INTRA- AND INTER-SPECIES COMMUNICATION IN THE MARINE ENVIRONMENT

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LONG-TERM GOALS

The long-term goal of this research is to explore the molecular mechanisms that marine bacteria use for intra-species and inter-species cell-cell communication. To do this we will investigate the functions controlled by the two quorum sensing signalling pathways of the model luminous bacterium *Vibrio harveyi*. We have shown that *V. harveyi* uses Signalling System 1 for intra-species communication and Signalling System 2 for inter-species cell-cell communication. We will identify target genes that are regulated exclusively by System 1 and System 2, and those target genes that are jointly controlled by both Systems. Our analysis of this regulatory network will lead to a molecular understanding of how bacteria integrate, process and transduce sensory information to control gene expression in response to inter-cellular communication.

OBJECTIVES

1. Identify the genes controlled by Signalling System 1 and Signalling System 2 in *V. harveyi*.
2. Examine the functions encoded by the genes identified in objective 1.
3. Determine the regulatory pathways that control intra- and inter-species communication in *V. harveyi*.
4. Test for production of autoinducers and autoinducer analogues (inhibitors) by other marine bacteria and lower marine eukaryotes.

APPROACH

Objective 1: Phage P1 was used to deliver a Mini-Mu*lac* transposon to the chromosome of *V. harveyi* strains that were defective in production of either the System 1 autoinducer (AI-1) or the System 2 autoinducer (AI-2). The two mutant banks were replica plated onto another set of petri plates containing Xgal and either AI-1 or AI-2. Colonies that exhibited altered *lacZ* activity in response to the addition of autoinducer were identified.

Objective 2: The AI-1 and AI-2 regulated fusions were sequenced following cloning of the genomic DNA flanking the Mini-Mu transposon. Database analysis was used to determine the identities of the genes.

Objective 3: The signalling pathways linking AI-1 and AI-2 detection to the specific target outputs were determined by introducing null or activated alleles of Lux signalling proteins into the *V. harveyi lac* insertion strains and measuring the activity of the fusions.

Objective 4: Cell-free culture fluids from 250 phytoplankton isolates were tested for AI-2 activity using a *V. harveyi* AI-2 reporter strain. Additionally, we determined the biosynthetic pathway for AI-2 and developed an *in vitro* method for AI-2 production. The structure of AI-2 was determined by generating a crystal and solving the structure of the *V. harveyi* LuxP sensor in complex with AI-2.

WORK COMPLETED

Objective 1: This objective has been completed. We identified 30 genes that are regulated by AI-1 and 11 genes that are regulated by AI-2 in *V. harveyi*. Two graduate students, Ken Mok and Jennifer Henke, have performed all of this work.

Objective 2: This objective has been completed. A variety of genes were identified including a Type III secretion system, two metalloproteases, chemotaxis genes, transcription factors and a number of unidentified ORFs. Ken Mok and Jennifer Henke performed this research.

Objective 3: This objective has been completed. The two-component systems we have already identified in previous work are the circuits that regulate the AI-1 and AI-2 targets. Importantly, this work led to the finding that AI-1 and AI-2 act synergistically to control gene expression. Furthermore, we demonstrated that although all the sensory information is channeled through a single response regulator, LuxO, the *V. harveyi* circuit retains the capacity to distinguish between AI-1 and AI-2 because each of these signals has a discrete and measurable impact on the activity of LuxO. Therefore, the autoinducer specific information flowing through LuxO is preserved because the circuit is designed to convert digital inputs into a single analog readout. Ken Mok performed this work in collaboration with a physicist Dr. Ned S. Wingreen of NEC Inc.

Objective 4: The phytoplankton samples were tested for autoinducer agonist and antagonist activity. None was observed. However, our recent structural identification of AI-2 (see below) facilitated the development of synthetic procedures to make AI-2 analogues and they are being tested for agonist and antagonist activity. A postdoctoral fellow, Stephan Schauder, determined the AI-2 biosynthetic pathway and developed the *in vitro* AI-2 biosynthesis procedure. Schauder, a fellow postdoc Xin Chen and Prof. F. Hughson (Molecular Biology Dept.) performed the structural analyses.

RESULTS

We have used a combination of genetic and biochemical analyses to identify the components of the *V. harveyi* quorum sensing circuit. Our model is shown for how *V. harveyi* accomplishes cell-cell communication is shown in Figure 1.

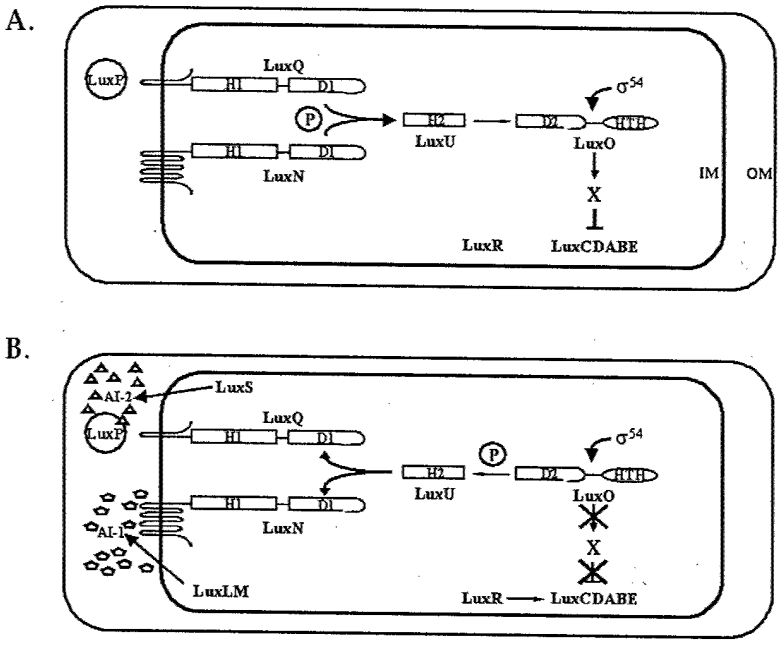

Figure 1. The *V. harveyi* Quorum-Sensing System. The low and high cell density states of the *V. harveyi* quorum-sensing system are shown (Panels A and B, respectively). H, D, IM, OM, and H-T-H denote histidine, aspartate, inner membrane, outer membrane and helix-turn-helix, respectively. The "P" in the circle signifies that signal transduction occurs by phosphorelay. Phosphate flow in the forward direction goes from histidine (H1) to aspartate (D1) to histidine (H2) to aspartate (D2). AI-1 and AI-2 are depicted as pentagons and triangles, respectively.

We identified a gene that we named *luxS* and showed that it was required for AI-2 production in *V. harveyi*. We further showed that *luxS* exists in over 40 species of Gram-negative and Gram-positive bacteria, and in every case, *luxS* is required for the production of AI-2. These findings led us to the idea that AI-2 is a signal molecule used by numerous bacteria for inter-species cell-cell communication. We found that 40-50 genes are controlled by the autoinducers in *V. harveyi*. We are particularly interested in those regulated by AI-2. To better understand AI-2 signalling, we determined the biosynthetic pathway for AI-2 and showed that LuxS is the AI-2 synthase. We demonstrated that AI-2 is produced from *S*-adenosylmethionine in three enzymatic steps. Specifically, when SAM is used as a methyl donor, *S*-adenosylhomocysteine (SAH) is produced. We showed that an enzyme called Pfs converts SAH to *S*-ribosyl homocysteine (SRH) and adenine, and subsequently, LuxS acts on SRH to make homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (Figure 2). The latter compound undergoes further rearrangements to form AI-2. We purified the Pfs and LuxS enzymes from diverse bacteria, and used them to synthesize AI-2 from SAH *in vitro*. In every case, AI-2 of identical specific activity was produced, and the other product of the LuxS reaction was homocysteine. These findings strongly suggest that AI-2 molecules from diverse species of bacteria are chemically identical. This result suggests that, unlike quorum sensing systems that are regulated by related homoserine lactone autoinducers, AI-2 is a unique, "universal" signal that could be used by a variety of bacteria for communication among and between species.

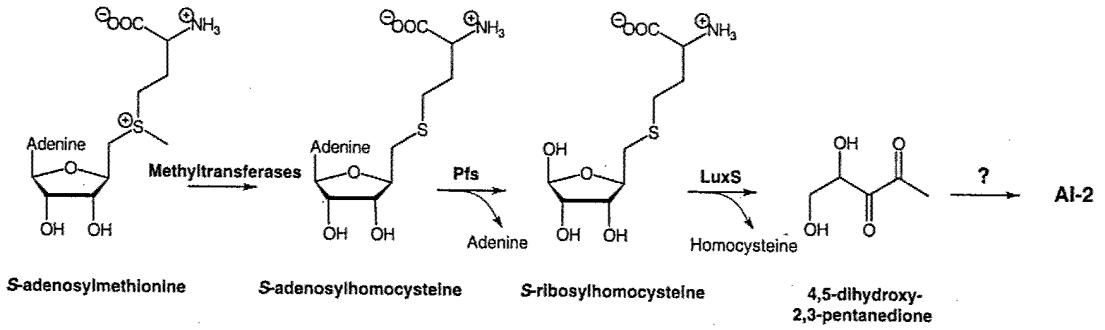

Figure 2. Biosynthesis of the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) from *S*-adenosylmethionine. *S*-adenosylmethionine donates methyl groups to various cellular substrates.

The X-ray structure of the LuxP-AI-2 complex was determined by multiple isomorphous replacement and refined using data to 1.5 Å resolution (Fig. 2a). The deep cleft between the two LuxP domains contains additional electron density, corresponding to the AI-2 ligand, that was clearly visible even in initial experimental electron density maps at 2.8 Å resolution. The quality of the additional electron density improved steadily as refinement of the protein model progressed until, at 1.5 Å resolution, it was straightforward to construct a model for AI-2 by positioning atoms in the difference electron density (Figure 3b-d).

In selecting atoms to place in the electron density, we considered the intensity of the observed density, issues of chemical valence and stability, potential hydrogen bonding partners, and the relationship of the proposed ligand to its presumptive precursor, DPD. The proposed AI-2 structure is a furanosyl borate diester stabilized within the LuxP binding site by numerous polar interactions (Fig. 3). Its molecular weight (194 Da) matches the mass spectrometric difference between holo-LuxP (containing bound AI-2) and apo-LuxP (lacking bound ligand). To confirm that the ligand contains boron, we performed ^{11}B NMR spectroscopy. The AI-2 molecule is shown in Figure 4.

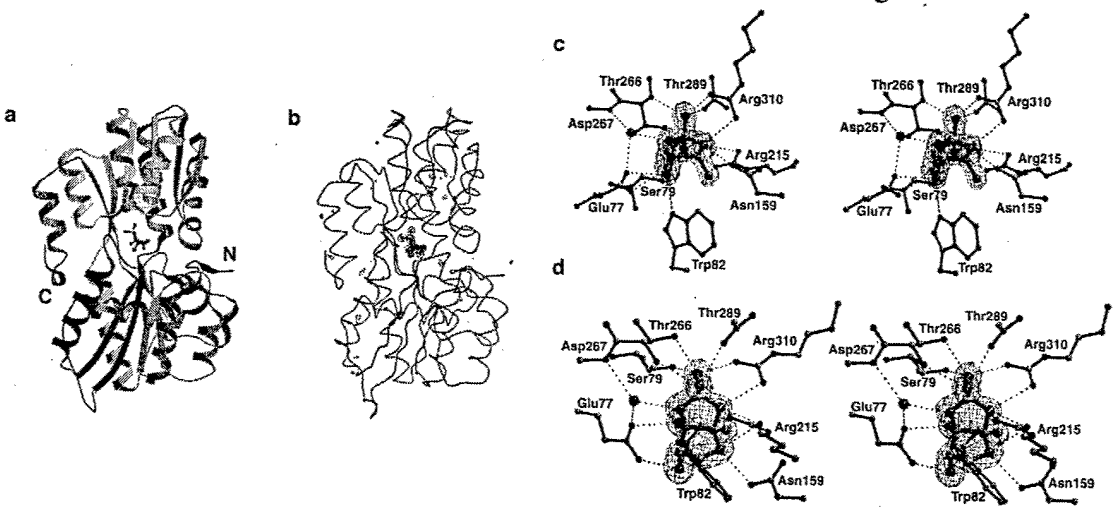

Figure 3. Structure of LuxP-AI-2 complex. a. Overview. b-d. F_o - F_c difference electron density (contoured at 4σ) calculated using phases derived from the model prior to AI-2 addition. The final refined

model for AI-2 is shown superimposed on this density. Boron, oxygen, nitrogen and carbon are colored yellow, red, blue and gray, respectively. Hydrogen bonds are shown in c and d as dashed red lines.

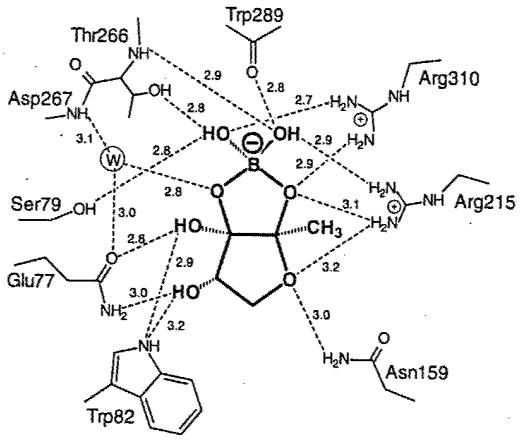

Figure 4. Structure of AI-2. AI-2 and the hydrogen bond network that stabilizes it in the LuxP binding site. O-O or O-N distances for potential hydrogen bonds are shown in Å.

To continue our investigation of intra- and inter-species communication in *V. harveyi*, we identified genes in addition to *luxCDABE* that are members of the *V. harveyi* quorum-sensing regulon, and we determined the mechanism of their regulation. We investigated the individual roles of AI-1 and AI-2 in quorum-sensing gene regulation and showed that AI-1 and AI-2 act synergistically. Because the *V. harveyi* quorum-sensing circuit responds to two signals, AI-1 and AI-2, there are at least four different input states: no autoinducer, AI-1 only, AI-2 only, and AI-1 + AI-2. Our measurements of light production showed that the circuit has the potential to distinguish between all four states. However, under the conditions of our experiments, the *V. harveyi* quorum-sensing circuit appears to function primarily as a coincidence detector that differentiates between the presence of both autoinducers and all other input states (Figure 5).

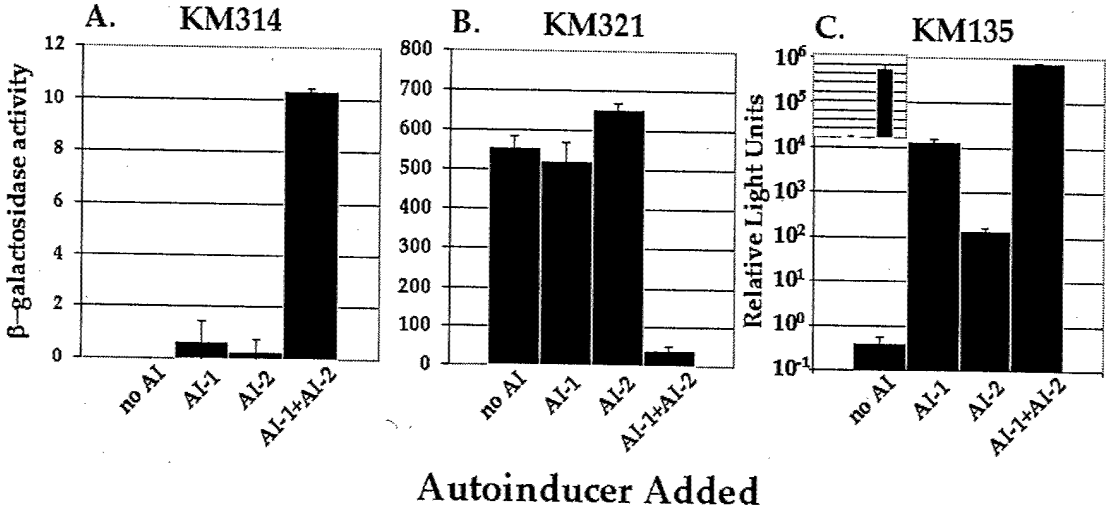

Figure 5. AI-1 and AI-2 Act Synergistically. β-galactosidase activities of the fusions to two quorum sensing targets in the AI-1$^-$, AI-2$^-$ strains (KM314, *hap-lacZ*) and (KM321, *popB-lacZ*) and light production of in the AI-1$^-$, AI-2$^-$ strain KM135 are shown in Panels A, B, and C, respectively. Note the logarithmic scale in Panel C; the inset shows the identical data plotted on a linear scale. Relative Light Units (RLU) are defined as counts min-1 ml-1 x 103 /cfu ml-1.

Examination of our data gives us a means to connect the responses of *V. harveyi* to autoinducers with the molecular circuitry of the Lux system. Our bioluminescence measurements show that the *V. harveyi* quorum-sensing system can discriminate between no autoinducer, AI-1 only, AI-2 only, and AI-1 + AI-2, demonstrating that the binary information encoded in the presence or absence of one or both autoinducers is preserved by *V. harveyi*. Since this and previous work clearly show that all of the sensory information transmitted through the *V. harveyi* circuit converges on LuxO, the information supplied by the presence or absence of each autoinducer must be internally represented by four widely separated levels of LuxO-P. Hence, the observation of four distinguishable levels of bioluminescence implies that a large decrease in [LuxO-P] must occur following the addition of each autoinducer. In effect, the *V. harveyi* quorum-sensing system converts two binary inputs, i.e., low/high concentrations of the two autoinducers, into a four-level output, i.e., the four discrete levels of LuxO-P. This "digital processing" can be accomplished if the presence of one autoinducer initiates a phosphatase activity that is much stronger than the remaining kinase activity, but not strong enough to completely deplete LuxO-P, as occurs when both autoinducers are present.

IMPACT

We have demonstrated that bacteria have evolved multiple chemical languages for communicating within and between species. Intra- and inter-species cell-cell communication allow bacteria to coordinate various biological activities and in so doing bacteria behave like multicellular organisms. The functions regulated by quorum sensing are diverse and reflect the unique needs of specific bacterial communities inhabiting distinct niches. Competing bacteria and susceptible eukaryotic hosts have evolved strategies for thwarting quorum-sensing bacteria by destroying the chemical signal molecules or producing signal antagonists that interfere with recognition of the true autoinducer molecule. In symbiotic associations, bacteria and most likely their eukaryotic hosts have evolved tactics that augment the quorum-sensing abilities of beneficial bacteria. These natural strategies that inhibit/enhance quorum-sensing are being used as models in the design of analogous synthetic therapies intended to manipulate quorum-sensing systems in bacteria. Research is now focused on the design and/or identification of molecules that are structurally related to autoinducers. Such molecules have potential use as anti-microbial drugs and anti-biofouling agents. Similarly, the biosynthetic enzymes involved in autoinducer production as well as the autoinducer detection apparatuses are viewed as potential targets for anti-bacterial and anti-biofouling compound design. Finally, biotechnological methods aimed at exploiting

useful bacterial quorum-sensing processes have potential uses in the improvement of industrial-scale production of natural products such as antibiotics.

TRANSITIONS

The *V. harveyi* autoinducer reporter strains constructed in this laboratory have been given to over 700 academic researchers for use in studying AI-1 and AI-2 signalling in a variety of bacteria. These same strains, the *luxS* gene, the AI-2 structure, the AI-2 biosynthetic procedure and the AI-2-LuxP crystallography data have been licensed from Princeton by Quorex Pharmaceuticals for the development of anti-microbial drugs. Additionally we have begun analysis of quorum sensing in *Vibrio cholerae*. Much of the work from these studies has also been licensed by Quorex to begin work on anti-Cholerae vaccines.

RELATED PROJECTS

1. *Vibrio cholerae* Quorum Sensing. We performed a genetic analysis to identify and characterize the genes encoding the *V. cholerae* quorum sensing circuit. We found that three parallel quorum sensing systems exist and, together, these communication systems converge to control virulence and biofilm formation. Graduate students Melissa Miller and Derrick Lenz performed the *V. cholerae* genetic analyses and virulence studies. This work has been published. We have begun to characterize how the *V. cholerae* quorum sensing circuit controls biofilm formation. To date, we have shown that low density promotes biofilm formation while high cell density and the presence of autoinducers inhibits biofilm formation. A postdoc, Brain Hammer has performed this research, and it has been submitted for publication.

2. The *V. harveyi* and *V. cholerae* repressors of luminescence (denoted X) in Figure 1 have been identified by a combined genetic and bioinformatic approach. In both bacterial species we have shown that the Lux repressor is an RNA binding protein called Hfq that acts in conjunction with a small RNA (sRNA) to control translation. We have identified the sRNA and it lies adjacent to *luxOU* in both the *V. harveyi* and *V. cholerae* chromosomes. We have shown that Hfq and the sRNA act to control translation of *luxR* in *V. harveyi*) and the homologous gene *hapR* (in *V. cholerae*). This work is being prepared for publication. Graduate students Kenny Mok and Derrick Lenz did this work.

3. *V. harveyi* Quorum sensing controls Type III secretion. As part of our analysis to identify genes regulated by quorum sensing in *V. harveyi*, we found that *V. harveyi* possesses a type III secretory system, and that its expression is controlled by autoinducers. We cloned and sequenced the 4 operons encoding the system. We made *lacZ* transcriptional reporter fusions to genes in each operon to show that expression of the apparatus is indeed controlled by quorum sensing. Finally, we prepared antibodies to effectors predicted to be secreted through the channel, and showed that their production and secretion is controlled by cell-cell communication. Jen Henke, a graduate student performed this work and she is currently writing it up for publication.

4. *Salmonella typhimurium* Quorum Sensing. LuxS and AI-2 regulate the expression of a previously unidentified operon encoding an ATP Binding Cassette (ABC) type transporter that we named the *lsr* (*luxS* regulated) operon. This transporter has homology to the ribose transporter of *E. coli*. A gene encoding a DNA binding protein that is located adjacent to the Lsr transporter structural operon is required to link AI-2 detection to operon expression. This gene, (*lsrR*), encodes a protein that represses *lsr* operon expression in the absence of AI-2. Mutations in the *lsr* operon render *S. typhimurium* unable to eliminate AI-2 from the extracellular environment, suggesting that the role of the Lsr apparatus is to transport AI-2 into the cells. A graduate student named Michiko Taga is performing the *S. typhimurium* work. This work has led to one published manuscript and another is in preparation. We have also crystallized the *S. typhimurium* LsrB binding protein-AI-2 complex and we are currently solving the structure to identify the *S. typhimurium* AI-2 molecule.

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PATENT APPLICATIONS

1. Compositions and Methods for Regulating Bacterial Pathogenesis (Approved)
2. LuxO-Sigma 54 Interactions and Methods of Use
3. Boron Containing Bacterial Quorum Sensing Signals
4. Inhibitors of Autoinducer Transposters
5. Compositions and Methods for the Prevention and Treatment of Infections by *Vibrio cholerae*

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14. ABSTRACT

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