Mutant analysis of luminescence and autoinduction in a marine bacterium

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ABSTRACT

The marine symbiotic bacterium *Vibrio fischeri* is striking for its ability both to emit light and to dramatically regulate light emission using a cell-to-cell signalling mechanism called autoinduction. The latter is mediated by a signal molecule called the "autoinducer". The mechanistic bases of both luminescence and autoinduction are well known in *V. fischeri*, but this knowledge is mostly derived from studies of the cloned luminescence and autoinduction genes expressed in *Escherichia coli*. In this study, luminescence and autoinduction mutations were systematically generated in *V. fischeri* to explore aspects of luminescence and autoinduction not addressable in *E. coli*, such as the adaptive significance of luminescence. Most dramatically, the mutants revealed the presence of multiple autoinducers and autoinducer synthases in *V. fischeri*. One of the autoinducers (autoinducer-2, or AI-2) was chemically purified and shown to be *N*-octanoyl-L-homoserine lactone. The genetic locus encoding the AI-2 synthase was cloned and designated *ain* (*a*uto*in*ducer). Manipulation of *ain* and AI-2 in *V. fischeri* demonstrated that the function of AI-2 appears to be to inhibit rather than to promote autoinduction.

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Chapter 1. Introductory remarks

The marine bacterium *Vibrio fischeri* has drawn scientific attention for more than fifty years because of its possession of three specialized properties: its ability to emit light, its participation in light organ symbioses with fishes and squids, and its ability to regulate light emission in a cell density-dependent manner involving an extracellular factor (autoinduction). This attention has been rewarded: *V. fischeri* is presently the best developed system for studying both bacterial luminescence and animal-microbe symbiosis, and the current model for autoinduction is based entirely on studies of the *V. fischeri* system. Much of the work on luminescence and most of the work on autoinduction relied on the *V. fischeri* luminescence (*lux*) genes cloned into *Escherichia coli*. Surprisingly, a mutant approach in *V. fischeri* has until recently not been systematically used to develop the system further, though recent construction of defined mutants of *V. fischeri* has already revealed novel aspects of regulation of luminescence (Dunlap and Kuo 1992). This dissertation constitutes an additional contribution to the ongoing mutant analysis of *V. fischeri*. The mutant approach applied in this study has allowed the experimental examination of the biological role of the luminescence system in *V. fischeri*, and has revealed exciting and heretofore unknown complexities in the mechanism of autoinduction, including the fact that *V. fischeri* possesses multiple autoinducers and autoinducer synthases. As a prelude to the experiments presented in the subsequent chapters, I will devote the rest of this chapter to a review and discussion of current ideas concerning luminescence (prelude to Chapter 2) and autoinduction (Chapters 3 and 4).

I. Luminescence

Luminescence is found in at least eleven species and four genera of bacteria found in a variety of mostly marine habitats (Nealson and Hastings 1991). All are members of the γ subgroup of purple bacteria, and all but one belong to the facultatively aerobic enteric group of bacteria. Two species of luminous bacteria are not marine; luminous strains of *Vibrio cholerae* are isolated from brackish and fresh waters while *Photorhabdus luminescens* participates in symbioses with certain soil nematodes. However, most species of luminous bacteria are marine, and the most intensively studied of the luminous bacteria - *V. fischeri*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, and *Vibrio harveyi* - have been isolated from both the water column and from animals, including the gastrointestinal tracts of those animals. The first three species also

occur in the light organs of certain fishes and squids in a species-specific manner. Colonization of animals results in high cell densities (10^{6-10} cells ml^{-1}; Nealson and Hastings 1991), far higher than in the surrounding sea water (10^{3-4} cells ml^{-1}).

A. Mechanism of luminescence

Bacterial luminescence is the product of an oxygenation reaction catalyzed by the heterodimeric flavoenzyme luciferase (see Figure). Purified luciferase can cleave O_2 and oxygenate both a long-chain aldehyde and a reduced flavin cofactor to the corresponding fatty acid and oxidized flavin (Cormier and Strehler 1953, McElroy et al 1953, Dunn et al 1973); a 4a-hydroperoxyflavin is postulated to combine with the aldehyde to form the light-emitting species (Eberhard and Hastings 1972, Suzuki et al 1983, Vervoort et al 1986). Luciferase can also cleave $O_2^{-}\cdot$ and H_2O_2 to yield light via the same light-emitting intermediate if a flavin with the appropriate oxidation state is present (Watanabe and Nakamura 1976, Hastings et al 1979, Kurfurst et al 1983). The fatty aldehyde itself is synthesized from the fatty acid, NADPH, and ATP by a fatty acid reductase complex (Riendeau and Meighen 1979). Each of the two flavin reductases purified from *V. fischeri* is capable of supplying the reducing power necessary for the light-emitting reaction (Zenno et al 1994, Zenno and Saigo 1994).

The two subunits of luciferase are encoded by the chromosomal genes *luxA* and *luxB*. The subunits of the fatty acid reductase complex are a reductase, an acyl transferase, and an acyl-protein synthetase, encoded by the genes *luxC*, *luxD*, and *luxE*, respectively (Boylan et al 1985). The *V. fischeri lux* genes are linked in the order *luxCDABE* and are cotranscribed from a single promoter; thus luciferase and the fatty acid reductase complex are coregulated (Engebrecht et al 1983, Engebrecht and Silverman 1984). In *V. fischeri* there are three other *lux* genes linked to *luxCDABE*: *luxG*, the function of which is unknown (Swartzman et al 1990) but which has sequence similarity to a known flavin reductase (Andrews et al 1992), and the regulatory genes *luxR* and *luxI*, which are described later in this chapter.

B. Regulation of luminescence

The luminescence of *V. fischeri* is regulated. One form of regulation is a cell density-dependent induction of luminescence called autoinduction, which I discuss in detail later in this chapter. In addition, elimination of the *lux* operon promoter reveals an autoinduction-independent modulation of luminescence (Dunlap and Kuo 1992). This

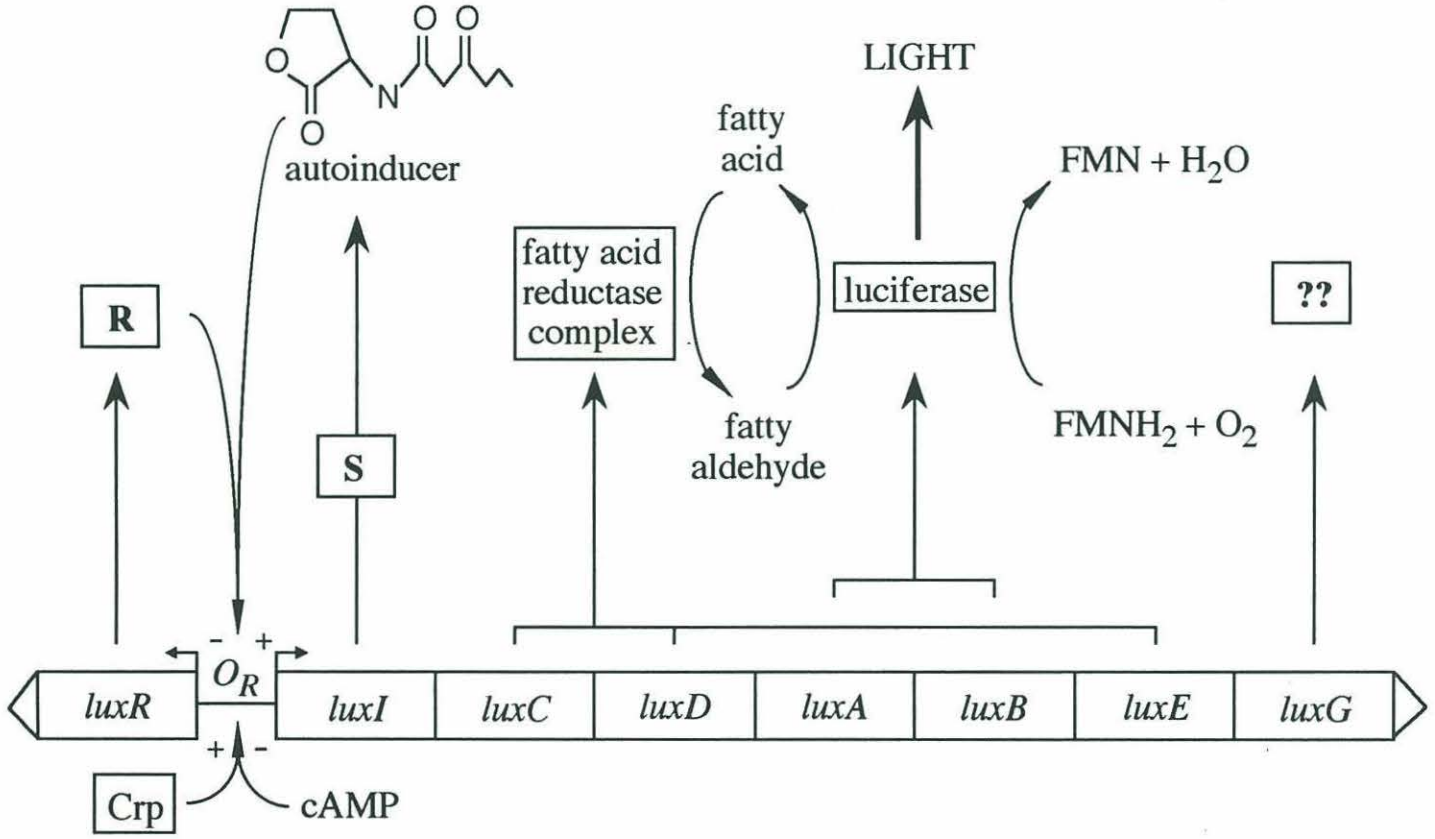

Figure. Classical model of the luminescence system of *Vibrio fischeri*

modulation is independent of any known *lux* regulatory elements, including the *lux* promoter, and has been proposed to reflect gene-copy changes over the course of batch culture growth (Dunlap 1992).

Luminescence is also subject to control by a number of environmental factors. Low O_2 tensions may increase luminescence and luciferase levels (Ruby and Nealson 1976, Nealson and Hastings 1977), as may pure O_2, though *V. fischeri* does not grow under the second condition (Colepicolo et al 1992). The mechanism of O_2 modulation of *luxAB* expression is unknown. Induction of luminescence is delayed by the presence of iron (Makemson and Hastings 1982), and iron delays the above-described autoinducer-independent modulation (Dunlap 1992, Dunlap and Kuo 1992). Glucose delays induction, even in the presence of cyclic AMP (cAMP; Ruby and Nealson 1976). Glucose also represses luminescence under phosphate-limited chemostat conditions, and the repression is reversed by cAMP (Friedrich and Greenberg 1983). Consistent with a model of catabolite repression, the *E. coli* cAMP receptor protein (Crp) activates *luxR* transcription in the presence of cAMP (Dunlap and Greenberg 1985), *luxR* has an upstream *E. coli* Crp-binding site (Engebrecht and Silverman 1987), and *V. fischeri* mutants analogous to *E. coli* catabolite repression mutants with respect to luminescence can be isolated (Dunlap 1989). Also, cAMP-Crp stimulates transcription of *luxICDABEG* (Dunlap and Greenberg 1985). However, cAMP-Crp control is insufficient to explain the influence of glucose, since glucose also delays autoinducer-independent modulation in the absence of the Crp-binding site (Dunlap and Kuo 1992).

C. Function of luminescence

Significant progress has been made in elucidating the mechanisms of luminescence and its regulation in *V. fischeri*, but the biological function of luminescence in this and other luminous bacteria remains obscure. The luminescence system involves at least five polypeptides, two of which (the luciferase subunits) can comprise up to 5% of cellular protein (Hastings et al 1965). Also, each light-emitting event ultimately consumes at least two reducing equivalents and one molecule of ATP. Also, the luminescence system is regulated, presumably to ensure that the *lux* gene products are synthesized only when needed. All of this nurtures the suspicion that the luminescence system is of adaptive significance to the bacteria. A few hypotheses which attempt to explain the adaptive value of the luminesence system are discussed here.

In one hypothesis, luciferase defends the cell against oxygen toxicity by scavenging O_2. This hypothesis is based on the ability of luciferase to reduce molecular

oxygen, even at low O_2 tensions. Oxygen metabolism produces $O_2^{-}\cdot$, H_2O_2, $HO\cdot$, and perhaps singlet O_2, all of which are highly reactive species that can reduce or oxidize lipids, nucleotides, and proteins, and thus inactivate enzymes, induce mutations, and kill cells (Kanfer and Turro 1981, Imlay and Linn 1988). To protect themselves from these threats, some organisms synthesize enzymes such as catalase and superoxide dismutase that scavenge H_2O_2 and $O_2^{-}\cdot$, respectively (Fridovich 1978). Such enzymes are often induced by oxidative stresses such as H_2O_2, $O_2^{-}\cdot$, or hyperbaric O_2 (Gregory and Fridovich 1973, Finn and Condon 1975). Their absence due to null mutation lowers resistance to oxidative stress (Loewen 1984, Farr et al 1986). In the oxygen scavenger hypothesis, luciferase is proposed to protect the bacterium from oxygen toxicity by scavenging molecular oxygen or its derivatives in a fashion analogous to catalase and superoxide dismutase (McElroy and Seliger 1962). In support of this hypothesis, pure O_2 induces luciferase synthesis in *V. fischeri* and *P. luminescens* (Colepicolo et al 1992). Both of the toxic radicals $O_2^{-}\cdot$ and H_2O_2 can be cleaved by purified luciferase to produce light (Watanabe and Nakamura 1976, Hastings et al 1979, Kurfurst et al 1983).

In a second hypothesis, luciferase mediates terminal electron transfers to O_2 when cytochrome oxidase cannot. This hypothesis is based on the high O_2 affinity of luciferase (Hastings 1952, Lloyd et al 1985). Oxidative phosphorylation depends on the heme enzyme cytochrome oxidase to transfer reducing equivalents from the electron transport chain to O_2. In the electron carrier hypothesis, luciferase is proposed to perform the electron transfer function of cytochrome oxidase under conditions where cytochrome oxidase might not function, such as low O_2 tension or low iron (Nealson and Hastings 1979, Makemson and Hastings 1982). Respiration and luminescence compete in *V. harveyi*, evidently for reductant (Ulitzur et al 1981, Grogan 1984), luciferase induction promotes growth of *V. harveyi* that are cytochrome oxidase-deficient due to iron limitation (Makemson and Hastings 1986), and iron limitation promotes luminescence (Makemson and Hastings 1982), as may low O_2 tensions (Nealson and Hastings 1977). Finally, some luminescence mutants survive poorly under anaerobic conditions (Dieterich and Nealson 1972, Nealson and Hastings 1979).

In another hypothesis, luminescence serves as a mechanism for the dispersal and propagation of luminous bacteria. In this hypothesis, light emission by bacterial aggregations or colonies on particles in the water column attract potential host animals (Nealson and Hastings 1979). Thus, the luminescence system supplies a means by which bacteria defecated from one host colonize new gastrointestinal tracts. Consistent with this hypothesis, fecal pellets and sediment trap particles freshly collected from oceanic

sources emit light (Andrews et al 1984), as do fecal pellets defecated by fish fed with luminous bacteria (Ruby and Morin 1979).

Additional hypotheses focus on the fact that luciferase is an oxygenase; its oxygenation of fatty aldehydes is proposed to be useful to the bacteria. The fatty acids might be oxidized further (Seliger 1987) or be covalently attached to the oxidized flavin for some unknown purpose (Kasai et al 1991). The fact that luciferase can oxygenate its substrates under low O_2 tensions has also been proposed to be advantageous to organisms living under hypoxic conditions (Seliger 1975).

The above-described hypotheses do not necessarily mutually exclude one another. The luminescence system may have multiple functions. Also, it is possible that the adaptive value of the luminescence system has changed over time. It has been suggested that luminescence enzymes evolved initially to protect the strictly anaerobic inhabitants of the primitive earth's anaerobic environment from the toxic effects of low but increasing concentrations of O_2, or to perform oxygenations under low O_2 tensions; it is suggested that subsequently luminescence was adapted to behavioral or other uses unrelated to oxygen metabolism, or became vestigial (McElroy and Seliger 1962, Seliger 1975, 1987).

II. Autoinduction

A growing bacterial cell exists in the presence of its clonal siblings. Perhaps unsurprisingly then, the environmental stimuli to which a bacterium responds sometimes originate from the bacterium's fellow cells. In the laboratory world of pure batch cultures, such stimuli manifest themselves in the observation that many biologically significant activities are cell density-dependent. In many cases the cell density dependence has been shown to be mediated by a protein, such as the peptide phermones of *Enterococcus faecalis* (Dunny et al 1978), the extracellular differentiation factor of *Bacillus subtilis* (Grossman and Losick 1988), and the morphogenetic C-factor of *Myxococcus xanthus* (Kim and Kaiser 1990). In other cases the cell density dependence is mediated by a small membrane-permeable signal molecule. Small signal molecules were first demonstrated to regulate cell differentiation in *Streptomyces griseus* and then to regulate luminescence in *V. fischeri*, where the mechanism was first named "autoinduction".

Autoinduction is a mode of gene regulation where a bacterium synthesizes a specific small molecule that diffuses freely into the medium and triggers a transcriptional activator of target genes in the same or another bacterium. The most striking aspect of autoinduction is its involvement of the extracellular compartment, for this implies that

autoinduction is a cell-cell signalling system. Because of this same extracellular aspect, expression of the genes targeted by autoinduction appears to be cell density-dependent. Thus we can view autoinduction as a method for the individual cell to measure local cell density.

A. Autoinduction in *Vibrio fischeri*

The term "autoinduction" was first used to describe the spectacular kinetics of light emission in batch cultures of *V. harveyi*, where luminescence per cell first declined until the cells reached the middle of the exponential growth phase (5×10^7 cells ml^{-1}), and then rose 10^{3-4}-fold over about 4 cell doublings (Nealson et al 1970) . The induction but not the initial decline was attributed to an induction of synthesis of luciferase. Without any known external stimulus, this enzyme induction presumably responded to a stimulus derived from the bacteria themselves, and so was termed "autoinduction".

At first autoinduction was not specifically associated with an extracellular signal. Kempner and Hanson (1968) had earlier performed "conditioned medium" experiments where cells were exposed to medium in which another culture had already been grown. Conditioned medium eliminated the initial decline of luminescence, leading Kempner and Hanson to propose the existence of an inhibitor of luminescence present in fresh medium but subsequently removed by the cells. Nealson et al (1970) rejected the inhibitor hypothesis but proposed that "conditioning" was the stimulus responsible for autoinduction. Finally, Eberhard (1972) showed that the conditioning was due to both removal of an inhibitor and release of a species-specific activator of luminescence, and thus that autoinduction is partly mediated by a signal or "autoinducer".

1. The signal

An autoinducer of luminescence should be isolatable from medium conditioned by a dense culture of *V. fischeri* fully induced for luminescence. From such a source, and using a naturally autoinducer-deficient strain of *V. fischeri* as an assay for the *V. fischeri* autoinducer, Eberhard et al (1981) purified a substance that was physically and biologically indistinguishable from synthetic *N*-3-oxohexanoyl-L-homoserine lactone (3-oxohexanoyl-HSL). The autoinducer was extractable from medium with ethyl acetate (consistent with the fatty acyl moiety) and labile to base (consistent with the lactone ring). Addition of the autoinducer to a culture of wild-type *V. fischeri* immediately induced luminescence, eliminating the initial decline.

The most novel feature of autoinduction is its extracellular aspect, which implies that the autoinducer moves from cell to medium. 3-oxohexanoyl-HSL is a small slightly polar molecule that might be capable of freely diffusing through both aqueous and lipid compartments of the cell. Kaplan and Greenberg (1985) showed that equilibration of radioactive autoinducer between cells and media was indeed extremely rapid. Dilution of the autoinducer in the medium to concentrations below 10 nM resulted in disappearance of autoinducer from cells and cessation of induction. This result can be explained by a simple model where the rate of diffusion of autoinducer (when autoinducer in medium < 10 nM) is greater than the rate of binding of to the putative autoinducer receptor. However, the data do not exclude the possibility that autoinducer might be actively transported across the membrane.

The synthetic precursors to the autoinducer are unknown. Engebrecht et al (1983) cloned a *V. fischeri* locus into *E. coli* which directed *E. coli* to synthesize both luciferase and an autoinducer activity. *E. coli* without *luxI* did not produce the autoinducer. Genetic complementation tests, protein synthesis analysis, and sequencing showed that a single 578 bp gene encoded a 22 kD polypeptide that was necessary and sufficient for *E. coli* to synthesize the autoinducer and release it into the medium (Engebrecht and Silverman 1984, 1987). Mutation of the gene, *luxI*, reduced the synthesis of luciferase; luciferase synthesis was restored by the addition of exogenous autoinducer. LuxI bore no sequence similarity to other known proteins. No attempt was made to show that *luxI* was necessary for autoinducer synthesis in *V. fischeri*. Significantly, *luxI* is part of the *luxICDABEG* operon, implying that the autoinducer is a positive autoregulator. Subsequently, autoregulation was demonstrated directly (Eberhard et al 1991).

That a single gene confers on *E. coli* the ability to produce the autoinducer implies that *E. coli* has most of the metabolic machinery needed for synthesis and that *luxI* encodes an enzyme that catalyzes a single final step in the synthetic pathway, presumably formation of the amide bond. Indeed, *E. coli* mutants deficient in the synthesis of the putative homoserine lactone precursor homoserine do not produce an autoinducer activity in the presence of *luxI* (Roberto Kolter, personal communication). So far, LuxI has not been purified, nor has it been demonstrated to have a synthase activity. Eberhard et al (1991) failed to synthesize the autoinducer by mixing a crude *V. fischeri* lysate with an activated 3-oxohexanoyl-group and homoserine lactone, but succeeded when homoserine lactone was replaced by *S*-adenosylmethionine. This intriguing result raises the possibility that lactonization occurs either concurrently with or after formation of the amide bond, perhaps catalyzed by a lactonizing activity common to both *E. coli* and *V. fischeri*. However, Kolter reports that an *E. coli metA* mutant, which synthesizes

homoserine but not *S*-adenosylmethionine, is capable of supporting autoinducer production (personal communication). This result is reconcilable with the Eberhard et al (1986) report if 1) exogenous homoserine lactone added to *V. fischeri* extracts is extremely labile and 2) *V. fischeri*, like *Aerobacter aerogenes* (Shapiro and Mather 1958), converts *S*-adenosylmethionine to homoserine lactone.

Nothing is known about the metabolic fate of the autoinducer, whether it is modified or degraded, nor whether such events are relevant to the kinetics of autoinduction.

2. The signal response

A simple autoinducer response mechanism could consist of 1) a receptor for the autoinducer and 2) a transcriptional activator that binds at or near the *luxICDABEG* promoter and interacts with RNA polymerase. Engebrecht and Silverman (1984, 1987) cloned and sequenced a single 750 bp gene encoding a 29 kD polypeptide that appeared to perform both tasks. Mutation of the gene, *luxR*, reduced the synthesis of luciferase; the phenotype was not rescued by the addition of exogenous autoinducer (Engebrecht et al 1983).

If LuxR is the signal receptor, it should bind to autoinducer. So far, active LuxR has not been purified. Random mutagenesis of *luxR* defined two functional domains, a "DNA-binding domain" and a "sensing domain" which included mutations that were suppressed by elevated levels of exogenous autoinducer (Slock et al 1990, Shadel et al 1990). Choi and Greenberg (1991) rendered LuxR function independent of autoinducer by deleting its amino-terminus, including the sensing domain, but leaving the DNA-binding domain intact; they argued that the amino-terminus masks the DNA-binding function of LuxR, and that autoinducer interaction with the sensing domain removes the mask.

The ligand requirements of LuxR are poorly defined. *V. fischeri* responded to a limited number of synthetic *N*-acyl-L-HSLs other than 3-oxohexanoyl-HSL (Eberhard et al 1986). These inducers varied principally in the structure of the fatty acyl moiety, while most changes in the lactone ring abolished activity. The results implied that the recognition constraints on fatty acyl structure are substantially less than those on the amino acyl moiety. Whether there are separate binding sites for the two moieties is unknown. The observation that LuxR is membrane-associated despite its lack of membrane-spanning domains (Kolibachuk and Greenberg 1993) raises the possibility that

the amino acyl moiety is recognized by LuxR while the fatty acyl moiety controls localization of the autoinducer to the cytoplasmic membrane and access to LuxR.

In addition, Eberhard et al (1986) observed nonlinear kinetics of the luminescence response, and thus proposed that LuxR binds to multiple autoinducer molecules (presumably in a cooperative fashion), and that there is cooperativity between LuxR molecules. This is consistent with genetic dominance experiments that imply the existence of a multimerization domain (Choi and Greenberg 1992b). It should be noted that Choi and Greenberg (1991, 1992b) do not show that the putative multimerization is necessary for activation of *luxICDABEG.*

The domain studies place the DNA-binding domain toward the carboxyl-terminus of LuxR. The carboxyl terminus of LuxR is similar to the carboxyl termini of the response regulator members of the family of two-component signal transduction proteins (Henikoff et al 1990). Members of this family possess a characteristic carboxyl-terminal "helix-turn-helix" motif that has been implicated in DNA binding. The presumptive *lux* operator is a 20 bp palindrome that is 30 bp upstream from the *luxICDABEG* transcriptional start site, mutation of which eliminated transcriptional activation by autoinducer (Devine et al 1989). Specific binding of purified LuxR to the operator has not been demonstrated. Finally, the extreme carboxyl-terminus appears to activate RNA polymerase, presumably after LuxR is bound to DNA (Choi and Greenberg (1992a).

While activating transcription of *luxICDABEG*, LuxR also represses its own synthesis. *luxR* is divergently transcribed from *luxICDABEG*, and is 147 bp from the *lux* operator (Engebrecht and Silverman 1983, 1987). Inbetween lies the putative Crp-binding site. Autorepression requires autoinducer (Engebrecht and Silverman 1986, Dunlap and Ray 1989), the DNA-binding domain and the extreme amino-terminus of LuxR (Choi and Greenberg 1991), the *lux* operator, and an undefined locus in the *luxIC* region (Shadel and Baldwin 1991). Whether autorepression is effected by steric hindrance of RNA polymerase or Crp binding by LuxR binding is unknown. LuxR may also stimulate its own synthesis at low autoinducer concentrations (Shadel and Baldwin 1991).

3. The current model for autoinduction

The above experiments, almost all with the cloned *lux* genes in *E. coli*, lead to a remarkably simple and elegant model for autoinduction (see Figure). At low cell density, *luxR* transcription is stimulated by cAMP-Crp, while *luxICDABEG* is transcribed at a low level, allowing the slow accumulation of LuxI and thus of autoinducer in both the cells

and the medium. At a higher cell density, autoinducer reaches a threshold concentration that allows it to bind to the sensor domain of LuxR, which then binds with the *lux* operator via its DNA-binding domain. Binding to the operator allows LuxR to both stimulate transcription from the *luxICDABEG* promoter and to block Crp access to the *luxR* promoter. Stimulated transcription of *luxI* initiates a positive feedback loop of autoinducer synthesis that is limited only by repression of *luxR* transcription.

The adaptive significance of this system is unclear. Most authors have assumed that the purpose of autoinduction is to measure cell density. One difficulty with this assumption is that it is not clear that there is any benefit to the organism of linking luminescence to cell density, especially since the adaptive significance of luminescence itself is not established. A second difficulty is that the actual occurrences of both luminescence and cell density of *V. fischeri* outside of the artifactual confines of the laboratory are poorly documented. It is possible that the membrane-permeable property of the autoinducer is an incidental function of its dual amino acyl-fatty acyl structure, and that the primary purpose of this structure is to reflect the status of amino acid and fatty acid metabolic pools. Since the adaptive significance of autoinduction per se is not a central concern of this dissertation, I shall from now on simply assume that a system to monitor local cell density is useful to the bacterium.

B. Autoinduction in other bacteria

For years autoinduction was treated as the unique specialization of a small group of bacteria. Recently, however, autoinduction systems homologous to that of *V. fischeri* have been discovered in other species of bacteria. Naturally, the *V. fischeri* model described above has been the intellectual paradigm for dissecting these other systems. In contrast, the autoinduction systems of *V. harveyi* and *S. griseus*, of which significant understanding has come only relatively recently, are not homologous to their *V. fischeri* counterpart.

1. Autoinduction systems which fit the *V. fischeri* paradigm

Agrobacterium tumefaciens is a plant pathogen well known for its ability to induce tumors in its host by injecting a fragment of a tumor-inducing plasmid (pTi) into host cells. pTi can be transfered from one bacterium to another, but only in the presence of a diffusible signal called "conjugation factor" (CF; Zhang and Kerr 1991). Synthesis of and response to CF require the pTi genes *traI* and *traR*, respectively, and *traR* activates

transcription of the conjugal transfer gene *traA* (Piper et al 1993, Fuqua and Winans 1994). CF is *N*-3-oxooctanoyl-L-HSL (Zhang et al 1993), TraI has 31% identity with LuxI, and TraR has 20% identity with LuxR.

Pseudomonas aeruginosa is an opportunistic human pathogen that exports an elastase, an alkaline protease, and an exotoxin as virulence factors. Expression of these enzymes is cell density-dependent, and requires *lasI* and *lasR* (Frank and Iglewski 1988, Gambello and Iglewski 1991, Passador et al 1993). LasR has 27% identity with LuxR while LasI has 35% identity with LuxI. *lasI* directs the synthesis of *N*-3-oxododecanoyl-L-HSL, which is required for LasR to activate transcription of the elastase gene *lasB* (Pearson et al 1994). A 20 bp palindrome similar to the *lux* operator is upstream from *lasB* (Gray et al 1994). Also, another *luxR* homologue, *rhlR*, regulates synthesis of elastase, a phenazine antibiotic, and rhamnolipid surfactants (Ochsner et al 1994).

Erwinia carotovora is a plant pathogen that exports a cellulase, a polygalacturonase, a protease, and lyases as virulence factors, as well as a carbapenem antibiotic. Synthesis of the carbapenem requires either a diffusible factor, which turns out to be 3-oxohexanoyl-HSL (Bainton et al 1992), or *carI*, which encodes a protein with 25% identity with LuxI (Swift et al 1993). Expression of the virulence factors is induced upon stationary phase, and requires *expI*, which encodes a protein with 30% identity with LuxI (Pirhonen et al 1993).

Published homologues of LuxR are: SdiA, which positively regulates cell division in *E. coli* (Wang et al 1991); RhiR, which is required for expression of the rhizosphere-specific *Rhizobium leguminosarum* genes *rhiABC* (Cubo et al 1992); and PhzR, which is required for the cell density-responsive regulation of phenazine synthesis in *Pseudomonas aureofaciens* (Pierson et al 1994). Other components of these autoinducer systems, if that is indeed what they are, have not been identified. Also, autoinducer activities have been detected in a number of other bacteria, though in most cases the target of autoinducer regulation is not known. An example of the latter is the autoinducer activity of *Enterobacter agglomerans*, which has a gene *eagI* homologous to *luxI* (Swift et al 1993). Finally, a homoserine lactone-based signal has been proposed to mediate the starvation response in *E. coli* (Huisman and Kolter 1994).

The above list of autoinduction system components in diverse proteobacteria leads to the suspicion that autoinduction may be a ubiquitous and perhaps fundamental mode of regulation in the proteobacteria. If so, one can expect autoinduction to be the mechanism of regulation in numerous other cell density-dependent processes. Perhaps the ability to sense local cell density has been crucial to the manifest success of the proteobacterial group. But despite the variety of autoinducer-regulated functions

identified so far, in no case is the adaptive value of connecting the autoinducer-regulated function with cell density unequivocally clear, though one might make the argument that extracellular virulence factors are effective only when employed in massive, instantaneous doses.

2. Autoinduction systems which do not fit the paradigm

The *V. harveyi* autoinducer is structurally similar to its *V. fischeri* counterpart. Cao and Meighen (1989) purified the *V. harveyi* autoinducer and found it to be physically and biologically indistinguishable from *N*-3-hydroxybutanoyl-L-HSL. Two autoinducer-deficient mutants responded much more to the D-3-hydroxy isomer than to the L-3-hydroxy isomer (Cao and Meighen 1993). This result suggested that the fatty acyl moiety of the autoinducer is diverted from the synthetic rather than degradative pathways of fatty acid metabolism, since classical fatty acid synthesis involves D-isomer intermediates while degradation involves only L-isomers. Blockage of fatty acid synthesis also inhibited autoinducer production.

In contrast to the autoinducer, the genes involved in synthesis of and response to the *V. harveyi* autoinducer appear to be completely different from their *V. fischeri* counterparts, *luxI* and *luxR*. Bassler et al (1993) cloned a locus that complemented a spontaneous mutant deficient in luminescence. Mutagenesis of the locus followed by mapping and recombination into the chromosome revealed two distinct loci corresponding to two distinct phenotypes, neither of which, curiously, was deficient in luminescence. The LuxM$^-$ phenotype synthesized a reduced but still significant amount of autoinducer activity, and responded to both wild-type-conditioned media and to synthetic autoinducer. The LuxN$^-$ phenotype responded to conditioned media but not to synthetic autoinducer. The authors concluded that the LuxM$^-$ and LuxN$^-$ phenotypes reflected losses of an autoinducer synthase and receptor, respectively. The ability of all mutants to condition media at a reduced but significant level suggested the presence of a second autoinducer of luminescence distinct from 3-hydroxybutanoyl-HSL. The ability of all mutants to respond to this putative second autoinducer implied a separate and specific receptor for the second autoinducer. Subsequently, mutants deficient in response to this as yet unidentified second autoinducer were isolated and called LuxQ$^-$ (Bassler et al 1994b).

Sequencing of the *luxMN* locus revealed three open reading frames. The LuxN$^-$ mutations fell within the *luxN* gene, which encodes a protein related to members of the two-component family of sensors and response regulators. LuxN has the "histidine

kinase" motif characteristic of the sensors, as well as the "Asp-57" phosphorylation site characteristic of the response regulators, but lacks the helix-turn-helix motif. The LuxM$^-$ mutations fell within two contiguous open reading frames, *luxL* and *M*, neither of which was similar to other known genes. Neither complementation tests nor protein synthesis analyses has been done with *luxLM*. The LuxQ$^-$ mutations fell within the genes *luxP* and *Q*; *luxQ* encodes a homologue of *luxN* and *luxP* encodes a homologue of *E. coli* and *Salmonella* ribose-binding proteins. Each of these genes possesses its own candidate promoter and ribosome-binding sites. None of these genes is a *luxI* or *luxR* homologue. Hybridization, random mutagenesis, and sequencing near the *luxCDABE* genes have failed to identify *luxI* or *luxR* homologues, though complementation of *V. fischeri lux* mutations has not yet been attempted.

A second luminescence-complementing locus was cloned, mutagenized, and sequenced. *luxO* encodes a protein related to the two-component response regulators, this time with both the Asp-57 and helix-turn-helix motifs (Bassler et al 1994a). Surprisingly, *luxO* mutants were fully and constitutively luminescent. This independence from autoinduction led to the conclusion that LuxO is a repressor of luminescence that is inactivated by autoinducer. However, the ability of *luxO* to complement luminescence-deficient mutants is difficult to reconcile with a repressor model.

Finally, the *S. griseus* autoinduction system, also found in variant form in some other *Streptomyces* sp., differs even more radically from that of *V. fischeri* (Horinouchi and Beppu 1994). *S. griseum* colonies produce an autoinducer that controls the timing of both sporulation and antibiotic production. This autoinducer or "A-factor" is 2-(6'-methylheptanoyl)-3*R*-hydroxymethyl-4-butanolide which, like the autoinducers described above is a γ-butyrolactone, but is not a *N*-acyl-homoserine lactone. Structurally similar autoinducers are produced by other *Streptomyces* sp. A-factor synthesis requires *afsA*, the product of which is not homologous to any known protein, including LuxI (Hara et al 1983, Horinouchi et al 1989). A-factor binds specifically to a protein in the absence of which sporulation and antibiotic production commence aberrantly early (Miyake et al 1989, Miyake et al 1990). Thus the A-factor receptor is a negative regulator rather than a positive regulator like LuxR. The primary structure of the autoinducer receptor of the closely related *Streptomyces virginiae* system has been reported to resemble not LuxR but the *E. coli* antiterminator NusG, but this report has since been retracted by its authors (Okamoto et al 1992, Horinouchi and Beppu 1994).

Despite the structural similarities between the autoinducers, the two *Vibrio* autoinduction systems clearly and dramatically differ from each other and from the *Streptomyces* systems. The dissimilarities render the conservatism of the autoinducers

themselves even more striking. This might be a case of convergent evolution. If so, the common γ-butyrolactone motif must be of specific utility to the bacteria. The physical-chemical properties of the structure may make it an ideal extracellular messenger (one that diffuses through both hydrophilic and hydrophobic compartments), or the structure may result from a need for autoinduction to reflect the state of fatty acid metabolism in the cell.

C. Why study autoinduction?

Autoinduction is interesting and important because 1) it is an apparently common regulator of significant cellular functions, possibly including many functions of which we are presently not aware. 2) it might inform us of the importance of intercellular communication as an environmental signal relevant to those cellular functions. 3) it is an interesting signal transduction system in its own right, involving a number of novel mechanistic elements. The study of autoinduction, until recently the exclusive province of students of vibrios and streptomycetes, is now of broader and more general interest than ever before. Since the *V. fischeri* system is through historical accident the best-known and effectively paradigmatic example of autoinduction, it is both imperative and convenient to explore the *V. fischeri* system even more thoroughly than has been done. To this end P. Dunlap brought basic genetic techniques to bear on the *V. fischeri* system, both confirming the classical model of autoinduction as developed with *V. fischeri* genes cloned into *E. coli*, as well as revealing novel elements in cell density-dependent regulation (Dunlap and Kuo 1992). In the course of these studies, Dunlap encountered phenomena that could be interpreted as evidence for a second autoinducer of luminescence in *V. fischeri* (personal communication). Since a second autoinducer has potentially significant ramifications with respect to the classical model of autoinduction, I have continued that line of research in my dissertation.

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Chapter 2. Construction and characterization of defined *lux* mutants of *Vibrio fischeri*

ABSTRACT

Luminescence in *Vibrio fischeri* requires O_2, luciferase (encoded by *luxAB*), reduced flavin mononucleotide, and the aldehyde product of a reaction catalyzed by a fatty acid reductase complex (encoded by *luxCDE*). Expression of the operon *luxICDABEG* is dependent on *luxR* and *luxI*; *luxI* is required for the synthesis of an autoinducer molecule (*N*-3-oxohexanoyl-L-homoserine lactone) while *luxR* is required for both response to the autoinducer and activation of transcription of *luxICDABEG*. The hypothesis that luminescence plays a role in protecting the cell from oxidative stress was examined by generating defined *lux* mutations in *V. fischeri*. The mutants were constructed using plasmid conjugation and gene replacement techniques, and then were tested for resistance to treatment with the toxic oxygen species H_2O_2. Both large *lux* deletion mutants (Δ*luxRICD* and Δ*luxDABEG*) and mutants with small nonpolar mutations in individual *lux* genes were constructed. These included mutants deficient in luciferase, fatty acid reductase complex, *luxI*, and *luxR*. The mutants did not differ from the *lux*$^+$ parent strain in resistance to H_2O_2 toxicity, indicating that luminescence is not involved in resistance to H_2O_2. The mutants also did not differ from the wild-type in any obvious phenotype, including growth rate, except in the predicted luminescence-related functions, and in the accumulation of a yellow pigment typical of *V. fischeri*. The distribution of pigment phenotypes among the mutants suggested that *luxG* is linked to the yellow pigment.

INTRODUCTION

Luminescence in the marine enteric symbiont *Vibrio fischeri* and other bacteria requires two enzymatic activities, those of a luciferase and a multienzyme fatty acid reductase complex. Luciferase catalyzes the light-emitting reaction, which involves the cleavage of molecular oxygen and the oxidation of reduced flavin mononucleotide (FMN) and a fatty aldehyde to the corresponding FMN and fatty acid (McElroy et el 1953, Cormier and Strehler 1953, Dunn et al 1973). The fatty acid reductase complex generates the aldehyde (Riendeau and Meighen 1979). The two subunits of luciferase are encoded by the genes *luxA* and *B*, while the subunits of the fatty acid reductase complex are encoded by the genes *luxC*, *D*, and *E* (Engebrecht and Silverman 1984). In *V. fischeri*, synthesis of these enzymes is activated by a putative transcriptional regulatory protein LuxR upon binding with a small membrane-permeable molecule, *N*-3-oxohexanoyl-L-homoserine lactone (autoinducer), synthesis of which requires the *luxI* gene (Eberhard et al 1981, Engebrecht et al 1983, Engebrecht and Silverman 1984). The *lux* genes are organized into a single chromosomal locus which is sufficient to support luminescence when cloned into *Escherichia coli*; in this locus *luxR* is divergently transcribed from the operon *luxICDABEG*, and between the two transcriptional units lies the *lux* operator (Engebrecht et al 1983, Devine et al 1989, Swartzman et al 1990). The function of *luxG* is unknown, but the gene product has sequence similarity to the *E. coli* flavin reductase Fre/FsrC (Andrews et al 1992).

Bacterial luminescence involves the consumption of reducing equivalents and ATP (to reduce FMN and synthesize aldehyde) and of O_2 (to reoxidize the reduced substrates), as well as the synthesis of at least seven gene products. The adaptive significance of these activities to *V. fischeri* and other luminescent bacteria is not understood. One hypothesis is that luciferase helps defend the cell against oxidative stress (McElroy and Seliger 1962, Colepicolo et al 1992). Aerobic metabolism generates, through successive single-electron reductions of O_2, superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$), each a highly reactive molecule that can oxidize or reduce lipids, proteins, and DNA, thus causing mutation and cell death (Fridovich 1978, Kanfer 1981, Imlay and Linn 1988). Oxidative stresses are exacerbated by increased partial pressures of O_2, exposure to radical-generating agents, and direct treatment with H_2O_2. Luciferase, which is capable of catalyzing the light-emitting reaction by cleaving O_2 , H_2O_2, or $O_2^{-}\cdot$ (Watanabe and Nakamura 1976, Hastings et al 1979, Kurfurst et al 1983), is hypothesized to protect the cell against oxidative stress by eliminating excess O_2 or one of the other toxic oxygen species.

Alternative hypotheses for the biological role of the luminescence system include the notion that luciferase is an alternate electron acceptor to cytochrome oxidase under conditions where the latter does not function (Seliger 1975, Nealson and Hastings 1979, Ulitzur et al 1981, Makemson and Hastings 1986, Guerrero and Makemson 1989), that luciferase is a sink for excess reducing equivalents that presumably arise when cells move from a nutritionally replete environment to growth-restricted conditions (from gut tract to sea water or light organ; Keynan et al 1963, Dunlap 1984), that the fatty acid product of the reaction has some metabolic significance independent of luminescence (Seliger 1987, Kasai et al 1990), and that luminescence attracts potential host animals to the free-living bacterium (Ruby and Morin 1979, Andrews et al 1984). None of these hypotheses has been directly tested through the analysis of luminescence mutants. Luminescence variants have been isolated from a number of species of bacteria (Rogers and McElroy 1955, Keynan and Hastings 1961, Nealson and Markovitz 1970, Cline and Hastings 1971). In one instance the variants were physiologically compared to the wild-type and reported to be less tolerant of excess reducing equivalents than the wild-type (Keynan et al 1963). The genotypes of the above variants have not been defined.

To address these hypotheses I have generated the first defined *lux* deletion mutants of *V. fischeri*, using a gene transfer system described previously (Dunlap and Kuo 1992). In this study the *lux* mutants were tested for resistance to H_2O_2 toxicity. My results suggest that the *lux* genes, luciferase, and luminescence are not involved in resistance to H_2O_2. I also show that the yellow pigment typical of *V. fischeri* (Baumann et al 1984) is dependent on a gene downstream of *luxICDABE*, presumably *luxG*.

MATERIALS AND METHODS

Strains, plasmids, and culture methods. *V. fischeri* and *E. coli* strains used in this study are described in Table 1 and Figure 1. MJ-100 is a spontaneously naladixic acid-resistant (Nx^r) isolate of *V. fischeri* MJ-1, which originated from the light organ of the fish *Monocentris japonicus* (Ruby and Nealson 1976). MJ-112 and MJ-141 are, respectively, *luxR* and *luxICD* mutants of MJ-100 (Dunlap and Kuo 1992). Of the *E. coli* strains, JM83 was used for most cloning procedures (Yanisch-Perron et al 1985), while S17-1 carries transfer functions (tra^+) that allow conjugation of mobilizable (mob^+) plasmids from S17-1 into a variety of proteobacteria, including *V. fischeri* (Simon et al 1986, Dunlap and Kuo 1992). pJE202 is the entire MJ-1 *lux* region cloned into the Inc(pMB1) plasmid pBR322 (Engebrecht et al 1983). pNL121 is the same *lux* region cloned into the mob^+, chloramphenicol resistant (Cm^r), Inc(p15A) plasmid pSUP102 (Simon et al 1986, Dunlap and Kuo 1992). pWH112 and pWH141 are derivatives of pNL121 with mutations in, respectively, *luxR* and *luxICD* (Dunlap and Kuo 1992). These and other plasmids are described in Table 1.

Except where otherwise indicated, MJ-100 and its derivatives were maintained on 1.5% agar plates of LBS medium (1% tryptone, 0.5% yeast extract, 2% NaCl, 0.3% glycerol, 50 mM Tris, pH 7.5; Dunlap 1989) with 20 mg l^{-1} Nx, and grown in liquid culture by shaking in 3 mL LBS at 28°C. Growth of liquid cultures was monitored by measuring optical density at 660 nm (A_{660}). For minimal medium experiments, cells were grown in an artificial seawater medium (300 mM NaCl, 50 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2$, 1 g l^{-1} NH_4Cl, 100 mg l^{-1} α-glycerophosphate, 20 mg l^{-1} ferric ammonium citrate, 25 mM HEPES, pH 7.5; Friedrich and Greenberg 1983) with 10 mM glucose but without Nx at 28°C. *E. coli* strains were grown on LB, LBS, or SOB medium with appropriate antibiotics at pH 7.5 and 28°C (conditions necessary for luminescence in *E. coli*). To select or screen for presence or absence of the appropriate genetic marker, antibiotics were added to medium to the following concentrations: 34 mg l^{-1} Cm, 50 mg l^{-1} ampicillin (Ap), 20 mg l^{-1} kanamycin (Km), 200 mg l^{-1} neomycin (Nm), 12 mg l^{-1} tetracycline (Tc). When used, 2 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside was applied to the surface of a plate, while synthetic autoinducer was added to liquid medium to 5 mg l^{-1}.

Construction of mutants: general procedures. All mutants were generated by enzymatically manipulating the cloned MJ-1 *lux* genes (cloned into pSUP102), transforming the plasmid bearing the resulting *lux* mutation into S17-1, transconjugating the plasmid into MJ-100, and screening for nonluminescent (Lux^-) recombinants.

Table 1. Strains and plasmids used in this study. Constructions are detailed in the Materials and Methods.

Strain or plasmid	Relevant genotypic and phenotypic characters	Source
E. coli JM83	*recA*	Yanisch-Perron et al 1985
E. coli S17-1	RP4 *tra*$^+$	Simon et al 1986
V. fischeri MJ-1		Ruby and Nealson 1976
MJ-100	MJ-1 that is spontaneously Nx^r	Dunlap and Kuo 1992
MJ-112	MJ-100 with *luxR*::Mu dI1734, Nm^r	Dunlap and Kuo 1992
MJ-141	MJ-100 with *luxI*::Mu dI1734', Δ*luxICD*, Nm^s	Dunlap and Kuo 1992
MJ-201	MJ-112 with Δ*luxRICD*, ΔMu dI1734, Nm^s	This study
MJ-203	MJ-100 with in-frame Δ*luxA*	This study
MJ-207	MJ-100 with Δ*luxRICD*	This study
MJ-208	MJ-100 with in-frame Δ*luxR*	This study
MJ-209	MJ-100 with nonpolar 4 bp insertion in *luxD*	This study
MJ-210	MJ-100 with Δ*luxDABEG*	This study
MJ-211	MJ-100 with nonpolar Δ*luxI*	This study
pBR322	Inc(ColE1), Ap^r Cm^r Tc^r	
pJE202	pBR322 with MJ-1 *luxRICDABEG*, Tc^s	Engebrecht et al 1983
pAK004	pJE202 with Δ*luxBEG*	This study
pACYC184	Inc(p15A), Cm^r Tc^r	
pSUP102	pACYC184 with RP4 *mob*$^+$	Simon et al 1986
pNL121	pSUP102 with MJ-1 *luxRICDABEG*, Tc^s	Dunlap and Kuo 1992
pWH112	pNL121 with *luxR*::Mu dI1734, Km^r	Dunlap and Kuo 1992
pWH141	pNL121 with *luxI*::Mu dI1734' Δ*luxICD*, Km^s	Dunlap and Kuo 1992
pWH201	pWH112 with Δ*luxRICD*, ΔMu dI1734, Km^s	This study
pAK203	pNL121 with in-frame Δ*luxA*	This study
pAK207	pNL121 with Δ*luxRICD*	This study
pAK208	pNL121 with in-frame Δ*luxR*	This study
pAK209	pNL121 with nonpolar 4 bp insertion in *luxD*	This study
pAK210	pNL121 with Δ*luxDABEG*	This study
pAK211	pNL121 with nonpolar Δ*luxI*	This study

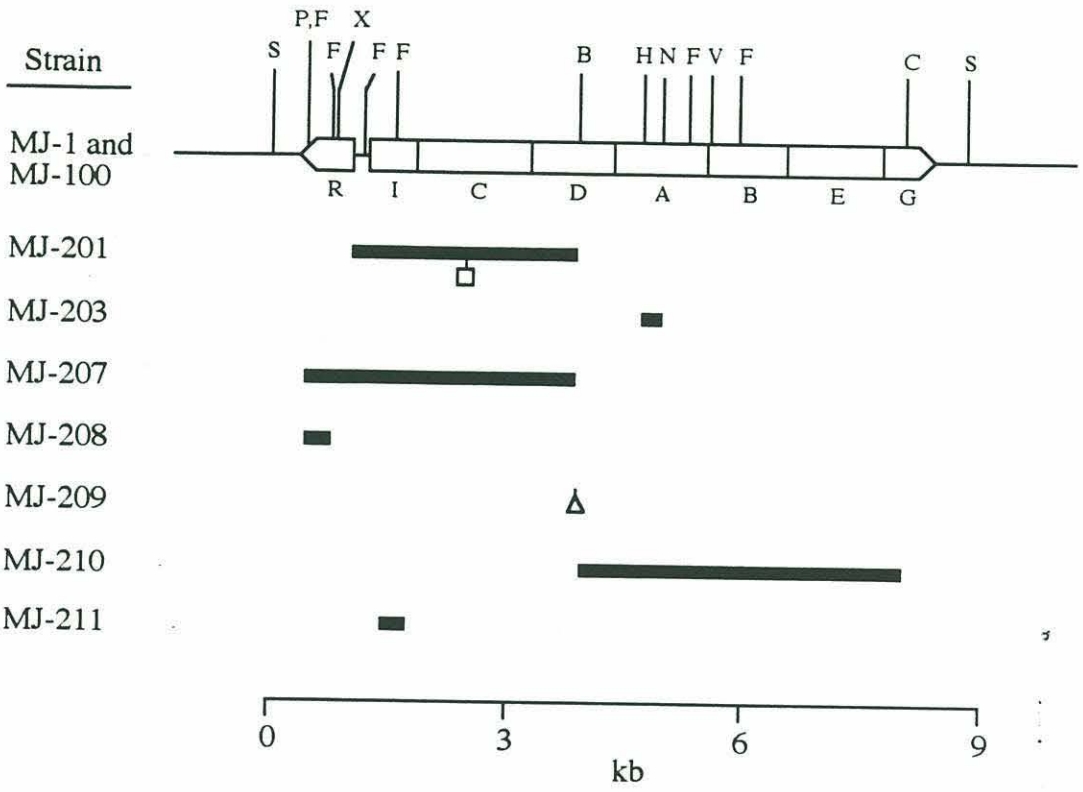

Figure 1. Physical and genetic maps of the *lux* region of *Vibrio fischeri* MJ-1 and MJ-1-derived *lux* deletion mutants constructed in this study. Letters above the map indicate restriction sites exploited in this study and recognized by the following enzymes: S, *Sal*I; P, *Pst*I; F, *Sfc*I; X, *Xba*I; B, *Bgl*II; H, *Xho*I; N, *Nhe*I; V, *Pvu*II; C, *Nco*I. Letters below the map indicate the *lux* genes. The *lux* promoters and operator are located between the *luxR* and *luxICDABEG* operons. Black bars (▬) indicate stretches of DNA deleted from each mutant. Triangle (Δ) indicates a 4 bp insertion into *luxD*. Square (□) indicates 1.4 kb Mu dI1734 DNA, including the Mu *c* gene and the promoter for *neo*, interrupting the *lux* region of MJ-201. The construction of each mutant is detailed in the Materials and Methods.

Restriction digests, blunt-ending reactions (using DNA polymerase I large (Klenow) fragment), and ligations followed standard procedures, as did transformations into *E. coli*. The desired ligation products were detected by culturing transformed *E. coli* in the presence of Cm at 28°C, screening for Lux$^-$ colonies, isolating plasmid, and performing an appropriate restriction analysis. To mate strains, the plasmid-bearing donor S17-1 and the recipient MJ-100 were separately cultured at 28°C in LBS without antibiotics to A_{660} = 0.5, and 10 μL each culture was aliquoted onto an LBS plate. After overnight incubation, the mixture was cultured on LBS in the presence of Cm and Nx. Doubly-resistant transconjugants were purified and then cultured in the absence of Cm. Because pSUP102-derived plasmids appear to be unstable in MJ-100 in the absence of selection (Dunlap and Kuo 1992), double recombinants (in which the enzymatically altered *lux* region on the plasmid had replaced the wild-type *lux* region on the MJ-100 chromosome) were recovered by screening for Lux$^-$ Cm^s Nx^r colonies. Recombinants arose at frequencies of 0.02-1.0%. In some cases loss of the plasmid was confirmed by examining plasmid extracts on agarose gels.

The putative mutants were then purified and characterized. Luminescence was quantified as described previously (Rosson and Nealson 1981) in 3 ml LBS cultures using a Pacific Instruments Digital Photometer Model 124. Luciferase activity was measured in crude lysates of centrifuged 1 ml cultures at A_{660} = 1 using a single-turnover assay as described previously (Rosson and Nealson 1981). Aldehyde deficiency was determined by exposing a colony of the strain in question to vapors of 95% *n*-decanal for 5 min, and scoring for luminescence (Nealson and Markovitz 1970). Autoinducer synthesis was assessed by measuring the ability of the strain in question to condition medium to prematurely induce luminescence in MJ-100 (Eberhard 1972). Conversely, ability to respond to autoinducer was assessed by culturing the strain in question in either medium that had been conditioned by MJ-100 and then filter-sterilized, or in unconditioned medium with synthetic autoinducer. Complementation tests were performed using the transformation or transconjugation protocols described above and scoring for luminescence. All mutants were verified by Southern analysis. *V. fischeri* genomic DNAs were extracted as previously described (Dunlap and Kuo 1992), DNA probes were prepared and purified on low-melting point agarose electrophoretic gels following standard procedures, and Southern hybridizations were conducted with a Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim).

Details pertaining to each mutant are presented in the following text, and in Table 1 and Figure 1.

Construction of the *luxRICD* mutant MJ-201. The *lux* deletion plasmid pWH201 was generated by treating pWH112 with *Bgl*II (one site in *luxD* and two sites in Mu dI1734) and ligase. pWH201 thus lacks 2.8 kb *lux* DNA, including *luxI*, *luxC*, the 5' ends of *luxR* and *luxD*, and the *lux* promoters and operator, but retains 1.4 kb Mu dI1734 DNA (including the Mu *c* gene and the promoter for *neo*; Castilho et al 1984). pWH201 was used to construct the *lux* deletion mutant MJ-201. MJ-201 emitted no detectable light, expressed a low but detectable level of luciferase activity (0.02x level of MJ-100), failed to respond to either exogenous autoinducer or aldehyde, and failed to prematurely induce luminescence in MJ-100. Southern hybridizations showed that the 2.8 kb fragment deleted from pWH201 was also lacking in MJ-201, and that the *lux* sequences flanking the deletion were intact (data not shown).

Construction of the *luxR* mutant MJ-208. Complete digestion of pNL121 with *Pst*I, followed by partial digestion with *Xba*I (one site in *luxR* and one in pSUP102), blunt-ending, and ligation yielded pAK208. This plasmid theoretically has a 450 bp (150 aa) deletion in *luxR* that is in-frame (according to the published sequence; Engebrecht and Silverman 1987). This *luxR* deletion plasmid complemented the mutant MJ-141 (which has a functional *luxR* but no functional *luxICD*) but not MJ-112 (which is defective in *luxR*). pAK208 was used to construct the *luxR* deletion mutant MJ-208. MJ-208 was complemented for luminescence by pWH141 (*luxR*$^+$ Δ*luxICD*) but not by pWH112 (*luxR*::Mu dI1734). Southern analysis showed that MJ-208 lacked the *Xba*I site in *luxR*. MJ-208 was expected to express *luxICDABEG*, but only at low levels, and in fact emitted 0.0006x the level of light emitted by MJ-100 at A_{660} = 1.5. Also as expected, addition of autoinducer to the medium had no effect on luminescence.

Construction of the *luxRICD* mutant MJ-207. pNL121 was digested with *Bgl*II and *Pst*I, blunt-ended, and ligated. The resulting plasmid, pAK207, possessed a 3.5 kb deletion extending from the *Pst*I site in *luxR* to the *Bgl*II site in *luxD*, and was used to construct MJ-207.

Construction of the *luxI* mutant MJ-211. pNL121 was first partially digested with *Sfc*I; singly-cut plasmid was purified by low-melting point agarose gel electrophoresis. A set of deletions was generated using the Erase-a-Base System (Promega); the procedure involved sequential treatment of the DNA with exonuclease III, S1 nuclease, Klenow, and T4 DNA ligase. The exonuclease digestion was conducted at 25°C for 0.5-2 min. Transformants were screened for responsiveness to autoinducer by picking poorly luminescent colonies and coculturing them with MJ-203 (*luxI*$^+$ Δ*luxA*; see below). Four plasmids with nonpolar mutations in *luxI* were detected in this manner, recovered, and shown by restriction analysis to carry ~200-300 bp deletions. One

plasmid with a ~250 bp deletion, pAK211, was used to construct the *luxI* deletion mutant MJ-211. At A_{660} = 1.5, MJ-211 emitted 0.0002x the amount of light emitted by MJ-100, but in the presence of synthetic autoinducer light emission was 0.2x the level of MJ-100. Thus exogenous autoinducer promoted luminescence in MJ-211 1000-fold at A_{660} = 1.5. The presence and size of the deletion in MJ-211 was confirmed by Southern analyses.

Construction of the luciferase (*luxA*) mutant MJ-203. Complete digestion of pNL121 with *Xho*I (one site in *luxA*) and partial digestion with *Nhe*I (one site in *luxA* and two sites in pSUP102) followed by blunt-ending and ligation yielded pAK203. pAK203 theoretically has a 168 bp (56 aa) deletion within *luxA* that is in-frame and therefore nonpolar (based on the published sequence; Foran and Brown 1988). To demonstrate that the deletion was indeed nonpolar, the *luxRICDAB'* plasmid pAK004 was constructed by digesting the *lux*$^+$ plasmid pJE202 with *Pvu*II (one site in *luxB* and one site in pBR322) and ligating, thus truncating *luxB* and deleting *luxE*. Neither pAK203 nor pAK004 alone was capable of supporting luminescence in JM83, but together the plasmids complemented one another for luminescence, demonstrating that pAK203 expressed both *luxB* and *luxE*. pAK203 was then used to construct the nonpolar *luxA* deletion mutant MJ-203. MJ-203 emitted no detectable light, nor expressed any detectable luciferase activity, but did condition medium in our autoinducer assay. Southern analysis showed that MJ-203 lacked the *Xho*I site in *luxA*.

Construction of the aldehyde (*luxD*) mutant MJ-209. pAK209 was constructed by digesting pNL121 with *Bgl*II, filling-in the resulting 4 bp overhangs with Klenow, and ligating. These manipulations theoretically generate a 4 bp insertion and thus a frameshift in *luxD*. S17-1 transformed with pAK209 was Lux$^-$. However, transformants were physiologically complemented by exogenous aldehyde, demonstrating that the insertion was nonpolar. pAK209 was used to construct the *luxD* mutant MJ-209, the luminescence of which was also responsive to aldehyde. Without aldehyde, MJ-209 emitted light at 0.0001x the level of MJ-100 at A_{660} = 1.5. Southern hybridization showed that MJ-209 lacked the *Bgl*II site in *luxD*.

Construction of the *luxDABEG* mutant MJ-210. Complete digestion of pNL121 with *Bgl*II, followed by partial digestion with *Nco*I (one site in *luxG*, one site in pSUP102), blunt-ending, and ligation, yielded pAK210, which lacked 4.0 kb DNA containing *luxABE*, the 3' end of *luxD*, and the 5' end of *luxG*. S17-1 transformed with pAK210 did not luminesce. pAK210 was used to construct the *lux* mutant MJ-210, which emitted no detectable light. A 8.8 kb *Sal*I fragment of pNL121 (containing all of *lux*) hybridized to a single 4.8 kb fragment of a *Sal*I-*Bgl*II digest of MJ-210 DNA while

hybridizing to 4.8 and 4.0 kb fragments of similarly digested MJ-100 DNA, indicating that MJ-210 lacked 4.0 kb *lux* DNA.

Measurement of resistance to H_2O_2. Strains were tested for H_2O_2 resistance in two ways. In the liquid culture test, cells were grown in minimal medium for > 12 hours. Before reaching stationary phase ($A_{660} < 1$) cultures were diluted into fresh minimal medium at least 100-fold. The dilute cultures were monitored for growth over the course of exponential phase; at $A_{660} = 0.2$, H_2O_2 was added to 40 μM, and growth was monitored for several more hours. In the top agar test, cells were grown in LBS without antibiotics for 24 hrs ($A_{660} > 2$) and then diluted with fresh LBS to $A_{660} = 0.5$; 100 μl of the dilute culture was mixed with 5 ml molten top agar (LBS with 0.75% low-melting point agarose at 37°C) and poured onto a hardened LBS agar plate. After 30 min, a concentration blank was placed on the center of the plate and 10 μl 30% H_2O_2 was applied to the blank. Plates were incubated at room temperature overnight and diameters of the resulting clear circular zones of growth inhibition were measured with a ruler. Alternatively, the zones of inhibition were outlined with a marker and photocopied onto paper, and the resulting images were cut out and weighed on a balance.

RESULTS

Low resistance of the *lux* mutant MJ-201 to H_2O_2. In all media tested, the Δ*luxRICD* mutant MJ-201 grew more slowly than did MJ-100. In the liquid culture assay for resistance to H_2O_2, the growth of both MJ-100 and MJ-201 was inhibited by the addition of H_2O_2, but cultures of MJ-100 were inhibited only temporarily while cultures of MJ-201 were inhibited much more strongly (Fig. 2a). This result was confirmed with the top agar assay for H_2O_2 resistance; H_2O_2 left a consistently and significantly larger zone of growth inhibition in a confluent layer of MJ-201 than in a layer of MJ-100 (data not shown).

Normal resistance of the *luxR* mutant to H_2O_2. To provide further evidence that the H_2O_2 sensitivity of MJ-201 was due to the low expression of *lux* genes, I tested the in-frame Δ*luxR* mutant MJ-208 for resistance to oxidative stress. Unlike MJ-201, MJ-208 exhibited a growth curve no different from that of MJ-100, and exhibited no greater sensitivity to H_2O_2 than did MJ-100 in either the liquid or the top agar assay (data not shown). Varying the concentration of H_2O_2 from 20 to 80 μM did not affect the result of the liquid assay. To confirm these results, H_2O_2-resistance of MJ-208 was quantitated in a third manner. Cells were cultured as in the top agar assay. However, instead of diluting the culture, I challenged 1 ml aliquots of the dense culture with a range of H_2O_2 concentrations (from 200 μM to 88.2 mM), incubated the cultures for 5 min, and then diluted the cultures for spreading on LBS plates. The viability of the cultures after treatment with H_2O_2, as measured by the number of colonies arising after H_2O_2 treatment relative to the number of colonies without H_2O_2 was not different between MJ-208 and MJ-100 (data not shown). These results suggest that low *lux* expression was in fact not responsible for the heightened sensitivity of MJ-201 to H_2O_2.

Normal resistance of the *lux* mutant MJ-207 to H_2O_2. One difference between the *luxR* mutant and MJ-201 is that the former presumably synthesises the fatty acid reductase complex at a low level, while the latter lacks *luxC* and *D*. Also, MJ-201 lacks *luxI* and the *lux* promoters and operator, which were retained in the construction of MJ-208. Finally, MJ-201 differs from MJ-208 in possessing exogenous insert DNA, consisting of the Mu *c* gene (encoding the Mu phage repressor) and the promoter of *neo*. To ask if the H_2O_2 sensitivity of MJ-201 was due to the absence of any of certain *lux* sequences or to the presence of the exogenous DNA, I constructed a mutant that was similar to MJ-201 in its lack of *luxI*, *luxC*, the 5' ends of *luxR* and *luxD*, and the *lux* promoters and operator, but differed from MJ-201 in its lack of any Mu DNA. The resulting Δ*luxRICD* mutant, MJ-207, grew at the same rate as MJ-100, and exhibited the

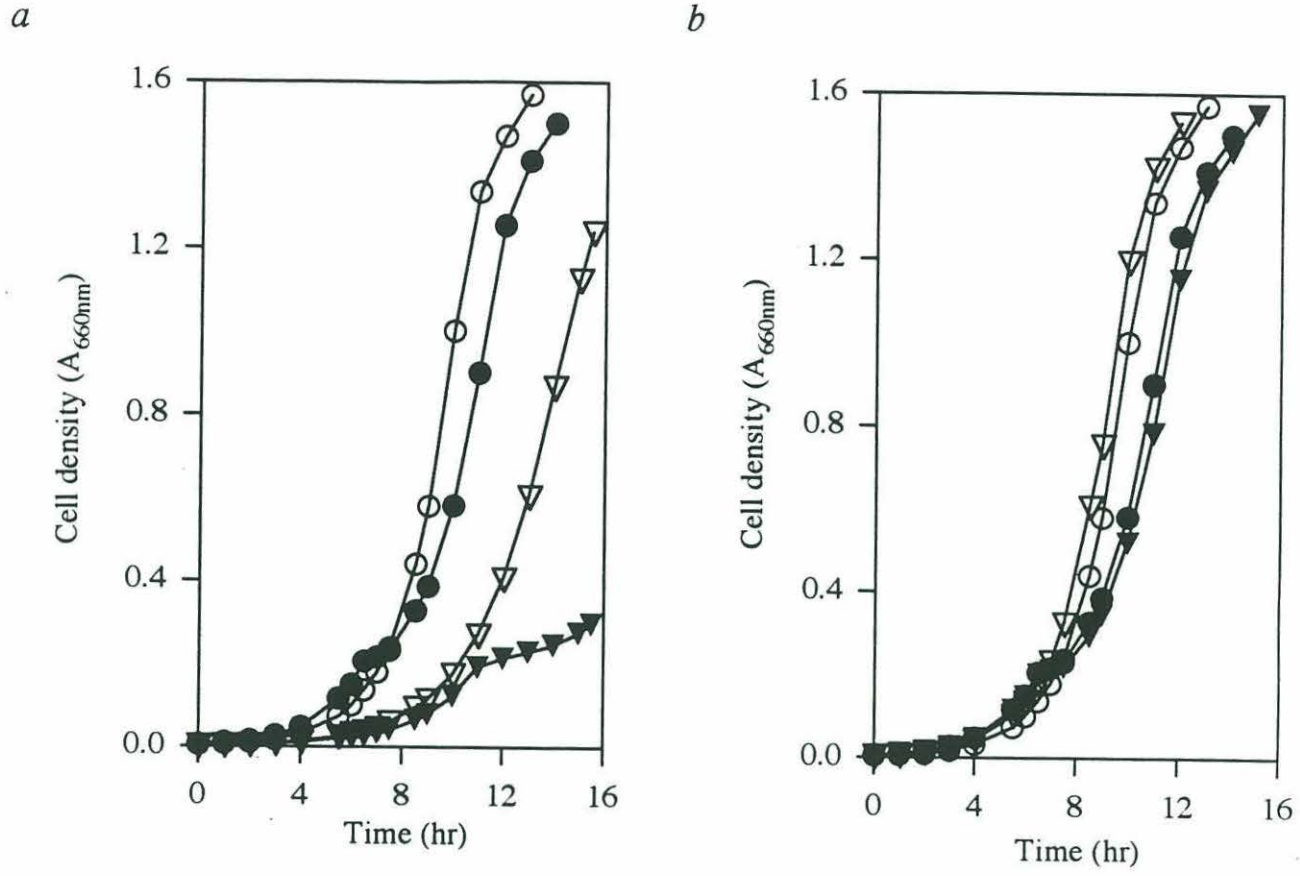

Figure 2. Growth and resistance to H_2O_2 of *lux* mutants. Strains were cultured in a minimal medium and treated with H_2O_2 at $A_{660} = 0.2$, as detailed in the Materials and Methods. Filled symbols denote H_2O_2-treated cultures, unfilled symbols denote untreated cultures. a) Strains areMJ-100 (O) and the Δ*lux* mutant MJ-201 (∇). b) Strains are MJ-100 (O) and the Δ*luxRICD* mutant MJ-207 (∇). MJ-201 differs from MJ-207 in its possession of Mu dI1734 DNA.

same resistance to H_2O_2 as that of the wild-type (Fig. 2b). I conclude that the low growth rate and low resistance of MJ-201 are due to the presence of Mu DNA rather than to *lux* mutation.

Pigment production by the *luxI* mutant. Each of the above mutants, but not the wild-type MJ-100, was deficient in the accumulation of a yellow pigment characteristic of *V. fischeri*. This pigment is visible in colonies and stationary-phase liquid cultures, and in the cell-free supernatants of such cultures. The fact that each of the mutants lacked *luxR* clearly indicates that the pigment-deficient phenotype is dependent on *luxR*. To confirm that pigment synthesis is coregulated with luminescence, I examined the nonpolar Δ*luxI* mutant MJ-211. MJ-211 emitted a relatively low level of light compared to the wild-type, and concomitantly failed to produce visible amounts of pigment. Both luminescence and pigmentation were rescued by growth in the presence of exogenous autoinducer (Table 2). Furthermore, neither luminescence nor pigmentation were rescued by the addition of exogenous autoinducer to the Δ*luxR* mutant MJ-208. These results demonstrate that pigment accumulation is, like luminescence, regulated by autoinduction.

Normal pigmentation of the luciferase and aldehyde mutants. One possible reason that pigmentation and luminescence are induced together is that pigment synthesis reflects the activities of one of the luminescence enzymes. To ask if the flavoenzyme luciferase is involved in pigment production, I examined the in-frame Δ*luxA* mutant MJ-203. The luciferase mutant did not obviously differ from MJ-100 in either growth characteristics or pigmentation. Similarly, the nonpolar *luxD* mutant MJ-209, while deficient in aldehyde production, was normally pigmented. These results do not implicate either luminescence or fatty acid reduction in pigmentation.

Deficient pigmentation of the *luxG* mutant. The above results do not exclude the possibility that pigment accumulation is directed by genes not involved in luminescence but which are nevertheless regulated by autoinducer and LuxR. One such gene is *luxG*, which is not necessary for luminescence in *E. coli* (data not shown). It is also possible that autoinducer and LuxR regulate pigmentation genes outside of the *lux* operon. To assess this possibility, I constructed a *luxG* mutant. There is no known phenotype or protein associated with *luxG*, so there is no way to phenotypically detect a *V. fischeri* mutant with a simple deletion internal to *luxG*. However, a *luxG* deletion could be detected if linked to the deletion of other genes with known functions. Thus I constructed MJ-210, which lacks *luxABE*, the 3' end of *luxD*, and the 5' end of *luxG*. MJ-210 was deficient in pigment production, implying that accumulation of the yellow pigment is directed by *luxG*.

Table 2. Yellow pigment production of *lux* mutants

Strain	Yellow pigment
MJ-100 (*lux*$^+$)	+
MJ-201 (Δ*luxRICD*)	-
MJ-207 (Δ*luxRICD*)	-
MJ-203 (Δ*luxA*)	+
MJ-209 (*luxD*$^-$)	+
MJ-211 (Δ*luxI*)	-
MJ-211 + autoinducer	+
MJ-208 (Δ*luxR*)	-
MJ-208 + autoinducer	-
MJ-210 (Δ*luxDABEG*)	-

DISCUSSION

In this study, defined mutations of the *lux* genes were constructed in *V. fischeri*. To test whether luminescence protects the cell against H_2O_2 toxicity, the effects of the mutations on cellular resistance to H_2O_2 were measured. Mutations in *lux* had no apparent effect on resistance to H_2O_2, nor on growth, but certain mutations did affect pigmentation.

There has been only one previous report of the construction of defined mutants of a luminescent bacterium (Dunlap and Kuo 1992). In this study I introduced enzymatically altered genes into *V. fischeri*, moved the altered genes into the chromosome through gene replacement, and documented the gene replacements by complementation and Southern analysis. This genetic methodology provides a powerful and general approach to studying the *V. fischeri lux* genes in *V. fischeri*, as opposed to in *E. coli*, as well as any other cloned *V. fischeri* genes. I stress the importance of performing such studies in *V. fischeri*, since it is known that aspects of *lux* regulation such as autoinducer-independent modulation differ between *V. fischeri* and *E. coli* (Dunlap and Kuo 1992).

Both the lower growth rate and the greater H_2O_2 senstivity of MJ-201 relative to MJ-100, MJ-208, and MJ-207 imply that the insertion of 1.4 kb Mu dI1734 DNA into the *lux* locus has a substantial physiological affect on *V. fischeri*. The foreign DNA includes the Mu *c* gene (encoding the Mu phage repressor) and the promoter of the Km^r determinant *neo*. The reason for the adverse effect of the Mu DNA is unknown; it is possible that the promoters for *c* or *neo* may affect the expression of flanking *V. fischeri* genes. Alternatively, it is possible that the expression of *c* function causes the observed phenotype. Given that Mu-derived constructs are commonly used in transposon mutagenesis and the generation of transcriptional and translational fusions, and that *neo* cassettes are often used to construct selectable insertions, the effect of exogenous DNA reported here may serve as a cautionary note.

This study is the first systematic use of a genetic approach to assess the physiological role of luminescence in bacteria. The mutant phenotypes confirmed the functions of the individual *lux* genes as established in studies with the cloned genes in *E. coli* (Engebrecht and Silverman 1984). Given the complexity and energetic requirements of luminescence (reducing power, O_2, ATP, protein synthesis), it is perhaps surprising that the *lux* mutants (excepting MJ-201) did not differ from MJ-100 in growth rate or yield. Estimates of the magnitude of the impact of luminescence on total cellular metabolism vary widely, from as high as 20% of respiration to as low as 0.007% of total

energy expenditure (Dunlap 1985, Makemson 1986, Makemson and Gordon 1989). My results support the view that luminescence is not an enormous energetic burden on the cell.

My inability to detect a clear effect of *lux* mutation on H_2O_2 resistance is inconsistent with the hypothesis that the function of luciferase is to detoxify H_2O_2. However, this result does not prove that luciferase function does not involve elimination of $O_2^{-}\cdot$ or excess O_2. The *luxA* mutant generated in this study could be employed to directly address these possibilities. Similarly, the *luxA* mutant could be used to test some of the other hypotheses of the adaptive function of luminescence, especially the hypothesis that luciferase acts as an alternate electron acceptor. Utilizing the *lux* mutants to investigate the role of luminescence in symbiosis may be more difficult, because the only known cultivable host of *V. fischeri*, the squid *Euprymna scolopes*, cannot establish a successful symbiosis with MJ-1, the strain used in this study (Ruby and McFall-Ngai 1992). However, the mutation approach used in this study might be applicable to still other strains of *V. fischeri* that can infect *E. scolopes* (McFall-Ngai and Ruby 1991).

Even though neither the deletions in *luxR* and *luxA* nor the broad deletion from *luxR* to *luxD* had a demonstrable effect on growth or H_2O_2 resistance, there was a clear pattern relating yellow pigment to the *lux* locus. The pattern is consistent with the notion that development of the yellow pigment is a product not of luciferase function or fatty acid reduction but of LuxG. *E. coli* with the intact *lux* operon does not appear to synthesize the yellow pigment, and I have not constructed a *V. fischeri* strain with a mutation in *luxG* alone. Before this study, LuxG had no demonstrated function, but has sequence similarity to Fre, an *E. coli* flavin reductase involved in the generation of the free radical functional group of ribonucleotide reductase (Fontecave et al 1987, Spyrou et al 1991, Andrews et al 1992). Known NAD(P)H-flavin oxidoreductases in luminescent bacteria are not encoded by *luxG* (Zenno et al 1994, Zenno and Saigo 1994). Similarly, the nature and function of the pigment are unknown. Its solubility in water and flourescence in ultraviolet have led to the suggestion that it is riboflavin (Giese 1943). Such a suggestion is tempting if LuxG is indeed a flavin reductase. The mutants and genetic techniques described in this study, together with chemical identification of the pigment, should clarify the functional role of *luxG*.

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Chapter 3. Multiple *N*-acyl-L-homoserine lactone autoinducers of luminescence in the marine symbiotic bacterium *Vibrio fischeri*

This chapter has appeared in the Journal of Bacteriology 176(24):7558-7565. What follows is a copy of the galley proofs. On the sixth page of the proofs, first column of text, "δ_1, $\delta_H = 4.42$" should read as "γ_1, $\delta_H = 4.42$".

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Multiple *N*-Acyl-L-Homoserine Lactone Autoinducers of Luminescence in the Marine Symbiotic Bacterium *Vibrio fischeri*†

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In *Vibrio fischeri*, the synthesis of *N*-3-oxohexanoyl-L-homoserine lactone, the autoinducer for population density-responsive induction of the luminescence operon (the *lux* operon, *luxICDABEG*), is dependent on the autoinducer synthase gene *luxI*. Gene replacement mutants of *V. fischeri* defective in *luxI*, which had been expected to produce no autoinducer, nonetheless exhibited *lux* operon transcriptional activation. Mutants released into the medium a compound that, like *N*-3-oxohexanoyl-L-homoserine lactone, activated expression of the *lux* system in a dose-dependent manner and was both extractable with ethyl acetate and labile to base. The *luxI*-independent compound, also like *N*-3-oxohexanoyl-L-homoserine lactone, was produced by *V. fischeri* cells in a regulated, population density-responsive manner and required the transcriptional activator LuxR for activity in the *lux* system. The *luxI*-independent compound was identified as *N*-octanoyl-L-homoserine lactone by coelution with the synthetic compound in reversed-phase high-pressure liquid chromatography, by derivatization treatment with 2,4-dinitrophenylhydrazine, by mass spectrometry, and by nuclear magnetic resonance spectroscopy. A locus, *ain*, necessary and sufficient for *Escherichia coli* to synthesize *N*-octanoyl-L-homoserine lactone was cloned from the *V. fischeri* genome and found to be distinct from *luxI* by restriction mapping and Southern hybridization. *N*-Octanoyl-L-homoserine lactone and *ain* constitute a second, novel autoinduction system for population density-responsive signalling and regulation of *lux* gene expression, and possibly other genes, in *V. fischeri*. A third *V. fischeri* autoinducer, *N*-hexanoyl-L-homoserine lactone, dependent on *luxI* for its synthesis, was also identified. The presence of multiple chemically and genetically distinct but cross-acting autoinduction systems in *V. fischeri* indicates unexpected complexity for autoinduction as a regulatory mechanism in this bacterium.

Autoinduction is an intercellular signalling and gene regulatory mechanism for population density-responsive control of luminescence in *Vibrio fischeri*, a bacterium that occurs at high population density in light-organ symbiosis (e.g., 10^{10} to 10^{11} cells ml^{-1}) and other habitats (23). Autoinducer (*N*-3-oxohexanoyl-L-homoserine lactone [*N*-3-oxohexanoyl-L-HSL]) is a self-produced, membrane-permeable compound that accumulates as *V. fischeri* population density increases (10, 20). At threshold concentrations, autoinducer, via the autoinducer receptor and transcriptional activator LuxR, triggers transcription of the luminescence (*lux*) operon, *luxICDABEG*, which contains genes for autoinducer synthase (*luxI*) and luminescence enzymes (13, 14, 30).

Besides *V. fischeri*, other species of proteobacteria that are symbionts or pathogens of higher organisms have recently been found to produce *N*-acyl-L-HSL autoinducers structurally similar or identical to the *V. fischeri* autoinducer. For example, *N*-3-hydroxybutanoyl-L-HSL mediates autoinduction of luminescence in *Vibrio harveyi*, *N*-3-oxooctanoyl-L-HSL mediates autoinduction of conjugation in *Agrobacterium tumefaciens*, and *N*-3-oxohexanoyl-L-HSL and *N*-3-oxododecanoyl-L-HSL mediate autoinduction of virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*, respectively (2, 4, 25, 32). In many cases, the genes for the autoinducer synthase and transcriptional activator exhibit substantial sequence similarity to *luxI* and *luxR*, respectively, of *V. fischeri* (16, 17, 24, 26, 27). Recently, evidence has been obtained for an HSL-based signal of starvation in *Escherichia coli* (19). The diversity of species that use autoinduction and the chemical and genetic similarities of their autoinduction systems indicate that autoinduction is an evolutionarily conserved signalling and regulatory mechanism of general importance in proteobacteria.

In this report, we demonstrate that *V. fischeri* produces a second, novel autoinducer, *N*-octanoyl-L-HSL, that activates *lux* operon transcription via LuxR. The synthesis of *N*-octanoyl-L-HSL is directed by a novel autoinducer synthase locus, *ain*, that is distinct from *luxI*. A third *V. fischeri* autoinducer, *N*-hexanoyl-L-HSL, dependent on *luxI* for its synthesis, was also identified. The presence of multiple chemically and genetically distinct autoinduction systems in *V. fischeri* indicates unexpected complexity for autoinduction as a signalling and gene regulatory mechanism within a single bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and physiological assays. The strains used in this study are derivatives of *E. coli* K-12 and *V. fischeri* MJ-1 and are listed in Table 1. *V. fischeri* MJ-100, a spontaneously nalidixic acid-resistant (Nx^r) derivative of MJ-1 (8), and its derivatives were maintained on solid LBS medium (8) with 20 μg of nalidixic acid ml^{-1}. *E. coli* S17-1 is a strain capable of conjugatively transferring the mobilizable chloramphenicol resistance (Cm^r) plasmid pSUP102 to a wide range of recipients (29), including *V. fischeri* (6, 8). *E. coli* strains were maintained on solid LB medium (1) with appropriate antibiotics to ensure plasmid maintenance.

Growth conditions, cell density and luminescence assays, and the light-measuring equipment and standard were as

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli K-12		
JM83	Δ(*lac-proAB*)	31
PD100	*zah-735*::Tn*10* Δ(*argF-lac*)*U169*, Tc^r	7
S17-1	RP4 tra$^+$	29
V. fischeri		
MJ-1	Lux$^+$	28
MJ-100	MJ-1, Nx^r	8
MJ-203	MJ-100, Δ*luxA* (168-bp *Xho*I-*Nhe*I in-frame deletion)	This study
MJ-207	MJ-100, Δ*luxRICD* (~3.5-kbp *Pst*I-*Bgl*II deletion)	This study
MJ-208	MJ-100, Δ*luxR* (450-bp *Pst*I-*Xba*I in-frame deletion)	This study
MJ-211	MJ-100, Δ*luxI* (~250-bp nonpolar deletion)	This study
Plasmids		
pSUP102	pACYC184, RP4 *mob*$^+$, Cm^r Tc^r	29
pJE202	pBR322 with ~8.8-kbp *Sal*I fragment from *V. fischeri* DNA (*luxR luxICDABEG*), Ap^r	13
pNL121	pSUP102 with ~8.8-kbp *Sal*I *lux* fragment of pJE202, Cm^r	8
pPD749	P*tac-luxR*, *lacI*^{q}, Ap^r	7
pJR551	pACYC184 with *luxR*::MuΔ (*c nerAB*) dI1681 (*lacZYA* Km^s) and *luxICDABEG* with a nonpolar point mutation in *luxI*, Cm^r	9
pAI002	pSUP102 with ~10.5-kbp fragment from *V. fischeri*, *ain*$^+$, Cm^r	This study
pAI004	pBR322 with ~2.7-kbp *Hin*dIII *V. fischeri* DNA fragment, *ain*$^+$, Ap^r	This study
pAK211	pNL121 with ~250-bp nonpolar deletion in *luxI*	This study
pAK411	pBR322 with ~8.5-kbp *lux* fragment of pAK211	This study
pAK208	pNL121 with 450-bp in-frame *Pst*I-*Xba*I deletion in *luxR*	This study
pAK408	pBR322 with ~8.3-kbp *Sal*I fragment of pAK208	This study

[a] Ap^r, ampicillin resistant; Km^s, kanamycin sensitive; Tc^r, tetracycline resistant.

previously described (8). *V. fischeri* was inoculated into liquid LBS or ASH medium (8) to an $A_{660} < 0.01$. Liquid cultures of *E. coli* were handled similarly except that LB medium (1) with 50 mM Tris (pH 7.5) and an appropriate antibiotic was used.

For the measurement of autoinducer activity, filter-sterilized supernatants of high-density cultures (A_{660} of 1.5 in LBS for *V. fischeri* and 2.0 for *E. coli*) (i.e., conditioned media) were mixed 1:1 with autoinducer assay medium (an LB-Tris medium) (9). Alternatively, supernatants were extracted with acidified ethyl acetate (100 μl of glacial acetic acid in 1 liter of ethyl acetate) and dried as described previously (10) and then redissolved in assay medium. Next, an exponential-phase culture ($A_{660} = 0.3$) of the autoinducer assay strain, *E. coli* PD100(pPD749, pJR551) (9), was added to a final A_{660} of 0.01, and subsequent luminescence and growth were monitored. The growth rates of the autoinducer assay strain within experiments were essentially similar. The responses of *V. fischeri* strains to an autoinducer were determined by inoculating the strain into a 1:1 mixture of unconditioned medium and medium conditioned by an appropriate autoinducer-producing strain.

Construction of *V. fischeri lux* mutants. Most of the deletions used in this study to construct *V. fischeri lux* mutants were generated in the subcloned *lux* genes (*luxR* and *luxICDABEG*) of pNL121 (8) by digestion with appropriate restriction enzymes (Fig. 1), blunt ending with Klenow fragment, ligating, transforming *E. coli* JM83, and selecting Cm^r transformants. An exception was the nonpolar *luxI* deletion of pAK211, which was constructed by partial digestion of pNL121 with *Sfc*I, removal of approximately 250 bp of *luxI* DNA with the Promega (Madison, Wis.) Erase-a-Base System, ligation, and screening for JM83 transformants that luminesced only in the proximity of MJ-203, an autoinducer-producing derivative of MJ-100 that contains a nonpolar deletion in *luxA*, the gene specifying the α-subunit of the light-emitting enzyme luciferase (Fig. 1). Plasmids and *E. coli* strains were manipulated by using standard procedures (1).

V. fischeri lux mutants (Fig. 1) were then constructed by conjugating modified pNL121 from S17-1 to MJ-100 by a previously described mating and transconjugant selection procedure (8) and screening the resulting Nx^r Cm^s recombinant *V. fischeri* strains to confirm the mutant phenotype. For example, the nonpolar *luxI* deletion mutant MJ-211 was constructed by conjugating pAK211 into MJ-100 and screening for colonies that were Lux$^-$ except in proximity to MJ-203. Mutant genotypes and phenotypes were verified by physiological measurements, complementation tests, and Southern hybridizations.

Southern hybridizations. DNA was transferred from 1% agarose gels to Zeta-Probe blotting membrane by standard procedures (1) and probed and developed with the Boehringer Mannheim (Indianapolis, Ind.) nonradioactive DNA labeling and detection kit. Probes were prepared by labeling purified fragments by random priming. Hybridizations and high-stringency washes were performed at 65°C.

RP HPLC of autoinducers. Synthetic *N*-acyl-L-HSLs, kindly provided by D. Lynn (University of Chicago), were dissolved in

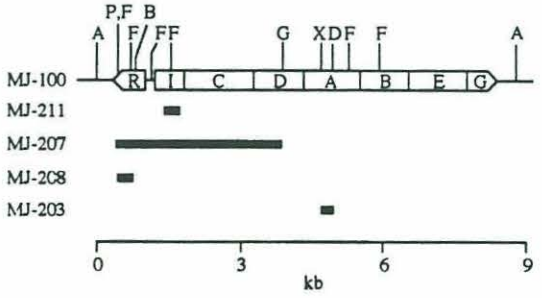

FIG. 1. The *lux* genes of *V. fischeri* and *V. fischeri lux* mutants constructed in this study. Solid bars indicate deletions. Restriction sites are denoted as follows: A, *Sal*I; P, *Pst*I; F, *Sfc*I; B, *Xba*I; G, *Bgl*II; X, *Xho*I; D, *Nhe*I. Letters inside boxes indicate *lux* genes.

ethanol and diluted into fresh medium. Samples were concentrated on an in-line C_{18} enrichment column and separated with a previously described reversed-phase high-pressure liquid chromatography (RP HPLC) system (21). Flow rate was 1 ml min^{-1}. Fractions were dried in a VR-I HetoVac with vacuum but without heat, dissolved in 10 μl of ethanol, mixed with 3 ml of autoinducer assay medium, and assayed for autoinducer activity with PD100(pPD749, pJR551) as described above. For gradient elutions, either 750 μl of *luxI*-conditioned medium, 3 ml of *ain*-conditioned medium, or an appropriate amount (1 ng to 2 μg) of a synthetic autoinducer compound (*N*-hexanoyl-L-HSL, *N*-3-oxohexanoyl-L-HSL, *N*-octanoyl-L-HSL, *N*-3-oxooctanoyl-L-HSL, *N*-3-oxodecanoyl-L-HSL, or *N*-3-oxododecanoyl-L-HSL) was applied to the column. Mobile phase was composed of sodium phosphate (10 mM, pH 7) and methanol, and the gradient was 0 to 100% (vol/vol) methanol over 8 min. Fractions (500 μl) were collected, and the luminescence of assay cultures was measured after a 2-h incubation. For isocratic separations, either 2 ml of *luxI*-conditioned medium, 500 μl of *ain*-conditioned medium, or 5 ng of synthetic compound was applied. Mobile phase was composed of sodium phosphate and methanol. Fractions (250 μl) were collected.

Purification of *V. fischeri* autoinducer-2 (AI-2). JM83(pAI004) (*ain*$^+$; Table 1) was cultured at 28°C in M9 medium (1) supplemented with 0.8% glycerol, 1 mM $MgSO_4$, 230 mg of L-proline $liter^{-1}$, and 150 mg of ampicillin $liter^{-1}$ to an $A_{660} > 3$, after which cells were removed from the medium by centrifugation in a Sorvall GS-3 rotor at 4°C and 16,000 × *g* for 30 min. Then a peristaltic pump was used to pass 4 liters of culture supernatant through two in-series 60-ml Varian Mega Bond Elut C_{18} columns at a flow rate of 2 ml min^{-1} at 4°C. Columns were eluted with the following series of mixtures of water and increasing amounts (vol/vol) of methanol: fraction 1, 100 ml of 0% methanol; fraction 2, 30 ml of 25% methanol; fraction 3, 20 ml of 50% methanol; fraction 4, 10 ml of 75% methanol; fraction 5, 10 ml of 75% methanol; fraction 6, 10 ml of 100% methanol; fraction 7, 40 ml of 100% methanol. Respective fractions from the two columns were pooled, and 10 μl of each pooled fraction was assayed for autoinducer activity, which was found to be present in fractions 5 and 6. These two fractions were pooled, rotoevaporated to dryness at 42°C, redissolved in 10% methanol, filtered, and eluted isocratically with 40% phosphate buffer–60% methanol by the HPLC system described above. Autoinducer-containing fractions were pooled, dried, dissolved in water, and eluted isocratically by RP HPLC with 50% phosphate buffer–50% methanol. The resulting fractions were assayed for autoinducer activity, which occurred as a single peak at 10 to 13 min. Active fractions were dried, dissolved in 200 μl of water, and extracted twice with 200 μl of acidified ethyl acetate. The resulting highly purified material was pooled, dried again, and stored at −20°C.

Mass spectrometry and NMR spectroscopy. For mass spectrometry, samples were analyzed with a VG AutoSpec-Q mass spectrometer by utilizing a desorption chemical ionization probe and electron impact ionization. Magnet scanning was at 3.4 s $decade^{-1}$ from 310 to 41 Da. Low-resolution spectra ($M/\Delta M$ = 4,000) were acquired in the centroid mode, whereas high-resolution spectra ($M/\Delta M$ > 10,000) were acquired in the continuum mode. For nuclear magnetic resonance (NMR) spectroscopy, ^1H spectra of samples dissolved in a deuterated solvent (methanol or chloroform) were obtained with a Bruker AC-300 NMR spectrometer.

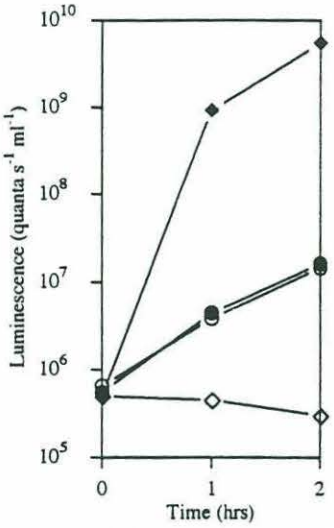

FIG. 2. Synthesis of autoinducer activity by *luxI* mutants of *V. fischeri*. Cell-free culture supernatants of MJ-100 (*lux*$^+$) and two *luxI* deletion mutants, MJ-211 and MJ-207, were assayed for autoinducer. The medium was not conditioned (◇) or was conditioned by MJ-100 (◆), MJ-207 (○), or MJ-211 (●).

RESULTS

Production of autoinducer activity by *luxI* mutants of *V. fischeri*. Previously, in a study of *lux* gene regulation with *lux*::*lacZ* (Mu dI) gene replacement mutants of *V. fischeri*, we observed that a mutant defective in *luxI*, expected to produce no autoinducer, nonetheless exhibited *lux* operon transcriptional activation (8). To examine the basis for that activation, we constructed a *V. fischeri luxI* deletion mutant, MJ-211, containing an approximately 250-bp nonpolar internal deletion in *luxI* (Fig. 1). The nonpolar nature of the deletion permitted transcription initiated from the *luxICDABEG* promoter to be monitored by luminescence, and the absence of foreign DNA (i.e., *E. coli lacZYA* and other genes of Mu dI present in *lux*::*lacZ* fusion mutants) avoided possible complications in interpreting results. Consistent with earlier results, luminescence in MJ-211 exhibited substantial *lux* operon transcriptional activation in the absence of added autoinducer. To determine if the *luxI* mutant actually produced autoinducer activity, cell-free supernatants of medium conditioned by the growth of MJ-211 were examined by a sensitive assay for autoinducers that activate expression of the *V. fischeri* luminescence system (9). Medium conditioned by MJ-211 activated *lux* operon expression (Fig. 2), suggesting that *V. fischeri* produced a second, *luxI*-independent autoinducer activity. To exclude the unlikely possibility that the remaining approximately 300 bp of *luxI* DNA in MJ-211 retained autoinducer synthase function, we constructed another mutant, MJ-207, in which the entire *luxI* gene and flanking sequences were removed (Fig. 1). Medium conditioned by MJ-207 gave results identical to those of MJ-211 (Fig. 2), demonstrating that the second autoinducer activity was independent of *luxI*. The autoinducer assay system did not respond to media conditioned by luminous bacteria closely related to *V. fischeri* (*Photobacterium leiognathi* LN-1a and *V. harveyi* B-392) or medium conditioned by *E. coli* JM83, indicating that the putative second autoinducer activity was due specifically to *V. fischeri* and not to a general conditioning effect.

The possibility, however, that the putative *luxI*-independent

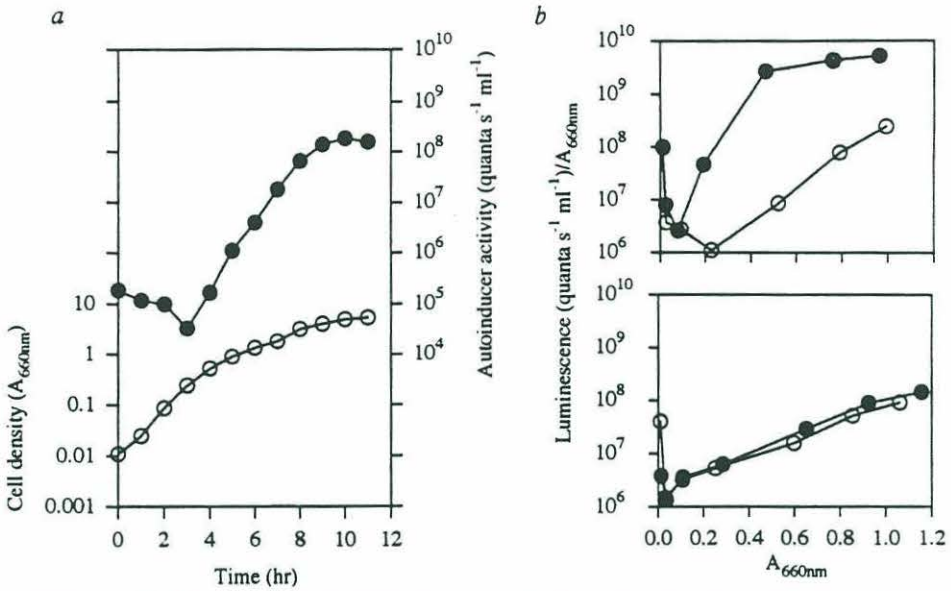

FIG. 3. Regulated synthesis of the *luxI*-independent autoinducer by *V. fischeri* and requirement of LuxR for *lux* operon transcriptional activation by the *luxI*-independent autoinducer. (a) Cells of *V. fischeri* MJ-211 (Δ*luxI*) were inoculated into 50 ml of ASH broth, and cell density (○) and autoinducer activity (●) were monitored during culture growth. Autoinducer levels were measured as the luminescence response of autoinducer assay strain PD100(pPD749, pJR551) by using 2-ml samples of cell-free growth medium of the MJ-211 culture at each time point (see Materials and Methods). (b) Responses of *luxI* deletion mutant MJ-211 (upper panel) and *luxR* deletion mutant MJ-208 (lower panel) to the *luxI*-independent autoinducer activity. Open symbols denote responses to unconditioned medium, and solid symbols denote responses to medium conditioned by MJ-207.

autoinducer activity did not result from the synthesis of a second autoinducer but from a spurious conditioning effect on the autoinducer assay system specific to *V. fischeri* existed. For example, *V. fischeri* cells might have released into the medium a compound, such as an iron chelator, that led to a change in the copy number of the *lux* gene-containing plasmids (5) in the *E. coli* autoinducer assay strain or a compound that activated transcription from plasmid-borne promoter sequences which continued on into the *lux* operon. To rigorously exclude this possibility, we used an approach modeled after that employed to extract and chemically characterize the first *V. fischeri* autoinducer, *N*-3-oxohexanoyl-L-HSL (*V. fischeri* AI-1) (10). We found the *luxI*-independent autoinducer activity, like AI-1, to be extractable from conditioned medium with acidified ethyl acetate. Furthermore, the *luxI*-independent activity, extracted and concentrated by rotoevaporation, was active in a dose-dependent manner and was destroyed by incubation at pH 12 for 10 min, like AI-1 (data not shown). Sensitivity to treatment with base suggested that the compound contained a lactone ring (10). Chemical similarities with AI-1 indicated that the *luxI*-independent activity was likely to be an autoinducer and not a spurious conditioning effect. This conclusion was supported by two additional similarities with AI-1, regulated synthesis of the *luxI*-independent activity and its requirement of LuxR for activity in the *lux* system, as described below.

Regulated synthesis of the *luxI*-independent autoinducer activity. In *V. fischeri*, the synthesis of AI-1 is regulated. The presence of *luxI* as part of the autoinducer-controlled *lux* operon results in an autocatalytic, positive-feedback loop for AI-1 synthesis, which leads to a rapid increase in the level of AI-1 once *lux* operon induction is triggered (5, 11, 13, 15). To determine if synthesis of the *luxI*-independent autoinducer activity also might be regulated, we assayed the cell-free supernatant of an culture MJ-211 for *luxI*-independent autoinducer activity during growth. The level of activity was low initially and remained constant during the first few hours of culture growth. After the culture attained an A_{660} of approximately 0.3 to 0.5, however, the level of activity increased rapidly, at a rate faster than the rate of increase in cell density (Fig. 3a). These results suggest that the production of the *luxI*-independent autoinducer activity, like the production of AI-1, is inducible.

Requirement of LuxR for activity of the *luxI*-independent autoinducer. The experiments described above were conducted with *V. fischeri* and *E. coli* strains that contained an intact *luxR* gene, leaving open the possibility that the activation of *lux* operon transcription by the *luxI*-independent autoinducer, like activation of *lux* operon transcription by AI-1, required LuxR. To test this possibility, we examined the effect of medium conditioned by MJ-207 on luminescence in MJ-208 (Fig. 1), a *V. fischeri* strain with a deletion in *luxR*. Medium conditioned by MJ-207 stimulated luminescence in MJ-211 (Δ*luxI*) but had no effect on luminescence in MJ-208 (Fig. 3b), which demonstrated that the *luxI*-independent autoinducer activated *lux* operon transcription via LuxR. The above results, as well as the known limited tolerance of LuxR for autoinducer compounds structurally different from AI-1 (12), led us to conclude that the *luxI*-independent compound was likely to be an *N*-acyl-L-HSL chemically similar to AI-1. For these reasons, we termed the compound *V. fischeri* AI-2.

A genetic locus that directs the synthesis of AI-2. The production of AI-2 by *luxI* deletion mutants indicated that another autoinducer synthase gene was present in the *V. fischeri* chromosome. To isolate the locus containing that gene, we transformed a plasmid-borne MJ-1 chromosomal library (6) into JM83(pAK411), which bears the *lux* genes with the same 250-bp nonpolar deletion in *luxI* as MJ-211 (Table 1). By conferring luminescence on cells in the presence of an autoinducer, pAK411 served as a reporter for DNA fragments that directed the synthesis of autoinducers active in the *V. fischeri*

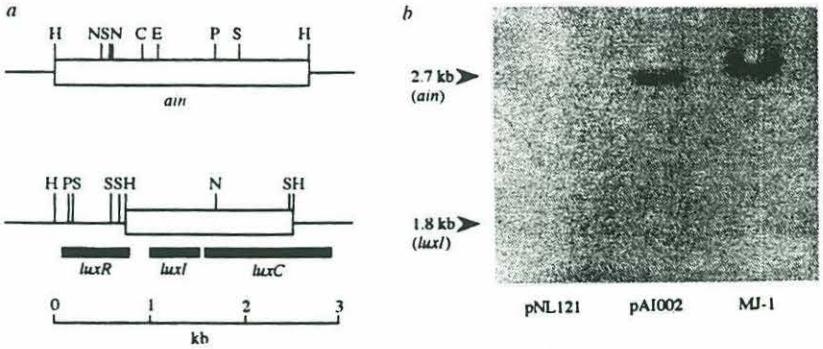

FIG. 4. Restriction map and Southern hybridization analysis of the novel *V. fischeri* autoinducer synthesis locus *ain*. The 2.7-kbp *Hin*dIII fragment containing *ain* was subcloned from the 10.5-kbp insert of pAI002, isolated from a genomic library of MJ-1 DNA that had been inserted into the *Bam*HI site of pSUP102 (6). (a) Physical maps of *ain* and *luxI*. Restriction sites are denoted as follows: H, *Hin*dIII; N, *Nsi*I; S, *Ssp*I; C, *Cla*I; E, *Eco*RV; P, *Pst*I. Black bars indicate *lux* genes. Open boxes indicate the 2.7- and 1.8-kbp *Hin*dIII fragments used as *ain* and *luxI* probes, respectively, in hybridization experiments. (b) Blot of *Hin*dIII digests of pNL121, pAI002, and MJ-1 genomic DNA, all probed with the 2.7-kbp *ain* fragment. In the reciprocal experiment, the 1.8-kbp *luxI* probe hybridized to same-sized *Hin*dIII fragments of pNL121 and MJ-1 DNA but did not hybridize to pAI002 (data not shown).

luminescence system. Two such fragments were found. Of approximately 50,000 transformant colonies screened for luminescence, 12 emitted high levels of light, and each of these was found by restriction analysis to have received a fragment containing *luxI*. Three others, however, emitted a low level of light that was consistent with the lower activation of luminescence by AI-2 in the autoinducer assay (Fig. 2); each had received a 10.5-kbp DNA fragment that by preliminary restriction analysis was distinct from *luxI*. This fragment contained a locus, designated *ain* (autoinducer), that was necessary and sufficient for JM83 to produce AI-2 (see below).

By subcloning, the *ain* locus was resolved to a 2.7-kbp *Hin*dIII fragment of the original 10.5-kbp clone. The detailed restriction map of the 2.7-kbp fragment differed unambiguously from that of the *luxI* region (Fig. 4a), and *ain* and *luxI* did not cross-hybridize (Fig. 4b), confirming that the two loci were distinct. The original 10.5-kbp *ain* clone did not, however, restore luminescence to JM83(pAK408), with a *lux* gene plasmid with a deletion in *luxR*. Therefore, the 10.5-kbp fragment did not express a protein that could substitute for LuxR in the *V. fischeri* luminescence system.

Chemical identity of AI-2. To chemically characterize AI-2, we fractionated media conditioned by *E. coli* and *V. fischeri* by RP HPLC and assayed fractions for autoinducer activity. From medium conditioned by JM83 containing *ain*, AI-2 was recovered in a fraction that eluted later (i.e., was more hydrophobic) than AI-1 from medium conditioned by JM83 containing *luxI* (Fig. 5a), indicating that AI-2 and AI-1 were chemically distinct compounds. Medium conditioned by MJ-207 yielded only AI-2, whereas medium conditioned by MJ-100 yielded AI-1, AI-2, and a second *luxI*-dependent autoinducer compound, AI-3 (data not shown). Furthermore, a *V. fischeri ain* gene replacement mutant failed to produce AI-2 but produced AI-1 and AI-3 (18). Thus, in *E. coli* as well as *V. fischeri*, the synthesis of AI-2 was dependent on the *ain* locus.

To chemically identify AI-2, we compared AI-2 chromatographically with several synthetic *N*-acyl-L-HSLs. Under RP HPLC gradient elution conditions, AI-2 separated unambiguously from most of the synthetic *N*-acyl-L-HSLs examined, including *N*-3-oxohexanoyl-L-HSL (AI-1), *N*-3-oxooctanoyl-L-HSL (the *A. tumefaciens* autoinducer), and *N*-3-oxododecanoyl-L-HSL (the *P. aeruginosa* autoinducer) (Fig. 5a). A fourth previously identified autoinducer, *N*-3-hydroxybutanoyl-L-HSL (the *V. harveyi* autoinducer), is a more hydrophilic compound that does not bind to the RP HPLC C_{18} columns (4) used here. Fractions of medium conditioned by *E. coli* containing *ain* in which the *V. harveyi* compound eluted if present (i.e., 0% methanol) exhibited no activity in the autoinducer assay. However, AI-2 did coelute under the gradient conditions employed with two synthetic compounds, *N*-octanoyl-L-HSL, which was shown previously to activate expression of the *V. fischeri* luminescence system and to interfere with the activity of AI-1 (12), and *N*-3-oxodecanoyl-L-HSL (Fig. 5a).

To differentiate between these two candidate compounds, a higher-resolution isocratic elution system that unambiguously separated them was used. Under these conditions, AI-2 was indistinguishable from *N*-octanoyl-L-HSL but distinct from *N*-3-oxodecanoyl-L-HSL (Fig. 5b). These results suggested that AI-2 was *N*-octanoyl-L-HSL.

As *N*-octanoyl-L-HSL, AI-2 presumably lacked a 3-oxo group. Since most other known autoinducers contain a 3-oxo group, we sought to directly confirm the absence of this group in AI-2. We used a keto-group detection procedure that involved derivatization with 2,4-dinitrophenylhydrazine and separation and detection of the resulting hydrazones by RP HPLC and UV absorbance (22). To our knowledge, this is the first application of this method to studies of autoinducer chemical structure. Each of these *N*-3-oxoacyl-L-HSLs was detected as its corresponding hydrazone by this analysis, but AI-2 was not detected, confirming its lack of a 3-oxo group (data not shown). We concluded that AI-2 is *N*-octanoyl-L-HSL, an *N*-acyl-L-HSL compound that is structurally related to but distinct from other known autoinducers.

To confirm the identification of AI-2 as *N*-octanoyl-L-HSL, we used mass spectrometry to compare AI-2 purified from medium conditioned by the growth of JM83(pAI004) (see Materials and Methods) with synthetic *N*-octanoyl-L-HSL. The low-resolution mass spectrum of AI-2, with a molecular ion at m/z = 227.2, was essentially identical to that of synthetic *N*-octanoyl-L-HSL, with minor contaminants (Fig. 6). The monotonically decreasing peaks at 156.1, 170.1, 184.1, 198.1, and 212.1 were consistent with a simple unbranched alkyl chain, confirming the absence of a 3-oxo or 3-hydroxy group. High-resolution mass spectrometry revealed that the m/z of the molecular ion was 227.1513, which corresponded to the elemental composition of *N*-octanoyl-L-HSL, $C_{12}H_{21}NO_3$ (calcu-

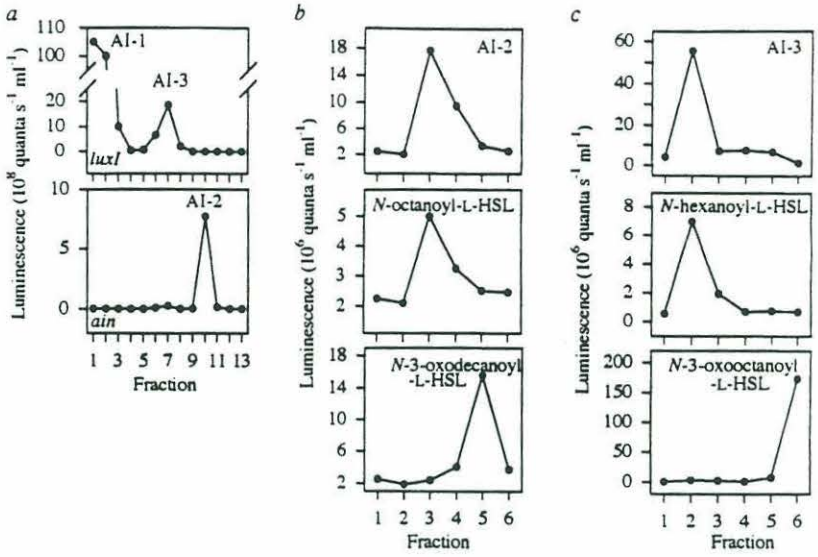

FIG. 5. Chromatographic identification of the novel *ain*-dependent autoinducer, AI-2, and a second *luxI*-dependent autoinducer, AI-3. (a) Gradient elution profiles of media conditioned by either *luxI*-expressing strain JM83(pJE202) (upper panel) or *ain*-expressing strain JM83(pAI004) (lower panel) and subjected to a methanol gradient. Synthetic *N*-acyl-L-HSLs eluted as follows: *N*-3-oxohexanoyl-L-HSL, fraction 2; *N*-hexanoyl-L-HSL and *N*-3-oxooctanoyl-L-HSL, fraction 7; *N*-octanoyl-L-HSL and *N*-3-oxodecanoyl-L-HSL, fraction 10; *N*-3-oxododecanoyl-L-HSL, fraction 12. (b) Isocratic separation (30% phosphate buffer–70% methanol) of JM83(pAI004)-conditioned medium and synthetic *N*-octanoyl-L-HSL and *N*-3-oxodecanoyl-L-HSL. (c) Isocratic elution (47% phosphate buffer–53% methanol) of JM83(pJE202)-conditioned medium and synthetic *N*-hexanoyl-L-HSL and *N*-3-oxooctanoyl-L-HSL.

lated m/z = 227.1521). The m/z of the base peak, 143.0576, also observed in mass spectra of AI-1 and the *P. aeruginosa* autoinducer (2, 10, 25), corresponded to the rearrangement product, $C_6H_9NO_3$ (calculated m/z = 143.0582).

To further confirm the structure of AI-2, we subjected the purified compound to NMR spectroscopy. The ^1H NMR spectrum of AI-2 matched that of synthetic *N*-octanoyl-L-HSL. The peak assignments of AI-2 in deuterated methanol were consistent with an unbranched octanoyl moiety for the following carbon designations (Fig. 7): a, δ_H = 2.20, t, 2H; b, δ_H = 1.59, t, 2H; c, δ_H = 1.29, broad s, 8H; d, δ_H = 0.87, t, 3H. Observed peaks diagnostic of HSL were as follows: α, δ_H = 4.55, m, 1H; β_1, δ_H = 2.50, m, 1H; β_2, δ_H = 2.25, m, 1H; δ_1, δ_H = 4.42, m, 1H; γ_2, δ_H = 4.26, m, 1H.

The biological activity of AI-2 also matched that of *N*-octanoyl-L-HSL. Over the concentration range tested, 10 to 1,000 nM, the autoinducer assay system responded identically to purified AI-2 and synthetic *N*-octanoyl-L-HSL (data not shown).

Chemical identity of a third *V. fischeri* autoinducer. Conducting RP HPLC analyses of conditioned media, we found that *V. fischeri* produced a third autoinducer activity, AI-3. AI-3 exhibited by RP HPLC a hydrophobicity that was intermediate between those of *N*-3-oxohexanoyl-L-HSL and *N*-octanoyl-L-HSL, indicating it was chemically distinct from AI-1 and AI-2. JM83 containing *luxI* produced this activity (Fig. 5a), but JM83 containing the *lux* genes with a deletion in *luxI* did not. Furthermore, neither JM83 containing only *ain* nor MJ-207 produced AI-3. Thus, AI-3, like AI-1, was dependent on *luxI* for its synthesis. The RP HPLC gradient elution profile of AI-3 was consistent with it being either *N*-hexanoyl-L-HSL, which had been previously shown to activate expression of the *V. fischeri* luminescence system and to inhibit the activity of AI-1 (12), or *N*-3-oxooctanoyl-L-HSL, the *A. tumefaciens* autoinducer (Fig. 5a). Under a higher-resolution isocratic elution

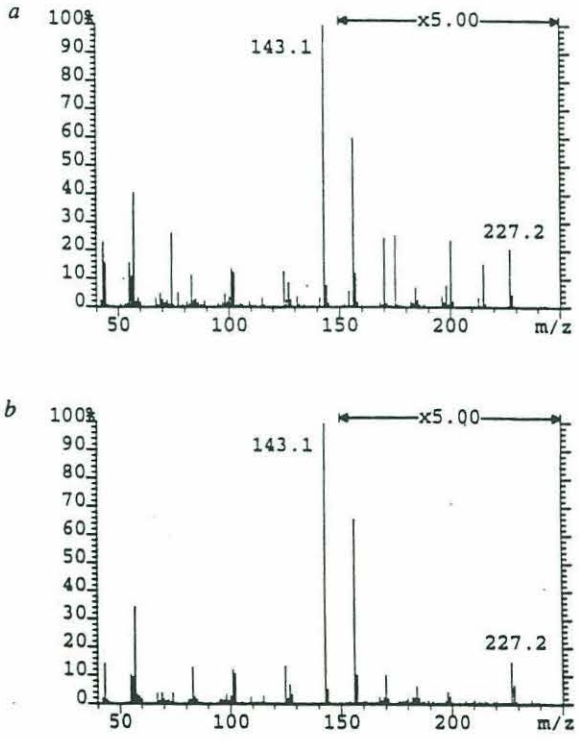

FIG. 6. Mass spectrometric analysis of *V. fischeri* AI-2. Low-resolution mass spectra of AI-2 purified from cell-free supernatant of *E. coli* JM83(pAI004) (a) and synthetic *N*-octanoyl-L-HSL (b). Peaks corresponding to the molecular ion (m/z = 227.2) and rearrangement product $C_6H_9NO_3$ (m/z = 143.1) are indicated. AI-2 was purified as described in Materials and Methods.

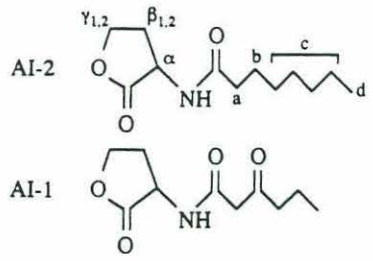

FIG. 7. Structural comparison of *V. fischeri* AI-2 (*N*-octanoyl-L-HSL) and AI-1 (*N*-3-oxohexanoyl-L-HSL). Carbon designations refer to proton assignments from NMR spectra described in the text.

regimen, the coelution of AI-3 with the synthetic compound identified it as *N*-hexanoyl-L-HSL (Fig. 5c). Thus, *V. fischeri* cells produced three chemically distinct autoinducers with activity in the *lux* system, AI-1 (*N*-3-oxohexanoyl-L-HSL) and AI-3 (*N*-hexanoyl-L-HSL), both of which are dependent on *luxI* for synthesis, and AI-2 (*N*-octanoyl-L-HSL), whose synthesis is dependent on the novel locus *ain*. The relative activities of autoinducer-containing RP HPLC fractions of medium conditioned by MJ-100 were 1.000, 0.033, and 0.167 for AI-1, AI-2, and AI-3, respectively.

DISCUSSION

In this study, we have identified a novel autoinducer of luminescence, *N*-octanoyl-L-HSL (AI-2), that is produced by the marine symbiotic bacterium *V. fischeri* and have isolated from the *V. fischeri* genome a novel locus, *ain*, that is involved in the synthesis of AI-2. This is the first report in which a second autoinducer from a bacterial species has been chemically identified and the gene directing its synthesis has been isolated. The *ain* locus and AI-2, acting via LuxR, constitute a third level of population density-responsive control of *lux* gene expression in *V. fischeri*, one that could supplement or inhibit transcriptional control by *N*-3-oxohexanoyl-L-HSL (AI-1) and enhance the mechanistically undefined effect of autoinducer-LuxR-independent modulation (8). We also have identified a second previously unrecognized autoinducer of luminescence in *V. fischeri*, *N*-hexanoyl-L-HSL (AI-3), whose synthesis, like that of AI-1, is dependent on *luxI*. Therefore, population density-responsive control of *lux* gene expression in *V. fischeri* is substantially more complex than previously envisioned.

The construction of *V. fischeri* mutants with defects in *luxI*, which eliminated the synthesis of AI-1 by these strains, led to the detection of AI-2, and isolation of the *ain* locus was facilitated by the use of a *lux* gene-containing plasmid with a nonpolar deletion in *luxI*, which served as a reporter for DNA fragments that directed the synthesis of autoinducers active in the *V. fischeri* luminescence system. These approaches may find applications with other autoinducer-utilizing bacteria to detect the presence of multiple autoinducers and to isolate the genes involved in the synthesis of them.

In this regard, other bacteria are likely to contain more than one autoinducer synthase gene and to synthesize more than one autoinducer. In *V. harveyi*, genetic evidence for an activity in addition to *N*-3-hydroxybutanoyl-L-HSL controlling luminescence has been obtained, although neither the chemical structure of the second activity nor the gene responsible for its synthesis has been identified (3). Furthermore, *A. tumefaciens* and *P. aeruginosa* apparently synthesize more than one autoinducer activity (25, 32).

Like autoinducers identified from other species of bacteria (2, 4, 25, 32), *V. fischeri* AI-2 and AI-3, as *N*-acyl-L-HSLs, are structurally similar to *V. fischeri* AI-1 (Fig. 7). Besides the chemical similarity of autoinducers, in many cases, the genes encoding the autoinducer synthases and autoinducer receptor proteins from these other bacteria exhibit sequence similarity to *V. fischeri luxI* and *luxR*, respectively (16, 17, 24, 26, 27). The *ain* locus, however, exhibits no obvious sequence similarity to *luxI* (18), suggesting that it arose independently. With respect to a possible AI-2-specific transcriptional activator analogous or homologous to *luxR*, we know at this point only that the original 10.5-kbp *ain* fragment did not restore luminescence to *E. coli* carrying a *lux* plasmid with a deletion in *luxR*, indicating that this fragment does not express a protein that can substitute for LuxR in the *V. fischeri* luminescence system. The putative protein might not be expressed by *E. coli* or might not recognize the *lux* operator, or its gene might be incomplete on the 10.5-kbp fragment or occur elsewhere in the *V. fischeri* chromosome.

The total amounts of each of the three autoinducers produced by *V. fischeri* were such that they exhibited activity in the luminescence system as AI-1 > AI-3 > AI-2, which is consistent with AI-1 as the primary autoinducer of luminescence. Besides controlling luminescence in *V. fischeri*, the different autoinducers encoded by the two autoinducer synthase genes also might function either separately or cooperatively to control various other cellular responses to the high population densities this bacterium encounters in light-organ symbiosis and other habitats (23). On the basis of the smaller effect of AI-2 on luminescence compared with those of AI-1 and AI-3, one possibility is that the activation of luminescence by AI-2 is incidental to its principal function, that of controlling a set(s) of genes distinct from those involved in light production. Identification of these genes and a transcriptional activator specific to AI-2 would add substantial insight into cellular responses to high population density in *V. fischeri*.

A striking feature of the *V. fischeri* luminescence system is the versatility of LuxR and LuxI seen in this study. LuxR recognized a wide range of chemically distinct *N*-acyl-L-HSLs, including the three different compounds produced by *V. fischeri* (AI-1, AI-2, and AI-3) (Fig. 5), the chemically distinct autoinducers produced by *A. tumefaciens* and *P. aeruginosa*, and three other compounds (*N*-3-oxodecanoyl-L-HSL, *N*-decanoyl-L-HSL, and *N*-nonanoyl-L-HSL) (data not shown). A reasonable explanation for this versatility is that LuxR recognizes both of the main structural elements of autoinducers, the HSL and hydrophobic acyl moiety, but its level of activity is strongly influenced by the length and composition of the acyl group (12). Similarly, LuxI catalyzed a critical step in the synthesis of two distinct autoinducers that differed only in the structure of the acyl group. This versatility presumably reflects the ability of LuxI to recognize two alternative fatty acyl substrates, as well as reflecting the presence of those substrates in *V. fischeri* and *E. coli*. The versatility of LuxR and LuxI, together with our demonstration of a second autoinducer system in *V. fischeri*, implies that autoinduction of luminescence and other bacterial population density-responsive activities may be the summation of an unexpectedly complex network of multiple cross-acting regulatory elements.

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Chapter 4. Negative regulation of luminescence by *N*-octanoyl-L-homoserine lactone, a *Vibrio fischeri* autoinducer

ABSTRACT

The symbiotic bacterium *Vibrio fischeri* synthesizes three different cell-cell signalling molecules, or autoinducers, that individually interact with LuxR to activate transcription of the luminescence genes. The autoinducer with highest activity, AI-1 (*N*-3-oxohexanoyl-L-homoserine lactone), requires *luxI* for its synthesis, while the autoinducer with lowest activity, AI-2 (*N*-octanoyl-L-homoserine lactone) requires *ain* for its synthesis. To determine the role of AI-2 in autoinduction, *ain* and *ain luxI* null mutants of *V. fischeri* were constructed. Light emission by the *ain luxI* mutant was very low but was stimulated by exogenous AI-2, confirming the ability of AI-2 to induce luminescence. However, the *ain* mutant exhibited a more rapid induction of luminescence relative to the wild-type strain. Also, induction in both the *ain* mutant and the wild-type was inhibited by physiological concentrations of exogenous AI-2. These results suggested that AI-2 is an inhibitor of luminescence induction. The cloned luminescence genes, which are functional in *Escherichia coli*, were used to show that the inhibitory property of AI-2 can be at least partly attributed to interference with AI-1-mediated induction of luminescence, but not to repression of *luxR* transcription. Thus AI-2, a minor inducer of luminescence, is also an inhibitor of AI-1 activity, and so mediates an additional level of control of luminescence.

INTRODUCTION

Autoinduction is a cell density-responsive intercellular signal and gene regulatory system first described in luminescent bacteria (Nealson et al 1970). Though subsequently discovered to regulate diverse cellular functions in diverse proteobacteria, including *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* (Zhang et al 1993, Pearson et al 1994), the mechanism of autoinduction is best understood in the marine light organ symbiont *Vibrio fischeri*, where it regulates luminescence. In a log-phase batch culture, luminescence per cell at first decreases, then rises rapidly 10^{3-4}-fold as the culture approaches stationary phase. This cell density-dependent activation of luminescence operon (*luxICDABEG*) transcription requires *luxI* and *luxR* (Engebrecht et al 1983). LuxI is the putative synthase of a membrane-permeable signal molecule (the autoinducer, *N*-3-oxohexanoyl-L-homoserine lactone, *N*-3-oxohexanoyl-L-HSL), and LuxR is putatively both the autoinducer receptor and the activator of *lux* transcription (Eberhard et al 1981, Engebrecht and Silverman 1984). The *lux* operator, to which LuxR presumably binds, is located between *luxR* and the *luxICDABEG* operon, which are divergently transcribed (Engebrecht et al 1983, Devine et al 1989). The putative AI-1-LuxR complex both activates transcription of *luxICDABEG* and represses transcription of *luxR*. This LuxR autorepression is probably effected by steric hindrance of Crp (cyclic AMP receptor protein)-mediated activation of *luxR* transcription (Dunlap and Greenberg 1988, Dunlap and Ray 1989, Shadel et al 1991).

The above model was established through study of *V. fischeri* genes cloned into *Escherichia coli*. Subsequent studies using *V. fischeri* mutants confirmed all elements of the model examined, but also revealed novel aspects of luminescence regulation. For example, the *V. fischeri lux* operon exhibits a modulation that is independent of and underlies autoinduction (Dunlap and Kuo 1992). Also, *V. fischeri* synthesizes three different autoinducers, called AI-1 (*N*-3-oxohexanoyl-L-HSL), AI-2 (*N*-octanoyl-L-HSL), and AI-3 (*N*-hexanoyl-L-HSL) (Kuo et al 1994). As with AI-1, synthesis of AI-2 is induced in a cell density-dependent manner. Also like AI-1, AI-2 interacts with LuxR to stimulate luminescence in *E. coli* and *V. fischeri*. Unlike AI-1, AI-2 is dependent not on *luxI* for its synthesis but on the distinct autoinducer synthesis locus *ain*.

Total AI-2 autoinducer activity in dense cultures of *V. fischeri* is only one-thirtieth that of AI-1 (Kuo et al 1994), implying that AI-2 is likely to make a very minor contribution to autoinduction. To rigorously demonstrate this suggestion, I constructed *ain* (AI-2$^-$) mutants of *V. fischeri*. Here I use these mutants to show that AI-2 primarily

inhibits rather than supplements autoinduction, and that this inhibiton can be attributed to inhibition of AI-1 activity.

MATERIALS AND METHODS

Strains and plasmids. MJ-100 is a spontaneously nalidixic acid (Nx)-resistant isolate of the wild-type *V. fischeri* strain MJ-1 (Dunlap and Kuo 1992, Ruby and Nealson 1976). MJ-211 is a nonpolar *luxI* deletion mutant derived from MJ-100 (Kuo et al 1994). It synthesizes AI-2 but not AI-1 or AI-3, and thus emits a low but visible amount of light that appears to be induced in a cell density-dependent manner (Fig. 1a). Other *V. fischeri* mutants are described below and in Table 1. *E. coli* PD100(pJR551,pPD749) is the basis for the most sensitive autoinducer assay of which we know (Dunlap and Ray 1989). *E. coli* JM83 (Yanisch-Perron et al 1985) was used for most cloning manipulations, which followed standard procedures (Ausubel et al 1987).

pAK411 contains the *lux* locus from MJ-1 but with a nonpolar deletion in *luxI* (identical to that of MJ-211), and cloned into the ampicillin resitance (Ap^r), Inc(pMB1) plasmid pBR322 (Kuo et al 1994). pAK411 allows JM83 to luminesce, but only in the presence of a source of autoinducer. pJR551 contains the *lux* region cloned into the chloramphenicol resistance (Cm^r), Inc(p15A) plasmid pACYC184, with a nonpolar mutation in *luxI* and a *luxR*::*lacZ* operon fusion (Dunlap and Ray 1989). pAK011 is a 11.8 kb *Bgl*II fragment from pJR551, containing *luxI*$^-$ and *luxR*::*lacZ*, cloned into the *Bam*HI site of pSUP102, a *mob*$^+$ derivative of pACYC184 (Simon et al 1986). pPD749 has *luxR* under the control of the lactose-inducible high-expression promoter P_{tac} (Dunlap and Greenberg 1988). pAI009 is a 2.7 kb *Hin*DIII fragment containing *ain* cloned from MJ-1 into pSUP102, and directs synthesis of AI-2 in JM83 (Gilson et al). Other plasmids were constructed using standard procedures (Ausubel et al 1987) and are described below and in Table 1. The *neo* cassette was the 1.8 kb *Bam*HI-*Hin*DIII fragment from Mu dI1734 (Castilho et al 1984) and conferred kanamycin resistance (Km^r) on *E. coli* and neomycin resistance (Nm^r) on *V. fischeri.*

Culture techniques. *E. coli* was cultured on LB medium with 50 mM Tris (pH 7.5). *V. fischeri* was maintained on LBS (Dunlap 1989) plates with Nx, while liquid cultures were grown in ASH medium without antibiotics (Dunlap and Kuo 1992). Growth was monitored by measuring absorbance at 660 nm (A_{660}) while luminescence was measured as described previously (Dunlap and Kuo 1992). β-galactosidase activity was assayed using standard procedures (Miller 1992) with four replicates (range < 10%). Autoinducer activity was assayed by measuring the luminescence response of PD100(pJR551,pPD749) in autoinducer assay medium, as described previously (Kuo et al 1994, Dunlap and Ray 1989). Except where indicated otherwise, antibiotics were used

Table 1. Strains and plasmids used in this study.

Strain or Plasmid	Relevant genotypic and phenotypic characters	Source
Strains		
V. fischeri		
MJ-1	Lux$^+$	Ruby and Nealson 1976
MJ-100	MJ-1, Nx^r	Dunlap and Kuo 1992
MJ-211	MJ-100, Δ*luxI* (~250 bp nonpolar deletion)	Kuo et al 1994
MJ-215	MJ-211, *ain*$^-$ (2 bp insertion)	This study
MJ-216	MJ-100, *ain*::*neo* (1.8 kb Nm^r cassette)	This study
E. coli		
JM83	Δ(*lac-pro*)	Yanisch-Perron et al 1985
PD100	*zah-735*::Tn10Δ(*argF-lac*)*U169*, Tc^r	Dunlap and Greenberg 1988
Plasmids		
pPD749	P_{tac}-*luxR*, *lacI*^{q}, Ap^r	Dunlap and Greenberg 1988
pJR551	pACYC184 with *luxR*:MuΔ(*c*, *nerAB*) dI1681 (*lacZYA*, Km^s) and *luxICDABE* with a nonpolar mutation in *luxI*, Cm^r	Dunlap and Ray 1989b
pSUP102	pACYC184 with RP4 *mob*$^+$, Cm^r Tc^r	Simon et al 1986
pAK011	pSUP102 with 11.8 kb *Bgl*II fragment from pJR551, *luxR*::*lacZ luxI*$^-$ *luxC*$^+$	This study
pAK411	pBR322 with *luxRICDABE* fragment with ~250 bp nonpolar deletion in *luxI*, Ap^r	Kuo et al 1994
pAI009	pSUP102 with *ain*	Gilson et al
pAI014	pAI009 with *ain*::*neo*, Km^r	This study
pAI015	pAI009 with 2 bp insertion in *ain*	This study

at the following concentrations: Nx, 20 mg l^{-1}; Ap, 150 mg l^{-1}; Cm, 34 mg l^{-1}; Km, 20 mg l^{-1}; Nm, 200 mg l^{-1}.

Genetic and chemical methods. *V. fischeri* autoinducer synthesis mutants were constructed using the *V. fischeri* gene transfer procedure described previously (Dunlap and Kuo 1992): generally, the targeted *V. fischeri* genes were cloned into pSUP102, engineered in JM83, verified by restriction analysis, and introduced to a Nx^r *V. fischeri* strain. The desired Nx^r Cm^s recombinants were identified with an appropriate screen, and confirmed by Southern hybridizations following established procedures (Kuo et al 1994), and suitable phenotypic tests (see below). In the case of autoinducer synthesis mutants, conditioned media were assayed for autoinducer activity. Where distinction between the different *V. fischeri* autoinducers was required, autoinducer assays were applied to fractions of conditioned media separated by high-pressure liquid chromatography (HPLC), as described previously (Kuo et al 1994). Where a culture was treated with exogenous AI-1 or AI-2, synthetic N-3-oxohexanoyl-L-HSL or *N*-octanoyl-L-HSL, respectively, was dissolved in chloroform, added to the culture tube, and dried before addition of culture medium and bacteria.

Construction of *ain* mutants. The *ain*$^-$ plasmid pAI015 was constructed by digesting pAI009 with *Cla*I (one site in *ain*, one site in vector; Gilson et al), blunt-ending, gel-purifying singly-cut plasmid, and ligating. The resulting plasmid theoretically has a frameshifting 2 bp insertion in *ain*. JM83(pAI015) was Cm^r AI^-. The *V. fischeri ain*$^-$ Δ*luxI* double mutant MJ-215 was constructed by introducing pAI015 to MJ-211 and screening for Nx^r Cm^s recombinants that emitted no light detectable by eye. Southern analysis confirmed that MJ-215 lacks the *Cla*I site in *ain*. MJ-215 synthesized no autoinducer activity detectable by the PD100(pJR551,pPD749) assay, but responded to exogenous AI-1 or AI-2 by emitting light (Fig. 1b). Finally, the luminescence defect of MJ-215 was rescued by introduction of pAI009 but not of pSUP102 or pAI015.

The *ain*::*neo* plasmid pAI014 was constructed by partially digesting pAI009 with *Cla*I, blunt-ending, purifying singly-cut plasmid by low melting-point agarose gel electrophoresis, and ligating with a *neo* cassette. JM83(pAI014) was Cm^r Km^r AI^-. The *V. fischeri ain*::*neo* mutant MJ-216 was constructed by introducing pAI014 to MJ-100 and screening for Nx^r Cm^s Nm^r recombinants. Southern analysis confirmed that MJ-216 lacks the *Cla*I site but possesses a 1.8 kb insert in *ain*. Autoinducer assays of HPLC fractions of conditioned medium confirmed that MJ-216 synthesizes AI-1 and AI-3 but not AI-2.

RESULTS

Mutation of both *ain* and *luxI* abolishes induction of luminescence. To assess the role of AI-2 in regulation of luminescence, I constructed a *ain luxI* double mutant of *V. fischeri* and observed its luminescence phenotype. The *ain luxI* mutant MJ-215 did not synthesize a detectable autoinducer activity and correspondingly emitted a very low level of light which was never induced (Fig. 1a), implying that *V. fischeri* synthesizes no autoinducers capable of inducing the *lux* operon other than AI-1, -2, and -3, and that the limited induction observed in the *luxI* mutant MJ-211 is due to its synthesis of AI-2. Also, luminescence in MJ-215 was stimulated by exogenous AI-2 (Fig. 1b). Growth rate of MJ-215 was indistinguishable from that of MJ-100 and MJ-211. These results confirm that AI-2 is an autoinducer of luminescence, though one with less than 0.02x the specific activity of AI-1 (Table 2).

Mutation of *ain* alone accelerates induction. To assess the contribution of AI-2 to autoinduction in the presence of the other autoinducers, I constructed a mutant deficient in AI-2 alone by inserting a *neo* cassette into *ain*. The resulting mutant MJ-216 was Lux$^+$; MJ-216 colonies were visually indistinguishable from those of MJ-100. However, the two strains differed in their kinetics of luminescence; in log-phase batch cultures, induction of luminescence appeared to take place earlier and more quickly in MJ-216 than in MJ-100 (Figure 2a). This surprising result suggested that AI-2 might inhibit rather than induce luminescence in *V. fischeri*, but did not exclude the possibility that the *neo* insertion might cause abnormal luminescence kinetics. My previous experience with chromosomal insertion mutants has led me to caution that exogenous DNAs can have gross and poorly understood effects on the growth physiology of *V. fischeri* (Kuo 1994). Indeed, MJ-216 grew significantly more slowly than did MJ-100 (at mid-exponential phase, doubling times of MJ-100 and MJ-216 were 40 and 130 min, respectively). Given the wild-type growth rate of the *ain luxI* mutant MJ-215, as well as the lack of effect of exogenous AI-2 on the growth rates of any *V. fischeri* strain tested (see below), I conclude that AI-2 itself does not affect growth physiology.

Exogenous AI-2 inhibits autoinduction. To determine if the accelerated induction of MJ-216 was due to the lack of AI-2 rather than the presence of exogenous DNA, induction was examined in the presence of exogenous AI-2. AI-2 inhibited luminescence of MJ-216 without altering growth rate (data not shown), implying that absence of AI-2 is responsible for part if not all of the accelerated autoinduction phenotype of MJ-216 (Fig. 2a). Exogenous AI-2 similarly inhibited autoinduction in the *ain*$^+$ *luxI*$^+$ strains MJ-100 and MJ-1 in a dose-dependent manner (Fig. 2b). Inhibition

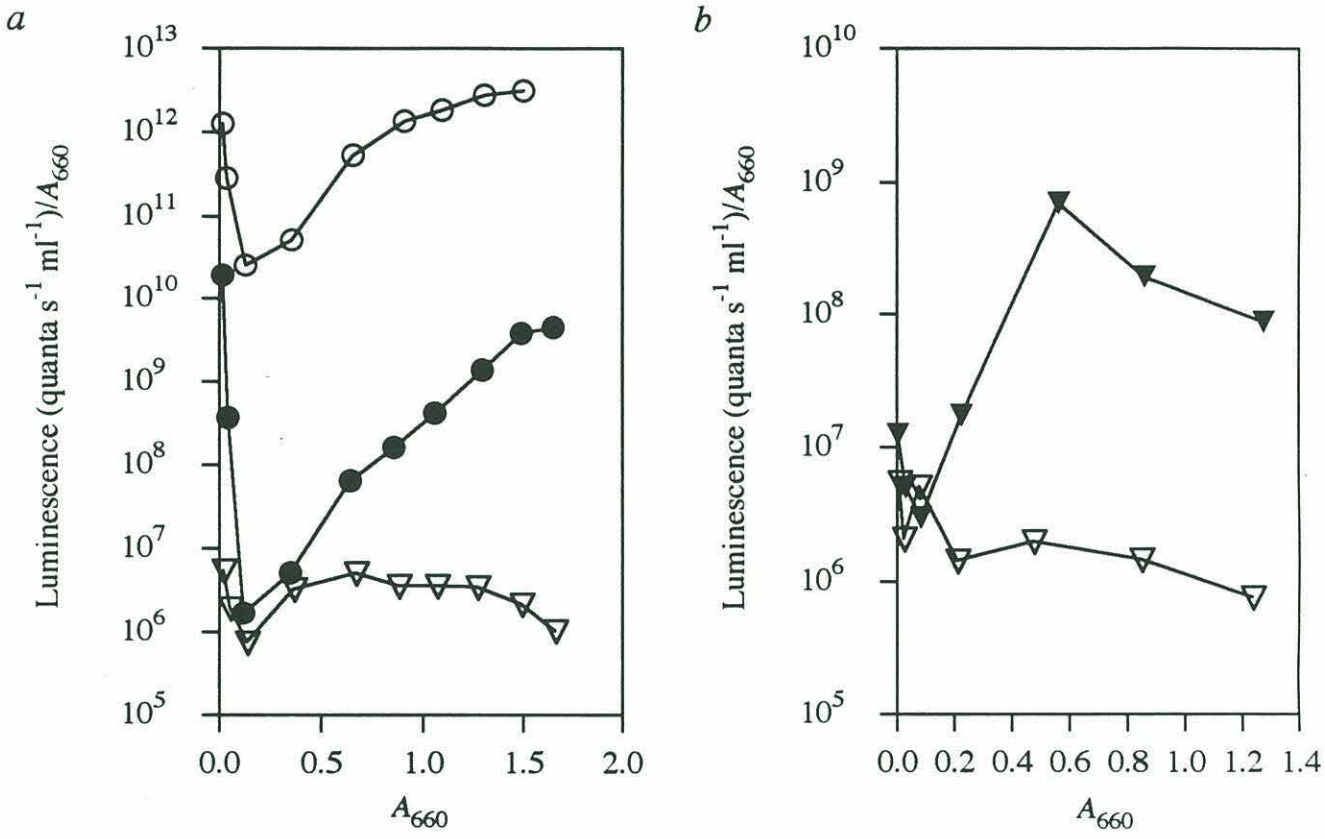

Fig. 1. The autoinducer mutant MJ-215. *a*. Comparison between MJ-100 (○), the *luxI* mutant MJ-211 (●), and the *ain luxI* mutant MJ-215 (▽). *b*. Luminescence of MJ-215 in the absence (▽) and the presence (▼) of 170 ng ml^{-1} AI-2.

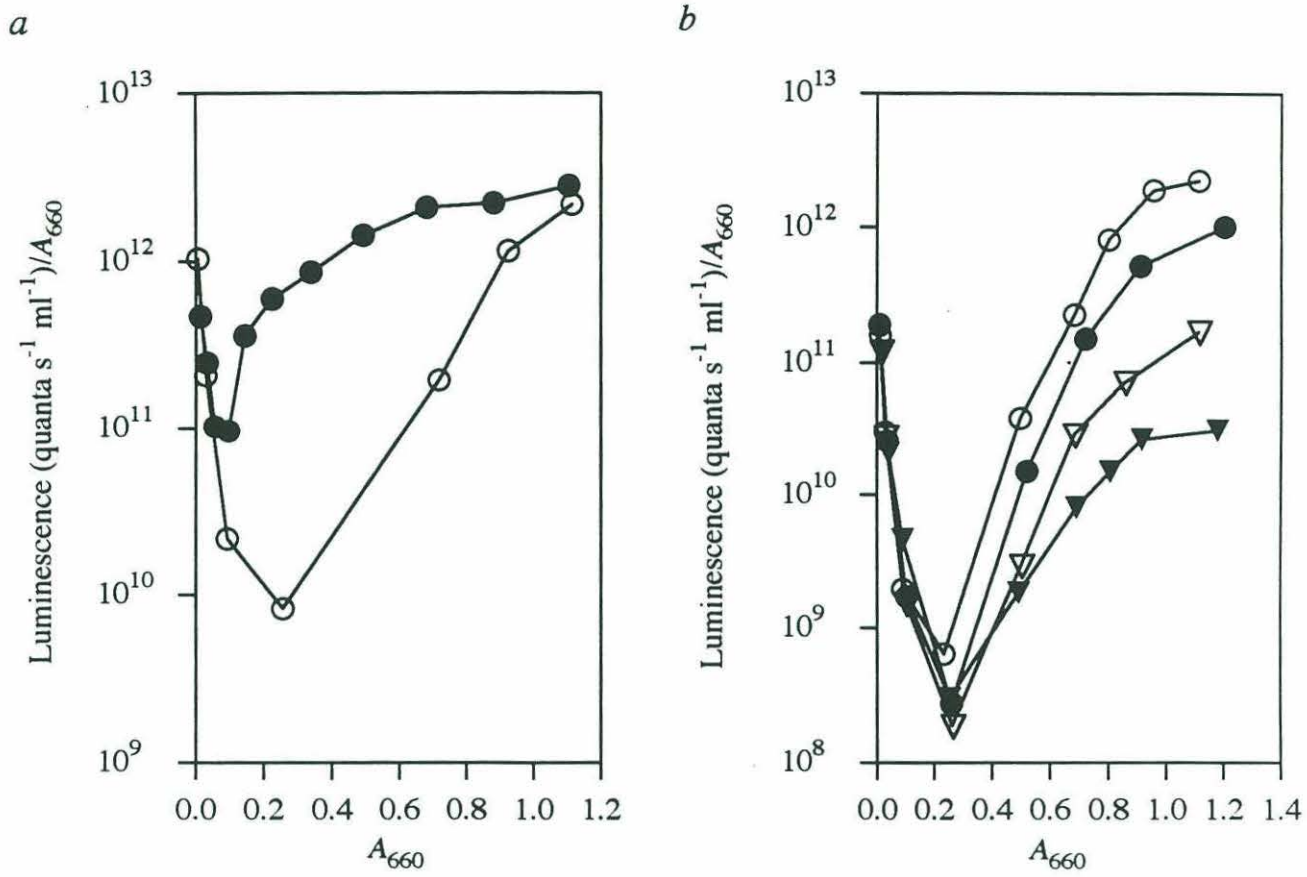

Fig. 2. Inhibition of autoinduction by *ain* and AI-2. *a.* Comparison between the *ain*$^+$ strain MJ-100 (O) and the *ain* mutant MJ-216 (●). *b.* Autoinduction in the wild-type strain MJ-1 in the presence of 0 (O), 1.7 (●), 17 (▽), and 170 (▼) ng ml^{-1} exogenous AI-2.

was observed with doses well within the range of AI-2 concentrations synthesized by *V. fischeri* (a dense culture of MJ-211 contained activity equivalent to ~15 ng ml^{-1} AI-2; data not shown). In none of these strains was growth rate affected by any concentration of AI-2. These AI-2 addition experiments, together with the MJ-216 phenotype, demonstrate that AI-2 is an inhibitor of autoinduction.

AI-2 inhibits activity of exogenous AI-1. While the above results demonstrate that AI-2 inhibits autoinduction, they do not demonstrate the mechanism of that inhibition. Given the structural and functional similarities between the *V. fischeri* autoinducers, AI-2 might occupy the same putative binding site on LuxR as does AI-1; it seems reasonable to propose that AI-2 inhibition requires AI-2-LuxR interaction. If so, AI-2 should inhibit AI-1 activity in an *E. coli* system containing only LuxR, exogenous AI-1, and *luxCDABE* as a target for AI-1-LuxR activation. *E. coli* JM83 was first transformed with the Δ*luxI* plasmid pAK411, which carries both *luxR* and *luxCDABE*, and then was treated with a fixed arbitrary amount of AI-1 (10 ng ml^{-1}) and a range of amounts of AI-2 (from 0 to 333 ng ml^{-1}). Induction of luminescence by AI-1 was reduced by AI-2 in a dose-dependent manner (Fig. 3a). This demonstration of AI-2-mediated inhibition in an *E. coli* system shows that LuxR, AI-1, and *luxICDABE* are the only elements necessary to observe AI-2-mediated inhibition. Qualitatively similar results were obtained with the *ain luxI* mutant MJ-215 treated with a fixed amount of AI-1 and a range of amounts of AI-2 (Fig. 3b).

AI-2 does not repress transcription of *luxR*. Since AI-2, like AI-1, induces luminescence via LuxR, I hypothesized that AI-2, also like AI-1, represses transcription of *luxR* via LuxR; such repression might contribute to AI-2-mediated inhibition of autoinduction. To detect such repression, I used the *luxR*::*lacZ* operon fusion plasmid pJR551 to directly measure *luxR* transcription in an *E. coli* system. Because pJR551 contains *luxCDABE*, I could simultaneously monitor luminescence and thus measure autoinducer activity. pPD749, which contains *luxR* fused to an exogenous promoter and thus expresses *luxR* unregulated by AI-1-LuxR, was also present in the *E. coli* system. By measuring β-galactosidase activities of *E. coli* PD100(pJR551,pPD749) with and without exogenous autoinducers, I demonstrated that AI-1 represses *luxR* but AI-2 does not. LuxR autorepression was not observed even with a concentration of AI-2 that stimulated luminescence to the same degree as AI-1 (Table 2). This shows that the level of *luxICDABEG* transcriptional activation is not linearly related to the level of *luxR* transcriptional repression. Qualitatively similar results were obtained by mobilizing the *luxR*::*lacZ* operon fusion plasmid pAK011 into the *ain luxI* mutant MJ-215, and then measuring *luxR* transcription in the presence of exogenous autoinducers (Table 2).

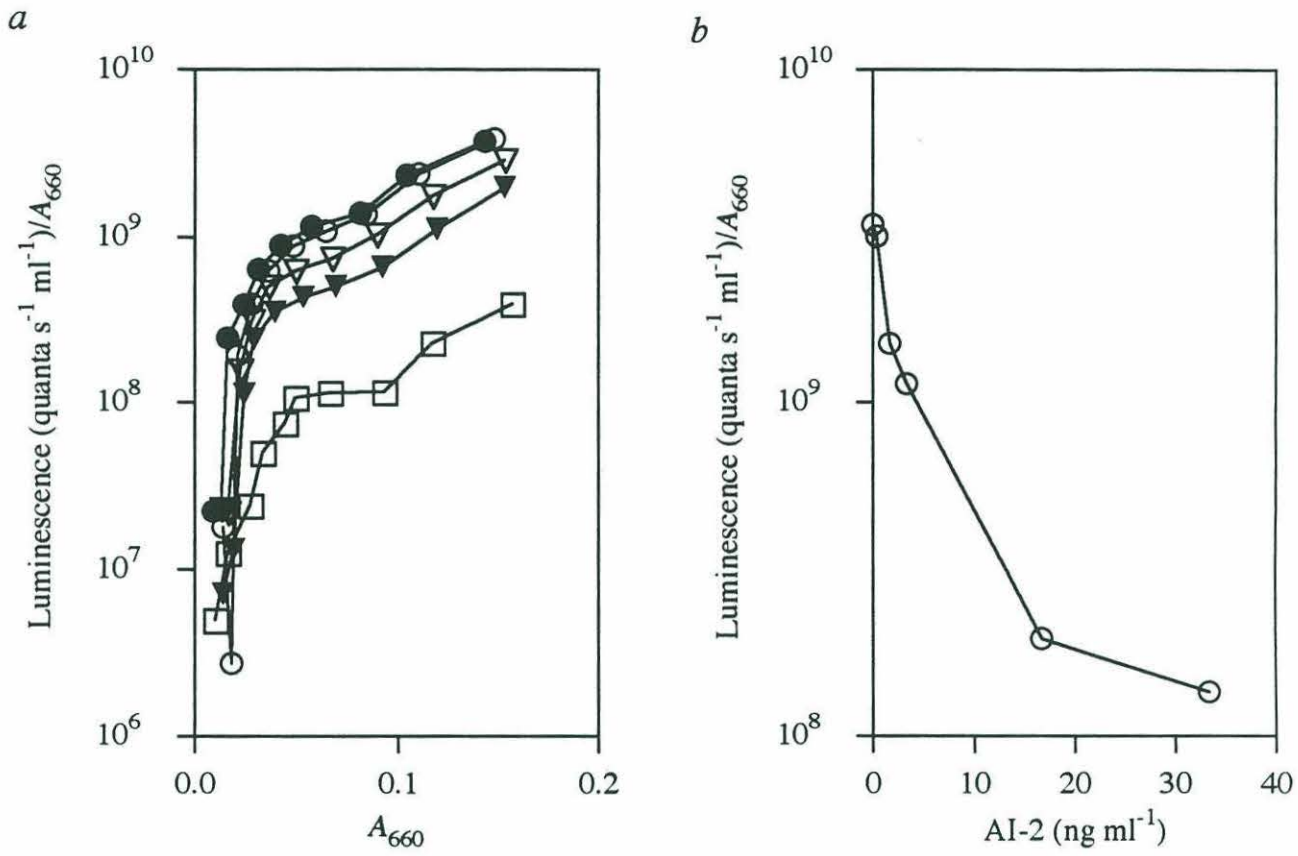

Fig. 3. Inhibition of AI-1 activity by AI-2. In each experiment cultures were treated with 10 ng ml^{-1} AI-1 and a range of AI-2 concentrations. *a.* Inhibition of AI-1 activity in *E. coli* JM83(pAK411,pSUP102), which has a nonpolar deletion in *luxI*. AI-2 was added to 0 (○), 0.33 (●), 3.3 (▽), 33 (▼), and 330 (□) ng ml^{-1}. *b.* Inhibition of AI-1 activity in the *ain luxI* mutant MJ-215. AI-1 was added to each culture to 10 ng ml^{-1}, and luminescence was measured at A_{660} = 0.1.

Table 2. Effect of AI-2 on transcription of *luxR*::*lacZ* operon fusions on plasmids pJR551 and pAK011 in *E. coli* and *V. fischeri*, respectively.

Strain[a]	Additions[b]	Luminescence quanta s^{-1} ml^{-1} A_{660}^{-1}	β-galactosidase activity Miller units
E. coli			
PD100(pJR551,pPD749)	None	2.22×10^8	310
	AI-1	8.12×10^{11}	190
	AI-2	7.36×10^{11}	320
V. fischeri			
MJ215(pAK011)	None	2.31×10^7	670
	AI-1	7.84×10^{10}	500
	AI-2	1.98×10^{10}	720

[a]*E. coli* was grown in autoinducer assay medium (Dunlap and Ray 1989) to $A_{660} = 1.0$. *V. fischeri* was grown in ASH medium with 17 mg l^{-1} Cm to $A_{660} = 1.1$.

[b]To *E. coli*, autoinducers were added to 33 ng ml^{-1} (AI-1) or 1.6 μg ml^{-1} (AI-2). To *V. fischeri*, autoinducers were added to 67 ng ml^{-1} (AI-1) or 1.7 μg ml^{-1} (AI-2).

To determine whether AI-2 represses *luxR* transcription when simultaneously inhibiting AI-1-mediated induction of *luxICDABEG* transcription, I measured *luxR* transcription from the *luxR*::*lacZ* fusion plasmid pAK011 in the presence of AI-1, AI-2, LuxR, and *luxCDABE*. JM83(pAK411,pAK011) was treated with 10 ng ml^{-1} AI-1 and 333 ng ml-1 AI-2, luminescence was monitored until significant inhibition of AI-1 activity was observed (at A_{660} = 0.27, induction of luminescence was inhibited by AI-2 to 12% of the level without AI-2), and β-galactosidase activity was measured. Under such conditions, no repression of *luxR* transcription was observed (Table 3), demonstrating that the level of AI-2-mediated inhibition of AI-1 activity is not related to the level of *luxR* transcription. I conclude that AI-2 does not inhibit autoinduction by repressing *luxR*.

Table 3. Effect of AI-2 on AI-1-mediated induction of luminescence from pAK411 and on transcription of a *luxR*::*lacZ* fusion on pAK011 in a single cell[a].

Additions[b]	Luminescence quanta s^{-1} ml^{-1} A_{660}^{-1}	β-galactosidase activity Miller units
None	8.04×10^6	53
AI-1	4.30×10^8	52
AI-2	1.01×10^7	53
AI-1 and AI-2	5.37×10^7	53

[a]JM83(pAK411,pAK011) was grown to $A_{660} = 0.27$.

[b]Autoinducers were added to 10 ng ml^{-1} (AI-1) or 333 ng ml^{-1} (AI-2).

DISCUSSION

I have demonstrated that an autoinducer of luminescence, AI-2, inhibits the AI-1-mediated autoinduction of luminescence in *V. fischeri*, and that this inhibition can be attributed to inhibition of AI-1 activity but not to repression of *luxR* transcription. I have also shown that in the absence of AI-1, -2, and -3, all autoinduction is abolished, revealing the autoinducer-independent modulation of luminescence described previously (Fig. 1a; Dunlap and Kuo 1992).

The wild-type growth rate of the *ain luxI* mutant MJ-215 and the lack of effect of exogenous AI-2 on growth rates of all strains tested lead me to conclude that neither *ain* nor AI-2 regulates growth. Thus the aberrantly long doubling time of the *ain*::*neo* mutant MJ-216 must be ascribed to its insert DNA. Similar affects have been previously observed with other exogenous DNAs inserted into the *lux* region of *V. fischeri* (Kuo 1994). Given the frequent and routine insertion of selectable markers in genetic studies, the effect of exogenous DNA reported here may serve as a cautionary note.

My demonstration that AI-2 has an inhibitory activity is consistent with the report that a number of synthetic *N*-acyl-L-HSLs, including *N*-octanoyl-L-HSL, both stimulated luminescence and inhibited AI-1 activity in the natural autoinducer-deficient *V. fischeri* strain B-61 (Eberhard et al 1986). Because the nature of the autoinducer deficiency of B-61 is unknown, it is not possible to use B-61 to examine the mechanisms underlying the observed inhibition. The use of defined autoinducer mutants and *E. coli* systems in this study has allowed me to examine potential mechanisms of inhibition as well as to demonstrate the presence of the inhibition in wild-type *V. fischeri*.

It is not yet understood why AI-2 has a lower specific activity than does AI-1. A simple explanation is that AI-1 and AI-2 have different binding affinities for LuxR, but that AI-1-LuxR and AI-2-LuxR are functionally equivalent. This explanation is consistent with the current model for autoinduction and LuxR autorepression, where AI-1-LuxR binds to the *lux* operator to simultaneously stimulate transcription from the *luxICDABE* operon and sterically prevent Crp-mediated activation of *luxR* transcription (Dunlap and Greenberg 1988, Dunlap and Ray 1989, Shadel et al 1991). I thus predicted that AI-2, which interacts with LuxR to stimulate luminescence (Kuo et al 1994), would similarly repress *luxR* transcription. I saw no such repression by 1.6 μg ml^{-1} AI-2, which induces *luxICDABE* to a level comparable to that of 33 ng ml^{-1} AI-1, which is sufficient for significant repression of *luxR*. Thus AI-2 can induce luminescence without simultaneously repressing *luxR* transcription. Therefore I suspect that the current model for LuxR action is overly simple. Also, I conclude that AI-1-LuxR and AI-2-LuxR

cannot be functionally equivalent. AI-1-LuxR and AI-2-LuxR must differ in their abilities to repress *luxR*, or to activate *luxICDABEG*, or both.

The proposal that AI-1-LuxR and AI-2-LuxR are functionally different provides an explanation for the seemingly contradictory finding that a molecule with demonstrable luminescence-inducing activity, AI-2, can also inhibit autoinduction of luminescence. My results are consistent with a mechanism of inhibition where AI-1 and AI-2 compete for the same putative binding site on LuxR, and where AI-2-LuxR is a significantly less potent activator of *luxICDABEG* transcription than is AI-1-LuxR. Because of the lower activity of AI-2-LuxR, substitution of some AI-1 molecules with AI-2 at LuxR results in lower total expression of *luxICDABEG* per cell. Because *luxI* expression is lowered, AI-2 also inhibits the synthesis of AI-1, and thus further decreases the rate of induction of luminescence. Because AI-2 itself activates a low level of *luxICDABEG* transcription, AI-2 never eliminates autoinduction or luminescence altogether.

The above model invokes only the autoinducers and LuxR, and no other regulatory elements, for its operation. However, the model requires the assumption that availability of AI-binding sites limits the amount of *luxICDABEG* transcription; such a limitation has not been experimentally demonstrated. Other possible mechanisms for inhibition have not been excluded, including the logical possibilities that AI-2 might degrade AI-1, block LuxI activity, inactivate LuxR, inhibit LuxR synthesis post-transcriptionally, or even interfere with the luminescence reactions. Also, the model leaves unanswered the question as to how AI-1-LuxR and AI-2-LuxR differ. LuxR has been proposed to act as a multimer (Choi and Greenberg 1992), and the putative interaction between the DNA-binding "helix-turn-helix" motif of LuxR with the palindromic *lux* operator is reminiscent of some regulatory proteins that bind operator DNA more or less effectively depending on their dimerization state (Gussin et al 1983). I speculate that AI-1-LuxR and AI-2-LuxR differ in their state of multimerization.

This model of autoinducer interactions contrasts sharply with the model proposed for the *V. harveyi* autoinducers (Bassler and Silverman 1994). In the latter, two autoinducers bind to distinct cognate receptors (LuxN and LuxPQ), each of which inactivates a single DNA-binding repressor of *luxCDABE* transcription (LuxO). My model does not preclude the existence in *V. fischeri* of a system analogous to that of *V. harveyi*, but none of the three *V. fischeri* autoinducers is known to derepress a repressor of luminescence.

The demonstration of a functional role of AI-2 in regulation of luminescence leaves unknown the functional role of AI-3. Total AI-3 activity in *V. fischeri* is intermediate between that of AI-1 and AI-2, and AI-3 is also a *N*-acyl-L-HSL (Kuo et al

1994). It seems reasonable to suppose that AI-3, like AI-2, serves as an inhibitor of autoinduction. It should be noted that an inhibitory role for either AI-2 or AI-3 does not exclude the possibility that these multiple autoinducers might serve other regulatory roles in the cell.

The synthesis by *V. fischeri* of an inhibitor of autoinduction leads me to suggest that fine-tuned control of the rate of luminescence induction is significant to the bacterium. It is possible that, during the rapid changes in population density that luminous bacteria presumably experience when moving from high density environments such as the light organ symbiosis (10^{6-10} cells ml^{-1}; Nealson and Hastings 1991) to the surrounding water column (10^{3-4} cells ml^{-1}) and back, the rate of *lux* autoinduction is optimized by the interactions between AI-1 and AI-2. That an autoinduction system may be modulated by an additional regulatory factor is not unprecedented; *modA* is a negative regulator of the *A. tumefaciens* autoinduction system that is presently regarded as a secondary or modulating element (Hwang et al 1994). However, the activity of ModA has so far not been shown to require an extracellular autoinducer. Nevertheless, the evolution of negative regulatory elements that inhibit autoinduction, such as AI-2 and perhaps ModA, points to the importance to bacteria of controlling the rate of autoinduction events during transitions between different population density states.

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Chapter 5. Concluding remarks

In this study I have used a mutational approach in *V. fischeri* to investigate novel aspects of luminescence and autoinduction. This was the first attempt to use defined *lux* mutants to determine the physiological role of luminescence. Also, these mutants potentially lend insight into the functions of *luxG* and the unidentified yellow extracellular pigment. Finally, I have employed these mutants to demonstrate the existence of a second autoinducer system, to isolate and identify the autoinducer, and to generate evidence for the second autoinducer's function in *V. fischeri*. I will discuss my ruminations on these observations in the remainder of this chapter. First, however, I wish to emphasize the importance of constructing mutations in *V. fischeri*. Until recently, all genetic studies of *V. fischeri* biology were restricted to the cloned *lux* genes in *E. coli*. By constructing *V. fischeri* mutants, I have encountered aspects of *V. fischeri* biology that could not have been observed using cloned genes in *E. coli*. Even more instructively, I have belatedly learned that an experimental "dialogue" between the mutants and the cloned genes, where results in one system are tested in the other system and vice versa, is productive and rewarding (and fun). My hope is that this and the few other studies utilizing *V. fischeri* mutants will provoke broader use of genetic techniques to more completely understand this bacterium, both in its own right and as a model for studies of luminescence, autoinduction, and symbiosis.

I. Luminescence

The *lux* mutants described in Chapter 2 confirmed most of the classical model for luminescence established in *E. coli* (the exception is autoinduction, discussed below), demonstrated that luminescence does not play an obviously significant role in bacterial growth, and provided a means to assess luminescence as a functional adaptation. In the last capacity, the *lux* mutants were used to test the hypothesis that luciferase is a defense against H_2O_2 toxicity. The lack of difference in H_2O_2 sensitivity between the *lux* mutants and the wild-type is inconsistent with the hypothesis. Many of the other "adaptationist stories" proposed for luminescence could be similarly examined with these mutants. Thus the hypothesis that luciferase serves to defend the cell against some other form of oxidative stress (or any other kind of stress) could be tested by subjecting the luciferase mutant to the stress. The notion that luciferase is an alternative electron acceptor to cytochrome oxidase could be examined by growing the luciferase mutant under conditions where cytochrome oxidase is not functional, such as iron depletion, or

under O_2 tensions too low for cytochrome oxidase function but high enough for luciferase function (luciferase has a higher affinity for O_2 than does cytochrome oxidase; Hastings 1952, Yonetani 1963). The idea that luminescence serves to lure potential host animals could be addressed by observing captive host behavior with respect to free-living mutant bacteria. The proposal that the purpose of the luminescence reaction is to generate the fatty acyl product could be investigated by following the fates of radiolabeled aldehydes when assimilated by the aldehyde mutant.

II. Pigmentation

The distribution of pigment phenotypes among the *lux* mutants generated in Chapter 2 points to a link between *luxG* and the extracellular yellow pigment of *V. fischeri*. However, such a link is not rigorously demonstrated without construction of a *V. fischeri* strain with mutation in *luxG* alone. *E. coli* JM83 with the cloned *lux* genes on pNL121 does not appear to synthesize the pigment; whether this is due to an unknown alteration in *luxG* on pNL121 (the *luxG* in question has not been sequenced) or to a lack of a necessary substrate in *E. coli* is unknown.

LuxG has sequence similarity to Fre, an *E. coli* NAD(P)H:flavin oxidoreductase that reduces the ferric iron of ribonucleotide reductase, a necessary step in deoxyribonucleotide synthesis (Fontecave et al 1987, Spyrou et al 1991, Andrews et al 1992). Specifically, three of four regions located at the active site are conserved between Fre and LuxG (Zenno and Saigo 1994). Thus it has been suggested that LuxG catalyzes the flavin reduction necessary for luminescence (Andrews 1992). However, none of the six NAD(P)H:flavin oxidoreductases so far purified from luminous bacteria are encoded by LuxG, though four (including one of the two from *V. fischeri*) are homologues of Fre (Zenno et al 1994, Zenno and Saigo 1994, Lei et al 1994). I know of no successful demonstration of flavin or iron reductase activity that is regulated by autoinducer, or is dependent on LuxG.

Similarly, the nature and function of the pigment are unknown. Its solubility in water and flourescence in ultraviolet have led to the suggestion that it is riboflavin (Giese 1943). The functional significance of an extracellular flavin is, however, unclear. Alternatively, the yellow color is reminiscent of a phenazine. Phenazines have a heterocyclic structure and redox functionality analogous to those of flavins (Morrison et al 1978). In this light, it is intriguing that two *Pseudomonas* sp. have been shown to synthesize extracellular phenazine antibiotics in a cell density-dependent manner, each requiring a *luxR* homologue for production (Ochsner et al 1994, Pierson et al 1994).

Interestingly, genes involved in the synthesis of riboflavin are located 3' to the *luxG* genes of *Photobacterium* sp. (Lee and Meighen 1992, Lee et al 1994). However, the *V. fischeri luxG* is followed by a transcriptional terminator that serves both the *luxICDABEG* operon and a convergently transcribed open reading frame (Swartzman et al 1990). Intriguingly, the latter has sequence similarity to the *Bacillus subtilis* riboflavin synthesis gene *ribG* (Lee et al 1993), but its transcriptional orientation is difficult to reconcile with the autoinducer-dependence of pigmentation (Chapter 2).

On a more theoretical level, it is not obvious how a flavin reductase might be involved in synthesis of a flavin. Elucidation of the enzymatic steps of riboflavin synthesis is not yet complete (Richter et al 1993, Lee et al 1994), but none of the known steps is obviously analogous to the reduction of ribonucleoside diphosphates to deoxyribonucleotides. Even if a *luxG*-specific mutation in *V. fischeri* results in pigment deficiency, LuxG is not necessarily a synthase of the pigment; alternatively, LuxG may influence pigment production through an indirect effect on intermediary metabolism. A variant altered in metabolic acidification was also altered in pigmentation (Giese 1943), and this pigment defect was rescued by strong buffer. It was unclear whether the acids were destroying the pigment directly, or were adversely affecting pigment production by reducing cellular metabolism or viability. In my own work I have not observed correlations amongst *lux* mutants between acidification of medium and pigmentation (data not shown).

III. Autoinduction

While the *luxI* and *luxR* mutants of Chapter 3 confirmed the primary role of AI-1 in inducing expression of the *lux* operon, as well as repression of *luxR*, they also revealed the presence of a second and separate autoinducer system in *V. fischeri*. The second autoinducer activity, AI-2, was shown to be *N*-octanoyl-L-HSL. Synthesis of AI-2 was directed by *ain*, which bears sequence similarity to *V. harveyi luxM* (see below) but not *V. fischeri luxI*. AI-2 was capable of interacting with LuxR to stimulate expression of *luxICDABEG*, but not to repress *luxR*. However, the *ain* mutants of Chapter 4 showed that the overall effect of AI-2 on regulation of luminescence is to inhibit induction of *luxICDABEG* by AI-1, thus slowing autoinduction. Finally, LuxI synthesized a third molecule AI-3 (*N*-hexanoyl-L-HSL) capable of inducing the *lux* operon via LuxR.

A. AI-2

AI-2 lowers the rate of induction of luminescence by AI-1, and thus modifies the autoinduction response. As to the purpose of inhibition, we can only speculate that the rapidity of autoinduction is relevant to its adaptive role. These results do not exclude the possibility that AI-2 regulates functions other than luminescence (see below).

The *ain* locus has been mapped and sequenced by L. Gilson; a single gene *ainS* is necessary for synthesis of AI-2 (Gilson et al). The carboxyl terminus of AinS has sequence similarity with *V. harveyi* LuxM (Bassler et al 1993) and not LuxI, while the amino terminus bears no resemblance to either LuxLM or LuxI. The fact that genes as divergent as *luxI* and *ainS* direct the production of structurally similar *N*-acyl-HSLs in *E. coli* lends support to the notion that autoinducer synthesis is catalyzed by a single step, probably formation of the peptide bond; presumably LuxI and AinS utilize the same homoserine lactone substrate but different fatty acyl substrates. Verification of this notion awaits purification of active autoinducer synthases.

The dissimilarity of LuxI and AinS also supports the notion that the structures of autoinducers are products of convergent evolution, and thus that the *N*-acyl-L-HSL motif is of adaptive significance to these bacteria, as proposed in Chapter 1. *V. fischeri* is the only example of a bacterium with both the LuxI- and AinS-types of putative autoinducer synthase; I would not be surprised if both types were to be discovered in *V. harveyi*, *P aeruginosa*, and *A tumefaciens*.

Sequencing revealed another open reading frame immediately 3' to *ainS* (Gilson et al). This gene possesses its own candidate promoter and ribosome-binding site. The sequence of the first fifth of the protein is known, and is similar to that of *V. harveyi* LuxN and LuxQ. Since LuxN and LuxQ are putative autoinducer receptors (Bassler et al 1994), the *ainS*-linked gene is an obvious candidate for an AI-2 receptor gene and has been named *ainR* (Gilson et al). If so, presence of this gene has no effect on either stimulation of luminescence (pAI002 containing *ainR* failed to complement a Δ*luxR* plasmid in *E. coli* in Chapter 3; other data not shown) or on AI-2-mediated inhibition of autoinduction (data not shown). It is possible that *ainR* is either insufficient to transduce the AI-2 signal to a response, or is not functional in *E. coli*. Alternatively, *ainR* together with AI-2 might regulate some unknown target other than *lux*. However, S. Callahan and I have so far not detected radiolabeled AI-2-regulated polypeptides on polyacrylamide gels (data not shown).

B. AI-3

The ability of *luxI* to direct the synthesis of two distinct autoinducers, AI-1 and AI-3, is striking. The same autoinducers are also recognized by LuxR. The fact that luxI is involved in the synthesis of two structurally similar molecules lends further credence to the notion that LuxI catalyzes a single peptide bond-formation step. Presumably there exist in both *E. coli* and *V. fischeri* at least two distinct pools of potential fatty acyl substrates for LuxI.

I have not explored AI-3 in depth, and so cannot state whether AI-3 is a supplementary autoinducer to AI-1, inhibits like AI-2, or performs yet another function. It should be possible to examine some of these propositions following the approach already used for investigating AI-2 function in Chapter 4. Given LuxI synthesized significantly less AI-3 activity than AI-1 activity (Chapter 3), it is possible that AI-3 merely represents an inefficiency, a product of imprecision in LuxI substrate recognition. Alternatively, AI-3 may be the product of LuxI utilization of an alternative fatty acyl pool, to be used when the 3-oxohexanoyl pool for AI-1 synthesis is limiting.

C. LuxR

Like AI-1, AI-2 induces *luxICDABEG* transcription, but unlike AI-1, AI-2 appears not to repress *luxR* transcription (Chapter 4). This result is inconsistent with the current model for autoinduction and LuxR autorepression, which views LuxR binding to the *lux* operator as the proximal cause for both *luxICDABEG* activation and *luxR* repression. The behavior of AI-2 logically separates *luxICDABEG* activation from *luxR* repression, implying that LuxR-operator binding is itself not sufficient for one or both effects.

The putative domain for LuxR multimerization overlaps two hydrophobic regions and the putative autoinducer-binding domain, suggesting that autoinducer-binding may promote multimerization (Choi and Greenberg 1992). I speculate that the autoinducer itself mediates dimerization, by acting as a bridge between the homoserine lactone binding site in one LuxR monomer and a hydrophobic pocket in another LuxR monomer, and that this dimerization is required for *luxR* repression but not for low levels of *luxICDABEG* activation. The "original" helix-turn-helix DNA-binding protein, λ repressor, binds weakly to its palindromic operator as a monomer and binds several orders of magnitude more strongly as a dimer (Gussin et al 1983). Similarly, monomeric LuxR may bind to its palindromic operator too weakly to repress *luxR*. In effect, the autoinducer-binding domain is identical to the dimerization domain, and AI-1 is a "dimerization factor". In this model, the differences in repression activity between AI-1

and AI-2 are due entirely to their differing abilities to mediate dimerization, which is a consequence of their differing fatty acyl structures. If one supposes that dimerization and stronger DNA binding also promote greater *luxICDABEG* activation, then this model can also explain differences in *luxICDABEG* inducing activity between different *N*-acyl-L-HSLs.

Definitive establishment of AI-LuxR and LuxR-LuxR interactions awaits purification of active LuxR, but it may be possible to use existing *E. coli* systems to test the above-described dimerization model. I suggest using the Choi and Greenberg (1992) genetic dominance system as a logical starting point. Choi and Greenberg report genetic dominance of the putative autoinducer-binding domain of LuxR over intact LuxR with respect to induction of luminescence (by AI-1). This observation can be explained by LuxR dimerization, or by titration of the autoinducer. My model predicts that 1) genetic dominance should be similarly observed with respect to repression of *luxR* transcription and 2) no genetic dominance should be observed if AI-2 is supplied in lieu of AI-1. The second observation would be consistent with LuxR dimerization but not with autoinducer titration. Thus AI-2 might prove to be a useful probe for analyzing the mechanism of LuxR action.

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