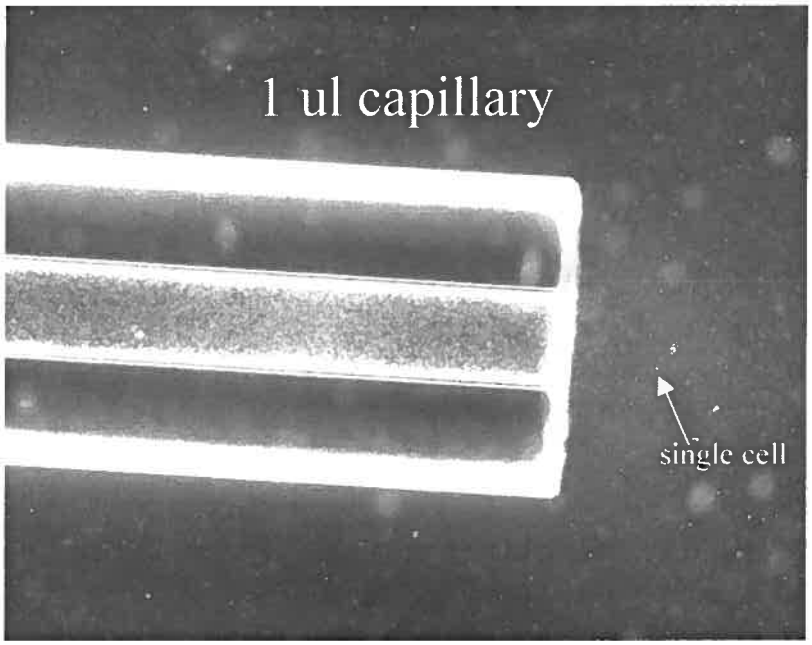
Quorum Scenting

or

Do bacteria exist that can chemotax towards acyl-homoserine lactones?



Kristen DeAngelis
University of California, Berkeley

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Introduction: Quorum Sensing + Chemotaxis = Quorum Scenting

Quorum sensing is a behavioral change in high-density bacterial populations, characterized by the ability to perform diverse functions such as extracellular enzyme production, swarming, luminescence, or virulence (Fuqua, 2001). The secretion of acyl-homoserine lactones (AHLs) in gram-negative bacteria allows detection of the concentration of AHLs to read as a proxy for cell density. The ability to sense one's bacterial density is thought to give the cell an advantage when performing a task too large to undertake alone. Although many species of bacteria are able to emit and respond to AHLs, it is unknown whether these quorum sensing compounds can act as a chemotactic agent for bacteria. Chemotactic agents can vary from simple assimilative compounds like sugars and amino acids (Falke, 2001), to more complex compounds like chitin (Bassler, 1991), and even toxins to be bioremedmdiated as in the case of *Ralstonia eutropha* chemotaxis towards its target herbicide (Hawkins, 2002).

It seems as though the ability to chemotax towards or away from a quorum sensing signal might make sense in the natural environment, since the signals are giving other cells information about their identity. If the receiving cell knows what the signal means, then this could provide an indication as to where the next biofilm is going to form, offering shelter for the motile bug, or where the next complex food source is, offering opportunity to secrete extracellular enzymes to eat some complex organic matter, or the location of a mounting attack on a host offering opportunity to infect. The goals of this study are to:

(1) Isolate QS bacteria from marine environments
(2) Test marine isolates for the ability to chemotax towards or away from AHLs
(3) Identify any bacteria that chemotax towards or away from AHLs.

Isolation and identification of bacteria able to chemotax towards or away from quorum sensing molecules would present a new paradigm for the role of QS molecules in the environment, how bacteria form a quorum, and perhaps also indicate how bacteria of different species chemically interact with each other.

Materials and Methods

Marine water samples from the nearby salt-water environments Eel Pond and Garbage Beach were collected and plated onto salt-water complete (SWC) media at 1:1, 1:10 and 1:100 dilutions. SWC media consisted of 750 ml sea water, 5 g Tryptone, 3 g yeast extract, 3 ml glycerol, pH 7.0 per L; plates included 20 g agar per L media. Colonies arose after one to two days and were picked for isolation onto new plates based on their ability to luminesce or swarm. Luminescence was identified by eye after examination in the dark for at least 5 minutes, and swarmers were identified by their fast colonization of the plate and highly irregular colony morphology. It was surmised that colonies exhibiting swarming or luminescent behavior might be more likely to possess quorum sensing systems, and therefore be more likely to respond to a chemotaxis assay for QS signal molecules. The culture was determined a pure isolate by colony morphology and wet mount, usually after one to three transfers on SWC plates. Isolates were then frozen at –80C in SWC media plus 25% glycerol. All isolates examined were amenable to freezing and could be grown with normal colony morphology from freezer stocks.

Once in pure cultures on plates, isolates were characterized based on colony morphology, cell morphology, and identified via sequencing of the 16s ribosomal DNA gene or by ARDRA with the enzyme *Hha*I of the ribosomal DNA gene. ARDRA was used for rough identification in addition to 16S ribosomal DNA sequencing because many sequences came back incomplete or undetermined. Similar ARDRA patterns indicated a high likelihood of similar isolates. Sequences of 16S rDNA were compared to Michigan State University's Ribosomal Database Project (http://rdp.cme.msu.edu/html) and also to the National Center for Biotechnology Information's GenBank database BLAST server (http://www.ncbi.nlm.nih.gov/BLAST). In all cases these two databases agreed well with each other and were used as an approximate identifier of isolated luminescent and swarmer bacteria.

To prepare isolate strains for the chemotaxis capillary assay cells had to be motile in the Taxis buffer, which consisted of 50% artificial seawater, 0.1% NH_4Cl, 0.002% K_2HPO_4, 150 mM HEPES buffer pH 7.5, and 1.5 ml glycerol per L (Bassler, 1991). The isolate strain was grown in liquid SWC media overnight from a single colony from a plate. This overnight culture was used to inoculate 30 ml of fresh SWC media in a 50ml flask. Isolates were grown until it was determined by wet mount at 100x that the cells were all motile in the culture, which usually occurred when a 600nm optical density of about 0.05 was reached. Once all cells were swimming, two aliquots of 1.5 ml each were centrifuged at 2500 rpm for two minutes. Most of the media supernatant was aspirated off and replaced with 1ml of Taxis buffer. Once resuspended in Taxis buffer, the cells were again checked for motility by wet mount. If most cells were still swimming, this prep was used for capillary chemotaxis assays.

For each chemotaxis capillary assay, the washed cells to be tested for chemotaxis were placed in a chamber formed by a 2mm capillary formed into a "U" shape between a microscope slide and a coverslip, as per Hawkins et al (2002). At the open end of the U-tube, chemotactic chemical was introduced to the cells in a 1 ul capillary. The diffusion of the chemotactic chemical into the washed cell suspension creates a gradient. If the chemotactic chemical is an attractant, the cells will swim up the capillary; if it is a repellant, they will swim away from the mouth of the capillary.

Isolates were examined for chemotaxis using a capillary mount on a glass slide under the light microscope. Capillary chemotaxis assays were performed with 50% SWC as the positive control and 1% EtOH as the negative control; all chemoattractant chemicals are diluted in Taxis buffer. The AHL 3oxoC6-HSL (Sigma, K3255), one of the cognate AHLs of *Vibrio fischeri*, was tested as chemoattractants to the luminescent and swarming marine isolates. This acyl-homoserine lactone was chosen for its availability and for its moderate solubility in water (stock solution 1M in 50% EtOH and water, chemotaxis solution 0.5 – 2 mM in 1% EtOH and Taxis buffer). As an additional test, the degradation product of the AHL signal, homoserine lactone, was also tested for chemoattractant properties.

Chemotaxis was observed under an inverted Zeiss light microscope at 100x. Time-lapse photos taken every 10 seconds for five minutes tracked the swimming of the cells towards or away from the mouth of the capillary tube. The chemotactic properties of the oxoC6-HSL were measured for their ability to change the strength of the response of the cells to the attractant 50% SWC. Chemotaxis was measured by recording the moment at which the "swell" of cells entering the capillary tube was the largest, which I called the maximum attraction time. Because the time-lapse was only every 10 seconds, maximum attraction times are reported as seconds in multiples of ten, and in cases where multiple replicates were tested of the same experiment, maximum attraction times are reported as averages of all trials ± standard error of the mean.

Results

Identification and isolation of luminescent and swarming bacteria proved quite easy, thus I was able to accumulate a total of fifteen isolates for chemotaxis analysis, thirteen luminescent and two swarmers. Four of the fifteen luminescent isolates came from classmates who isolated them during the luminescence and growth curve lab exercise. Sequencing of these fifteen 16S rDNA genes of these isolates showed that we isolated three isolates of *Vibrio alginolyticus*, four isolates of *Vibrio fischeri*, two isolates of *Pseudoalteromonas atlantica* (one of which was not luminescent but was a swarmer), and one isolate of *Vibrio wodanis*. Five of the ten isolates were not sequenced because of inaccurate template concentration. Of the remaining five, two did not PCR well enough to perform ARDRA, and the other three fell into the same ARDRA-type class that was different from the other sequenced isolates. So although ARDRA analysis and 16S rDNA sequencing agreed well, it was not possible to classify these three remaining ARDRA-types with respect to sequenced 16S rDNA genes; these isolates were called "ARDRA type 1" to distinguish them as unidentified clones of the same ARDRA-type.

Most of the analysis of chemotaxis for oxoC6-HSL was performed using *Vibrio alginolyticus* isolate K2a. This isolate was chosen from a preliminary screen, which showed that the behavior of the motile cells was different in the presence and absence of oxoC6-HSL. At levels of 2mM oxoC6-HSL it appeared that this chemical was a mild repellant of the cells, but it was very difficult to measure the repulsion. So in a compromise it was thought that the cells could be shown oxoC6-HSL at the same time as a strong attractant, 50% SWC media, and the decrease in attraction by oxoC6-HSL addition could be measured more easily. The *V. alginolyticus* isolate moved even faster towards the oxoC6-HSL in 50% SWC than it did to 50% SWC alone (Figure 2). Average time recorded for maximum attraction times for 50% SWC was 159 seconds (standard error 7.4 sec, n = 8), and 2mM oxoC6-HSL in 50% SWC was 93.3 seconds (standard error 3.3 sec, n = 4). This is a significant difference in attraction time. In examining different levels of oxoC6-HSL it was determined that there was no real difference between 0.5 mM, 1 mM and 2 mM oxoC6-HSL in 50% SWC (Figure 1). When the oxoC6-HSL signal was added alone, there seemed to be no chemoattraction and perhaps only slight chemorepulsion after about three minutes exposure. Cells that were shown 1% ethanol in Taxis buffer as a negative control exhibited no response of attraction or repulsion. Cells shown 0.5, 1 or 2 mM homoserine lactone, a degradation product of oxoC6-HSL in 50% SWC showed the same chemotactic attraction as to 50% SWC alone.

Other isolates were tested for changes in attraction to 50% SWC with addition of oxoC6-HSL (Table 2). Another isolate that had a similar response to oxoC6-HSL was isolate K3, possibly also *V. alginolyticus* as determined by ARDRA. This isolate showed a maximum attraction time of 210 seconds to 50% SWC but only 100 seconds when oxoC6-HSL was also added. Though this experiment was not repeated on this isolate, we can be fairly confident of the numbers because of the replicates performed on the sister isolate K2a. Another unidentified isolate, HCQ, showed the opposite response as *V. alginolyticus*; it had a much faster reaction time to 50% SWC alone (80 sec) than it did to 50% SWC plus oxoC6-HSL (140 sec). A third unidentified isolate, RP1, showed no change in response between attractant (160 sec) and attractant plus signal (180 sec). These last two isolates had the same ARDRA patterns but because of this physiological difference in chemotaxis may turn out not to be the same species.

Discussion

Chemotaxis studies on the K2a isolate identified as *Vibrio alginolyticus* appeared to be more attracted to 50% SWC with addition of 2mM oxoC6-HSL, while addition of oxoC6-HSL alone had no or slightly chemorepulsive effect. I propose two hypotheses to explain this apparent contradiction in chemotactic behavior of this isolate. The first hypothesis is that the AHL has its own methyl-accepting chemotaxis protein. This MCP that senses AHLs would likely be a cytoplasmic MCP since it is thought that there is no membrane-bound transporter for AHLs but that their ampiphilic nature makes them freely diffusible through cell membranes such that the concentration inside of the cell is an exact reflection of the concentration of signal outside the cell. In this case the contradiction in behaviors could be explained by the interacting of two phosphorelay cascades that arises from two competing MCPs being activated by chemotactic compounds.

The second hypothesis to explain *V. alginolyticus* isolate K2a behavior is that the QS signal cascade is branched, with one signal cascade that feeds into the changes in gene expression characteristic of QS "on" cells, and the other signal cascade inserting into the chemotaxis phosphorelay downstream of the MCPs. *V. alginolyticus* has not been analyzed for a quorum sensing system, but its close relative *V. harveyi* has two quorum sensing systems (Mok, 2003). The signal receptors LuxN and LuxQ area two-component system receptor kinases which phosphorylate downstream activators to induce changes in gene expression when the cognate signal molecule binds. It is possible that these sensor kinases can also function to change the phosphorylation state of CheA or CheW, which then effects the direction of flagellar motor rotation. Having two different kinases affecting the phosphorylation of CheA or CheW would result in modification of behavior of the cells motility in the face of each chemotactic agent alone compared to in combination.

Since the isolate does not respond to the degradation product of the signal homoserine lactone, we may further conclude that perhaps the change in behavior of the cells is a combination of chemotaxis towards the attractant and activation of the quorum sensing system in *V. alginolyticus*. Proper identification of isolates and classification of their QS systems may be the key to understanding the role of QS in affecting chemotaxis. However, lower concentrations of oxoC6-HSL seemed to attenuate the increased response to 50% SWC, although at such low replication rate this effect did not seem to be significant. However, if this were a genuine trend then it could indicate that at physiological levels of signal necessary to induce QS-controlled gene expression, which are in the micromolar range, there would be no effect on chemotaxis towards 50% SWC. This experiment could easily be performed and would be most informative by examining a cognate and non-cognate AHL to the *V. alginolyticus* isolate.

My beginning hypothesis was that *V. fischeri* would be able to chemotax towards one or both of its cognate AHLs if this mechanism exists, while *V. harveyi* would not. These isolates would have made particularly good studies for QS chemotaxis because they are likely to be motile, their quorum-sensing mechanisms are well characterized, and they do not respond to each other's cognate AHLs (Bassler, 1997). The fact that identification of the organisms came too late either prevented me from attempting to isolate a *V. fischeri* specifically, *V. fisheri* was isolated but not positively identified. Nonetheless, it seems that *V. alginolyticus* is able to respond to very high levels of 2 mM oxoC6-HSL in the presence of 50% SWC, a strong attractant. Future studies could use these isolates to grow and extract, using ethyl acetate, AHLs from culture to also be used in the chemotaxis assays.

Acknowledgements

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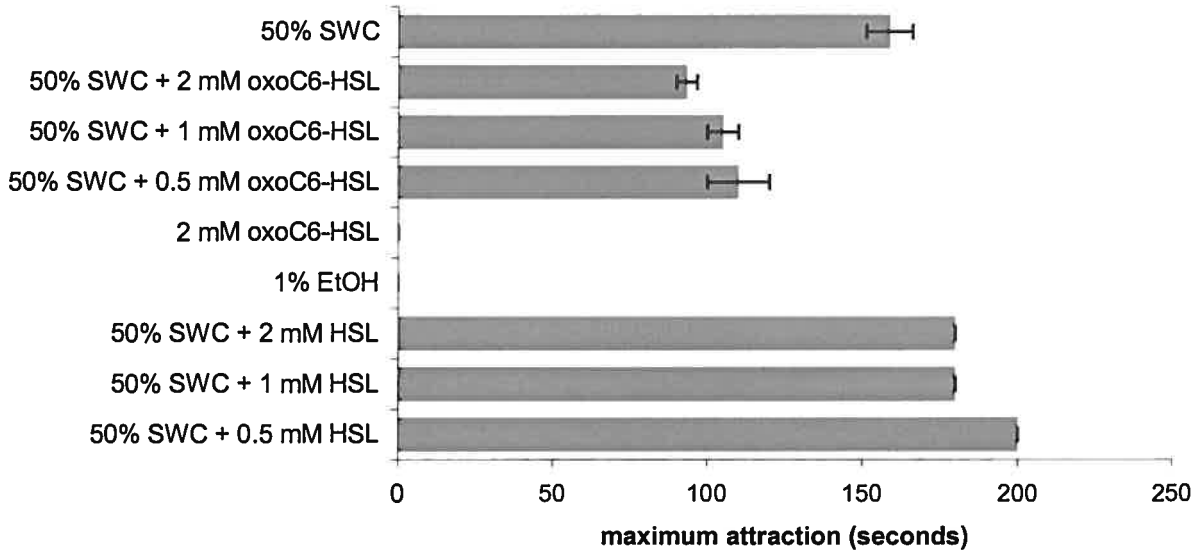
Figure 1. Chemotaxis of *Vibrio alginolyticus* marine isolate K2a towards QS signal oxoC6-HSL.

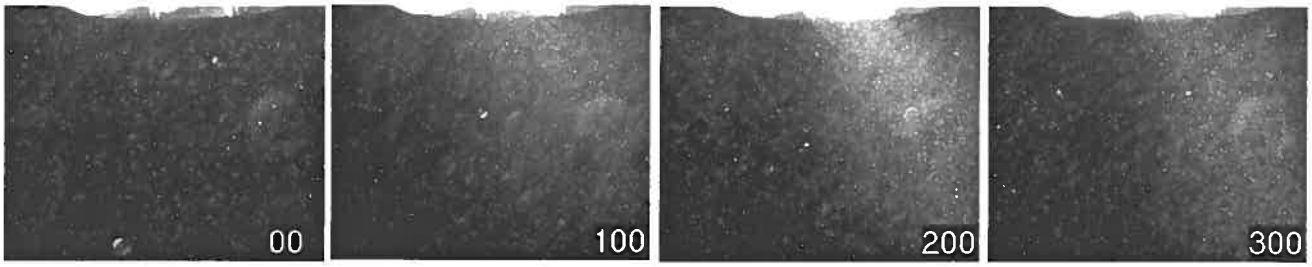


K2a *Vibrio alginolyticus*		
chemoattractant*	response	max attraction (sec+/-std err, (n))
50% SWC	++	159 +/- 7.4 (6)
50% SWC + 2 mM oxoC6-HSL	+	93.3 +/- 3.3 (4)
50% SWC + 1 mM oxoC6-HSL	+	105 +/- 5.0 (2)
50% SWC + 0.5 mM oxoC6-HSL	+	110 +/- 10 (2)
2 mM oxoC6-HSL	0	0 (2)
1% EtOH	0	0 (8)
50% SWC + 2 mM HSL	++	180 (1)
50% SWC + 1 mM HSL	++	180 (1)
50% SWC + 0.5 mM HSL	++	200 (1)

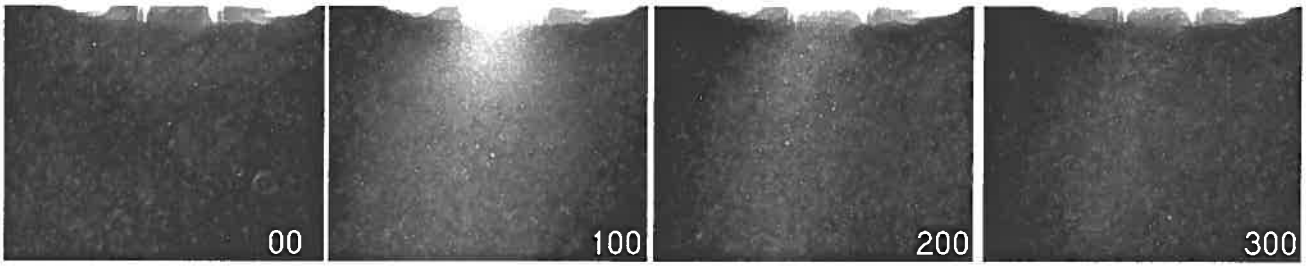
Chemotaxis capillary assays were performed on the luminescent marine isolate K2a, identified by 16s rDNA sequencing as *Vibrio alginolyticus*. All chemoattractants are in Taxis buffer, which is 50% ASW, 0.1% NH4Cl, 0.002% K2HPO4, 50 mM HEPES pH7.5, 1.5 ml glycerol per Liter. Above shows a graphical depiction of the data, listed in the table below as mean maximum attraction times, standard error where appropriate, and number of replicates in parentheses. It seems clear that while there was no attractive effect of the oxoC6-HSL signal alone, that the 50% SWC strong attractant became an even stronger attractant when introduced with signal present.

Figure 2. Time-lapse images of chemotaxis capillary assay for *V. alginolyticus* isolate K2a.

a. Attraction to 50% SWC (sec)



b. Attraction to 50% SWC + 2 mM oxoC6 HSL (sec)



Time-lapse photos were taken of chemotaxis towards the strong attractant 50% SWC media (a) and 50% SWC media plus 2mM oxoC6-HSL (b). Photos were taken every ten seconds for five minutes; shown are excerpts from the 0, 100, 200, and 300 second timepoint. At the top of each image is the capillary opening which emits a gradient of chemotactic agent into the surrounding buffer containing the cells to be evaluated. From these photos it can clearly be seen the maximum attraction of cells at 200 seconds in (a) and 100 seconds in (b), which is defined as the point when the most cells are gathered outside of the mouth of the capillary tube. After this time, the cells begin movement into and up the capillary tube as they consume the attractant and shift the gradient.

Table 2. Maximum attraction time of marine isolates to the QS signal molecule oxoC6-HSL.

			Measurement of maximum attraction time (seconds)	
Isolate	ID (by RDP & NCBI BLAST)	location	oxoC6-HSL + 50% SWC max attraction (sec)	50% SWC max attraction (sec)
K2a	*Vibrio alginolyticus*	Garbage Beach	**93.3**	**157**
K2b	*Vibrio alginolyticus*	Garbage Beach	nd	nd
K3	*Vibrio alginolyticus (?)*	Garbage Beach	**100**	**210**
WMH	*Vibrio fischeri*	Eel Pond	nd	nd
RP2	*Vibrio fischeri*	Eel Pond	nd	nd
Vpa	*Vibrio parahaemolyticus*	culture collection	nd	nd
HCQ	ARDRA type 1	Eel Pond	**140**	**80**
RP1	ARDRA type 1	Eel Pond	**160**	**180**

Other marine luminescent or swarming isolates were tested for changes in chemotactic behavior towards 50% SWC in the presence of oxoC6-HSL. Of the three other isolates that were tested, K3 reacted more quickly in the presence of signal, just like K2a, HCQ reacted less quickly, and RP1 had the same reaction.