

# **STUDIES ON ENVIRONMENTAL RELEVANCE OF QUORUM SENSING SIGNAL DECAY**

Thesis by

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## Abstract

Signal degradation impacts all communications. Although acyl-homoserine lactone (acyl-HSL) quorum sensing signals are known to be degraded by defined laboratory cultures, little is known about their stability in nature. Here, we show for the first time that acyl-HSLs are biodegraded in soils sampled from diverse US sites and by termite hindgut contents. Furthermore, high-affinity acyl-HSL degraders were enriched in oligotrophic biofilm reactors that were inoculated with a typical turf soil.

When amended to soil samples at physiologically relevant concentrations,  $^{14}\text{C}$ -labeled acyl-HSLs were mineralized to  $^{14}\text{CO}_2$  rapidly and, at most sites examined, without lag. A lag-free turf soil activity was characterized in further detail. Heating or irradiation of the soil prior to the addition of radiolabel abolished mineralization, whereas protein synthesis inhibitors did not. Mineralization exhibited an apparent  $K_m$  of  $1.5\ \mu\text{M}$  acyl-HSL, ca. 1000-fold lower than that reported for a purified acyl-HSL lactonase. Under optimal conditions, acyl-HSL degradation proceeded at a rate of  $13.4\ \text{nmol} \cdot \text{h}^{-1} \cdot \text{g}$  of fresh weight soil $^{-1}$ . Bioassays established that the final extent of signal inactivation was greater than for its full conversion to  $\text{CO}_2$ , but that the two processes were well coupled kinetically. An MPN of  $4.6 \times 10^5$  cells  $\cdot \text{g}$  of turf soil $^{-1}$  degraded physiologically relevant amounts of hexanoyl-[1- $^{14}\text{C}$ ]HSL to  $^{14}\text{CO}_2$ . It would take chemical lactonolysis months to match the level of signal decay achieved in days by the observed biological activity. The results implicate a real-world challenge for acyl-HSL-producing bacteria to outpace

biological acyl-HSL degradation and thus to successfully engage in cell-cell communications in soils and other environments.

Furthermore, high-affinity acyl-HSL degraders were enriched in oligotrophic biofilm reactors that were inoculated with the turf soil. When supplied at physiological concentration, acyl-HSL was observed to be >95% consumed at certain stage. Greater species diversity was formed in the acyl-HSL-supplied reactor than the otherwise acyl-HSL-absent reactor. The most abundant bacterial species belonged to six different phyla based on 16S rDNA, including *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Acidobacteria*, *Planctomycetes* and *Bacteroidetes*. Six bacterial strains were successfully isolated from the biofilm reactors and designated Soil Oligotrophic Degradors which are *Variovorax* strains SOD31 and SOD32, *Mesorhizobium* strains SOD33 and SOD35, *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36. Unambiguous growth of the six isolates on physiological amount of acyl-HSLs under oligotrophic condition has been demonstrated. In further investigation of *Variovorax* strain SOD31, the bacterium exhibited an acyl-HSL-limiting growth kinetics with a half-saturation constant of 1.7  $\mu\text{M}$ , which is in high agreement with what we observed from the turf soil. The results suggests that strain SOD31 may be an active contributor to the soil's activity of degrading acyl-HSL.

Rapid signal decay might serve either to quiet signal crosstalk that might otherwise occur between spatially separated microbial aggregates or as a full system reset. Depending on the context, biological signal decay might either promote or complicate

cellular communications and the accuracy of population density based controls on gene expression in species-rich ecosystems. The successful isolation and cultivation of high-affinity acyl-HSL degraders capable of utilizing physiological concentration of acyl-HSL under oligotrophic conditions will allow determination of the distribution of signal-producing and –degrading microbes in soils and other environment. This advance will lead to improved understanding of signal decay influences quorum sensing and community function and structure in naturally occurring microbial communities.



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## **Chapter 1**

### **Introduction and Overview**



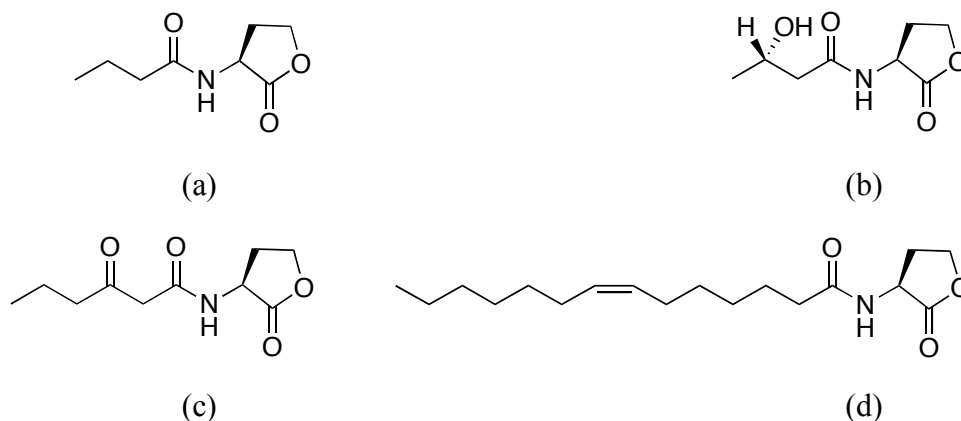
## 1.1 Introduction

### 1.1.1 Quorum sensing and signaling molecules of acyl-homoserine lactones

Over the last three decades, it has become apparent that a diversity of *Proteobacteria* employ acyl-homoserine lactones (acyl-HSLs) as signal molecules and regulate expression of specific genes in response to their own population density (7, 8, 10, 20, 26, 31). This has been termed quorum sensing. In the quorum sensing bacteria, specific synthase (LuxI-type protein) catalyzes the production of acyl-HSLs, which are dedicated signaling compounds and can diffuse freely in and out of the cell (14, 23), or transport via a combination of diffusion and active efflux (23). At low population densities, signals diffuse or are pumped out of the cells and are essentially diluted to extinction. However, when increased numbers of these cells produce the signals within a confined space, the signals can accumulate and reach a critical concentration. They bind to the regulator (LuxR-type) protein and activate expression of specific genes or gene clusters. The overall desired behavior is that at low population densities, these genes are “off,” and at high population densities, these genes are “on.”

The known, naturally occurring acyl-HSLs contain a homoserine lactone moiety and an acyl-side chain (Fig. 1.1), which ranges in length from 4 to 16 carbons, may contain a degree of unsaturation, and may be modified with a keto or hydroxyl functional group at carbon position 3 (9). Past laboratory studies have shown that the nature of the side chain can impart markedly different *in vitro* chemical and biochemical stabilities (13, 16, 35). A given microbial species that makes acyl-HSLs typically makes one of a dozen or so

known acyl-HSL signals. The actual structure of the signal depends on the specific acyl-HSL synthase. In some cases, a species will make two or more signals because it has correspondingly distinct synthases (12, 32, 33).



**Figure 1.1. Examples of naturally occurring acyl-homoserine lactones. (a),** Butanoyl-HSL; **(b),** 3-hydroxybutanoyl-HSL; **(c),** 3-oxohexanoyl-HSL; **(d),** 7,8-*cis*-tetradecenoyl-HSL.

### 1.1.2 Chemical and biological degradation of acyl-HSLs

In the environment, acyl-HSLs decompose chemically as a function of the pH, occurring most rapidly under alkaline conditions and generating the corresponding acyl-homoserine, which is not active, or only very poorly active, as a quorum signal (11, 29). The half-life is predicted by Eberhart's equation,  $t_{1/2}(\text{acyl-HSL}) = 10^{(7-\text{pH})}$  days (25). For instance, the half-life is around 30 days at pH 5.5, 24 hours at pH 7.0 and 2 hours at pH 8.0. The pHs, ranging from 5 to 9, are by no means rare in terrestrial soils or other

environments. Therefore it stands to reason that pH can either be a very substantial factor, or at other times be an almost inconsequential factor on quorum signal stability and its effect on quorum sensing.

Acyl-HSLs have been documented to be biologically degraded via several distinct mechanisms. The two major mechanisms of microbially mediated degradation were first reported in 2000 (5, 16). Using a mechanism similar to pH hydrolysis, the acyl-HSLs can be inactivated by acyl-HSL lactonases produced by *Bacillus*, *Agrobacterium*, and *Arthrobacter* species (3, 4, 17, 22, 24, 28). In a second mechanism, the amide bond of acyl-HSLs can be cleaved by acyl-HSL acylases produced by *Varivorax*, *Ralstonia*, and *Pseudomonas* species, generating a fatty acid and homoserine lactone (13, 15, 16, 18). All of these products have very little or no activity as quorum sensing signals. Recently a *Rhodococcus erythropolis* strain has been shown to possess a novel oxidoreductase activity (27). Moreover, mammalian sera or tissues (1, 6, 21, 34) and *Lotus corniculatus* plantlets (2) have been shown capable of degrading acyl-HSLs as well. Refer to Chapter 2 for detailed review.

## 1.2 Overview of the thesis

**Chapter 2** is a review on acyl-HSL decay as intrinsic to bacterial cell-cell communication, detailing the following aspects: (i) Summary of microbes and organisms that engage in acyl-HSL signal degradation, and homologues to known acyl-HSL degrading enzymes; (ii) Mechanisms of acyl-HSL degradation, e.g., pH-, temperature- and acyl-HSL chain structure-dependent chemical hydrolysis, and biochemical hydrolysis

by acyl-HSL lactonases, acylases, oxidoreductase and mammalian sera or tissues; (iii) Specificity of acyl-HSL degrading enzymes, as regards to acyl-HSL structure, compounds that are degraded, and their stereospecificity; (iv) Acyl-HSL stability in natural environments, including acyl-HSL degradation by natural soil communities, mutual beneficial effects demonstrated in defined cocultures, microbial aggregates insulation by signal decay, and influence of pH, temperature and acyl-HSL chain structure; (v) Coevolution of quorum sensing bacteria with hosts and acyl-HSL-degrading bacteria.

Despite many microorganisms and mechanisms being discovered as regards to acyl-HSL degradation, such efforts were carried out in laboratory pure culture (3, 4, 17, 22, 24, 27, 28), simple synthetic communities by using defined cocultures (22, 28), and laboratory soil microcosms seeded with recombinant strains (19). The stability of acyl-HSLs in particular environments, i.e., soils, over short and long periods is poorly understood. In **Chapter 3**, we have begun to examine such issues and taken the approach of synthesizing radiolabeled acyl-HSLs to examine their fate when amended at low concentrations to buffered bulk soil slurries. We have shown that acyl-HSL-inactivating microbes are indeed active in the environment and that physiologically relevant concentrations of quorum signals are subject to a rapid biodegradation in bulk soils.

The apparent  $K_m$  of the degradation activity upon acyl-HSL quorum signals in the soil is ca. 1000-fold lower than that of a purified acyl-HSL degrading enzyme from *Bacillus cereus* (30), suggesting that acyl-HSL degrading organisms currently available in culture may not be representative sources of the observed soil activity. Moreover, when the bacterial protein synthesis inhibitor chloramphenicol was added to soil slurries,

mineralization of acyl-HSL was significantly stimulated. Additionally, there was significant difference observed between the extent of acyl-HSL inactivation versus its conversion to CO<sub>2</sub>. These results suggest that bacteria might be converting a significant fraction of acyl-HSL decay products into cell material. This prompted us to perform enrichments for microbes exhibiting the low K<sub>m</sub> activity observed in soils.

In **Chapter 4**, we have applied soil-inoculated biofilm reactors to enrich high-affinity acyl-HSL degraders capable of utilizing physiological amount of acyl-HSL under oligotrophic conditions. When supplied at physiological concentration, acyl-HSL was observed to be significantly consumed through the reactors. Six bacterial strains have been successfully isolated and identified. A *Variovorax* strain SOD31 exhibited an acyl-HSL-limiting growth kinetics with a half-saturation constant of 1.7 μM, which is in highly agreement with what we observed from the turf soil and suggests that strain SOD31 may be an active contributor to the soil's activity of degrading acyl-HSL.

Major conclusions of this thesis, and recommendations for future work are presented in **Chapter 5**. The results presented here demonstrated the occurrence of rapid signal decay and potential contributors in natural environments. It is consistent with the notion that acyl-HSLs must have restricted stability in natural environments for them to accurately function since highly stable molecules would accumulate over time and constantly exist at inducing concentrations despite fluctuations in population density. This research reinforces the importance of signal decay as intrinsic to bacterial cell-cell communication, as well as providing bases for further studies towards how signal decay may influence community function and structure in naturally occurring microbial communities.

### 1.3 References

1. **Chun, C. K., E. A. Ozer, M. J. Welsh, J. Zabner, and E. P. Greenberg.** 2004. Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. *Proc. Natl. Acad. Sci. USA* **101**:3587-3590.
2. **Delalande, L., D. Faure, A. Raffoux, S. Uroz, C. D'Angelo-Picard, M. Elasri, A. Carlier, R. Berruyer, A. Petit, P. Williams, and Y. Dessaux.** 2005. N-hexanoyl-L-homoserine lactone, a mediator of bacterial quorum-sensing regulation, exhibits plant-dependent stability and may be inactivated by germinating *Lotus corniculatus* seedlings. *FEMS Microbiol. Ecol.* **52**:13-20.
3. **Dong, Y. H., A. R. Gusti, Q. Zhang, J. L. Xu, and L. H. Zhang.** 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. *Appl. Environ. Microbiol.* **68**:1754-1759.
4. **Dong, Y. H., L. H. Wang, J. L. Xu, H. B. Zhang, X. F. Zhang, and L. H. Zhang.** 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**:813-817.
5. **Dong, Y. H., J. L. Xu, X. Z. Li, and L. H. Zhang.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. USA* **97**:3526-3531.
6. **Draganov, D. I., J. F. Teiber, A. Speelman, Y. Osawa, R. Sunahara, and B. N. La Du.** 2005. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J. Lipid Res.* **46**:1239-1247.

7. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry-US* **20**:2444-2449.
8. **Fuqua, C., and E. P. Greenberg.** 2002. Listening in on bacteria: Acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell Biol.* **3**:685-695.
9. **Fuqua, C., and E. P. Greenberg.** 1998. Self perception in bacteria: Quorum sensing with acylated homoserine lactones. *Curr. Opin. Microbiol.* **1**:183-189.
10. **Fuqua, C., M. R. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. *Ann. Rev. Genet.* **35**:439-468.
11. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.* **50**:727-751.
12. **Gray, K. M., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1994. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. *J. Bacteriol.* **176**:3076-3080.
13. **Huang, J. J., J. I. Han, L. H. Zhang, and J. R. Leadbetter.** 2003. Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* **69**:5941-5949.
14. **Kaplan, H. B., and E. P. Greenberg.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**:1210-1214.

15. **Leadbetter, J. R.** 2001. Plant microbiology: Quieting the raucous crowd. *Nature* **411**:748-749.
16. **Leadbetter, J. R., and E. P. Greenberg.** 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *J. Bacteriol.* **182**:6921-6926.
17. **Lee, S. J., S. Y. Park, J. J. Lee, D. Y. Yum, B. T. Koo, and J. K. Lee.** 2002. Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **68**:3919-3924.
18. **Lin, Y. H., J. L. Xu, J. Y. Hu, L. H. Wang, S. L. Ong, J. R. Leadbetter, and L. H. Zhang.** 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Mol. Microbiol.* **47**:849-860.
19. **Molina, L., F. Constantinescu, L. Michel, C. Reimmann, B. Duffy, and G. Defago.** 2003. Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. *FEMS Microbiol. Ecol.* **45**:71-81.
20. **Nealson, K. H., T. Platt, and J. W. Hastings.** 1970. Cellular control of synthesis and activity of bacterial luminescent system. *J. Bacteriol.* **104**:313-317.
21. **Ozer, E. A., A. Pezzulo, D. M. Shih, C. Chun, C. Furlong, A. J. Lulis, E. P. Greenberg, and J. Zabner.** 2005. Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing. *FEMS Microbiol. Ecol.* **253**:29-37.



22. **Park, S. Y., S. J. Lee, T. K. Oh, J. W. Oh, B. T. Koo, D. Y. Yum, and J. K. Lee.** 2003. AhlD, an N-acylhomoserine lactonase in *Arthrobacter sp.*, and predicted homologues in other bacteria. *Microbiology-Sgm* **149**:1541-1550.
23. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**:1203-1210.
24. **Reimmann, C., N. Ginet, L. Michel, C. Keel, P. Michaux, V. Krishnapillai, M. Zala, K. Heurlier, K. Triandafillu, H. Harms, G. Defago, and D. Haas.** 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology-Sgm* **148**:923-932.
25. **Schaefer, A. L., B. L. Hanzelka, M. R. Parsek, and E. P. Greenberg.** 2000. Detection, purification and structural elucidation of acylhomoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Meth. Enzymol.* **305**:288-301.
26. **Swift, S., J. A. Downie, N. A. Whitehead, A. M. L. Barnard, G. P. C. Salmond, and P. Williams.** 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. *Advances in Microbial Physiology* **45**:199-270.
27. **Uroz, S., S. R. Chhabra, M. Camara, P. Williams, P. Oger, and Y. Dessaux.** 2005. N-acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. *Microbiology-Sgm* **151**:3313-3322.

28. **Uroz, S., C. D'Angelo-Picard, A. Carlier, M. Elasri, C. Sicot, A. Petit, P. Oger, D. Faure, and Y. Dessaux.** 2003. Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology-Sgm* **149**:1981-1989.
29. **Voelkert, E., and D. R. Grant.** 1970. Determination of homoserine as lactone. *Anal. Biochem.* **34**:131-137.
30. **Wang, L. H., L. X. Weng, Y. H. Dong, and L. H. Zhang.** 2004. Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). *J. Biol. Chem.* **279**:13645-13651.
31. **Winans, S. C., and B. L. Bassler.** 2002. Mob psychology. *J. Bacteriol.* **184**:873-883.
32. **Wood, D. W., F. C. Gong, M. M. Daykin, P. Williams, and L. S. Pierson, 3rd.** 1997. N-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J. Bacteriol.* **179**:7663-7670.
33. **Wood, D. W., and L. S. Pierson, 3rd.** 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**:49-53.
34. **Yang, F., L. H. Wang, J. Wang, Y. H. Dong, J. Y. Hu, and L. H. Zhang.** 2005. Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. *FEMS Lett.* **579**:3713-3717.
35. **Yates, E. A., B. Philipp, C. Buckley, S. Atkinson, S. R. Chhabra, R. E. Sockett, M. Goldner, Y. Dessaux, M. Camara, H. Smith, and P. Williams.** 2002. N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain

length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect. Immu. **70**:5635-5646.

## **Chapter 2**

### **Acyl-HSL Signal Decay:**

### **Intrinsic to Bacterial Cell-Cell Communications**

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## 2.1 Introduction

Many members of the bacterial phylum *Proteobacteria* employ low molecular weight chemical signaling molecules in the coordination of their group behaviors. This process of cell to cell communication is known as quorum sensing and it was first described as the mechanism that underlies light production by the squid symbiont *Vibrio fischeri* (Nealson *et al.* 1970, Nealson and Hastings 1979). The structure of the signaling molecule of *Vibrio fischeri*, N-3-(oxohexanoyl) homoserine lactone (3OC6HSL), was determined (Eberhard *et al.* 1981) and was the first representative of a large family of molecules, the acyl-homoserine lactones (acyl-HSLs). These dedicated signaling molecules all have a homoserine lactone moiety but can differ in the substituents and length of the acyl chain, which can vary from four to sixteen carbons (Fuqua *et al.* 2001). Their function as signaling molecules employed by diverse gram negative bacteria has been studied extensively. The canonical way in which acyl-HSLs are utilized in quorum sensing requires the synthesis of acyl-HSL signals by synthases, which are encoded by homologues of the *luxI* gene and signal response regulator proteins, which are encoded by homologues of the *luxR* gene (Engebrecht *et al.* 1983, Gambello and Iglewski 1991, Piper *et al.* 1993). The LuxR protein homologues bind to acyl-HSL signaling molecules and activate the transcription of genes that have proven advantages to express when cell numbers are high. The way these molecules function to coordinate group behaviors at the genetic and biochemical levels has been intensively studied over the last 30 years. The synthesis of acyl-HSLs (Hanzelka *et al.* 1997, Schaefer *et al.* 1996), interactions with response regulators (Stevens and Greenberg 1997), acyl-HSL quorum sensing controlled

regulons (Wagner *et al.* 2003), and, in recent years the degradation of acyl-HSL signaling molecules, have been investigated. The later topic is the subject of this review.

Acyl-HSL quorum signal degradation is important given that the presence and concentration of these signaling molecules are key to several microbial group behaviors. Acyl-HSL mediated quorum sensing has been found to underlie a host of microbial group behaviors from antibiotics and toxins production to swarming motility and biofilm formation (Fuqua *et al.* 2001, Swift *et al.* 2001). Quorum sensing would not be effective as a gene regulation mechanism if signaling molecule concentrations did not accurately portray cell numbers. The critical concentration for activation of quorum responses varies for different quorum sensing microbes from ca. 5 nM to 2  $\mu$ M *in vitro* (Fuqua *et al.* 1995, Kaplan and Greenberg 1985, Pearson *et al.* 1995, von Bodman *et al.* 1998, Whiteley *et al.* 1999). Since the quorum sensing process depends on the concentration of signaling molecules accurately reflecting cell population density, signal molecules stability and their potential for degradation are key areas of study if we aim to understand how this process functions in nature.

## **2.2 Acyl-HSL degrading organisms, enzymes and homologs**

Since the year 2000 with the first reports of microbially-mediated signal degradation (Dong *et al.* 2000, Leadbetter and Greenberg 2000), the search and study of microbes and organisms that engage in acyl-HSL signal degradation has been fruitful (Table 2.1). Database searches have identified numerous homologs of known acyl-HSL lactonase and acylase enzymes in a wide range of species suggesting that this activity could be widespread (Table 2.2). Organisms with homologs to known acyl-HSL degrading

**Table 2.1. Demonstrated mechanisms and proteins involved in acyl-HSL degradation by diverse bacteria and eukaryotes**

Species	Mechanism	References
<b>Bacteria</b>		
<i>Proteobacteria</i>		
<i>α-proteobacteria</i>	<i>Agrobacterium tumefaciens</i> A6 <i>A. tumefaciens</i> C58	Lactonase AttM Lactonases AttM & AiiB
<i>β-proteobacteria</i>	<i>Variovorax paradoxus</i> VAI-C <i>Ralstonia</i> sp. XJ12B	Acylase ND* Acylase AiiD
<i>γ-proteobacteria</i>	<i>Pseudomonas aeruginosa</i> PAO1 <i>Pseudomonas</i> sp. PAI-A <i>Klebsiella pneumoniae</i> KCTC2241 <i>Acinetobacter</i> sp. C1010	Acylases PvdQ/PA2385 & QuiP/PA1032 Acylase ND Lactonase AhIK ND
<i>Firmicutes Bacilli</i> (low-G+C Gram-positive)	<i>Bacillus</i> sp. 240B1 <i>B. thuringiensis</i> <i>B. cereus</i> <i>B. mycoides</i> <i>B. stearothermophilus</i> <i>B. anthracis</i>	Lactonase AiiA Lactonase AiiA homologues Lactonase AiiA homologues Lactonase AiiA homologues Lactonase ND Lactonase AiiA homologues
<i>Actinobacteria Actinobacteria</i> (high-G+C Gram-positive)	<i>Rhodococcus erythropolis</i> W2 <i>Arthrobacter</i> sp. IBN110 <i>Streptomyces</i> sp. M664	Acylase & oxidoreductase ND Lactonase AhID Acylase AhIM
<b>Eukarya</b>	Mammalian sera/tissues Alga <i>Laminaria digitata</i> Legume <i>Lotus corniculatus</i>	(Chun <i>et al.</i> 2004, Draganov <i>et al.</i> 2005, Ozer <i>et al.</i> 2005, Yang <i>et al.</i> 2005) (Brochardt <i>et al.</i> 2001, Michels <i>et al.</i> 2000) (Delalande <i>et al.</i> 2005)

\*ND, Not determined.

**Table 2.2. Homologs of acyl-HSL lactonases and acylases**

Organism with Homolog	Protein (as annotated)	% AA Similarity	Acyl-HSLase Homolog	Phylum
<b>Acyl-HSL Lactonase Homologs</b>				
<i>Deinococcus radiodurans</i> R1	Hypothetical protein	57%	AiiB	<i>Deinococcus-Thermus</i>
<i>Moorella thermoacetica</i> ATCC 39073	Beta-lactamase-like	53%	AiiB	<i>Firmicutes</i>
<i>Thiomicrospira crunogena</i> XCL-2	Beta-lactamase-like	51%	AiiB	<i>Proteobacteria</i>
<i>Bradyrhizobium japonicum</i> USDA 110	AttM/AiiB family protein	51%	AiiB	<i>Proteobacteria</i>
<i>Natronomonas pharaonis</i>	Hydrolase	50%	AiiB	<i>Euryarchaeota</i>
<i>Photorhabdus luminescens</i> subsp.	Hypothetical protein plu2238	88%	AttM	<i>Proteobacteria</i>
<i>Yersinia intermedia</i> ATCC 29909	Zn-dependent hydrolases	87%	AttM	<i>Proteobacteria</i>
<i>Nocardioides</i> sp. JS614	Beta-lactamase-like	56%	AttM	<i>Actinobacteria</i>
<i>Clostridium beijerincki</i> NCIMB 8052	Putative metallohydrolase	56%	AhID	<i>Firmicutes</i>
<i>Staphylococcus haemolyticus</i>	Hypothetical protein	52%	AhID	<i>Firmicutes</i>
<i>Aspergillus oryzae</i>	Hypothetical protein	49%	AhID	<i>Fungi</i>
<i>Haloarcula marismortui</i>	Hypothetical protein	45%	AhID	<i>Euryarchaeota</i>
<i>Sulfolobus solfataricus</i>	Conserved hypothetical protein	55%	AhID	<i>Crenarchaeota</i>
<i>Thermoplasma volcanium</i>	Zn-dependent hydrolase	50%	AhID	<i>Euryarchaeota</i>
<i>Paracoccus denitrificans</i> PD1222	Beta-lactamase-like	56%	AiiA	<i>Proteobacteria</i>
<i>Archaeoglobus fulgidus</i> DSM 4304	Hypothetical protein	54%	AiiA	<i>Euryarchaeota</i>
<i>Desulfovibrio desulfuricans</i>	Metallo-beta-lactamase family	52%	AiiA	<i>Proteobacteria</i>
<i>Bacillus licheniformis</i> ATTC 14580	Putative hydrolase	51%	AiiA	<i>Firmicutes</i>
<i>Leptospira interrogans</i> sv. Copenhageni	Metallo-beta-lactamase	51%	AiiA	<i>Spirochaetes</i>
<i>Ralstonia eutropha</i> JMP134	Beta-lactamase-like	51%	AiiA	<i>Proteobacteria</i>
<i>Frankia</i> sp. EAN1pec	Beta-lactamase-like	50%	AiiA	<i>Actinobacteria</i>
<i>Rubrobacter xylanophilus</i> DSM 9941	Beta-lactamase-like	50%	AiiA	<i>Actinobacteria</i>
<i>Deinococcus geothermalis</i> DSM 11300	Beta-lactamase-like	50%	AiiA	<i>Deinococcus-Thermus</i>
<i>Rhizobium etli</i>	Hypothetical protein	49%	AiiA	<i>Proteobacteria</i>
<i>Canis familiaris</i> (Dog)	Serum paraoxonase/arylesterase	91%	PON1	<i>Metazoa</i>
<i>Bos taurus</i> (Cow)	Similar to paraoxonase 1	90%	PON1	<i>Metazoa</i>
<i>Oryctolagus cuniculus</i> (Rabbit)	Serum paraoxonase/arylesterase 1	90%	PON1	<i>Metazoa</i>
<i>Tetraodon nigroviridis</i> (Pufferfish)	Unnamed protein product	78%	PON1	<i>Metazoa</i>
<i>Xenopus laevis</i> (African Clawed Frog)	MGC80011 protein	77%	PON1	<i>Metazoa</i>

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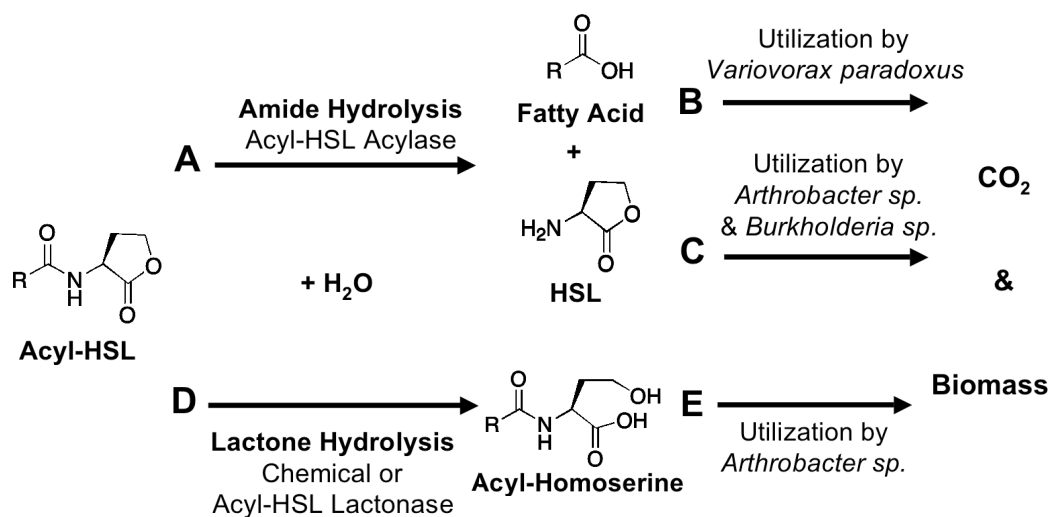
Organism with Homolog	Protein (as annotated)	% AA Similarity	Acyl-HSLase Homolog	Phylum
<i>Streptomyces coelicolor</i>	Gluconolactonase precursor	53%	PON1	<i>Actinobacteria</i>
<i>Trichodesmium erythraem</i>	Senescence marker protein-30	53%	PON1	<i>Cyanobacterium</i>
<i>Aspergillus fumigatus</i> (Green Mold)	Hypothetical protein	50%	PON1	<i>Fungi</i>
<i>Homo sapiens</i> (Human)	Paraoxonase/arylesterase	94%	PON2	<i>Metazoa</i>
<i>Pan troglodytes</i> (Chimpanzee)	Similar to paraoxonase/arylesterase	94%	PON2	<i>Metazoa</i>
<i>Gallus gallus</i> (Domestic Chicken)	Similar to paraoxonase 2	84%	PON2	<i>Metazoa</i>
<i>Pongo pygmaeus</i> (Bornean Orangutan)	Paraoxonase 1	83%	PON2	<i>Metazoa</i>
<i>Xenopus tropicalis</i> (Western Clawed Frog)	Paraoxonase 1	80%	PON2	<i>Metazoa</i>
<i>Platichthys flesus</i> (Wild Flounder)	Paraoxonase (arylesterase)	73%	PON2	<i>Metazoa</i>
<i>Danio rerio</i> (Zebrafish)	Similar to paraoxonase 2	72%	PON2	<i>Metazoa</i>
<i>Strongylocentrotus purpuratus</i> (Urchin)	Similar to paraoxonase 2	54%	PON2	<i>Metazoa</i>
<i>Caenorhabditis briggsae</i> (Nematode)	Hypothetical protein CBG10483	50%	PON2	<i>Metazoa</i>
<b>Acyl-HSL Acylase Homologs</b>				
<i>Deinococcus radiodurans</i> R1	Aculeacin A acylase	68%	AiiD	<i>Deinococcus-Thermus</i>
<i>Hahella chejuensis</i> KCTC 2396	Protein related to penicillin acylase	51%	AiiD	<i>Proteobacteria</i>
<i>Nocardioides sp.</i> JS614	Peptidase S45, penicillin amidase	52%	AiiD	<i>Actinobacteria</i>
<i>Psychrobacter cryohalolentis</i> K5	Peptidase S45, penicillin amidase	53%	AiiD	<i>Proteobacteria</i>
<i>Ralstonia metallidurans</i>	Penicillin amidase	81%	AiiD	<i>Proteobacteria</i>
<i>Ralstonia solanacearum</i> GMI1000	Conserved hypothetical protein	89%	AiiD	<i>Proteobacteria</i>
<i>Rubrivivax gelatinosus</i> PM1	Protein related to penicillin acylase	48%	QuiP	<i>Proteobacteria</i>
<i>Azotobacter vinelandii</i> AvOP	Peptidase S45, penicillin amidase	82%	QuiP	<i>Proteobacteria</i>
<i>Gloeobacter violaceus</i> PCC 7421	Probable penicillin amidase	49%	QuiP	<i>Cyanobacteria</i>
<i>Nostoc punctiforme</i> PCC 73102	Related to penicillin acylase	48%	QuiP	<i>Cyanobacteria</i>
<i>Pseudomonas fluorescens</i> PfO-1	Penicillin amidase family protein	79%	QuiP	<i>Proteobacteria</i>
<i>Pseudomonas putida</i>	Penicillin amidase family protein	69%	PvdQ	<i>Proteobacteria</i>
<i>Pseudomonas syringae</i>	Penicillin amidase family protein	74%	PvdQ	<i>Proteobacteria</i>
<i>Streptomyces lavendulae</i> subsp.	Penicillin V acylase precursor	52%	PvdQ	<i>Actinobacteria</i>
<i>Actinoplanes utahensis</i>	Aculeacin A acylase	54%	AhIM	<i>Actinobacteria</i>
<i>Shewanella baltica</i> OS155	Peptidase S45, penicillin amidase	48%	AhIM	<i>Proteobacteria</i>

enzymes hail from all three domains of life (*Bacteria*, *Eucarya*, and *Archaea*) and dwell in a wide range of environments and conditions, from mesophilic and mesothermic to extreme haloalkaliphilic, thermophilic and acidophilic environments, suggesting that within a wide range of environments there may be biotic interactions with acyl-HSLs. A variety of these interactions involve acyl-HSLs in host-symbiont relationships (Nealson *et al.* 1970) and pathogenic infections (de Kievit and Iglewski 2000, Parsek and Greenberg 2000). Given such diverse and close interactions, it is perhaps not surprising that eukaryotic hosts have evolved mechanisms to interact with acyl-HSLs (Chun *et al.* 2004, Telford *et al.* 1998). Acyl-HSL degrading activity by paraoxonase (PON) enzymes was discovered from mammalian cells in epithelial and colon cells, which are cells in the front lines of contact with potential pathogens (Chun *et al.* 2004). Many species containing such homologs reside in environments for which known acyl-HSL producers have been found. The co-localization of acyl-HSL producing and degrading organisms in environments suggests the range of community interactions that could exist. Acyl-HSL production by a haloalkaliphilic archaeon that activates an *Agrobacterium* biosensor is believed to regulate the production of an extracellular protease (Paggi *et al.* 2003). Other haloalkaliphilic species are known to have acyl-HSL degrading lactonase homologs: *Natronomonas* sp. and *Haloarcula* sp. (Table 2.1). Also the discovery of nine long chain acyl-HSLs produced by the acidophilic archaeon *Acidithiobacillus ferrooxidans* (Farah *et al.* 2005) provides evidence for the presence of acyl-HSLs in acidic environments. Given the greater stability of long chain acyl-HSLs under acidic conditions (Yates *et al.* 2002), these molecules would be stable in this environment and could be subject to degradation by other organisms such as *Thermoplasma* or *Sulfolobus* sp., which can exist in such

environments and have known acyl-HSL degrading homologs. These locations would be interesting environments to study microbial production and degradation of acyl-HSLs.

Several methods have been successful to identify organisms that degrade acyl-HSLs from the environment, including enrichment cultures which select organisms that utilize acyl-HSLs as the sole source of carbon and energy (Huang *et al.* 2003, Leadbetter and Greenberg 2000), as well as the screening techniques by which environmental isolates are either grown initially in undefined medium followed by examination of cultured organisms for acyl-HSL degrading abilities via bioassay (D'Angelo-Picard *et al.* 2005), or incubated initially in acyl-HSL signal followed by screening based on bioassay or growth on signal (Uroz *et al.* 2003). These methods have identified bacteria that degrade signal via an acylase mechanism, by which the acyl-HSL is cleaved at the amide bond (Leadbetter and Greenberg 2000, Uroz *et al.* 2003), or that utilize the lactonase mechanism, by which the microbes hydrolyze the homoserine lactone ring (Park *et al.* 2003) (Fig. 2.1). As more techniques are devised to screen acyl-HSL degrading organisms in the soil rhizosphere (Jafra and van der Wolf 2004, Uroz *et al.* 2003) and other environments, the challenge will be to understand how acyl-HSL degradation is used by the microbes identified and to examine their degradation abilities in light of the biology of the natural communities in which these microbes reside.

The ability of both acyl-HSL degrading acylase and lactonase enzymes to interfere with the quorum sensing of pathogens that employ acyl-HSL signals in pathogenicity or virulence has been tested in a number of ways: co-culture of acyl-HSL degrading and



**Figure 2.1 Key mechanisms by which acyl-HSLs can be inactivated and further degraded.** **A**, Cleavage of the amide bond by bacterial AHL acylase yields the corresponding fatty acid and homoserine lactone (HSL) (Leadbetter 2001, Lin *et al.* 2003). The acyl-HSL amide is chemically stable under conditions of non-extreme temperature and pH. **B**, The fatty acid that is released is known to be utilized by organisms such as *Variovorax paradoxus* (Leadbetter and Greenberg 2000). **C**, The HSL that is released is known to be utilized as an energy nutrient by diverse *Arthrobacter* and *Burkholderia* species (Yang *et al.* 2006). **D**, Cleavage of the lactone ring by bacterial AHL lactonase yields the corresponding acyl-homoserine (Dong *et al.* 2000, Park *et al.* 2003, Zhang *et al.* 2002). The lactone ring is also subject to chemical hydrolysis; the chemical half life of the ring is ca.  $10^{[7-pH]}$  days (Eberhard *et al.* 1981, Schaefer *et al.* 2000). **E**, The acyl-homoserine degradation product generated by lactonolysis is known to be utilized as an energy nutrient by *Arthrobacter* species (Flagan *et al.* 2003).

acyl-HSL producing bacteria (Dong *et al.* 2000, Park *et al.* 2003, Uroz *et al.* 2003), expression of acyl-HSL degrading enzymes in quorum sensing pathogens (Dong *et al.* 2000, Lin *et al.* 2003, Reimmann *et al.* 2002) and expression of acyl-HSL degrading enzymes in transgenic plant hosts (Dong *et al.* 2001). These methods have been effective to attenuate pathogenicity of acyl-HSL-utilizing pathogens such as *Erwinia* and *Pseudomonas* species. When an extracellularly excreted acyl-HSL acylase enzyme of *Streptomyces*, AhlM, was added to *Pseudomonas aeruginosa* cultures, decreased virulence factors were observed (Park *et al.* 2005). Expressing an acyl-HSL lactonase homologue of *Bacillus* sp., AiiA, in *Pseudomonas aeruginosa* prevented accumulation of C4HSL and reduced the accumulation of 3OC12HSL, and thus reduced virulence gene expression and swarming motility of *P. aeruginosa* (Reimmann *et al.* 2002). Expression of AiiA in a non acyl-HSL degrading soil bacterium, *P. fluorescens* reduced potato soft rot as a preventative against infection and as a curative measure; the recombinant strains also interfered with *P. chlorotaphis* which produces compounds effective as a natural biocontrol against fungal pathogens (Molina *et al.* 2003). Applications of acyl-HSL degrading enzymes have been reviewed extensively (Dong and Zhang 2005).

Signal degradation via the lactonase mechanism by *Agrobacterium tumefaciens* (Zhang *et al.* 2002) demonstrates how this process can be an integral part of an acyl-HSL mediated quorum sensing circuit. AttM, the acyl-HSL degrading lactonase of *Agrobacterium tumefaciens*, is growth-phase regulated and controls the bacterium's exit from its quorum state, making this process an essential component of the microbe's quorum sensing system (Zhang *et al.* 2002). It is possible that bacteria such as *Ralstonia* sp. which contain homologues to the acyl-HSL acylase AiiD, or *Pseudomonas*

*aeruginosa*, which quorum senses with two known acyl-HSL mediated quorum sensing systems and has two acyl-HSL degrading acylases (Huang *et al.* 2003, Huang *et al.* 2006), may also utilize acyl-HSL degradation in the modulation of their quorum sensing systems. Inactivation by eukaryotes infected by bacterial pathogens that employ quorum sensing in their virulence is a new area of study for which regulation of this activity will be interesting and important.

### **2.3 Mechanisms of acyl-HSL degradation**

Over the past six years, diverse acyl-HSL degradation mechanisms have been documented. Besides chemical hydrolysis, the rates of which are subject to pH, temperature and acyl-HSL side chain structure, many bacteria and even eukaryotes are found to be able to rapidly degrade acyl-HSL via different mechanisms (Table 2.1).

#### **2.3.1 Chemical hydrolysis**

In the environment, acyl-HSLs decompose chemically under alkaline conditions (Voelkert and Grant 1970). The lactone ring is hydrolyzed, generating the corresponding acyl-homoserine.

##### **2.3.1.1 pH-dependent chemical hydrolysis**

The Eberhard equation is prevalingly accepted to determine the half-lives of acyl-HSLs at defferent pHs: acyl-HSL  $t_{1/2} = 10^{[7 - \text{pH}]}$  days (Eberhard *et al.* 1981, Schaefer *et al.* 2000). For instance, when studying the acyl-HSL concentration variation during growth

of a plant pathogen *Erwinia carotovora* subsp. *carotovora*, 3OC6HSL decreased ca. 18.7% after 9 hours into stationary phase in LB medium amended with 100 mM potassium phosphate buffer to maintain pH at ca. 6.7, which was slightly higher than that calculated using Eberhard's equation (12.2%) (Byers *et al.* 2002).

Since LB is poorly buffered, the pH of aerobically grown cultures of both *Pseudomonas aeruginosa* (which employs both C4HSL and 3OC12HSL as its quorum sensing signals), and *Yersinia pseudotuberculosis* (which employs 3OC6HSL and C6HSL), increased from ca. 7.0 to greater than 8.5 after 24 h of growth (Yates *et al.* 2002). Under such conditions, acyl-HSLs become largely degraded due to pH-dependent hydrolysis. After synthetic 3OC12HSL was incubated with stationary-phase cell-free culture supernatant of *Pseudomonas aeruginosa*, high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) revealed the corresponding hydrolyzed product of 3-oxododecanoyl-homoserine. Furthermore, by acidifying the culture supernatants to pH 2.0, recycling of the lactone ring of C4HSL was observed. However, carbon-13 nuclear magnetic resonance spectroscopy showed that such ring reformation was not a simple reversal of the ring cleavage process. The pH must first approach the pK of the carboxyl group (ca. pH 2) before the hydroxyl group can close the ring. Ring recycling has also been observed by Uroz *et al.* (2005).

A similar pattern of decreasing half-life with increasing pH was observed by Delalande *et al.* (2005), however, the half-lives were much shorter at low pHs yet much longer at high pHs than those predicted by the Eberhard equation. For instance, at 20°C,

the half-life was only 8 days at pH 5.5 as opposed to 30 days theoretically, while it was 12 hours at pH 8.0 as opposed to 2 hours theoretically.

### **2.3.1.2 Temperature-dependent hydrolysis**

Formerly reported, 3OC6HSL is temperature-stable and may be boiled without loss of activity (Schaefer *et al.* 2000) and can even endure heating at 140°C in air for 10 min (Eberhard *et al.* 1981) as a thin dry film. In contrast, Byers *et al.* (2002) found that 3OC6HSL was subject to rapid inactivation by boiling. Substantial loss of the molecule (ca. > 50%) was observed when synthetic 3OC6HSL was boiled for 10 min. The reason for such contradiction was probably because the latter experiment was performed in aqueous solutions where hydrolysis could readily occur.

In addition, nonenzymatic temperature-dependent hydrolysis was demonstrated at different temperatures (Yates *et al.* 2002). The experiments were carried out at given temperature with known quantities of acyl-HSLs hydrolyzed in unbuffered aqueous solutions. The resulted pH decrease was measured, from which the concentration of released corresponding acyl-homoserine and thus the acyl-HSL releasing rate were calculated. Much greater hydrolysis was observed at 37°C than at 22°C with all the acyl-HSLs tested (C4HSL, C6HSL, 3OC6HSL and C8HSL) albeit to different extents. A similar pattern of such half-life decreasing as temperature increases was also observed by Delalande *et al.* (2005).

On the other hand, it is well accepted that acyl-HSLs should be stored at low temperature (i.e., -20°C) (Schaefer *et al.* 2000). The 3OC6HSL concentration was found to be the same when assayed 1 day or 1 week after being stored at -20°C (Byers *et al.*



2002). Acyl-HSLs degradation rates appear to be greatly reduced at such low temperature.

### **2.3.1.3. Acyl-HSL chain structure-dependent hydrolysis**

When investigating temperature influence on acyl-HSLs hydrolysis rates as described above, Yates *et al.* found out that the rates also depended on the side chain length and substitution at the third carbon position (2002). For the four acyl-HSLs tested (C4HSL, C6HSL, 3OC6HSL, C8HSL), at both 22°C and 37°C, the longer the acyl side chain, the slower the hydrolysis was, while 3OC6HSL hydrolyzed faster than C6HSL which had no substitution at the third carbon position. Consistently, by using carbon-13 nuclear magnetic resonance spectroscopy, the authors found that the ring opening of HSL, C3HSL, and C4HSL decreased accordingly. As pointed out by the authors, the lactone ring is made less electrophilic because the carbonyl group gains more electrons as the acyl chain length increases, and thus less susceptible to attack by hydroxide ions. While an oxo-substitution at the third carbon position does the opposite.

### **2.3.2 Biochemical hydrolysis by acyl-HSL lactonases**

That acyl-HSLs can also be degraded biochemically was first reported in 2000 by a protein designated AiiA, produced by *Bacillus* species (Dong *et al.* 2000). The enzymatic activity was later shown to be that of an acyl-HSL lactonase (Dong *et al.* 2001) using a mechanism similar to chemical hydrolysis, cleaving the ester bond of the lactone ring and releasing the corresponding acyl-homoserine (Fig. 2.1D).

The purified AiiA protein from the *Firmicute* bacterial isolate *Bacillus* sp. 240B1 effectively inactivated three acyl-HSLs tested, i.e., 3OC6HSL, 3OC8HSL and 3OC10HSL (Dong *et al.* 2000). By using HPLC and ESI-MS, digestion of 3OC6HSL with AiiA resulted in only one product of the open-ring form of the molecule, i.e., 3-oxohexanoyl-homoserine (Dong *et al.* 2001). Similar results were observed with all the acyl-HSLs tested (C4HSL, 3OC8HSL and 3OC12HSL). Following this discovery, 20 bacterial isolates among 800 from soil and plant samples were found capable of enzymatic degradation of acyl-HSLs (Dong *et al.* 2002). Eight isolates were identified belonging to *Bacillus thuringiensis*, with acyl-HSL inactivating activities ranging from 480 to 680 pmol/h/unit of OD<sub>600</sub>. Nine genes, exhibiting high levels of homology to *aiiA*, were cloned from strains belonging to *B. thuringiensis*, *B. cereus* and *B. mycoides*. Two *Bacillus* sp. A23 and A24 have also been isolated from rhizosphere soils, whose 16S rRNA genes are > 99% identical with those from *Bacillus cereus* group, and identified encoding acyl-HSL lactonase (*aiiA* homologues) (Reimann *et al.* 2002). They both degraded synthetic C4HSL and C6HSL rapidly and almost completely. Recombinant *P. aeruginosa* PAO1 expressing the *aiiA* homologue from A24 dramatically reduced 3OC12HSL and completely prevented C4HSL accumulation, thus markedly decreasing expression of several virulence factors and swarming motility. In addition, *aiiA* homologue genes were found in 16 subspecies of *B. thuringiensis*, of which all wild-type strains showed acyl-HSL degrading activities albeit to different extent (Lee *et al.* 2002). The *B. anthracis* AiiA lactonase has also been shown capable of efficiently cleaving acyl-HSLs (Ulrich 2004).

The acyl-HSL degradation enzyme encoded by *attM*, a homologue of *aiiA*, has been identified in *Agrobacterium tumefaciens* A6 (Zhang *et al.* 2002). By using HPLC and ESI-MS, the only reaction product of 3OC8HSL digested by purified AttM was determined to be 3-oxooctanoyl-homoserine, thus confirming AttM to be an acyl-HSL lactonase. Furthermore, *attM* is negatively controlled by a transcription factor, AttJ. Expression of *attM* is growth phase-dependent, which is initially suppressed by AttJ but enhanced substantially at stationary phase with 3OC8HSL largely degraded. Therefore, *A. tumefaciens* A6 adopts a unique signal turnover system to exit from conjugation-related quorum sensing. Besides *attM*, another *attM*-paralogous gene, *aiiB*, has been identified in *A. tumefaciens* C58 (Carlier *et al.* 2003). Serving as a lactonase as well, *aiiB* is encoded on pTi plasmid, as apposed to *attM* lying on pAt plasmid. It was observed that AiiB was less efficient than AttM in degrading synthetic acyl-HSLs (i.e., C6HSL, 3OC6HSL, C7HSL, C8HSL and 3OC8HSL). Recombinant *Ewringia carotovora* subsp. *atroseptica* expressing either *attM* or *aiiB* reduced accumulation of its cognate quorum sensing signal 3OC8HSL to 2 to 3 orders of magnitude less than wild type with AttM being more effective. Interestingly, *attM*, encoded by the *attKLM* operon, was further demonstrated to be involved in an assimilative pathway of  $\gamma$ -butyrolactone (GBL) in *A. tumefaciens* C58 (Carlier *et al.* 2004). The expression of the *attKLM* operon was activated in the presence of GBL and *A. tumefaciens* C58 did not accumulate 3OC8HSL as it did when the *attKLM* operon was not induced (growing on mannitol). The *attKLM* operon-induced *A. tumefaciens* C58 also became able to inactivate exogenous C6HSL and 3OC8HSL. Thus it demonstrated a genetic link between the GBL degradation pathway and acyl-HSL mediated quorum sensing system.

*Arthrobacter* strain IBN110, a soil isolate, was found to be able to degrade various acyl-HSLs (Park *et al.* 2003). Using a whole-cell assay, the strain degraded C4HSL, C6HSL, 3OC6HSL, C8HSL, C10HSL, 3OC12HSL, with C8HSL to be the most effectively degraded. The acyl-HSL degrading lactonase was identified and designated AhlD. By using HPLC and ESI-MS, the digestion product of C6HSL by AhlD was confirmed to be hexanoyl-homoserine. Acyl-HSL degrading activities were also observed in *Bacillus stearothermophilus* KCTC3067, and *Klebsiella pneumoniae* KCTC2241 in which another acyl-HSL lactonase of AhlK was identified.

### 2.3.3 Biochemical degradation by acyl-HSL acylases

Through another distinctive mechanism, the amide bond of acyl-HSLs can be cleaved by acyl-HSL acylases (Fig. 2.1A), first discovered in a soil isolate *Variovorax paradoxus* VAI-C that utilizes acyl-HSLs as sole source of energy and nitrogen, releases homoserine lactone (HSL) as a product of these reactions and metabolizes the acyl moiety as energy substrate (Leadbetter and Greenberg 2000). *V. paradoxus* VAI-C was able to grow on 3OC6HSL as sole source of carbon, energy and nitrogen, although its growth rate was largely increased when NH<sub>4</sub>Cl was amended (ca. 5 times faster). The bacterium could grow on the full spectrum of saturated acyl-HSLs tested as sole energy source. Furthermore, a linear relationship was observed between the molar growth yields on those molecules (C4HSL, C6HSL, C8HSL, C10HSL and C12HSL) and their acyl chain length. Homoserine lactone (HSL) was detected in the culture fluid by quantitative amino acid analysis as a major degradation product that could serve as nitrogen source for growth. By using radiolabeled C4HSL (<sup>14</sup>C at the ring carbon position one) as energy

source, 56% of recovered radiolabel was in  $^{14}\text{CO}_2$ , thus demonstrating subsequent cleavage of the ring. Moreover, when the radiolabeled C4HSL was used as nitrogen source, more complete utilization of the molecule was observed, i.e., 95% of recovered radiolabel was in  $^{14}\text{CO}_2$ .

The first gene encoding a protein with acyl-HSL acylase activity, designated *aiiD*, was later identified in a *Ralstonia* strain XJ12B, isolated from a mixed-species biofilm (Lin *et al.* 2003). The bacterium was capable of growing on C4HSL and 3OC12HSL as sole energy source, while purified AiiD exhibited significantly less inactivation on 3OC6HSL than other long-chain acyl-HSLs tested (i.e., 3OC8HSL, 3OC10HSL and 3OC12HSL). By using HPLC and ESI-MS, as well as 5-dimethylamino-1-naphthalenesulphonyl chloride (dansyl chloride) derivation to increase the hydrophobicity of amino acids for HPLC separation, HSL (dansylated) was detected as the major product of digested 3OC10HSL by purified AiiD, thus confirming AiiD to be an acyl-HSL acylase.

A close homologue of the *aiiD* gene was identified in *Pseudomonas aeruginosa* PAO1, *pvdQ*, and was shown to be an acyl-HSL acylase (Huang *et al.* 2003). *P. aeruginosa* PAO1 and a soil isolate *Pseudomonas* strain PAI-A were found capable of degrading long-chain ( $\geq 8$  carbons) acyl-HSLs as sole source of carbon and energy (Huang *et al.* 2003). By using liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC/APCI-MS), HSL was directly detected as acyl-HSL degradation product from cell-free culture supernatants. *Escherichia coli* cells expressing *pvdQ* degraded acyl-HSLs and released HSL, which was also specific towards long-chain ( $\geq 8$  carbons) acyl-HSLs. However, *pvdQ* was found to be sufficient but not necessary for

the acyl-HSL degradation, as two *pvdQ* knockout mutants were still able to degrade 3OC12HSL. Recently, a second acyl-HSL acylase has been identified in *P. aeruginosa*. Designated QuiP, it is the product of *P. aeruginosa* gene PA1032 (Huang *et al.* 2006). Acyl-HSL degradation and stoichiometric amounts of HSL accumulation were determined by LC/APCI-MS from culture supernatants of *E. coli* expressing recombinant *quiP*. Not surprisingly, QuiP has a specificity for long-chain ( $\geq 7$  carbons) acyl-HSLs that is similar to PvdQ.

An acyl-HSL acylase designated AhlM has been identified in a *Streptomyces* strain M664 (Park *et al.* 2005). By using HPLC and ESI-MS, as well as *o*-phthaldialdehyde (OPA) derivation for HPLC separation, HSL (OPA derivative) was detected as the digestion product of C10HSL by purified AhlM. The enzyme exhibited much more effective degrading activity with long-chain acyl-HSLs ( $\geq 8$  carbons), i.e., substantial decrease for C6HSL and 3OC6HSL and virtually no activity for C4HSL. Moreover, AhlM was more active against C8HSL than 3OC8HSL that has an oxo-substitution at the third carbon position.

*Rhodococcus erythropolis* W2, an isolate from a tobacco rhizosphere (Uroz *et al.* 2003), was shown to degrade acyl-HSLs by both acylase and oxidoreductase (see below) activities (Uroz *et al.* 2005). The bacterium was capable of utilizing diverse acyl-HSLs as sole carbon and energy source with a preference for short-chain acyl-HSLs (Uroz *et al.* 2005). By using HPLC and LC-MS, as well as dansyl chloride derivation for HPLC separation, HSL (dansylated) was detected as the product from any of the 3OC10HSL, 3OC6HSL or 3OHC10HSL being degraded by crude cell extracts of *R. erythropolis* W2, thus confirming existence of acyl-HSL acylase activity. There were also isolates

identified belonging to the genera *Pseudomonas*, *Comamonas*, *Variovorax* and *Rhodococcus* able to degrade acyl-HSLs (Uroz *et al.* 2003).

#### 2.3.4 Biochemical degradation by acyl-HSL oxidoreductase

Besides the acyl-HSL acylase activities described above, *Rhodococcus erythropolis* W2 has been shown to possess a novel oxidoreductase activity (Uroz *et al.* 2005).

Resting *R. erythropolis* W2 whole cells in phosphate buffer saline (PBS) much more efficiently degraded acyl-HSLs with the oxo-substituent at the third carbon position of the side chain than those without. By using HPLC and LC-MS, 3-oxo-substituted long-chain ( $\geq 8$  carbons) acyl-HSLs were detected to be initially converted to their corresponding 3-hydroxy derivatives by whole W2 cells, but not the crude cell extracts, thus demonstrating a novel oxidoreductase activity associated with whole cells. Although 3OC6HSL was not reduced to the corresponding 3-hydroxy counterpart, it was still almost completely utilized by *R. erythropolis* W2 growing cells by acyl-HSL acylase as described above.

Interestingly, the oxidoreductase activity is not specific for acyl-HSLs. It also reduced 3-oxo-6-phenylhexanoyl-homoserine lactone (3O6PhC6HSL) and 3-oxododecanamide (3OC12NH<sub>2</sub>) to their corresponding hydroxy counterparts. It is not stereospecific either since both D- and L-isomers of 3OC12HSL were converted to 3OHC12HSL.

### 2.3.5 Inactivation reactions involving oxidized halogen antimicrobials

Oxidized halogens are widely used for microbial control in natural and industrial systems, which have been found capable of rapidly inactivating acyl-HSLs with oxo-substitution at the third carbon of the side chain (Brochardt *et al.* 2001, Michels *et al.* 2000).

The nonenzymatic degradation pathway of acyl-HSLs by oxidizing hypochlorite and stabilized hypobromite has been illustrated (Michels *et al.* 2000). Both LC/DAD (photodiode array UV spectroscopy) and LC/APCI-MS results showed that when 3OC6HSL was oxidized by stabilized hypobromite at pH 8, it was rapidly dibrominated at the  $\alpha$ -carbon position (i.e., between the two carbonyl groups of the side chain), and the last 4 carbons (i.e., from the  $\beta$ -carbon which is next to the halogenated carbon) of the side chain were shorn off, thus being converted to the  $\alpha,\alpha$ -dibromoethanoyl homoserine lactone (DBEHL) and releasing a 4 carbon butyric acid. The following step, which was the same as pH-hydrolysis, DBEHL was slowly hydrolyzed to the corresponding opening form, i.e.,  $\alpha,\alpha$ -dibromoethanoyl-2-(4-hydroxy)butanoic acid (hyd-DBEHL). Furthermore, at acidic pHs (pH 6 and 3) to slow the reaction rate, mono and dibrominated compounds,  $\alpha$ -monobromo-3OC6HSL and  $\alpha,\alpha$ -dibromo-3OC6HSL, were detected as precursors to DEBHL, thus demonstrating a stepwise modification of 3OC6HSL. Same results were obtained for hypochlorite as well. However, such reactions were only observed with oxo-substituted acyl-HSLs at the third carbon of the acyl chain, as compared to straight-chain acyl-HSLs.

Hypochlorous and hypobromous acids (HOCl and HOBr) were found to rapidly react with acyl-HSLs with a 3-oxo group and eliminate the molecules' function as



quorum sensing signals, while straight-chain acyl-HSLs were not affected (Brochardt *et al.* 2001). Furthermore, deactivation of 3OC6HSL was observed by HOBr in the marine alga *Laminaria digitata*. The formation of HOBr is catalyzed by bromoperoxidase in the presence of bromide and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which are found in seawater and produced by *L. digitata* respectively. Interestingly, the reaction between 3OC6HSL and stabilized hypobromite in a *P. aeruginosa* biofilm medium occurred. DEBHL and hydrolyzed DBEHL were detected as described above, despite the much higher level of biofilm components.

### **2.3.6 Inactivation by eukaryotes**

#### **2.3.6.1 Inactivation by mammalian sera or tissues**

All three paraoxonase (PON) genes, *PON1*, *PON2* and *PON3*, are highly conserved in mammalian animals; PONs or PON-like proteins can be found in all animal species (Draganov *et al.* 2000, Draganov *et al.* 2005). PON1 is synthesized in the liver and secreted into the blood; PON3, which is also expressed mostly in the liver and some in the kidney, is found ca. 100 times less than PON1 in the serum, whereas PON2 is cell-associated and expressed in many tissues, including brain, liver, kidney and testis, but not found in serum (Draganov and La Du 2004, Draganov *et al.* 2000, Draganov *et al.* 2005, Ng *et al.* 2001, Reddy *et al.* 2001). Recently discovered acyl-HSL inactivation activities by mammalian sera or tissues are most likely caused by PON enzymes, based on their enzymatic characteristics, e.g., Ca<sup>2+</sup>-dependent and sensitive to ethylenediaminetetraacetic acid (EDTA) (Chun *et al.* 2004, Draganov *et al.* 2005, Ozer *et al.* 2005, Yang *et al.* 2005).

Human airway epithelia has been shown capable of degrading several acyl-HSLs tested, including C6HSL, 3OC12HSL and C12HSL, but not C4HSL or 3OC6HSL and such activity is cell membrane-associated (Chun *et al.* 2004). Furthermore, diverse mammalian cells from different animals and/or organs were shown possessing variable acyl-HSL inactivating capacity on 3OC12HSL but not C4HSL, including cell lines from human colon carcinoma, human bronchoalveolar carcinoma, human cervix, human kidney, human lung fibroblasts cultures, monkey kidney and canine kidney (with a decreasing capability) but not Chinese hamster ovary. Later on, by using proton-nuclear magnetic resonance (NMR), HPLC and ESI-MS, such activity was shown to be lactonase activity, i.e., hydrolyzing the lactone ring of 3OC12HSL to the corresponding 3-oxododecanoyl homoserine (Ozer *et al.* 2005).

All three purified human PONs (PON1, PON2 and PON3), which were extracted from recombinant commercial *Trichoplusia ni* High Five insect cells with optimized procedures, have been shown to be lactonases/lactonizing enzymes through enzymatic studies and capable of hydrolyzing acyl-HSLs (Draganov *et al.* 2005). All three purified PON enzymes degraded the DL-acyl-HSLs tested (except PON3 on 3OC6HSL), i.e., 3OC6HSL, C7HSL, C12HSL and C14HSL which were especially much more effectively hydrolyzed by PON2. Furthermore, the hydrolysis was found to be stereoselective since only half of the DL-acyl-HSL pool was hydrolyzed whereas all of the L-3OC6HSL was completely hydrolyzed by PON2. In addition, all such activities were fully inhibited in the presence of EDTA.

The rabbit serum has been shown to be able to degrade a full range of acyl-HSLs from C4HSL to 3OC12HSL, with a preference to long-chain acyl-HSLs (Yang *et al.*

2005). HPLC and ESI-MS results showed lactonase-like activity of the rabbit serum. Likewise, such inactivation could be completely inhibited by EDTA and fully rescued by adding  $\text{Ca}^{2+}$ , which resembles those of PONs. Furthermore, mammalian sera samples from mouse, rabbit, horse, goat, human and bovine, but not chicken or fish, demonstrated strong yet comparable inactivation activity on 3OC12HSL, with mouse and rabbit showing relatively higher activities. Such activity could be inhibited by EDTA as well. PON1, PON2 and PON3 genes from mouse were cloned and expressed in Chinese hamster ovary (CHO) cell line, whose acyl-HSL inactivation activities were significantly increased to comparable levels despite the difference of the three PONs, thus confirming PONs could degrade acyl-HSLs.

Human and mouse sera have also been shown capable of rapidly inactivating 3OC12HSL, which can be largely inhibited by EDTA as well (Ozer *et al.* 2005). HPLC and ESI-MS results further confirmed a lactone ring-hydrolyzing activity. Interestingly, mice serum lacking *Pon1* almost completely lost 3OC12HSL-degrading ability, while adding back purified human PON1 was sufficient to rescue such ability. Therefore, PON1 is most likely responsible for the acyl-HSL inactivation in mouse serum. Furthermore, CHO cells transfected with recombinant adenoviruses expressing three human PON enzymes all very effectively degraded 3OC12HSL, with PON2 the most active.

### **2.3.6.2 Inactivation by plants**

Legume *Lotus corniculatus* plantlets have been shown capable of degrading several acyl-HSLs, i.e., C6HSL, 3OC6HSL, 3OC8HSL and 3OC10HSL, whereas C6HSL appeared to be stable in the gnotobiotic root system of wheat and corn (Delalande *et al.*

2005). Both bioassay and HPLC results demonstrated C6HSL disappearance from the medium. The acyl-HSL inactivation ability of the *Lotus* crude extracts was lost upon boiling, thus supporting the view that it was due to enzymatic activities. The underlying mechanisms are not clear.

#### 2.4 Specificity of acyl-HSL degrading enzymes

Several bacteria have acyl-HSL degrading enzymes that have activity against a broad range of acyl-HSLs whereas others are more specific. *Variovorax*, *Ralstonia* and *Bacillus* sp. contain enzymes that degrade a variety of both long and short chain length acyl-HSLs (Dong *et al.* 2000, Leadbetter and Greenberg 2000, Lin *et al.* 2003, Park *et al.* 2003) whereas *Streptomyces* and *Pseudomonas* seem to have a preference for long chain acyl-HSLs (Huang *et al.* 2003, Huang *et al.* 2006, Park *et al.* 2005). Some bacteria have preferences for substitution at the third carbon position: *Rhodobacter* sp. degraded C6HSL better than 3OC6HSL (Uroz *et al.* 2005) whereas *Variovorax* sp. degraded C6HSL more readily (Leadbetter and Greenberg 2000).

Given that some acyl-HSL synthases produce mixture of acyl-HSLs (Fuqua *et al.* 2001, Gonzalez and Marketon 2003, Marketon and Gonzalez 2002), it is interesting to speculate that enzymes that degrade acyl-HSLs could have a wider specificity for degradation to ensure that acyl-HSLs that could potentially be bioactive are degraded when their functions are not needed. In bacteria that produce acyl-HSL signals and use the degradation mechanism to regulate quorum sensing, the degrading enzymes might have widened specificity because there is not selection against this or because relaxed specificity selected the ability to degrade non-cognate acyl-HSLs that might have

activity. Acyl-HSL degrading enzymes with broad specificity could enable non acyl-HSL producing strains to degrade a wide range of signaling molecules produced by other organisms.

Acyl-HSL acylases identified have been shown to have homology to other acylases and the N-terminal nuclease hydrolase family of proteins (Lin *et al.* 2003). Many acyl-HSL acylase enzymes are similar to penicillin amidase proteins, but they differ in the range of substrates they can degrade. AhLM from *Streptomyces* was able to degrade penicillin G by deacetylation indicating that this enzyme has broader specificity (Park *et al.* 2005), whereas *Ralstonia* sp. acylase does not degrade penicillin and many penicillin acylases do not degrade acyl-HSL. Lactonases identified have a range of acyl-HSL substrate specificity but other functions of these enzymes have not been identified (Dong and Zhang 2005). *In vitro* protein studies of the substrate kinetics for these enzymes may suggest what natural substrates these enzymes could degrade in nature.

Biologically synthesized acyl-HSLs are presumed to be in the L-form (Watson *et al.* 2002). Consistently, bioassay revealed that L-isomers were essential as the autoinducers in quorum sensing, wherea no effect was seen with D-isomers of acyl-HSLs, which were neither agonists nor antagonists (Ikeda *et al.* 2001). Another way of acyl-HSL inactivation could be conversion to the D-form (Roche *et al.* 2004), which is less active in *E. carotovora* quorum sensing (Bainton *et al.* 1992). Several acyl-HSL degrading enzymes identified are not stereospecific for acyl-HSLs. *P. aeruginosa* PAO1 degrades both D- and L-forms of the long chain acyl-HSL, i.e., C10HSL (Huang *et al.* 2003). *Rhodococcus erythropolis* strain W2 was not stereospecific either in that it converted D-3OC12HSL to 3OHC12HSL (Uroz *et al.* 2005).

## 2.5 Acyl-HSL stability in natural environments

It is not unreasonable to argue that acyl-HSLs must have restricted stability in natural environments since highly stable molecules would accumulate over time and constantly exist at inducing concentrations despite fluctuations in population density, thus losing their function as signals of such. Therefore, investigations into acyl-HSL stability in natural environments appear to be truly essential to understand such cell-cell communications.

The first study documented on acyl-HSL degradation by natural microbial communities was performed by using radiolabeling approach, and indeed demonstrated rapid acyl-HSL biodegrading activities (Wang and Leadbetter 2005).  $^{14}\text{C}$ -labeled acyl-HSLs were mineralized to  $^{14}\text{CO}_2$  when amended to fresh soil samples at physiological concentrations in nongrowth buffer and mostly without lag. The acyl-HSL degradation rate under optimal conditions,  $13.4 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{g soil}^{-1}$ , is more than 2 orders of magnitude faster than theoretical pH-hydrolysis as described earlier. To outpace such rapid degradation, an acyl-HSL-producing bacterium would have to reach a population density of greater than  $10^9$  or  $10^{10} \text{ cells} \cdot \text{g soil}^{-1}$  in the species-rich soil communities, based on the results that acyl-HSL synthesis rates appear to be no more than  $10^{-18} \text{ moles} \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$ , i.e., when grown optimally *in vitro* (Dong *et al.* 2000, Huang *et al.* 2003, Lin *et al.* 2003, Reimann *et al.* 2002, Zhang *et al.* 2002). Moreover, the degradation activity by the soil communities can consume acyl-HSLs to an extent of less than 20 pM, which is far below the threshold concentrations required by many known acyl-HSL-producing

bacteria to elicit a quorum response *in vitro*, i.e., from 5 nM to 2  $\mu$ M (Fuqua *et al.* 1995, Kaplan and Greenberg 1985, Pearson *et al.* 1995, von Bodman *et al.* 1998, Whiteley *et al.* 1999).

pHs ranging from 5 to 9 are prevailingly common in many environments. Although acyl-HSLs are subject to pH-hydrolysis, rapidly decomposing to the corresponding acyl-homoserines at circumneutral and higher pHs, they are quite stable for weeks or months at acidic pHs  $\leq 6$ , however, none of acyl-HSLs, acyl-homoserines or HSL is known to accumulate in the environment. The many acyl-HSL-degrading activities described above serve to explain part of this concern; studies have also been conducted to investigate utilization of the metabolized intermediates of acyl-HSLs (Fig. 2.1).

Acyl-homoserines, which are the open-ring hydrolyzed products of acyl-HSLs, are known to be rapidly utilized as sole energy and nitrogen sources by an *Arthrobacter* strain VAI-A (Flagan *et al.* 2003); while homoserine lactone (HSL), which is the amide bond cleaved moiety of acyl-HSLs, can be utilized as a nitrogen source by *Variavorax paradoxus* VAI-C and *Arthrobacter* strain VAI-A (Flagan *et al.* 2003, Leadbetter and Greenberg 2000), and as an energy source by several *Arthrobacter* strains (HSL-1, HSL-2 and HSL-3) and a *Burkholderia* strain HSL-4 (Yang *et al.* 2006). HSL lactonase activity has also been found in *Pseudomonas aeruginosa*, but it is not observed to be utilized as a growth nutrient (Huang *et al.* 2003). More importantly, mutual beneficial effects were clearly demonstrated in defined cocultures of *Arthrobacter* strain VAI-A and *Variavorax* strain VAI-C using 3OC6HSL as sole carbon source (Flagan *et al.* 2003). Not only was the growth rate of VAI-A markedly enhanced by using viable cell counts, but also the biomass yield of cocultures increased dramatically than that of either of the

monocultures (i.e., 6.3- and 1.8-fold greater for VAI-A and VAI-C respectively) and that of the sum of their individual yields (i.e., 1.4-fold greater) under identical conditions. When 3OC6HSL was utilized as sole carbon and nitrogen sources, the effects were even more superior; the growth rates of each strain significantly exceeded those of the monocultures and the biomass yield results were similar to the above described. Similarly, when any of the four isolates (*Arthrobacter* strains HSL-1, HSL-2, HSL-3 and *Burkholderia* strain HSL-4) was cocultured with *Ralstonia mannitolytica* strain SDV (encoding an acyl-HSL acylase) with C10HSL as sole energy source, the growth yields remarkably increased yet to variable extent (i.e., 11-26% depending on the isolates) than that of the monoculture of SDV (Yang *et al.* 2006). Furthermore, degradation of acyl-HSLs by soil communities exhibited an apparent  $K_m$  of 1.5  $\mu\text{M}$ , which is ca. 1000-fold lower than that of a purified acyl-HSL lactonase from *Bacillus cereus* (Wang *et al.* 2004), suggesting that the observed degradation activities of soils are not mainly accounted for by acyl-HSL-degrading organisms currently available in culture (Wang and Leadbetter 2005).

On the other hand, soils are not well-mixed systems; bacteria typically grow as microcolonies in soils. Although little is known of the natural distribution of quorum sensing bacteria or acyl-HSL-degrading bacteria in soils, there has been evidence that signal degradation could serve to insulate microbial aggregates (Molina *et al.* 2003). By using *Chromobacterium violaceum* CV026 as an acyl-HSL biosensor, which produces the purple pigmented violacein, acyl-HSLs production and diffusion were confirmed (as the biosensor being bright purple) from the acyl-HSL-producing bacterium *Erwinia carotovora* 852, when the *E. carotovora* strain was spotted 16-17 mm away from the *C.*



*violaceum* line on LB agar plates. However, no induction of violacein was observed when a recombinant strain *Pseudomonas fluorescens* P3, expressing the *aiiA* gene from *Bacillus* sp. A24 that encodes a lactonase, was spotted in between the *E. carotovora* and the biosensor at a distance of 6-7 mm from the biosensor line, clearly demonstrating degradation of surrounding acyl-HSLs by the P3 strain. Slightly weaker degradation activity was also observed by the wild-type *Bacillus* sp. A24. Therefore, such insulation of microbial aggregates from extraneous signals could disrupt beneficial or deleterious cross talk between spatially separated microbial populations that might otherwise occur (Blosser and Gray 2000, Lewenza *et al.* 2002, McDougald *et al.* 2003, Pierson *et al.* 1998).

Acyl-HSL-dependent quorum sensing is significantly influenced by the local temperature and pH. Lower temperature increases the half-lives of acyl-HSLs (Yates *et al.* 2002). One reason why many marine bacteria use acyl-HSLs as quorum sensing signals despite the high pH of seawater (ca. pH 8) is probably due to the low water temperature. Such concern should also be taken into account when culturing bacteria. For instance, LB is commonly used with aeration in laboratories, however alkalization tends to happen due to release of ammonia from degradation of peptides as carbon and energy sources. On the contrary, anaerobic metabolism lowers the pH by producing weak acids. For example, in natural environment, it has been observed that soft rot, caused by quorum sensing pathogen *Erwinia carotovora*, occurs more readily with limiting oxygen, probably because low pH helps *E. carotovora* to accumulate quorum sensing signals (Perombelon and Kelman 1980, Yates *et al.* 2002).

Long-chain acyl-HSLs appear to have greater advantages over short-chain acyl-HSLs in the environments. The longer the acyl side chain, the more stable they are at high pHs; once hydrolyzed, they can reform the ring and regain biological activity at circumneutral or mild acidic conditions (depending on the pK of the carboxyl group) (Yates *et al.* 2002). However, given their hydrophobic properties, long-chain acyl-HSLs would be more difficult to diffuse through cells and may be rapidly partitioned into organic compounds so that their function as quorum sensing signals would be in question. Moreover, many acyl-HSL-degrading bacteria or enzymes have a specificity or preference towards long-chain acyl-HSLs (Huang *et al.* 2003, Huang *et al.* 2006, Lin *et al.* 2003, Park *et al.* 2005). For example, *P. aeruginosa* PAO1 only degrades acyl-HSLs with chain length  $\geq 8$  carbons (Huang *et al.* 2003) and *Streptomyces* sp. M664 much more effectively degrades acyl-HSLs with chain length  $\geq 8$  carbons (Park *et al.* 2005). Although that sounds paradoxical, it may be the reason why some bacteria employ both short- and long-chain acyl-HSLs, e.g., *P. aeruginosa* which produces both C4HSL and 3OC12HSL to control many of the same virulence genes (Latifi *et al.* 1996, Passador *et al.* 1993, Pearson *et al.* 1994, Winson *et al.* 1995).

## **2.6 Coevolution of quorum sensing bacteria with hosts and acyl-HSL-degrading bacteria**

Both plants and animals have sophisticated mechanisms to defend pathogens (Dangl and Jones 2001) and in return, many bacterial pathogens have evolved strategies to overcome host defense, such as the population density-dependent quorum sensing

system. The pathogens elicit their virulence genes when enough population is achieved to overwhelm the host's defense response. It is possible that such antagonistic coexistence could lead to complicated coevolution of the pathogens and hosts.

As diverse bacterial species have been characterized to use quorum sensing to gain advantages over other competitors, it is not surprising to see those many mechanisms that bacteria and eukaryotes have evolved to degrade acyl-HSLs and thus disrupt quorum sensing systems, as demonstrated by pure laboratory cultures, defined cocultures, laboratory microcosms and soil microbial communities (for reviews, see Dong and Zhang 2005, Zhang 2003, Zhang and Dong 2004). Besides, several other organisms have been shown capable of inhibiting or interfering quorum sensing via different mechanisms. For example, halogenated furanones from the marine red alga *Delisea pulchra* disrupt acyl-HSL-mediated quorum sensing responses (Givskov *et al.* 1996, Manefield *et al.* 1999, 2001, 2002, Rasmussen *et al.* 2000). Triclosan, an inhibitor of the enoyl-acyl carrier protein (ACP), reduced acyl-HSL biosynthesis *in vitro* (Hoang and Schweizer 1999, Liu *et al.* 2002). Pea *Pisum sativum*, crown vetch *Coronilla varia* and alga *Chlamydomonas reinhardtii* have been shown to exude substances that mimic bacterial acyl-HSLs and interfere with quorum sensing (Teplitski *et al.* 2004, 2000).

During this process, the hosts may have again evolved more complex defense mechanisms. One example is that in response to infection of a pathogen *Erwinina carotovora*, host plants can increase the pH of the apoplastic fluid around infection area by activating a rapid proton influx into the plant cells and making the intercellular pH be ca. 8, thus remarkably enhancing acyl-HSL hydrolysis and weakening virulence

responses of *E. carotovora* (Baker *et al.* 1986, 1990, Byers *et al.* 2002, Nachin and Barras 2000).

Microorganisms usually evolve resistance mechanisms to counter antimicrobials. A good therapeutic agent would have to be able to eliminate the target bacterial virulence response without inhibiting the physiology of the bacterium; halogenated furanones from *Delisea pulchra*, which can interfere with quorum sensing, have been observed to have no appreciable inhibitory effects on growth of pathogenic bacteria over many generations (Bauer and Teplitski 2001). However, very likely acyl-HSL degraders, or transgenic plants expressing acyl-HSL-degrading bacterial enzymes, may promote generation of bacterial strains capable of inhibiting such acyl-HSL degradation or to be acyl-HSL-independent for expression of virulence factors. In fact, such acyl-HSL-independent strains of *Erwinia carotovora* subsp. *carotovora* have been readily isolated (Whitehead *et al.* 2001).

## **2.7 Conclusions**

In the past thirty years, the synthesis of acyl-HSLs and their role in the context of quorum sensing system have been studied intensively. It is only in the past six years have we realized that acyl-HSL degradation is just as important to functional quorum sensing system as production. For microbes that degrade acyl-HSL signals but are not known to accumulate acyl-HSLs or have quorum sensing systems, acyl-HSL degradation could provide a means to compete with acyl-HSL-producing, quorum sensing neighbors in the environment. Wang and Leadbetter (2005) have shown that acyl-HSL degradation by

microbial consortia can proceed at physiological concentrations and to the extent below what is sensed by known quorum sensing bacteria, indicating that acyl-HSL degradation poses challenges to quorum sensing bacteria in natural communities. Acyl-HSL degrading microbes that use acyl-HSLs as either a sole carbon source or carbon and nitrogen source (Huang *et al.* 2003, Leadbetter and Greenberg 2000, Lin *et al.* 2003) could gain an additional advantage in soil environments where nutrients are limited.

Surveys of many environments from the rhizosphere to marine habitats have identified an increasing number of bacteria that produce acyl-HSLs (Cha *et al.* 1998, D'Angelo-Picard *et al.* 2005, Wagner-Dobler *et al.* 2005). The possibility that acyl-HSL mediated quorum sensing is widespread suggests that we may be seeing tip of the iceberg in terms of microbes and organisms with acyl-HSL degrading potential. Therefore we have much to learn about the ways in which degradation is used and how it influences quorum sensing microbes and communities. As more bacterial-bacterial and bacterial-eukaryotic interactions are studied, we may get closer to an understanding of the complexity of community interactions with acyl-HSLs.

## 2.8 References

Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. (1992). A general role for the *lux* autoinducer in bacterial cell signaling: control of antibiotic synthesis in *Erwinia*. *Gene* **116**, 87-91.

- Baker, C. J., Atkinson, M. M., Roy, M. A., and Collmer, A. (1986). Inhibition of the hypersensitive response in tobacco by pectate lyase. *Physiol. Mol. Plant Pathol.* **29**, 217-225.
- Baker, C. J., Mock, N., Atkinson, M. M., and Hutcheson, S. (1990). Inhibition of the hypersensitive response in tobacco by pectate lyase digests of cell-wall and of polygalacturonic acid. *Physiol. Mol. Plant Pathol.* **37**, 155-167.
- Bauer, W. D., and Teplitski, M. (2001). Can plants manipulate bacterial quorum sensing? *Aust. J. Plant Physiol.* **28**, 913-921.
- Blosser, R. S., and Gray, K. M. (2000). Extraction of violacein from *Chromobacterium violaceum* provides a new quantitative bioassay for N-acyl homoserine lactone autoinducers. *J. Microbiol. Meth.* **40**, 47-55.
- Brochardt, S. A., Allain, E. J., Michels, J. J., Stearns, G. W., Kelly, R. F., and McCoy, W. F. (2001). Reaction of acylated homoserine lactone bacterial signaling molecules with oxidized halogen antimicrobials. *Appl. Environ. Microbiol.* **67**, 3174-3179.
- Byers, J. T., Lucas, C., Salmond, G. P. C., and Welch, M. (2002). Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *J. Bacteriol.* **184**, 1163-1171.
- Carrier, A., Chevrot, R., Dessaux, Y., and Faure, D. (2004). The assimilation of gamma-butyrolactone in *Agrobacterium tumefaciens* C58 interferes with the accumulation of the N-acyl-homoserine lactone signal. *Mol. Plant-Microbe Interactions* **17**, 951-957.
- Carrier, A., Uroz, S., Smadja, B., Fray, R., Latour, X., Dessaux, Y., and Faure, D. (2003). The Ti plasmid of *Agrobacterium tumefaciens* harbors an *attM*-paralogous gene,

- aiiB*, also encoding N-acyl homoserine lactonase activity. *Appl. Environ. Microbiol.* **69**, 4989-4993.
- Cha, C., Gao, P., Chen, Y. C., Shaw, P. D., and Farrand, S. K. (1998). Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol. Plant-Microbe Interactions* **11**, 1119-1129.
- Chun, C. K., Ozer, E. A., Welsh, M. J., Zabner, J., and Greenberg, E. P. (2004). Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. *Proc. Natl. Acad. Sci. USA* **101**, 3587-3590.
- D'Angelo-Picard, C., Faure, D., Penot, I., and Dessaux, Y. (2005). Diversity of N-acyl homoserine lactone-producing and -degrading bacteria in soil and tobacco rhizosphere. *Environ. Microbiol.* **7**, 1796-1808.
- Dangl, J. L., and Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature* **411**, 826-833.
- de Kievit, T. R., and Iglewski, B. H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infect. Immu.* **68**, 4839-4849.
- Delalande, L., Faure, D., Raffoux, A., Uroz, S., D'Angelo-Picard, C., Elasri, M., Carlier, A., Berruyer, R., Petit, A., Williams, P., and Dessaux, Y. (2005). N-hexanoyl-L-homoserine lactone, a mediator of bacterial quorum-sensing regulation, exhibits plant-dependent stability and may be inactivated by germinating *Lotus corniculatus* seedlings. *FEMS Microbiol. Ecol.* **52**, 13-20.
- Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L., and Zhang, L. H. (2002). Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. *Appl. Environ. Microbiol.* **68**, 1754-1759.

- Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., and Zhang, L. H. (2001). Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**, 813-817.
- Dong, Y. H., Xu, J. L., Li, X. Z., and Zhang, L. H. (2000). AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. USA* **97**, 3526-3531.
- Dong, Y. H., and Zhang, L. H. (2005). Quorum sensing and quorum-quenching enzymes. *J. Bacteriol.* **43**, 101-109.
- Draganov, D. I., and La Du, B. N. (2004). Pharmacogenetics of paraoxonases: a brief review. *N-S Arch. Pharmacol.* **369**, 78-88.
- Draganov, D. I., Stetson, P. L., Watson, C. E., Billecke, S. S., and La Du, B. N. (2000). Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. *J. Biol. Chem.* **275**, 33435-33442.
- Draganov, D. I., Teiber, J. F., Speelman, A., Osawa, Y., Sunahara, R., and La Du, B. N. (2005). Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J. Lipid Res.* **46**, 1239-1247.
- Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H., and Oppenheimer, N. J. (1981). Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry-US* **20**, 2444-2449.
- Engbrecht, J., Nealson, K., and Silverman, M. (1983). Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* **32**, 773-781.



- Farah, C., Vera, M., Morin, D., Haras, D., Jerez, C. A., and Guiliani, N. (2005). Evidence for a functional quorum-sensing type AI-1 system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **71**, 7033-7040.
- Flagan, S., Ching, W. K., and Leadbetter, J. R. (2003). *Arthrobacter* strain VAI-A utilizes acyl-homoserine lactone inactivation products and stimulates quorum signal biodegradation by *Variovorax paradoxus*. *Appl. Environ. Microbiol.* **69**, 909-916.
- Fuqua, C., Burbea, M., and Winans, S. C. (1995). Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the *tram* gene. *J. Bacteriol.* **177**, 1367-1373.
- Fuqua, C., Parsek, M. R., and Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. *Ann. Rev. Genet.* **35**, 439-468.
- Gambello, M. J., and Iglewski, B. H. (1991). Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J. Bacteriol.* **173**, 3000-3009.
- Givskov, M., DeNys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P. D., and Kjelleberg, S. (1996). Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling. *J. Bacteriol.* **178**, 6618-6622.
- Gonzalez, J. E., and Marketon, M. M. (2003). Quorum sensing in nitrogen-fixing rhizobia. *Microbiol. Mol. Biol. Rev.* **67**, 574-579.
- Hanzelka, B. L., Stevens, A. M., Parsek, M. R., Crone, T. J., and Greenberg, E. P. (1997). Mutational analysis of the *Vibrio fischeri* LuxI polypeptide: critical regions of an autoinducer synthase. *J. Bacteriol.* **179**, 4882-4887.

- Hoang, T. T., and Schweizer, H. P. (1999). Characterization of *Pseudomonas aeruginosa* Enoyl-Acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. *J. Bacteriol.* **181**, 5489-5497.
- Huang, J. J., Han, J. I., Zhang, L. H., and Leadbetter, J. R. (2003). Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* **69**, 5941-5949.
- Huang, J. J., Petersen, A., Whiteley, M., and Leadbetter, J. R. (2006). Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* **72**, 1190-1197.
- Ikeda, T., Kajiyama, K., Kita, T., Takiguchi, N., Kuroda, A., Kato, J., and Ohtake, H. (2001). The synthesis of optically pure enantiomers of N-acyl-homoserine lactone autoinducers and their analogues. *Chem. Lett.*, 314-315.
- Jafra, S., and van der Wolf, J. M. (2004). Fast screening method for detection of acyl-HSL-degrading soil isolates. *J. Microbiol. Meth.* **57**, 415-420.
- Kang, B. R., Lee, J. H., Ko, S. J., Lee, Y. H., Cha, J. S., Cho, B. H., and Kim, Y. C. (2004). Degradation of acyl-homoserine lactone molecules by *Acinetobacter* sp strain C1010. *Can. J. Microbiol.* **50**, 935-941.
- Kaplan, H. B., and Greenberg, E. P. (1985). Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**, 1210-1214.
- Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996). A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the

transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol. Microbiol.* **21**, 1137-1146.

Leadbetter, J. R. (2001). Plant microbiology: Quieting the raucous crowd. *Nature* **411**, 748-749.

Leadbetter, J. R., and Greenberg, E. P. (2000). Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *J. Bacteriol.* **182**, 6921-6926.

Lee, S. J., Park, S. Y., Lee, J. J., Yum, D. Y., Koo, B. T., and Lee, J. K. (2002). Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **68**, 3919-3924.

Lewenza, S., Visser, M. B., and Sokol, P. A. (2002). Interspecies communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*. *Can. J. Microbiol.* **48**, 707-716.

Lin, Y. H., Xu, J. L., Hu, J. Y., Wang, L. H., Ong, S. L., Leadbetter, J. R., and Zhang, L. H. (2003). Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Mol. Microbiol.* **47**, 849-860.

Liu, B. Q., Wang, Y. Q., Fillgrove, K. L., and Anderson, V. E. (2002). Triclosan inhibits enoyl-reductase of type I fatty acid synthase in vitro and is cytotoxic to MCF-7 and SKBr-3 breast cancer cells. *Cancer Chemoth. Pharm.* **49**, 187-193.

Manefield, M., de Nys, R., Kumar, N., Read, R., Givskov, M., Steinberg, P., and Kjelleberg, S. A. (1999). Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by

displacing the AHL signal from its receptor protein. *Microbiology-Sgm* **145**, 283-291.

Manefield, M., Rasmussen, T. B., Henzter, M., Andersen, J. B., Steinberg, P., Kjelleberg, S., and Givskov, M. (2002). Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology-Sgm* **148**, 1119-1127.

Manefield, M., Welch, M., Givskov, M., Salmond, G. P. C., and Kjelleberg, S. (2001). Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. *FEMS Microbiol. Lett.* **205**, 131-138.

Marketon, M. M., and Gonzalez, J. E. (2002). Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. *J. Bacteriol.* **184**, 3466-3475.

McDougald, D., Srinivasan, S., Rice, S. A., and Kjelleberg, S. (2003). Signal-mediated cross-talk regulates stress adaptation in *Vibrio* species. *Microbiology-Sgm* **149**, 1923-1933.

Michels, J. J., Allain, E. J., Borchardt, S. A., Hu, P. F., and McCoy, W. F. (2000). Degradation pathway of homoserine lactone bacterial signal molecules by halogen antimicrobials identified by liquid chromatography with photodiode array and mass spectrometric detection. *J. Chromatogra. A* **898**, 153-165.

Molina, L., Constantinescu, F., Michel, L., Reimann, C., Duffy, B., and Defago, G. (2003). Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. *FEMS Microbiol. Ecol.* **45**, 71-81.

- Nachin, L., and Barras, F. (2000). External pH: An environmental signal that helps to rationalize *pel* gene duplication in *Erwinia chrysanthemi*. *Mol. Plant-Microbe Interactions* **13**, 882-886.
- Nealson, K. H., Platt, T., and Hastings, J. W. (1970). Cellular control of synthesis and activity of bacterial luminescent system. *J. Bacteriol.* **104**, 313-317.
- Nealson, K. K. H., and Hastings, J. J. W. (1979). Bacterial bioluminescence: its control and ecological significance. *Microbiol. Rev.* **43**, 496-518.
- Ng, C. J., Wadleigh, D. J., Gangopadhyay, A., Hama, S., Grijalva, V. R., Navab, M., Fogelman, A. M., and Reddy, S. T. (2001). Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *J. Biol. Chem.* **276**, 44,444-44,449.
- Ozer, E. A., Pezzulo, A., Shih, D. M., Chun, C., Furlong, C., Lusic, A. J., Greenberg, E. P., and Zabner, J. (2005). Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing. *FEMS Microbiol. Ecol.* **253**, 29-37.
- Paggi, R. A., Martone, C. B., Fuqua, C., and De Castro, R. E. (2003). Detection of quorum sensing signals in the haloalkaliphilic archaeon *Natronococcus occultus*. *FEMS Microbiol. Ecol.* **221**, 49-52.
- Park, S. Y., Kang, H. O., Jang, H. S., Lee, J. K., Koo, B. T., and Yum, D. Y. (2005). Identification of extracellular N-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching. *Appl. Environ. Microbiol.* **71**, 2632-2641.

- Park, S. Y., Lee, S. J., Oh, T. K., Oh, J. W., Koo, B. T., Yum, D. Y., and Lee, J. K. (2003). AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. *Microbiology-Sgm* **149**, 1541-1550.
- Parsek, M. R., and Greenberg, E. P. (2000). Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signaling mechanism involved in associations with higher organisms. *Proc. Natl. Acad. Sci. USA* **97**, 8789-8793.
- Passador, L., Cook, J. M., Gambello, M. J., Rust, L., and Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127-1130.
- Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H., and Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* **91**, 197-201.
- Pearson, J. P., Passador, L., Iglewski, B. H., and Greenberg, E. P. (1995). A second N-acyl-homoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1490-1494.
- Perombelon, M. C. M., and Kelman, A. (1980). Ecology of the soft rot *Erwinias*. *Annu. Rev. Phytopathol.* **18**, 361-387.
- Pierson, E. A., Wood, D. W., Cannon, J. A., Blachere, F. M., and Pierson, L. S. (1998). Interpopulation signaling via N-acyl-homoserine lactones among bacteria in the wheat rhizosphere. *Mol. Plant Microbe Interact.* **11**, 1078-1084.

- Piper, K. R., von Bodman, B. S., and Farrand, S. K. (1993). Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**, 448-450.
- Rasmussen, T. B., Manefield, M., Andersen, J. B., Eberl, L., Anthoni, U., Christophersen, C., Steinberg, P., Kjelleberg, S., and Givskov, M. (2000). How *Delisea pulchra* furanones affect quorum sensing and swarming motility in *Serratia liquefaciens* MG1. *Microbiology-SGM* **146**, 3237-3244.
- Reddy, S. T., Wadleigh, D. J., Grijalva, V., Ng, C., Hama, S., Gangopadhyay, A., Shih, D. M., Lusic, A. J., Navab, M., and Fogelman, A. M. (2001). Human paraoxonase-3 is an HDL-associated enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. *Arterioscl. Throm. Vascular Biol.* **21**, 542-547.
- Reimann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafillu, K., Harms, H., Defago, G., and Haas, D. (2002). Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology-Sgm* **148**, 923-932.
- Roche, D. M., Byers, J. T., Smith, D. S., Glansdorp, F. G., Spring, D. R., and Welch, M. (2004). Communications blackout? Do N-acylhomoserine lactone-degrading enzymes have any role in quorum sensing? *Microbiology-Sgm* **150**, 2023-2028.
- Schaefer, A. L., Hanzelka, B. L., Parsek, M. R., and Greenberg, E. P. (2000). Detection, purification and structural elucidation of acylhomoserine lactone inducer of

*Vibrio fischeri* luminescence and other related molecules. *Meth. Enzymol.* **305**, 288-301.

Schaefer, A. L., Val, D. L., Hanzelka, B. L., Cronan, J. E., and Greenberg, E. P. (1996). Generation of cell-to-cell signals in quorum sensing: Acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc. Natl. Acad. Sci. USA* **93**, 9505-9509.

Stevens, A. M., and Greenberg, E. P. (1997). Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. *J. Bacteriol.* **179**, 557-562.

Swift, S., Downie, J. A., Whitehead, N. A., Barnard, A. M. L., Salmond, G. P. C., and Williams, P. 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. Pages 199-270. *Adv. Microb. Physiol., Vol 45*.

Telford, G., Wheeler, D., Williams, P., Tomkins, P. T., Appleby, P., Sewell, H., Stewart, G. S., Bycroft, B. W., and Pritchard, D. I. (1998). The *Pseudomonas aeruginosa* quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. *Infect. Immu.* **66**, 36-42.

Teplitski, M., Chen, H. C., Rajamani, S., Gao, M. S., Merighi, M., Sayre, R. T., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. (2004). *Chlamydomonas reinhardtii* secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. *Plant Physiol.* **134**, 137-146.

Teplitski, M., Robinson, J. B., and Bauer, W. D. (2000). Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population



- density-dependent behaviors in associated bacteria. *Mol. Plant-Microbe Interactions* **13**, 637-648.
- Ulrich, R. L. (2004). Quorum quenching: Enzymatic disruption of N-acylhomoserine lactone-mediated bacterial communication in *Burkholderia thailandensis*. *Appl. Environ. Microbiol.* **70**, 6173-6180.
- Uroz, S., Chhabra, S. R., Camara, M., Williams, P., Oger, P., and Dessaux, Y. (2005). N-acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. *Microbiology-Sgm* **151**, 3313-3322.
- Uroz, S., D'Angelo-Picard, C., Carlier, A., Elasri, M., Sicot, C., Petit, A., Oger, P., Faure, D., and Dessaux, Y. (2003). Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology-Sgm* **149**, 1981-1989.
- Voelkert, E., and Grant, D. R. (1970). Determination of homoserine as lactone. *Anal. Biochem.* **34**, 131-137.
- von Bodman, S. B., Majerczak, D. R., and Coplin, D. L. (1998). A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. *Proc. Natl. Acad. Sci. USA* **95**, 7687-7692.
- Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., and Iglewski, B. H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.* **185**, 2080-2095.
- Wagner-Dobler, I., Thiel, V., Eberl, L., Allgaier, M., Bodor, A., Meyer, S., Ebner, S., Hennig, A., Pukall, R., and Schulz, S. (2005). Discovery of complex mixtures of

novel long-chain quorum sensing signals in free-living and host-associated marine alphaproteobacteria. *Chembiochem* **6**, 2195-2206.

Wang, L. H., Weng, L. X., Dong, Y. H., and Zhang, L. H. (2004). Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). *J. Biol. Chem.* **279**, 13645-13651.

Wang, Y. J., and Leadbetter, J. R. (2005). Rapid acyl-homoserine lactone quorum signal biodegradation in diverse soils. *Appl. Environ. Microbiol.* **71**, 1291-1299.

Watson, W. T., Minogue, T. D., Val, D. L., von Bodman, S. B., and Churchill, M. E. A. (2002). Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Molecular Cell* **9**, 685-694.

Whitehead, N. A., Welch, M., and Salmond, G. P. C. (2001). Silencing the majority. *Nature Biotechnology* **19**, 735-736.

Whiteley, M., Lee, K. M., and Greenberg, E. P. (1999). Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **96**, 13904-13909.

Winson, M. K., Camara, M., Latifi, A., Foglino, M., Chhabra, S. R., Daykin, M., Bally, M., Chapon, V., Salmond, G. P. C., Bycroft, B. W., Lazdunski, A., Stewart, G. S. A. B., and Williams, P. (1995). Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **92**, 9427-9431.

- Yang, F., Wang, L. H., Wang, J., Dong, Y. H., Hu, J. Y., and Zhang, L. H. (2005). Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. *FEMS Lett.* **579**, 3713-3717.
- Yang, W. W., Han, J. H., and Leadbetter, J. R. (2006). Utilization of homoserine lactone as a sole source of carbon and energy by soil *Arthrobacter* and *Burkholderia* species. *Arch. Microbiol.* **185**, 47-54.
- Yates, E. A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S. R., Sockett, R. E., Goldner, M., Dessaux, Y., Camara, M., Smith, H., and Williams, P. (2002). N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect. Immun.* **70**, 5635-5646.
- Zhang, H. B., Wang, L. H., and Zhang, L. H. (2002). Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* **99**, 4638-4643.
- Zhang, L. H. (2003). Quorum quenching and proactive host defense. *Trends In Plant Science* **8**, 238-244.
- Zhang, L. H., and Dong, Y. H. (2004). Quorum sensing and signal interference: diverse implications. *Mol. Microbiol.* **53**, 1563-1571.

## **Chapter 3**

### **Rapid Acyl-homoserine Lactone Quorum Signal Biodegradation in Diverse Soils**

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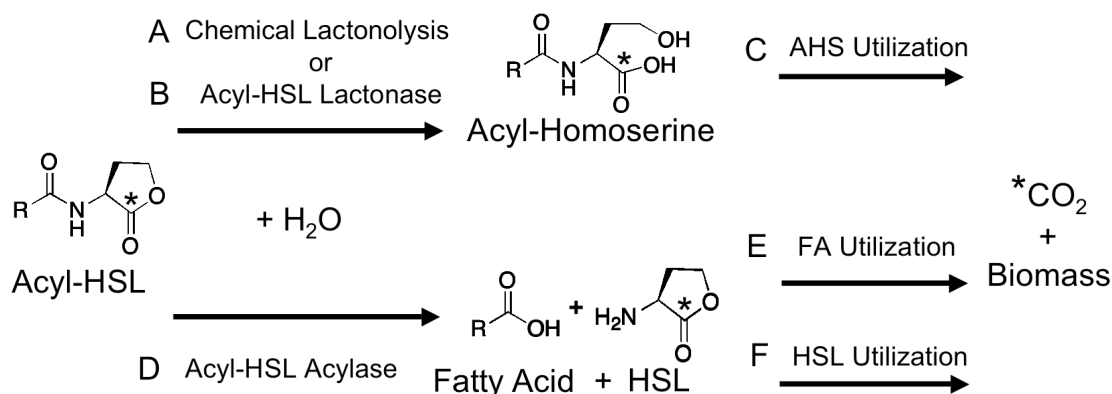
### 3.1 Introduction

Over a 30-year period, it has become apparent that a diversity of *Proteobacteria* employ acyl-homoserine lactones (acyl-HSLs) as dedicated signal molecules in quorum sensing controlled gene expression (16, 21, 23, 44, 59, 66). Among these are strains isolated from soil belonging to the genera *Agrobacterium* (24, 72), *Burkholderia* (36), *Chromobacterium* (7, 40), *Pseudomonas* (25, 67, 68), *Ralstonia* (18), *Rhizobium* and other related genera involved in legume symbioses (6, 49, 54, 57), *Rhodobacter* (50, 56), and *Serratia* (1, 9, 52). These soil bacteria can use quorum sensing to regulate the production of biologically active secondary metabolites in soils such as cyanide (7, 51), phenazines (19, 48, 58, 68), prodigiosin (27, 61), violacein (40), and carbapenems (42). Quorum sensing by soil bacteria can benefit agriculture: the acyl-HSL-controlled production of phenazines and other anti-fungal metabolites by certain pseudomonads is now well established to underlie their “biocontrol” activities (26, 39, 60, 67). Other quorum sensing species, such as certain species of *Agrobacterium*, *Burkholderia*, *Erwinia*, *Pseudomonas*, and *Ralstonia*, are known to use quorum sensing mechanisms during plant pathogenesis (49). Acyl-HSL regulation can also control the production of compounds that alter the properties of soils and soil aggregates. *Serratia* and *Pseudomonas* species are known to regulate the production of diverse surfactants using acyl-HSL signaling (11, 27, 36), and exopolysaccharide production is also known to be quorum controlled in several bacterial species (2).

Over the past 4 years, research has documented a diversity of soil microbes capable of rapidly biodegrading acyl-HSLs (14, 28, 38, 43, 45, 63) by cleaving either the

amide or lactone bonds of these molecules (33). The two routes by which acyl-HSLs are known to be degraded are shown in Fig. 3.1. The potent negative effects of enzyme-based acyl-HSL degradation on signal accumulations and quorum-sensing have been demonstrated during pure-culture laboratory studies (14, 38, 51, 71). Such effects have also been examined in simple synthetic communities using defined co-cultures (45, 63), laboratory soil microcosms seeded with recombinant strains (43), and transgenic plants expressing bacterial proteins (13). However, the stability of acyl-HSLs in natural environments over short and long periods is poorly understood, and we are not aware of any studies demonstrating signal decay in naturally occurring microbial communities.

Do bacteria actually express signal degrading activities in bulk soils in nature, and can they act on physiologically relevant concentrations of these molecules in the field? What is the biochemical stability of acyl-HSLs in soils? To our knowledge, no reports have documented acyl-HSL degradation activities in natural environmental samples. Here we have begun to examine such issues. We have taken the approach of synthesizing radiolabeled acyl-HSLs to examine their fate when amended at low concentrations, relevant to quorum sensing, to buffered bulk soil slurries and other samples.



**Figure 3.1. Pathways of acyl-HSL degradation.** Acyl-HSLs are known to be inactivated by hydrolysis at either the lactone ring or the acyl-amide linkage. Lactone hydrolysis occurs chemically as a function of increased pH (**A**) (55) or due to the activity of acyl-HSL lactonases produced by strains of *Agrobacterium*, *Arthrobacter*, *Bacillus*, and *Klebsiella* (**B**) (13, 45, 71). (**C**) The resultant acyl-homoserine (AHS) hydrolysis product is known to be utilized by *Arthrobacter* strain VAI-A (17). (**D**) Amide hydrolysis is known to be catalyzed by acyl-HSL acylases produced by *Pseudomonas*, *Ralstonia*, and *Variovorax* species (28, 34, 38). (**E**) The fatty acid (FA) released is utilized as an energy source by the strains producing the acylase enzyme. (**F**) The homoserine lactone (HSL) released by the acylase can be utilized as a N source by *Variovorax* and *Arthrobacter* in a process that involves the mineralization of the lactone ring (17, 34), and as an energy source by several *Arthrobacter* and *Burkholderia* strains (69).

## 3.2 Materials and methods

### 3.2.1 Site descriptions

Sites sampled during the course of this study included soils from the suburban Caltech campus in Pasadena, Calif., agricultural soils from USDA plots in Pullman, Wash., NSF Long Term Ecological Research plots near Michigan State University's Kellogg Biological Station, Hickory Corners MI, and the hindgut contents of a dampwood termite, *Zootermopsis*.

The activity best characterized in this study was present in bulk soils sampled from well-watered and well-fertilized turf grass in front of Beckman Auditorium on the Caltech campus. Bulk soil samples from this and the other Caltech sites were collected from the upper 5 cm. Other Caltech sites examined included the soil collected from under the canopies of Coastal Redwood (*Sequoia sempervirens*), Olive (*Olea europaea*), Rose (*Rosa hybrid tea*), and Wisteria (*Wisteria sinensis*), and from surface sediments from a shallow, lily-laden pond.

Samples from USDA agricultural plots in Pullman, Wash., were collected by Linda Thomashow and included bulk soils collected from fields of winter wheat and spring wheat. Spring Wheat plots exhibiting *Take-All* (a fungal pathogen) suppressive and nonsuppressive activities were examined. *Take-All* suppression is known to be mediated by acyl-HSL quorum sensing regulated antibiotic production by biocontrol pseudomonads (60, 67, 68).



The descriptions of sites and soils from Kellogg Biological Station's NSF Long-Term Ecological Research (KBS-LTER) plots are available online at <http://lter.kbs.msu.edu/ExperimentalDesign.html>. KBS LTER samples were taken from the main experimental agricultural sites as well as from successional and other forest sites. At the main site, annual crops consist of a rotation of corn, soybean, and wheat. Samples from two distinct subplots were examined: treatment 1 (r1), and treatment 4 (r1). The former was chisel-plowed and received standard levels of common chemical inputs. The latter was an organic-based (no chemical inputs at any time) system planted with a winter leguminous cover crop and receiving additional post-planting cultivation to control weed. Other LTER sites examined included perennial systems planted with alfalfa (treatment 6, r1) and poplar trees (treatment 5, r1). Samples from the never-plowed treatment 8 site, which is 200 meters south of the others and serves as the surrogate native soil for soil organic matter studies, were examined. In the successional and other forest plots, samples were taken from three sites: a 40- to 60-year-old successional forest, which was formerly an agricultural field, a 40- to 60-year-old conifer plantation, and a late successional forest that has never been cut.

Analysis of Caltech campus soils and termite hindgut homogenates commenced within ca. 1 h of collection. Soils from the USDA and KBS-LTER plots were collected in April and May 2004, respectively, delivered via overnight mail, and analyzed immediately upon receipt. For the case of the soils collected from KBS-LTER plots, samples were maintained on ice from the time of collection until analyses commenced. For all soils, samples were passed through a 2-mm-pore-size sieve after collection and

before further analysis. The water content of each soil was determined in triplicate, as was the pH using the CaCl<sub>2</sub> method (5) with a soil-to-solution ratio of 1 : 2.5.

Specimens of *Zootermopsis* were collected from decaying *Ponderosa* pine logs located near the Chilao Flats Campground in the Angeles National Forest of Southern California and maintained in the laboratory in plastic containers on a ponderosa diet. Ten guts were extracted and homogenized in 10 ml of a buffered salt solution (pH 7.0) in a ground-glass homogenizer, and then equally distributed between two reaction tubes.

### 3.2.2 Synthesis and purification of <sup>14</sup>C-labeled acyl-homoserine lactones

*Escherichia coli* BL21 (DE3) expressing recombinant EsaI (65), the *Pantoea stewartii* acyl-HSL synthase, was obtained from S. Beck von Bodman and used to generate oxohexanoyl-L-[1-<sup>14</sup>C]HSL and hexanoyl-L-[1-<sup>14</sup>C]HSL from L-[1-<sup>14</sup>C]methionine as previously described (34). Briefly, cells were grown in 18 mm tubes in 5 ml of LB containing ampicillin (400 µg · ml<sup>-1</sup>). Isopropyl-β-thiogalactoside (1 mM) was added after 2 h of growth at 37°C. Cells were then harvested by centrifugation when the culture had reached an optical density (600 nm) of 1.0. The cells were resuspended in 2 ml of ampicillin-containing minimal media (65) to which 2 µCi of L-[1-<sup>14</sup>C]methionine (55 mCi · mmol<sup>-1</sup>; American Radiolabeled Chemicals, Inc., St. Louis, Mo.) was added and incubated for an additional 5 h. After removing the cells by centrifugation, the culture fluid was extracted with 2 equal volumes of acetic acid acidified (0.01% v/v) ethyl acetate (55). After evaporating away the ethyl acetate to dryness, the residue was dissolved in 500 µl of 50% methanol (balance water) and loaded onto reversed-phase high-performance liquid chromatography system (Ultrasphere ODS Hypersil 5 µm, 125-

by 4.6-mm Column; Beckman Coulter System Gold). The HPLC system included an in-line  $\beta$ -particle detector ( $\beta$ -RAM; IN/US Systems). The identity of HPLC-purified acyl-HSLs was confirmed using liquid chromatography and atmospheric chemical ionization mass spectrometry (28). The EsaI acyl-HSL synthase, when expressed in *E. coli*, catalyzed the synthesis of nearly equimolar amounts of oxohexanoyl-HSL and hexanoyl-HSL. The specific activities of the purified  $^{14}\text{C}$ -acyl-HSLs were determined by using a combination of bioassay quantification methods (55) and quench-corrected liquid scintillation counting (Beckman Coulter LS 6500). Authentic acyl-HSL standards were obtained from Sigma or were a generous gift from Bernhard Hauer. Acyl-HSLs were stored in acetic acid acidified ethyl acetate at  $-20^\circ\text{C}$  until use.

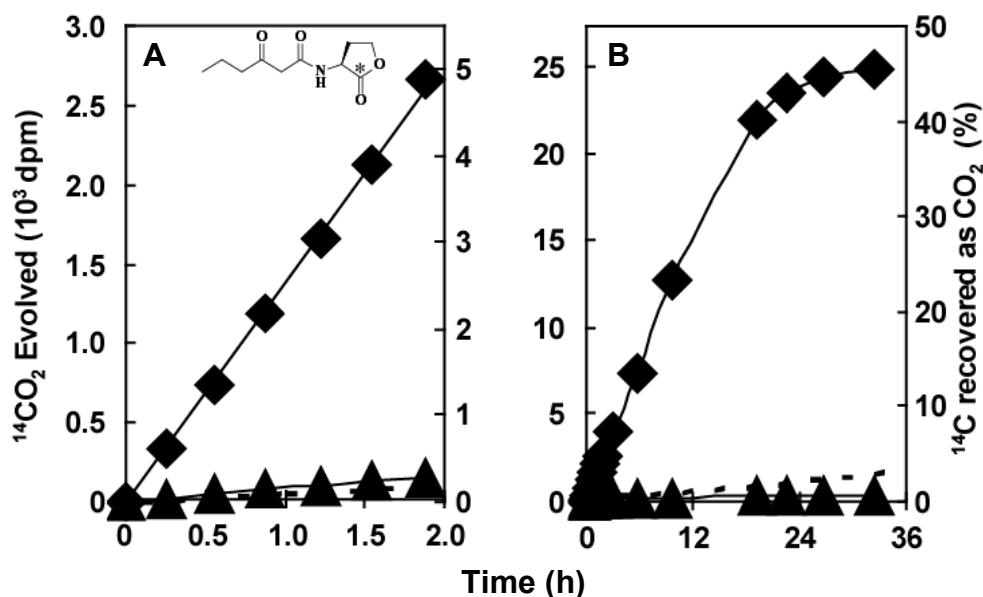
### **3.2.3 Mineralization of $^{14}\text{C}$ -labeled acyl-homoserine lactones and quantification of $^{14}\text{CO}_2$ .**

Purified oxohexanoyl-L-[1- $^{14}\text{C}$ ]HSL and hexanoyl-L-[1- $^{14}\text{C}$ ]HSL were used as substrates in 250 soil slurry reactions. The [ $^{14}\text{C}$ ]acyl-HSL substrate was dispensed from an acetic acid acidified ethyl acetate solution into a sterile, dry 18-mm tube. The ethyl acetate was evaporated away to dryness. Five milliliters of sterile buffer of the appropriate pH was dispensed into each tube. Immediately after this, the amount of radioactivity was measured. Two hundred milligrams of fresh soil was added to each tube. Soil slurries were incubated under once-flow-through aeration;  $^{14}\text{CO}_2$  released from the radiolabeled substrate was collected and quantified by bubbling the reactor outgas through a triple tandem array of phenylethylamine-alkaline traps (4, 34) replaced at every

time point. The rates and yields of  $^{14}\text{CO}_2$  emissions from soil slurries incubated were determined using liquid scintillation counting of radioactivity collected in the  $\text{CO}_2$  traps as previously described (4, 34). Acidification of the reaction fluid was performed at the end of experiment and was not found to release further radioactivity. Assays under each set of conditions were performed at least in duplicate and at  $21^\circ\text{C}$  unless otherwise noted.

The  $\text{CO}_2$  traps at no less than seven time points were collected during the first 2 h of incubation for each of the 250 soil slurry reactions comprising this study (e.g., Fig. 3.2A). Mineralization by 200 reactions was further monitored for several days. All slurries described in this study contained 200 mg fresh weight soil in 5 ml of a buffered salt solution at a pH as noted. For sterilization controls, soils were either autoclaved for 40 min at  $120^\circ\text{C}$  or were  $\gamma$ -irradiated (Mark I-68A  $^{137}\text{Cs}$  Irradiator), receiving  $1.80 \times 10^6$  rad.

When noted, the protein synthesis inhibitors chloramphenicol or cycloheximide was amended to slurries ( $100 \mu\text{g} \cdot \text{ml}^{-1}$ , final concentration) prior to the addition of radiolabel. However, whether such treatments were effective in inhibiting protein synthesis was not determined, and thus the results must be interpreted with some uncertainty. The interpretation of inhibition controls (e.g., examining rates of [ $^3\text{H}$ ]leucine or [ $^{35}\text{S}$ ]methionine incorporation into new proteins) would be complicated in samples such as soils because of their great species richness. Whereas a 99% inhibition (or similar lack thereof) of protein synthesis might be interpreted in a clear-cut manner during a pure-culture study, such would be difficult to interpret in a sample containing thousands of different species. The organisms responsible of signal decay might be among the dozens to hundreds of species accounting for the remaining 1%.



**Figure 3.2. Acyl-HSL mineralization by untreated and heat-inactivated turf soil.** The left and right y axes describe each data point as disintegrations per minute (dpm) and percent recovery, respectively. Dashed lines represent the theoretical amount of  $^{14}\text{CO}_2$  that would be released if the initial degradation step was not biological and governed by a rate-limiting, abiotic hydrolysis of the acyl-HSL lactone ring, with subsequent biological mineralization of the resultant acyl-homoserine product (17, 55, 70). **(A)** Release of  $^{14}\text{CO}_2$  over the initial 2 h of incubation of a representative soil slurry with oxohexanoyl-L-[1- $^{14}\text{C}$ ]HSL at pH 5.5; ( $\blacklozenge$ ), untreated turf soil; ( $\blacktriangle$ ), autoclaved turf soil. An influence similar to that of the autoclaved soil on the degradation activity was observed when the soil had been  $\gamma$ -irradiated (not plotted). **(B)** Mineralization by the same samples over a 34 h incubation period.

### 3.2.4 Interpretation of acyl-HSL degradation rates from CO<sub>2</sub> release

Since only one carbon position in the acyl-HSL molecules synthesized and used in these analyses contained radiolabel, the specific activity of the <sup>14</sup>CO<sub>2</sub> that was released was equivalent to the experimentally determined specific activity of the parent [<sup>14</sup>C]acyl-HSL substrate. The recovery of each mole of <sup>14</sup>CO<sub>2</sub> would (at a minimum) be interpreted as reflecting the inactivation of at least an equimolar amount of acyl-HSL signal. Signal inactivation occurring in soil slurries might have been judged to be more extensive (see below) if any radiolabel had been incorporated into cell material or other biodegradation products, but no attempts were made in this study to perform a complete <sup>14</sup>C inventory.

### 3.2.5 Determination of optimal conditions for acyl-HSL degradation

The pH optimum was determined at an incubation temperature of 21°C in reaction vessels containing 200 mg soil and buffered with 5 ml of 20 mM sodium phosphate at the desired pH. The temperature optimum for acyl-HSL degradation was determined at an incubation pH of 6.0. The temperature variation was measured by monitoring a thermometer inserted into control reaction vessels, and found to be ≤ 1°C. The influence of initial acyl-HSL substrate concentration on signal decay rates was determined at an incubation pH of 6.0 and temperature of 21°C. Radiolabeled acyl-HSLs were diluted with their corresponding, unlabeled acyl-HSL to a specific activity of 0.35 mCi · mmol<sup>-1</sup> for experiments involving elevated concentrations of acyl-HSL. Origin

software (OriginLab) was used for kinetic line fitting and for determining Michaelis-Menten characteristics.

### 3.2.6 Bioassay determination of acyl-HSL inactivation rates in turf soil

Concurrent with measuring the rates of mineralization of oxohexanoyl-L-[1-<sup>14</sup>C]HSL ([<sup>14</sup>C]3OC6HSL) in turf soil, the rates of its disappearance were measured using the bacterial luciferase assay strain (55). For each reaction, 10 g of the same freshly collected turf soil that had been sieved through 2-mm-diameter pores used in the mineralization assays was added to 500 ml of sterile sodium phosphate buffer (20 mM; pH 6.0) in a 2-liter Fernbach flask. For control reactions, either no soil was added or the soil-buffer slurry was autoclaved for 40 min at 120°C and cooled to room temperature prior to initiation of the assay. The reaction mixtures were amended with non-radioactive 3OC6HSL to a final concentration of 1 µM and stirred gently with magnetic stirring bars at 21°C. At each time point, triplicate 1-ml samples were taken from the flasks for subsequent bioassay analysis (46). The overall volume loss due to sampling over the course of the experiment was less than 10% of the initial total volume. Kinetic data were fitted using Microsoft Excel to zero-order or first-order half-life decay kinetics.

Sorption controls were performed specifically to examine the possibility that any observed acyl-HSL disappearance as measured using bioassays might reflect reduced bioavailability or extractability rather than inactivation per se. Purified oxohexanoyl-L-[1-<sup>14</sup>C]HSL was added to 18-mm reaction vessels. After the ethyl acetate was evaporated, 5 ml of sodium phosphate buffer (20 mM; pH 6.0) was added to the tube. Samples were

taken immediately to measure the initial radioactivity by using a scintillation counter. To this reaction, 200 mg of autoclaved soil was added to the buffer, and the reaction tube was shaken for 30 minutes. After this, the soil was separated from the fluid by centrifugation at 13,500 rpm with an Eppendorf model 5415C for 15 minutes. The fluid was extracted with acidified ethyl acetate, and the radioactivity in the various fractions was determined. Under these precise incubation conditions, the sorption of 1  $\mu\text{M}$  3OC6HSL to the turf soil was found to be experimentally insignificant.

### **3.2.7 The most-probable-number (MPN) of soil microbes mineralizing $^{14}\text{C}$ -labeled acyl-HSLs**

For most probable number (MPN) enumeration of acyl-HSL-mineralizing microbes, a suspension of freshly collected turf-grass soil was serially diluted into a MES (morpholineethanesulfonic acid)-buffered (10 mM; pH 5.5) growth medium containing 50 mg of yeast extract liter<sup>-1</sup>, 50  $\mu\text{M}$  glucose, and 50  $\mu\text{M}$  succinate. Two-milliliter aliquots from each dilution were transferred to 18-mm-diameter tubes containing  $102 \pm 2$  nM (final concentration) radiolabeled hexanoyl-L-[1- $^{14}\text{C}$ ]HSL, and tubes were closed with a butyl rubber stopper. Tubes in the series contained from  $2 \times 10^{-1}$  to  $2 \times 10^{-10}$  g fresh soil. The dilution series from the soil sample were performed in triplicate and were allowed to incubate for 18 days prior to analysis of the  $^{14}\text{CO}_2$  released. Cultures were considered positive for acyl-HSL mineralization if  $^{14}\text{CO}_2$  recovery was greater than 25% of that of the initial substrate, and were scored with an MPN table. The MPN values derived here would be abundance underestimates if the microbes responsible for the high



rates of acyl-HSL mineralization observed in soil slurries were either unable to use the energy substrates provided or otherwise unable to thrive in the cultivation medium, which is consistent with what is known regarding many microbial populations (32).

### 3.3 Results

#### 3.3.1 Acyl-HSL mineralization by bulk soil slurries

Upon the introduction of acyl-[1-<sup>14</sup>C]-HSLs into the vast majority of the ca. 250 soil slurry reactions performed in this study, <sup>14</sup>CO<sub>2</sub> was released at a linear rate and without any evident lag. Samples from two sites mineralized the radiolabel after a lag of several hours (discussed below). A representative curve of the initial degradation kinetics is presented in Fig. 3.2A. It has previously been shown that the biological degradation of acyl-homoserines by soil *Arthrobacter* isolate VAI-A (which does not utilize acyl-HSLs) is limited by, and thus does not supersede in rate, the half-life kinetics of the alkaline chemical hydrolysis of the acyl-HSL lactone bond (17). The acyl-HSL degradation observed far outpaced that would have occurred if the initial acyl-HSL inactivation event had been chemical and not biochemical, followed by a biological decomposition of the corresponding acyl-homoserine hydrolysis product (Fig. 3.2A and B, dashed lines). After the initial, linear kinetic phase, mineralization was observed to decelerate to a plateau in all ca. 200 reactions monitored for extended time periods (e.g., Fig. 3.2B). By the time of plateau, at least 33% of the acyl-HSL radiolabel had been recovered as <sup>14</sup>CO<sub>2</sub>. To confirm that an enzymatic (either abiotic or cell-associated) activity was responsible for

acyl-HSL mineralization, soil was autoclaved and cooled prior to the addition of radiolabel (Fig. 3.1A and B), or was  $\gamma$ -irradiated (plot not shown). Release of label as  $^{14}\text{CO}_2$  was markedly diminished by such treatments, decreasing release rates by over 99%, suggesting that the activity was catalyzed by free enzymes or microorganisms.

Soils and pond sediments ranging in pH from 5.1 to 7.3 were sampled from 6 sites across the Caltech campus and were screened for acyl-HSL mineralization. On a weight-for-weight basis, the initial rates of degradation between any of 2 of these sites differed by no greater than a factor of 5 (data not plotted); the fastest degradation was observed in samples collected from a turf-grass soil, which was chosen for characterization in detail. Soil from this site had a pH of 6.6 at the time of collection.

Soils from USDA agricultural plots and KBS-LTER plots were obtained and screened for acyl-HSL mineralization as well (Table 3.1). Most of the sites were observed to exhibit significant mineralization activities without lag. One soil sample, the corn-soybean-wheat rotation soil with standard chemical inputs from KBS-LTER, and the termite gut homogenates exhibited 4- and 6-h lags, respectively. These were the only lags in mineralization observed during the course of this study. From both samples, mineralization proceeded at a linear rate after the lag. Although the reasons are not clear, the results suggest that signal decay might actually be induced by the exogenous acyl-HSL at those sites. The total  $^{14}\text{C}$  recovered as  $\text{CO}_2$  was  $64\% \pm 7\%$  for the soils and  $36\% \pm 1\%$  for the termite guts.

**Table 3.1. Initial rates of release of  $^{14}\text{CO}_2$  from hexanoyl-L-[1- $^{14}\text{C}$ ]HSL by soil communities and other environmental samples.**

Sample Type	Sample pH	Hexanoyl-HSL
		mineralization at pH 6.0 and 32°C (nmol · h <sup>-1</sup> · g of fresh weight <sup>-1</sup> ) <sup>a</sup>
Turf Soil (Calif.)	6.6	13.4 ± 0.9
Spring Wheat Soil, <i>Take-All</i> Suppressive (Wash.)	4.8	4.4 ± 0.5
Spring Wheat Soil, Non-Suppressive (Wash.)	4.9	4.9 ± 0.8
Winter Wheat Soil (Wash.)	5.4	4.9 ± 0.4
Corn-Soybean-Wheat Rotation Soil, standard chemical inputs (Mich.)	5.8	≤0.1 <sup>b</sup>
Corn-Soybean-Wheat Rotation Soil, organic inputs only (Mich.)	5.7	2.2 ± 0.1
Poplar Soil (Mich.)	6.0	2.4 ± 0.3
Alfalfa Soil (Mich.)	6.0	3.7 ± 0.2
Surrogate Native Soil (Mich.)	4.8	2.5 ± 0.4
Late Successional Forest Soil (Mich.)	4.8	2.5 ± 0.1
Early Successional Forest Soil (Mich.)	4.8	2.2 ± 0.3
Conifer Plantation Soil (Mich.)	4.3	2.1 ± 0.1
<i>Zootermopsis angusticollis</i> Termite Guts (Calif.)	~7	≤0.1 <sup>c</sup>

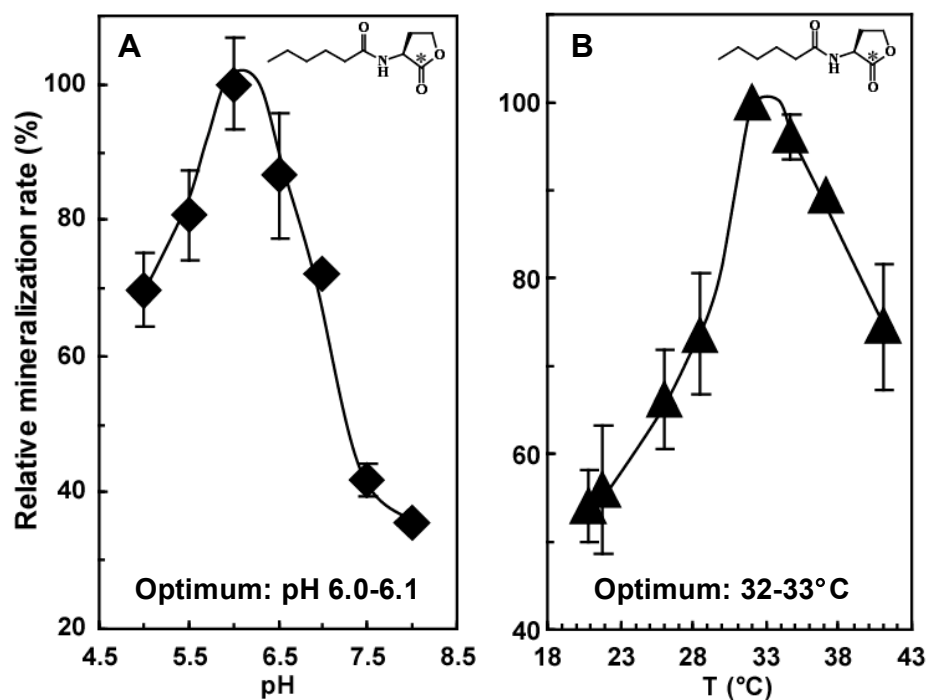
<sup>a</sup> Rates represent linear kinetics observed over the initial 2 h of incubation with 10.3 ± 0.5 μM [ $^{14}\text{C}$ ]C6HSL at 32°C and pH 6.0, conditions found to be optimal for C6HSL mineralization by Caltech turf soil. Reactions were performed at least in duplicate.

<sup>b</sup> After a 4-h lag, mineralization proceeded at a linear rate of 2.0 ± 0.1 nmol · h<sup>-1</sup> · g of fresh weight<sup>-1</sup>.

<sup>c</sup> After a 6-h lag, mineralization proceeded at a linear rate of 10.1 ± 0.3 nmol · h<sup>-1</sup> · g of fresh weight<sup>-1</sup>.

### 3.3.2 Influence of pH and temperature on quorum signal degradation

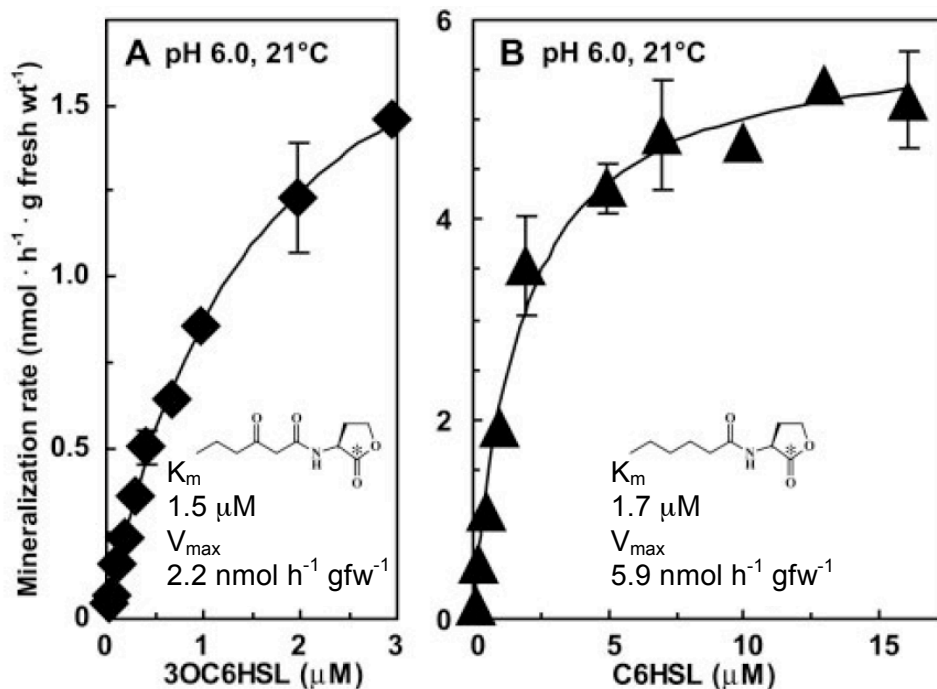
The optimal incubation pH for hexanoyl-HSL mineralization by turf-soil (original pH, 6.6) was determined to be 6.0 (Fig. 3.3A); the slowest  $^{14}\text{CO}_2$ -release rates were observed at pH values of  $\geq 7.5$ , conditions under which the relative contribution of chemical acyl-HSL inactivation events would be expected to increase substantially (70). The optimal incubation temperature for mineralization was determined to be 32°C (Fig. 3.3B), with significant mineralization occurring at the full range of temperatures tested between 21°C and 42°C.



**Figure 3.3. The influence of incubation pH and temperature on hexanoyl-HSL mineralization. (A)** When incubated at 21°C, the maximum rate of mineralization was observed at a pH of 6.0. **(B)** When incubated at pH 6.0, the maximum rate was observed at 32°C. Symbols and error bars denote the average  $\pm$  standard deviation of rates over the initial 2 h of incubation determined from duplicate reactions.

### 3.3.3 Degradation kinetics of two acyl-HSL signals

The acyl-side chains of acyl-HSLs of known, naturally occurring acyl-HSLs range in length from 4 to 16 carbons, may contain a degree of unsaturation, and may be either unmodified or modified with a keto or hydroxyl functional group at carbon position 3 (22). Past laboratory studies have shown that the nature of the side chain can impart markedly different *in vitro* chemical and biochemical stabilities (28, 34, 70). We performed a limited examination of the influence of acyl-side chain structure on acyl-HSL stability by comparing the rates at which hexanoyl-HSL and oxohexanoyl-HSL were degraded by turf-grass soil. Hexanoyl-HSL was observed to be degraded more rapidly than oxohexanoyl-HSL at all substrate concentrations tested. Varying the initial concentrations of both acyl-HSLs markedly influenced their rates of degradation with apparent Michaelis-Menten kinetics (Fig. 3.4A and B). Although hexanoyl-HSL was degraded at nearly three times the rate of oxohexanoyl-HSL, the activities for these acyl-HSLs essentially shared equivalent apparent  $K_m$  half-saturation constants, 1.7 and 1.5  $\mu\text{M}$  acyl-HSL. At the deduced temperature and pH incubation optima of 32°C and pH 6.0, the maximum acyl-HSL degradation rate observed during the course of these studies was  $13.4 \pm 0.9$  nmol of hexanoyl-HSL degraded  $\cdot \text{h}^{-1} \cdot \text{g}$  of fresh weight soil<sup>-1</sup> using 10  $\mu\text{M}$  acyl-HSL as initial substrate ( $n = 3$ ).

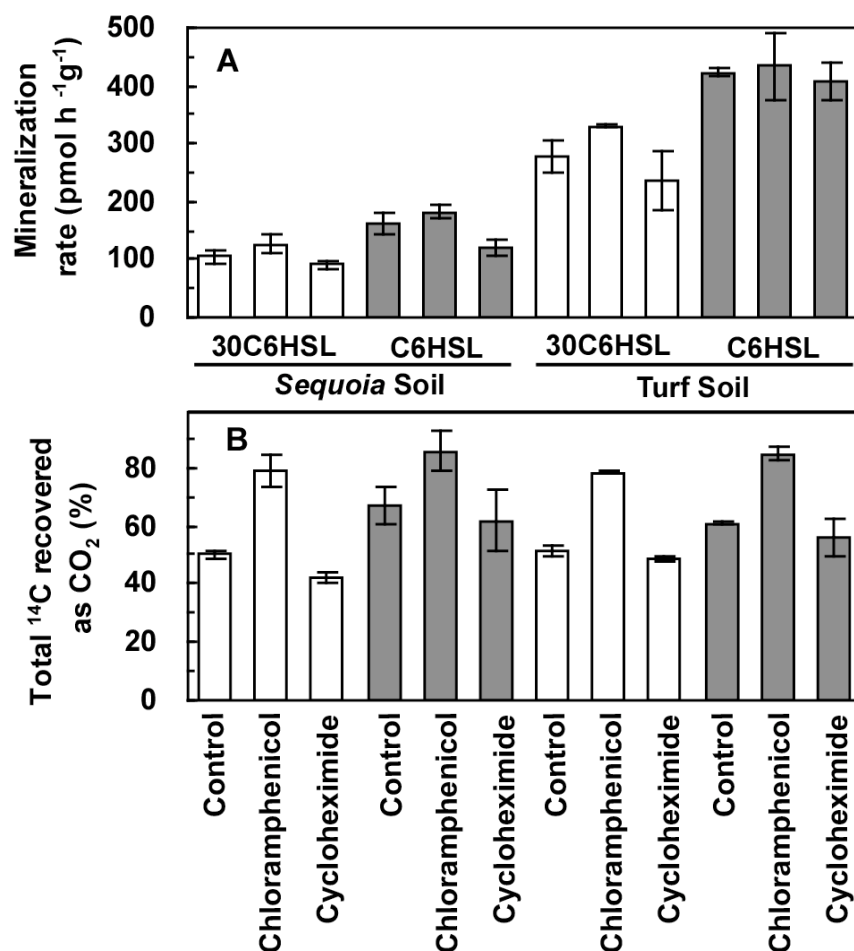


**Figure 3.4. Acyl-HSL mineralization by soil exhibits apparent Michaelis-Menten saturation kinetics.** The influence of the initial concentration of oxohexanoyl-HSL (A) or hexanoyl-HSL (B) on acyl-HSL degradation kinetics at 21°C and pH 6.0. Symbols and error bars denote the average  $\pm$  standard deviation of rates over initial 2 h of incubation determined from duplicate reactions.

### 3.3.4 Influence of protein synthesis inhibitors on acyl-HSL mineralization

That  $^{14}\text{CO}_2$  was released without lag from most of the soils examined suggested that the biochemical machinery involved in acyl-HSL mineralization was already in place at the time those soils had been collected, i.e., that the addition of radiolabel had not served to induce bacterial or fungal microbiota in those particular samples to express the activity during the course of the *ex situ* assay. To explore this issue further, the prokaryotic and eukaryotic protein synthesis inhibitors chloramphenicol and cycloheximide were amended to slurries prior to the addition of radiolabeled oxohexanoyl-HSL or hexanoyl-HSL. Addition of the inhibitors did not abolish the activity. The initial  $^{14}\text{CO}_2$  release rates from chloramphenicol- or cycloheximide-treated slurries were statistically equal between the controls and the antibiotic-treated reactions (Student's *t* test;  $P > 0.01$ ), irrespective of the two soil types and two acyl-HSLs examined (Fig. 3.5A). This observation is consistent with the conclusion that the degradation potential observed in the *Sequoia* and turf soil slurries was an endogenous feature of the soils at the time of their collection. However, whether or not the inhibitors had actually influenced the target populations as intended was not confirmed (for rationale, see Materials and Methods).

In striking contrast, the final extent of degradation was markedly influenced by the addition of one but not the other of the two inhibitors (Fig. 3.5B). Recoveries of  $^{14}\text{CO}_2$  from radiolabeled substrates were significantly stimulated in all reactions to which the bacterial protein synthesis inhibitor chloramphenicol had been added (i.e., *Sequoia* redwood or turf soils amended with either [ $^{14}\text{C}$ ]3OC6HSL or [ $^{14}\text{C}$ ]C6HSL) relative to the



**Figure 3.5. The influence of bacterial and eukaryotic protein synthesis inhibitors on acyl-HSL mineralization by *Sequoia* and turf soils.** Incubation was at 21°C and a pH of 6.0 with an initial concentration of  $293 \pm 27$  nM acyl-HSL. **(A)** Rates over initial 2 h of incubation of acyl-HSL mineralization. **(B)** Final recoveries of acyl-HSL radiolabel as <sup>14</sup>CO<sub>2</sub> after extended incubation.



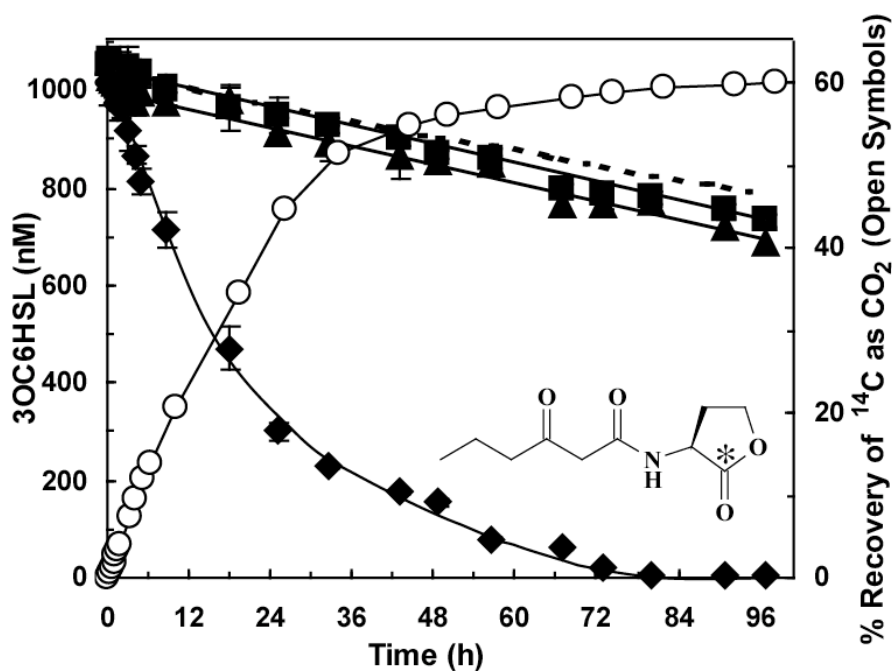
control and cycloheximide treatments (Student's *t* test;  $P > 0.01$ ). This result is not inconsistent with the possibility that bacteria, when treated with chloramphenicol, were redirecting ca. one-fifth of the original acyl-HSL-derived carbon that would normally have been routed to *de novo* protein synthesis pathways to their pre-existing mineralization apparatus. Alternatively, a chloramphenicol-resistant subpopulation with acyl-HSL mineralization capacity might have become free to thrive on this substrate in the absence of competition, thus contributing to the higher recoveries. Such issues are now being investigated further.

### **3.3.5 Comparison of acyl-HSL disappearance with CO<sub>2</sub>-product appearance in turf soil**

That chloramphenicol treatments (see above) stimulated the final extent of radiolabel recovery as <sup>14</sup>CO<sub>2</sub> suggests that there could be other major product(s) generated during acyl-HSL degradation. To examine this possibility further, the rate and extent of disappearance (i.e., inactivation) of biologically active 3OC6HSL was compared concurrently with its recovery as <sup>14</sup>CO<sub>2</sub>. In reactions containing 1 μM initial 3OC6HSL, turf soil was observed to inactivate the signal molecule with similar kinetics as and to be well coupled with its conversion to <sup>14</sup>CO<sub>2</sub>, albeit at 3- to 4-fold greater an initial rate (Fig. 3.6). Heat inactivation of the soil-buffer mixture prior to the addition of the signal, or no addition of soil to the mixture resulted in dramatically slower rates of signal inactivation (Fig. 3.6), well modeled by the reported chemical half-life of this molecule at the given incubation pH (55). Curiously, there was a remarkable difference

observed between the extent of 3OC6HSL inactivation versus the extent of its conversion to  $^{14}\text{CO}_2$ . By hour 96, the amount of biologically active acyl-HSL had decreased to below the 20 picomolar detection limit (i.e., after acyl-HSL extraction, concentration, determination, and subsequent back calculation). By extrapolation, it would take ca. 3 months for chemical inactivation alone to achieve similar results under similar incubation conditions. In contrast to the essential totality with which biological action had inactivated the signal molecule, only 60% was recovered as  $^{14}\text{CO}_2$  (Fig. 3.6). This result provides further (albeit indirect and circumstantial) evidence to suggest that substantial portions of acyl-HSL molecules, when supplied in small amounts that are physiologically relevant to quorum sensing, might be being converted into cell material by bacterial populations active in the soil.

Monitoring for substrate acyl-HSL disappearance versus the appearance of an intermediate or final product, such as  $\text{CO}_2$ , can be complicated by issues of sorption, i.e., if the signal molecule was being tightly bound to soil particles and unavailable for assay rather than being inactivated. The issue of sorption was examined directly in heat-inactivated soil. For this specific line of experiments (i.e., incubation pH and temperature and acyl-HSL:soil:buffer ratio), 3OC6HSL was not observed to sorb to soil strongly ( $\leq 6\%$  sorbed) and thus remained in solution for accurate concentration determinations via bioassay. Similarly, acidification of reaction mixtures at the end of soil slurry experiments (i.e., to convert particulate or dissolved [ $^{14}\text{C}$ ]carbonates to gaseous  $^{14}\text{CO}_2$ ) did not serve to further stimulate the recovery of radiolabel, suggesting that the differences in extent of acyl-HSL disappearance versus its recovery as  $\text{CO}_2$  were real and reflected the formation of at least one other major product, perhaps biomass.



**Figure 3.6. Acyl-HSL disappearance versus <sup>14</sup>CO<sub>2</sub> release.** Shown are acyl-HSL disappearance from freshly collected soil (◆), heat-killed soil (▲), and soil-free (■) reactions, and the release of <sup>14</sup>CO<sub>2</sub> (○) from [<sup>14</sup>C]oxohexanoyl-HSL in samples of the same freshly collected soil. Reactions were performed at pH 6.0 and 21°C using an initial concentration of 1 μM oxohexanoyl-HSL. The viable soil exhibited an initial rate of 3OC6HSL degradation of 2.1 nmol · h<sup>-1</sup> · g of fresh weight<sup>-1</sup> (◆; i.e., by bioassay), and 0.5 nmol · h<sup>-1</sup> · g of fresh weight<sup>-1</sup> (○; i.e., by <sup>14</sup>CO<sub>2</sub>-release). Dashed-lines represent the theoretical chemical degradation of the starting acyl-HSL as a function of its reported half-life, 240 h. Over the full 96-h incubation period, acyl-HSL decrease from both the heat-killed (▲) and soil-free controls (■) exhibited first order exponential decay kinetics, with an apparent half-life of ~185 hours. The endogenous 3OC6HSL content of the soil at the time of its collection was ≤20 pM, the bioassay detection limit after acyl-HSL extraction and concentration.

### 3.3.6 MPN of acyl-HSL-mineralizing microbes

Since the apparent  $K_m$  for acyl-HSL degradation by soils was ca. 1000-fold lower than (i.e., remarkably superior to) the  $K_m$  determined for the purified acyl-HSL lactonase from *Bacillus cereus* (64), and because of the strong circumstantial evidence (see above) that suggests that bacteria might be converting a significant fraction of acyl-HSL decay products into cell material, we wished to begin to examine which not-yet-cultivated organisms capable of metabolizing oligotrophic amounts of carbon might be responsible for the observed soil degradation activities. An MPN enumeration of turf-soil microbes capable of mineralizing low concentrations of hexanoyl-HSL was performed. After 18 days incubation, an MPN of  $2.3 \times 10^7$  heterotrophic cells  $\cdot$  g of fresh weight soil<sup>-1</sup> was observed to develop in the enrichments. Two percent of the cells recovered in dilutions tubes (MPN =  $4.6 \times 10^5$  cells  $\cdot$  g of fresh weight soil<sup>-1</sup>) released  $\geq 25\%$  of the initial 100 nM hexanoyl-[1-<sup>14</sup>C]HSL radiolabel as <sup>14</sup>CO<sub>2</sub> over the same period. Attempts are currently under way to isolate and study the most abundant of the organisms utilizing oligotrophic amounts of acyl-HSLs from these enrichments.

## 3.4 Discussion

The results of this study show that acyl-HSL-inactivating microbes are indeed active in the environment and that physiologically relevant concentrations of quorum signals are subject to a rapid biodegradation in bulk soils. Moreover, the apparent  $K_m$  of the degradation activity for acyl-HSL quorum signals in the soil is ca. 1000-fold lower

than that of a purified acyl-HSL degrading enzyme from *Bacillus cereus* (64), suggesting that acyl-HSL degrading organisms currently available in culture may not be representative sources of the observed soil activity.

### **3.4.1 Possible impact of signal decay on quorum sensing over long and short timescales**

As introduced previously (34), quorum-sensing systems can not be expected to function as monitors of population density over the long term if the signal molecules employed were inherently stable. The population sizes of quorum-sensing bacteria in nature are likely often in flux. A nascent quorum-sensing population or single cell should begin its growth with a slate clean of signals for the regulatory circuit to function properly, i.e., as a reflection of population density. That is, local quorum signal concentrations should illuminate “current events,” as opposed to being a “historical record” of microbial societies long since vanished. Biological signal decay accomplished an effective “system reset” in days that what would have taken chemical inactivation to accomplish over a long season (Fig. 3.2 and 3.6). This clearly would considerably shorten the timescale required for re-equilibration of a site after the dissipation of an active quorum-sensing population.

However, rapid signal decay might also challenge quorum regulated activities in nature, a possibility strongly supported by recent studies performed in defined multi-culture sand mesocosms (43), as well as other studies on “quorum quenching” (12-15, 35, 38, 45, 51, 64). The maximum biological degradation rate observed in this study,  $13.4 \pm$

0.9 nmol C6HSL · h<sup>-1</sup> · g of fresh weight soil<sup>-1</sup>, is ca. 450-fold greater than the initial chemical degradation rate expected under incubation at similar pH, i.e., as predicted by the equation, acyl-HSL  $t_{1/2} = 10^{[7-pH]}$  days (55). For instance, the acyl-HSL chemical half-life is ca. 60 hours in a turf soil at pH 6.6. Clearly, acyl-HSL degradation can greatly outmatch chemical inactivation events in these and similar soils (Table 3.1). Would such high rates be expected to have a meaningful impact on the short-term accumulation of acyl-HSLs by either growing or resting populations of quorum-sensing bacteria in the environment? We have observed that the phytopathogen *Pantoea stewartii* and the opportunistic pathogen *Pseudomonas aeruginosa* synthesize acyl-HSLs at rates no greater than 10<sup>-18</sup> moles · cell<sup>-1</sup> · h<sup>-1</sup>, i.e., when grown optimally *in vitro* (unpublished data). Although acyl-HSL synthesis rates are not readily accessible from other published reports, this rate appears to be reasonably representative of the observed upper limits for a variety of quorum-sensing bacteria (14, 28, 38, 51, 71). Moreover, many known acyl-HSL-producing bacteria require a local accumulation of acyl-HSLs to reach (depending on the species in question) levels from 5 nM to 2 μM for a quorum response to occur *in vitro* (2, 20, 46), whereas the biological activity revealed here can consume acyl-HSLs to levels of less than 20 pM, i.e., > 100-fold less than is sensed by the keenest of the known quorum-sensing bacteria. Assuming a well mixed system, acyl-HSL-producing bacteria could be considerably challenged to outpace biological acyl-HSL degradation, becoming hampered in their ability to engage in cell-cell communications until reaching population densities of greater than 10<sup>9</sup> or 10<sup>10</sup> cells · g of soil<sup>-1</sup>. Such high population densities would indeed be quite a high cell load for a single species living in a soil, as soils

typically contain  $<10^{11}$  total microbial cells  $\cdot$  g of soil<sup>-1</sup> representing thousands of different species.

However, soils are not well-mixed systems, and their water content can be extremely variable. Bacteria typically grow as microcolonies in soils. The results of this study do not indicate whether cell-associated or abiotic signal hydrolases infiltrate acyl-HSL-producing aggregates. Little is known of the natural distribution of quorum-sensing bacteria in soils. However, infiltration of acyl-HSL producing aggregates by signal-degrading organisms or enzymes might not be absolutely necessary for this activity to have an impact on quorum regulated events. Quorum signals are known to diffuse readily through biomass (29, 47), and signal binding by LuxR-type transcriptional activators is known to be a reversible process in some but not other quorum-sensing bacteria (62). Strong signal decay and signal sinks directly adjacent to acyl-HSL producing populations have previously been modeled and predicted to influence signal accumulations within biofilms, increasing the effective population size required to engage in quorum-sensing (8).

### **3.4.2 Signal degradation could serve to insulate microbial aggregates**

The possibility that beneficial or deleterious “cross talk” between spatially separated microbial populations (even those belonging to different species) might occur in nature has previously been suggested (3, 37, 41, 48). The proximity of acyl-HSL mineralization activities to nascent or well-developed aggregates of quorum-sensing bacteria in soil is not yet known, but signal degradation might be expected to serve to

insulate (for better or worse) such populations from signal molecules produced by cells elsewhere, disrupting both *intra*- and *inter*-species communications that might otherwise occur between spatially separated microcolonies. Disruption of quorum-sensing by signal degrading bacteria has already been demonstrated to occur in simple plant mesocosms seeded with defined microbial co-cultures left unmixed after inoculation (43). Insulation of microbial aggregates from extraneous signals thus could preserve and even accentuate the spatial and chemical heterogeneity and the biological microstructure of complex microbial ecosystems. The quorum-sensing systems of soil bacteria likely have evolved and continue to evolve in the context of a significant challenge by signal decay (33), and may already be tuned to meet it. Clearly, biological signal degradation should be taken into account during the design and interpretation of studies aimed at reaching a better understanding of microbial cell-cell communications and quorum-sensing controlled processes in species-rich environments such as soils.

### **3.4.3 Future directions**

A recent review has raised the teleological question of why and for what intended evolutionary function have enzymes capable of signal decay evolved (53). Certainly, approaching with clarity the design and interpretation of experiments aimed at revealing “why” an activity occurs is significantly more challenging than establishing that or how it occurs (31). This challenge notwithstanding, we hope to continue to better answer some of the provocative questions that have been raised about biological acyl-HSL degradation. It is not at all beyond reason that abiotic (no longer cell-associated) soil



hydrolases of broad substrate specificity might be responsible for several of the activities revealed in this study (10, 30), especially those present in samples wherein linear kinetics commenced without lag and appeared insensitive to protein synthesis inhibitors. While the activity of abiotic enzymes would yield the same expected potential impacts on quorum-sensing populations, it would be difficult to invoke that the activities of such enzymes necessarily benefit the cells that had at one time produced them. The activity could essentially be accidental.

However, this is not to say that the enzymes are certainly abiotic. In samples from two sites examined (Table 3.1), signal decay progressed only after an initial lag of several hours and was not likely incidental. If *de novo* protein synthesis was required in those samples, then abiotic enzymes alone would not likely account for signal decay. Signal decay might actually be a direct response to, i.e., induced by exposure to acyl-HSLs at those sites, suggesting that the activity is regulated and presumably of some benefit to the cells in being so. Finally, the results of the MPN cultivation experiments performed in this study suggest that it may be achievable to isolate pure cultures of microbes catalyzing the low  $K_m$  activities revealed in this study, cells perhaps capable of reaping some definite and definable nutritional reward during their metabolism of trace levels of an intriguing class of biological molecules. Their cultivation and identification would open the door to future experimental lines, including the use of oligonucleotide-based fluorescence *in situ* hybridization techniques to map the distribution of signal producing and degrading microbes in soils and other environments.

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### **3.5 References**

1. **Ang, S., Y. T. Horng, J. C. Shu, P. C. Soo, J. H. Liu, W. C. Yi, H. C. Lai, K. T. Luh, S. W. Ho, and S. Swift.** 2001. The role of RsmA in the regulation of swarming motility in *Serratia marcescens*. *J. Biomed. Sci.* **8**:160-169.
2. **Beck von Bodman, S., D. R. Majerczak, and D. L. Coplin.** 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. *Proc. Natl. Acad. Sci. U.S.A.* **95**:7687-7692.
3. **Blosser, R. S., and K. M. Gray.** 2000. Extraction of violacein from *Chromobacterium violaceum* provides a new quantitative bioassay for N-acyl homoserine lactone autoinducers. *J. Microbiol. Methods* **40**:47-55.

4. **Brune, A., E. Miambi, and J. A. Breznak.** 1995. Roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites. *Appl. Environ. Microbiol.* **61**:2688-2695.
5. **Carter, M. R. (ed.).** 2000. Soil sampling and methods of analysis. CRC Press, Boca Raton, Fla.
6. **Cha, C., P. Gao, Y. C. Chen, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol. Plant-Microbe Interact.* **11**:1119-1129.
7. **Chernin, L. S., M. K. Winson, J. M. Thompson, S. Haran, B. W. Bycroft, I. Chet, P. Williams, and G. S. Stewart.** 1998. Chitinolytic activity in *Chromobacterium violaceum*: substrate analysis and regulation by quorum sensing. *J. Bacteriol.* **180**:4435-4441.
8. **Chopp, D. L., M. J. Kirisits, B. Moran, and M. R. Parsek.** 2003. The dependence of quorum sensing on the depth of a growing biofilm. *Bull. Math. Biol.* **65**:1053-1079.
9. **Christensen, A. B., K. Riedel, L. Eberl, L. R. Flodgaard, S. Molin, L. Gram, and M. Givskov.** 2003. Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. *Microbiology* **149**:471-483.
10. **Conrad, R.** 1996. Soil microorganisms as controllers of atmospheric trace gases (H<sub>2</sub>, CO, CH<sub>4</sub>, N<sub>2</sub>O, NO). *Microbiol. Rev.* **60**:609-640.
11. **De Kievit, T., P. C. Seed, J. Nezezon, L. Passador, and B. H. Iglewski.** 1999. RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. *J. Bacteriol.* **181**:2175-2184.

12. **Dong, Y.-H., A. R. Gusti, Q. Zhang, J.-L. Xu, and L.-H. Zhang.** 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. *Appl. Environ. Microbiol.* **68**:1754-9.
13. **Dong, Y.-H., L.-H. Wang, J.-L. Xu, H.-B. Zhang, X.-F. Zhang, and L.-H. Zhang.** 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**:813-817.
14. **Dong, Y.-H., J.-L. Xu, X.-Z. Li, and L.-H. Zhang.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. U.S.A.* **97**:3526-31.
15. **Dong, Y.-H., X.-F. Zhang, J.-L. Xu, and L.-H. Zhang.** 2004. Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. *Appl. Environ. Microbiol.* **70**:954-60.
16. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Neilson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry-US* **20**:2444-2449.
17. **Flagan, S., W.-K. Ching, and J. R. Leadbetter.** 2003. *Arthrobacter* strain VAI-A utilizes acyl-homoserine lactone inactivation products and stimulates quorum signal biodegradation by *Variovorax paradoxus*. *Appl. Environ. Microbiol.* **69**:909-916.
18. **Flavier, A. B., L. M. Ganova-Raeva, M. A. Schell, and T. P. Denny.** 1997. Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *J. Bacteriol.* **179**:7089-7097.

19. **Fray, R. G., J. P. Throup, M. Daykin, A. Wallace, P. Williams, G. S. Stewart, and D. Grierson.** 1999. Plants genetically modified to produce N-acylhomoserine lactones communicate with bacteria. *Nat. Biotechnol.* **17**:1017-20.
20. **Fuqua, C., M. Burbea, and S. C. Winans.** 1995. Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the traM gene. *J. Bacteriol.* **177**:1367-1373.
21. **Fuqua, C., and E. P. Greenberg.** 2002. Listening in on bacteria: acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell Biol.* **3**:685-695.
22. **Fuqua, C., and E. P. Greenberg.** 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Curr. Opin. Microbiol.* **1**:183-189.
23. **Fuqua, C., M. R. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**:439-68.
24. **Fuqua, W. C., and S. C. Winans.** 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J. Bacteriol.* **176**:2796-2806.
25. **Gray, K. M., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1994. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. *J. Bacteriol.* **176**:3076-3080.
26. **Hamdan, H., D. M. Weller, and L. S. Thomashow.** 1991. Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces*

- graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79 and M4-80R. Appl. Environ. Microbiol. **57**:3270-3277.
27. **Horng, Y. T., S. C. Deng, M. Daykin, P. C. Soo, J. R. Wei, K. T. Luh, S. W. Ho, S. Swift, H. C. Lai, and P. Williams.** 2002. The LuxR family protein SpnR functions as a negative regulator of N-acylhomoserine lactone-dependent quorum sensing in *Serratia marcescens*. Mol. Microbiol. **45**:1655-71.
28. **Huang, J. J., J.-I. Han, L.-H. Zhang, and J. R. Leadbetter.** 2003. Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. Appl. Environ. Microbiol. **69**:5941-5949.
29. **Kaplan, H. B., and E. P. Greenberg.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J. Bacteriol. **163**:1210-1214.
30. **Klose, S., and M. A. Tabatabai.** 2002. Response of amidohydrolases in soils to chloroform fumigation. J. Plant Nutr. Soil Sci. **165**:125-132.
31. **Lander, A. D.** 2004. A calculus of purpose. PLoS. Biol. **2**:712-4.
32. **Leadbetter, J. R.** 2003. Cultivation of recalcitrant microbes: cells are alive, well, and revealing their secrets in the 21st century laboratory. Curr. Opin. Microbiol. **6**:274-281.
33. **Leadbetter, J. R.** 2001. News and Views: plant microbiology - quieting the raucous crowd. Nature **411**:748-749.
34. **Leadbetter, J. R., and E. P. Greenberg.** 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J. Bacteriol. **182**:6921-6.

35. **Lee, S. J., S. Y. Park, J. J. Lee, D. Y. Yum, B. T. Koo, and J. K. Lee.** 2002. Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **68**:3919-24.
36. **Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: Identification of the LuxRI homologs CepRI. *J. Bacteriol.* **181**:748-756.
37. **Lewenza, S., M. B. Visser, and P. A. Sokol.** 2002. Interspecies communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*. *Can. J. Microbiol.* **48**:707-16.
38. **Lin, Y.-H., J.-L. Xu, J. Hu, L.-H. Wang, S. L. Ong, J. R. Leadbetter, and L.-H. Zhang.** 2003. Acyl-homoserine lactone acylase from *Ralstonia* str. XJ12B represents a novel and potent class of quorum quenching enzymes. *Mol. Microbiol.* **47**:849-860.
39. **Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl. Environ. Microbiol.* **58**:2616-2624.
40. **McClellan, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. Stewart, and P. Williams.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* **143**:3703-3711.

41. **McDougald, D., S. Srinivasan, S. A. Rice, and S. Kjelleberg.** 2003. Signal-mediated cross-talk regulates stress adaptation in *Vibrio* species. *Microbiology* **149**:1923-33.
42. **McGowan, S. J., M. T. Holden, B. W. Bycroft, and G. P. Salmond.** 1999. Molecular genetics of carbapenem antibiotic biosynthesis. *Antonie Van Leeuwenhoek* **75**:135-141.
43. **Molina, L., F. Constantinescu, L. Michel, C. Reimann, B. Duffy, and G. Defago.** 2003. Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. *FEMS Microbiol. Ecol.* **45**:71-81.
44. **Nealson, K. H., T. Platt, and J. W. Hastings.** 1970. Cellular control of the synthesis and activity of the bacterial luminescence system. *J. Bacteriol.* **104**:313-322.
45. **Park, S.-Y., S.-J. Lee, T.-K. Oh, J.-W. Oh, B.-T. Koo, D.-Y. Yum, and J.-K. Lee.** 2003. AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. *Microbiology* **149**:1541-50.
46. **Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* **92**:1490-1494.
47. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**:1203-1210.



48. **Pierson, E. A., D. W. Wood, J. A. Cannon, F. M. Blachere, and L. S. Pierson.** 1998. Interspecies signaling via N-acyl-homoserine lactones among bacteria in the wheat rhizosphere. *Mol. Plant-Microbe Interact.* **11**:1078-1084.
49. **Pierson, L. S., D. W. Wood, and E. A. Pierson.** 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. *Annu. Rev. Phytopathol.* **36**:207-225.
50. **Puskas, A., E. P. Greenberg, S. Kaplan, and A. L. Schaefer.** 1997. A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. *J. Bacteriol.* **179**:7530-7537.
51. **Reimmann, C., N. Ginet, L. Michel, C. Keel, P. Michaux, V. Krishnapillai, M. Zala, K. Heurlier, K. Triandafillu, H. Harms, G. Defago, and D. Haas.** 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology* **148**:923-32.
52. **Riedel, K., T. Ohnesorg, K. A. Krogfelt, T. S. Hansen, K. Omori, M. Givskov, and L. Eberl.** 2001. N-acyl-L-homoserine lactone-mediated regulation of the lip secretion system in *Serratia liquefaciens* MG1. *J. Bacteriol.* **183**:1805-9.
53. **Roche, D. M., J. T. Byers, D. S. Smith, F. G. Glansdorp, D. R. Spring, and M. Welch.** 2004. Communications blackout? Do N-acylhomoserine-lactone-degrading enzymes have any role in quorum sensing? *Microbiology* **150**:2023-8.
54. **Rodelas, B., J. K. Lithgow, F. Wisniewski-Dye, A. Hardman, A. Wilkinson, A. Economou, P. Williams, and J. A. Downie.** 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. *viciae*. *J. Bacteriol.* **181**:3816-3823.

55. **Schaefer, A. L., B. L. Hanzelka, M. R. Parsek, and E. P. Greenberg.** 2000. Detection, purification and structural elucidation of acylhomoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Meth. Enzymol.* **305**:288-301.
56. **Schaefer, A. L., T. A. Taylor, J. T. Beatty, and E. P. Greenberg.** 2002. Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *J. Bacteriol.* **184**:6515-21.
57. **Schripsema, J., K. E. E. d. Rudder, R. B. v. Vliet, P. P. Lankhorst, E. d. Vroom, J. W. Kijne, and A. A. N. v. Brussel.** 1996. Bacteriocin *small* of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-homoserine lactone molecules, known as autoinducers, and as quorum sensing co-transcription factors. *J. Bacteriol.* **178**:366-371.
58. **Stead, P., B. A. Rudd, H. Bradshaw, D. Noble, and M. J. Dawson.** 1996. Induction of phenazine biosynthesis in cultures of *Pseudomonas aeruginosa* by L-N-(3-oxohexanoyl)homoserine lactone. *FEMS Microbiol. Lett.* **140**:15-22.
59. **Swift, S., J. A. Downie, N. A. Whitehead, A. M. L. Barnard, G. P. C. Salmond, and P. Williams.** 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology, *Adv. Microbial. Physiol.* **45**:199-270.
60. **Thomashow, L. S., and D. M. Weller.** 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J. Bacteriol.* **170**:3499-3508.

61. **Thomson, N. R., M. A. Crow, S. J. McGowan, A. Cox, and G. P. Salmond.** 2000. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Mol. Microbiol.* **36**:539-56.
62. **Urbanowski, M. L., C. P. Lostroh, and E. P. Greenberg.** 2004. Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J. Bacteriol.* **186**:631-7.
63. **Uroz, S., C. D'Angelo-Picard, A. Carlier, M. Elasri, C. Sicot, A. Petit, P. Oger, D. Faure, and Y. Dessaux.** 2003. Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology* **149**:1981-9.
64. **Wang, L. H., L. X. Weng, Y. H. Dong, and L. H. Zhang.** 2004. Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase. *J. Biol. Chem.* **279**:13645-51.
65. **Watson, W. T., F. V. Murphy, T. A. Gould, P. Jambeck, D. L. Val, J. E. Cronan, S. B. von Bodman, and M. E. A. Churchill.** 2001. Crystallization and rhenium MAD phasing of the acyl-homoserine lactone synthase EsaI. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **57**:1945-1949.
66. **Winans, S. C., and B. L. Bassler.** 2002. Mob psychology. *J. Bacteriol.* **184**:873-83.
67. **Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L. S. Pierson, 3rd.** 1997. N-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J. Bacteriol.* **179**:7663-7670.

68. **Wood, D. W., and L. S. Pierson, 3rd.** 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**:49-53.
69. **Yang, W.-W., Han, J. H., and Leadbetter, J. R.** 2006. Utilization of homoserine lactone as a sole source of carbon and energy by soil *Arthrobacter* and *Burkholderia* species. *Arch. Microbiol.* **185**: 47-54.
70. **Yates, E. A., B. Philipp, C. Buckley, S. Atkinson, S. R. Chhabra, R. E. Sockett, M. Goldner, Y. Dessaux, M. Camara, H. Smith, and P. Williams.** 2002. N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect. Immun.* **70**:5635-46.
71. **Zhang, H.-B., L.-H. Wang, and L.-H. Zhang.** 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U.S.A.* **99**:4638-4643.
72. **Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate.** 1993. *Agrobacterium* conjugation and gene regulation by N-acyl-L-homoserine lactones. *Nature* **362**:446-448.

## **Chapter 4**

# **Biofilm-Reactor Based Enrichment of Bacteria Utilizing Oligotrophic Concentrations of Acyl-homoserine Lactone Quorum Sensing Signal Molecules**

## 4.1 Introduction

In Chapter 3, we demonstrated that many soil microbial communities are poised to inactivate and mineralize physiological amount of acyl-HSLs rapidly. These results provide insights into the stability of acyl-HSLs in natural environments, as well as further experimental directions.

Firstly, the apparent  $K_m$  of soil degradation activity for these signal molecules was 1,000 times lower than (i.e., remarkably superior to) that exhibited by a purified acyl-HSL-degrading enzyme from *Bacillus cereus* (51), which suggests that acyl-HSL-degrading organisms currently available in culture may not be representative sources of the observed soil activity.

Secondly, two pieces of evidence suggest that bacteria might be converting a significant fraction of acyl-HSL decay products into cell material. Remarkable difference was observed between the extent of acyl-HSL inactivation (based on bioassay) and its conversion to  $^{14}\text{CO}_2$  (based on radiolabeling approach at the same time). Over an extended period of incubation, the amount of biologically active acyl-HSL decreased to below 20 pM. In contrast to such essential totality with which biological action had inactivated the signal molecule, only 60% was recovered as  $^{14}\text{CO}_2$ , suggesting that there could be at least one other major product generated during acyl-HSL degradation, perhaps biomass. Consistent with this hypothesis, when the bacterial protein synthesis inhibitor chloramphenicol was added to soil slurries, recoveries of  $^{14}\text{CO}_2$  from radiolabeled acyl-HSL substrates were significantly stimulated in all reactions tested relative to the controls. This result supported the possibility that bacteria, when treated

with chloramphenicol, were redirecting ca. one-fifth of the original acyl-HSL-derived carbon that would normally have been routed to *de novo* protein synthesis pathways to their pre-existing mineralization apparatus. These two pieces of evidence (albeit indirect and circumstantial) suggest that substantial portions of acyl-HSL molecules, when supplied in small amounts that are physiologically relevant to quorum sensing, might be converted into cell material by bacterial populations active in the soil.

Thirdly, based on an MPN enumeration of turf-soil microbes in a medium that contained carbon sources on the order of  $10^2$   $\mu$ M, an MPN of  $2.3 \times 10^7$  heterotrophic cells  $\cdot$  g of fresh weight soil<sup>-1</sup> was observed to grow in the enrichments. Among them, 2% of the cells were able to significantly mineralize acyl-HSL, i.e., releasing  $\geq 25\%$  of the initial 100 nM acyl-HSL radiolabel as <sup>14</sup>CO<sub>2</sub> over the same period. The results suggest that it may be achievable to isolate pure cultures of microbes catalyzing the low  $K_m$  activities exhibited by soil communities.

The above results and rationale prompted us to perform enrichments for microbes exhibiting the low  $K_m$  activity observed in soils, to examine which not-yet-cultivated organisms capable of metabolizing oligotrophic amounts of carbon might be responsible for the observed soil degradation activities.

Here we have taken the approach of employing once-flow-through biofilm reactors using a dilute, mineral basal medium containing oligotrophic amount of acyl-HSL. The concept is that if a sessile population of oligotrophic cells can access fairly low concentrations of growth nutrients in the medium flowing past them, they will in principle grow their biomass over time. Therefore, it became possible to further isolate bacteria capable of degrading oligotrophic amount of acyl-HSLs.

## 4.2 Materials and methods

### 4.2.1 Bacterial strains and defined medium composition

The bacterial strains used (isolation described below) were *Variovorax* strains SOD31 and SOD32, *Mesorhizobium* strains SOD33 and SOD35, *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36. A defined medium was used for both the biofilm reactors and growth of bacterial isolates. One liter of the defined medium consists of 10 mg of NaCl, 5 mg of KCl, 4 mg of MgCl<sub>2</sub> · 6H<sub>2</sub>O, 1 mg of CaCl<sub>2</sub> · 2H<sub>2</sub>O, 10 μM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0) (2), 0.1 μM NH<sub>4</sub>Cl, 0.1 μM Na<sub>2</sub>SO<sub>4</sub>, and 26.6 μg of HCl-dissolved trace elements (22). This basal medium was autoclaved (30 min at 121°C unless noted), and after cooling, filter-sterilized vitamins were added. The final vitamin composition of 1-liter medium was 0.1 μg of riboflavin and 1 μg each of the following: L-ascorbic acid, D-calcium-pantothenate, folic acid, niacinamide, nicotinic acid, 4-aminobenzoic acid, pyridoxal-HCl, thiamine-HCl, lipoic acid, biotin and cyanocobalamin. Then 100 μl of sterile soil extract was added to 1-liter of the autoclaved, vitamin-amended medium.

Soil extract, which is commonly used as a nonselective medium for soil bacteria (2, 17), was obtained as the following. Bulk soil was freshly sampled from the upper 5 cm of well-watered and well-fertilized turf grass in front of Beckman Auditorium on the Caltech campus, and passed through a 2-mm-pore-size sieve after collection. Five hundred grams of such sieved soil was added to 500 ml of distilled water in a 2-liter glass



flask that had been autoclaved. The flask was then shaken for 24 h at 30°C, put into a 60°C water bath for another 24 h, and autoclaved for 2 h. One gram of freshly sampled and sieved soil, handled in the same way as above, was added to the flask and it was shaken for 24 h at 30°C and autoclaved for 1 h. The suspension was then paper-filtered (VWR International, Cat. No. 28331-060) and 315 ml fluid was obtained. The pH of the fluid was circumneutral and adjusted to 6.0 with 0.1 M HCl. Finally it was autoclaved to sterilize. The final extract was dark-brown colored and contained soil organic matter, mostly humic substances and other nonhumic substances, e.g., polysaccharides, proteins, sugars, amino acids, and other small molecules (17).

Hexanoyl-HSL (C6HSL) was dispensed from an acetic acid-acidified ethyl acetate solution into a dry 18-mm diameter glass tube. Ethyl acetate was evaporated away to dryness. Five milliliters of the basal medium was dispensed into the tube to dissolve the hexanoyl-HSL. Afterwards, this 5 ml of hexanoyl-HSL containing defined medium was filter sterilized through a 0.2 µm-pore filter (Pall Life Sciences Corp., Cat. No. PN4454) and provided to the biofilm reactors as specified (see below) to a final concentration of 1.5 µM.

For cultivation on solid media of acyl-HSL agarose plates, 10 g l<sup>-1</sup> of agarose (Fisher Scientific, Cat. No. ICN820721) was amended to the basal medium and the rest of procedure was the same as above. For the diluted yeast extract plates, 0.5 g l<sup>-1</sup> yeast extract (Becton, Dickinson and Co., Cat. No. 212750) and 10 g l<sup>-1</sup> agar (Becton, Dickinson and Co., Cat. No. 214230) were dissolved in water, autoclaved and spread onto plates.

#### 4.2.2 Description of biofilm reactors

One set of the biofilm reactors consists of eight reactors, representing four different conditions with each condition duplicated (Fig. 4.1 and Table 4.1). Four 4-liter medium bottles were provided and each bottle led to two duplicate reactors. The C6HSL was provided to two of the medium bottles and thus four of the reactors, two duplicates of which were inoculated with microorganisms being investigated, and the other two duplicates of which served as control. The other two medium bottles, and thus the other four reactors, were not provided with C6HSL, two duplicates of which were inoculated with microorganisms being investigated for comparison. Based on weight, each column was filled with ca.  $1.74 \times 10^4$  beads; total surface area was  $0.062 \text{ m}^2$  and available volume was 9.8 ml.

For the two medium bottles containing C6HSL, instead of using the silicone venting closure (P1), a size 7 rubber stopper (Cole-Parmer Instrument Co., Cat. No. C-62991-22) was inserted tightly, with a sterile 21G needle (P25) pierced through and a  $0.2 \text{ }\mu\text{m}$ -pore filter (P26) securely fitted to balance air pressure and keep the medium sterile. The purpose was to compensate chemical degradation of acyl-HSL. Based on the theoretical half life of acyl-HSL, i.e.,  $t_{1/2} = 10^{[7-\text{pH}]}$  days (8), acyl-HSL degrades ca. 15% every 60 h at pH 6.0 and thus supplementary acyl-HSL needed to be added through the filter, which was replaced afterwards.

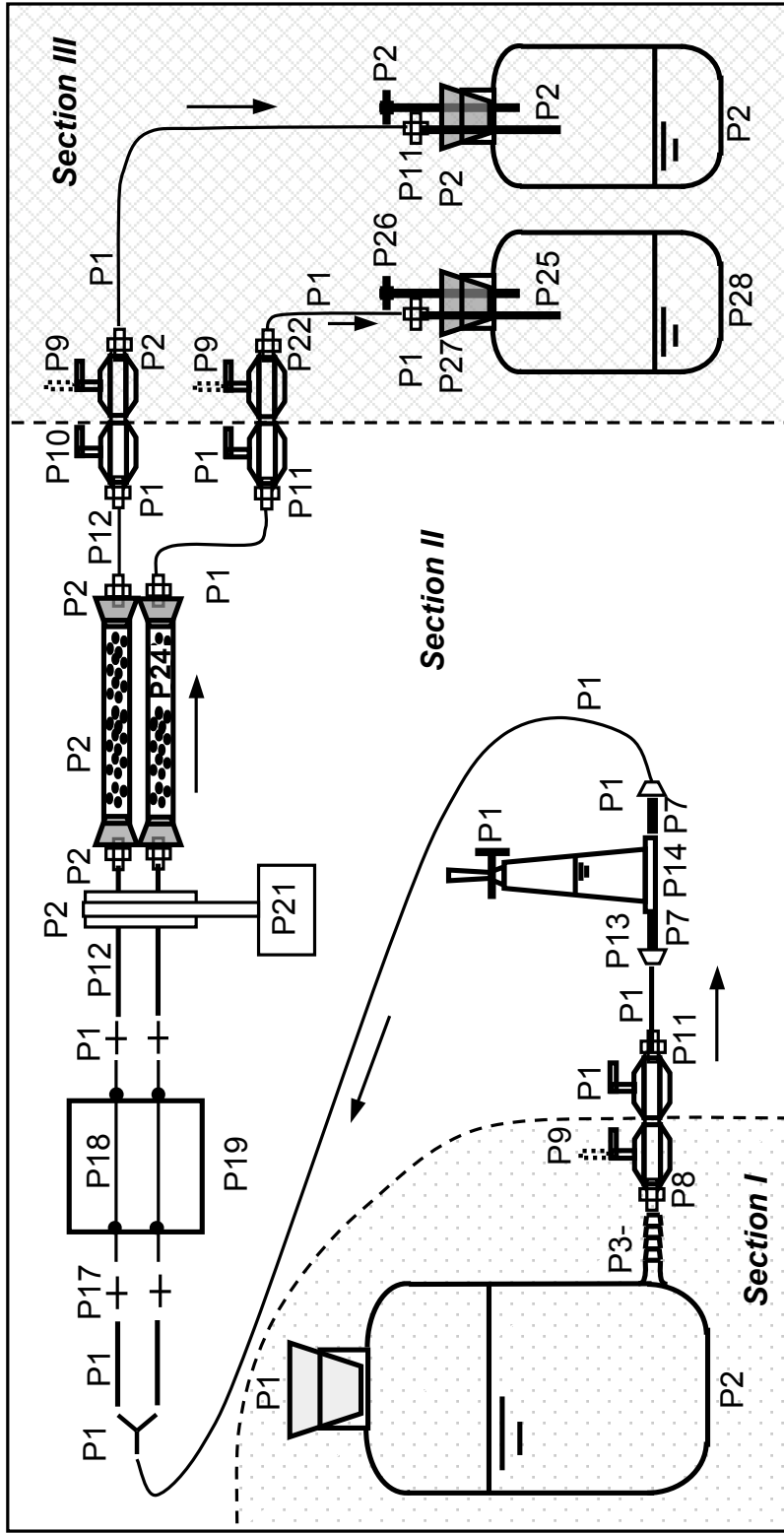
After assembly, the whole system was autoclaved to sterilize, except the following.

- (i) The electronic equipments (P19, P20 and P21) were excluded that had no direct contact with the medium.
- (ii) The venting closures (P1) and rubber stoppers (P27) were

autoclaved separately and applied after cooling, while the bottles (P2 and P28) were capped with aluminum foil during autoclaving because the venting closures and rubber stoppers tended to expand much less than the glass bottles at high temperature and thus be drawn into the bottle after cooling. (iii) The needles (P25) and syringe filters (P15 and P26) were self-sterile and were inserted after cooling, while P14 in section II and P11 in section III were wrapped with aluminum foil during autoclaving.

The soil inoculum used in this study was freshly sampled and sieved through 2-mm-pore-size sieve in the same way as the soil extract was prepared (see above) from the turf grass that was previously shown capable of degrading acyl-HSL (52). One gram of such sieved soil was added to 20 ml of the defined medium in a 50 ml sterile centrifuge tube and vortexed (VWR International Mini Vortexer, VM-3000) for a few minutes. Then 5 ml of the suspension was added directly into each of the four reactors (P23) described above. The system was kept quiescent for 1 h before the flow started.

Section II was kept intact during the whole incubation period except for replacement of the filter (P15) periodically. Section I was disassembled when the medium was drained close to the tubulation (P2) and a new set was readily replaced. So was section III when the outflow collecting bottles (P28) were full, following the same autoclaving procedure described above.



**Figure 4.1. Biofilm reactors for enrichment of bacteria capable of degrading physiological amount of acyl-HSL under oligotrophic conditions.** Each system comprises eight reactors, representing four different conditions with each condition duplicated. Shown are two reactors as duplicates originated from one medium bottle. Acyl-HSL is provided to four of the reactors, two of which are inoculated with microorganisms being investigated. The other four reactors are not supplied with acyl-HSL, two of which are inoculated with microorganisms being investigated. Refer to Table 4.1 for part description.

**Table 4.1. Biofilm reactor part description. Refer to Fig. 4.1 for assembling.**

Part	Description	Vendor	Catalogue No.
P1	Sterile silicone venting closure	Cole-Parmer Instrument Co.	C-06299-00
P2	Single neck 4-liter glass bottle with drian tubulation	Tudor Scientific Glass	TG-2285-105
P3	Platinum silicone tubing, 3/8" ID	Cole-Parmer Instrument Co.	C-95802-18
P4	Reducing conneter, 3/8" x 1/4"	Cole-Parmer Instrument Co.	C-30703-54
P5	Platinum silicone tubing, 1/4" ID	Cole-Parmer Instrument Co.	C-95802-12
P6	Reducing connector, 1/4" x 1/8"	Cole-Parmer Instrument Co.	C-30703-50
P7	Platinum silicone tubing, 1/8" ID	Cole-Parmer Instrument Co.	C-95802-04
P8	Female luer x 1/8" hose barb	Cole-Parmer Instrument Co.	C-06359-37
P9	Three-way stopcock, female luer x male luer lock	Cole-Parmer Instrument Co.	C-31507-12
P10	One-way stopcock, female luer x male luer lock	Cole-Parmer Instrument Co.	C-31507-01
P11	Male luer x 1/16" hose barb	Cole-Parmer Instrument Co.	C-06359-09
P12	Silicone tubing, 1/16" ID	Saint-Gobain Plastics Corp.	ABW00002
P13	Reducing connector, 1/8" x 1/16"	Cole-Parmer Instrument Co.	C-30622-23
P14	Autoclavable 4-place bubble trap	BioSurface Technologies Corp.	FC 34
P15	Sterile 13 mm syringe filter, 0.2 µm-pore	Pall Life Sciences Corp.	PN4454
P16	Three-way Y connector, 1/16"	Cole-Parmer Instrument Co.	C-30703-90
P17	Straight connector 1/16"	Cole-Parmer Instrument Co.	C-30622-49
P18	Two stop silicone pump tubes, 1.65 mm ID	Cole-Parmer Instrument Co.	C-07616-38
P19	High-precision multi-channel pump	Watson-Marlow Pumps	205U
P20	Aluminium fitting	Customized at Caltech	NA
P21	Heating strip	Barnstead Thermolyne	CN45515
P22	Female luer x 1/16" hose barb	Cole-Parmer Instrument Co.	C-06359-27
P23	Liquid chromatography column with luer lock, 1.5 cm x 10 cm	Sigma-Aldrich	C4169-5EA
P24	1.0 mm Zirconia/Silica beads	BioSpec Products, Inc.	11079110z
P25	Sterile 21G needle, 1 1/2" long	Becton-Dickinson Corp.	305167
P26	Sterile 25 mm syringe filter, 0.2 µm-pore	Fisher Scientific	09-719C
P27	Rubber stopper, size 6 1/2	Cole-Parmer Instrument Co.	C-62991-20
P28	Two-liter glass bottle	VWR International	89000-242

### 4.2.3 Acyl-HSL concentration measurement

One milliliter of sample from each reactor was taken every day through the steel 3-way stopcocks (P9 in both section I and section III), which were flamed to sterilize every time before and after sampling. Each sample was centrifuged in a tabletop centrifuge (Eppendorf 5415C) for 2 min at 13,000 rpm and the supernatant was transferred to a 2-ml microcentrifuge tube and amended with acetic acid (0.1% v/v) and stored at -20°C. Acyl-HSL concentrations in the samples, which represented what were in the medium as well as outflows, were measured by using liquid chromatography and atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS, Hewlett Packard 1100 Series) every week following a modified procedure previously described (14). The analyses were performed at Caltech's Environmental Analysis Center. Briefly, 250  $\mu$ l of each sample was put into a 2 ml LC/MS glass vial (Hewlett Packard, Cat. No. 5182-0546) with snap on cap (Hewlett Packard, Cat. No. 5182-0542), mixed with the same volume of methanol, and loaded onto the LC/APCI-MS. The column used was a C8 reverse phase column (Alltech Associates, Inc., Part No. 72568, 150 mm  $\times$  4.6 mm). The eluent was methanol and water (acidified with acetic acid, 0.1% v/v) at a constant ratio of 75:25 (v/v) over a 6-minute run. The flow rate was 1 ml/min. Authentic acyl-HSL standards were obtained from Sigma or were a generous gift from Bernhard Hauer. Standards over a range of 100 nM to 5  $\mu$ M were prepared each time the samples were measured.

Acyl-HSL consumption rate ( $R$ , nmoles  $\text{h}^{-1} \text{ml}^{-1}$ ) through the reactor is calculated as the following:

$$R = (C_i - C_o) \times f / V$$

where  $C_i$  is acyl-HSL concentration in the medium ( $\mu\text{M}$ ),  $C_o$  is acyl-HSL concentration in the outflow ( $\mu\text{M}$ ),  $f$  is the flow rate ( $\text{ml h}^{-1}$ ) and  $V$  is available reactor volume, which is 9.8 ml.

#### **4.2.4 Bacterial isolation from the biofilm**

At the end of the incubation of the biofilm reactors, each column was disconnected from the system and its content was transferred into a 50-ml sterile centrifuge tube and vortexed for a few minutes. Then 3 ml of the top layer liquid suspension was transferred into another 15-ml sterile centrifuge tube. The column content was washed three times afterwards, i.e., each time 3 ml of fresh defined medium was added into the same 50-ml centrifuge tube, it was vortexed for a few minutes and then 3 ml of the top layer liquid suspension was transferred into the 15-ml centrifuge tube and combined with previous transfers. At the end, the combined liquid suspension was concentrated by centrifugation at 13,000 rpm for 3 min for a final volume of 3 ml. One hundred microliter of such prepared suspension was made 10-fold serial dilutions and the left was used for DNA extraction (see below). Then 100  $\mu\text{l}$  of each dilution was spread onto freshly made solid acyl-HSL agarose plates and incubated at 30°C for 10 days.

Based on colonial morphology (e.g., shape, appearance, size, elevation, margin type, pigmentation), 6 bacterial strains were identified. Pure strains were obtained by

repeated streaking on freshly made solid acyl-HSL agarose plates. Colonies were also streaked onto the diluted yeast extract agar plates for phenotype comparison. For prolonged maintenance of the strains, liquid cultures in the acyl-HSL-containing defined medium was amended with 10% (v/v) DMSO (dimethyl sulfoxide, Sigma-Aldrich, Co.) and kept frozen at -80°C.

#### **4.2.5 DNA extraction**

The processed content from each column was washed another three times following the same procedure as above, except that each time after adding 3 ml of fresh defined medium, it was sonicated for 30 min in order to enhance detachment of the biofilm from the silica beads surface. The obtained liquid was combined with the above-prepared suspension that was left after being made dilutions for bacterial isolation. The liquid was concentrated by centrifugation at 13,000 rpm for 3 min in a 2 ml microcentrifuge tube. The supernatant was discarded and DNA was extracted from the pellet (39). The following reagents were added to the microcentrifuge tube: 0.5 g of 0.1 mm zirconium/silica beads (Biospec Products, Inc.), 100 µl of 5% (w/v) polyvinylpolypyrrolidone in 120 mM sodium phosphate at pH 8.0 (2), 50 µl of 20% (w/v) SDS (sodium dodecyl sulfate) and 500 µl Tris-equilibrated phenol at pH 8.0. The sample was disrupted by three cycles of bead beating at high speed for 30 seconds (MiniBeadbeater-8, Biospec Products, Inc.) and incubation in ice-water bath for another 30 seconds. Afterwards, the sample was centrifuged at 14,000 rpm for 1 min; 400 µl of the top aqueous layer was carefully transferred to a fresh 1.5 ml microcentrifuge tube,



avoiding cell debris and phenol, and DNA was extracted using the DNeasy extraction kit (QIAGEN, Inc., Valencia, Calif.) following the manufacturer's recommendations.

DNA from pure bacterial isolates was obtained from overnight cultures in  $0.5 \text{ g l}^{-1}$  yeast extract liquid medium using DNeasy extraction kit as well.

DNA samples were quantified by using a DyNA Quant 200 fluorometer and Hoechst dye 33258, as described by the manufacturer (Hoefer Pharmacia Inc., San Francisco, Calif.) and stored at  $-20^{\circ}\text{C}$  until used as templates for polymerase chain reaction (PCR, see below).

#### **4.2.6 Nucleotide sequence analysis of 16S ribosomal DNA from biofilm communities and bacterial isolates**

The nucleotide sequences of PCR-amplified fragments of the 16S ribosomal DNA (rDNA) of both the biofilm communities and bacterial isolates were determined using a modification of previously described techniques (22, 39).

For each sample of the biofilm communities,  $2 \mu\text{l}$  of diluted template DNA (in TE buffer, ca.  $0.1 \text{ ng}$ ) was added to  $18 \mu\text{l}$  of PCR solution containing  $10 \mu\text{l}$  FailSafe™ 2× PCR PreMix D (Epicentre Technologies),  $0.6 \mu\text{l}$  each of the two universal bacterial primers ( $20 \mu\text{M}$ , QIAGEN, Inc., Valencia, Calif.), 27-forward [ $5'$ -AGA GTT TGA TCC TGG CTC AG- $3'$ ] and 1492-reverse [ $5'$ -GGT TAC CTT GTT ACG ACT T- $3'$ ] primers (21),  $0.6 \mu\text{l}$  of expand high fidelity *Taq* polymerase (Roche Diagnostics) and  $6.2 \mu\text{l}$  of sterile water (Fluka Chemical Corp.). PCR amplification of community 16S rDNA was performed in a thermal cycler (Mastercycler, Eppendorf Scientific, Inc.) and consisted of

the following schedule: 94°C for 2 min followed by 25 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, and finally 72°C for 10 min. PCR products were ligated into the pCR4-TOPO cloning vector with the TOPO TA cloning kit (Cat. No. K4575-01, Invitrogen, San Diego, Calif.). Ninety-six *Escherichia coli* colonies carrying the insert-containing plasmids were picked and re-streaked onto LB plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Using a 96-well PCR plate (Fisher Scientific, Cat. No. 05-500-63) with each well containing 25 µl of TE buffer, the 96 colonies were picked again and transferred into the wells and mixed by swirling. The PCR plate was sealed with sterile Mylar plate sealer (MP Biomedicals) and kept at -20°C for further sequencing, after 1 µl of cell suspension from each well was transferred to another 96-well PCR plate (second plate) for restriction fragment length polymorphisms (RFLP) analysis. The PCR solution was added to each well of the second PCR plate, which consisted of the following reagents: 10 µl FailSafe™ 2× PCR PreMix H, 1 µl each of the two standard T3 and T7 primers (10 µM, QIAGEN, Inc., Valencia, Calif.), 0.2 µl of *Taq* polymerase (New England Biolabs, Inc.) and 6.8 µl of sterile water for a total of 20 µl reaction. PCR amplification consisted of the following schedule: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2.5 min, and finally 72°C for 10 min. After double digestion with *MspI* and *RsaI* enzymes following the manufacturer's recommendations (New England Biolabs, Inc.), RFLP pattern was visualized via gel electrophoresis using 2% (w/v) low-melting-point agarose (Fisher Scientific, Cat. No. ICN820721) and analyzed manually. As noted in the text, each library consisted of the valid clones that the expected ca. 1.4 kb PCR products and complete digestion were obtained. Based on RFLP results, most abundant clone types

were sequenced as noted in the text. The PCR amplification for sequencing was the same as for RFLP analysis except that expand high fidelity *Taq* polymerase (Roche Diagnostics) was used.

For each bacterial isolate, 16S rDNA was PCR-amplified and cloned into competent *E. coli* cells following the same procedure as above, except that ca. 10 ng of bacterial DNA was initially used as template. Then the insert-containing plasmids were obtained from transformed *E. coli* cells by using a QIAprep Spin Miniprep Kit (QIAGEN, Inc., Valencia, Calif.).

Sequencing was performed by Laragen, Inc., (West Los Angeles, Calif.) using T3 and T7 primers and primers previously designed to target internal regions of the 16S rDNA genes of most bacteria (21), 533-forward [5'-GTG CCA GC(A/C) GCC GCG GTA A-3'] and 1100-reverse [5'-AGG GTT GCG CTC GTT G-3']. Sequences were read, edited and assembled using DNASTAR Lasergene programs. Chimera check was performed at the Ribosomal Database Project-II release 8.1 (Center for Microbial Ecology at Michigan State University, Mich.) and Bellerophon (16). The ARB freeware package (<http://www.arb-home.de/>) was used for multiple sequence alignments and phylogenetic analysis (31). The 16S rRNA-based phylogenetic tree was constructed using Tree-Puzzle 5.0 maximum-likelihood analysis (40, 45) with 10,000 puzzling-steps, and laid out in Treeview (33). Sequences obtained by using NCBI BLAST and the RDP-II sequence match function were selected in tree construction on the basis of their close similarity or close clustering with the clones and isolates.

#### 4.2.7 Growth studies

Growth studies of bacterial isolates of SOD31 through SOD36 were performed by culturing the bacteria in the defined medium containing appropriate amounts of hexanoyl-HSL. At each hexanoyl-HSL concentration examined, including zero which served as the control, duplicate cultures of 50 ml each were incubated in 125-ml screw-capped flasks at 30°C being shaken. At each time point, 100 µl of sample from each culture was taken to measure cell density, which was calculated from viable cell counts by plating samples from 10-fold serial dilutions of cultures onto duplicate diluted yeast extract plates. During the course of incubation, volume loss from sampling was less than 5% of initial total volume.

#### 4.2.8 Other analysis

Microscopic examinations were performed by using a Zeiss Stemmi 2000 stereomicroscope (low magnification) and a Zeiss Axioplan research microscope (higher magnification; phase-contrast microscopy).

#### 4.2.9 Nucleotide accession numbers

The GenBank accession numbers for the sequences generated in this study are: *Variovorax* strain SOD31, EF125930; *Variovorax* strain SOD32, EF125931; *Mesorhizobium* strain SOD33, EF125932; *Bradyrhizobium* strain SOD34, EF125933;

*Mesorhizobium* strain SOD35, EF125934; *Labrys* strain SOD36, EF125935; Clone A1, EF125936; Clone A2, EF125937; Clone A3, EF125938; Clone A4, EF125939; Clone A5, EF125940; Clone A6, EF125941; Clone A7, EF125942; Clone A8, EF125943; Clone A9, EF125944; Clone A10, EF125945; Clone A11, EF125946; Clone A12, EF125947; Clone B1, EF125948; Clone B2, EF125949; Clone B3, EF125950; Clone B4, EF125951; Clone B5, EF125952; Clone B6, EF125953; Clone B7, EF125954; Clone B8, EF125955; Clone B9, EF125956.

The GenBank accession numbers for other 16S rDNA sequences used in the construction of Fig. 4.5 are: *Variovorax paradoxus* VAI-C, AF250030; *Pelomonas saccharophila*, AF396932; *Azoarcus tolulyticus*, L33688; *Dokdonella fugitiva*, AJ969432; *Mesorhizobium loti*, AY509218; *Labrys methylaminiphilus*, DQ337554; *Bradyrhizobium japonicum*, AF208508; *Methylobacterium organophilum*, D32226; *Arthrobacter pascens*, AJ576068; *Nakamurella multipartita*, Y08541; *Gemmatimonas aurantiaca*, AB072735; *Acidobacterium* str. Ellin342, AF498724; *Gemmata obscuriglobus*, X54522; *Hongiella* sp., AY576756; *Sphingoterrabacterium koreensis*, AB267721; *Prototheca zopfii*, X74006.

### **4.3 Results and discussion**

#### **4.3.1 Acyl-HSL consumption through biofilm reactors inoculated with fresh soil sample**

Four reactor conditions were examined. The two variables were (i) inoculation (or not) of the otherwise sterile reactor system with a soil suspension and (ii) addition (or not) to the basal media with acyl-HSL to a final concentration of 1.5  $\mu\text{M}$ .

For the four reactors to which acyl-HSL was supplied, acyl-HSL consumption rates, which might vary at different growth stages and flow rates, were obtained by quantifying the acyl-HSL concentrations through the reactors using LC/MS during the course. The two uninoculated control reactors showed no signs of acyl-HSL degradation, at the end of which acyl-HSL concentrations maintained at ca. 1.5  $\mu\text{M}$  (Fig. 4.2A). In contrast, the two soil-inoculated biofilm reactors were observed to markedly decrease acyl-HSL concentration in the effluent (Fig. 4.2A and B).

Initially, 5 ml of soil suspension that contained ca. 200 mg soil was added into the reactor and the flow started after 1 h. Right at the beginning of the flow, acyl-HSL was consumed remarkably, with an acyl-HSL consumption rate of 0.7  $\text{nmoles h}^{-1} \text{ ml}^{-1}$  through the reactor. The flow rate at the early stage of ca. 7 days was chosen relatively high and acyl-HSL consumption kept dropping, which was probably because a portion of the soil community could not retain on the surface inside the column and was washed out.

After 10 days, the acyl-HSL concentration in the effluent was below 100 nM that was the detection limit of LC/MS and thus acyl-HSL was over 95% consumed. Such nearly total consumption of acyl-HSL was maintained during the following 20 days despite increase of the flow rate. The maximum acyl-HSL consumption rate happened at 40 d with a flow rate of 8.7  $\text{ml h}^{-1}$ . Before that, the fast acyl-HSL consumption increase stage was reasonable that the biofilm was growing since more nutrients were supplied along with increase of the flow rate. After that, although the flow rate increased, the acyl-

HSL consumption rate dropped. That was probably because the biofilm's ability to attach to the surface was weakened by the faster flow and more cells were washed out. During the last stage after 56 days, acyl-HSL consumption followed the same trend as the flow rate.

#### **4.3.2. Isolation of bacteria from the biofilm**

During the ca. 3 month incubation of biofilm reactors, among the four soil-inoculated reactors, greater density of biomass was observed where acyl-HSL containing medium was employed compared to the otherwise acyl-HSL-absent reactor. Complex microbial communities were observed by phase-contrast microscopy, including a variety of bacterial and eukaryotic microbes. At the end of incubation, samples of the liquid suspension, including a variety of 10-fold serial dilutions, were spread onto acyl-HSL agarose plates. No microorganisms were recovered from the uninoculated reactors, no matter acyl-HSL was supplied or not to the reactors. On the contrast, several colony types appeared from soil-inoculated reactors.

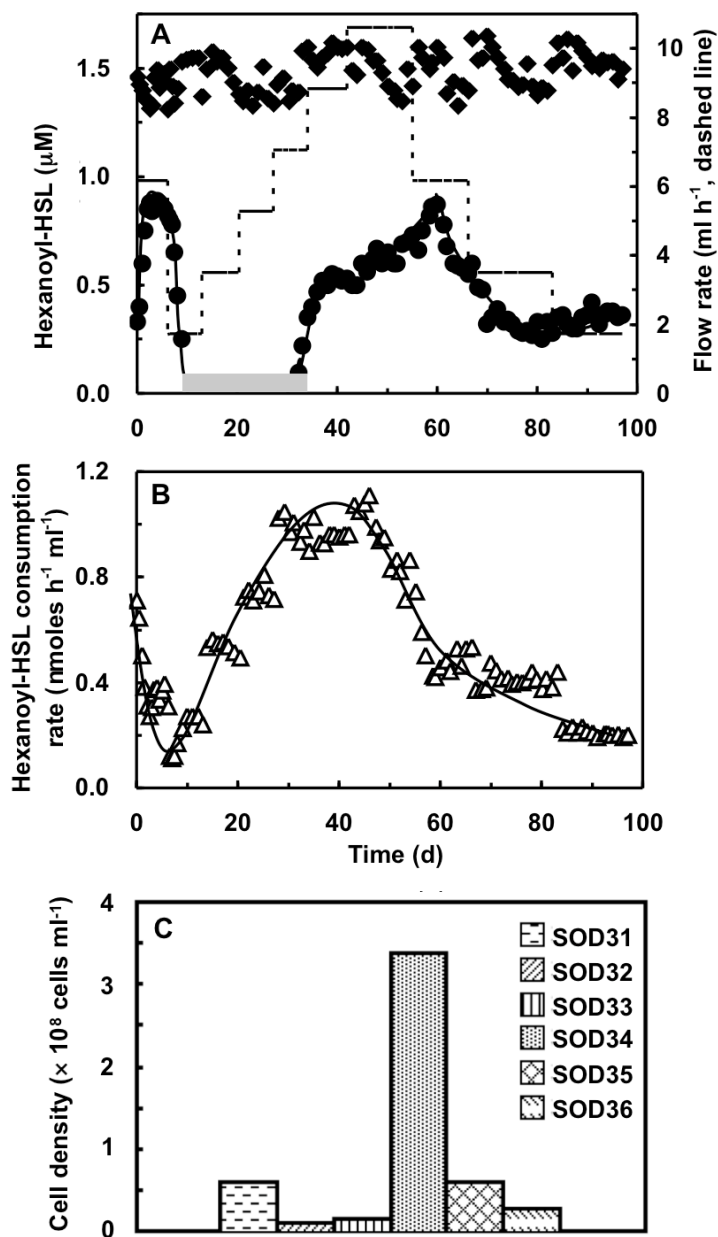
Six pure bacterial strains were isolated by repeated streaking on acyl-HSL agarose plates and were designated Soil Oligotrophic Degradors as SOD31 through SOD36. Their relative abundance in the biofilm suspension from a representative inoculated and acyl-HSL-supplied reactor is shown in Fig. 4.2C, from which the total cell density was more than 10-fold higher than what recovered from a representative inoculated but acyl-HSL-absent reactor ( $4.1 \times 10^7$  cells ml<sup>-1</sup> recovered). SOD34 was the most abundant bacterium. All of the six isolates were able to grow in the low nutrient

defined medium containing 1.5  $\mu\text{M}$  acyl-HSL, as well as rich media of 0.5  $\text{g l}^{-1}$  yeast extract or LB media, so that they are facultative oligotrophs (20, 43, 44, 50).

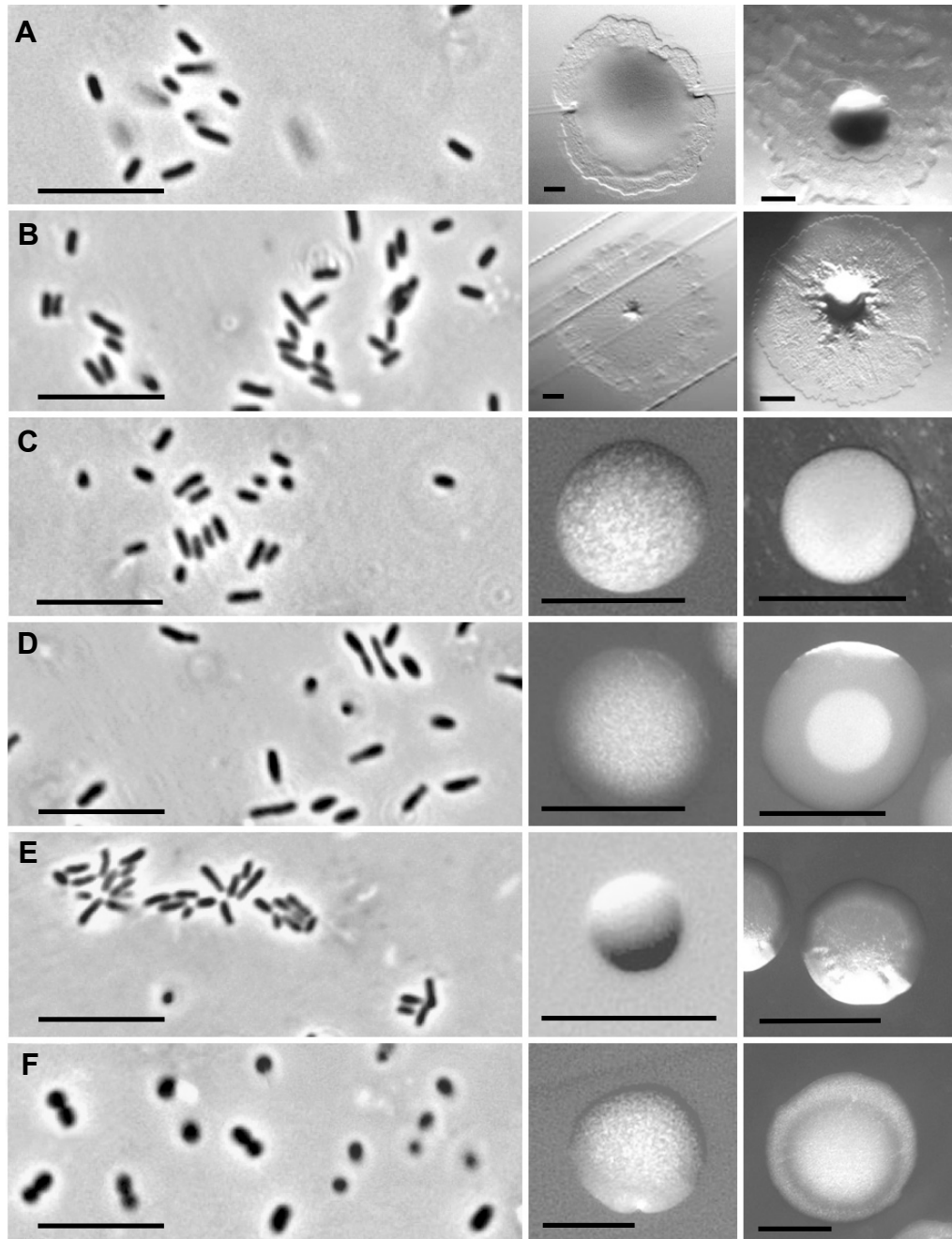
Cell and colonial morphology of the six isolates are shown in Fig. 4.3. Growing cells of all the six bacteria were observed to be motile albeit to different extent. Cells of four bacteria were bacillus. Among them, SOD31 cells were the largest, measuring  $1.8 \times 0.8 \mu\text{m}$  in dimension and SOD32 cells were slightly smaller; SOD33 and SOD35 cells were remarkably smaller, measuring  $1.2 \times 0.8 \mu\text{m}$  and  $1.0 \times 0.6 \mu\text{m}$  respectively in dimension. SOD34 cells were coccobacilli and measured  $1.5 \times 0.9 \mu\text{m}$  in dimension. SOD36 cells were coccus or diplococcus that were ca.  $1.2 \mu\text{m}$  in diameter.

They formed pale white to light brown colonies on acyl-HSL agarose plates and diluted yeast extract plates. None of the isolates had pigment. Colonies of SOD31 and SOD32 were umbonate and significantly larger than the other four isolates. They both had spreading edge, while SOD31 had much smoother central dome than SOD32. SOD33 raised creamy white, thick and smooth colonies on both plates. Colonies of SOD34 were flat, smooth and edged; the microscopic color changed distinctly from light brown in the center to gray at the edge, which was much wider on diluted yeast extract plate than on acyl-HSL agarose plate. SOD35 was the smallest one; colonies on acyl-HSL agarose plate had a convex raised surface and smooth, entire and rounded margin, while colonies on diluted yeast extract plate were dry and wrinkled. SOD36 colonies were white and smooth with regular or slightly undulated margin, and formed a semi-transparent circle inside the colony on the diluted yeast extract plate.





**Figure 4.2. Hexanoyl-HSL consumption through representative reactors inoculated with fresh soil samples. (A)** Data show the hexanoyl-HSL concentration, measured by LC/MS, at the end of uninoculated ( $\blacklozenge$ ) and inoculated ( $\bullet$ ) reactors. During the incubation period, hexanoyl-HSL level in the medium reservoirs were maintained at  $1.5 \pm 0.1 \mu\text{M}$ . The dashed line shows the variation of the flow. Shaded box highlights where hexanoyl-HSL concentration was below the LC/MS detection limit of 100 nM. **(B)** Hexanoyl-HSL consumption rates through the inoculated reactor. **(C)** Relative abundance of bacterial isolates SOD31 through SOD36 recovered from the biofilm of the inoculated reactor (to which acyl-HSL was supplied) at the end of incubation with a total of  $5.1 \times 10^8 \text{ cells ml}^{-1}$  recovered. The cell abundance ratio of SOD31 through SOD36 is ca. 10 : 2 : 3 : 56 : 10 : 4.



**Figure 4.3 Cell and colonial morphology of bacterial isolates SOD31 through SOD36 (A through F).** Left panels are phase-contrast micrographs of growing cells; bars are 10  $\mu\text{m}$ . Middle panels are photographs of colonies grown on acyl-HSL agarose plates; bars are 0.2 mm. Right panels are photographs of colonies on diluted yeast extract agar plates; bars are 0.5 mm.

### 4.3.3 Phylogeny analysis of 16S rDNA from the bacterial isolates and the biofilms

DNAs were purified from two representative soil-inoculated reactors that acyl-HSL was amended to one but not the other of them. The 16S rDNA amplification reactions were performed using the universal bacterial primer set. PCR inhibition was observed with higher concentrations of DNA template possibly due to humic substances (17) that contained in the defined medium and retained inside the reactor column. Humic substances are ubiquitous in soil and water which are a mixture of complex polyphenolics produced during the decomposition of organic matter and have been found inhibiting PCR reactions (19, 46, 54). The DNA samples had to be highly diluted, i.e., 0.1 ng DNA as template per 10  $\mu$ l of PCR reaction. PCR reaction successfully yielded products of the anticipated ca. 1.4 kb size. These were ligated into cloning vehicles and transformed into *Escherichia coli* cells. From them, two libraries of 86 and 89 insert-containing plasmid clones were collected respectively. After sorting of the libraries by comparing RFLP patterns, the rank abundances of the DNA types recovered in the two libraries are presented in Fig. 4.4.

The most abundant clones from each library were targeted for sequencing and further analyses. Sequences of the 16S rDNAs of the bacterial isolates of SOD31 through SOD36 were obtained as well. Genera to which these strains or clones belong were first suggested through web-based similarity searching against the GenBank and Ribosomal Database Project databases, and further supported by phylogenetic analysis. A Tree-

Puzzle likelihood method was employed for the construction of the phylogram presented in Fig. 4.5.

All the six isolates belong to the *Proteobacteria* phylum. SOD31 and SOD32 are *Variovorax* strains, belonging to the  $\beta$ -*Proteobacteria* class, and the 16S rDNA of them are over 97% identical with a soil isolate of *V. paradoxus* VAI-C, from which the acyl-HSL biodegradation mechanism by acyl-HSL acylases was first discovered (23). The bacterium could grow on the full spectrum of saturated acyl-HSLs tested as sole energy source and a linear relationship was observed between the molar growth yields on those molecules and their acyl chain length. The other 4 isolates belong to the  $\alpha$ -*Proteobacteria* class. SOD33 and SOD35 are *Mesorhizobium* strains and their 16S rDNA are 99% identical. SOD34 is a *Bradyrhizobium* strain and SOD36 is a *Labrys* strain.

Bacteria in the genera *Mesorhizobium* and *Bradyrhizobium*, as well as *Rhizobium*, *Allorhizobium*, *Azorhizobium* and *Sinorhizobium* have the ability to establish nitrogen-fixing root- or stem-nodule symbioses with leguminous plants (30, 42). Quorum sensing has been implicated to be important for the symbiotic interaction between these bacteria and their respective host plants (9), e.g., maintenance of viability, exopolysaccharides synthesis, plasmid transfer and nodulation (3, 11, 13, 28, 29, 32, 38, 55). Of particular interest, several *Bradyrhizobium* sp. strains have been shown to be involved in acyl-HSL-like quorum sensing mechanism (29, 36); nevertheless, a unique signal of *bradyoxetin* (2-{4-[[4-(3-aminooxetan-2-yl)phenyl](imino)methyl]phenyl} oxetan-3-ylamine) has been shown to mediate population-density-dependent control of nodulation gene expression in *B. japonicum* (27, 28). Recently, a LuxI/LuxR-type quorum sensing system has been identified in *Mesorhizobium tianshanense* that controls symbiotic nodulation (57).

As to the different clone types from the biofilm reactors, clone A1 through clone A12 were from the soil-inoculated reactor where acyl-HSL was supplied in the medium, while clone B1 through clone B9 were from the otherwise acyl-HSL-absent reactor. Greater species diversity was observed in biofilm reactors where acyl-HSL containing medium was employed. Clone A1 through clone A12 distributed among 5 different phyla, i.e., *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Acidobacteria* and *Bacteroidetes*, with a majority belonging to *Proteobacteria*, accounting for 60% of the top 12 clones, or 30% of the total library. Clone B1 through clone B9 distributed among 3 phyla, i.e., *Proteobacteria*, *Planctomycetes* and *Bacteroidetes*, with a majority belong to *Bacteroidetes*, accounting for 58% of the top 9 clones, or 38% of the total library.

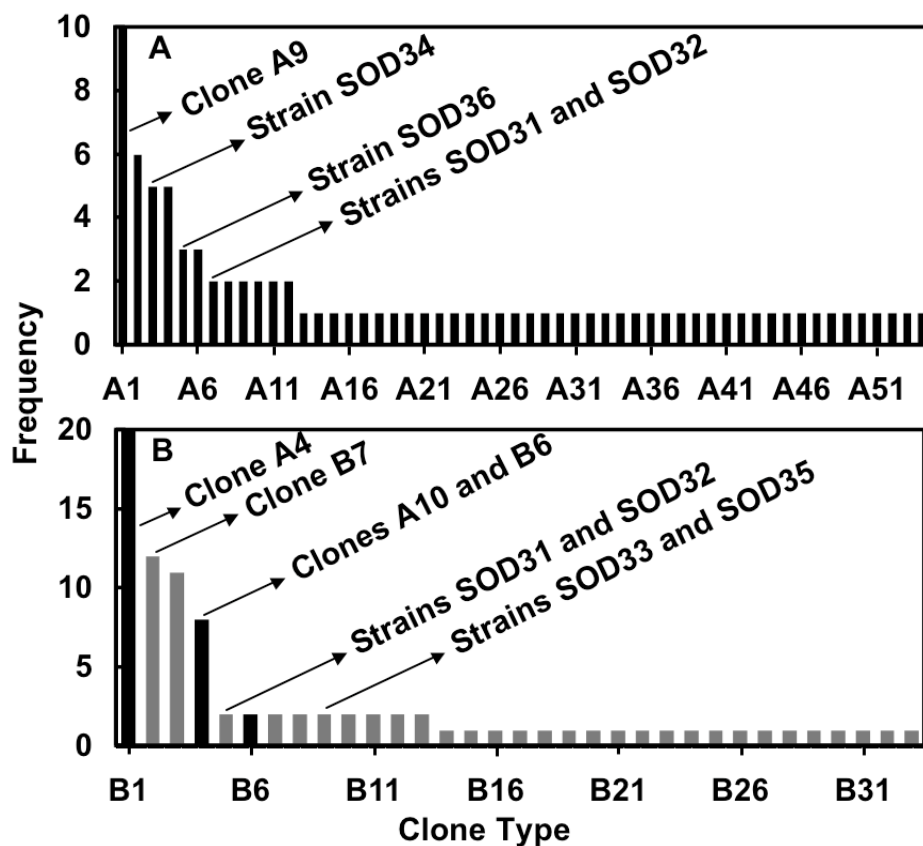
The facts that a diversity of *Proteobacteria* employ acyl-HSLs as signaling molecules in quorum sensing but not other bacterial phyla, and that the six isolates and the majority clones from the acyl-HSL-supplied reactor all belong to *Proteobacteria*, implicate the possible co-localization of acyl-HSL-producing and -degrading organisms and existence of community interactions in the natural soil environments. These isolates might target at certain subpopulation of acyl-HSL-producing microorganisms in the soil.

A majority of known acyl-HSL-degrading bacteria belong to the *Proteobacteria* phylum. Besides the *V. paradoxus* VAI-C, several bacteria were discovered employing acyl-HSL acylases to degrade acyl-HSL. The first gene encoding a protein with acyl-HSL acylase activity, designated *aiiD*, was later identified in a *Ralstonia* strain XJ12B, isolated from a mixed-species biofilm (25). A close homologue of the *aiiD* gene was identified in *Pseudomonas aeruginosa* PAO1, *pvdQ*, and was shown to be an acyl-HSL acylase (14). Recently, a second acyl-HSL acylase has been identified in *P. aeruginosa*.

Designated QuiP, it is the product of *P. aeruginosa* gene PA1032 (15). There were also isolates identified belonging to the genus of *Comamonas* able to degrade acyl-HSLs (49). Moreover, a few *Proteobacteria* bacteria were found using acyl-HSL lactonases to degrade acyl-HSLs. *Agrobacterium tumefaciens* A6 employs AttM to degrade acyl-HSL and adopts a unique signal turnover system to exit from conjugation-related quorum sensing (56). Besides *attM*, another *attM*-paralogous gene, *aiiB*, has been identified in *A. tumefaciens* C58 (1) which serves as a lactonase as well. Another acyl-HSL lactonase of AhlK has been identified in *Klebsiella pneumoniae* KCTC2241 (35).

Several bacteria from the *Actinobacteria* phylum have been identified capable of degrading acyl-HSLs. An acyl-HSL acylase designated AhlM has been identified in a *Streptomyces* strain M664 (34). *Rhodococcus erythropolis* W2, an isolate from a tobacco rhizosphere (49), was shown to degrade acyl-HSLs by both acylase activity and oxidoreductase activity (48) through which whole W2 cells converted 3-oxo-substituted long-chain ( $\geq 8$  carbons) acyl-HSLs to their corresponding 3-hydroxy derivatives. Another acyl-HSL lactonase of AhlD has been identified in a soil isolate *Arthrobacter* strain IBN110 (35).

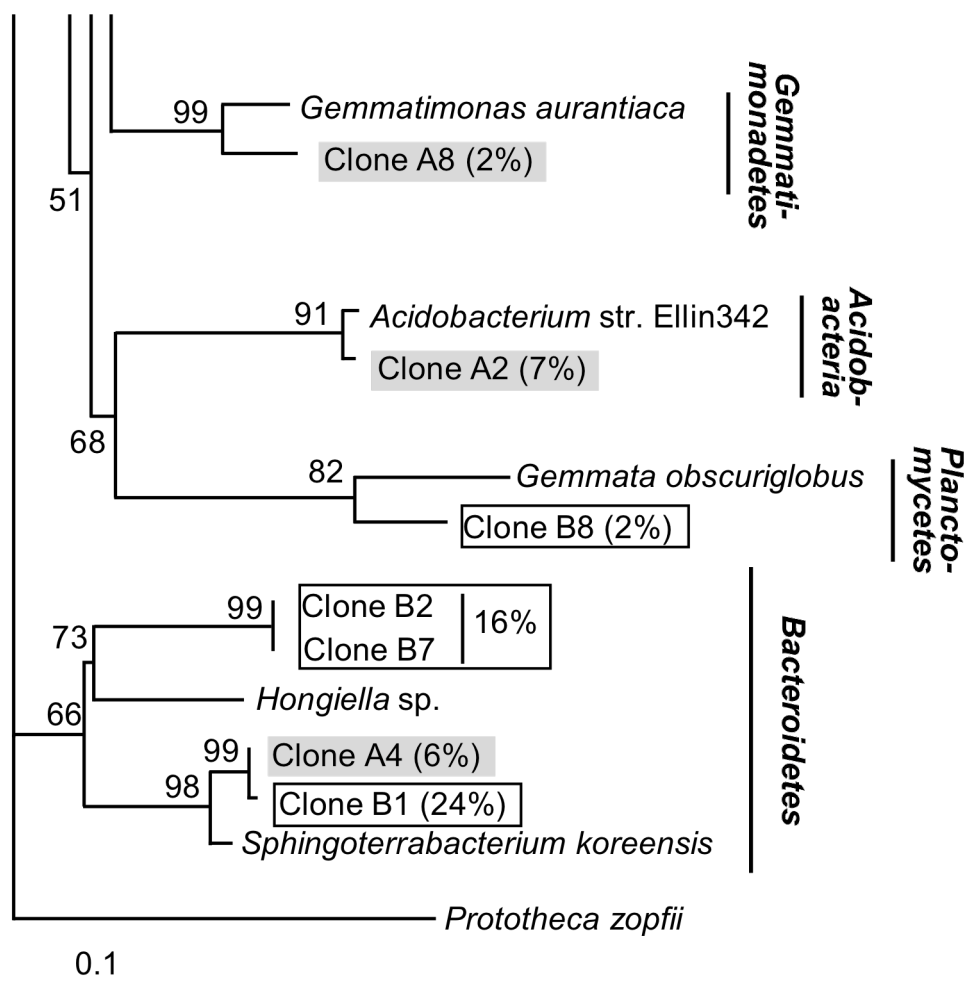
Among the other 4 phyla of *Gemmatimonadetes*, *Acidobacteria*, *Planctomycetes* and *Bacteroidetes*, no members are known to degrade acyl-HSLs. Interesting, although many *Bacillus* species, belonging to the *Firmicute* phylum, have been identified capable of degrading acyl-HSL by lactonase (5, 24, 37, 47), including *Bacillus* sp. 240B1 from which the first acyl-HSL lactonase AiiA was identified (6, 7), none of the most abundant clones from either of the biofilm reactors were from *Bacillus* species.



**Figure 4.4 Rank abundances of recovered 16S rDNA clone types amplified from soil-inoculated biofilm reactors. (A)** Frequency at which 54 distinct DNA types were encountered in an 86-clone inventory amplified from the biofilm of a representative reactor with hexanoyl-HSL amended in the medium. The 16S rDNA genes of the most abundant 12 clones, accounting for 51% of total, were sequenced. **(B)** Frequency at which 33 distinct DNA types were encountered in an 89-clone inventory amplified from the biofilm of a representative reactor where hexanoyl-HSL was absent. The 16S rDNA genes of the most abundant 9 clones, accounting for 66% of total, were sequenced. The arrows point out the corresponding clones or isolates that are  $\geq 97\%$  identical on DNA level.







**Figure 4.5. Maximum likelihood phylogeny of 16S rDNA genes cloned from the biofilm communities and six bacterial isolates.** Shaded boxes highlight the 12 clones from a representative soil-inoculated reactor with hexanoyl-HSL amended in the medium, corresponding to Fig. 4.4A. The 9 bordered clones were from a representative soil-inoculated reactor where hexanoyl-HSL was absent, corresponding to Fig. 4.4B. Bacterial strains of SOD31 through SOD36 were isolated from the biofilm reactors and recovered on solid acyl-HSL agarose plates. Construction of the phylogram used 1035 unambiguously aligned nucleotide positions in a 10,000-step Tree-Puzzle maximum likelihood analysis. Percentages denote frequency of the encounter of particular clone types within the inventory. The bar represents evolutionary distance as 0.1 changes per nucleotide position as determined by measuring the lengths of the horizontal lines connecting the species. Bootstrap values are placed to the immediate left of each node, which provide support for the robustness of the adjacent nodes. *Prototheca zopfii* was used as the outgroup in the construction of the unrooted tree. See Materials and Methods for GenBank accession numbers.

#### 4.3.4 Growth studies of the bacterial isolates

Pure cultures of *Variovorax* strains SOD31 and SOD32, *Mesorhizobium* strains SOD33 and SOD35, *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36 were grown in the defined medium supplied with 1.5  $\mu\text{M}$  hexanoyl-HSL. Control cultures of all the strains were incubated at the same time, i.e., no acyl-HSL was supplied. Cell densities were measured during the course by plate counting. Table 4.2 shows typical culture yields of the six isolates.

**Table 4.2. Culture yields of isolates SOD31 through SOD36 in defined medium containing 1.5  $\mu\text{M}$  hexanoyl-HSL. Control cultures contained no hexanoyl-HSL.**

Strain	Yield on 1.5 $\mu\text{M}$ acyl-HSL ( $\times 10^6 \text{ ml}^{-1}$ )	Yield from control cultures compared to yield on 1.5 $\mu\text{M}$ acyl-HSL (%)
SOD31	9.0	20
SOD32	3.1	30
SOD33	6.7	45
SOD34	47.2	35
SOD35	6.2	40
SOD36	27.4	50

Growth yields of the six isolates on 1.5  $\mu\text{M}$  acyl-HSL reached  $10^6$  to  $10^7$  cells  $\text{ml}^{-1}$  after about a week. *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36 had much higher yields than the other four strains. All of the six isolates had growth yields on 1.5  $\mu\text{M}$  acyl-HSL at least 2-fold higher than those of the controls, i.e., yields from the minus

acyl-HSL medium. Therefore, unambiguous growth on acyl-HSL of the six isolates has been demonstrated.

As to the apparent growth of the six isolates in minus acyl-HSL control cultures, we tested the possibility of growth on soil extract, which was one of the components of the medium. As is known, soil extract contains many organic matters, mostly humic substances and other easily degradable molecules, including proteins, amino acids and sugars (17). We compared the yields of the six isolates in the defined medium all supplied with 1.5  $\mu$ M acyl-HSL; nevertheless one variable was addition (or not) to the medium with soil extract.

For all the six isolates, the yields from the regular medium, i.e., containing soil extract, were consistent and virtually equal to what we observed as presented in Table 4.2 (data not shown). Interestingly, *Mesorhizobium* strains SOD33 and SOD35 could not grow without soil extract. The reasons were unclear, which might be the missing of some nutrients essential from the soil extract for the bacterial growth. For the other 4 strains, i.e., *Variovorax* strains SOD31 and SOD32, *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36, the yields decreased 10% to 25% without soil extract. From Table 4.2, yields on other possible nutrients except acyl-HSL, i.e., the “control” yields, accounted for 20% to 50% of the “overall” yields, i.e., in the medium containing both acyl-HSL and soil extract. However, yields on soil extract seemed only contributing partially to the “control” yields such that there might be other nutrients supporting their growth (see below for further information).

Among all the six isolates, *Variovorax* strain SOD31 had the largest difference between yields on 1.5  $\mu\text{M}$  acyl-HSL and controls. *Bradyrhizobium* strain SOD34 had the highest yield on 1.5  $\mu\text{M}$  acyl-HSL and it was the most abundant one recovered from the biofilm as well. Therefore, SOD31 and SOD34 were chosen for further growth studies.

*Variovorax* strain SOD31 was grown in the defined medium containing various concentrations of acyl-HSL up to 150  $\mu\text{M}$ . At each concentration, cultures were grown in duplicates. Another batch of experiments were performed following exactly the same procedure except that soil extract was excluded from the medium; no significant difference was observed as to the patterns of the growth curves, growth rates and yields (see below). Representative growth curves shown in Fig. 4.6A-left were grown in the medium without soil extract. *Variovorax* strain SOD31 was able to degrade acyl-HSL over the full range tested, from 0.75 to 150  $\mu\text{M}$ . And the culture yields were nicely proportional to acyl-HSL concentrations (Fig. 4.6B-left).

There was an exponentially growing phase before 1.8 d. The doubling time of the cultures at all concentrations was virtually the same, i.e.,  $2.1 \pm 0.2$  h. The yield during this stage accounted for 4% to 54% to the overall yield at different acyl-HSL concentrations (with greater contribution at low acyl-HSL concentration), while the control culture reached maximum cell density at 1.8 d. After 1.8 d, the cultures entered another slower exponentially growing phase and there was still remarkable growth before cultures reached maximum cell density. By comparing to the control culture where there was no acyl-HSL in the medium, it is reasonable that during the second logarithmic-phase, the cultures were actually growing on acyl-HSL.

As to the growth occurred during the first logarithmic-phase before 1.8 d, besides soil extract, vitamins and other unknown substrates in the medium that might be easier degradable for *Variovorax* strain SOD31 probably contributed to the growth as well, for instance, impurities from the compounds dissolved in the medium. Therefore, at such low nutrients level, these substrates might become substantial sources for growth.

Growth rates of *Variovorax* strain SOD31 on acyl-HSL were calculated based on the second logarithmic-phase from 1.8 d (Fig. 4.6C-left). The bacterium exhibited an acyl-HSL-limiting growth kinetics. The half-saturation constant was  $1.7 \pm 0.2 \mu\text{M}$ , which is in highly agreement with what we observed from soil communities in Chapter 3. SOD31 has by far the most superior affinity to degrade acyl-HSL and may be an active contributor to the soil's activity of degrading acyl-HSL.

*Bradyrhizobium* strain SOD34 was also cultivated in the defined medium containing various concentrations of acyl-HSL up to  $150 \mu\text{M}$ , following exactly the same procedure as with *Variovorax* strain SOD31. Representative growth curves are shown in Fig. 4.6A-right where soil extract was not supplied in the medium as well.

Remarkably distinct from *Variovorax* strain SOD31, the cultures of *Bradyrhizobium* strain SOD34 achieved a maximum yield at  $30 \mu\text{M}$  of initial acyl-HSL concentration (Fig. 4.6B-right). The control growth could be due to vitamins and other unknown substrates as discussed above. With initial acyl-HSL concentrations higher than  $100 \mu\text{M}$ , growth yields were even lower than the control cultures. Only one logarithmic-phase was observed and the growth rates decreased with higher concentrations of acyl-HSL (Fig. 4.6C-right).

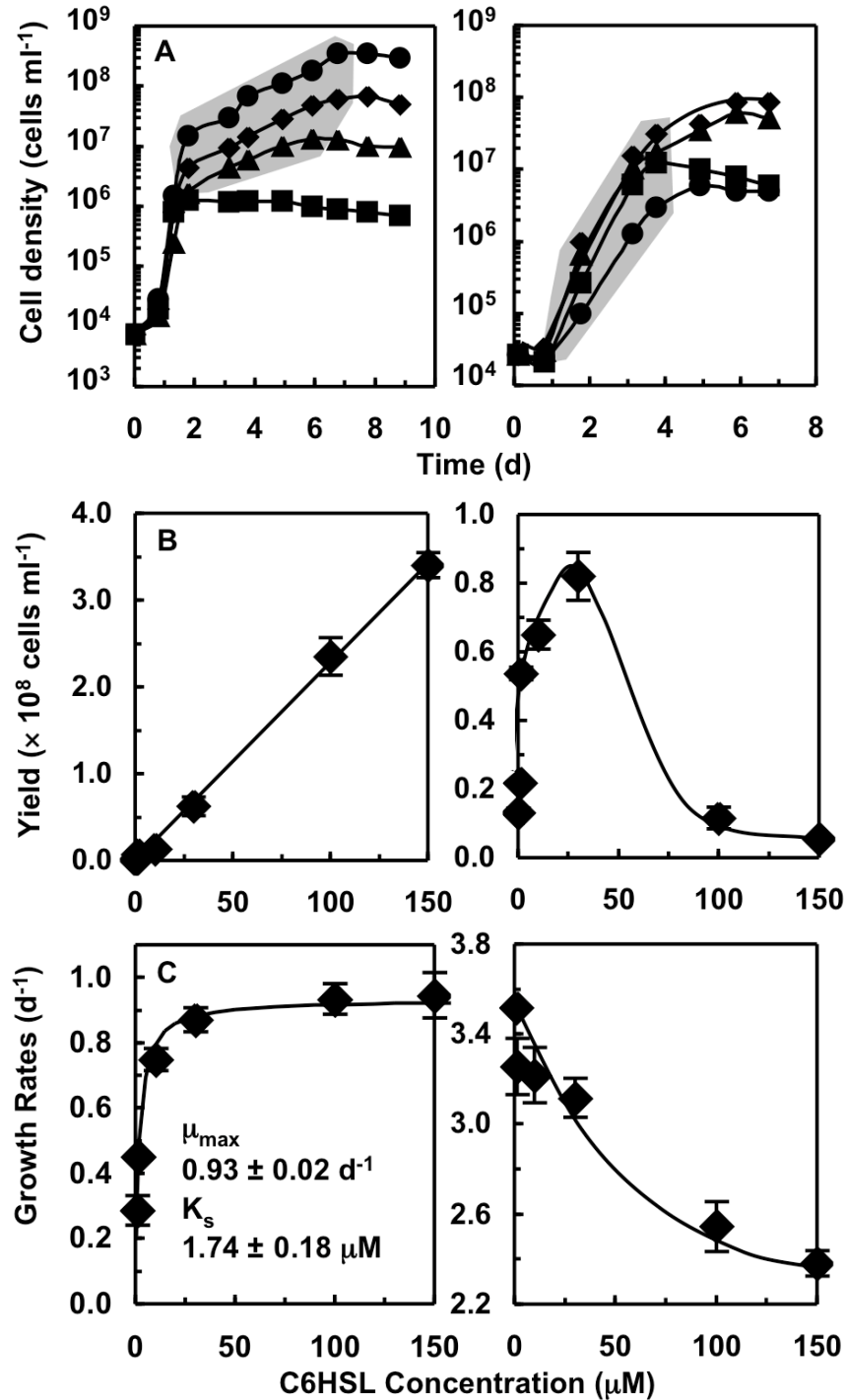
Such result of growth inhibition by acyl-HSL is curious and unusual. Several reports have provided us insights into the underlying mechanisms such as the growth of some strains of *Rhizobium leguminosarum* biovar *viciae* being inhibited by 3-hydroxy-7-*cis*-tetradecenoyl-homoserine lactone (3OHC14:1HSL). This acyl-HSL is produced by a LuxI homologue of CinI (26). It is previously known as the "*small*" bacteriocin (10, 12, 41) which is among the family of proteinaceous toxins produced by certain bacteria to inhibit the growth of similar bacterial strain(s). *R. leguminosarum* bv. *viciae* has a symbiotic plasmid pRL1JI that carries genes required for nodulation and symbiotic nitrogen fixation on legumes such as pea, vetch and lentil (3). pRL1JI has been identified of having high frequency of transfer between different rhizobial strains (18). Besides CinI, the *R. leguminosarum* bv. *viciae* strain A34 has been found having three other acyl-HSL synthases (3, 38, 55), two of which are encoded on pRL1JI, i.e., TraI and RhiI.

BisR and TraR, which are LuxR-type regulators also encoded on pRL1JI, are critical in this phenomenon. The mechanism has been found specifically poised to detect 3OHC14:1HSL made by different cells, e.g., potential recipients of pRL1JI, via a quorum-sensing relay involving BisR and TraR in donor cells (4). Interestingly and consistently to this mechanism, *cinI* is normally repressed by BisR in donor cells and thus 3OHC14:1HSL production is very low (53). When potential recipients are present and produce 3OHC14:1HSL by CinI, BisR induces expression of *traR* in response to 3OHC14:1HSL, which may occur at very low concentrations of 3OHC14:1HSL (ca. 1 nM). TraR subsequently induces the plasmid transfer in a quorum-sensing-dependent manner in response to the TraI-made acyl-HSLs, i.e., 3-oxooctanoyl-homoserine lactone

(3OC8HSL) and octanoyl-homoserine lactone (C8HSL). In this process, both BisR and TraR and multiple acyl-HSLs (3OHC14:1HSL, 3OC8HSL and C8HSL) are required.

Through such complicated mechanisms, the donor cells (carrying pRL1JI) induce plasmid transfer as long as 3OHC14:1HSL from potential recipients is present ( $\geq 1$  nM), even though the cells may still be at low density. The overall behavior becomes growth inhibition (of the donor cells) by acyl-HSL, with 3OHC14:1HSL switching the bacteria into stationary phase yet at low density (10). In another *Rhizobium* sp. NGR234, it has also been found that expressing TraR resulted in 3OC8HSL-dependent inhibition of growth (11).

Originally in the biofilm reactors, acyl-HSL was always supplied at 1.5  $\mu\text{M}$  level. At such concentration, the growth rate of *Bradyrhizobium* strain SOD34 was ca. 3.2  $\text{d}^{-1}$  while that of *Variovorax* strain SOD31 was only ca. 0.5  $\text{d}^{-1}$ . Therefore, the reason why SOD34 was the most abundant one recovered was probably because SOD34 was able to grow faster and accumulate more biomass than SOD31 inside the reactor columns.



**Figure 4.6. Representative growth curves (A), yields (B) and rates (C) of SOD31 (left panels) and SOD34 (right panels) utilizing hexanoyl-HSL at various concentrations.** Cultures were grown in duplicates at each hexanoyl-HSL concentration. Plots in panel A show cell density of cultures in the medium containing 0 (■), 10 (▲), 30 (◆) and 150 (●) μM of hexanoyl-HSL. Shaded boxes highlight the exponentially growing phase during which the growth rates in panel C are calculated.



#### **4.3.5 Acyl-HSL consumption through biofilm reactors to which mixture of the bacterial isolates of SOD31 through SOD36 was inoculated**

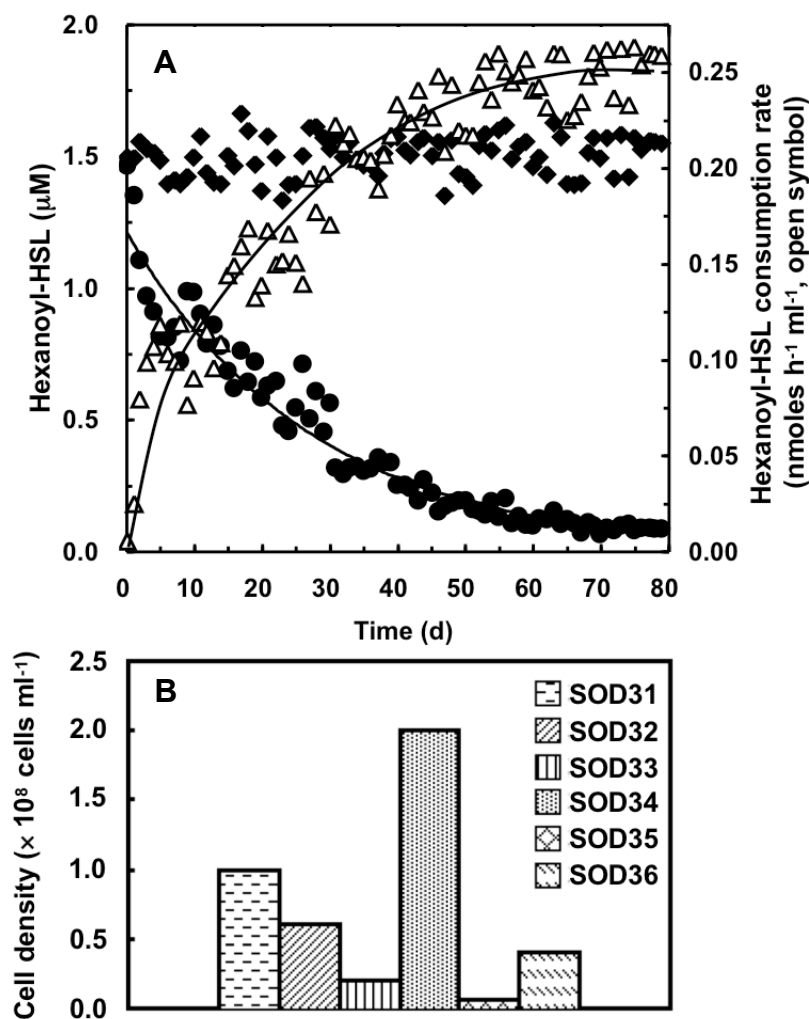
Mixture of the six isolates, i.e., *Variovorax* strains SOD31 and SOD32, *Methyrrhizobium* strains SOD33 and SOD35, *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36, were inoculated into a new set of biofilm reactors to test their ability to degrade acyl-HSL following the same procedure as the soil-inoculated reactors, except that initially 5 ml of culture suspension containing ca.  $2 \times 10^6$  cells ml<sup>-1</sup> of each of the six isolates was used as inoculum. Same as the soil-inoculated reactors, acyl-HSL concentration in the medium was maintained at 1.5  $\mu$ M when applied.

Figure 4.7A shows acyl-HSL concentrations in the outflows from representative reactors and acyl-HSL consumption rate from the bacteria-inoculated reactor. The flow rate was maintained at 1.7 ml h<sup>-1</sup>. No apparent acyl-HSL consumption occurred through the uninoculated control reactor. In contrast, through the bacteria-inoculated reactor, during the incubation period, acyl-HSL consumption rate steadily kept increasing. After two and a half months, acyl-HSL was ca. 95% consumed and acyl-HSL consumption rate reached 0.26 nmoles h<sup>-1</sup> ml<sup>-1</sup>.

Formerly in soil-inoculated reactors, only after 10 days at the same flow rate of 1.7 ml h<sup>-1</sup>, acyl-HSL was over 95% consumed (Fig. 4.2A), and acyl-HSL consumption rate was ca. 0.27 nmoles h<sup>-1</sup> ml<sup>-1</sup> (Fig. 4.2B), which was very close to the maximum value observed here through bacterial-inoculated reactor. Since these six isolates were probably a tiny portion of the whole soil community, their ability to degrade acyl-HSL was much

weaker. Nonetheless, the results clearly demonstrated their ability to grow and utilize acyl-HSL to significant extent over a period of reasonable length.

And at the end of incubation, biofilm suspensions from bacteria-inoculated reactors were obtained and relative abundance of the six isolates was measured (see Materials and Methods). Shown in Fig. 4.7B are data from the acyl-HSL supplied reactor. *Bradyrhizobium* strain SOD34 was the most abundant one, which was the same case as the soil-inoculated reactors. Compared to soil-inoculated reactor, the portion of SOD31 and SOD32 increased significantly. From the acyl-HSL-absent reactor, the total cell density was  $1.7 \times 10^8$  cells ml<sup>-1</sup> recovered and the cell abundance ratio of SOD31 through SOD36 was 10 : 9 : 4 : 35 : 2 : 10.



**Figure 4.7. Hexanoyl-HSL consumption through representative reactors inoculated with mixture of the bacterial isolates of SOD31 through SOD36. (A)** Data show the hexanoyl-HSL concentration at the end of uninoculated (◆) and inoculated (●) reactors, which were measured by LC/MS, and hexanoyl-HSL consumption rate (Δ) through the inoculated reactor. During the incubation period, hexanoyl-HSL level in the medium reservoirs was maintained at  $1.5 \pm 0.1 \mu\text{M}$ . Flow rate was maintained at  $1.7 \text{ ml h}^{-1}$ . **(B)** Data show relative abundance of bacterial isolates of SOD31 through SOD36 recovered from the biofilm of the inoculated reactor (to which acyl-HSL was supplied) at the end of incubation with a total of  $4.3 \times 10^8 \text{ cells ml}^{-1}$  recovered. The cell abundance ratio of SOD31 through SOD36 is 10 : 6 : 2 : 20 : 1 : 4.

#### 4.4 Conclusions

Here we show that for the first time high-affinity acyl-HSL degraders were enriched in oligotrophic biofilm reactors, to which fresh soil samples were inoculated that in the previous chapter acyl-HSL degradation activities have been observed. When supplied at physiological concentration, acyl-HSL was observed to be significantly consumed through the reactors. During certain stage, the acyl-HSL concentration in the effluent was below the detection limit of 100 nM and thus acyl-HSL was over 95% consumed.

Six pure bacterial strains have been successfully isolated, including *Variovorax* strains SOD31 and SOD32, *Mesorhizobium* strains SOD33 and SOD35, *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36. They all belong to the *Proteobacteria* phylum; so do the majority of the clones from the soil-inoculated and acyl-HSL-supplied reactor based on culture-independent community 16S rDNA analysis. Along with the fact that a diversity of *Proteobacteria* employ acyl-HSLs as signaling molecules in quorum sensing but not other bacterial phyla, these results implicate the possible co-localization of acyl-HSL-producing and -degrading organisms and existence of community interactions in the natural soil environments.

All of the six isolates had growth yields on 1.5  $\mu$ M acyl-HSL at least 2-fold higher than those of the controls, i.e., yields from the minus acyl-HSL medium. Therefore, unambiguous growth of the six isolates on acyl-HSL has been demonstrated. Furthermore, *Variovorax* strain SOD31 was able to degrade acyl-HSL over the full range

tested, from 0.75 to 150  $\mu\text{M}$ . And the culture yields were nicely proportional to acyl-HSL concentrations. The bacterium exhibited an acyl-HSL-limiting growth kinetics with a half-saturation constant of 1.7  $\mu\text{M}$ , which is in highly agreement with what we observed from the turf soil and suggests that strain SOD31 may be an active contributor to the soil's activity of degrading acyl-HSL.

After being inoculated into a new set of biofilm reactors, mixed culture of the six isolates remarkably degraded the supplied acyl-HSL as well as keeping a steadily increasing acyl-HSL consumption rate. After two and a half months, acyl-HSL was ca. 95% consumed. The results clearly demonstrated their ability to grow and utilize acyl-HSL to significant extent over a period of reasonable length.

By using biofilm reactors to mimic the natural environments, e.g., soils, where oligotrophic conditions usually occur (20, 44, 50), and with bacterial strains successfully isolated that are able to degrade acyl-HSL at physiologically relevant concentration, we gain more insights into how signal decay may influence quorum sensing and community function and structure in naturally occurring microbial communities.

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#### 4.5 References

1. **Carlier, A., S. Uroz, B. Smadja, R. Fray, X. Latour, Y. Dessaux, and D. Faure.** 2003. The Ti plasmid of *Agrobacterium tumefaciens* harbors an *attM*-paralogous gene, *aiiB*, also encoding N-acyl homoserine lactonase activity. *Appl. Environ. Microbiol.* **69**:4989-4993.
2. **Carter, M. R. (ed.).** 2000. Soil sampling and methods of analysis. CRC Press, Boca Raton, Florida.
3. **Cubo, M. T., A. Economou, G. Murphy, A. W. B. Johnston, and J. A. Downie.** 1992. Molecular characterization and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *Rhizobium leguminosarum* biovar *viciae*. *J. Bacteriol.* **174**:4026-4035.
4. **Danino, V. E., A. Wilkinson, A. Edwards, and J. A. Downie.** 2003. Recipient-induced transfer of the symbiotic plasmid pRL1JI in *Rhizobium leguminosarum* bv. *viciae* is regulated by a quorum-sensing relay. *Mol. Microbiol.* **50**:511-525.
5. **Dong, Y. H., A. R. Gusti, Q. Zhang, J. L. Xu, and L. H. Zhang.** 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. *Appl. Environ. Microbiol.* **68**:1754-1759.

6. **Dong, Y. H., L. H. Wang, J. L. Xu, H. B. Zhang, X. F. Zhang, and L. H. Zhang.** 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**:813-817.
7. **Dong, Y. H., J. L. Xu, X. Z. Li, and L. H. Zhang.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. USA* **97**:3526-3531.
8. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Neilson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry-US* **20**:2444-2449.
9. **Gonzalez, J. E., and M. M. Marketon.** 2003. Quorum sensing in nitrogen-fixing rhizobia. *Microbiol. Mol. Biol. Rev.* **67**:574-579.
10. **Gray, K. M., J. P. Pearson, J. A. Downie, B. E. A. Boboye, and E. P. Greenberg.** 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: Autoinduction of a stationary phase and rhizosphere-expressed genes. *J. Bacteriol.* **178**:372-376.
11. **He, X. S., W. Chang, D. L. Pierce, L. O. Seib, J. Wagner, and C. Fuqua.** 2003. Quorum sensing in *Rhizobium* sp. strain NGR234 regulates conjugal transfer (*tra*) gene expression and influences growth rate. *J. Bacteriol.* **185**:809-822.
12. **Hirsch, P. R.** 1979. Plasmid-determined bacteriocin production by *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **113**:219-228.
13. **Hoang, H. H., A. Becker, and J. E. Gonzalez.** 2004. The LuxR homolog ExpR, in combination with the sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J. Bacteriol.* **186**:5460-5472.

14. **Huang, J. J., J. I. Han, L. H. Zhang, and J. R. Leadbetter.** 2003. Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* **69**:5941-5949.
15. **Huang, J. J., A. Petersen, M. Whiteley, and J. R. Leadbetter.** 2006. Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* **72**:1190-1197.
16. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**:2317-2319.
17. **James, N.** 1958. Soil extract in soil microbiology. *Can. J. Microbiol.* **4**:363-370.
18. **Johnston, A. W. B., J. L. Beynon, A. V. Buchananwollaston, S. M. Setchell, P. R. Hirsch, and J. E. Beringer.** 1978. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. *Nature* **276**:634-636.
19. **Kreader, C. A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.* **62**:1102-1106.
20. **Kuznetsov, S. I., G. A. Dubinina, and N. A. Lapteva.** 1979. Biology of oligotrophic bacteria. *Annu. Rev. Microbiol.* **33**:377-387.
21. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, Inc., New York.
22. **Leadbetter, J. R., and J. A. Breznak.** 1996. Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov.,



- isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl. Environ. Microbiol. **62**:3620-3631.
23. **Leadbetter, J. R., and E. P. Greenberg.** 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J. Bacteriol. **182**:6921-6926.
24. **Lee, S. J., S. Y. Park, J. J. Lee, D. Y. Yum, B. T. Koo, and J. K. Lee.** 2002. Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. Appl. Environ. Microbiol. **68**:3919-3924.
25. **Lin, Y. H., J. L. Xu, J. Y. Hu, L. H. Wang, S. L. Ong, J. R. Leadbetter, and L. H. Zhang.** 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. Mol. Microbiol. **47**:849-860.
26. **Lithgow, J. K., A. Wilkinson, A. Hardman, B. Rodelas, F. Wisniewski-Dye, P. Williams, and J. A. Downie.** 2000. The regulatory locus cinRI in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. Mol. Microbiol. **37**:81-97.
27. **Loh, J., R. W. Carlson, W. S. York, and G. Stacey.** 2002. Bradyoxetin, a unique chemical signal involved in symbiotic gene regulation. Proc. Natl. Acad. Sci. USA **99**:14446-14451.
28. **Loh, J. T., J. P. Y. Yuen-Tsai, M. G. Stacey, D. Lohar, A. Welborn, and G. Stacey.** 2001. Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes. Mol. Microbiol. **42**:37-46.

29. **Lohrke, S. M., C. J. Madrzak, H. G. Hur, A. K. Judd, J. H. Orf, and M. J. Sadowsky.** 2000. Inoculum density-dependent restriction of nodulation in the soybean-*bradyrhizobium japonicum* symbiosis. *Symbiosis* **29**:59-70.
30. **Long, S. R.** 2001. Genes and signals in the rhizobium-legume symbiosis. *Plant Physiol.* **125**:69-72.
31. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* **32**:1363-1371.
32. **Marketon, M. M., and J. E. Gonzalez.** 2002. Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. *J. Bacteriol.* **184**:3466-3475.
33. **Page, R. D. M.** 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**:357-358.
34. **Park, S. Y., H. O. Kang, H. S. Jang, J. K. Lee, B. T. Koo, and D. Y. Yum.** 2005. Identification of extracellular N-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching. *Appl. Environ. Microbiol.* **71**:2632-2641.
35. **Park, S. Y., S. J. Lee, T. K. Oh, J. W. Oh, B. T. Koo, D. Y. Yum, and J. K. Lee.** 2003. AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. *Microbiology-Sgm* **149**:1541-1550.

36. **Pongsilp, N., E. W. Triplett, and M. J. Sadowsky.** 2005. Detection of homoserine lactone-like quorum sensing molecules in *Bradyrhizobium* strains. *Curr. Microbiol.* **51**:250-254.
37. **Reimmann, C., N. Ginet, L. Michel, C. Keel, P. Michaux, V. Krishnapillai, M. Zala, K. Heurlier, K. Triandafillu, H. Harms, G. Defago, and D. Haas.** 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology-Sgm* **148**:923-932.
38. **Rodelas, B., J. K. Lithgow, F. Wisniewski-Dye, A. Hardman, A. Wilkinson, A. Economou, P. Williams, and J. A. Downie.** 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. *viciae*. *J. Bacteriol.* **181**:3816-3823.
39. **Salmassi, T. M., and J. R. Leadbetter.** 2003. Analysis of genes of tetrahydrofolate-dependent metabolism from cultivated spirochaetes and the gut community of the termite *Zootermopsis angusticollis*. *Microbiol-Sgm* **149**:2529-2537.
40. **Schmidt, H. A., K. Strimmer, M. Vingron, and A. von Haeseler.** 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18**:502-4.
41. **Schripsema, J., K. E. E. deRudder, T. B. vanVliet, P. P. Lankhorst, E. deVroom, J. W. Kijne, and A. A. N. vanBrussel.** 1996. Bacteriocin *small* of *Rhizobium leguminosarium* belongs to the class of *N*-acyl-1-homoserine lactone molecules, known as autoinducers and as quorum sensing co-transcription factors. *J. Bacteriol.* **178**:366-371.

42. **Schultze, M., and A. Kondorosi.** 1998. Regulation of symbiotic root nodule development. *Annual Review of Genetics* **32**:33-57.
43. **Schut, F., E. J. Devries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button.** 1993. Isolation of typical marine bacteria by dilution culture: Growth, maintenance, and characteristics of isolates under laboratory conditions. *Appl. Environ. Microbiol.* **59**:2150-2160.
44. **Schut, F., R. A. Prins, and J. C. Gottschal.** 1997. Oligotrophy and pelagic marine bacteria: facts and fiction. *Aqua. Microb. Ecol.* **12**:177-202.
45. **Strimmer, K., and A. von Haeseler.** 1996. Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* **13**:964-969.
46. **Tsai, Y. L., and B. H. Olson.** 1992. Rapid method for separation of bacterial-DNA from humic substances in sediments for polymerase chain reaction. *Appl. Environ. Microbiol.* **58**:2292-2295.
47. **Ulrich, R. L.** 2004. Quorum quenching: Enzymatic disruption of N-acylhomoserine lactone-mediated bacterial communication in *Burkholderia thailandensis*. *Appl. Environ. Microbiol.* **70**:6173-6180.
48. **Uroz, S., S. R. Chhabra, M. Camara, P. Williams, P. Oger, and Y. Dessaux.** 2005. N-acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. *Microbiology-Sgm* **151**:3313-3322.
49. **Uroz, S., C. D'Angelo-Picard, A. Carlier, M. Elasri, C. Sicot, A. Petit, P. Oger, D. Faure, and Y. Dessaux.** 2003. Novel bacteria degrading N-acylhomoserine lactones

- and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology-Sgm* **149**:1981-1989.
50. **Wainwright, M., F. Barakah, I. Al-Turk, and T. A. Ali.** 1991. Oligotrophic microorganisms in industry, medicine and the environment. *Sci. Progress* **75**:313-322.
51. **Wang, L. H., L. X. Weng, Y. H. Dong, and L. H. Zhang.** 2004. Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). *J. Biol. Chem.* **279**:13645-13651.
52. **Wang, Y. J., and J. R. Leadbetter.** 2005. Rapid acyl-homoserine lactone quorum signal biodegradation in diverse soils. *Appl. Environ. Microbiol.* **71**:1291-1299.
53. **Wilkinson, A., V. Danino, F. Wisniewski-Dye, J. K. Lithgow, and J. A. Downie.** 2002. N-acyl-homoserine lactone inhibition of rhizobial growth is mediated by two quorum-sensing genes that regulate plasmid transfer. *J. Bacteriol.* **184**:4510-4519.
54. **Wilson, I. G.** 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **63**:3741-3751.
55. **Wisniewski-Dye, F., J. Jones, S. R. Chhabra, and J. A. Downie.** 2002. *railR* genes are part of a quorum-sensing network controlled by *cinI* and *cinR* in *Rhizobium leguminosarum*. *J. Bacteriol.* **184**:1597-1606.
56. **Zhang, H. B., L. H. Wang, and L. H. Zhang.** 2002. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* **99**:4638-4643.

57. Zheng, H. M., Z. T. Zhong, X. Lai, W. X. Chen, S. P. Li, and J. Zhu. 2006. A LuxR/LuxI-type quorum-sensing system in a plant bacterium, *Mesorhizobium tianshanense*, controls symbiotic nodulation. J. Bacteriol. **188**:1943-1949.

## **Chapter 5**

### **Conclusions and Outlook**

## 5.1 Summary and conclusions

In the past thirty-five years, the synthesis of acyl-HSLs and their role in the context of quorum sensing system have been studied intensively. It is only in the past six years have we realized that acyl-HSL degradation is just as important to functional quorum sensing system as acyl-HSL production. Quorum sensing would not be effective as a gene regulation mechanism if signaling molecule concentrations did not accurately portray cell numbers, therefore, signal molecules stability and their potential for degradation are key areas of study to understand how this process functions in nature.

Acyl-HSL degradation is important both ecologically and practically. For microbes that degrade acyl-HSL signals but are not known to accumulate acyl-HSLs or have quorum sensing systems, acyl-HSL degradation could provide a means to compete with acyl-HSL-producing, quorum sensing neighbors in the environment. Furthermore, several quorum sensing species, such as species of *Agrobacterium*, *Burkholderia*, *Erwinia*, *Pseudomonas*, and *Ralstonia*, are known to use quorum sensing mechanisms during plant, animal and even human pathogenesis, e.g., *Crown gall*, *Stewart's Wilt of Corn* and *Cystic fibrosis* diseases (1, 9, 10). On the other hand, certain soil bacteria involved in quorum sensing can benefit agriculture: the acyl-HSL-controlled production of phenazines and other anti-fungal metabolites by certain pseudomonads is now well established to underlie their "biocontrol" activities (4, 8, 13, 19). Reinforcement or inhibition of acyl-HSL-degradation provides the possibility to control undesired quorum-regulated activities or boost desirable quorum sensing.



Although significant amount of information on degradation of acyl-HSLs has been generated by using defined laboratory cultures, little is known about their stability in nature. The results presented in this thesis demonstrated the occurrence of rapid quorum sensing signal decay and potential contributors in natural environments.

In **Chapter 3**, we have developed and implemented a method to assess the acyl-HSL signal mineralization potential of freshly collected soil samples from diverse US sites. We have synthesized acyl-[1-<sup>14</sup>C]HSLs by cultivating bacterial cultures that make signals in L-[1-<sup>14</sup>C]methionine-containing medium (11), and purified them via HPLC. When soil samples were amended with radioactive acyl-HSLs at physiologically relevant concentrations, <sup>14</sup>CO<sub>2</sub> was released rapidly and, at most sites examined, without lag. The results demonstrated unambiguous turnover of acyl-HSLs, implicating signal-degrading microorganisms as actually being active in the natural world.

Furthermore, we observed that a typical turf soil exhibited an apparent  $K_m$  of 1.5  $\mu$ M acyl-HSL, ca. 1000-fold lower than (i.e., remarkably superior to) that reported for a purified acyl-HSL lactonase from *Bacillus cereus* (17). From a number of soil studies, a majority of microbes are oligotrophs that grow in low nutrient situations capable of scavenging and mineralizing extremely low level of organic carbon with oligotrophic metabolisms (6, 12, 16). In **Chapter 4**, we show that for the first time high-affinity acyl-HSL degraders were enriched in oligotrophic biofilm reactors, to which fresh soil samples were inoculated that in the previous chapter acyl-HSL degradation activities have been observed.

Six pure bacterial strains have been successfully isolated and designated Soil Oligotrophic Degradors as SOD31 through SOD36. They all belong to the *Proteobacteria*

phylum, including *Variovorax* strains SOD31 and SOD32, *Mesorhizobium* strains SOD33 and SOD35, *Bradyrhizobium* strain SOD34 and *Labrys* strain SOD36. Unambiguous growth on acyl-HSL of the six isolates has been demonstrated. A *Variovorax* strain SOD31 exhibited an acyl-HSL-limiting growth kinetics with a half-saturation constant of 1.7  $\mu\text{M}$ , which is in highly agreement with what we observed from the turf soil and suggests that strain SOD31 may be an active contributor to the soil's activity of degrading acyl-HSL.

Depending on the context, biological signal decay might either promote or complicate cellular communications and the accuracy of population density based controls on gene expression in species-rich ecosystems. By demonstrating the occurrence of rapid quorum sensing signal decay and potential contributors in natural environments, we gain more insights on how signal decay may influence quorum sensing in natural environments.

## **5.2 Recommendations for future research**

This research reinforces the importance of signal decay as intrinsic to bacterial cell-cell communication, as well as providing bases for further studies towards how signal decay may influence community function and structure in naturally occurring microbial communities.

### **5.2.1 Identification of highly potent enzymes to degrade acyl-HSLs**

We demonstrated that a *Variovorax* strain SOD31 exhibited an acyl-HSL-limiting growth kinetics with a half-saturation constant of 1.7  $\mu\text{M}$ . To date, this is the most potent signal-degrading bacterium. Therefore, high affinity signal-degrading enzymes could be purified from this strain, which may have wide application on the control of pathogenic quorum sensing behavior.

A cosmid library needs to be built from genomic DNA of *Variovorax* strain SOD31. High-throughput screening of the cosmid clones will be performed for their activity to degrade acyl-HSLs using a bioassay method (3). The goal is to identify genes in *Variovorax* strain SOD31 encoding the signal-degrading enzymes. The identified genes will be sequenced and further investigated for the minimum functional size by deletion analysis. The minimum gene sequences will be subcloned into proper vectors to construct corresponding plasmids. The plasmids containing the identified genes will be transformed into *E. coli* cells and the corresponding proteins will be purified. By homology analysis of the amino acid sequence of purified proteins, knowledge on the purified proteins may be obtained according to their similarity to existing proteins.

The purified proteins can be tested for their activity to degrade acyl-HSLs. Novel and highly potent enzymes may be obtained to degrade acyl-HSLs, which can be applied to control pathogenic quorum sensing behavior.

### **5.2.2 Acyl-HSL signal degradation pathways by purified enzymes**

Currently several acyl-HSL degradation mechanisms have been discovered as reviewed in Chapter 2. It is possible that novel mechanisms exist to degrade acyl-HSLs.

Therefore, the degradation pathway undertaken by purified enzymes from *Variovorax* strain SOD31 needs to be elucidated.

The purified enzymes will be incubated with several representative acyl-HSLs at certain temperature for certain amount of time respectively (2). After digestion, the reaction mixture will be extracted and separated with high performance liquid chromatography (HPLC). The separated products will be analyzed by mass spectrometry (MS) and tandem MS. If enough quantity of products can be obtained, nuclear magnetic resonance (NMR) can be used to identify products. After elucidating the digestion product, the degradation pathway will be proposed. This will complement our knowledge on the signal degradation mechanism.

It may also be desirable to solve crystal structures of the purified signal-degrading enzymes (5, 7, 18). This will give us detailed 3-dimensional structural information on these enzymes. By co-crystallizing one enzyme with acyl-HSLs, the enzyme catalytic sites responsible for degrading acyl-HSL can be obtained, which can elucidate the possible signal-degrading mechanism at atomic level. By site-directed mutations of amino acid in the catalytic sites, we can identify the amino acids that are essential for the catalytic activity of the enzyme. This detailed structural information along with a powerful directed enzyme evolution strategy may further help us to engineer the enzymes to achieve better signal-degrading activities.

### **5.2.3 Visualization of the distribution pattern of signal-producing and -degrading bacteria using fluorescence in situ hybridization**

In nature, for the acyl-HSL signals to be appropriately functional as accurate reflection of the quorum sensing bacterial population, there must exist an elaborate balance between the bacteria that synthesize the signals and their possible acyl-HSL-degrading neighbors. It would be intriguing to study the crosstalk between acyl-HSL synthesizers and degraders. To initiate the investigations into this topic, it would be interesting to study the distribution pattern of signal-producing and -degrading bacteria.

Fluorescence in situ hybridization (FISH) is widely used to visualize the distribution of specific species in microbial ecosystems (14). The 16S rRNA-directed oligonucleotide DNA probes, which are specific to certain bacteria, e.g., *Variovorax* strain SOD31, and labeled at the 5' with proper fluorophores such as fluorescein or 6-carboxyfluorescein (FAM), can be used to detect the corresponding bacteria. After fixation and permeabilization, FISH labeled bacteria can be visualized by confocal laser scanning microscopy. Colocalization of signal-producing and -degrading bacterial may demonstrate their relative distribution pattern.

Taking advantage of the *Variovorax* strain SOD31 that has remarkably high affinity to degrade acyl-HSL and possibly maintains a population in the turf soil, a soil sample can be carefully laid out on a glass slide and *Variovorax* strain SOD31 may be FISH labeled. Certain pattern of SOD31 distribution might be revealed. The idea is to see whether signal-degrading bacteria can cluster around signal-producing bacteria.

Alternatively, by using a similar system to the biofilm reactors in Chapter 4, a glass microchamber can be applied instead of the columns that can be disconnected from the system for confocal microscopy visualization without disturbing the structure of the biofilm formed. *Pantoea stewartii*, which is a plant pathogen, produces 3-oxo-hexanoyl-HSL for quorum sensing, and is not known to degrade its own signal (15). We have observed that the bacterium may accumulate the signal to ca. 5  $\mu$ M in laboratory cultures in rich medium. The bacterium can be grown in the chamber supplied with relatively rich medium, e.g., yeast extract medium, for acyl-HSL to be accumulated in the chamber. Then culture of the signal-degrading bacterium *Variovorax* strain SOD31 can be added to the chamber for a period of time for them to utilize acyl-HSL. Then FISH will be performed on the bacterium in the chamber. The distribution pattern of these two bacteria may be observed under confocal microscope. Such may provide us information upon interspecies interactions.

### 5.3 References

1. **de Kievit, T. R., and B. H. Iglewski.** 2000. Bacterial quorum sensing in pathogenic relationships. *Infect. Immu.* **68**:4839-4849.
2. **Dong, Y. H., L. H. Wang, J. L. Xu, H. B. Zhang, X. F. Zhang, and L. H. Zhang.** 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **411**:813-817.

3. **Dong, Y. H., J. L. Xu, X. Z. Li, and L. H. Zhang.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acad. Sci. USA **97**:3526-3531.
4. **Hamdan, H., D. M. Weller, and L. S. Thomashow.** 1991. Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. tritici by *Pseudomonas fluorescens* 2-79 and M4-80R. Appl. Environ. Microbiol. **57**:3270-3277.
5. **Kim, M. H., H. O. Kang, B. S. Kang, K. J. Kim, W. C. Choi, T. K. Oh, C. H. Lee, and J. K. Lee.** 2005. Crystallization and preliminary crystallographic analysis of Bacillus thuringiensis AHL-lactonase. Biochimica Biophysica Acta-Proteins Proteomics **1750**:5-8.
6. **Kuznetsov, S. I., G. A. Dubinina, and N. A. Lapteva.** 1979. Biology of oligotrophic bacteria. Annu. Rev. Microbiol. **33**:377-387.
7. **Liu, D. L., B. W. Lepore, G. A. Petsko, P. W. Thomas, E. M. Stone, W. Fast, and D. Ringe.** 2005. Three-dimensional structure of the quorum-quenching N-acyl homoserine lactone hydrolase from Bacillus thuringiensis. Proc. Natl. Acad. Sci. USA **102**:11,882-11,887.
8. **Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. **58**:2616-2624.

9. **Parsek, M. R., and E. P. Greenberg.** 2000. Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signaling mechanism involved in associations with higher organisms. *Proc. Natl. Acad. Sci. USA* **97**:8789-8793.
10. **Pierson, L. S., D. W. Wood, and E. A. Pierson.** 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. *Annu. Rev. Phytopathol.* **36**:207-225.
11. **Schaefer, A. L., E. P. Greenberg, and M. R. Parsek.** 2001. Acylated homoserine lactone detection in *Pseudomonas aeruginosa* biofilms by radiolabel assay. *Meth. Enzymol.* **336**:41-47.
12. **Schut, F., R. A. Prins, and J. C. Gottschal.** 1997. Oligotrophy and pelagic marine bacteria: facts and fiction. *Aqua. Microbial. Ecol.* **12**:177-202.
13. **Thomashow, L. S., and D. M. Weller.** 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis var. tritici*. *J. Bacteriol.* **170**:3499-3508.
14. **Thurnheer, T., R. Gmur, and B. Guggenheim.** 2004. Multiplex FISH analysis of a six-species bacterial biofilm. *J. Microbiol. Methods* **56**:37-47.
15. **von Bodman, S. B., D. R. Majerczak, and D. L. Coplin.** 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. *Proc. Natl. Acad. Sci. USA* **95**:7687-7692.
16. **Wainwright, M., F. Barakah, I. Al-Turk, and T. A. Ali.** 1991. Oligotrophic microorganisms in industry, medicine and the environment. *Sci. Progress* **75**:313-322.



17. **Wang, L. H., L. X. Weng, Y. H. Dong, and L. H. Zhang.** 2004. Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). *J. Biol. Chem.* **279**:13,645-13,651.
18. **Watson, W. T., F. V. Murphy, T. A. Gould, P. Jambeck, D. L. Val, J. E. Cronan, S. B. von Bodman, and M. E. A. Churchill.** 2001. Crystallization and rhenium MAD phasing of the acyl-homoserinelactone synthase EsaI. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **57**:1945-1949.
19. **Wood, D. W., F. C. Gong, M. M. Daykin, P. Williams, and L. S. Pierson, 3rd.** 1997. N-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J. Bacteriol.* **179**:7663-7670.