Acyl-homoserine lactone degrading marine bacteria: Do they exist?

Grace E Lim
Microbial Diversity 2001
Marine Biological Laboratory

Home Institution: Scripps Institution of Oceanography
9500 Gilman Dr, Mail Code 0208
La Jolla, CA 92093
glim@ucsd.edu

Abstract

Acyl-homoserine lactones (acyl-HSL) are small molecules that are used in density dependent regulation in some bacteria. These molecules freely diffuse from the cell into the outside environment. Hence, it may be advantageous for other bacteria to utilize these molecules as a carbon source. The aim of this study is to look for acyl-HSL-utilizing bacteria in the marine environment. One strain, a *Pirulella*-like bacterium, has been isolated in (almost) pure culture. This bacterium forms rosettes of ovoid cells. It can use acyl-HSL as a sole carbon and nitrogen source. Growing this strain in the presence of ampicillin demonstrate its lack of peptidoglycan in its cell wall, a feature consistent with its placement in the order Planctomycales.

Introduction

Acyl-homoserine lactones (acyl-HSLs) are low molecular weight compounds which serve as cell-cell signaling compounds in some Gram negative bacteria (most notably in the Proteobacteria division). These bacteria produce and use acyl-HSLs to regulate density-dependent expression of some sets of genes, and hence, this phenomenon is also known as quorum sensing. At low cell densities, acyl-HSLs are at a low concentration and hence are not able to turn on gene expression. At high cell densities, acyl-HSLs accumulate and activates gene expression. Quorum sensing was first elucidated in the bioluminescence pathway of *Vibrio fisheri* (Eberhard, 1981), and is now known in many other organisms and regulates a wide variety of genes. Though there is diversity in the downstream phenotypes, and in the types of acyl-HSLs, the mechanism for quorum sensing remains largely conserved. Phenotypes regulated by quorum sensing include swarming motility of *Serratia liquifaciens*, biofilm formation in *Pseudomonas aeruginosa*, virulence factors in *Erwinia carotovora* and plasmid transfer in *Agrobacterium tumafaciens*. The homoserine lactone moiety remains conserved among all known Gram negative quorum sensing bacteria, and variation among acyl-HSLs occur along the acyl side chain.

Since acyl-HSLs turn on a variety of phenotypes which confer fitness to the bacterium, it is conceivable that other bacterial species would get rid of these acyl-HSLs to gain a competitive advantage over the acyl-HSL-producing cells. There are two possible mechanisms for this, both of which have been demonstrated. The first mechanism is for a bacterium to degrade acyl-HSLs without using it as a carbon/nitrogen source. A soil *Bacillus sp.* has been found to carry out this pathway (Dong, 2000). It uses a lactonase enzyme to cleave the lactone ring. The gene that encodes this enzyme was been cloned into potato and tobacco and was shown to confer resistance to *E. carotova*, which mediates infection via a quorum sensing mechanism (Dong, 2001). Another mechanism that a bacterium might use to get rid of acyl-HSLs is to use it as a carbon and or nitrogen source. The acyl chain provides a hydrocarbon that can provide energy by β-oxidation, and the lactone ring when cleaved is essentially a

serine amino acid. This mechanism has been demonstrated in *Variovorax paradoxus*, a β-proteobacterium found in soil (Leadbetter, 2000).

The only known examples of acyl-HSLs are known from the terrestrial environment, while the marine environment remains unexplored. The marine environment harbors numerous species of bacteria which produce acyl-HSLs. For example, the squid *Euprymna scolopes*, the pinecone fish, and the angler fish contain *Vibrio spp.* which use acyl-HSLs to regulate their bioluminescence phenotype. They obtain an inoculum of the bacterium from the surrounding seawater. Hence, seawater may be a dilute solution of acyl-HSLs since their producers are commonly found in seawater. Acyl-HSLs are not very stable in seawater since they hydrolyze quickly in even mildly basic solution. The lactone ring is prone to alkaline hydrolysis. At pH 8, the half-life of an acyl-HSL is less than three hours (Schaefer, 2000). Nevertheless the hydrolyzed acyl-HSL can still server as a carbon or nitrogen source for other bacteria.

I hypothesize that there are bacteria in the marine environment that can use acyl-HSLs as a carbon source. To test this hypothesis, I will attempt to enrich for these bacteria by setting up enrichments with acyl-HSL as a sole carbon source.

Materials and Methods

ENRICHMENT PROCEDURES. Seawater from Stony Beach, Woods Hole, marine sediment from Eel Pond and a microbial mat from Sippewisset salt marsh were collected in July 2001 and used as inocula to enrich for acyl-HSL degraders. The amount of inoculum used in each enrichment is listed below (Table 1). The sediment and mat samples were vortexed with an equal amount of seawater (SW) base, and the suspension was used as the inoculum. 3mL of liquid medium was used for each enrichment and it consisted of an artificial carbon-, nitrogen- and sulfur-free seawater base supplemented with trace elements, 12-vitamin solution and vitamin B_{12} (recipes can be found in the appendix). To this basal medium, sodium hydroxide was added to the correct pH, and 5mM ammonium chloride and/or 0.5mg/mL (about 3mM) N-(β-ketocaproyl)-DL-homoserine lactone (VAI, Figure 1) were added to certain enrichments (Table 2) The racemic mixture was used instead of the biologically active pure L isomer since it was available from Sigma immediately. In later transfers and Sippewisset mat enrichment, 1mM sodium sulfate and either 5mM MOPS or MES were added to serve as a sulfur source and a buffering agent respectively in the medium. An enrichment with glucose as a carbon source was used as a positive control. All incubations for liquid cultures were carried out at room temperature with shaking.

Sample	Date collected	Amount used as inoculum
seawater (Stony Beach)	July 3, 2001	0.5mL
marine sediment (Eel Pond)	July 3, 2001	0.1mL
microbial mat (Sippiwisett)	July 16, 2001	0.3mL

Table 1: Inocula used in enrichments

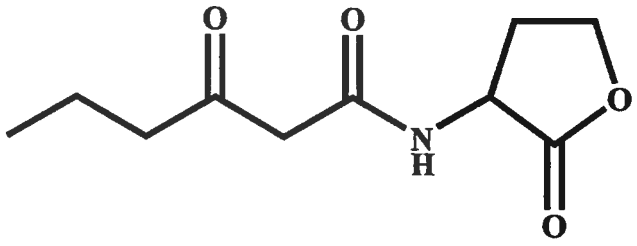

Figure 1

Enrichment	pH	5mM NH_4Cl added	3mM VAI added
1	6	no	yes
2	6	yes	yes
3	7	no	yes
4	7	yes	yes
5	6	yes	no
6	7	yes	no

Table 2

Enrichments were shaken at room temperature, and growth was monitored by microscopy. Optical density was not measured since virtually all of the cultures did not have visible turbidity. Transfers were made every 2-5 days depending on growth.

ISOLATION ON PLATES. Streak, spread and pour plates were made from initial, 1st, or 2nd transfers of the seawater or sediment enrichments. Media used were SWC, 2216 agar, and seawater base with acyl-HSL. Plates were incubated aerobically at room temperature. The following table (Tables 3) shows the various plating performed. Isolated colonies were picked and visualized microscopically. Morphotypes which resembled those in liquid enrichments were inoculated back into liquid medium.

Inoculum	Inoculum Size	Plating Method	Medium
sediment, 1st transfer, HSL-N, pH 6	10^0; 15µL	spread	SW base + HSL + NH_4Cl
sediment, 1st transfer, HSL-N, pH 7	10^0; 15µL	spread	SW base + HSL+ NH_4Cl
sediment 1st transfer, HSL, pH 7	10^0; 15µL	spread	SW base + HSL
seawater, initial, HSL, pH 7	10^0; 15µL	spread	SW base + HSL
sediment, 1st transfer, HSL-N, pH 6	10^0; 15µL	spread	SW base+ NH_4Cl
sediment, 1st transfer, HSL-N, pH 7	10^0; 15µL	spread	SW base+ NH_4Cl
sediment 1st transfer, HSL, pH 7	10^0; 15µL	spread	SW base
seawater, initial, HSL, pH 7	10^0; 15µL	spread	SW base

sediment, 2nd transfer, HSL-N, pH 7	10^{0}; 10µL	pour	buffered SW base + HSL + NH_4Cl
sediment, 2nd transfer, HSL, pH 7	10^{0}; 10µL	pour	buffered SW base + HSL
sediment, 2nd transfer, HSL-N, pH7	10^{0}; 10µL	pour	buffered SW base + NH_4Cl
seawater, 1st transfer, HSL, pH7	10^{0}; 10µL	pour	buffered SW base + HSL
sediment, 2nd transfer, HSL, pH 7	10^{-2}; 20µL, 10^{-3}; 20uL	spread	2216 agar and Seawater Complete agar (SWC)
sediment, 2nd transfer, HSL-N, pH 7	10^{-2}; 20µL, 10^{-3}; 20uL	spread	2216 agar and Seawater Complete agar (SWC)
seawater, 1st transfer, HSL, pH7	10^{-2}; 20µL, 10^{-3}; 20uL	spread	2216 agar and Seawater Complete agar (SWC)
seawater, 1st transfer, HSL-N, pH 7	10^{-2}; 20µL, 10^{-3}; 20uL	spread	2216 agar and Seawater Complete agar (SWC)
seawater, initial, HSL, pH 6	10^{0}; loopful	streak	SWC
seawater, initial, HSL-N, pH 6	10^{0}; loopful	streak	SWC
seawater, initial, HSL, pH 7	10^{0}; loopful	streak	SWC
seawater, initial, HSL-N, pH 7	10^{0}; loopful	streak	SWC
sediment, 1st transfer, HSL-N, pH 6	10^{0}; loopful	streak	SWC
sediment, 1st transfer, HSL-N, pH 7	10^{0}; loopful	streak	SWC
sediment, 1st transfer, no HSL-N, pH 6	10^{0}; loopful	streak	SWC
sediment, 1st transfer, no HSL-N, pH 7	10^{0}; loopful	streak	SWC

Table 3

REFEEDING ENRICHMENTS WITH VAI. The enrichments did not get very turbid (highest OD was 0.06 in seawater, 1st transfer, HSL pH 7). We hypothesized that the hydrolyzed VAI could not be used as a carbon source, hence we added more VAI (3mM) to an aliquot of the culture. The spiked culture and the original culture were incubated at room temperature for 48hr.

EFFECT OF VAI CONCENTRATION ON GROWTH. Since it was ambiguous whether VAI could serve as a sole carbon source in certain bacteria, a VAI concentration dependence experiment was performed. A pH 7, buffered SW base with ammonium chloride was used, and various amounts of VAI were added. The amounts were 0.125mg/mL, 0.25mg/mL, 0.375mg/mL, 0.5mg/mL and 0.625mg/mL. 1mL of medium was used for each concentration. A glucose (at 5mM) control was also set up. The inoculum used was the 2nd transfer of the seawater enrichment grown on HSL-N pH 7. This experiment was also performed in duplicate using the "pure" liquid culture (from isolated colony grown in liquid). A no carbon source control was also set up.

TRANSFER OF ISOLATED COLONY INTO LIQUID MEDIUM. Some colonies were picked and re-inoculated into liquid medium. In the first attempt, similar looking colonies from the same plate were picked (using a pulled Pasteur pipet connected to a hose and pipet tip and using mouth suction) and inoculated into a buffered SW base medium with VAI and medium without VAI. In the second attempt, a single colony was picked, resuspended in 10μL of seawater base, and 3μL of the suspension inoculated into each of the following media: buffered SW with 3mM VAI, or with 5mM glucose, or without any carbon source.

EFFECT OF AMPICILLIN ON GROWTH. To test if the bacterium was a *Planctomycete*-like organism, 0.2mg/mL ampicillin (sodium salt) was added to buffered SW base with either 3mM VAI, 5mM glucose or no carbon source.

Results

ENRICHMENTS. Cultures did not turn visually turbid, and the highest turbidity recorded was around 0.06 OD. Hence, enrichments were examined microscopically daily or every other day for growth. During the first couple of days in the initial enrichment, there was a wide diversity of cell morphologies. There were motile spirilla, motile rods, stationary rods and cocci, and they were present in the no carbon source control as well. There was probably still a moderate amount of carbon source in the inoculum that bacteria that did not utilize HSL could thrive on. In subsequent enrichments, the number of different cell morphologies decreased and there were three dominant morphologies that were present in enrichments with VAI as a carbon source and not in the no carbon souce control. They were ovoid cells that seem to divide by budding and formed rosettes (nicknamed pudgy rods) (these ovoid cells were also sometimes motile), rods that clumped together in no particular fashion but seem to be associated with the pudgy rods (nicknamed slender rods), and bulgy filaments (nicknamed butt-ugly filaments) (Figure 2). The enrichments at pH 6 had a lot less growth than those at pH 7, especially in subsequent transfers. The pudgy rods that form rosettes were found in all the different enrichments but predominated in the enrichments with ammonium chloride at pH 7 with seawater as an inoculum. The motile pudgy rods also seem to be aerotactic. The seawater enrichment without an added nitrogen source at pH 7 contained many slender rods, but they could not be separated from the pudgy rods. Some slender rods were also present in the sediment enrichment at pH 7 with added nitrogen, again associated together with the pudgy rods. The latter might be producing an extracellular matrix which traps other bacteria. The butt-ugly filaments were present in the seawater enrichments at pH 7 without added nitrogen. The microbial mat enrichment contained pudgy rods but there were many other morphotypes present as well. Eukaryotes were abundant.

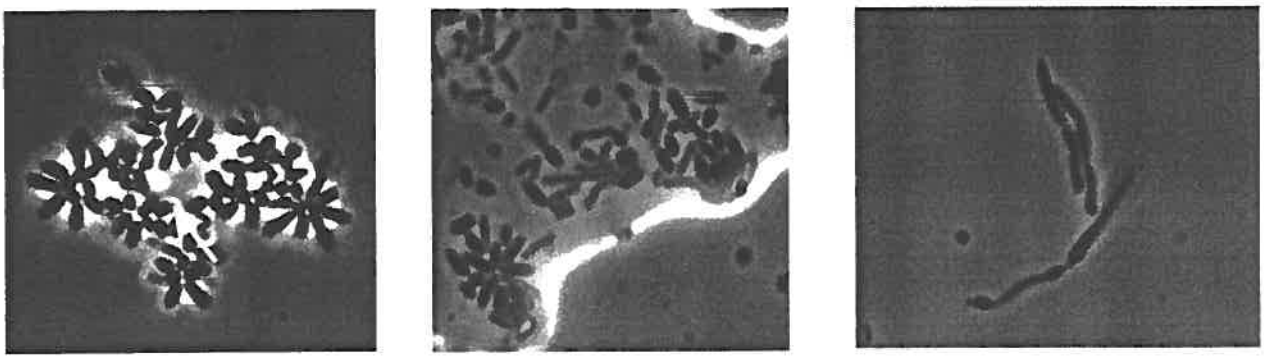

Figure 2: (from left to right) pudgy rods forming rosettes, slender rods associated with pudgy rods (arrowed), ugly filaments

ISOLATION ON PLATES. Spreading or streaking the various enrichments on complex media (SWC and 2216) did not seem to work. The colonies that grew up did not give the right cell morphologies under the microscope. Spread plates on SW base + VAl did not work well either, the colonies that came up were very tiny (though microscopically, they showed to be rosettes. However, it was near impossible to get a single colony so I was never sure which colony was the right one.). Pour plates with VAl as the sole carbon source worked well, and the majority of the colonies that came up were white translucent and extremely tiny, with a diameter of about 1mm. These colonies, when examined under the microscope, showed to be rosette formers. Similar colonies came up on the plates without a carbon source but they were much fewer in number (Figure 3), however, the cells did not seem to form rosettes.

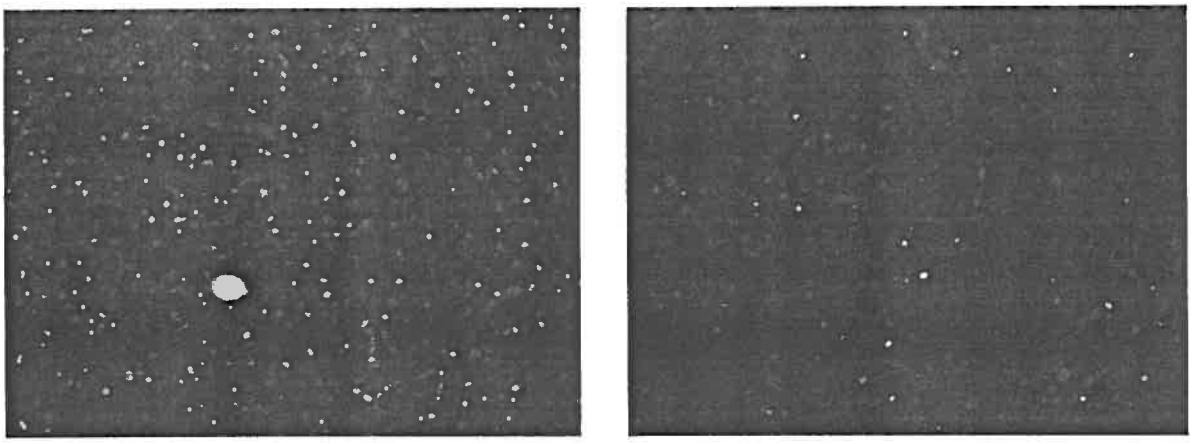

Figure 3: on left, pour plate with VAl as a sole carbon source showing numerous tiny white colonies and a big white colony; on right, pour plate without any carbon source.

REFEEDING ENRICHMENTS WITH VAl. The re-fed cultures did not show an increase in growth.

EFFECT OF VAI CONCENTRATION ON GROWTH. In the first experiment with the enrichment as the inoculum, growth was highest in the tube which contained 0.375mg/mL of VAI. Growth petered out at higher concentrations. In the second experiment (with the "pure" culture as inoculum), turbidity was seen in tubes containing 0.375mg/mL of VAI and higher. There was no increase or decrease in growth with increasing VAI concentration above 0.375mg/mL.

TRANSFER OF ISOLATED COLONY INTO LIQUID MEDIUM. In the first attempt, rosette formers were seen in the VAI-containing tube inoculated with the tiny colony from the VAI pour plate, while no growth was seen in the VAI-containing medium inoculated with a colony from the plate without carbon source (Figure 4). In the second attempt, turbidity was seen in tubes with either VAI or glucose. Upon microscopic examination, microcolonies were observed. In tubes inoculated from one colony, the rods looked longer but were still motile. The other colony produced pudgy rods, as previously observed.

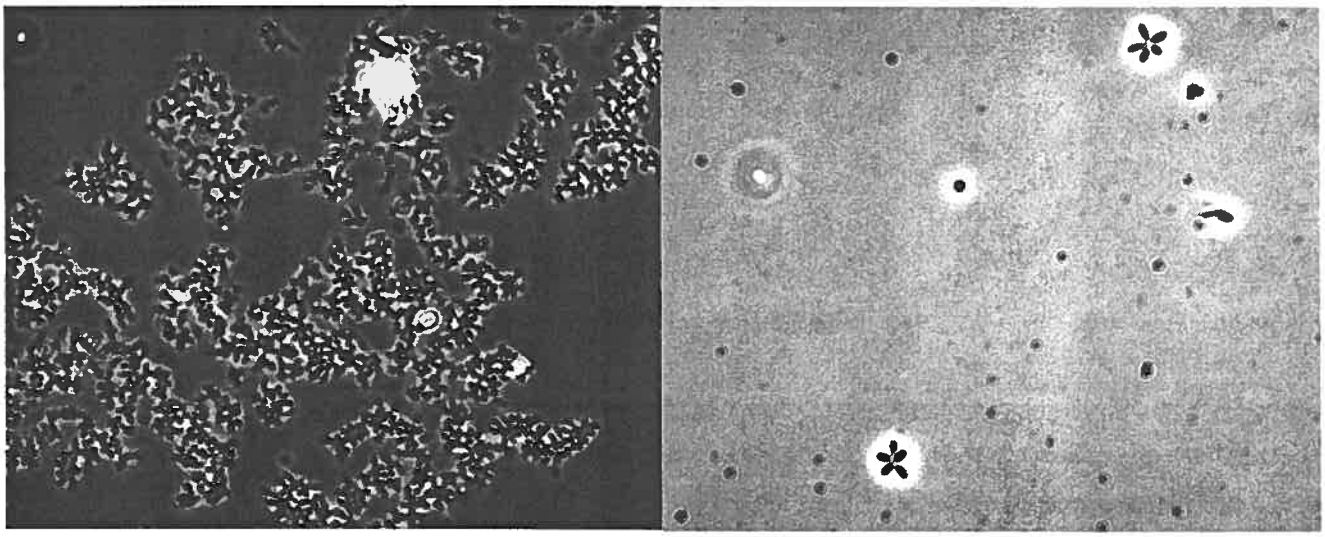

Figure 4: Rosettes from "pure" liquid culture. On left, a large clump of cells; on right, 2 rosettes.

EFFECT OF AMPICILLIN ON GROWTH. The cultures took a couple of days to turn slightly turbid, and growth seemed somewhat slower with ampicillin. However, there was still growth and the morphology was still that of rosettes. The cells however, looked more elongated and less healthy. There were few motile rods in the tube with VAI but motile rods were abundant on cells grown with glucose.

Discussion, Conclusion and Future Direction

In this study, acyl-HSL degrading bacteria have been successfully been enriched from the marine environment. Despite the lower pHs used (pH 6 or pH 7 instead of pH 8), some bacteria were still able to

grow in the enrichments. The rosette forming bacterium looked like a Planctomycete-like organism, and it resembled *Pirelulla marina* (The Prokayotes) (Figure 5), which belongs to the order Planctomycales. The physiology of this organism also agrees with my observations. Firstly, this organism is an oligotroph, which may explain why I see rosette formers in my enrichment tubes without any added carbon source. *P. marina* is also motile, and motility of pudgy rods were seen in my enrichments. This bacterium also produces a "holdfast substance", which allows cells to adhere to surfaces and to each other. This explains why there are other colony types found within the clumps.

Growth was better at pH 7 than at pH 6, and there are a couple of explanations for this. Firstly, the bacteria may prefer living at pH 7, since it is closer to its natural pH. Secondly, more VAI is hydrolyzed at pH 7 than at pH 6, and the hydrolyzed VAI may be a better carbon source than the bona fide VAI.

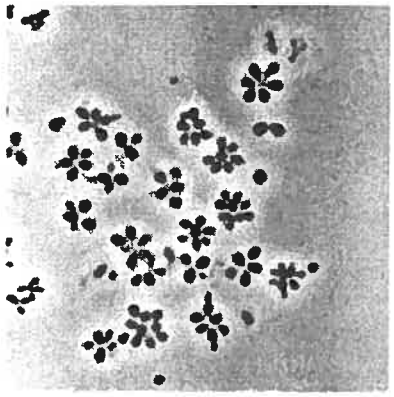

Figure 5: *Pirelulla marina* (from The Prokaryotes)

Growth was observed in media containing ampicillin, an antibiotic which interferes with peptidoglycan synthesis. This is consistent with the physiology of *P. marina*, which has a proteinaceous cell wall. Growth was a little sluggish and I am not sure why. Concentrations of up to 0.625mg/mL of VAI did not inhibit growth of the *Pirelulla*-like bacterium, despite the first experiment using a mixed culture.

Next steps include : (1) Obtaining a 16S rDNA sequence of this VAI-utilizing bacterium. (2) Obtaining this bacterium in pure culture. (3) Testing the salt and pH tolerance of this organism. (4) Conduct growth curve experiments with the pure L-isomer of VAI. (5) Investigate the chemotaxis and aerotaxis of this organism.

Acknowledgments

I am grateful to A. Schaefer and E. P. Greenberg for insight into this project, the course directors, faculty and TAs for making the lab so conducive to scientific inquiry.

References

Dong, Y-H. et al. 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acac. Sci. USA 97:3526-3531.

Dong Y-H., Wand, L-H. et al. 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactone. Nature 411: 813-817.

Eberhard, A. A. et al. 1981. Structural identification of autoinducer of *Photobacterium fisheri* luciferase. Biochemistry 20:2444-2449.

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

Schaefer, A. L. et al. 2000. Detection, purification and structural elucidation of acylhomoserine lactone inducer of Vibrio fisheri luminescence and other molecules. Methods Enzymol. 305:288-301.