*), and sweet pea (*Lathyrus*
odoratus) did not secrete substances that affected the Chromobacterium reporters. There seemed to be some variation in the amounts of CV026-active substances released by pea seedlings of the same variety, and some variation between several pea cultivars tested (data not shown). Much greater seedling-to-seedling variability was observed among crown vetch seedlings (data not shown). Crown vetch cultivars are relatively new and are not as homogeneous as the pea varieties. It is therefore possible that such variability in AHL mimics is encountered in nature, similarly to the variability in halogenated furanone production by geographically distant populations of *D. pulchra* (Wright et al., 2000). It is also not unlikely that synthesis or release of CV026-inhibitory substances requires additional environmental cues, as observed for QS signal-mimics produced by *M. truncatula* (Mathesius et al., submitted).

**Current uncertainties and directions for future research.** Currently, there is a great deal of scientific and commercial interest in the chemical manipulation of AHL signaling among medical and environmental researchers (Finch et al., 1998; Zhu et al., 1998; Kline et al., 1999; Fray et al., 1999; Bauer and Robinson, 2002). Industrial researchers focus on the use of plant-signals in disrupting biofilms formed on submerged surfaces. Many of such molecules were detected in a screen for antifouling agents of marine microalgae (Hellio et al., 2001).

In the medical area, the major thrust is to find natural or synthetic compounds that will interfere with quorum sensing in pathogenic bacteria, thus disrupting their virulence. Thus far such studies have focused on the halogenated furanones from *Delisea* and on synthetic analogs of bacterial AHLs. Plant scientists (Fray et al., 1999; Dong et al., 2000) have introduced and successfully expressed genes coding for both AHL-synthases and AHL-degrading enzymes in plants. Transgenic AHL-degrading plants attenuated bacterial infections (Dong et al., 2000), while AHL-producing plants functionally complemented LuxI mutants (Fray et al., 1999). Broad ecological consequences of introducing QS-enzymes into plants are unclear, and may very well be detrimental to
plants' health. For example, enteric bacteria (including rhizosphere nitrogen fixer *Klebsiella pneumoniae*) that do not produce their own AHLs, still have functional AHL-receptors and rely on AHLs from other bacteria to modify their gene expression (Michael et al., 2001; B. Ahmer and E. Triplett, personal communication).

Global effects of AHLs on bacterial metabolism also need to be considered. It has always been assumed that AHLs control a number of genes involved in secondary metabolism (rev. in Chapter 1). However based on our proteomics studies presented in Chapter 5, wild type bacteria adjust multiple types of behaviors when exposed to AHLs. Further studies with AHL-mimics will require structural identification of the molecules. Based on the initial studies, more hydrophobic substances from plants and *C. reinhardtii* could be chromatographed on reverse phase C$_{18}$ silica columns using water:acetonitrile or water: methanol gradients. The hydrophilic substances in pea exudates which did not bind to the C$_{18}$ column, were successfully retained and eluted from a NH$_2$-bonded silica column using water:methanol and water:acetonitrile gradients, as discussed in Chapter 3. The compounds appear to be stable for several months when stored at -20°C, which facilitates all the identification experiments.
BIBLIOGRAPHY


