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Quorum Regulation of Luminescence in *Vibrio fischeri*

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Abstract

Luminescence in *Vibrio fischeri* is controlled by a population density-responsive regulatory mechanism called quorum sensing. Elements of the mechanism include: LuxI, an acyl-homoserine lactone (acyl-HSL) synthase that directs synthesis of the diffusible signal molecule, 3-oxo-hexanoyl-HSL (*V. fischeri* autoinducer-1, VAI-1); LuxR, a transcriptional activator protein necessary for response to VAI-1; GroEL, which is necessary for production of active LuxR; and AinS, an acyl-HSL synthase that catalyzes the synthesis of octanoyl-HSL (VAI-2). The population density-dependent accumulation of VAI-1 triggers induction of *lux* operon (*luxICDABEG*; genes for luminescence enzymes and for LuxI) transcription and luminescence by binding to LuxR, forming a complex that facilitates the association of RNA polymerase with the *lux* operon promoter. VAI-2, which apparently interfers with VAI-1 binding to LuxR, operates to limit premature *lux* operon induction. Hierarchical control is imposed on the system by 3':5'-cyclic AMP (cAMP) and cAMP receptor protein (CRP), which are necessary for activated expression of *luxR*. Several non-*lux* genes in *V. fischeri* are controlled by LuxR and VAI-1. Quorum regulation in *V. fischeri* serves as a model for LuxI/LuxR-type quorum sensing systems in other Gram-negative bacteria.

Introduction

Over the past few years, awareness of a new paradigm in bacterial gene regulation, called quorum sensing, has developed. Classically defined, quorum sensing involves the production, via proteins of the LuxI family, of an acyl-homoserine lactone (acyl-HSL) signal molecule and the response to that signal via transcriptional regulatory proteins of the LuxR family (Fuqua *et al*., 1996; Dunlap, 1997; Hastings and Greenberg, 1999). There is also growing recognition of quorum sensing, via other types of signals and other types of regulatory proteins, in Gram-positive bacteria and potentially also in eukaryotic organisms (Kleerebezem *et al*., 1998; Gomer, 1999). Originating primarily in studies of luminescence autoinduction in marine bacteria (Greenberg, 1997), LuxI/LuxR type quorum sensing systems have now been identified in over 25 species of Gram-negative bacteria, including several plant and animal pathogens. The activities regulated by quorum sensing include, for example, luminescence, conjugative plasmid transfer, and the production of antibiotics and extracellular enzymes. The growing number of species identified as using quorum sensing and the chemical and genetic homologies of the quorum sensing systems indicate that quorum regulation is an evolutionarily conserved mechanism for regulating gene expression that is widespread among bacteria. One of the first and presently the most thoroughly studied of the LuxI/LuxR type quorum sensing systems is that of *Vibrio fischeri*. Studies of luminescence in *V. fischeri* have been pursued in many laboratories over the past thirty years, and by defining the major themes and working out the details of quorum regulation in this bacterium, those studies have generated a physiological, chemical and molecular-genetic foundation for the identification and analysis of quorum sensing systems in other bacteria. New information continues to emerge on the *V. fischeri* system, which serves as a guide and a reference point for studies of quorum regulation in other species. This paper provides an overview and current perspective on the quorum sensing system of *V. fischeri*.

Luminescence Induction

Light production by *V. fischeri* cells changes in a striking manner during batch culture growth. Initially high if strongly luminous cells are used as the inoculum, luminescence drops many fold during the first several cell divisions and then begins a sharp rise as the culture reaches late exponential phase, attaining a peak in early stationary phase. Synthesis of luciferase, the light-producing enzyme, generally follows the pattern of light production, with luciferase activity remaining at a fairly constant level in the culture for the first several cell divisions and then inducing rapidly as the culture enters late exponential phase. Experimental analysis of this dramatic pattern of light production in *V. fischeri* and *Vibrio harveyi* led to demonstrations that the cells produce and release into the medium a diffusible luminescence inducing factor, autoinducer, which accumulates during growth and triggers induction of luciferase when it attains a threshold concentration (Nealson *et al*., 1970; Eberhard, 1972; Nealson, 1977; Nealson and Hastings, 1979; Rosson and Nealson, 1981). Originally called autoinduction to reflect the self-produced nature of the inducing factor, this form of regulation is now also referred to as quorum sensing (Fuqua *et al*., 1994) to reflect its relationship to population density.

An Ecological Rationale for Quorum Regulation

Quorum sensing apparently functions to enable *V. fischeri* cells to discriminate between different habitats and

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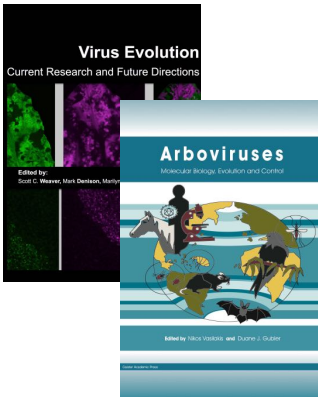

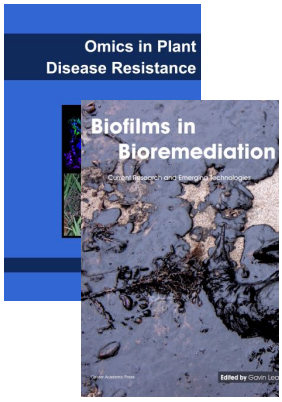

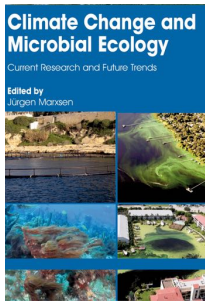

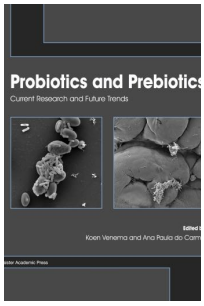

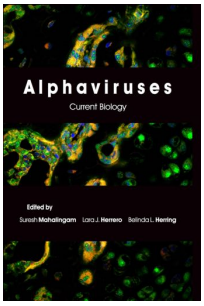

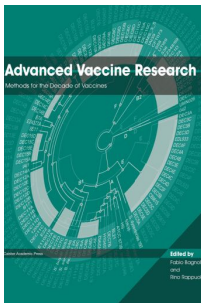

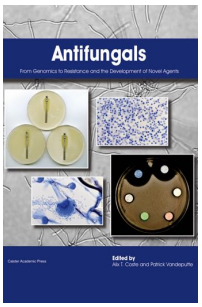

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adaptively produce or not produce light. In seawater, where the population density of *V. fischeri* cells is low (<10^2 cells/ml; Ruby and Nealson, 1978; Ruby *et al*., 1980; Baumann and Baumann, 1981; Lee and Ruby, 1992), diffusion would not allow the inducer to accumulate to the threshold concentration necessary for induction (Rosson and Nealson, 1981). Presumably, luminescence would not be beneficial under these conditions, if one assumes that the function of luminescence is to be seen by higher organisms or is in some other way related to association with higher organisms (see Dunlap, 1991, for a discussion); the light produced by fewer than 100 cells dispersed throughout 1 ml of seawater, even if those cells are fully induced, presumably would be too weak to be seen by animals. In light organ symbiosis with fish and squid, where the density of *V. fischeri* cells is high (ca. 10^9 to 10^{10} cells/ml; Dunlap and Greenberg, 1991; Ruby, 1996), autoinducer concentrations exceed those necessary to induce luminescence (Ruby and Nealson, 1976; Ruby and Asato, 1993; Boettcher and Ruby, 1995), which is produced at a sufficiently high level to be used by the animal in visually related behaviors (*e.g*., Singley, 1983; McFall-Ngai, 1990; Dunlap and Greenberg, 1991). Because luminescence requires energy, both for the luminescence reaction and for synthesis of luciferase (see Dunlap and Greenberg, 1991, for a discussion), it is logical to assume that luminescence would be induced only under those conditions when it enhances the survival or growth of *V. fischeri*. This notion implies that light production has a benefit for the bacteria, one that offsets its cost, and that a high level of light production may be in some way physiologically adaptive in the symbiotic state (Dunlap, 1985).

Two Major Advances

Studies of luminescence regulation in *V. fischeri* during the early 1980's resulted in two major advances, the chemical identification of the inducing factor (Eberhard *et al*., 1981) and the cloning of the luminescence genes (Engebrecht *et al*., 1983). These advances opened up the quorum sensing mechanism in *V. fischeri* to detailed molecular and genetic analysis, and as a consequence they established the foundation for the discovery of quorum sensing systems in other bacteria. Eberhard *et al*. (1981) extracted and purified the autoinducing compound from medium conditioned by the growth of *V. fischeri*, identified it as 3-oxohexanoyl-L-homoserine lactone (*V. fischeri* autoinducer-1, VAI-1) and demonstrated that the chemically synthesized compound has full biological activity. Initially carefully conserved and doled out sparingly, VAI-1 recently became commercially available at low cost, reflecting its importance as a tool in quorum sensing research.

Shortly after VAI-1 was chemically identified, Engebrecht *et al*. (1983) isolated a fragment of the *V. fischeri* MJ-1 chromosome that conferred on *Escherichia coli* the ability to produce light and to regulate light production in a population density-responsive manner. The fragment was found to contain genes for the luminescence proteins and genes necessary for the regulated expression of those proteins, including a gene necessary and sufficient for synthesis of the *V. fischeri* autoinducer by *E. coli*. The *V. fischeri lux* genes are organized in two divergently transcribed units, *luxR* and *luxICDABEG* (the *lux* operon) (Engebrecht and Silverman, 1984). The first gene of the *lux* operon, *luxI*, and the single gene of the divergent transcriptional unit, *luxR*, are regulatory. *luxI* was defined as required for cells to synthesize VAI-1, whereas *luxR* specifies a protein necessary for cells to activate *lux* operon transcription in response to VAI-1 (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984). The *luxA* and *luxB* genes specify the α and ß subunits of luciferase, and *luxC*, *luxD*, and *luxE* encode the polypeptides of the *lux*-specific fatty acid reductase complex (reductase, acyl transferase, and acyl protein synthetase), which are necessary for the synthesis and recycling of the aldehyde substrate for luciferase (Boylan *et al*., 1985; 1989). The last *lux* operon gene, *luxG*, which is followed by a strong transcriptional terminator (Swartzman *et al*., 1990), encodes a probable flavin reductase of the Fre/LuxG family of NAD(P)H-flavin oxidoreductases (Zenno and Saigo, 1994). The LuxG protein is not required for high levels of light production in *V. fischeri* (A. Kuo and P.V. Dunlap, unpublished data), so another flavin reductase activity in this species (Zenno *et al*., 1994) apparently can compensate for the loss of *luxG*.

The *V. fischeri lux* Regulatory Region

The *luxR* gene and the *lux* operon are separated by a 219 bp regulatory region that contains the promoters for these two transcriptional units. The *luxR* promoter consists of -10 and -35 regions similar to consensus sequences for *E. coli* "housekeeping" promoters and a consensus binding site for 3':5'-cyclic AMP (cAMP) receptor protein (CRP) at position -59 from the *luxR* transcriptional start. The *lux* operon promoter has a -10 region but has no -35 region identifiable by comparison with *E. coli* consensus sequences. At this position, centered 40 bp upstream of the *lux* operon transcriptional start, is a 20-bp region of dyad symmetry, termed the *lux* box. This region is required for activation of *lux* operon transcription and has been implicated as the binding site for LuxR (Baldwin *et al*., 1989; Devine *et al*., 1988; 1989; Engebrecht and Silverman, 1987; Shadel *et al*., 1990; Stevens *et al*., 1994; Stevens and Greenberg, 1997; Egland and Greenberg, 1999). Identical or similar sequences have been identified in the *lux* operon promoters of various *V. fischeri* strains as well as in the promoters controlling expression of *lasB* and *rhlI* in *Pseudomonas aeruginosa*, *traA* and *traI* of the octopine-type Ti plasmid, and *traI* of the nopaline-type plasmid, respectively, of *Agrobacterium tumefaciens*, *solI* of *Ralstonia solanacearum*, and *cepI* of *Burkholderia cepacia* (Engebrecht and Silverman, 1987; Devine *et al*., 1988; 1989; Shadel *et al*., 1990; Fuqua *et al*., 1994; Fuqua and Winans, 1994; Gray *et al*., 1994; Hwang *et al*., 1994; Latifi *et al*., 1995; Flavier *et al*., 1997; Lewenza *et al*., 1999). The *lux* box represents a conserved regulatory sequence; its presence upstream of a bacterial gene is interpreted as consistent with autoinducer-mediated control of that gene (Gray *et al*., 1994). The presence in *V. fischeri* of a *lux* box preceeding each of several recently isolated non-*lux* genes that are positively regulated by LuxR and VAI-1 (Callahan and Dunlap, manuscript in preparation) supports that interpretation.

A Molecular Model for Quorum Regulation of *lux* Operon Expression

The organization and function of the *lux* genes described above leads to a simple model for quorum regulation of the *lux* operon. A central aspect of the model is the membrane-permeant nature of VAI-1; the concentration of VAI-1 within cells rapidly equilibrates with that outside cells (Kaplan and Greenberg, 1985). According to the model, when *V. fischeri* cells fully induced for luminescence are transferred at a low population density to fresh medium, or in nature are released, for example, from the light organ of a fish or squid into seawater, VAI-1 rapidly diffuses away from the cells. The cellular concentration of VAI-1 then drops below the threshold necessary for activation of *lux* operon transcription, and synthesis of the *lux* operon proteins decreases sharply. As the population of *V. fischeri* cells grows in the medium, or increases following colonization of a squid or fish light organ (*e.g.*, Ruby, 1996), VAI-1 gradually accumulates within the medium and within cells. Presumably, de novo synthesis of VAI-1 results from the activity of extant LuxI protein and from a small amount of new LuxI produced from basal, unactivated *lux* operon transcription, with the test tube or animal light organ tubule providing a diffusion barrier. When VAI-1 attains the threshold level for interaction with LuxR, the LuxR/VAI-1 complex then interacts with RNA polymerase, facilitating its association with the *lux* operon promoter and transcription of the *lux* operon (Engebrecht and Silverman, 1984; Stevens *et al.*, 1994; Stevens and Greenberg, 1997) (Figure 1). An autocatalytic increase in synthesis of VAI-1 ensues, since *luxI* is part of the *lux* operon (Engebrecht *et al.*, 1983; Friedrich and Greenberg, 1983; Eberhard *et al.*, 1991), leading to a rapid increase in luciferase synthesis and luminescence.

With respect to synthesis of LuxR, transcription from the *luxR* promoter is activated by cAMP and CRP (described below). High levels of LuxR protein and VAI-1 apparently could place a cap on *lux* operon expression. VAI-1 and LuxR repress *luxR* expression post-transcriptionally and transcriptionally (Engebrecht and Silverman, 1986; Dunlap and Greenberg, 1988; Dunlap and Ray, 1989; Shadel and Baldwin, 1991; 1992a). Conversely, *luxR* expression can be activated by low levels of VAI-1 and LuxR in both a cAMP/CRP-dependent and cAMP/CRP-independent manner (Shadel and Baldwin, 1991; 1992a; 1992b).

LuxR Protein

According to the model outlined above, LuxR protein binds VAI-1 and activates transcription from the *lux* operon promoter. The role for LuxR as an autoinducer-binding transcriptional activator is based in part on the requirement for LuxR in *E. coli* containing the *lux* operon and in *V. fischeri* to produce light in response to VAI-1 (Engebrecht *et al.*, 1983; Engebrecht and Silverman, 1984; Dunlap and Kuo, 1992). Consistent with this role also is the presence of a *lux* box (described above) in the *lux* operon promoter, as well as genetic and physiological evidence that LuxR binds

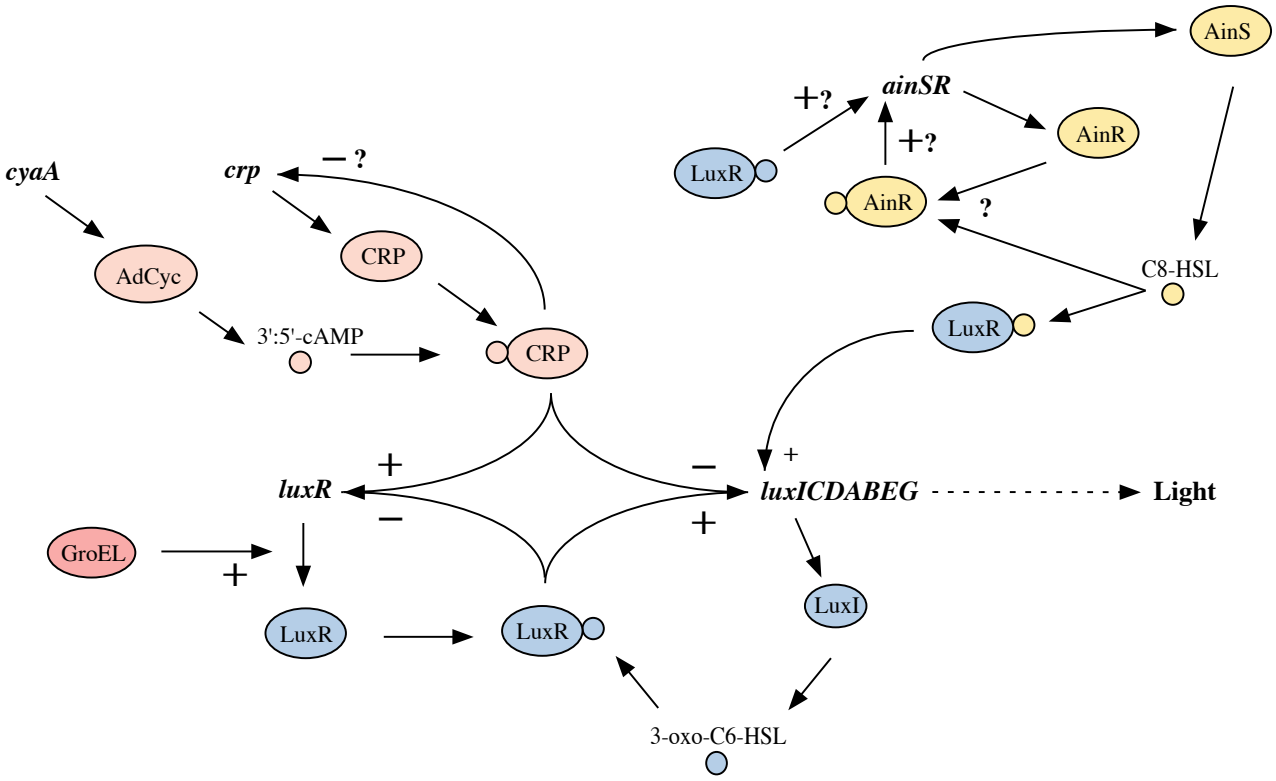

Figure 1. Regulatory Elements and Circuitry Controlling Luminescence in *V. fischeri*
Along with the quorum sensing system, consisting of LuxR, LuxI, and AinS, hierarchical control by cAMP/CRP is also depicted, as is the influence of GroEL on LuxR. Abbreviations: AdCyc, adenylate cyclase; 3-oxo-C6-HSL, 3-oxo-hexanoyl-homoserine lactone; C8-HSL, octanoyl-homoserine lactone. Transcriptional activation and repression are indicated by + and -, respectively.

VAI-1 (Shadel *et al*., 1990; Slock *et al*., 1990; Adar *et al*., 1992; Adar and Ulitzur, 1993; Gray *et al*., 1994; Hanzelka and Greenberg, 1995). However, the mechanism by which LuxR activates *lux* operon transcription has not been examined in detail until recently. Furthermore, it is theoretically possible that LuxR/VAI-1 operates not directly to activate transcription but indirectly. For example, LuxR/VAI-1 could function by relieving a repression of *lux* operon expression by LexA or another repressor protein (e. g., Ulitzur, 1989; Ulitzur and Dunlap, 1995); the *lux* operon *lux* box exhibits substantial similarity to the LexA protein binding site found in *E. coli* promoters controlled by the SOS response (Ulitzur and Kuhn, 1988; Baldwin *et al*., 1989). For these reasons and because *V. fischeri* LuxR serves as a model for new members of the emerging LuxR family of proteins (Fuqua *et al*., 1996; Dunlap, 1997), substantial emphasis has been placed recently on understanding the structure and function of LuxR.

The *V. fischeri* LuxR protein, consisting of 250 amino acid residues, apparently associates with the cytoplasmic membrane (Engebrecht and Silverman, 1984; Kolibachuk and Greenberg, 1993). Synthesis of active LuxR and the binding of VAI-1 by the amino-terminal portion of LuxR (see below) are enhanced by GroEL (Figure 1), indicating that chaperonin-mediated folding is necessary to stabilize the protein in active form (Adar *et al*., 1992; Dolan and Greenberg, 1992; Adar and Ulitzur, 1993; Hanzelka and Greenberg, 1995). The carboxy-terminal portion of LuxR (see below) exhibits remarkable thermal stability *in vitro*, regaining full activity after being heated to 70 °C for 30 min; either the protein is not denatured when heated or it folds back, upon cooling, into active form (Stevens and Greenberg, 1997) in the absence of GroEL.

Detailed analysis of full length LuxR has proven difficult; the overexpressed protein forms highly insoluble inclusion bodies (Kaplan and Greenberg, 1987). Deletion and mutational analyses of LuxR, however, have substantially overcome that impass. LuxR is composed of two modules or domains, an amino-terminal regulatory domain and a carboxy-terminal transcriptional activating domain. The amino-terminal domain, which binds VAI-1, regulates the activity of the carboxy-terminal domain, which associates with DNA and activates *lux* operon transcription. In the absence of VAI-1, the amino-terminal domain blocks the ability of the carboxy-terminal domain to associate with DNA. Binding of VAI-1 by the amino-terminal domain is interpreted as altering that interaction, permitting the carboxy-terminal domain then to bind to the *lux* regulatory region and activate *lux* operon transcription (Slock *et al*., 1990; Shadel *et al*., 1990; Choi and Greenberg, 1991; 1992a; Stevens *et al*., 1994; Hanzelka and Greenberg, 1995; Egland and Greenberg, 1999). Alternatively, the amino-terminal domain might prevent a proposed transcriptionally functional multimerization of LuxR to occur. In the presence of VAI-1, that interaction would be altered, permitting a LuxR dimer then to form and associate with the dyadically symmetric *lux* box (Choi and Greenberg, 1992b; Fuqua *et al*., 1996). Regardless, DNase I protection analysis and *in vitro* transcription assays with purified *lux* regulatory region DNA and purified proteins have demonstrated that the LuxR carboxy-terminal domain polypeptide and RNA polymerase bind synergistically to the *lux* box-*lux* operon promoter region and that the binding of these two proteins in this region is sufficient for *lux* operon mRNA synthesis (Stevens *et al*., 1994; Stevens and Greenberg, 1997). The *in vitro* nature of these results with purified DNA and proteins argues against the hypothesis for a repressor protein that blocks *lux* operon transcription, the activity of which is alleviated by LuxR/VAI-1, and it argues against an hypothesized indirect effect of autoinducer, one in which autoinducer is an effector for another protein that activates LuxR (Sitnikov *et al*., 1995). Instead, these results support a more simple model, one in which a mutually dependent interaction of the carboxy-terminal domain of LuxR, RNA polymerase, the *lux* box and the *lux* operon promoter region facilitates RNA polymerase binding to and initiating transcription from the *lux* operon promoter (Stevens and Greenberg, 1997).

Homologs of the *V. fischeri* LuxR protein have been identified in several other species of bacteria that use quorum sensing. The amino acid residue identities to *V. fischeri* LuxR range from 18% to 38% in these other species (Ochsner *et al*., 1994; Fuqua *et al*., 1996; Dunlap, 1997; Flavier *et al*., 1997; Milton *et al*., 1997; Puskas *et al*., 1997; Swift *et al*., 1997; Nasser *et al*., 1998; Lewenza *et al*., 1999). The similarities between the LuxR homologs tend to occur in the amino-terminal domain and in the helix-turn-helix portion of the C-terminal domain (Fuqua *et al*., 1994). The C-terminal domain of LuxR exhibits similarity to the DNA-binding region of members of the UphA/FixJ family of proteins (Fuqua *et al*., 1994; Fuqua *et al*., 1996). Furthermore, the deduced translation products of *luxR* genes from different strains of *V. fischeri* exhibit greater than 80% identity (Gray and Greenberg, 1992).

LuxI Protein and Synthesis of VAI-1

The first gene of the *lux* operon, *luxI*, directs the synthesis of a 25 kDa protein that is necessary and sufficient for *E. coli* and *V. fischeri* to produce VAI-1 (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984; Kuo *et al*., 1994; 1996). Questions central to understanding quorum sensing in *V. fischeri* have included whether LuxI is directly responsible for synthesis of VAI-1, and, if so, whether LuxI catalyzes VAI-1 synthesis from 3-oxo-hexanoyl-CoA and *S*-adenosylmethionine (SAM) (Eberhard *et al*., 1991; Hanzelka and Greenberg, 1996), or possibly from other substrates.

Recently, *in vitro* analysis of LuxI resolved these questions. Schaefer *et al.* (1996), relying on the finding that in *V. fischeri*, *luxI*, in addition to directing the synthesis of VAI-1, also directs the synthesis of hexanoyl-HSL (VAI-3) (Kuo *et al*., 1994), for which the probable substrates were more readily available than for VAI-1, demonstrated that a purified maltose binding protein-LuxI fusion protein catalyzes the synthesis of VAI-3 from hexanoyl-acyl carrier protein (hexanoyl-ACP) and SAM. No additional factor and no energy source was required for the activity. These results, in demonstrating the acyl-HSL synthase activity of LuxI, established that LuxI functions directly in autoinducer synthesis (Schaefer *et al*., 1996) and not through other cellular enzymes as had been proposed (Salmond *et al*., 1995). Acyl-HSL synthase activity has also been demonstrated for the product of *traI*, a gene necessary for synthesis of autoinducer in *A. tumefaciens*; purified hexahistidinyl-TraI protein utilized 3-oxo-octanoyl-ACP and SAM to synthesize 3-oxo-octanoyl-HSL (More *et al*., 1996). Also, the enzymatic activity of purified *P. aeruginosa* RhlI,

using SAM and either butanoyl-CoA or hexanoyl-CoA to produce the corresponding acyl-HSLs, was demonstrated recently (Jiang *et al*., 1998).

Several proteins with sequence similarity to LuxI (25 to 35% identity) have been identified in various other species of bacteria producing acyl-HSLs (Fuqua *et al*., 1996; Dunlap, 1997; Milton *et al*., 1997; Swift *et al*., 1997; Flavier *et al*., 1997; Nasser *et al*., 1998; Lewenza *et al*., 1999). Also, the deduced LuxI sequences of different *V. fischeri* strains exhibit a high degree of identity (Gray and Greenberg, 1992). A recent mutational analysis defined two regions of LuxI important for its activity as an acyl-HSL synthase, amino acid residues 25-70, proposed as the active site, and 104-164, possibly involved in substrate specificity; these regions contain many of the residues conserved in the members of LuxI family of proteins (Hanzelka *et al*., 1997).

Heirarchical Control of Quorum Sensing in *V. fischeri*

The LuxI/LuxR quorum sensing mechanism in *V. fischeri* is controlled by the intracellular signal cAMP and CRP (Figure 1). cAMP/CRP are required for transcription of *luxR*. The dominance of this control over the quorum sensing mechanism is revealed by the absence of luminescence induction in *cya*-like and *crp*-like mutants of *V. fischeri* (Dunlap, 1989). Early studies identified a transient repression of luminescence by glucose (Ruby and Nealson, 1976; Friedrich and Greenberg, 1983) that was suggestive of cAMP control of *lux* gene expression. Isolation of the *V. fischeri lux* genes (Engebrecht *et al*., 1983) then made obvious and feasible the use of *E. coli cya* and *crp* mutants with which to examine that possibility. CRP and cAMP were shown to be necessary for induction of luminescence, first in *E. coli* and later in *V. fischeri*, and to function by activating transcription from the *luxR* promoter (Dunlap and Greenberg, 1985; 1988; Dunlap and Ray, 1989; Dunlap, 1989; Dunlap and Kuo, 1992). Activation of *luxR* transcription apparently potentiates the quorum sensing response by building up the cellular level of LuxR protein. A consensus cAMP/CRP-binding site is present in the *luxR* promoter region (Devine *et al*., 1988; Engebrecht and Silverman, 1987), and CRP has been shown to bind to the region encompassing this site (Shadel *et al*., 1990; Stevens *et al*., 1994; Stevens and Greenberg, 1997). Additional regulatory complexity is indicated by the 2 to 10-fold repression of *lux* operon transcription by cAMP/CRP, which mirrors the repression of *luxR* expression by VAI-1/LuxR (Dunlap and Greenberg, 1985; Dunlap and Greenberg, 1988; Dunlap and Kuo, 1992).

Control of quorum regulation by cAMP/CRP might serve to funnel information on the nutritional and growth rate status of the cell into the quorum sensing mechanism. The dominance of cAMP/CRP control suggests that it functions to stimulate the quorum sensing response under conditions of nutritional and growth rate limitation, when cAMP levels in *V. fischeri* cells presumably are high, and to delay it or block it completely under conditions of rapid growth, when cellular levels of cAMP presumably would be low. Various culture conditions influence growth and luminescence in *V. fischeri* in opposite ways, enhancing one while suppressing the other, including glucose, oxygen and iron (Ruby and Nealson, 1976; Nealson and Hastings, 1977; Haygood and Nealson, 1985a; 1985b; Hastings *et al*., 1987), but how those conditions are coupled to *lux* operon induction is not yet understood (Dunlap, 1992a; 1992b). If those conditions influence the cellular level of cAMP, for example, that influence, via its effect on cAMP/CRP control of *luxR* expression, would provide a mechanistic link.

Other quorum sensing systems apparently also are subject to a dominant control by cAMP/CRP. In *P. aeruginosa*, *lasR* encodes a LuxR homolog that is at the top of a quorum sensing heirarchy in that species; expression of *lasR* is controlled by Vfr, a CRP homolog, from a consensus cAMP/CRP binding site in the *lasR* promoter (Albus *et al*., 1997). Similarly in *Erwinia chrysanthemi*, expression of the LuxR homolog *expR* is positively controlled by CRP from a cAMP/CRP binding site in the *expR* promoter region (Nasser *et al*., 1998; Reverchon *et al*., 1998).

A Second Quorum Sensing System in *V. fischeri*

The quorum sensing system of *V. fischeri* contains a second autoinducer synthase gene, *ainS*, which directs the synthesis of a second autoinducer, octanoyl-HSL (VAI-2) (Figure 1). VAI-2 via LuxR negatively modulates *lux* operon transcription. Evidence for the second quorum sensing system developed from construction of a non-polar *luxI* deletion mutant of *V. fischeri*, MJ-211. Despite the mutation, which eliminated the ability of cells to produce VAI-1 and which was expected to result in a non-luminous phenotype, the mutant produced a low but detectable level of light. Furthermore, light production during growth of the mutant in batch culture showed the same pattern of decline and rise back as the parental strain, a pattern characteristic of quorum regulation. The luminescence behavior of the mutant suggested that *V. fischeri* cells produce a *luxI*-independent inducing factor. Purification and identification of that factor as octanoyl-HSL (VAI-2) confirmed the presence of the second quorum sensing system, as did isolation of a fragment of *V. fischeri* DNA distinct from *luxI* that was necessary and sufficient for *E. coli* to produce VAI-2 (Kuo *et al*., 1994).

Genetic analysis of that fragment revealed the presence of two genes, *ainS*, which is required for production of VAI-2, and *ainR*, which might encode a regulatory protein (Gilson *et al*., 1995). The deduced amino acid sequence of *ainS* exhibited no significant similarity to LuxI or other members of the LuxI family of autoinducer synthesis proteins (Gilson *et al*., 1995), now defined enzymatically as acyl-HSL synthases (Schaefer *et al*., 1996; More *et al*., 1996). The C-terminal half of AinS is homologous (34% identity), however, to the *V. harveyi* LuxM protein (Gilson *et al*., 1995). LuxM and the upstream gene LuxL have been implicated in synthesis of a *V. harveyi* quorum sensing signal for luminescence (Bassler *et al*., 1993). The AinS-LuxM homology led Gilson *et al*. (1995) to propose that AinS and LuxM are members of a new family of autoinducer synthesis proteins. Open issues, however, have been whether AinS exhibits enzymatic activity and if so, whether it uses substrates similar to those used by the LuxI family of acyl-HSL synthases. A recent study has resolved those issues by demonstrating *in vitro* the acyl-HSL synthase activity of a purified maltose binding protein-AinS fusion protein, which uses SAM and octanoyl-ACP to produce octanoyl-HSL (Hanzelka *et al*., manuscript

in preparation).

VAI-2 can operate *in vivo* as a negative modulator of *lux* operon transcription. By itself, VAI-2 stimulates luminescence a small amount and does so in a LuxR-dependent manner (Eberhard *et al*., 1986; Kuo *et al*., 1994), indicating the ability of VAI-2 via LuxR to weakly activate *lux* operon transcription (Figure 1). Furthermore, VAI-2 interferes with the luminescence stimulating activity of VAI-1, suggesting a competitive inhibition by VAI-2 of the interaction between VAI-1 and LuxR (Eberhard *et al*., 1986; Kuo *et al*., 1996). Consistent with these observations, a *V. fischeri* mutant defective in *ainS* induces luminescence at a lower population density and more rapidly than the parent strain (Kuo *et al*., 1996). The inhibitory activity of VAI-2 has been hypothesized to help prevent premature induction of luminescence, i.e., at low population densities (Kuo *et al*., 1996).

Various aspects of VAI-2 and *ainSR* remain unresolved at this time (Figure 1). Perhaps most intriguing is whether VAI-2 via LuxR, or possible via AinR, regulates the expression of genes other than *lux* (Kuo *et al*., 1994). As described below, LuxR and VAI-1 have been found recently to activate the production of at least seven non-Lux proteins in *V. fischeri*, but so far no protein whose production is dependent on VAI-2 has been identified (Callahan and Dunlap, manuscript in preparation). A regulatory role for AinR also has not been identified, but one seems possible given the partial identity of AinR with the amino terminus of the *V. harveyi luxN* gene product, which has been proposed to function as a sensor/receptor for *V. harveyi* autoinducer (Bassler *et al*., 1993). How *ainSR* transcription is controlled also remains undefined; however, the *ainSR* regulatory region contains a *lux* box, which implies transcriptional control by LuxR and either VAI-1 or VAI-2 (Gilson *et al*., 1995).

Quorum Regulation of Non-*lux* Genes in *V. fischeri*

Previously, luminescence was the only activity known to be regulated in *V. fischeri* by LuxR and acyl-HSLs. Recent studies have identified, however, several *V. fischeri* genes other than those of the *lux* operon that are quorum regulated. An important step in identifying these genes was the construction of a mutant entirely defective in the synthesis of *V. fischeri* autoinducers. The identification of VAI-2 and the cloning of the *ainS* gene made this step both necessary, in demonstrating the presence of a second signal, one with demonstrated cross activity on *lux* operon transcription, and obvious, by providing the genetic locus for that activity. A double acyl-HSL synthase mutant, MJ-215, defective in both *luxI* and in *ainS*, was constructed by replacement of the wild-type *ainS* gene with a mutated form in the *luxI* deletion background. The double mutant does not induce luminescence and makes neither VAI-1 nor VAI-2; it responds, however, to exogenously added VAI-1 with the production of a high level of light (Kuo *et al*., 1996), demonstrating that the LuxR and luminescence functions are intact.

Two complementary approaches have been used with this strain to identify quorum regulated non-*lux* genes in *V. fischeri*. The first approach, 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of total cellular proteins, permitted visualization of proteins produced under the control of *V. fischeri* acyl-HSLs. With this method, five non-Lux proteins whose production is dependent on VAI-1 and LuxR, and their genes, have been identified (Callahan and Dunlap, manuscript in preparation). The second approach employed *lacZ*-fusion technology via transposon MudI, and that approach has led to the identification of two non-*lux*, quorum sensing regulated genes, both of which differ from those identified by 2-D PAGE analysis (Callahan and Dunlap, unpublished data). The identification of these seven genes reveals the presence of a LuxR/VAI-1-dependent quorum sensing regulon in *V. fischeri*; the luminescence system is one phenotypically distinct part of this regulon (Callahan and Dunlap, manuscript in preparation). Recent evidence indicates that the quorum sensing regulon is composed of additional genes (Callahan and Dunlap, unpublished data), which would be consistent with the large number of quorum-regulated genes identified in certain other bacteria (*e.g*., Van Delden and Iglewski, 1998). Analysis of the functions of the proteins coordinately produced under quorum sensing control by *V. fischeri* may help define the nature of the environmental conditions important in this bacterium's adaptation to high population density and host association.

Perspective

Autoinduction of luminescence, thought at one time to be a peculiar attribute of a few obscure marine bacteria, is now recognized as a model quorum sensing system with wide relevance in basic and applied research on bacterial gene regulation and host association. One can wonder, from the perspective available today and with awareness of the years of dedicated effort by individuals and small groups during the 1960's, 1970's and 1980's in defining the mechanism of luminescence gene regulation, what other neglected or unknown bacterial activities, if given similar effort, would lead to new paradigms in bacterial physiology and genetics. Certainly, the long-neglected marine environment would appear to be a good hunting ground for such activities.

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